Comparative Grain Development in Temperate Grasses

Thesis submitted for the Degree of Doctor of Philosophy at the University of Leicester

by

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September 2012

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ABSTRACT

Temperate or pooid cereal crops, such as wheat, barley and oat, represent a major world food source much of which is attributable to the storage capabilities of the cereal endosperm and influences of domestication selection. *Brachypodium distachyon* has recently become established as a genome-sequenced model system and the first wild member of the Pooideae be studied in detail. We provide the first detailed description of grain development in *Brachypodium* assessing its suitability as a model for grain development in crop species. Cellular and molecular mapping of developing *Brachypodium* endosperm domains reveals significant differences in aleurone differentiation reflecting differences in grain filling and endosperm storage reserves. We extend this survey of grain morphology and endosperm organisation to a wider sample of the Pooideae incorporating both wild and cultivated species. Focusing on the functionally important aleurone domains, distinct patterns of grain tissue organisation are described. Results indicate that organizational features are correlated to species' ecological and grain quality characters and that the modified aleurone region, absent in *Brachypodium*, may be a feature of only a subset of cereals, specifically the Triticeae tribe. A more systematic candidate gene approach focusing on transcription factors was initiated in attempts to find the genes underpinning this variation. The identification of orthologous key regulatory genes with both similar and contrasting patterns of expression provides information on the differences and conservation of grain developmental pathways amongst the Pooideae. To investigate function of candidate genes, publically available insertional mutants for major MADS-box and YABBY genes were obtained and characterized, while simultaneously attempting to establish genetic transformation protocols to enable RNAi analyses of other candidate genes.

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ABBREVIATIONS

As	Acetosyringone
Approx.	Approximately
ANOVA	Analysis of Variance
AP-buffer	Alkaline Phosphatase buffer
BCIP	5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt
BETL	Basal Endosperm Transfer Layer (Maize)
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CaCl	Calcium Chloride
CaMV35s	Cauliflower Mosaic virus 35S promoter
CEC	Compact Embryonic Callus
CER	Controlled Environment Room
Cu	Copper Sulphate
°C	degrees centigrade
DAA	Days After Anthesis
DAG	Days after Germination
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
cDNA	complementary DNA
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
EtOH	Ethanol
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
GFP+ve	Tissue where fluorescence is detected
GFP-ve	Tissue where fluorescence is not detected
GPWG	Grass Phylogeny Working Group
cm	centimetre
DAPI	4',6-diamidino-2 phenylindole dihydrochloride
DEPC	diethyl pyrocarbonate
dNTP	deoxynucleotide triphosphate
FST	Flanking Sequence Tag
GDU	Grain Dispersal unit
HCl	Hydrochloric Acid
IFR	Institute of Food Research
ISH	In-situ Hybridisation
JIC	John Innes Centre (Norwich)
LB	Left Border
M	Molar
MRI	Magnetic Resonance Imaging
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NaPO	Sodium Phosphate
NBT	Nitroblue Tetrazolium
NEB	New England Biolabs
ng	nanograms
nm	Nanometer
MA	Modified Aleurone region

MgCl	Magnesium Chloride
mRNA	messenger RNA
MSB	Murashige & Skoog Basic
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
RB	Right Border
RT-PCR	reverse transcriptase-PCR
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SSC	Saline Sodium Citrate
TDNA	Transfer DNA
Tris-HCl	Tris(Hydroxymethyl) aminomethane hydrochloride
TE	Tris-EDTA buffer
TBS	Tris Buffered Saline
TZ	Tetrazolium Chloride
UTR	untranslated region
UV	Ultra Violet
VIGS	Virus Induced Gene Silencing
WT	wild type
µM	Micromolar
µl	Microlitre
%	percentage

SCOPE & AIMS.

This thesis compares aspects of cereal grain morphology, development and genetic regulatory mechanism between major domesticated and closely related wild temperate grass species with emphasis placed upon the comparison between wild and cultivated endosperm morphology.

Chapter 1 provides a comprehensive introduction to the subjects and areas under investigation and a review of relevant literature. This information identifies the relevancy of this research and provides a basis for interpretation of the findings of the following chapters.

Chapter 2 details the conditions, materials and techniques used throughout this investigation.

Chapter 3 focuses on grain development in *Brachypodium* providing a detailed comparison to the wheat from a macro-histological through to molecular level. These comparisons build a detailed picture of temperate cereal grain development focused on pertinent features of endosperm development aiming to identify key features distinguishing wild and cultivated species. By identifying and characterising significant differences at a structural and molecular level we will gain insight into the effects of domestication and cultivation on the grain's development and can assess the suitability of *Brachypodium* as model for grain development in wheat, and its potential in relation to other temperate cereal species.

Chapter 4 represents a comprehensive survey of grain morphology and endosperm organisation across a representative selection of wild and cultivated species of the core Pooideae. Employing a wide range of cytological and

molecular techniques these investigations compare aspects of plant ecology and morphology influential to the grain, key differences in histological organisation between wild and cultivated species, and features of endosperm differentiation at both histological and molecular levels. This survey provides a cross-species comparative analysis aiming to identify key aspects relevant to grain morphology, evolution and the effects of domestication, and identifies species valuable in future molecular analyses.

Chapter 5 examines transcription factor candidate genes relating to endosperm development in *Brachypodium* in relation to orthologues in major domesticated species. Here we aim to positively identify and provide preliminary expression pattern characterisations for candidate gene markers underpinning grain morphological variation identified in previous chapters.

Chapter 6 describes preliminary characterisation for *Brachypodium* TDNA insertional mutants disrupting a C-class and a YABBY gene at molecular and phenotypic levels. Investigating these mutant lines we describe optimization and streamlined procedure for use in an ongoing program of *Brachypodium* genetic investigation. Finally we detail an attempt to establish an *Agrobacterium*-mediated transformation protocol using embryonic callus.

CHAPTER1

INTRODUCTION

INTRODUCTION

1.1 Temperate Cereal Grains

1.1.1 Modern Cereals

Worldwide the grains of cultivated cereals are an important food supply, forming the basis of the human diet and the bulk of calorific intake (http://faostat.fao.org). The grains of eight main species: wheat, maize, rice, barley, sorghum, oats, rye, and millet together account for 56% of the food energy and 50% of the protein consumed by humans globally (Cordain, 1999, Gibbons & Larkins, 2005). Just three of these species: wheat, maize and rice together comprise at least 75% of the world's grain production and are the foundation of the modern human diet (Gustafsen et al, 2009; Murphy, 2007). Wild grasses have provided a source of starch in the human diet since the middle Stone Age (Mercader, 2009) but it is only since the domestication of cereals approximately 10,000 years ago that they have formed the major component. Domestication improved the yield and productivity of grasses and allowed for the transformation of a roaming, hunter-gatherer human population into a sedentary agrarian society (Doebley et al, 2006; Glemin & Bataillon, 2009).

The domestication process has significantly altered the physiology, development, and morphology of these cereals and modern domesticated grasses are now quite distinct from their wild progenitors (Shewry, 2009; Zohary, 2004). Modern agriculture has become centered around grasses and the dependence of the human population upon cereal grains (grass seeds) has prompted one author to say we have become "canaries" (Harlan, 1992). Such an analogy is imperfect

however: the human genetic makeup is still that of a Paleolithic hunter-gatherer with nutritional requirements optimally adapted to wild meats, fruits and vegetables, not to cereal grains (Cordain, 1999). There is still a significant element of nutritional compromise and discord in our cereal grain diet that the ongoing domestication process and modification of cereals through scientific research is seeking to address. Nevertheless, population size and lifestyle has led us down a path of absolute dependence upon cereals and it is clear that their continued development and exploitation will be an enormously important part of shaping the modern world and confronting the problems it faces like food security and energy. It is without doubt that within the many grass species (approximately 10,000; Hubbard, 1954; Clayton & Renvoize, 1986) growing, and often dominating, in many different habitats around the world there is great, varied and often untapped, potential. A detailed knowledge and understanding of the evolution, development and key features of grasses will be crucial in exploiting all they have to offer and critical in meeting the demands of an ever-increasing world population.

1.1.2 The Pooideae Subfamily

As the largest member of the major BEP (Bambusoideae, Ehrhartoideae, Pooideae) sub-clade, the subfamily Pooideae represents a major lineage of the Poaceae family encompassing almost all of the world's major temperate zone cereal, biofuel and forage crop species (Fig 1.1; Glemin & Bataillon, 2009; Bremer 2002; Shewry, 2009). Wheat is the dominant temperate zone crop along with three other important species, barley, oat, and in the more northerly latitudes, rye. In addition to these economically important cereal crops, significant forage species,

such as *Lolium* and *Festuca*, turf species such as rye grass, and invasive weed genera such as *Elymus* and *Bromus*, all belong to the Pooideae (Hubbard, 1954). The various members of the Pooideae show significant diversification through the cooler climates and encompass all of the world's "cool season" grasses, with all species being C3 photosynthetic (Glemin and Bataillon, 2009). High levels of morphological, physiological and ecological diversity seen in this group have lead to difficulties in classification (Hsiao et al, 1995). Various molecular analyses have started to resolve this situation amongst both the Pooideae and the larger Poaceae where family level phylogenies have been produced using a range of molecular markers such as chloroplast restriction sites and gene sequences, ribosomal proteins and polymerase genes, and other nuclear genes such as *GBSS1* (Granule Bound Starch Synthase 1) or phytochrome (reviewed in Kellogg, 1998). To overcome the limitations of sample size etc, data from several of these studies was combined in a collaborative effort by the Grass Phylogeny Working Group (GPWG) to produce a comprehensive phylogeny. This phylogeny forms a widely accepted subfamilial classification of grasses (Summarised in fig 1.1; GPWG, 2001). However, there still exists some conflict in the allocation of the main tribal groups of the Pooideae and to the evolutionary relationships between them (Catalan et al, 1997: Hsiao et al, 1995). Morphological studies have questioned the distinction between the Aveneae and Poeae tribes, and have in some cases linked the genus *Bromus* to the Poeae, although this depends heavily upon inflorescence architecture (Doring et al, 2007; Catalan et al, 1997). Of particular difficulty has been the placement of *Brachypodium* in relation to the Bromeae and Triticeae tribes, with which it shares several putative synapomorphies (Catalan et al, 1995).

Simple starch grains, an elongate caryopsis with linear hilum and hairy ovary tip

are all features shared by *Brachypodium* and the Bromeae and Triticeae tribes.

Similarities in inflorescence architecture along with hairy lodicules has convinced

some authors to group *Brachypodium* with the Triticeae, whilst the similarity of the

caryopsis, in particular the prominent nucellar layer, has lead to some grouping

Brachypodium with Bromeae (Hayek, 1925). The identification of distinctive

characters including karyotype, seed storage proteins, polysaccharide content etc

along with molecular data such as that of Soreng et al (1990), Catalan et al (1997),

have ultimately placed *Brachypodium* in a distinct tribe sister to the Bromeae and

Triticeae tribes.

 The "core Pooids" form a prominent and important sub-group of the Pooideae

comprised of the Poeae, Aveneae, Triticeae, and Bromeae tribes (Doring et al,
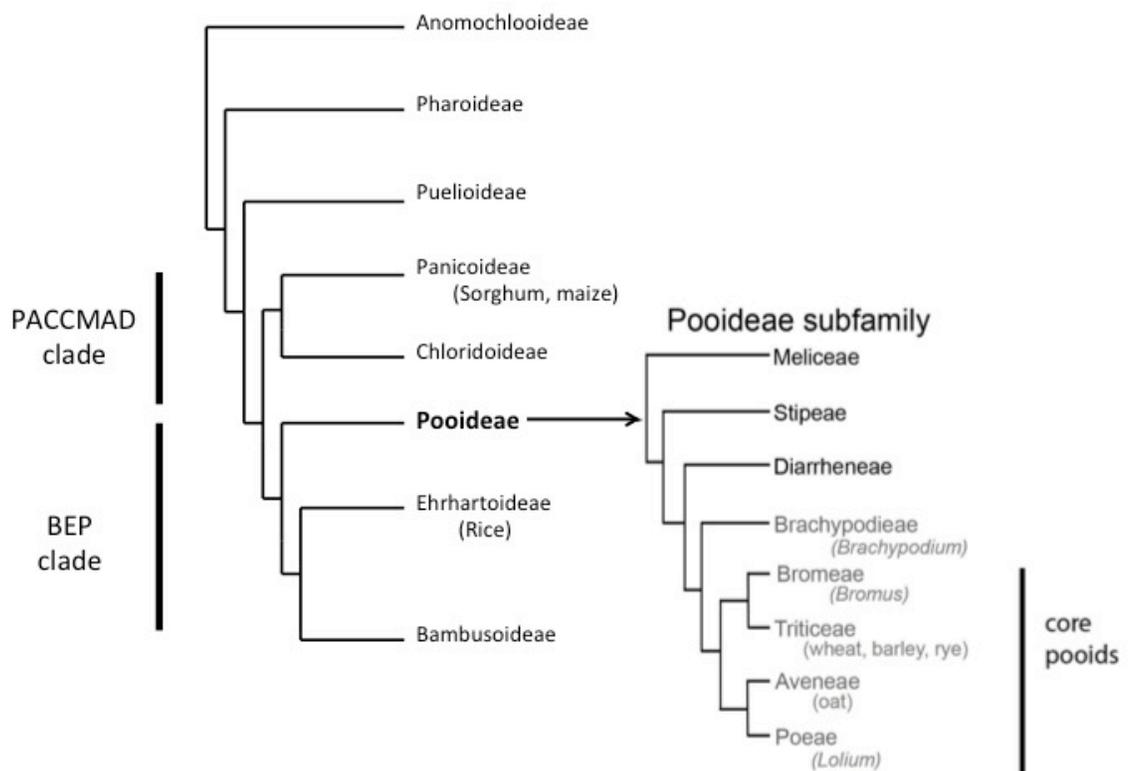
Figure 1.1: Phylogeny of the Poaceae and important sub-groups

2007: Catalan et al, 1997) This sub-group was first identified and named in a study by Davis and Soreng (1993), who found evidence to support this grouping based upon chloroplast restriction site variation. Karyological investigations have since shown this group is united in having large chromosomes of x=7 (Catalan et al, 1997). The Festucoid grasses sit just outside of this group and show significant differences to the core-pooids, but it is an interesting contrast that the Brachypoideae, also sitting just outside, shows all of the core-pooid apomorphies other than chromosome size (Catalan et al, 1995). Using sequence information from the 3' end of the chloroplast *NdhF* gene in 48 representatives of the 12 Pooideae tribes of the findings of Catalan et al (1997) support the well defined nature of this group and a study by Hsiao et al (1995), using the ITS region of nuclear ribosomal sequences also identifies this group. Thus the core pooids forms an important and defined group containing all of the significant domesticated cereal crops of the temperate world.

1.2 Domestication.

Plant domestication is one of the most dramatic demonstrations of the human ability to manipulate the evolutionary processes of speciation, natural selection, and adaptation, and was a defining event for the emergence of human civilization (Darwin 1883; Gustafson et al, 2009). Agriculture marks the shift from a world of hunter–gatherer communities to complex plant-cultivating societies. In the freedom and stability of these early agrarian societies technologies and skills such as writing, currency, arts, the study of science, social structure and the emergence of the state were able to emerge (Purugganan & Fuller, 2009; Brown et al, 2009).
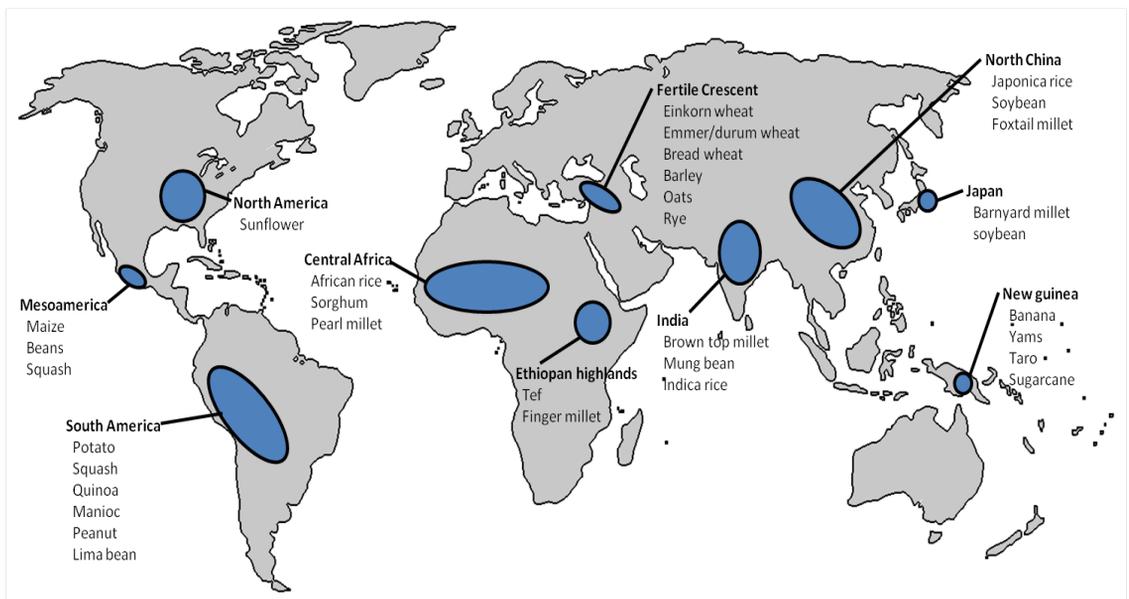
Figure 1.2: Distribution of the World's Major Domestication Centers and Staple Crops to Have Emerged From them.
(Adapted from Glemin & Bataillon, 2009)

Grass seeds offer a valuable foodstuff as many possess a large, starch rich endosperm that is a rich source of energy and often seeds are borne aloft, on stems where they are visible and easily collectable. Archeological evidence has shown that the grains of wild cereals form a seasonally significant part of the diet of early human hunter-gatherers in and around the Fertile Crescent (Vavilov 1940; Weiss et al, 2006: Glemin & Bataillon, 2009). This area is one of the major sites around the world where the earliest evidence of crop use and the beginnings of agriculture have been uncovered, referred to as "domestication centres" (Diamond 2002; Glemin & Bataillon, 2009). Figure 1.2 indicates the main domestication centres around the world and the major crops to have emerged from them in independent domestication events. Grasses have dominated in the majority of these domestication centres but nowhere more so than in the old world domestication centre, the Fertile Crescent. Often referred to as the "seat of agriculture", all of the major pooid cereal crops have emerged from this relatively small and botanically rich area in central Asia, spanning areas of what is now Turkey, Syria, Iraq and Iran, (Charmet, 2011; Salamini et al, 2002). Some of the earliest evidence of plant domestication can be seen at sites like Abu Hureya in Syria and in the Karacadag region where cereal cultivation is thought to have first occurred around 10,000 years ago with barley, rye and wheat species such as einkorn and emmer (Heun et al, 1997; Hillman 2000; Salamini et al, 2002).

Grasses are typically colonizing species and their dominance amongst the major crop species is perhaps an indication of their frequent capacity for phenotypic plasticity and adaptability. This flexibility makes them well suited to the strong selective pressures of domestication and many grasses also show predilection for

polyploidisation that can offer important evolutionary advantages (Adams &

Wendel, 2005; Hegarty & Hiscock, 2008; Vaughan et al, 2007). The position of the

Fertile Crescent, latitudinally central to the world's major land mass allowing a

gradual dispersal to climatically similar regions, in conjunction with the pooid

grasses' diversity and adaptability has made it not only one of the earliest sites of

domestication but it is also the one from which the domesticated species and

agricultural practice was able to rise and spread most quickly (Diamond, 2002).

1.2.1 *The Domestication Process*

During its early stages the domestication process in cereals was heavily focused

on the grain and closely associated traits such as harvesting (Glemin & Bataillon,

2009; Charmet 2011). Most of the changes that occur in this process are

deleterious in the wild, and the process begins when wild-harvested seeds are

distributed and sown by their harvester (Davies & Hillman, 1992). Harvesting and

eating wild seeds a human forager is a seed predator whose activities promote

wild-type traits: only seeds falling to the ground escape predation and so contribute

to future generations. Wherever collected seeds are distributed or sown then the

direction of selection is immediately reversed. In this sown population only

collected seeds contribute to the next generation (Glemin & Bataillon, 2009).

Where a cycle of harvest and sowing of collected seeds is maintained a seed-

retentive phenotype will come to dominate as they contribute most highly to future

generations. Ultimately plants become dependent upon harvest for seed dispersal

whilst harvesters become dependent upon the increased yield and availability of

the seed resource. Precise identification of a point of sufficient distinction between

a wild and domesticated plant can be difficult. A generally accepted definition for the term "domesticated" is where a plant has accumulated sufficient deleterious mutations and adaptations to be unable to compete in its native environment and shows dependence upon human intervention for its propagation (Darwin 1883; Charmet 2011). Whilst domestication may be described as complete once this threshold is reached it does not mark the end of the domestication process, which is ongoing and continual (Brown et al, 2009; Gepts 2004). Post-domestication evolution can be better described as crop "improvement". Such ongoing selection and modification refers to the conscious selection practices occurring where the benefit of agriculture is recognised and can include previously un-selected traits such as grain colour, storage characteristics, processing quality etc. The domestication process is necessarily ongoing as once established there generally follows regular cropping in large monocultures where selection must be maintained in order to maintain productivity against insect pests and pathogens etc (Carver, 2009; Murphy 2007).

  Recognition of the advantages of cultivation and of conscious, deliberate selection practice is a distinguishing feature human plant domestication and extends its influence to almost all aspects of plant physiology and morphology. Environmental manipulation in favor of the crop species is a defining aspect of the early, deliberate stages of domestication (Davies & Hillman, 1992; Glemin & Bataillon, 2009). Clearing the ground to sow seed and reduce competition leads to important traits associated with seed bed competition that distinguishes domesticates from their wild counterparts. Conscious and deliberate selection also favors physical factors such as increased plant vigor, seed size, disease resistance

and higher yield etc (Zohary & Hopf, 2001). As domesticated plants are moved into different regions and their range of cultivation increases there is selection not only for desirable traits as a crop but also for adaptations favorable to the local climate and conditions. It is this type of selection and the expansion of crops from their domestication origin that gives rise to the many local landraces and varieties of crop plants (Diamond 2002). Finally, it is important to note that the domestication process is not an unwavering path to defined and ideal end point. Just as evolution provides constant selection according to the demands of the changing environment the domestic environment is constantly changing according to varying demands, harvesting practices and disease etc (Murphy 2007) with domesticated plants experiencing a similar selection.

1.2.2 The Domestication Syndrome

The selective pressures of domestication lead to a distinct and predictable set of phenotypic changes distinguishing a crop plant from its wild ancestors, often referred to as the "domestication syndrome" (Fuller 2007; Brown et al 2009; Zohary & Hopf, 2001). Domestication traits seen in plants such as potatoes or peas are quite different to those in cereal crops, apples or cotton, although it is recognised that the selective forces driving the changes are very similar and parallels can be drawn between them, such as the dependence upon anthropized environments, harvesting etc. Perhaps the best example of this parallel evolution is the reduced seed head shattering seen in wheat, rice and maize crops, all of which arose in separate domestication events, although other traits such as rapid germination, synchronous growth and lack of dormancy are equally ubiquitous traits seen in

both dicot and monocot domesticates (Frary & Doganlar 2003). In cereal crops, whilst exerting a global influence over plant morphology and physiology, domestication is primarily focused on floral reproductive structures, producing plants with grains more amenable to harvesting and processing, increased grain size and nutritional quality. The main features of the domestication syndrome of particular relevance to grain development are as follows:

- **Seed retention**: Reduced seed-head shattering is a key feature of all major cereal crops thought to occur unconsciously during early stages of domestication with evidence of this non-shattering phenotype often used as diagnostic trait of domesticated forms by archeologists (Glemin & Bataillon, 2009). The importance of variation in this trait as opposed to simple loss-of-function mutation, and complex selection for optimal seed retention efficiency as compared to harvest-threshing energy requirements has been revealed in recent studies (Zhou et al, 2012).

- **Increased grain size**: Increased fruit size is not a cereal specific trait and can be seen in other domesticated crops species such as tomatoes, apples and beans (Cong et al, 2008; Zohary & Hopf, 2001). Selection for larger grains occurs quickly in domesticated populations both intentionally and indirectly through various harvesting and processing methods.

- **Seedling competition**: Conscious cultivation practices provide strong selection for rapid germination and seedling vigour in response to seed-bed competition (Glemin & Bataillon, 2009). This environment strongly also

selects for reduced dormancy characteristic via the loss of glumes and other germination inhibitors.

- **Loss of seed dispersal agents**: Awns, barbs, hairs and other floral appendages with function in wild-grain dispersal are often, but not always, lost in domesticated populations (Elbaum et al, 2007), their loss often desirable in relation to post-harvest processing and in increasing energy use efficiency of the crop.

- **Loss of sensitivity to environmental cues**: Reduced response to stimuli such as day length or vernalisation requirements in domesticates allows for convenient, seasonally-independent sowing, synchronous seedling germination, uniformity of growth and capacity for cropping in response to demand (Glemin & Bataillon, 2009).

- **Uniformity and synchronicity of growth/maturity**: These traits facilitate reduced and single-pass harvest practice, effective timing of fertiliser application and greatly facilitate efficient harvesting (Glemin & Bataillon, 2009).

- **Enhanced culinary biochemistry**: The unique grain biochemistry and altered starch balance of wheat and maize have both been important targets of selection in modern varieties (Gibbon & Larkins 2005; Shewry 2009).

This description of domestication syndrome traits is not exhaustive but can be seen to span both grain and floral traits, and wider aspects of whole-plant morphology and physiology. Any changes that do occur under domestication however will be doing so because of an ultimate impact upon the grain production

or harvest. It is crucial in assessing grain morphology and development not to consider the grain in isolation to the plant but to take into account factors of plant architecture, physiology and ecology, all of which are influenced under domestication and may have profound influence upon the grain.

1.2.3 Wheat Domestication

Wheat is unsurpassed in its success amongst the worlds cereal grains; it has risen to become one of the most productive and nutritionally valuable grasses on the planet, unrivalled in both its range and extent of cultivation and use (Shewry, 2009, Lagudah & Appels, 1992, Davies & Hillman, 1992; Charmet, 2011; Zohary & Hopf, 2001). Bread wheat offers the highest yield potential of almost any grass and the grains of elite genotypes may show between 60% to 80% starch with as much as 17% to 28% protein (Avivi 1978; Avivi et al, 1983; Nevo et al, 1986; Levy & Feldman 1987). A key factor in the success of wheat is the unique gluten content of the flour, allowing it to be processed into a large range of breads, pasta and noodles (Shewry 2009). Alongside the powerful shaping forces of domestication the effects of hybridisation and subsequent polyploidy have also been a critical and defining aspect of wheat's evolutionary history and development (Nevo et al, 2002; Gustafsen et al, 2009).

1.2.4 Polyploidy in Domestication

Despite its recent origins and the reduced genetic diversity associated with the domestication process, over 25,000 different types of wheat can be recognised

(Shewry 2009). This capacity for diversification and adaptability in wheat is generally thought to be attributable to polyploidy (Dubcovsky & Dvorak, 2007). Polyploidisation contributes significantly to overall plant biodiversity and evolutionary history, as many as 47%–70% of flowering plants, are polyploids or are of polyploid origin (Paleopolyploids). Polyploidy has been shown to have particular evolutionary significance amongst the pooid grasses (Soltis & Soltis,2004; Adams & Wendel, 2005; Heslop-Harrison, 2010). The majority of wheat cultivation around the world is comprised of *Triticum aestivum*, an allohexaploid of genomic constitution BBAADD. This species has arisen in agriculture and no wild hexaploid wheat exists (Shewry, 2009; Matsuoka 2011). This hexaploid form arose as a result of a two-stage hybridisation, first between two diploid progenitors to produce a tetraploid and then a second natural hybridisation to produce the hexaploid genome. Figure 1.3 illustrates the hybridisations and genome evolution of polyploid wheat. Wheat evolution has become a textbook example of crop evolution by allopolyploidisation with the inter-specific hybridisations having been well investigated and documented (Murphy 2007; Kihara, 1924, Petersen et al, 2006).

  Triticum uratu is widely accepted as the AA genome donor to hexaploid wheat (Gustafson et al, 2009; Matsuoka 2011). *T. uratu* is a wild species, progenitor to domesticated *T. monococcum*, Einkorn wheat, a relic crop species still cultivated in regions of Europe and Turkey (Shewry, 2009; Gustafsen et al, 2009, Charmet, 2011). The origin of the BB genome has been the subject of considerable debate but it is generally accepted to have originated from a member of the sitopsis section of the *Aegilops* genus and that the closest living ancestor is *Aegilops*
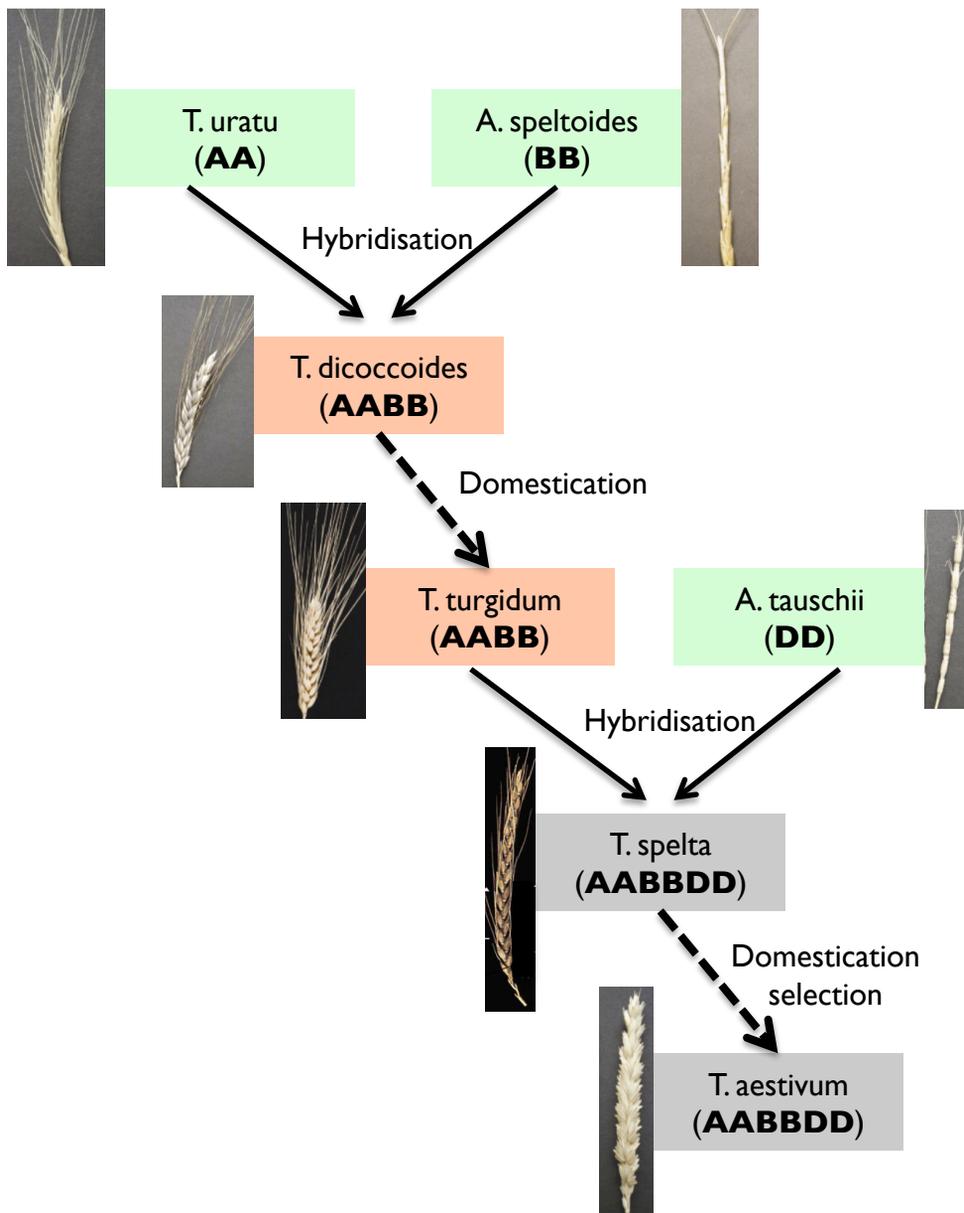
Figure 1.3: Genome Evolution of Polyploid Wheat.
Figure depicts the evolutionary relationships and species contributing to the modern hexaploid wheat genome. Mature spikelet images are shown beside each species name and ploidy is shown. Hybridisation between the two diploid and most likely wild progenitors gave rise to the first tetraploid, *T. dicoccoides*. Under domestication *T. dicoccoides* gave rise to *T. turgidum* or Durum which turn hybridised with a third wild diploid species, *A. tauschii* giving rise to the first hexaploid wheat. Polyploid wheat exists only under cultivation. The figure depicts a common view that hulled *T. spelta* arose as the first hexaploid wheat and via further domestication selection, although it is not clear if *T. spelta* does evolutionarily precede *T. aestivum* (Charmet, 2011). Some spikelet images reproduced from Feuillet et al, 2008

speltoides (Petersen et al, 2006; Cox, 1998; Daud & Gustafson, 1996; Shewry, 2009). A natural hybridisation between *T. uratu* and *A. speltoides* is thought to have occurred early or even before the beginning of the neolithic revolution to produce a wild tetraploid wheat *T. dicoccoides* (AABB) (Gustafsen et al, 2009). Domestication selection give rise to *T. dicoccum* as the first cultivated tetraploid, Emmer wheat, which together with Einkorn represent the earliest cultivated forms, their genetic relationship indicating they both originated from a fertile crescent region of south-east turkey around 10 000 years ago (Nesbitt, 1998, Dubcovsky & Dvorak, 2007; Heun et al, 1997). Tetraploid wheats still form significant crop species; subsequent domestication selection gave rise *T. turgidum* varieties, Durum or hard wheats, varieties of which are cultivated as the source of pasta flour, accounting for about 5% of current wheat cultivation (Shewry, 2009).

The origin of the DD genome has been well studied and it is widely accepted that *Aegilops tauschii* represents the contributor (Petersen et al, 2006; Dvorak et al, 2012). As farming practice spread, a cultivated tetraploid wheat was bought into the range of *A. tauschii* and hybridisation occurred, likely in field margins, giving rise to the hexaploid bread wheat progenitor (Petersen et al, 2006; Shewry, 2009). It is thought that this allopolyploid was formed via a somatic doubling of a triploid hybrid between these two species around 8000-9000 years ago (Huang et al, 2002; Feldman, 2001). It is likely that early farmers, now alert to the benefits of crop cultivation, recognised some superior properties in this hexaploid plant, possibly through heterosis or some other desirable trait, and so selected it for cultivation.

Hexaploid wheats are represented by two species, *T. aestivum* and *T. spelta*, the main difference between the two as a cereal crop being a free threshing characteristic and grain biochemistry. *T. spelta*, or Spelt wheat, is a relatively minor crop cultivated as a livestock feed or speciality crop for the health food market due to lower gluten levels (Stallknecht et al, 1996). It has been the subject of numerous investigations and controversy as to whether the hulled *T. spelta* evolutionarily precedes *T. aestivum,* as generation of synthetic hexaploid lines typically results in the hulled phenotype (Dvorak et al, 2012). Free-threshing or naked grains present a fitness benefit as hulled grains typically show a greater dormancy and can also be seen to produce a higher quality flour (Doebley et al, 2006; Charmet 2011). Recent studies using disomic wheat lines have produced evidence to suggest that spelt was derived from a hybridisation with free-threshing tetraploid progenitor (Dvorak et al, 2012). However, there is no evidence to suggest that naked grains were selected over hulled grains on the basis of yield during early domestication and so it seems dormancy characteristics were decisive in bringing the naked grain phenotype to dominate in bread wheat (Charmet, 2011).

The three genomes comprising that of hexaploid wheat each consist of seven pairs of homologous chromosomes with high genetic similarity that is probably a consequence the diploid donor species having been derived from a monophyletic ancestor (Feldman & Sears, 1981). Chromosome similarity between different genomes is known as homeology and can be seen between members of the Triticeae including *Triticum*, *Aegilops*, *Agropyron*, and *Hordeum* genera (Feldman & Sears, 1981). These similarities offer high levels of genetic compatibility with

great potential for gene transfer and crop improvement through recovery of ancestral traits.

Examining it's history it is possible to see the evolutionary complexity presented by hexaploid wheat. Across this history of hybridisations the domestication process has acted at different stages and to varying extent in the primary, secondary and tertiary gene pools of the modern wheat genome, an understanding of which is crucially important to wheat crop research and improvement (Gustafson et al, 2009; Kellogg et al, 1998). Domestication is neither a defined nor linear process, and in shifting anthropogenic landscapes selection pressures during one stage may be very different and quite contrary to those at another (Murphy 2007). The unique and desirable character of wheat occurs as a function of the complex and multi-layered interplay between the genomes working in concert with broad and changing demands of domestication selection.

1.2.5 The Wheat Grain – Domestication and Post-Harvest Processing

Processing requirement is a major factor in relation to the wheat crop with significant impact upon the end use, economic and nutritive value of the crop (Evers & Millar, 2002). As a consequence of morphology wheat grains must undergo unique and intensive processing to produce a usable and valuable end product. As with all cereal crops, grain must first be threshed free of the seed head after harvest. The importance of variation in this seed-head shattering trait, as opposed to simple loss-of-function mutation, along with complex selection for an optimal seed retention efficiency in relation to threshing energy requirements has

been revealed in recent studies (Zhou et al, 2012). The manner in which grains separate from the seed head is then influential to subsequent processing requirements. The grains of barley, spelt, einkorn and emmer wheat are hulled, i.e. palea and lemma are retained upon threshing and grains must be dehusked prior to milling (Kent & Evers, 1994). *T.aestivum* is one of the few cereals to display a free-threshing phenotype and this trait massively reduces their energy requirement at harvest as naked grains can enter straight into the milling process to produce clean white flours (Kent & Evers, 1994).

 Grains must be milled to separate the bran which includes the amylase-producing outer layer of the endosperm from the starchy central endosperm region that is the flour. The creased morphology of the wheat grain represents an obstacle to this process, necessitating a very physical and energy demanding process with initial "breaking" followed by grinding of the grains to ensure that all of these peripheral layer materials are separated from the valuable flour. These requirements are in contrast to more rounded grains such as rice, where a simple abrasive polishing process is sufficient to separate the aleurone component from that of the central endosperm (Evers & Millar, 2002).

 The free-threshing phenotype and grain biochemistry are significant factors to the success of wheat but both appear to have emerged as post-domestication crop improvements. There is no evidence to suggest that either of these traits played anything more than a minor role during early domestication, and this phase of the process looks to have been driven very largely by wheat's productivity and environmental adaptability (Shewry 2009).

1.2.6 Molecular Genetics of Crop Domestication

Advances in molecular biology over recent years have revolutionized cereal crop research and development, and our understanding of the domestication process at a genetic level. The accumulation of genomic resources providing markers for population and genetic studies comparing crop species and their wild relatives, cloning technologies, genome sequence availability and mapping technologies have allowed the identification of loci and genes important in the plant developmental pathways and morphological changes associated with domestication (Vaughan et al, 2007; Century et al, 2008; Burger et al, 2008). Molecular markers have been instrumental in the analysis of genetic similarities between modern bread wheat progenitors and DNA fingerprinting techniques (Heun et al, 1997) have been used to generate strong evidence linking diploid wheat progenitor populations and tracing them to a specific geographic location, "the cradle of agriculture" in the Karadag mountains. The Golden Rice project also offers a good example of the value of gene introduction in cereal crops producing a "biofortified" grain with significantly increased nutritional quality, addressing the major issue of vitamin deficiencies in rice grain dominated diets (www.goldenrice.org). Molecular genetics is now at the forefront of domestication research, producing a great deal of information and opening many new lines of enquiry and research in areas of crop improvement and evolutionary history, particularly within the cereal crops.

An important feature to have emerged as genes associated with major domestication traits have been identified is the dominance of transcriptional

regulators amongst them (Doebley et al, 2006). Functioning as an evolutionary process, domestication incorporates strong developmental selection and morphological change and it can be expected that transcription factors would be influential. Recent research has highlighted the significant role of transcription factors in adaptive evolution and plant morphological development (Rieseberg & Blackman, 2010; Doebley & Lukens, 1998), and have shown them to be key players in the genetic differences distinguishing wild and cultivated species (Doebley et al, 1997; Li et al., 2006; Cong et al., 2008). In two recent reviews of domestic crop evolution (Glemin & Bataillon 2009; Doebly et al, 2006) transcriptional regulators are specifically identified as having played a central role in domestication. Table 1.1 lists a number of important transcription factors identified, for the most part, in relatively recent studies. Few of these genes are directly influential in grain traits, the majority being involved in floral morphology and development or vegetative characteristics. It is known that grain traits are under strong selection during early domestication, and whilst directly influential developmental genes have not been identified the impact of an altered floral morphology on grain architecture and morphology should not be overlooked.

  Some of the first major domestication genes to be identified were in maize and this species shows the most drastic morphological change between its wild progenitor (*Teosinte*) and domesticated form amongst the cereals. *TEOSINTE BRANCHED1* (*TB1*) and *TEOSINTE GLUME ARCHITECTURE1* (*TGA1*) are two genes identified as highly significant to these changes in plant and floral morphology (Preston et al, 2012; Doebley et al, 1995). *TB1* is a striking example of a transcription factor with a critical role in domestication traits, functioning as a

Major Genes involved in Crop domestication and significant improvements						
Gene name	Species in which identified	TF Family	Function	Mutation Function +/-	Nature of Causative Mutation	Date
DOMESTICATION GENES						
tb1	Zea mays	TCP	Inflorescence structure	gain	regulatory	1999
tga1	Zea mays	SBP	Seed casing	loss	AA substitution & regulatory	2005
Rc	Oryza sativa	HLH	Grain color	loss	Disrupted coding sequence	2006
qSH1	Oryza sativa	homeobox	Grain shattering	loss	regulatory	2006
sh4	Oryza sativa	Myb	Grain shattering	loss	AA substitution	2006
Q	Triticum aestivum	AP2-like	Inflorescence structure	loss/gain	AA substitution & regulatory	2006
vrs1	Hordeum vulgare	homeobox	Two-row to six-row barley	loss	regulatory	2007
PROG1	Oryza sativa	C2-H2-ZF	Growth habit	loss	AA substitution	2008
fas	Solanum lycospersicum	Yabby	Carpel number	gain	regulatory	2008
fw2.2	Solanum lycospersicum	Yabby	Fruit size	loss	regulatory	2008
MAJOR IMPROVEMENT GENES						
opaque-2/blz1	Z mays/Hordeum vulgare	bZIP	storage protein gene activation	loss	regulatory	1989/98
BPBF	Hordeum vulgare	DOF	GA response	--	--	1998
blz2	Hordeum vulgare	bZIP	Activation of storage protein genes	--	--	1999
GAMYB	Hordeum vulgare	Myb	GA response	--	--	2002
ZmMRP-1	Zea mays	Myb	Transfer cell differentiation	--	--	2002
SAD	Hordeum vulgare	DOF	GA response	--	--	2003
DL	Oryza sativa	Yabby	Caryopsis identity	gain	regulatory	2004
ramosa1	Zea mays	C2-H2-ZF	Inflorescence structure	loss	regulatory	2005
ramosa2	Zea mays	Lob	Inflorescence structure	loss	regulatory	2006
FUL	Brassica	MADS-box	Fruit dehiscence	gain	regulatory	2006
HvWRKY38	Hordeum vulgare	WRKY	GA response	--	--	2008
NUD	Hordeum vulgare	ERF	Adhering hull	loss	deletion	2008
GT1	Zea Mays	C2-H2-ZF	Tillering/carpel dev	gain	regulatory	2010
IND	Brassica	homeobox	Fruit dehiscence	loss	regulatory	2010

Table 1.1: Transcription factors involved in cereal domestication and post-domestication improvement

Regulatory genes known to have experienced selection under domestication or to show important function in relation to domesticated crops. In relation to function loss/gain and mutation nature, -- indicates data unavailable. Date indicates the first major publication characterising these genes.

major regulator of apical dominance highly influential to plant morphology through a repressive effect on cell cycle, causing a reduced outgrowth of axillary meristems and branch elongation (Doebley et al, 1997; Century et al, 2008).   In contrast to the highly branched form of the wild progenitor *Teosinte*, maize plants show a single stalk with short branches tipped by ears, these difference being largely controlled by *TB1*. The altered plant architecture and seed head morphology allows for greater productivity and significant harvest advantages (Century et al, 2008).

TGA1 has even more closely felt effects on the grain, and has been found to influence the development of glumes, the hardened cases immediately surrounding the spikelet (Dorweiler & Doebey, 1997). These structures have important implications in seed protection and dispersal; altered morphology would likely have significant influences over grain characteristics (Preston et al, 2012). Furthermore *TGA1* is a member of the SBP-family of transcriptional regulators, members of which have been seen to regulate the expression of other transcription factors including MADS-box genes (Cardon et al, 1999). Some evidence suggests *TGA1* sits at the top of a cascade of transcriptional regulation and in this way was seen to have an influence over a range of traits including cell lignification, organ growth and organ size (Doebley, et al, 1996; Dorweiler and Doebley, 1997).

The wheat *Q* gene is perhaps the single most influential gene in a cereal grain species domestication and is largely responsible for wheat's success as a cereal crop (Simons et al, 2006; Faris et al, 2005). *Q* encodes an AP2-like transcription factor with a profound influence over a suite of important early domestication and agricultural traits including free-threshing characteristics, rachis fragility, spike

architecture, flowering time and plant height (Simons et al, 2006; Zhang et al, 2011). Q is expressed only in polyploid wheat at higher levels then the wild (q) allele and its regulation is complex, homeoalleles being co-regulated such that the versions from the composite genomes all contribute to the final domesticated phenotype (Doebley et al, 2006; Zhang et al, 2011). In addition to differences in gene dosage, the Q allele mutation results in a single amino acid substitution altering both protein dimerization and regulatory properties of the transcription factor and subsequent gene expression (Simons et al, 2006; Faris et al, 2005; Doebly et al, 2006).

A single amino acid substitution has also been identified as highly significant in rice early domestication. Sh4 is a major rice QTL shown to account for 60% of variation in seed-head shattering between Indica and O. nivara (Zhang et al, 2009). Research showed this to be a consequence of a single amino acid substitution in the MYB binding domain of the gene leading to loss of function in abscission zone development. Similarly qSH1 was seen to explain 69% of variation in shattering between Indica and japonica rice cultivars, and occurs as a consequence of a single nucleotide substitution upstream of the homeobox gene that results in altered gene expression and so loss of abscission zone development (Zhang et al, 2009). The traits of greatest significance in establishing domestication largely relate to floral and harvesting characteristics and are often identified as caused by small, single locus mutations in regulatory genes causing altered expression patterns (Century et al, 2008; Kovach et al, 2007). Where traits are influenced by such small mutations the result is a strong selection allowing them to readily and rapidly establish in a population, and it is often assumed that phenotypes under

monogenic and simple control can be more quickly domesticated (Gross & Olsen, 2010).

   Molecular level understanding of the domestication process has also allowed the identification of the associated genetic bottleneck effect and the significantly reduced genetic diversity of our major cereal crops (Doebley et al, 2006). Population bottlenecks have reduced genetic diversity among cultivated cereals with perhaps as little as 10-20% of wild variation being used in modern wheat cultivars as compared to the wild ancestors, this reduced variation represents a significant obstacle to the improvement and adaptability of elite variety germplasm (Langridge et al, 2006; Tanksley & McCouch, 1997). Approaches such as targeted breeding using marker assistance, rehybridization, and GM are now aiming to reintroduce some of this variation, and the identification of ancestral species has allowed for the identification of valuable, compatible sources of the required traits. We have seen that selection pressures in the domestic environment are continual and these approaches represent a new direction and pace for the ongoing domestication process.

   1.2.7 *Grass Ecology*

   Ecology represents an important factor in plant reproductive adaptation. Ecologists working during the seventies proposed a triangular model of primary strategies as framework of plant behaviour in response to environmental factors, specifically conditions of high/low stress, disturbance and competition (Grime, 1977, 1979; Craine 2005). Extremes of three primary strategies evolved to tolerate

these environmental conditions define the points of the triangle; *competitor*, *stress-tolerator* and *ruderal*, with ecological scoring used to classify plant strategy determined by position intermediate to these points (see fig 1.3). Permutations of this triangular model have been widely used to great effect in plant ecology to identify and describe plant strategy, largely replacing the similar but less encompassing r-K continuum system. Whilst plant strategy is largely defined by the characteristics of the environment, this in turn largely defines the plant's habit, and it can be seen that habit is highly influential to grain characteristics.

Disturbance in the context of Grime's triangular model refers to the destruction or removal of vegetation and plants adapted to tolerate this condition are described as ruderals (Grime 1977). The agricultural environment features repeated and severe disturbance and represents perhaps the most extreme form of the ruderal environment, with domesticated species often being those best adapted to tolerate it. Ruderal species direct a large proportion of their photosynthate into high levels of seed production and under conditions of stress favor seed production over vegetation (Grime, 1977).

Typically annual or short perennial in lifecycle, ruderals show the ability to exploit productive environments and seeds often carry the capacity for rapid germination upon favorable conditions. Thus,
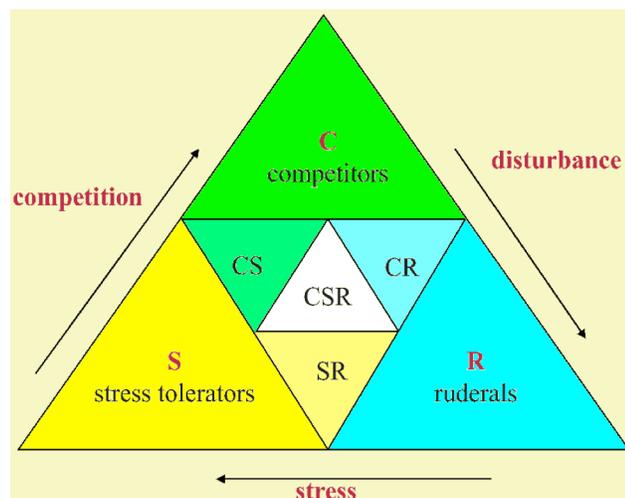
Figure 1.4: Strategy domains within Grime's triangular model (Grime, 1979)

many of the traits that can be seen in ruderal species are those highly desirable and selected for in a domesticated species.   Under domestication the environment is typically manipulated in favor of the crop and so the capacity for environmental stress tolerance has become greatly reduced in many domesticated cereals. Demand to increase crop production along with reduced input farming practices, problems such as pests and reduction to cropping area through salination has meant that this aspect of environmental adaptation has become increasingly significant again; resistant traits are significant targets of crop breeding and improvement programs (Huang et al, 2002). Alongside the dominance of ruderal-strategy traits aspects of the competitor and stress-tolerance strategy are also highly significant in relation to grain biology.

1.3 *Brachypodium* & Cereal Research

1.3.1 *Cereal Research and the Monocot Model*

 Temperate cereal research is often hampered by the genetic size and complexity of these species and has led to such research being dominated by a model systems approach (Mur et al, 2011). Prior to the availability of the *Brachypodium* genome, rice has formed the main monocotyledonous model system in-terms of genomic resources while maize provided an extensive resource of developmental genetics. Whilst research based in rice has revealed much about the developmental biology of the grasses and the molecular basis of the domestication process it does not form an ideal model for temperate grasses. As a semi-aquatic tropical species rice has specialised cultivation requirements and lacks temperate-

agriculture important traits such as vernalisation requirements and frost tolerance (Ozdemir et al, 2008). Furthermore characteristics such floral and grain structure are very different to those of wheat and barley and an alternative model was sought. In 2001 *Brachypodium distachyon* was first proposed as model for the temperate grasses (Draper et al, 2001) and the genome sequence was published in 2010; in just over a decade *B. distachyon* has been sequenced, developed and has quickly risen to become a valuable model system (Vogel et al, 2010; Mur et al, 2011; Vogel & Bragg, 2009).

1.3.2 *Brachypodium Distachyon*

Commonly called purple false brome, *Brachypodium distachyon* is a relatively small, fast-growing, annual grass native to the Middle East and countries around the Mediterranean basin, a range largely overlapping the fertile crescent area and ancestral range of the major small grain cereals (Opanowicz et al, 2008; Salamini et al, 2002). Typical of dry, ephemeral pasture and scrubland habitats *B. distachyon* is a pioneer species and a successful colonizer of disturbed environments (Hubbard, 1954; Schippmann, 1991). Mainly anthropogenic dispersal has allowed *B. distachyon* to expand well beyond its natural range and become naturalised in temperate areas of Australasia, North and South America, South Africa and Asia (Schippmann, 1991; Garvin et al, 2008). Although recognized as an invasive species, *B. distachyon* is relatively insignificant other than as a minor weed of areas of temperate cereal production (Bakker et al, 2009). The *Brachypodium* genus does have some notoriety for the difficulties in establishing the proper placement within the Pooideae however (Catalan et al,

1995: Kellogg 2001). Some of the earliest work on resolving the phylogenetic relationships of the Brachypoideae was initiated by Professor Clive Stace working at Leicester university in the 1980s. Since then studies such as that of Catalan et al (1995), using RFLP analysis and Hsiao et al (1995) using ITS sequencing has placed *Brachypodium* as the sole genus of the tribe Brachypoideae. As a sister to the core pooids and showing all of their apomorphies except that of chromosome size *Brachypodium* is well expected to offer a good physiological and genetic resemblance of these species (Huo et al., 2009). The genus *Brachypodium* has traditionally been seen to encompass around 18 species, both annual and perennial, with a cosmopolitan distribution (Khan & Stace, 1998). In a recent, detailed investigation of the three cytotypes regarded as the single *B. distachyon* species Catalan et al (2012) identified phenotypic and cytogenetic differences allowing their taxonomic separation into three distinct species following chromosome number. The diploid 2n=10 cytotype retains the *B. distachyon* name whilst 2n=20 cytotype was renamed *B. stacei* (in honour C. Stace's early work on the genus). These species represent two different diploid taxa whilst the third new species, the 2n=30 cytotype, was renamed *B. hybridum* and represents an allotetraploid derived from them.

The proposition and selection of *B. distachyon* as a sequencing target and model system reflects the number of advantages it offers, in particular over rice, as an experimental system. Diploid *B. distachyon* offers one of the smallest genomes to be found in the entire grass family, ~272 Mbp along with many other physical and phylogenetic attributes making it attractive as a temperate grass model (Draper et

	Brachypodium distachyon	*Arabidopsis thaliana*	*Triticum aestivum*	*Oryza sativa*
Number of chromosomes	10 (2n)	10 (2n)	42 (2n)	24 (2n)
Genome size (1C)	335 Mb	164 Mb	16 700 Mb	441 Mb
Reproductive strategy	Self fertile	Self fertile	Self fertile	Self-fertile
Life cycle (weeks)	10-18	10-11	12 (Spring Wheat) 40+ (Winter Wheat)	20-30
Height at maturity (m)	0.3	0.2	Up to 1	1.2
Transformation	available	available	possible	available
Growth requirements	simple	simple	simple	specialized

Table 1.2: Advantages of *B. distachyon* as a model system

al, 2001; Garvin, 2008; Huo et al, 2009; Opanowicz et al, 2008; Brkljacic et al, 2011), some which are summarised in table 1.2. *B. distachyon's* small genome favors insertional mutagenesis and efficient positional cloning of genes and being inbreeding facilitates the maintenance of homozygous lines (Vogel & Bragg, 2009). A small stature, rapid life cycle and simple growing conditions are particularly useful features for research, especially where extensive or field-based growth facilities may not be available. Densities of up to 1000 plants/m^2 can be achieved in growth chambers or glasshouses, a space which by comparison may accommodate just 50 wheat plants, or around 36 rice plants (Vogel & Bragg, 2009). Wheat and *Brachypodium* diverged around 32-39 million years ago whilst wheat and rice diverged more than 50 million years ago (Vogel et al., 2006; Mur et al, 2011). *Brachypodium* displays many of the agronomic traits that are of great importance in the temperate cereals, such as freezing tolerance, resistance to

certain pathogens and dormancy mechanisms that cannot be seen in rice (Ozdemir et al, 2008; Peraldi et al, 2011). Significantly, *Brachypodium* offers a more similar general grain structure to that of the temperate species (Ozdemir et al., 2008, Opanowicz et al., 2011) than does rice. Furthermore, the *Brachypodium* genome offers the first example of that of a wild grass, unaltered or shaped by domestication pressures felt by other species such as wheat. It is anticipated that *Brachypodium*, a member of the Pooideae closely related to wheat and from similar ancestral conditions, will offer a great deal of valuable comparison and insight into the domestication process in temperate cereals (Shewry, 2009; Opanowicz et al., 2011).

1.3.3 The Brachypodium Toolkit

In 2006 the US Department of Energy in conjunction with the Joint Genome Institute embarked upon the project to sequence the genome of the *B. distachyon* diploid ecotype Bd21 selected from the collection held by the USDA as the community standard reference line. Much of the initial funding for this sequencing project came from the USDA and centered on the potential of *Brachypodium* as a model for the bioenergy crops (Bevan et al, 2010). In 2010 the complete and annotated *Brachypodium* genome was released (Vogel et al, 2010) and a sophisticated and growing collection of tools and facilities are now available.

With efficient, stable transformation methods having been developed for both Bd21 and other genotypes (Vogel & Hill, 2008; Alves et al, 2009) the production of a growing collection of T-DNA insertion lines is underway through the BrachyTag project with confirmed lines already available to the research community (Thole et

al, 2009). This project is discussed in greater detail in chapter 6. Tilling populations have been generated by INRA in France and by the Boyce Thompson Institute and are being phenotyped. These resources will be available to the research community through a searchable database (http://urgv.evry.inra.fr/UTILLdb; Brkljacic et al, 2011). In addition to stable transformation methods, VIGS (Virus Induced Gene Silencing) techniques have been optimised, paving the way for rapid functional testing of candidate genes (Demircan et al, 2010; Pacak et al, 2010) and the availability of Affymetrix microarrays (NASC) will facilitate gene expression and transcriptomic analyses (Brkljacic et al, 2011). Germplasm collections have increased significantly with a large collection of 195 diploid lines from diverse sites in Turkey and numerous collections from varied environments in Northern Spain amongst which researchers report considerable variation in agronomically important traits such as seed size and set, plant architecture and flowering time (Vogel et al, 2009; Filiz et al., 2009). With the advent of efficient and simple crossing protocols (http://brachypodium.pw.usda.gov) overcoming the difficult cleistogamy of these diploid lines the production of recombinant inbred lines and mapping families can move forward and will add to those already developed (Garvin et al, 2008).

  With these resources *B. distachyon* has already become a demonstrably valuable and successful model for temperate grass research. *Brachypodium* sequence data has proved invaluable in the identification and cloning of agronomically important genes in wheat and barley (Opanowicz et al., 2008) and the syntenic relationship to wheat was instrumental in the characterisation and functional analysis of the PH1 locus (the rice sequence, although available, was too divergent in this area:

Griffiths et al, 2006). High levels of synteny, or gene order, amongst the closely related grass genomes (Bossolini et al, 2007, Huo et al, 2009) has made the model system approach a powerful tool in cereal research. Comparative genomic analyses have revealed evidence of generally good syntenic relationships at the whole genome level between *Brachypodium* and other members of the Pooideae (Hasterok et al, 2006; Huo et al, 2009; Ma et al, 2010) and researchers have identified similar patterns of gene loss or duplication in groups involved in starch synthesis and flowering time (Comparot-Moss & Denyer, 2009; Higgins et al, 2010). In a study of globulin genes and their conservation amongst temperate and tropical grasses (Gu et al, 2010) a duplication in the Triticeae results in the HMW glutenin gene, critical to the unique bread-making properties of wheat, that cannot be seen in any of the tropical grass genomes, but it can be identified in *Brachypodium* (Gu et al, 2010). There are however, some challenges to the significance of the relationship between *Brachypodium* and the Triticeae: genomic rearrangements and duplications occurring in the wheat/barley lineage after the divergence of the Brachypoideae appear to have made alignment at the macro level somewhat fragmentary (Bossolini et al, 2007; Mur et al, 2011; Wicker et al, 2011). Gene order appears to be more highly conserved between rice and *Brachypodium* but at the nucleotide level sequence conservation between *Brachypodium* and members of the Pooideae is generally much higher than can be seen in rice (Bossolini et al, 2007; Mur et al, 2011; Vogel et al, 2006). This sequence level similarity favors *Brachypodium* in functional genomic experimentation and markers created in *Brachypodium* typically have a higher conversion rate to temperate species (Mur et al, 2011). Recent research has

shown *Brachypodium* as an excellent resource for promoters for transgenic research in heterologous cereal species (Coussens et al, 2012). With wheat genome sequencing underway and the Barley genome sequence due for release imminently *Brachypodium* will be valuable in the assembly of these large Pooid genomes (Mur et al, 2011) and as a cross-platform, fast and simple functional genomic model, an alternative to direct research especially relevant to the pooid species.

1.4 GRAIN DEVELOPMENTAL BIOLOGY

1.4.1 Grain Structure: Wheat as a Typical Temperate Grain

The grain, or caryopsis, is the single seeded fruit characteristic of the grasses. It is a composite organ with three genetically distinct compartments; the pericarp and associated maternal tissues, the embryo and the endosperm. Maternal tissues comprises the outer nucellus, surrounding inner and outer integuments and the carpel wall, which develops into the pericarp after fertilisation. The maternal layers, although prominent in developing grains, become largely crushed at maturity and in particular the pericarp, becomes toughened as the grain ripens. These tissues form a watertight layer with a largely protective function during grain development and dispersal (Percival 1921). The diploid embryo and the triploid endosperm are the products of the double fertilisation event where the embryo derives from the egg cell and the endosperm emerges from the large central cell (Nawaschin 1898; Raghavan 2005). The embryo occupies a position at the proximal region of the grain whilst in all cultivated cereals and most grasses, the

prominent and persistent endosperm forms the largest compartment in the grain.

1.4.2 *Endosperm Functional Domains*

Endosperm is the major nutrient store of the grain and is comprised of discrete and differentiated regions functioning in both grain development and germination. These domains are described below and illustrated in figure 1.5 using wheat as an example.

Peripheral aleurone: This layer has critical functional importance in the cereal grain and great significance to the grain's nutritive, processing and end-use properties (Evers & Millar, 2002). Aleurone are typically the only living cells in the mature endosperm and are responsible for the production of hydrolases involved in the mobilisation of central endosperm starch and storage protein reserves upon germination (Berger 1999). In wheat the peripheral aleurone is comprised of a single layer of cuboidal cells, distinct from the thin-walled, irregular and larger cells of central starchy endosperm, extending around the outer edge of the endosperm. The layer is broken only by a cellularly distinct region overlaying the crease in the centre of the grain, termed the modified aleurone (Drea et al 2005b). Aleurone is typically removed in the milling process to separate the amylase producing activity of these cells from the flour (Evers & Millar, 2002).

Modified aleurone: This significant domain is an important feature of the grain's crease region and is known to function in grain filling (Olsen et al, 1992: Wang et al, 1994a). The endosperm has no direct vascular connection to the parent plant and so this region, comprised of transfer cells, works in conjunction with those of the nucellar projection to form a highly efficient transfer route into the developing
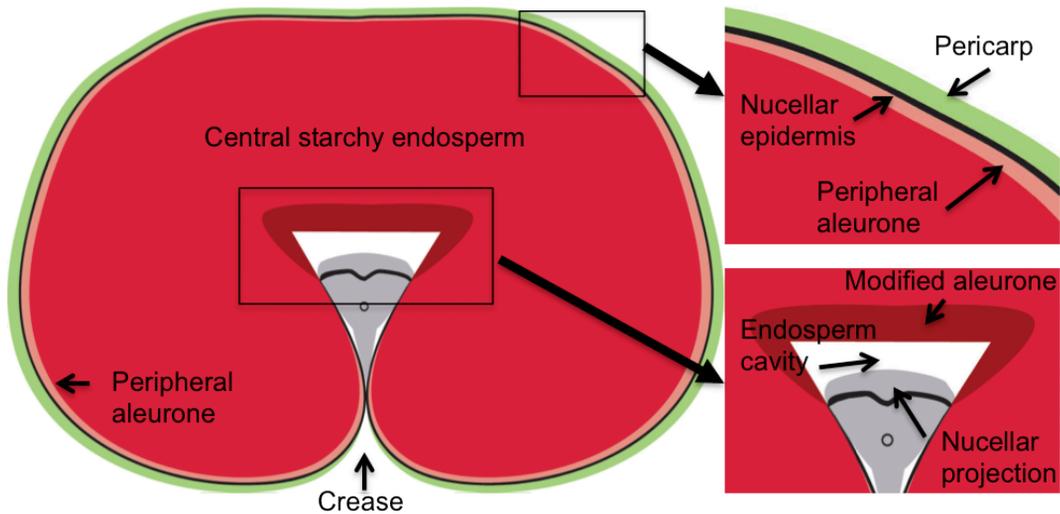
Figure 1.5: Mature Wheat Grain Structure Schematic

  Cartoon illustrates a transverse central section of a mature wheat grain and details the major composite layers. The central starchy endosperm forms the main storage tissues of the grain. The box at the center of the grain highlights the modified aleurone and nucellar projection tissues which function predominantly in grain filling. The box at the edge of the grain highlights the outer maternal layers and peripheral aleurone layer in the endosperm which functions predominately in the germinating grain to mobilize storage reserves,.

endosperm (Bewley & Black, 1994; Wang et al, 1994a,1994b, 1994c). Solutes

move from the phloem into the nucellar projection to be transported to the

endosperm cavity where they diffuse across a mucopolysaccharide filling this

space before uptake into the modified aleurone (Cochrane & Duffus, 1980).   The

transfer cells of the modified aleurone then facilitate a rapid dispersal of these

solutes into the central endosperm to fuel the synthesis of storage compounds

(Bewley & Black, 1994). The value of the modified aleurone in efficient grain filling

appears to be significant as it is a feature of all the developed and commercially

important grains. The modified aleurone is also the interface between maternal

and filial tissues and so, as the point of entry for solutes coming from the parent

plant into the grain, appears also have a significant defensive role against imported

pathogens etc (Drea et al, 2005b).

Starchy endosperm: This dominant compartment of the endosperm forms the

main storage reserve of the cereal grain. Central endosperm cells are responsible

for the manufacture and accumulation of starch and storage protein reserves

during grain development, the composition and level of these compounds having

been significant targets of selection during domestication (Berger 1999). Central

endosperm cells of most major temperate crops are densely packed with starch

granules and protein bodies at maturity, although radial gradients in composition

and level across the endosperm have been seen in some species, and undergo

PCD at maturity (Olsen et al, 1999; Young & Gallie, 1999).

  Wheat's apical position amongst the domesticated cereals makes it

representative of an advanced, in many ways "Ideal" cereal grain with enormous

agricultural importance that has resulted in grain structure, development and

physiology being well described and documented in scientific literature. Wheat offers a typical example of "creased" grain morphology, a feature of all of the domesticated temperate cereals, very different to what is seen in rice or maize. Features of the crease region are fundamental to grain development in the patterning and filling of the grain but have largely negative effects in relation to grain processing requirements (Drea et al, 2005b). In temperate cereals the generally accepted conduit for sugar and amino acid supply to the developing endosperm is through the nucellar projection and modified aleurone (Wang et al., 1994a). Rice shows two pathways involved in assimilate flow to the developing endosperm; one analogous to the nucellar projection pathway of wheat but lacking any transfer-differentiated cells in the endosperm and the other via the nucellar epidermis (Oparka & Gates, 1981; Ellis & Chaffey, 1987; Krishnan & Dayanandan, 2003). Maize, in turn, has a pronounced transfer cell layer within the endosperm i.e., the basal endosperm transfer layer or BETL (Costa et al, 2004; Hueros et al, 1999; Gomez et al, 2009).

1.4.3 Development of the Cereal Endosperm

Cereal grain development occurs as a consequence of a complex and coordinated spatio-temporal pattern of development involving many different processes. Endosperm development has been especially well studied in the temperate cereal crops, particularly in barley, and appears to follow a frequently similar developmental pattern between pooid species (Sabelli & Larkins 2009). Endosperm development can be divided into the 4 main phases, i) a early syncytial stage, ii) a short cellularisation phase, iii) a differentiation and grain filling stage and

finally iv) cell death and drying down. The developmental time frame can be variable according factors such as temperature and variety (Olsen et al, 1992).

 The endosperm originates from a triploid central cell resulting from the fusion of one sperm nuclei with the diploid central cell during fertilisation (Keissel-back, 1949) progressing with a period of endoreduplication within the fertilised central cell to produce a syncytium with nuclei distributed to the edges of the cell, around a large central vacuole (Mares et al, 1975; Raghavan 2005). The cellularisation phase begins with the initiation of cell walls between nuclei at the periphery of the central cell, producing open-ended alveolar like structures facing into the large central vacuole. Further cell wall formation and mitotic division follows giving rise to an initial peripheral layer of cells and a new internal layer of aveoli. Cellularisation progresses with concerted sequence of anticlinal and periclinal cell divisions until the central vacuole is abolished and the endosperm has become fully cellularised (Evers 1970; Olsen 2001; Costa et al, 2004).

 Differentiation of the endosperm subdomains begins soon after cellularisation is complete, most obviously with the peripheral aleurone layer. Cell division becomes restricted to the endosperm periphery and becomes increasingly biased to the anticlinal plane, allowing the surface to keep pace with the increasing endosperm volume (Olsen et al, 1992; Becraft 2007). In maize this transition to anticlinal cell division initiates near the embryo and progresses across the endosperm surface but in the contrasting morphology of the pooid cereals it is unclear if a similar pattern exists (Becraft & Yi 2011; Becraft 2007). The modified aleurone region transfer cells can also be seen to develop soon after endosperm cellularisation has occurred whilst the accumulation of starch and storage proteins occurs in the

central endosperm and reaches a peak during mid-late development. As the grain

approaches maturity and grain filling nears completion the cells of the central

endosperm begin to undergo programmed cell death (Young & Gallie 2000; Sabelli

& Larkins 2009). Peripheral aleurone cells accumulate dehydrins and undergo

processes that allow them to survive desiccation in the mature dry grain, presumed

to be similar to those of the embryo (Olsen et al, 1992) and desiccation marks

grain maturity.

  Aleurone development is a well studied and characterised area of grain

development as (besides its practical significance) it has been identified as a

valuable and simple system in which to study cell fate, specification and

differentiation (Becraft 2007). Aleurone cells have just a single fate choice

between starchy endosperm and aleurone identity and are often easy to isolate

and culture in vitro. Aleurone cell maturation and typical characteristics (cuboidal,

thick-walled and vacuolated) are similar to epidermal cells and it is generally

considered appropriate to view the aleurone as directly analogous to the epidermal

layer; many of the genes identified as affecting aleurone development also have

effects in epidermal development (Becraft & Yi, 2011). Studies involving in-vitro

endosperm cell culture, aleurone specific reporter gene constructs and maize

genotypes accumulating anthocynains in the aleurone layer, have shown positional

signalling to be crucial in peripheral aleurone cell specification (Gruis et al 2007,

2006). Compelling evidence is seen in studies of isolated maize endosperm cells

cultured in-vitro developing and maintaining a peripheral aleurone layer (but

notably not transfer cells) whilst largely lacking in other histological organisation.

aleurone cells are also seen to develop around occasional voids occurring inside

the endosperm culture (Gruis et al, 2006). Despite a relative abundance of research into aleurone development, relatively few specific markers and developmental regulators have been identified.

Grain development outside the key cultivated crop species is poorly characterised as compared to other plant organs and few regulatory genes have been identified (Sabelli & Larkins 2009). A great deal of work has examined the evolution and development of floral organ identity and inflorescence architecture in analyses across the cereal phylogeny, extending to both uncultivated and wild relatives (Doebley et al, 1997; Vollbrecht et al, 2005; Whipple et al, 2007). However, less is known about the evolution of grain form and function, particularly with regard to its tissue organisation and the ability of the endosperm to store rich reserves.

1.4.4 Grain Storage Reserves

The major function of the cereal endosperm is to provide a storage reserve and nutritional supply to the germinating seedling. This reserve may be as several different forms in the grain.

Starch: the dominant endosperm storage reserve amongst the domesticated cereals with grains of elite wheat varieties comprises as much as 70-80% starch at maturity (Gustafsen et al, 2009). Starch degradation provides a source of carbohydrate to the developing seedling upon germination and this rich reserve is also highly valuable to human nutrition, being the primary reason for which cereals were domesticated. Starch synthesis in plants has been well investigated and documented (for review see Smith, 1999; Emes et al, 2003; James et al, 2003;

Tomlinson & Denyer, 2003; Morell & Myers, 2005). Cereal starch production and accumulation occurs predominantly in the central endosperm, although transitory starch production is seen in the maternal pericarp, embryo and aleurone layers during grain development (Radchuck et al, 2009). Central endosperm starch reserves occur in amyloplastic starch granules of varying size shape and composition that is often characteristic for individual species or families e.g. rice and oat have a compound granule morphology, Triticeae species are rounded and maize are angular (Shapter et al, 2008; Preiss, 2004; Bewley & Black, 1994). Starch granules are comprised of linear and branched glucose polymers, amylose and amylopectin respectively of varying levels with amylopectin usually found in the highest concentration (Preiss, 2004; Shapter et al, 2008). Starch granule morphology has significant impacts upon its physiochemical properties and size is influential to potential use in food and industrial applications (Da Silva et al, 1997; Shapter et al, 2008).

Storage proteins: Whilst starch forms the dominant carbohydrate storage reserve of the cereal grain storage proteins also form an important nutritional reserve and source of amino acids (Gustafsen et al, 2009; Shewry & Halford 2002). The dominance of cereal grains in the human diet has made them the major source of protein, with composition an important and distinctive feature of different species influencing nutritional value and processing properties (Shewry & Halford 2002; Gu et al, 2010). Various different classes of seed storage protein exist with prolamins and globulins being the dominant classes amongst the cultivated Pooideae species (Larre et al 2010; Shewry & Halford 2002).

The prolamins are a highly significant class specific to the grasses, the dominant storage protein fraction in Triticeae and maize (zeins), their occurrence is restricted to the starchy endosperm region of the grain (Larre et al, 2010). Prolamins comprise the important gluten fraction of wheat flour and can be divided into two distinct polymeric and monomeric fractions, referred to as the glutenins and gliadins respectively (Tosi et al, 2009).

Globulins are found across the flowering plants and are typically embryo storage proteins but in cereal grains they occur in both embryo and endosperm (Shewry & Halford 2002; Bewley & Black, 1994). They can be divided into two major classes distinguished by molecular mass, the 7s and 11-12s globulins, s-value referring to the sedimentation coefficient, with most, if not all, cereals containing both of classes to greater or lesser extent (Bewley & Black, 1994). The 11-12s globulins are related to legumin proteins found in most dicot seeds and form the dominant storage protein class in oat and rice, where they represent a good source of dietary protein that is not deficient in any specific amino acids as, is the case with prolamins (Gibbons & Larkins 2005; Burgess & Miflin 1984). 7s globulins have been identified in all major cereal grains where they are laid down through mid to late grain development and are almost entirely restricted to the embryo and aleurone layers, making these tissue quite protein rich (Shewry & Halford 2002). These tissues being all that remain alive in a mature grain may also suggest they have some germination associated function. 7s globulin function appears to be solely in storage and is not crucial, as a null mutant in maize shows normal grain development and germination (although globulins are not the dominant storage protein in maize) (Kriz and Wallace, 1991).

Cell walls: For some species cell wall components, predominantly in the form of hemicelluloses, can also form an important fraction of seed carbohydrate storage reserves (Bewley & Black, 1994).

1.5 Genes regulating Temperate Grain Development

The specification of organs and of the domains within them is a key process in plant development defined by patterns of spatial and temporal gene expression. The significance of transcription factors in altering plant morphology and habit during domestication has been described earlier in this section as evidence of the powerful evolutionary implications of transcriptional regulation (Century et al, 2008: Riechmann & Ratcliffe, 2000; Zhang, 2003; Qu and Zhu, 2006).

1.5.1 MADS-box genes

These are perhaps the most well known and studied group of transcriptional regulators playing critical roles in diverse developmental processes including floral organogenesis and fruit development (Lopez-Dee et al, 1999, Zhao et al, 2006, Yamaguchi & Hirano, 2006; Shore & Sharrocks, 1995). MADS-box genes have been identified in plants, animals and fungi and are divided into two major lineages, type I and type II, or MIKC-type (Hileman et al, 2006). The MADS domain in both lineages is a highly conserved 58-60 amino acid region almost always found on or close to the N-terminus forming an alpha helical DNA binding domain responsible for nuclear localisation, recognition and protein dimerization (Hileman et al, 2006; Shore & Sharrocks, 1995). The MIKC-type genes are the most well studied in the plants and posses 3 additional functional domains in addition to the MADS (M)

domain: the weakly conserved intervening domain (I), the well conserved keratin-like coiled-coil domain (K) and the variable C-terminal domain (C) involved in transcriptional activation
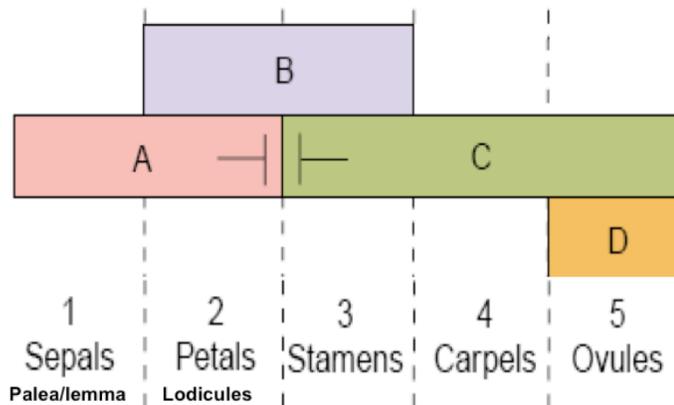
Figure 1.6: Floral ABCD model (Adapted from Ferrario et al, 2004).

(Zhao et al, 2006). Protein-protein interactions are mediated largely by the I and K domains and the formation of higher order protein complexes is an important part of MADS-box gene function (Honma & Goto 2001; Hileman et al 2006; Favaro et al, 2003). Phylogenetic analyses have identified 13 subfamilies amongst the eudicots of related function and expression pattern with 11 of these families identifiable amongst monocots (Becker & Theissen, 2003: De Bodt et al, 2003; Lee et al, 2003). The MADS-box genes are a functionally mobile and diverse group; studies have found a number of gene duplications and losses between species such as tomato and *Arabidopsis* and orthologous genes often do not share analogous function, whilst paralogs may adopt similar roles (Hileman et al, 2006: Zachgo et al. 1997; Davies et al. 1999; Kramer et al. 2004; Causier et al. 2005).

1.5.1i The "ABC" Model And Floral Organ Development

This model was first proposed in 1991 by Coen and Meyerowitz based upon studies of two floral homeotic mutants of the model eudicots, Arabidopsis and *Antirrhinum majus* (Snapdragon). This model describes how the combinatorial

action of 3 (MADS-box) gene classes, A-, B-, and C- across the 4 floral organ

whorls (Sepal, petal, stamen, carpel) acts to regulate floral development, as shown

in figure 1.5. A-class genes alone specify sepals in the outer floral whorl; A- and B-

class gene expression in the second whorl together specify petal development; In

the third whorl expression of B- and C-class genes specifies stamen development

whilst C-class genes specify carpels in the inner whorl.   Whilst devised upon the

action of these three gene classes the model has been extended to include D and

E classes with ovule-specification and wider cofactor function respectively

(Theissen, 2001). Ovules have come to be regarded as separate floral organs and

D-class mutants show disrupted ovule development and conversion to carpelloid

organs (Ferrario et al, 2004). E-class (or SEP) gene function was added to the

model in 2000, this gene class showing a somewhat divergent expression pattern

spanning the four innermost whorls with function as cofactors for organ identity and

in floral meristem determinacy, seen in both monocots and dicots (Ferrario et al,

2004: Rounsley et al, 1995; Pelaz et al, 2000; Honma & Goto, 2001; Pelaz et al,

2001; Favaro et al., 2003; Vandenbussche et al., 2003b).

1.5.1ii MADS-Box Genes in Fruit and Seed Development

A-class genes are typified by Arabidopsis *APETALA* (*AP1*). Phylogenetic

reconstructions have found AP1 to group with *CAULIFLOWER* (*CAL*) and

FRUITFUL (*FUL*) and are members of *the AP1/FUL*-like MIKC gene subfamily

(Preston & Kellogg, 2007: Kater et al, 2006). In *Arabidopsis* AP1 functions in sepal

and petal identity whilst *FUL* has a role in valve identity and seed-head shattering

mechanisms. *FUL* overexpression in *Brassica* was sufficient to abolish pod shatter (Ostergaard et al, 2005) and along with *AP1* also functions in floral meristem identity (Kater et al, 2006). Grasses seem only to posses the *FUL*-like genes although a whole genome duplication at the base of the monocot lineage has given rise to two distinct clades (Litt & Irish, 2003; Preston & Kellogg, 2007). Investigating *FUL* function across a range of species Preston and Kellogg (2007) found *FUL1* to be more typical of E-class genes, involved in organ identity across all spikelet organs whilst *FUL2* is more typically A-class function, often involved in the two outer whorl floral organ identities.

  C-class gene function has been extensively studied (Kramer et al, 2004) and *AGAMOUS* (*AG*), the primary C-class gene in *Arabidopsis*, has a critical role in stamen and carpel specification, with additional function in floral meristem determinacy and repressing A-class gene function (Gustafson-Brown et al, 1994). *Arabidopsis AG* mutants show conversion of stamens to petals and reiteration of new *ag* flowers in place of carpels in the centre of the flower. *AG* homologs in both eudicots and monocots show some conservation of function, duplications have introduced some variation and genes have diversified to become involved in various aspects of flower and fruit development in eudicots (Yamaguchi et al, 2006; Kramer et al, 2004).

  The dicot *AG* subfamily was split during early angiosperm evolution into two lineages: *AG*-like and *AGL11*-like, showing more C- or D-class like function respectively. In *Arabidopsis* the family is comprised of four members; AG, *SHATTERPROOF1* (*SHP1*), *SHP2*, the C-lineage genes, and *SEEDSTICK* (*STK*) representing the D-lineage (Pinyopich et al, 2003). Function of these genes is often

variable and overlapping amongst the dicots (Heijmans et al, 2012). The *SHP*
genes function with AG in the specification of carpel identity, along with
specification of the carpel dehiscence zone from which they get their name
(Pinyopich et al, 2003; Liljegren et al, 2000). *STK* (previously *AGL11*) functions in
funiculus growth and seed abcission but was also found to be involved in
specifying ovule identity (Pinyopich et al, 2003). Following the proposal of the ABC
model it has been determined that ovules can be considered as separate and
distinct floral organs, rather more similar to a meristematic axis than simply
modified leaflets (Matthews & Kramer, 2012). In line with the ABC nomenclature,
ovule-specifying genes are termed D-class. D-class gene function and regulation
in the dicots appears quite variable and often redundant to that of closely related
C-lineage genes (Heijmans et al, 2012). In *Arabidopsis* this D-class function was
partially shared by the *SHP* genes and *shp1 shp2 stk* triple mutants showed
disrupted seed and ovule development, with ovules converted into leaf-like or
carpelloid organs (Ferrario et al, 2004; Pinyopich et al, 2003). In *Petunia* two
distinct D-lineage genes are identified, *FBP7* and *FBP11*, both show ovule specific
expression and have been shown to confer ovule identity redundantly with other
AG family members, particularly *PETUNIA MADS BOX GENE3*, a member of the
C-lineage (Heijmans et al, 2012). Ectopic expression leads to ovule formation on
sepals and petals suggesting that their expression alone can be sufficient to
specify ovule identity (Columbo et al, 1995).

 Investigations focused on the C- and D-class genes in the monocots have
identified four *AG*-lineage genes in rice designated *MADS58*, *MADS3, MADS21*
and *MADS13* (Kang et al, 1995; Lopez-dee 1999; Lee et al, 2003). Despite some

doubts, most studies place *OsMADS58* and *OsMADS3* into the *AG* lineage (C-class) and *OsMADS13* and *OSMADS21* into the *AG11*-like lineage (D-class). *OsMADS58* and *OsMADS3* are considered to be paralogous and initial functional analyses conclude that subfunctionalisation had occurred between them (Yamaguchi et al, 2006). An initial investigation suggests *MADS3* plays a dominant role in stamen and lodicule specification, whilst *MADS58* was effective in carpel development and floral meristem determinacy, with a reduced role in stamen development (Yamaguchi et al 2006). A subsequent investigation of these gene's function has produced some contrasting findings to suggest that rice C-class genes show greater conservation of function to that of the dicots, and are discussed in more detail in relation to results shown in chapter 6 (Dreni et al, 2011). Studies have also revealed *DROOPING LEAF*, a YABBY transcription factor as having a critical role in the specification of carpel identity in rice (Yamaguchi et al, 2004); this gene's function is discussed in more detail shortly.

OsMADS13 and *OsMADS21* are confirmed as STK or D-lineage genes in rice (Kramer et al, 2004), both showing a high degree of similarity to those of eudicots (Yamaguchi & Hirano, 2006). *OsMADS13* plays a critical role in ovule identity (Dreni et al, 2007, Lopez-Dee et al, 1999), knockout resulting in a completely female sterile phenotype with ovules converted into reiterated ectopic carpelloid structures. This carpel-inside-carpel phenotype indicates it is also playing a role in floral meristem determinacy along with *MADS3* and *MADS58* (Dreni et al, 2011). *OsMADS21* expression was found to be lower and function is not obvious; silencing shows no aberrant phenotype individually or in combination with *OsMADS13* (Lee et al, 2003: Dreni et al, 2007).

Whilst the ABCD class genes are necessary for floral organ specification their loss is insufficient to convert floral organs to leaves and ectopic expression of B- and C- class genes does not result in organ formation (Zahn et al, 2005); an observation that lead to the identification of the E-class or *SEPELLATA* (*SEP*) genes as critical cofactors of floral organ identity genes. Five *SEP* genes have been identified in rice and of these *LEAFY HULL STERILE* (*LHS1*) is one of the few whose function has been well characterised, the mutant showing leafy palea, lemma and lodicules, along with disruption to stamen and pistil number (Jeon et al, 2000; Christiansen & Malcomber 2012). *SEP* genes have also been implicated in floral meristem determinacy but high levels of redundancy amongst this group have made functional investigation difficult (Gao et al, 2010; Uimari et al, 2004). The role, if any, of these genes in later floral and grain development has not been investigated but their prominence and association with earlier development suggests they may have some function.

1.5.2 YABBY Genes

YABBY genes belong to a small, plant specific family of transcription factors notable for their role in adaxial/abaxial developmental patterning and floral organ specification (Floyd & Bowman, 2007; Vollbrecht et al, 2000). Genes of this family are characterised by a zinc finger domain close to the N-terminus and a helix-loop-helix domain towards the C-terminus (The YABBY domain) similar to that of High Mobility Group proteins found in all eukaryotes (Bowman, 2000). YABBY genes were first identified in Arabidopsis with *CRABS CLAW* (*CRC*), found to be involved

carpel and nectary development (Bowman & Smyth, 1999). *CRC* acts immediately

downstream of *AG* to promote carpel identity and is distinct amongst the YABBY

family in having a role restricted to carpel development (Gomez-Mena et al, 2005;

Matthews & Kramer 2012). *DROOPING LEAF* (*DL*) is the rice orthologue of

AtCRC, and has a critical role in specifying carpel identity, mutants showing

complete homeotic transformation of carpels into stamens. *DL* also has critical

function in specification of the leaf mid-rib, from which it gets its name (Yamaguchi

et al., 2004). *OsDL* is expressed in carpel primordia and developing carpels but its

expression pattern in these organs is non-polar in contrast to both Arabidopsis and

to maize orthologues where expression is localized to abaxial or adaxial domains.

The expression of *OsDL* is independent of *MADS58* or *MADS3* and normal

expression levels are seen in *MADS58* and 3 RNAi plants (Dreni et al, 2012).

Yamaguchi et al (2004) concludes that there is conservation of function between

CRC and *DL*, but that *DL* has acquired additional function during grass evolution.

Studies of the *Arabidopsis* YABBY gene family have catergorized six genes

according to sequence whilst Toriba et al (2007) identifies 8 members of the

YABBY gene family in rice (Bowman 2000). Duplications have occurred but the

high levels of conservation of this gene family across the angiosperm phylogeny

argues for some critical function and their divergent evolution may underlie some of

the difference between monocot and dicot leaf and flower form (Eckardt, 2004).

FILAMENTOUS FLOWER (*FIL*) was identified in *Arabidopsis* as a gene with

major effects in floral morphology, mutants often producing flowers comprised of

just a filament and sepal-like structure (Komaki et al, 1988; Sawa et al, 1999). Two

further *FIL*-like genes can be identified in *Arabidopsis* (*YABBY2* & *YABBY3*) these

genes showing differing expression levels but are all restricted to the abaxial region of organs and organ primordia (Jang et al, 2004). Rice and maize orthologues of *FIL*-group genes have been identified but have been found to differ in their expression pattern and function as compared to dicots (Bowman 2000; Dai et al, 2007; Jang et al, 2004). Rice *YAB3*, orthologous to *AtYAB3*, has been seen to function in a leaf development regulatory network involving *WOX3* and *KNOX* genes and also in floral meristem, stamen and carpel development (Jang et al, 2004: Dai et al, 2007). Although detailed functional analysis of all monocot *FIL*-like genes is yet to be completed it is clear these genes have important function in meristem behaviour but show significant diversification (Juarez et al, 2004; Dai et al, 2007; Jang et al, 2004). In *Arabidopsis* the primary function of these genes is in organ polarity, a function that is shared but reversed in maize whilst in rice this is a function in which they appear not to be involved.

The applied significance of YABBY gene function in relation to fruit development and domestication has been shown in studies such as that of Cong et al (2008). Investigations of the evolution of fruit size in tomatoes it was found that an increase in locule (compartments) number of tomato fruit, and subsequent increase in fruit size, was the result of up-regulation in a transcription factor bearing the YABBY domain. Morphological characteristics of profound agricultural importance may be determined by the activity of these genes and the importance of YABBYs in a broad range of fundamental plant developmental processes is now becoming clear (Kumaran et al, 2002; Tanaka et al, 2012).

1.5.3 Other Relevent Gene Families

Smaller but equally significant classes of transcriptional regulators have great significance in seed development often showing grain, and sometimes endosperm, specific expression, placing them into a minority class of genes that function importantly and solely in grain development. Several specific and highly conserved motifs in gene promoters are shown to be important in endosperm specific gene expression (Onate et al, 1999: Albani et al, 1997; Rubio-Samoza et al, 2006a). The bipartite "endosperm box" region is the most prominent of these sequences, located around 300bp upstream of the transcription activation site is a conserved cis-acting motif found in the promoters of most seed storage proteins (Onate et al, 1999). The endosperm box is comprised of two protein-binding motifs, a GCN-4 like motif (GLM; 5'-ATGAG/CTCAT) and a prolamin box (PB: 5'-TGTAAAG) that either together or individually function to define endosperm specific expression (Schmidt, et al, 1992: Albani et al, 1997; Zhao et al, 1994: Rubio-Samoza et al, 2006b). The components of the endosperm box were first identified in promoters of maize storage protein genes, α-zeins, and it is in this species that their role was first extensively studied (Gibbon & Larkins, 2005). The components of the endosperm box have been identified in many different grass species and have been found to be well conserved amongst the Pooideae, particularly in the promoters of prolamins in the domesticates studied to date (Onate et al, 1999).

1.5.3i bZIPS

These transcriptional regulators activate endosperm specific gene expression through recognition of the endosperm box. bZIP proteins contain a basic and a

leucine repeat region, bZIP binding motifs, that are involved in DNA binding and protein interacting capabilities (Vicente Carbajosa et al, 1998). The bZIP family of transcription factors are typified by maize *OPAQUE2* (*O2*) and its wheat orthologue *SPA*, the family having a highly conserved intron/exon structure and nuclear localisation signal (Varagona et al, 1992: Vicente Carbajosa et al, 1998). Much of what is known about cereal grain storage protein expression regulation stems from work on *ZmO2*, which has been found to exert a profound influence over grain nutritional quality and processing characteristics. Specifically, *O2* functions as a transcriptional activator of storage protein synthesis genes, in particular the 22-kD a-zeins, a member of the prolamins and the major storage protein class of the maize endosperm (Schmidt, 1993: Kodrzycki et al., 1989; Schmidt et al, 1990, 1992). *O2*-mutants show significantly reduced levels of zein proteins producing a softer, opaque endosperm as compared to a harder and more vitreous wild-type endosperm and increased non-zein protein classes with higher lysine content and increased nutritional quality of flour (Wang & Larkins, 2001; Schmidt et al, 1992). It is well established that the zein rich maize endosperm offers a nutritionally unbalanced source of dietary protein being lacking in essential amino acids including lyseine and tryptophan. The importance of the O2 mutation was recognized with mutants showing nearly double the content of lyseine and tryptophan as compared to normal maize but was concomitant to agronomically inferior traits of soft endosperm, brittleness and insect susceptibility (Gibbons & Larkins, 2005). Quality Protein Maize (QPM) refers to maize genotypes developed through an extensive and ongoing breeding and research program aimed at incorporating the desirable low zein content of the O2 mutation with improved

agronomic traits.   QPM was recognized as having great significance in reducing malnutrition in developing countries where maize forms the dominant component of the human diet, in particular sub-saharan Africa (Sofi et al, 2009) and the use of new approaches for the continued improvement of QPM varieties remains an important research target (Gibbons & Larkins, 2005)

1.5.3.ii MYB Genes

MYB domain proteins are found throughout the metazoans typically containing 3 repeats (R1, R2 and R3) of the ~52 amino acid DNA binding domain, but significant members with both one and three repeats have been reported (Gomez et al, 2002; Martin & Paz-Ares, 1997; Jin & Martin, 1999; Kranz et al, 2000).   The single repeat class of MYB (R1MYB) proteins appears to have particular significance in relation to endosperm development and aleurone function. Their DNA recognition domain is atypical to that of the R2-R3 and R1-R2-R3 members and may bind DNA in a manner more similar to homeodomain proteins (Gomez et al, 2002; Mercy et al, 2003; Rubio-Samoza et al, 2006b). *ZmMRP-1* is a well characterised member of this class known to function in the specification of the maize Basal Endosperm Transfer Layer (BETL). The BETL is a distinctly differentiated region of transfer cells at the chalazal end of the maize kernel and appears to be functionally homologous to the modified aleurone layer in the wheat grain (Hueros et al, 1999). *MCB1* encodes another R1 MYB of the SHAQKYF sub-family in Barley functioning as a transcriptional repressor of amylase synthesis genes induced by GA in germinating aleurone (Rubio-Samoza et al, 2006b). *HvMYBS3* is another member of the same family with a similar expression pattern

and transcriptional repression function (Mercy et al, 2003; Rubio-Samoza et al, 2006a). The R1MYBs have significant roles in seed regulatory networks and function in this area is heavily influenced by interactions between members of the MYB protein classes.

  Transcription factors are key players in regulating grain development and morphological differences distinguishing wild and cultivated cereal species. This description provides background to some of the major genetic regulatory factors involved in temperate cereal grain development alongside which non-regulatory genes of critical function have also been isolated. However, relative to the complexity and involvement of the process comparatively few specific determinants of grain and endosperm development and differentiation have been identified (Sabelli & Larkins, 2009).

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Suppliers

Materials and chemicals were obtained from various suppliers, mainly Sigma-Aldrich, Melford, Thermo Fisher Scientific, BioGene, Promega, Duchefa Biochemie and Roche. DNA and RNA isolation and purification kits were purchased from Qiagen, Bioline and Sigma-Aldrich. Enzymes and other reagents were obtained from Invitrogen, New England Biolabs, and Bioline.

2.2 Seed Material

Seed material for the majority of the wild species was obtained through a specialist commercial seed supplier (www.Herbiseed.com). For the cultivated and some wild crop species seeds were obtained through the John Innes Centre germplasm resources centre. Seeds for *B. hybridum* and *B. stacei* were kindly supplied by the Doonan Laboratory (JIC). Initial seed stocks were increased through Leicester-grown seed. In all cases, upon maturity of the first flush of flowers watering was stopped and plants allowed to dry before seed was collected.

2.3 Seed Germination and Plant Growth

Typical growing conditions for all *Brachypodium*, wheat, barley and oat species comprised a 2 day vernalisation at 4ºC in darkness before germination on moist filter paper in petri dishes. Vernalisation was sometimes extended according to individual species' requirements. Germination was performed under daylight conditions at approx. 21ºC where day length exceeded 12 hours, otherwise under fluorescent light with 16 hour photoperiod. Seedlings were transplanted to compost at between 5-10 days after germination, dependent upon vigour. For

Brachypodium a free draining compost mix of 2:2:1 compost/grit/perlite/sharp sand was used. For all other species a 3:1:1 compost/perlite/sand was used. Plant material examined in chapters 1, 2 and 3 was grown under greenhouse conditions from transplanting, with supplemental heating and lighting, to give a 18 hour photoperiod. For mutant analyses and tissue culture detailed in chapter four, germination was performed as above but plants were largely grown under Controlled Environment Room (CER) conditions, as described by Alves et al (2009) and detailed separately within the chapter.

2.3.1 Brachypodium Grain Staging

Developing *Brachypodium* grains were staged using anthesis as a reference point, identified by visual observation of anther position and dehiscence through the transparent palea using a dissecting microscope. Developing grains were all collected from the lowest 6 florets of plant primary flush spikelets at various time-points correlating to DAA (Days After Anthesis).

2.4 Whole Grain Measurement

Measurements of grain dimensions were collected from dry, mature and dehulled grains (with the exception of the barley species where it was not practical to remove the hull) using Clarke CM145 digital vernier callipers on 20 grain samples. Grain mass was recorded using a Kern EW420-3NM electronic balance on a 50 grain sample.   Error amongst grain mass was calculated from ten x 5 grain samples.

2.5 Cytological Analyses

2.5.1 Light Microscopy

External and Macro-morphological analysis of grain features was performed using a Motic SMZ-168 dissecting microscope equipped with a Canon EOS 1000D digital camera. Images were collected using EOS utility software 2.4.0.1 and Canon Digital Photo professional.

Bright and darkfield microscopy for observation and imaging of grain stained sections was performed using a GX optical L3200 compound microscope equipped with a GT-vision GXCAM-5 5MP digital USB camera and GXCAPTURE software. Image analysis and measurement was performed using image-J software.

2.5.2 Mature Grain Profile Observations

Morphology observations and images for mature grain profiles were taken from dry grains cut transversely at the central point and observed using light microscopy as detailed above. Very brittle, fragile and powdery grains were cut within a drop of 80% glycerol to reduce breakage. Sections prepared in this way were covered with a cover slip to reduce glare and imaged immediately to ensure features were not distorted by moisture uptake.

2.5.3 Vital Staining

For Tetrazolium chloride (TZ) staining thin sections were made by hand of mature grains imbibed in distilled water overnight. Sections were taken as thinly as possible from the central point of the grain from at least three biological replicates. Freshly cut sections were immersed in 1ml of 0.5% TZ solution and incubated at 35°C for 3-6 hours according to intensity of staining. TZ solution was prepared

fresh and pH tested before use to ensure it fell between 6.5-7 (optimal staining requires a close to neutral pH). Stained sections were mounted in 80% glycerol and photographed immediately using dissecting and compound microscopes.

Evans Blue vital staining was performed as described previously (Young and Gallie, 1999) with minor modification. Briefly, sections were prepared as above. Freshly cut sections were immersed in a 0.1% Evans Blue solution for 4 minutes and then washed in several changes of distilled water for 3 hours with gentle agitation. Sections were immediately mounted in distilled water and photographed as above.

2.5.4 Starch Staining

Longitudinal and serial transverse sections from along the whole grain length were taken as described for vital staining. Sections were immersed in a 50% Lugol solution (Sigma) for 1 minute, washed in several changes of distilled water, mounted in 80% glycerol and immediately photographed as above.

2.5.5 Toluidine Blue Staining

14 µm thick transverse central grain or other tissue sections on glass slides, fixed and sectioned as described for mRNA in-situ hybridisation, were cleared of wax in 2 x 20 minute histo-clear II (National diagnostics) baths with agitation before rehydration through 100, 70 and 50% ethanol series (10 minutes each). Slides were immersed for 1 minute in 0.05% Toluidine blue in 0.1M phosphate buffer, pH6.8, and then rinsed through several changes of deionised water. Slides were then allowed to air dry and permanently mounted in Entellan (Merck, Rahway, NJ).

2.5.6 Calcofluor & DAPI Staining

Sections prepared as for toluidine blue staining were stained with DAPI solution (Partec) for 20 minutes followed by calcofluor (0.2%) for 10 minutes and glycerol mounted. Sections were viewed using a Nikon E800 fluorescence microscope equipped with digital image capture.

2.5.7 Scanning Electron Microscopy

Mature dry grains were imbibed overnight in distilled water.   Imbibed grains were trimmed at the distal end to facilitate penetration of fixative into the tissue and transferred to freshly prepared F.A.A fixative (Formaldehyde 3.7%, Acetic acid 5%, ethanol 50%) Grains were exposed to 3 cycles of moderate vacuum (~500 mbar) to ensure penetration of fixative and fixed overnight at 4°C with agitation. Fixed grains were transferred to 70% EtOH. Samples were dehydrated through a series of 80, 90 100% ethanol with 12-24 hours in each before critical point drying in a Bal-Tec 030 Critical Point Drier using CO_2 and following manufactures instructions. Samples were coated in gold using a Polaron SC7640 Sputter Coater for 90 seconds at ~2.0 kV. Samples were analyzed on a Hitachi S3000H scanning electron microscope equipped with digital image capture.

2.5.8 Cell Size & Wall Measurements

All measurements were recorded using Image-j software on LM or SEM images from previously detailed analyses. For cell size measurements the cell longest axis was recorded and cells were identified along a horizontal transect representing the central point of the endosperm in transverse sections, n=10 across at least 3 biological replicates. Cellular differentiation of the MA region for

chapter 1 table 3 was determined buy visually distinct differences in cell size and shape, tissue structure.   Cell wall measurements were recorded from SEM images and great care was taken to identify areas where a 90 degree section of a single thickness cell wall was visible.

2.6 Statistical Analysis

Principle component analysis was performed using the multibase excel plugin (http://www.numericaldynamics.com) on a normalised data set. ANOVA and Tukey's test was performed using the StatPlus:mac Le data analysis toolpack add-in for Microsoft Excel (http://www.analystsoft.com).

2.7 Grain Development Analyses

2.7.1 Total Starch Assay

Whole mature grain samples were ground in a pestle and mortar with +30% total sample weight of acid washed sand and liquid nitrogen to aid grinding and passed through nylon mesh to remove large particles and fibres. For each sample 0.036g (Actual sample weight 0.025g +30% adjustment for sand) was dispersed in 40µl 95% EtOH, suspended in 1ml 1M NaOH, and heated to 105ºC for 30 minutes. Samples were vortex mixed, spun for 1 min at 1300 rpm and the supernatant decanted. 100µl extract was then added to 1ml 0.1M Trichloracetic acid, mixed and diluted with 10ml H_2O. 2.8ml of each extract plus 200µl Lugols solution (Sigma) was immediately read against appropriate blanks at 620nm. A calibration curve was prepared from known starch concentrations.

2.7.2 Imbibition Weight Change Assay

3 x 15 grain samples for *B. distachyon* and *T. aestivum* "Cadenza" were thoroughly wetted before being placed onto wet filter paper at 20ºC and continuous light. Change in weight was measured over imbibition (From first wetting) until germination i.e. point of radical protrusion. At each time-point grains were removed from the filter paper and blotted onto absorbent paper to remove surface water before weighing using an electronic balance. Average values for the three replicates were recorded.

2.8 Nucleic Acid Analysis

2.8.1 Tissue Collection, Disruption & Storage

All plant material used for DNA or RNA extraction was snap frozen in liquid nitrogen quickly after collection and stored at -80C. Frozen grain and leaf tissues were typically ground using a Silamat amalgam mixer (Ivoclar Vivadent, UK) for 15 seconds with acid-washed glass beads, or for more robust spikelet samples, using a pestle and mortar and liquid N_2.

2.8.2 Total RNA Isolation

Total RNA was extracted from snap frozen ground tissues using either Trizol reagent (Invitrogen) according to manufacturers instructions, or for the more starch rich mature grain tissue the Spectrum plant total RNA extraction kit was used. High starch levels in mature grain tissues can interfere with Trizol extraction to give low yield.   For the majority of RNA extracted DNAse treatment was performed using NEB DNase I, in 1x reaction buffer (10mM Tris-HCl, 2.5mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6) for 10 minutes at 37ºC with a heat inactivation at 75ºC for 10

minutes.

Growth stage specific samples for chapters 3 and 5 were collected according to grain size: A "Pre-anthesis Spikelet" stage correlates to the whole spikelet where the lowest (Proximal-most) floret is approaching anthesis, a "young grain" stage immediately after anthesis (~1-3 DAA), a "Mid-Length" developmental stage (caryopses ~ half of their full lengthwise development, between 3-8 DAA), a later "Full-Length" caryopsis stage (as grains reached full proximal-distal development, correlating to ~8-12 DAA) and a fully developed or "Mature Grain" stage (but before desiccation, correlating to ~18-20 DAA). These developmental stages follow that of Opanowicz et al (2010) and were collected from three biological replicates. Leaf samples were collected from mature healthy leaves.

2.8.3 Genomic DNA Isolation

Genomic DNA extraction was performed using an approx. 300ng sample of young leaf tissue using a typical phenol:chloroform extraction protocol. Ground tissues were incubated in extraction buffer (200mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA & 0.5% SDS) at 50ºC for 20-30 minutes. This was followed by phenol:chloroform extraction and precipitation using isopropanol. DNA was resuspended in sterile water for direct use in PCR reactions.

2.8.4 RNA Quantification & cDNA Synthesis

A 3µl aliquot of total RNA was separated via electrophoresis in a 1% agarose gel to assess quality and final concentration was measured using a NanoDrop ND-1000 Spectrophotometer. Approximately 300ng RNA was used in 10 µl cDNA synthesis reactions using BioscriptTM Reverse Transcriptase (Bioline) using the

poly(T) primer 5'GACTCGAGTCGACATCGA(T).

2.8.5 PCR

Oligonucleotide primers were designed to amplify gDNA or cDNA products either manually or using Pubmed primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Primers were designed with a length ranging between 18 - 25 bp and a melting temperature (T_m) between 45 - 65°C. Sequence for all primers used here are shown in the appendix. Primers were obtained from Invitrogen and for all reactions were used at 10μm concentration. Reactions were performed using bioline MyTaq 2x complete PCR mix. PCR conditions were typically similar for both Genomic and cDNA amplification, being 1 μl of template in 10 μl reactions with the following cycle: 94°C 5 minutes, then 30 cycles of 94°C 30 seconds, 53°C 45 seconds, 72°C 1 minute followed by 72°C 6 minutes. Reaction conditions were varied in some cases to according to product size, primer T_m and individual gene copy numbers. All PCRs were performed in at least duplicate to ensure replicability of results.

2.8.6 Gel Electrophoresis

PCR products were loaded into 1% w/v agarose gel with EtBr and electrophoresis was performed at 100v alongside 1Kb or 100bp DNA ladders. Gels were examined and photographed using a UVP Bio-docit UV imager.

2.9 RNA In-Situ Hybridisation

2.9.1 Tissue Collection & Fixation

For RNA ISH experiments, developing grains were staged according to length as described above. Caryopses were trimmed and fixed in formalin–acetic acid–

alcohol (FAA; 3.7% formaldehyde, 5% acetic acid, 50% ethanol) and vacuum

infiltrated in 3 x 5 minute cycles. After overnight fixation, samples were transferred

to 70% EtOH and stored at 4ºC before transferal to the Tissue Tek vacuum

infiltration processor supplied by Bayer (Newbury, UK) for an automated

dehydration/infiltration process as follows: 70% ethanol for 1 h at 35 °C; 80%

ethanol for 1.5 h at 35 °C; 90% ethanol for 2 h at 35 °C; 100% ethanol for 1 h at 35

°C; 100% ethanol for 1.5 h at 35°C; 100% ethanol for 2 h at 35 °C; 100% xylene for

0.5 h at 35 °C; 100% xylene for 1.0 h at 35 °C; 100% xylene for 1.5 h at 35 °C; and

molten paraffin wax (supplied by VWR International, Poole, UK) for 2 h at 60 °C. All

steps were performed under vacuum (>50 cm Hg).

2.9.2 Embedding & Sectioning

Samples were then transferred to the Tissue Tek embedding console (Bayer) for

embedding in paraffin blocks. Wax sections of 14µm thickness were cut on a

mechanical Microtome (Bright 5030; Bright instrument co., England), organized

sequentially on poly-lysine-coated slides (Grace Biolabs, supplied by Stratech

Scientific, Soham, UK) on a drop of distilled water and dried down at 38°C

overnight.

2.9.3 RNA Probe Synthesis & Testing

For *Brachypodium* ISH gene specific fragments were amplified from cDNA using

T7 appended primers and were used to generate digoxyenin-labelled RNA probes.

PCR amplified fragments form the probe templates and were cleaned of PCR

reaction components using a Qiagen PCR purification kit. For ISH involving wheat

DNA clean probe templates including a T7 promoter region were available through previous work, details of their preparation can be found in Drea et al (2005).

Using these probe templates *In-vitro* transcription was performed in 10 µl reactions for 2 h at 37°C in the presence of digoxigenin-UTP (Dig-UTP)-nucleotides (0.35 mM) using Roche T7 polymerase. Products were precipitated in 4 M Lithium chloride and 3 vol absolute ethanol for <1 h at -18°C. Reactions were centrifuged at 13000 rpm for 10 min and pellets resuspended in 30 µl TE (100 mM Tris, 10 mM EDTA) buffer. 100x dilution of probes were made in water and 1 µl of spotted on nitrocellulose membrane, along with control RNA of known concentrations for dot-blot probe testing. Blots were developed as follows: 1 min wetting in TBS (10 mM Tris, 250 mM NaCl),30 min in blocking solution (1% in TBS, Roche), 30 min in anti-DIG-alkaline phosphatase (1:5000 in TBS, Roche); 5 min wash in TBS; 5 min in AP-buffer (0.1 M Tris, 0.1 M NaCl, and 50 mM $MgCl_2$, pH 9.5) and developed in AP-Buffer containing NBT (nitroblue tetrazolium:0.1 mg/ml) and BCIP (5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt: 0.075 mg/ml) in darkness. Blots were developed until strong colour products could be seen and probe strength/efficiency was judged by comparison to control RNA concentrations.

2.9.4 Slide Pretreatment

Slide clearing and pre-treatment was performed using the following steps: Histo-clear clearing agent for 20 min with agitation (twice); 100% ethanol, then through a 90%, 80%, 50%, ethanol series (10 min each), PBS rinse (3 mM NaH_2PO_4, 7 mM Na_2HPO_4, 130 mM NaCl) 3–4 min; proteinase K treatment (2–3 µg/ml in 100 mM

Tris, 10 mM EDTA pH7.5) for 30 min at 37°C; glycine (0.2% in PBS) 2 min; PBS

rinse 3–4 min; acetic anhydride (0.5% in 0.1 M triethanolamine pH 8) 10 min with

stirring; PBS rinse 3–4 min; back through the ethanol series, 50%, 80% and 100%.

Slides were allowed to completely air dry and were stored at 4°C until

hybridisation.

2.9.5 Hybridisation & Washing

Protocol used here follows that of Drea et al 2005 with only minor modification.

For hybridization probes were diluted in hybridization solution (300 mM NaCl, 10

mM Tris, pH 6.8, 10 mM, NaPO$_4$, 5 mM EDTA, 50% [v/v] formamide, 5% [w/v]

dextran sulfate, 0.5 mg/mL tRNA, Denhardt's solution (Denhardt's solution is

0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), and 0.1 mg/mL salmon

testis DNA). Typically a 1:50 dilution was used but this was variable according to

probe strength based upon dot blot results. Immediately prior to hybridization

probes were denatured at 80°C for 3 minutes and immediately put on ice. Probes

were applied to slides and covered with glass cover slips and hybridization was

performed overnight in a 50°C incubator. Cover slips were removed and slides

were washed through the following series: 3 x 0.2% SSC (Saline Sodium Citrate)

preheated to 50°C with gentle agitation, 1% blocking solution (Roche) in 1 x TBS

for 1hr, 1% BSA (Bovine Serum Albumin) in 1 x TBS + 0.03% TRITON for 1 hr, 1 x

TBS containing a 1:3000 dilution of anti-digoxigenin–alkaline phosphatase and

0.05% Tween 20 for 1 h, followed by 3 x 10 minute washes in 1 x TBS with gentle

agitation and a final 5 min in AP-buffer

2.9.6 *Slide Development & Mounting*

The colour reaction was developed in AP buffer containing NBT (0.1 mg/mL) and BCIP (0.075 mg/ mL) for up to 48 hrs. Once sufficient colour product was developed slides were washed several times in distilled water to stop the reaction, followed by sequential washes in 70 and 100% ethanol. Slides were then allowed to air dry and permanently mounted in Entellan (Merck, Rathway, NJ).

2.10 Phylogenetic Analyses

All sequence alignments were performed using CLUSTALW within the Geneious Pro v5.4 software (Drummond et al, 2011) with a Gap open cost of 20 and a Gap extend cost of 0.75. Alignments were edited by hand within the same software. Phylogenetic trees were also drawn in this software package and were all prepared as unrooted, neighbour-joining cladograms, using the jukes-cantor model and no outgroup. For chapter 3 sequence comparison Wheat cDNA sequences were used in BLASTN searches against the *Brachypodium* genome 8x release (www.modelcrop.org) to identify potential markers with high nucleotide sequence similarity and single copy status.

CHAPTER 3.

GRAIN DEVELOPMENT IN

BRACHYPODIUM DISTACHYON

GRAIN DEVELOPMENT IN *BRACHYPODIUM DISTACHYON*

3.1 Introduction

Since publication of the genome sequence in 2010 *Brachypodium distachyon* has become widely recognised as an important model system for temperate cereals (Draper et al, 2001; Opanowicz et al, 2008; Vogel et al, 2010). Little is known about the grain structure and development of *B. distachyon* however, and how it compares to known developmental patterns of the domesticated cereals. The emergence of *B. distachyon* as a model system provides both the first experimentally available system in which to examine temperate cereal grain development and the first opportunity to make a comparison between closely related wild and domesticated small-grain species. Recent reviews such as those of Charmet (2011) and Vaughan et al (2007) cite the advantages and continued need for urgent progress in the genetic manipulations of crops such that problems of climate change, need for increased productivity etc can be tackled. These same reviews suggest that an understanding of the mechanisms and progression of the domestication process in cereals is key to this progress. The investigation described in this chapter aims to provide a foundational overview of temperate cereal grain development at both the physical and molecular level, to provide a comparison of the contrast between a wild and domesticated grain and a basis for further exploration of the effects of domestication on cereal grains.

The comparison made here is largely between the community standard *B. distachyon* line Bd21, and elite modern bread wheat, *T. aestivum* "Cadenza", referred simply to as *Brachypodium* and wheat respectively throughout this chapter. Where appropriate comparison to significant features and detail of other

important species has been included. In this chapter we provide a detailed characterization of grain development in *B. distachyon* at the cellular and molecular level against the backdrop of published data for wheat, one of the most closely related and important cultivated cereals, whose development has been well characterised. We compare key physical and physiological features of structure and development focusing in particular on the development of the endosperm and those features of known important function in domesticated species. This characterization and comparison reveals that whilst broadly similar there exists significant differences in grain structure, cellular differentiation and gene expression patterns that reflect significant developmental differences between *Brachypodium* and wheat.

3.2 Results & Discussion

3.2.1 Plant & Flower Structure in Brachypodium Distachyon

 Initially, brief and basic observations on the habit and floral morphology of *Brachypodium* were made, these details providing an important backdrop and influence for the grain and its development. *B. distachyon* is typical of dry, disturbed and open habitats on sandy and rocky soils (Piep, 2007) and is native to areas and climatic conditions similar to those of ancestral wheat species (Opanowicz *et al*, 2011). *Brachypodium* demonstrates considerable plasticity in its growth habit according to growing conditions and to competition, evident in the differences seen between wild-grown and cultivated plants (See fig 3.1 A). Under CER and greenhouse conditions with supplemental lighting in Leicester (21°C, 18 hrs light) *Brachypodium* typically flowers at around 6 weeks from germination, the

life cycle complete in around 13 weeks. Casual observations reveal that quite

different growth and flowering habits could be seen according to lighting,

competition and water regime, and plant height was variable. Densely spaced and

competing plants typically show a more decumbent habit and with longer

internodes as compared to solitary plants, which generally showed reduced tillering

and a more upright habit. *Brachypodium* appears to be sensitive to wet rooting

conditions; a mildly waterlogged or even just continually moist rooting environment

also produces plants with a long, thin decumbent habit and with reduced flowering.

Shorter day-length could be seen to delay flowering and maturity.

Brachypodium produces a simple spike-like racemose inflorescence (Fig 3.1 B).

Under CER and greenhouse conditions in Leicester inflorescences are typically

comprised of between 3 and 7 spikelets. The inflorescence matures acropetally as

the spikelets emerge but within each spikelet maturation occurs basipetally (Khan

& Stace 1998). Similarly, maturation in the wheat spikelet occurs basipetally but at

the spike level, it is those spikelets occupying the central position that mature first.

Both terminal and lateral spikelets in *Brachypodium* are indeterminate with several

diminished and underdeveloped florets at the apex of the spikelet. Floret number

within the *Brachypodium* spikelet was highly variable with a typical range of

between 4-20. Each spikelet is subtended by two glumes and the individual

spikelet of *Brachypodium* was noted as superficially resembling the spike of wheat

(Fig 3.1 B). In wheat the spikelet is a regular arrangement of five florets producing

up to 4 grains, the most distal floret remaining unfertilised and under developed.

Studies of natural variation in *Brachypodium* have identified accessions with high

spikelet numbers and floral morphology more similar to that of wheat (J. Doonan
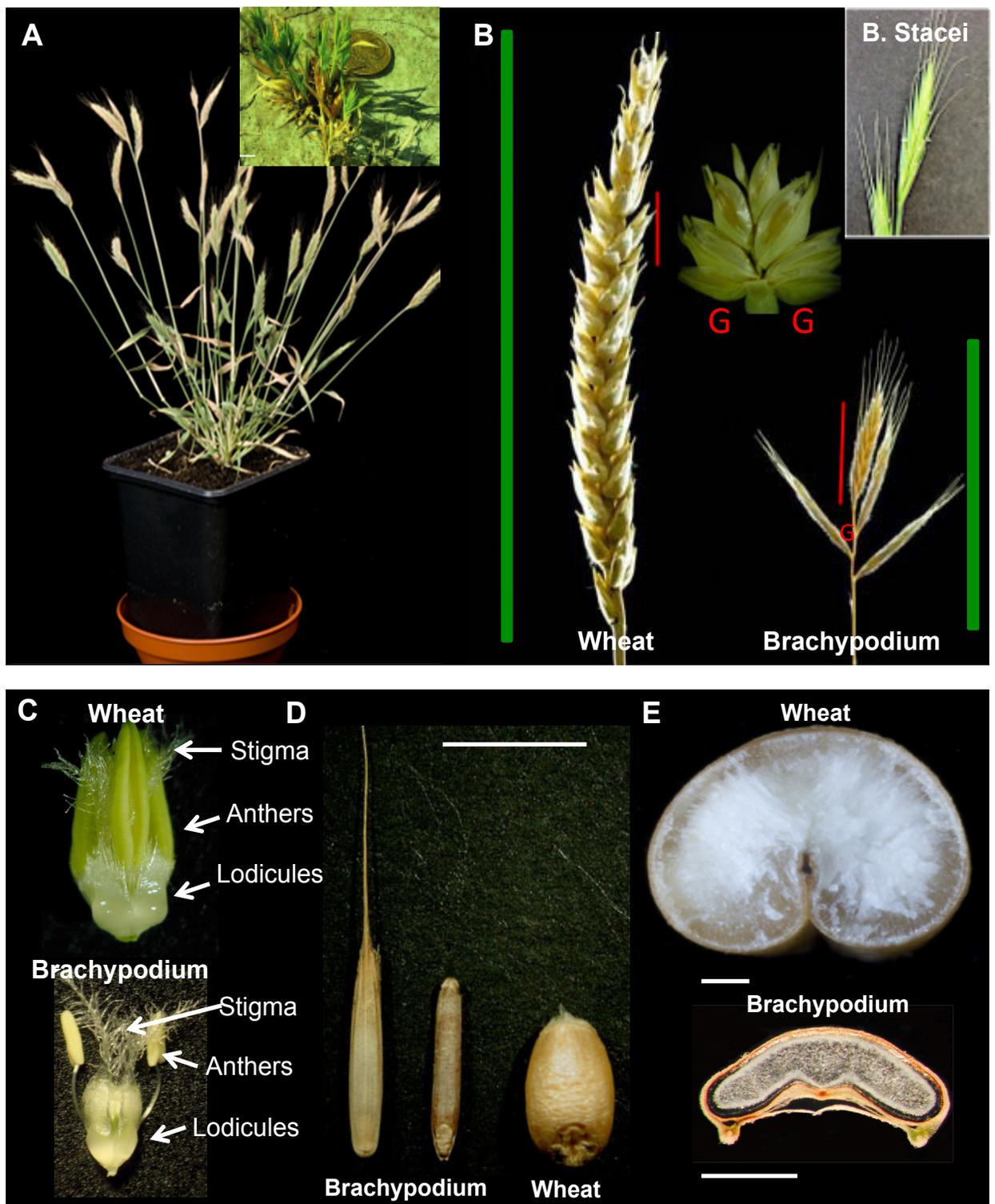
Figure 3.1: *Brachypodium* and Wheat, Flower and Grain External Morphology.
Panel A illustrates Brachypodium growth plasticity with a typical Bd21 plant grown under greenhouse conditions and a wild *B. distachyon* (Inset: taken from Mur *et al* 2011). (B) Wheat, cultivar "cadenza", and *B. distachyon* flower spike comparison. Green bars indicate flower spike level of floral organization, whilst red bars show spikelet. Mature spikes are shown in scale to each other. G in red indicates position of the glumes Inset shows spikelets of *B. stacei* with anthers exerted at anthesis. (C) *B. distachyon* and wheat floral organs at anthesis, where *B. distachyon* shows 2 anthers compared to 3 in wheat. (D) mature *B. distachyon* grain (left) husked and dehusked with *T. aestivum* "cadenza" (right). Scale shows 7mm (White bars). (E) Transverse central section of dry, mature wheat (top) and *B. distachyon* grain. Scale bars show 0.5mm

pers comm.). *Brachypodium* florets have awned lemmas and disarticulate freely at maturity. Disarticulation occurs at the distal end of the rachilla leaving it attached and lying flat against the concave adaxial surface of the grain, this is in contrast to shattering that can be seen in most pre-domestication wheat species (e.g. *T. uratu,T. dicoccoides, T. tauschii*) where disarticulation typically occurs at the proximal end of the rachilla (Nesbitt, 2001).

B. distachyon flowers are cleistogamous and anthers are short as compared to other *Brachypodium* species (Khan & Stace, 1998); they were very rarely observed exerting outside of the floret although expansion of the lodicules was observed around anthesis. In closely related species such as *B. stacei* or *B. sylvaticum* however anthers can be seen to exert from the floret (See fig 3.1 B inset). *Brachypodium* flowers are unusual in possessing just two anthers; grass florets, including those of many other members of the *Brachypodium* genus and wheat, typically contain three anthers (Hubbard 1954; Fig 3.1 C). *Brachypodium* Florets contain a single carpel and ovule typical of the grasses with two feathery stigmas and a mild ventral groove. In figure 3.1 C it can be seen that lodicule size in comparison to the other floral organs is comparable to that of wheat, but palea and lemma are not seen to spread at anthesis. This persistent closure of the flower, whilst promoting self-fertilisation, presents an obstacle in performing crosses.

Upon shattering the *Brachypodium* grain dispersal unit comprises the caryopsis with closely adhered palea and lemma. The *Brachypodium* lemma is notably thicker and more robust than the papery lemma in wheat, which is as expected, wheat having evolved the free-threshing character. Ecological studies have

suggested that granivorous insects may play a part in *Brachypodium* seed dispersal and possibly also predation (Arnan et al, 2010; Willot et al, 2000). Investiture into tough outer layers may provide part of the grain, and specifically the embryo's, protection mechanism. We also note however that lemma is awned and approximately equal in length to the grain in the Bd21 accession examined here although variability can be seen in its length. It is equipped with backward facing hairs and barbs along its length and so is likely to have evolved with dispersal function. Awns of this morphology are generally thought to play a role in positioning and moving grains into a favourable position in the soil, perhaps to avoid predation (Elbaum et al, 2007). It seems likely that *Brachypodium* is fairly general in its dispersal strategy and from examining only the morphology of the grain dispersal unit the primary method of dispersal is not clear. To avoid possible effects of physical or chemical restraint upon seedling germination the palea and lemma were removed from all grains grown in this study.

The *Brachypodium* grain is slender and ellipsoid (dehusked- see fig 3.1 D), carrying hairs at its apex. In comparison to wheat the *Brachypodium* grain is similar in length but contrasts starkly in both width and depth. The *Brachypodium* plant is considerably smaller in stature compared to wheat and so it can be seen that the grain is comparatively large. Grain size and shape in *Brachypodium* in comparison to wheat and other temperate cereals is discussed at length in the following chapter, and so is not repeated here.

***3.2.2** Comparison of Mature Grain Transverse Profiles Reveals Significant*

Differences in Grain Architecture Between Brachypodium and Wheat

Comparison of transverse sections of *Brachypodium* and wheat grains

immediately reveals a number of important similarities and differences in grain

profile. Most striking is that the *Brachypodium* grain does not demonstrate the

distinctly indented hilum or "crease" that is the major feature of wheat grains but

has a flatter and only superficially creased profile (see fig 3.1 E, 3.2 A & B). The

adaxial surface of the *Brachypodium* grain has only a fairly shallow concavity

giving the grain a flat to crescent shaped profile in stark contrast to the rounded

and heavily creased profile of the wheat grain, although their similarity and analogy

can be identified.

To determine the cellular basis for these differences in grain profile, various

cytological analyses of mature grains were performed. These analyses span both

maternal and filial tissue but focused on the endosperm. Along the centre of the

Brachypodium grain, above the shallow indentation on the adaxial surface, runs

the main vascular trace extending for almost the entire length of the grain. The

nucellar projection extending from the vasculature towards the endosperm is vastly

reduced in *Brachypodium* as compared to wheat, most notably later in

development (Fig. 3.2 A & B). A prominent feature of the mature *Brachypodium*

grain is the persistent nucellar epidermis that is largely obliterated by maturity in

wheat (fig 3.2 C & D). This layer is especially visible at the lobes of the

Brachypodium grain although is somewhat more compressed on the grain abaxial

surface. Reflecting the flat shape of the caryopsis, the *Brachypodium* endosperm

Figure 3.2: Comparative Grain Development & Structure

i) Developing *Brachypodium* and wheat grains from anthesis to maturity, showing similarity in their external appearance. Scale bars show 500 μm.

ii) Comparison of structure and features of grain transverse sections. A & B, Toluidine blue stained mature wheat and *Brachypodium* sections respectively. Peripheral aleurone is well defined in wheat as compared to *Brachypodium*, indicated with a red arrow and labeled al. C & D, Bright-field micrograph of peripheral grain cell layers highlighting differences in nucellar epidermis thickness. Red arrows indicate nucellar epidermis layer. E & F, separation of the aleurone from the central endosperm can be seen in wheat (E) whilst retention of the aleurone layer and separation of only outer, maternal layers is seen in the *Brachypodium* endosperm (F). Red arrows indicate aleurone layer. al; aleurone layer. nu; nucellar epidermis. Scale bars for A & B 500 μm, C-F 20 μm.

forms a crescent-shaped structure unlike the lobed structure that encloses the crease and nucellar projection in the rounder wheat grains. The endosperm cavity that is a significant feature of the wheat grain appears to be absent in *Brachypodium* (Fig. 3.2 A & B).

Distinct differences in endosperm cell size and cell wall thickness are apparent between *Brachypodium* and wheat. Peripheral aleurone cells in wheat are distinct cuboidal cells with thick walls, clearly differentiated from the larger, thin-walled cells of the central endosperm. Initial investigation using toluidine blue staining reveals these characteristics and shows the *Brachypodium* peripheral aleurone layer to be significantly less distinct from the central endosperm as compared to wheat (Fig. 3.2 A & B). Comparative differences between central endosperm and aleurone cell size were also noted. Wheat aleurone cells are typically around 40 μm in diameter, approximately a third of the size of central endosperm cells (see fig. 3.11 iii). In *Brachypodium* however this size difference is not seen and aleurone cells are typically equally sized, or just slightly smaller, to those of the central endosperm. Notably, the cells of the *Brachypodium* endosperm appear quite large in relation to the overall grain size and heavily vacuolated, and central endosperm cell walls were thick, a feature described in more detail in later sections.

3.2.3 Developmental Comparison Reveals Temporal and Regional Differentiation Differences Between Brachypodium and Wheat.

To gain further insight into the comparative differences between *Brachypodium* and wheat grain we examined development. Grains were staged using anthesis (0DAA (Days After Anthesis)) as a reference point and growth followed across

development. Externally, developmental progression between these species appears similar, as shown in figure 3.2i.   We used various cytological techniques including calcofluor and DAPI staining to chart development and identify key points such as endosperm cellularisation, filling etc. Results of these investigations are summarised in figure 3.3 and 3.4i. In *Brachypodium* the fertilised caryopsis (0DAA) is about 1 mm long and elongates rapidly between 2-6 DAA to reach a maximum length of 7-8mm by 6DAA (Fig. 3.3 F).   Using sectioned material to examine nuclear proliferation and lateral growth, 4 key stages of grain development were established (illustrated schematically in Fig. 3.4 i) corresponding to (i) syncytial endosperm and circular central vacuole 0-3DAA (Fig. 3.3 A); (ii) elongated, flat vacuole with thin sliver of endosperm (at which time cellularisation begins; 3-5DAA) (Fig. 3.3 B); (iii) cellularised endosperm with adaxial indentations flanking the nucellar projection 5-13DAA (Fig. 3.3 C); and (iv) fully-filled endosperm with smaller peripheral aleurone cells discernable at 13-18DAA (Fig. 3.3 D & E).   The entire process, from anthesis through to fully filled ripe grains takes about 24 days as opposed to an average of 35 days in wheat (depending on cultivar and growing conditions). In comparison Guillon et al 2012 record grain development for the variety Bd21-3 as complete in 34-36 days although plants were grown under a shorter day-length than used here.

  Developmental stages were compared with corresponding stages in wheat grains (Fig. 3.4 i). Although early post-fertilisation stages are similar in both species with endosperm nuclei proliferating and migrating around the periphery (Fig 3.3 A & B; Wegel et al 2005), there are clear differences evident before cellularisation. In wheat (Drea et al 2005b) and barley (Olsen et al, 1992) nuclei form a highly regular
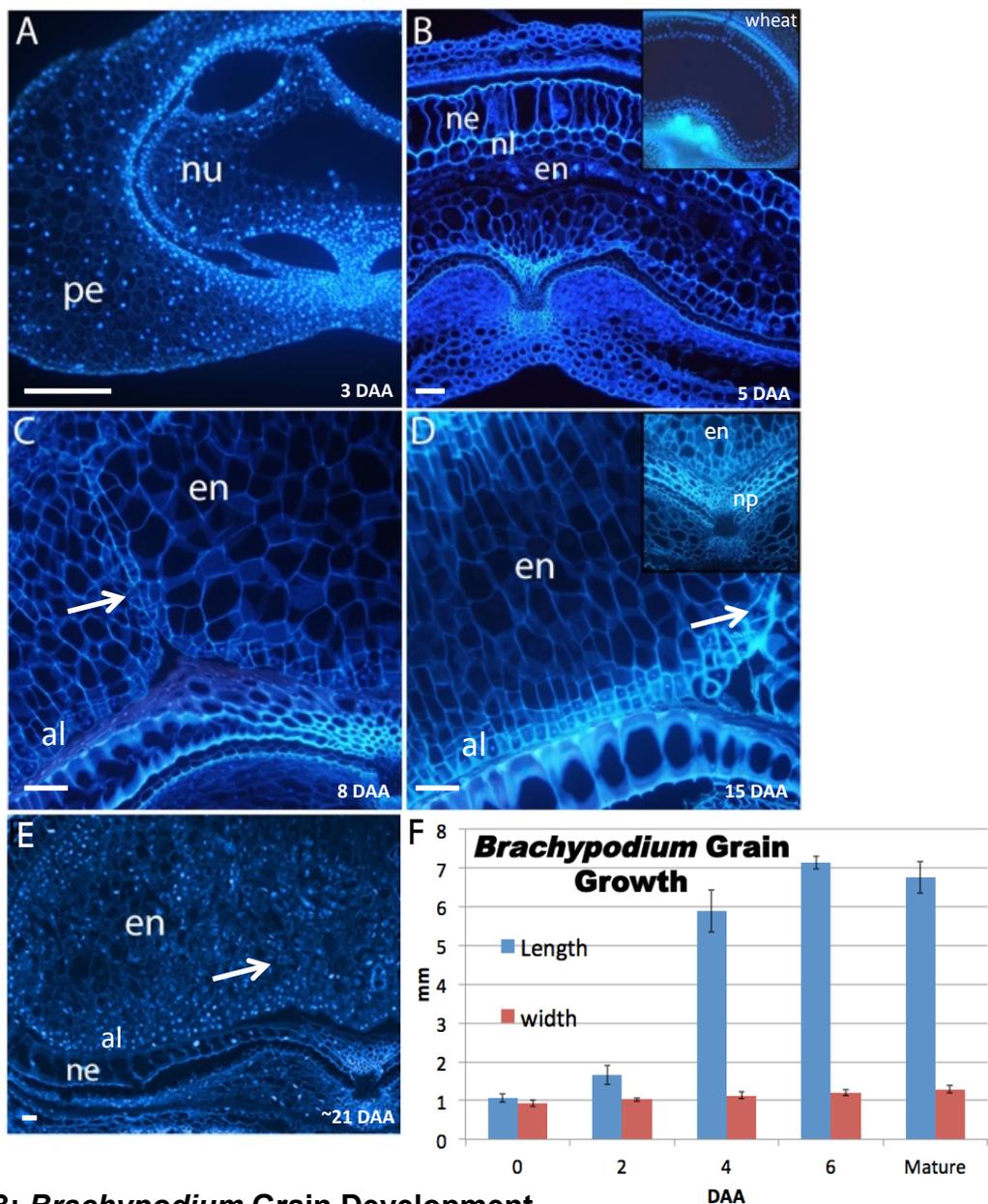
Figure 3.3: *Brachypodium* Grain Development.
Calcofluor and DAPI staining of central transverse sections of *Brachypodium* grain across development. A, 3DAA (Days After Anthesis); B, 5DAA, Inset depicts a wheat grain at a similar developmental stage. A comparative difference is shown with nuclei of the wheat synctial endosperm located around the edges of the large central vacuole in contrast to the flattened syncytial endosperm of *Brachypodium*. C, 8DAA & D 15DAA, both stained with calcofluor only, inset shows detail of the nucellar projection region; E ~ 21DAA. Arrows in C & D indicate internalised cells showing aleurone morphology, and in E, loss in the mature grain. pe, pericarp; nu, nucellar tissue; ne, nucellar epidermis; nl, nucellar lysate; np, nucellar projection; en, endosperm; al, aleurone. Scale Bars show 50μm.
F displays measurement of grain length and width during early development, showing rapid grain elongation. n=6 and error bars represent ± one standard deviation.

arrangement around the periphery of the central vacuole that is seen in wheat just prior to cellularisation. Cell division then re-orientates anticlinally in wheat and barley to produce internal layers of nuclei and cellularisation progresses. *Brachypodium's* nuclear distribution in the vacuole appears more random at this point in development (5DAA; Fig. 3.3 B with wheat inset). The *Brachypodium* grain becomes flattened dorso-ventrally and cellularisation can be seen as complete around 6-7DAA, comparing well with the findings of Guillon et al (2012) who also record endosperm cellularisation at 7 days after fertilisation. The endosperm at this stage in development is represented by just a sliver of material making it difficult to precisely identify when cellularisation occurs/completes. By about 8DAA the endosperm is obviously cellular; further cell division has increased its depth and smaller (presumptive) aleurone cells form around the periphery. Deep adaxial indentations become apparent in the endosperm flanking the nucellar projection (Fig. 3.3 C). The cells that line these indentations appear to show differentiation and similar aleurone identity to those in other areas of the endosperm, judged by their size, thicker cell walls and increased calcofluor fluorescence. As development progresses endosperm increases in size and these differentiated cells become internalised as the endosperm lobes and central region expand. No trace of these differentiated cells can be seen in mature endosperm (Fig 3.3 E, arrowed) suggesting they have re-differentiated and adopted central endosperm cell identity, suggesting mechanisms involving peripheral sensing determinants for aleurone cell identity, as have been described in maize (Gruis et al, 2006), are acting. Between 8 and 15DAA the nucellar tissue adjacent to the adaxial endosperm becomes increasingly compressed. At about 15DAA, small square

cells around the edge of the central endosperm are clearly visible and appear uniformly distributed abaxially, adaxially and laterally (Fig. 3.3 D and inset). The nucellar projection is much reduced as compared to wheat (inset).

To map changes in cell cycle activity during grain development, RNA in situ hybridisation was performed with a histone-4 marker (*H4*). *H4* is expressed in a DNA-replication dependent manner at a temporally specific stage of the cell cycle (During S-phase) and is a good indicator of cell cycle activity (Fobert et al, 1994). Hybridisation using this probe provides an indication of cellularisation and tissue differentiation (Drea et al, 2005b). At 3DAA, there are high levels of *H4* transcript in the outer pericarp layer and the two integuments, inner and outer, as well as the syncytial endosperm (Fig. 3.5 A). In wheat at this stage, histone transcript preferentially accumulates in the adaxial nucellar projection (Drea et al, 2005b) whereas in *Brachypodium* the transcript is mainly present in syncytial endosperm. The inner integument layer, marked naturally by the deposition of mucilage and subsequent oxidation resulting in a brown coloration, is visible at a much earlier stage in *Brachypodium* than in wheat grains. By 4DAA, *H4* transcript is further restricted to the outer integument (Fig. 3.5 A inset). The endosperm of *Brachypodium* at about 5DAA (Fig. 3.5 B) is restricted to a slim sliver of material between robust nucellar layers in a flat grain. Histone signal is evenly distributed throughout the endosperm, indicating that at this point the endosperm is not yet cellularised (Fig. 3.5 B). After the endosperm has clearly cellularised (8DAA), histone transcript is strongest along the periphery as in wheat, and is now distinctly patchy (Fig. 3.5 C). This patchy expression pattern is due to asynchronised cell cycle progression in neighboring cells combined with the tight S-phase specificity
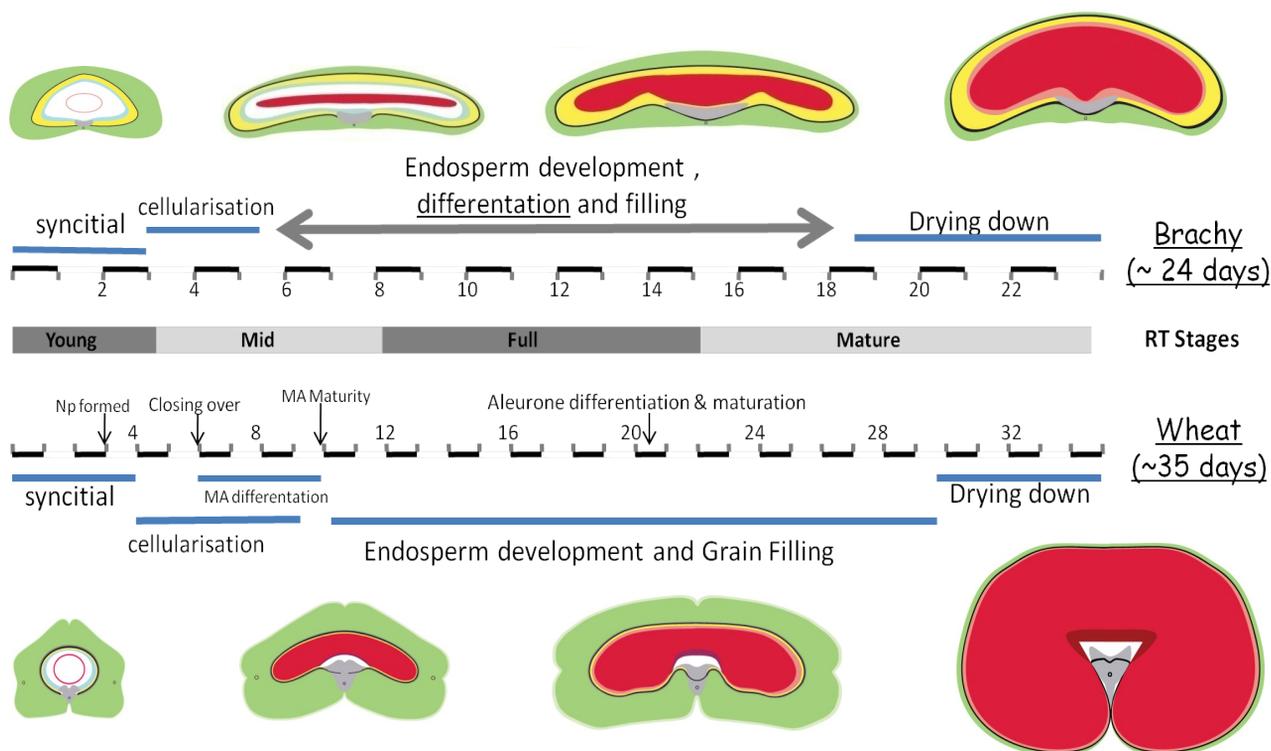
Figure 3.4: Comparative Progression of Grain Development in *Brachypodium* and Wheat.

Time-line of grain development from anthesis to maturity in focusing on the key stages in endosperm development. Grain illustrations are not to scale and grain tissues are indicated by colour: pericarp, green; integuments; black; nucellar epidermis, yellow; nucellar lysate, blue; nucellar projection, grey; aleurone, pink; central endosperm, red; MA, modified aleurone. Numbers of the scale indicate DAA (days after anthesis). Stages indicated as young, mid, full & mature correspond to the material used for RT-PCR analyses.
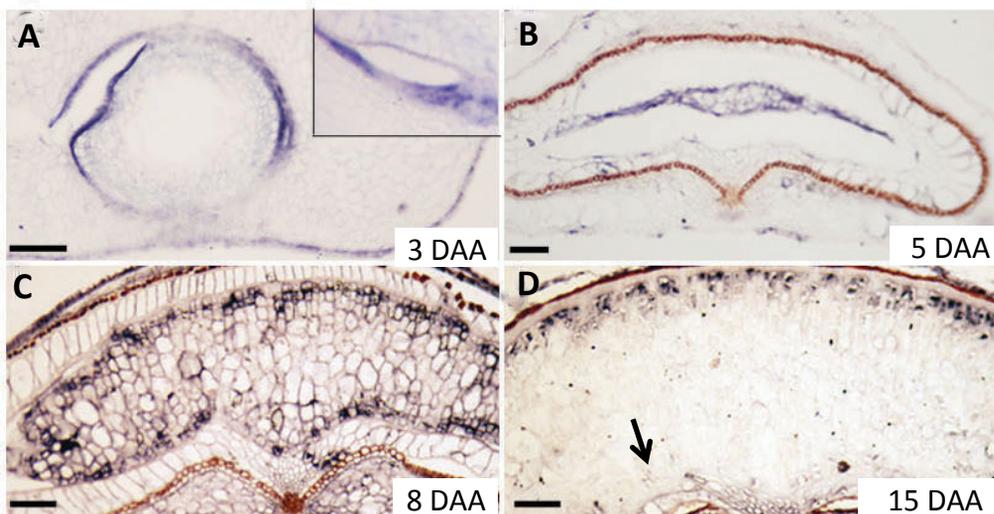
Figure 3.5: Cell-cycle Activity in the Developing *Brachypodium* Grain
Histone4 (H4) mRNA ISH on *Brachypodium* transverse central grain sections
at 3DAA (Days After Anthesis: A), 5DAA (B), 8DAA (C), 15DAA (D). The
BdH4 marker reveals mitotically active tissues. Expression is seen in the
integument layers during early development (A). Inset in (A) shows detail of
abaxial integument region close to the vasculature, where strong spotty
expression could be seen. Expression is seen throughout the endosperm pre-
cellularisation (B) becoming restricted to the endosperm periphery (C) and
abaxial region (D). Arrow indicates absent adaxial expression at 15 DAA.
Scale bars show 50µm.

of histone gene expression (Fobert et al,1994). Unlike the equivalent wheat developmental stage there is no specific exclusion of transcript from the region presumed to equate to the modified aleurone overlying the crease (Fig. 3.5 C). The disappearance of *H4* transcript can indicate tissue differentiation (Drea et al, 2005b) and the observed differences in the timing give an indication that the aleurone region adjacent to the crease develops quite differently in these two species. Subsequently, as grain filling is completed, histone expression is preferentially localised to the abaxial endosperm as shown by cross section of a grain at approximately 10DAA (fig. 3.5 D). Taken together, these data indicate there are distinct differences in the timing of differentiation in different regions of the *Brachypodium* grain and that these differ in a number of respects from wheat, particularly regarding the differentiation of the modified aleurone layer.

3.2.4 Molecular Mapping of Maternal and Filial Expression Domains

Cytological analysis suggested that *Brachypodium* grain structure and development was similar in many ways to that of wheat, or barley, but the timing and nature of cell-type specification differed. To gain further insight into these cytological differences, the molecular events associated with grain development were aligned using key marker genes. Selected markers relate largely to those specific for distinct functional domains identified in a study of wheat grain development and expression patterns (Drea et al, 2005b), and publications examining *Brachypodium* storage proteins (Laudencia-Chingcuanco & Vensel, 2008). Expression patterns were compared and related to the cytological and structural observations.

A BLASTN survey of a range of wheat grain domain- and tissue-specific ESTs (Drea et al, 2005b) against the *Brachypodium* genome was initially performed to compare tissue-level of genetic similarities. This survey provides a valuable comparison of the level of spatial genetic homology in the grain between *Brachypodium* and wheat, and is results are shown in figure 3.6 i). Notably this survey revealed generally lower levels of sequence similarity in the grouping expressed in the aleurone layers as opposed to groupings expressed in other tissues. The defensive functions of the modified aleurone in wheat and occurence of less spatially specific function in *Brachypodium* may be responsible for the homology seen amongst the modified aleurone specific sequences. The high level of sequence similarity in the integuments is also an interesting point of comparison and may be indicative of conservation of function in the maternal layers.

Those *Brachypodium* genes with greatest similarity to known tissue-specific markers in wheat (specifically, those with high sequence similarity at the nucleotide level but were not members of extensive gene families so as to maintain specificity) were selected from this survey. We used RT-PCR to check expression levels of *Brachypodium* orthologs of these potential markers across grain development before committing to probe design and synthesis. Only those with high expression levels were chosen for in-situ hybridization. Figure 3.6 ii) shows the results of this survey, being the genes used as markers. C13 endopeptidase or nucellain marks the nucellar lysate and nucellar epidermis - tissues that are degraded and compressed relatively early in wheat grain development (Linnestad et al, 1998; Drea et al, 2005b); pyruvate orthophosphate dikinase (*PPDK*) marks the single aleurone layer specifically in wheat though its actual function is elusive

i)

Chart (i): percent orthologues with p-value > e-05

Wheat expression patterns/domains	percent
Cell Cycle n=63	79.4 %
Aleurone n=3	33.3 %
Modified Aleurone n=76	47.4 %
Central Endosperm n=64	56.3 %
Nucellar n=24	58.3 %
Integuments n=10	90.0 %
Uniques n=8	50.0 %

ii)
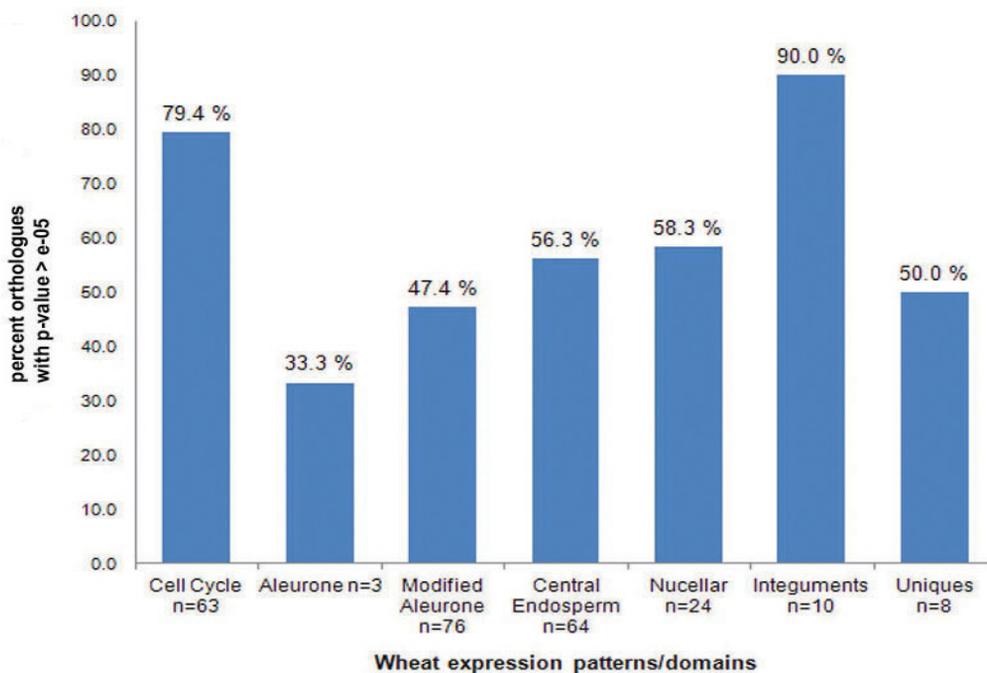
	Brachypodium orthologue	Gene annotation	Function & orthologues	Expected expression domains
leaf / stem / root / inflor / grains (young / mid / full / mature)	Bradi1g05980	BdH4	Cell Cycle	Spotty
	Bradi5g16960	BdC13	endopeptidase	Nucellar lysate
	Bradi3g29810	BdAlGal	Ethylene synthesis	Modified aleurone
	Bradi2g25740	BdPPDK	Photosynthesis	Aleurone
	Bradi1g13040	BdGLO1	Storage protein HvGLO1	-
	Bradi2g38060	BdGLO2	Storage protein	central endosperm
	BdGAPDH	BdGAPDH	Housekeeping	-

Figure 3.6: Grain-expressed Genes in *Brachypodium* and Wheat.

(i) Comparative homology of domain specifically expressed genes in wheat to *Brachypodium*. Chart shows a summary of BLASTN results for wheat sequences showing tissue-specific gene expression patterns against the *Brachypodium* genome (data from Drea et al., 2005b: chart taken from Opanowicz et al, 2011). Vertical bars show the number of wheat sequences returning significant hits at the nucleotide level.
(ii) RT-PCR profile of genes used in-situ hybridisation across *Brachypodium* plant and grain development. Table shows gene identifier, function in wheat and, where applicable, expected expression domain in *Brachypodium* based upon what is seen in wheat.

given its involvement in photosynthesis converting PEP to pyruvate (Drea et al, 2005b; Hong-Gyu et al, 2005; Chastain et al, 2006); an alpha-galactosidase specifically marks the modified aleurone in wheat (Drea et al, 2005b). *BdGLO1* and *BdGLO2* were chosen because they have been shown as the most abundant storage proteins in *Brachypodium* grain (Laudencia-Chingcuanco & Vensel, 2008) and provide useful markers for storage protein deposition in the endosperm during grain filling. Both *BdGLO* genes are expressed exclusively in the grain (10DAA onwards) when the endosperm is fully cellularised. Of the other genes shown all were found to have strong expression in the grain, although are not exclusively grain expressed. In all cases, except *BdGLO2* and *H4*, a BLASTN query with the starting wheat sequence resulted in a single highly significant (e>10^{-6}) result for *Brachypodium*. More than one homolog for *H4* and *BdGLO2* was expected on the basis of previous analyses (Drea et al., 2005b; Laudencia-Chingcuanco & Vensel, 2008). The presence of only one copy of the *PPDK* is interesting given that maize, rice and Sorghum all have two *PPDK* genes, with maize and rice producing two proteins (chloroplast and cytosolic forms) from one of the genes (Imaizumi et al., 1997). The *Brachypodium PPDK* gene has a similar structure to the rice and maize gene, *OsPPDK* and *ZmPPDK1*, where a 5' exon located distantly upstream provides a putative targeting peptide for chloroplast localisation (Imaizumi et al., 1997). Probes of approximately 400bp length were designed and specificity was initially confirmed via BLAST analysis. Cell-cycle or other genes of known expression pattern were used as control hybridisations. Results of these hybridisations are shown and described in figures 3.7 and 3.8, and are discussed in more detail through following paragraphs.

3.2.5 Endosperm Differentiation in Brachypodium and Wheat

In wheat the aleurone layer is a crucially important, functionally and structurally distinct layer extending around the periphery of the central endosperm (Evers & Millar, 2002). Aleurone cells themselves are typically quite obviously different and distinctly differentiated from those of the central endosperm. The molecular and cytological observations collected so far suggest the *Brachypodium* aleurone layer is different to that of wheat, tending to be more irregular, from 1 to 3 or more cells deep, and indistinct from the central endosperm. Figure 3.2 F shows that the *Brachypodium* aleurone layer appears to be structurally integrated with the central endosperm and remains firmly attached to the endosperm during physical disruption of the grain, in contrast to the situation in wheat where the aleurone tends to adhere to the maternal tissues during disruption (Fig. 3.2 E). This is a significant attribute of the wheat grain exploited during the milling process in order to separate the alpha-amylase producing activity of the aleurone cells from the starchy central endosperm (Evers & Millar, 2002).

The indistinct and variable cell morphology of the *Brachypodium* aleurone layer makes it difficult to determine the timing of its differentiation from the central endosperm using cytological examination. Several genes are strongly and specifically expressed in the aleurone and serve as good markers to follow its early development and subsequent differentiation. To investigate the timing of aleurone differentiation at the molecular level, the temporal and spatial expression of presumptive aleurone-specific marker genes was examined. *PPDK* is expressed in the developing wheat grain, initially in pericarp tissue before becoming specific and

strongly expressed in the peripheral aleurone layer (Drea et al, 2005b). Wheat endosperm expression of *PPDK* coincides with differentiation of the peripheral aleurone layer. In situ hybridisation using a *Brachypodium* PPDK probe found a weaker and more indistinct expression pattern. *BdPPDK* transcript accumulated in the pericarp tissue at early stages (Fig. 3.7 C) as was the case in wheat, but the expression in the peripheral endosperm was both weaker and occurred later during development than is the case in wheat (Fig. 3.7 D-F). Transcripts were detected only after cellularisation was complete, suggesting aleurone differentiation occurs later or less distinctly in *Brachypodium*. Furthermore, in wheat *PPDK* accumulates specifically in the peripheral aleurone and is absent from the modified aleurone but in *Brachypodium* the distinction was less obvious. *BdPPDK* expression was weak and patchy in the grain abaxial region, and it was unclear if expression was genuinely detectable in this area.

Since the *BdPPDK* expression pattern did not definitively mark the aleurone as it does in wheat, we used another aleurone marker, *BdGLO1*, an orthologue of which is expressed in the aleurone of barley (Heck et al., 1993). Figure 3.8 shows the *BdGLO1* transcript is detected strongly and specifically the peripheral aleurone and the embryo of the mature *Brachypodium* grain. *BdGLO1* transcript accumulation in the abaxial aleurone was found to extend into between 1 and 4 cell layers in an irregular manner, contrasting to a more discrete localisation in the adaxial layer (Fig. 3.8 D-E). This irregular definition of the peripheral aleurone at both a molecular and cellular level is quite unlike what is seen in wheat and other cultivated cereals. Staining adjacent to the embryo in 3.8A is thought to be non-specific, being seen with various probes, as shown in appendix figure A3e.
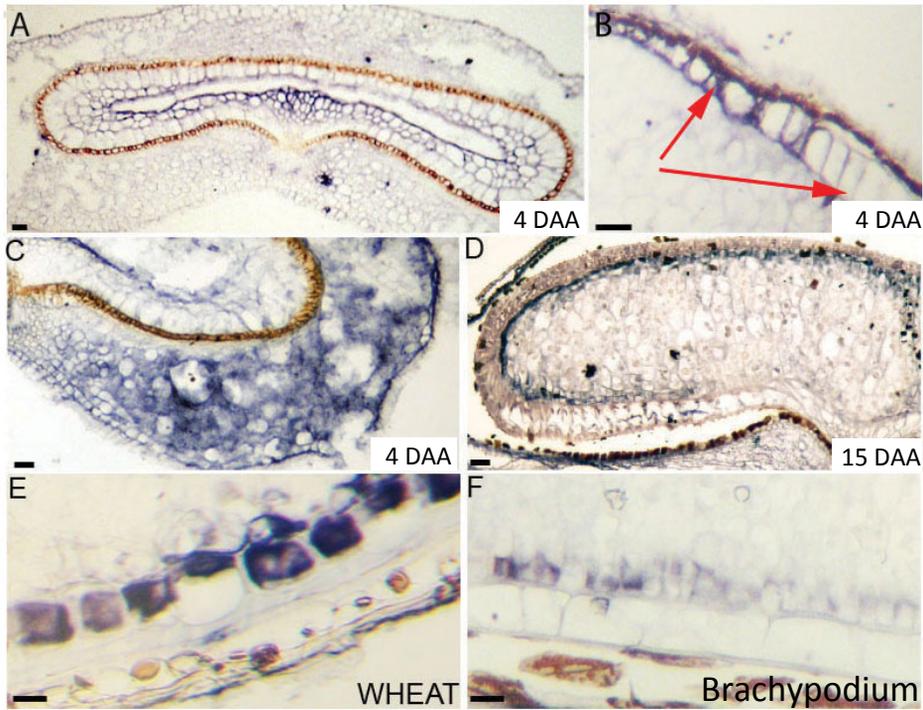
Figure 3.7: RNA ISH Analysis of *Brachypodium* Nucellar and Aleurone Tissues

Expression of BdC13 in (A) nucellar lysate of a 4DAA grain and (B) abaxial nucellar epidermis in a 15DAA grain cross section. (C) BdPPDK expression in the pericarp of 4DAA grain and (D) in the peripheral endosperm of 15DAA grains. E and F show specific comparison of PPDK expression in wheat and *Brachypodium*. (E) shows a strong and distinct aleurone specific expression in wheat alongside *Brachypodium* (F), where expression is weak and indistinct. Scale bars show 20µm.
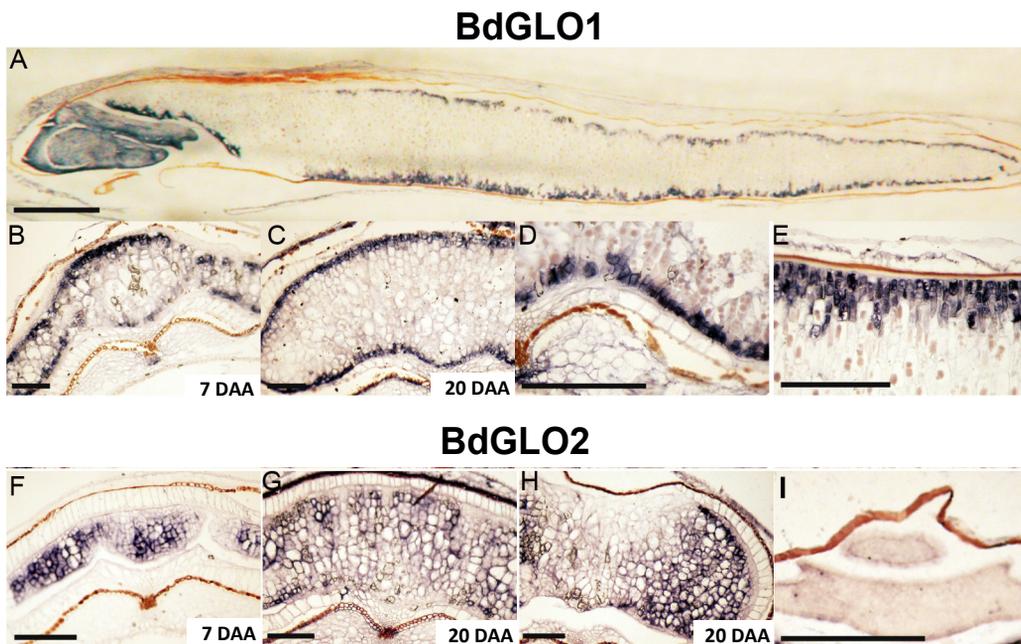
BdGLO1

BdGLO2

Figure 3.8: Molecular Mapping of Endosperm Domains in *Brachypodium* using RNA ISH for Globulin Gene Expression

A-E show expression patterns for BdGLO1 in the *Brachypodium* grain, F-I show BdGLO2 expression. (A) *BdGLO1* expression in mature grain longitudinal section, revealing peripheral aleurone domain and differences between grain abaxial and axial side, and embryo expression. Scale bar, 500µm. (B-E) *BdGLO1* expression at (B) 7DAA and (C) 20DAA grain cross sections. (D-E) show detail of GLO1 expression in adaxial and abaxial regions respectively in the 20 DAA grain. More irregular and deeper expression can be seen on the grain adaxial side as compared to the more discreet adaxial localisation.   F-H show *BdGLO2* expression in grain cross sections at (F) 7DAA and (G & H) 20DAA. Exclusion of GLO2 expression can be seen in the aleurone layer. I shows absence of expression of BdGLO2 in embryo, in contrast to the strong embryo expression for GLO1 shown in A. Scale bars B-I show 50µm.

A striking feature of *BdGLO1* expression was continuous peripheral pattern of expression extending into the adaxial region above the vasculature, detected from about 7-8DAA (Fig. 4.7 C). This continuous band of expression suggesting that the aleurone is not differentiated into discrete cell types as in wheat and barley, and that a molecularly distinct modified aleurone region is absent in *Brachypodium*.

 In comparison to *BdGLO1* expression patterns, in-situ hybridisation of *BdGLO2*, a member of a family of storage protein genes related to the 12S globulins of oat, was performed (Laudencia-Chingcuanco & Vensel, 2008). Storage protein genes provide useful markers for the central endosperm area and in wheat are typically excluded from peripheral and modified aleurone regions. As expected, transcripts were seen to be localised to the central endosperm and excluded from the peripheral layers (Fig. 3.8 F-H). The peripheral extent of *BdGLO2* expression in the central endosperm was seen to reflect and follow the irregular line as could be seen with *BdGLO1*. In addition, *BdGLO2* was not detected in the embryo (Fig 3.8 I), in contrast to the strong expression of *BdGLO1* (Fig. 3.8 J). These detailed spatial and temporal expression patterns agree with the RT-PCR results (fig 3.6ii) indicating a slightly earlier onset of expression for *BdGLO2* and indicates that specification of aleurone cell identity occurs after central endosperm proliferation has ceased.

3.2.6 The Modified Aleurone Domain is Absent in Brachypodium

 Based upon the results of our in-situ hybridization experiments and cytological observations we focused our analysis on the possible absence of localised

specialisation within the *Brachypodium* aleurone layer. We continued the approach of RNA in-situ hybridization examining accumulation of an alpha-galactosidase transcript found to be specifically expressed in the wheat modified aleurone (Drea et al, 2005b). RT-PCR for the *Brachypodium* orthologue of the Alpha-Galactosidase gene reveals high levels of expression throughout grain development (Fig 3.6 ii). We performed RNA in-situ hybridization for this gene across all stages of grain development but were unable to detect any specifically localized signal. This failure to detect expression is likely due to a non-functional probe. We did not pursue a reattempt or optimization of this experiment but it is an aim for future research, where we would seek to confirm the efficacy of our probe in northern analysis and potentially design alternatives for use in future experiments.

  To further investigate differentiation and regionalization of the endosperm we used vital staining as a novel approach to identify aleurone domains in the mature grain endosperm. Our aim was to establish the extent and definition of the *Brachypodium* peripheral aleurone layer, to determine if the presumptive modified aleurone comprised of either live or dead cells. Aleurone and embryo are the only living cells in the mature cereal grain and in wheat both the modified aleurone and central endosperm and are comprised of dead cells (Fig. 3.9; Young & Gallie, 1999). Vital staining was performed on sections of mature imbibed grains using two complementary and opposing techniques; Tetrazolium chloride (TZ) a compound that reacts with active enzymes (Dehydrogenases) to produce an orange color product (Formazon) and so marks only living, respiring cells (Peters, 2007) and Evans blue dye, which is absorbed by cells without a functioning membrane and so
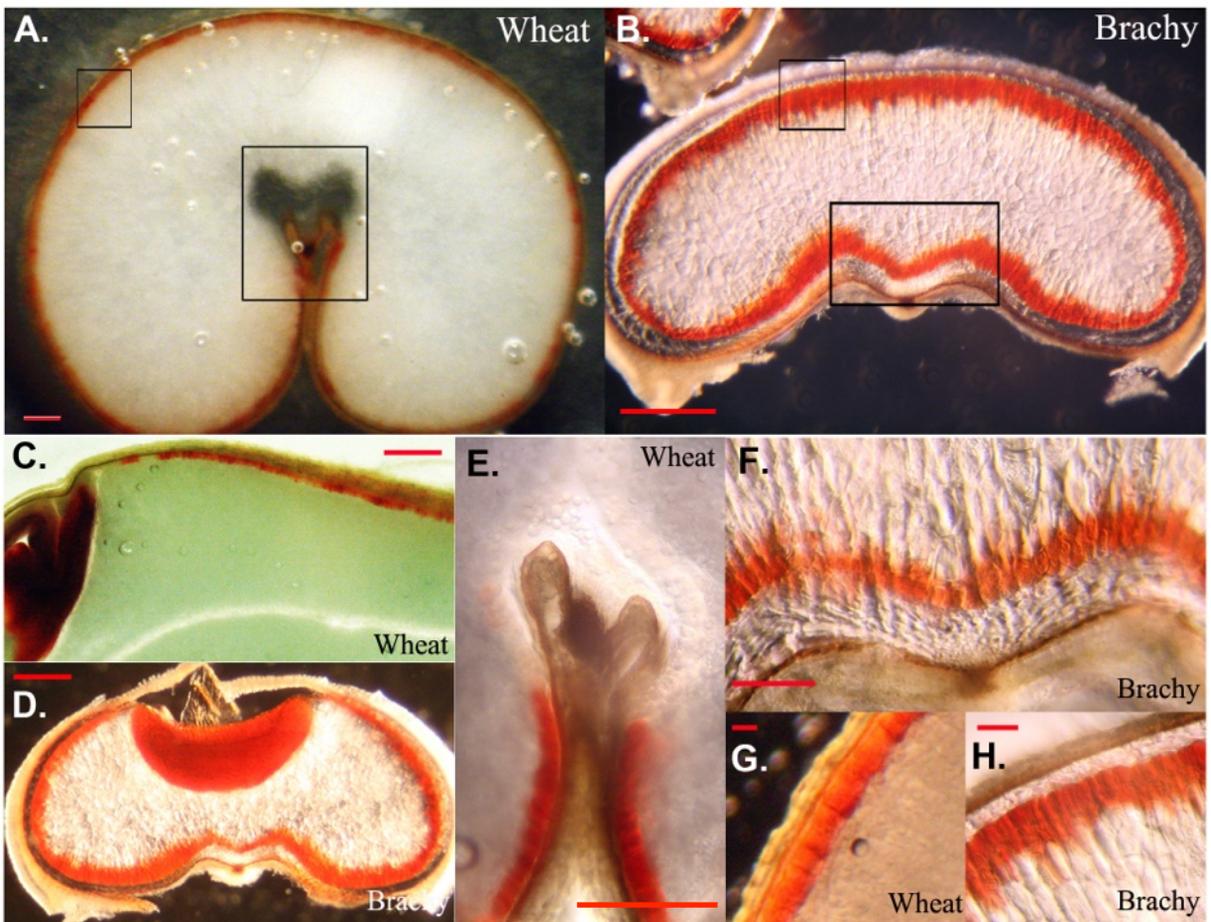
Figure 3.9: Vital Staining for Endosperm Cellular Domains.
  Tetrazolium chloride staining in (A) mature wheat and (B) *Brachypodium* central grain cross-sections, scale bars show 200 μm. Boxed regions shown in greater detail in images E-H. (G) Wheat peripheral aleurone staining shows a discrete layer whilst (H) *Brachypodium* peripheral aleurone layer staining is irregular. Vital staining pattern is identical to that seen for *BdGLO1*. (E) Wheat modified aleurone region is clear of staining, indicating this tissue is dead. (F) Corresponding *Brachypodium* aleurone region is stained, indicating it is a living tissue, with characteristics similar to the adjacent peripheral aleurone. (C & D) Strong live embryo staining is seen. Scale bars show 400 μm. A complementary Evans blue staining panel can be seen in appendix panel A3c.

marks only dead cells (Young & Gallie, 1999). TZ staining offers the clearest

results and figure 3.9 C and D show staining in the embryo and in peripheral

aleurone layer in *Brachypodium* and wheat respectively. Figure 3.9 A, C and E

show staining is absent in the wheat modified aleurone. In *Brachypodium* staining

can be seen in the region adjacent to the vascular bundle and is similar in

character to the staining around the rest of the endosperm periphery (Fig. 3.9 B

and F). Staining with Evans blue dye was seen to produce an identical pattern, a

panel of Evans blue staining can be found in the appendix figure A3c.

  Together these observations relating to the aleurone layer are powerfully

indicative that the modified aleurone domain is absent in *Brachypodium*. The

presence of a continuous peripheral layer of living aleurone cells, the lack of clear

cellular or molecular differentiation in the presumptive modified aleurone region,

strongly suggests that this region is not homologous to its equivalent in wheat.

3.2.7 *Maternal Tissue Organisation in Brachypodium Grains Differs from Wheat Throughout Development*

  The order and identity of tissue layers in the mature *Brachypodium* grain

resembles that of wheat, but one very striking difference revealed by cytological

analysis is the persistence of the nucellar epidermis layer. This layer, clearly visible

in figures 3.2 and 3.9 is a dominant feature of the grain across development and

forms a thick, hard and vitreous layer at maturity (See fig. 3.1 E). The nucellar

epidermis reaches 50-60µm at the thickest point in the lateral adaxial region (and

in longitudinal sections is very pronounced at the distal or stigma end of the grain)

and thinnest at the central abaxial point, where it is comparable to the thickness of

the nucellar epidermis in wheat (Fig. 3.2 C-D). In contrast the wheat nucellar epidermis layer is lost very early in grain development, becoming compressed and reduced even by 9DAA, and obliterated soon after.

In rice, similar to *Brachypodium*, the nucellar epidermis is also persistent through grain development. Rice lacks a basal differentiated transfer region of the endosperm, such as the modified aleurone or maize BETL and may utilize an alternative nucellar epidermis-mediated transport route (Ellis & Chaffey, 1987). The nucellar epidermal cells in rice are thought to be specialised for transport of nutrients (Oparka & Gates, 1981, Ellis & Chaffey 1987); they are elongated in the principal direction of assimilate flow (i.e. circumferentially around the endosperm) and symplastically connected to adjacent cells by numerous plasmodesmata. During the later stages of rice caryopsis development the nucellar epidermis along with integument and pericarp becomes compressed and ultimately collapses blocking assimilate flow to the endosperm and inhibiting further grain filling via this pathway (Ellis & Chaffey, 1987).

Similarity can be identified between *Brachypodium* and rice nucellar epidermal layers suggesting a similar mechanism of grain filling may exist between the two. The *Brachypodium* nucellar epidermis is well developed along the whole caryopsis, and minimally compressed by the expanding endosperm indicating it could play a part in assimilate transport into the caryopsis. This observation is supported by the apparent absence of a distinct transfer layer equivalent to the wheat modified aleurone region. Electron microscopy also indicates that *Brachypodium* nucellar epidermal cells are also rich in plasmodesmata (Opanowicz et al, 2011), a feature that may be important in providing a nutrient transfer function.

We also hypothesize that the thickened cell walls of the nucellar epidermis contribute to alternative carbon storage and provide a skeletal, reinforcing function that may be involved in predation defence. In relation to carbon storage, following paragraphs discuss the possibility of cell wall carbohydrates forming the primary storage reserve in *Brachypodium* grains. Hard grains (due to the thick cell walls) may have adaptive significance in relation to insect predation (Oliveras et al, 2008). Ants are a major grain predator for *Brachypodium* in some regions, and some species feed almost exclusively on grass grains. Whilst a correlation with particular grain composition is not clearly defined, in some studies granivorous ants generally favor elongated rather than round grains and grains that have obvious awns or hairs projecting (Pulliam & Brand, 1975). Mymechory often forms an important seed dispersal strategy in inhospitable (open, dry, fire-prone) habitats, such as Mediterranean scrub (Lengyel et al, 2009). Mechanical properties, such as hardness afforded by thick cell walls, can protect grains against predation while positively influencing dispersal (Oliveras et al, 2008). It is possible that the tough lemma and palea described earlier may also contribute to this defence. Just as squirrels quickly learn to "chip" an acorn, to remove the embryo and so stop it germinating during storage it seem likely that ants would quickly acquire a similar habit and so eat the nutritious and much softer embryo end of the grain. This may be foiled by the defence afforded by the lemma that remains attached to the seed. Retaining a palea tough enough to resist the ant's jaws would seem to overlap the need to develop a mechanically resistant endosperm however? Of course this line of defence is limited as the lemma cannot become so tough as to restrict germination, and other factors may be involved in the acquisition of robust nucellar

and endosperm cell walls.

We also examined nucellar cell wall degradation during grain development. In wheat the expression of a endopeptidase or nucellain gene (C13) could be seen to coincide with degradation of the nucellus and nucellar epidermis during early grain development (Drea et al, 2005b). We identified and examined expression of this gene's orthologue in *Brachypodium*, the RT-PCR and RNA in-situ hybridization data being shown in shown in figure 3.6ii and 3.7 A and B. We detected expression in the nucellar lysate layers of *Brachypodium* grains from 4DAA (Fig. 3.7 A), comparable to the expression pattern observed in wheat (Drea et al 2005b). Expression was not detected in the nucellar epidermis except in later stages of development when transcript was present in the central abaxial region where the layer is at its narrowest point in the grain (Fig. 3.7 B), cells are thinnest and obviously degraded/compressed. The expression of this endopeptidase could correlate with specific degradation of cellular components and compression of the layer in this region.

3.2.8 *Brachypodium Central Endosperm is Starch Poor But Rich in Cell Wall Material*

Starch forms the major storage product of all cultivated cereals, and accounts for up to 75% of the wheat grain (Bewley & Black, 1994). We detected low levels of starch in the *Brachypodium* grain, revealed with Lugol's staining on mature grain sections where *Brachypodium* shows significantly less-intense staining as compared to wheat (Fig. 3.10 A, B & I). Domesticated cereals typically show starch contents of between 50-70% whilst the Brachypodium starch content has

been shown to be less than 10% of the whole grain (Guillon et al, 2012). Wheat starch is evenly distributed across the endosperm whilst in *Brachypodium* it shows some concentration in the central endosperm region with a gradient moving towards the endosperm periphery. This gradient of starch was most clearly visible in cells isolated from mature endosperm (Fig. 3.10 J), this image being kindly supplied by M. parker (IFR). To investigate starch distribution in *Brachypodium*, endosperm longitudinal and serially sectioned whole-grains were examined. The results of this investigation, shown in figure 3.10 A-H reveal a distinct proximal-distal starch gradient in the mature grain. A notably greater concentration of starch could be seen in the proximal endosperm (close to the embryo) compared to the distal end, which at it's extreme appears devoid of starch. Figure 3.10 F-H also reveals that in distal half of the grain starch was largely absent from endosperm lobes and confined to the central zone.

Examination at a subcellular level reveals that *Brachypodium* and wheat grains differ in the number and size of starch granules (Fig. 3.10 L). Wheat endosperm cells were tightly packed with variable sized starch grains (Tomlinson et al, 2003), the equivalent *Brachypodium* cells having few and small granules, as judged by SEM (Fig. 3.10 L). Wheat starch granules show bimodal granule morphology (Shapter et al, 2008: Tomlinson et al, 2003) and we observed both classes here, the larger granules being typically ovoid with a distinct equatorial band and in a size range of 15-20 µm, and the more numerous smaller class that were typically quite spherical and ~ 5µm diameter. In contrast *Brachypodium* starch granule morphology is simple and small, with sizes ranging from 3-7µm diameter.

Figure 3.10: Starch Distribution in the *Brachypodium* Endosperm

A & B show longitudinal half-grain Lugol's stained grain sections for wheat and *Brachypodium* respectively. Images C-H show a Lugol's stained proximal-distal series of sections from an individual grain, revealing the distinct starch gradient. Overall starch levels can be seen to be low, but are most abundant in the proximal half of the grain. A transverse gradient can also be seen to exist, visible in F and more clearly displayed in the isolated endosperm fragment shown in J. ce, central endosperm; al, aleurone. Scale bars C-H show 0.5 mm.

Image I shows abundant starch staining in 14µm thick cross-section of wheat, inset details exclusion from the aleurone layer. K shows thin free-hand section of *B. stacei* grain. Starch is more abundant than observed in equivalent *Brachypodium* section and inset again shows exclusion from the aleurone. Exclusion indicates that aleurone layer in *B. stacei* shows the same irregular definition as seen in *B. distachyon*.

L shows high magnification SEM for starch granules in both grains. *Brachypodium* shows a distinct small and simple starch granule morphology as compared to the more abundant bimodal granules in wheat. Scale bars show 10 µm.
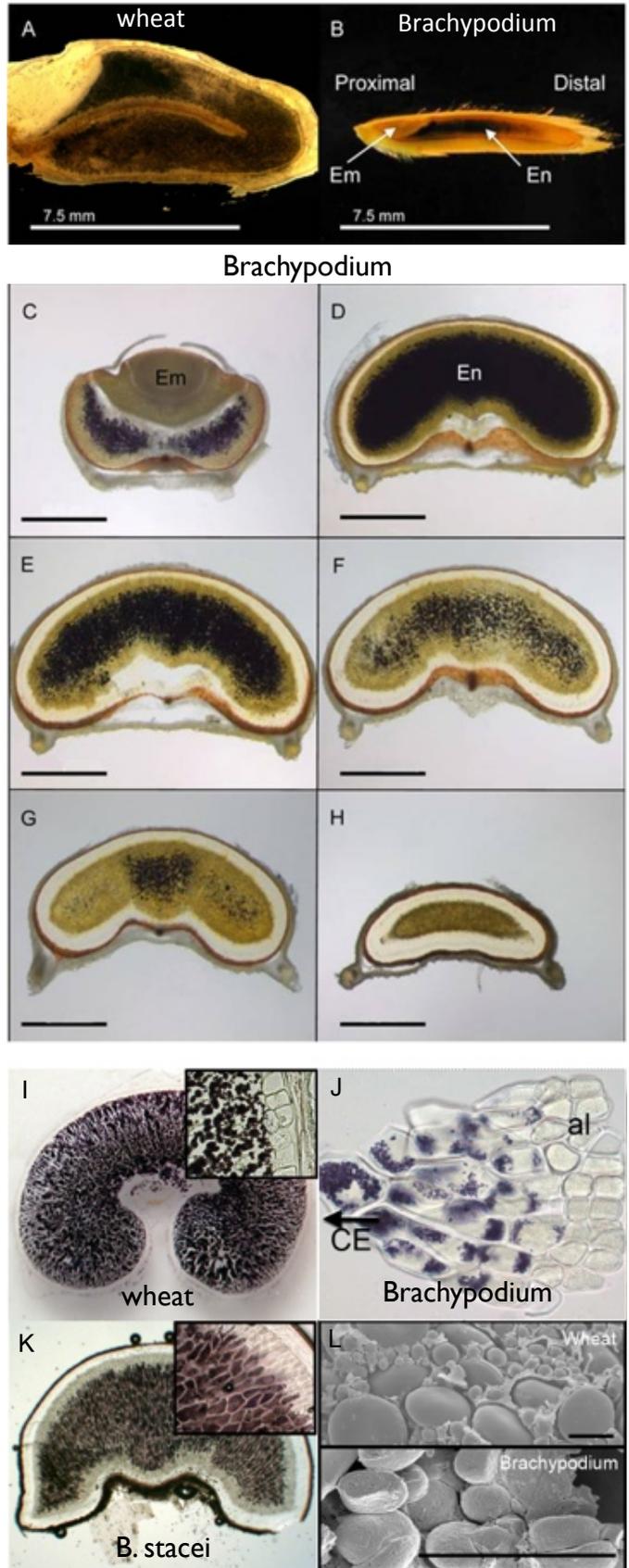
i)
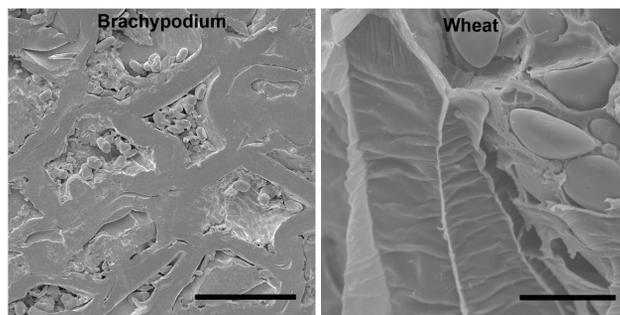
Figure 3.11: Endosperm Cell Wall Sizes and Depletion

i) Comparative SEM for central endosperm shows notably thicker cell wall and smaller cell size in *Brachypodium* as compared to wheat. Scale bars show 50 μm

ii) Brightfield and fluorescence images of *Brachypodium* endosperm cell wall degradation in early stages of grain germination. (A-B) 3DAG (days after germination) (C-D) 6DAG. ne, nucellar epidermis; ce, central endosperm. Scale bar, 50μm.
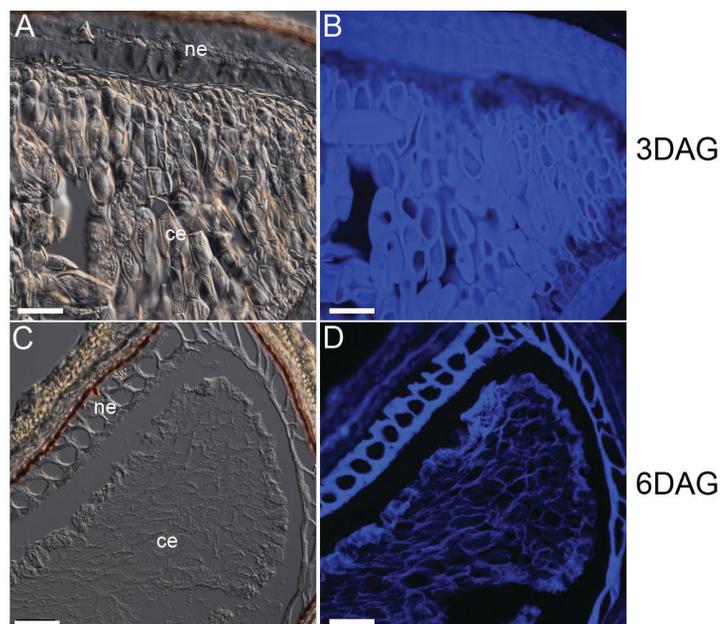
iii) Measurements for endosperm cell size, cell wall and nucellar epidermis thickness in mature *Brachypodium* and wheat grains.

ii)

iii)	*Brachypodium* mature grain	Wheat mature grain
central endosperm- cell wall thickness	2.73 ± 0.42	2 ± 0.06
Central endosperm- cell size	28.5 ± 4.68	131.2 ± 0.75
Aleurone layer - cell wall thickness	2.07 ± 0.51	4.9 0.08
Aleurone layer - cell size	12.46 ± 4.3	40.8 ± 0.06

n = 10 and errors represent ± 1 SD

A striking feature of the *Brachypodium* central endosperm is thickness of the endosperm cell walls, which were often over twice as thick as those of the much larger wheat endosperm. This feature can be seen clearly in the SEM (Fig 3.11i). Measurements were taken from SEMs of mature *Brachypodium* and wheat grains to quantify the differences in both cell size and cell wall thicknesses, shown in figure 4.11iii. Wheat aleurone and central endosperm cells are significantly larger than those of *Brachypodium*. Cell wall thickness is significantly greater in *Brachypodium* however, where *Brachypodium* endosperm cells often exceed 4µm diameter in comparison to wheat where thickness rarely exceeds 2µm. Examination along a proximal-distal axis found cell wall thickness to be consistent across the whole of the endosperm.

Based upon these observations and the findings of other investigators of *Brachypodium* grain development (Guillon et al, 2011, 2012) we hypothesized that low starch levels in conjunction with abundant cell wall material suggests cell wall carbohydrates form the major storage reserve in the *Brachypodium* endosperm. (1-3)(1-4)-β-D glucan has recently been reported as the major carbohydrate present in the *Brachypodium* grain and is present at unusually high levels as compared to domesticated pooid cereals (Guillon et al, 2012). High levels of hemicellulose in the thick cell walls of species such as coffee or Fenugreek provide the major carbohydrate storage reserve and make these seeds extremely hard (Sutherland et al, 2004). Similarly, a significant hemicellulose content has been detected in the *Brachypodium* endosperm cell walls (Opanowicz et al, 2011) providing further support for this hypothesis.

3.2.9 Endosperm Storage Reserve Utilisation in B. distachyon

In order to investigate the endosperm storage reserve utilisation and composition we examined starch and cell wall degradation along with partial and full endosperm removal in germinating *Brachypodium* seedlings. Results shown in figure 3.12ii reveal that near complete removal of the endosperm results in an arrest to seedling growth at around 3 DAG (Days After Germination). Removal of the distal endosperm portion results in slowed seedling growth at 5 DAG whereas seedlings with a complete endosperm are seen to still show significant increase in growth after 6 and 7 DAG. This suggests that the distal endosperm section is contributing to seedling nutrition at 5 days post-germination, and that this contribution originates from a region of the endosperm that is known to contain little starch. Figure 3.12i shows Lugol's stained longitudinal sections of germinating seedlings and the endosperm to reveal starch degradation. Starch can be seen most abundantly in the area immediately adjacent to the embryo in the early stages of germination, but by 48 hours has been reduced. At 96 hours starch is not detectable with Lugol's staining, and endosperm structure is obviously degraded. In comparison to results shown in 3.12ii we can see that seedling growth is still showing significant increase at 7 DAG, five days after starch can no longer be detected. This result indicates that a stored reserve other than starch is contributing to seedling nutrition and that the likely source of reserves fueling growth at this stage originates from cell walls. A more detailed analysis using Calco-fluor staining shows a progressive depletion of cell wall material during germination, shown in figure 3.11ii, with walls become attenuated to 2.38±0.74µm at 6 days post-germination, suggesting mobilisation of cell wall components during germination.

i)

24 hrs

48 hrs

96 hrs

ii)
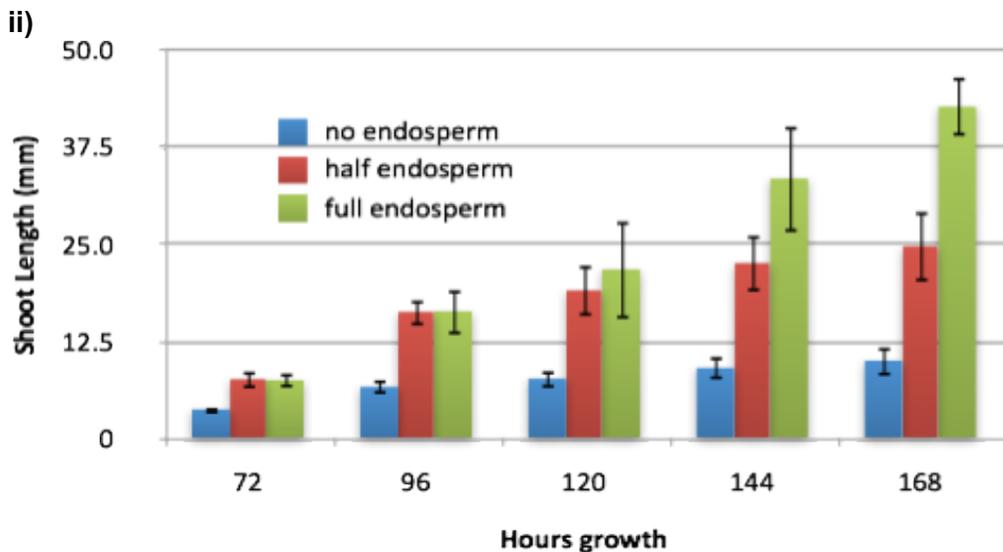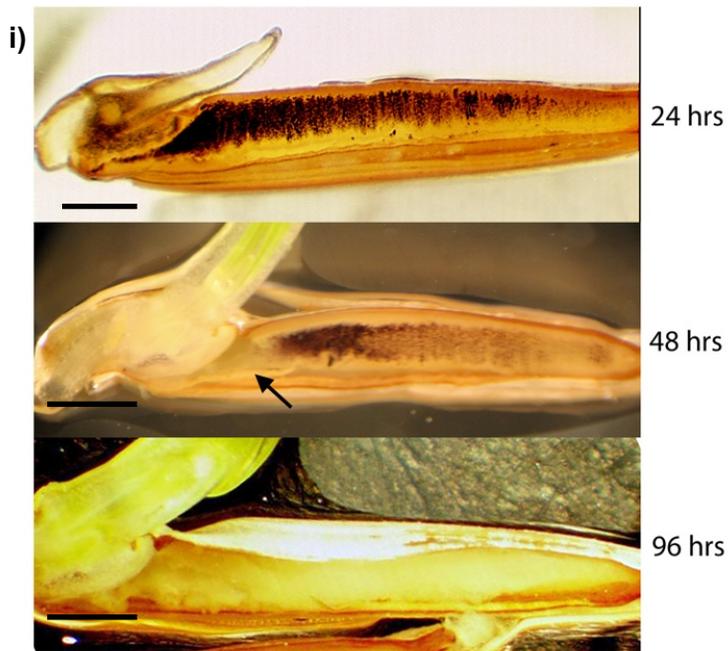
Figure 3.12: Starch Depletion and Utilisation During Germination.

i) Endosperm starch depletion at germination is shown with Lugol's staining for starch in half-grain longitudinal sections of germinating seedlings. Rapid depletion of starch can be seen over the early stages of germination, occurring most rapidly in proximity to the embryo, indicated by an arrow at 48hrs AG (after germination). Degradation of the endosperm structure is visible at 3 days AG. Scale bar shows 1mm.
ii) Results of partial and full endosperm excision and the effects on shoot length in germinating seedlings. Seedlings subject to majority endosperm removal show little increase in shoot length at 3 DAG. Seedlings with an intact endosperm show increased seedling shoot growth at 7 DAG as compared to those where the distal portion has been removed. These results indicate cell wall material in the distal endosperm portion is contributing to seedling nutrition.

A final feature of comparison was the distinct shape change observed in the endosperm over developmental maturity, desiccation and germination. Figure 3.13i reveals these differences showing that between late maturity and desiccation the *Brachypodium* grain becomes obviously shrunken and contracted, shape being somewhat restored at imbibition. The wheat grain, in contrast, does not show an equivalent change. To examine if differences in water uptake upon imbibition were responsible for these changes we measured the change in grain weight over 24 hours from the beginning of imbibition. We reasoned that the differing grain compositions and storage profiles may be influential to water requirements. Figure 3.13iii shows the pattern of water uptake is similar between the two with only a slightly greater percentage water uptake in *Brachypodium* as compared to wheat. The endosperm cavity area of the wheat grain shows the most obvious changes at imbibition, it is known to contain a mucopolysaccharide material seen to become engorged at imbibition (Bewley & Black, 1994). Images in figure 3.13ii, supplied by M. Parker (IFR), show this change in imbibed wheat grains. This function of this pectinaceous material is unclear, possibly involved in a simple "plugging" and protective function in the germinating wheat grain (M. Parker pers. Comm.) or may have a function in assimilate flow from nucellar projection to endosperm during grain filling. The *Brachypodium* shape change appears to be more widely dispersed across the whole area of the endosperm. It is a possibility that in the wheat grain the high starch level and "packing" of central endosperm cells with abundant starch granules (see Fig. 3.10) may be responsible for maintaining the wheat endosperm shape upon desiccation. These observations suggest that whilst a similar percentage of water uptake exists between the two differences in spatial
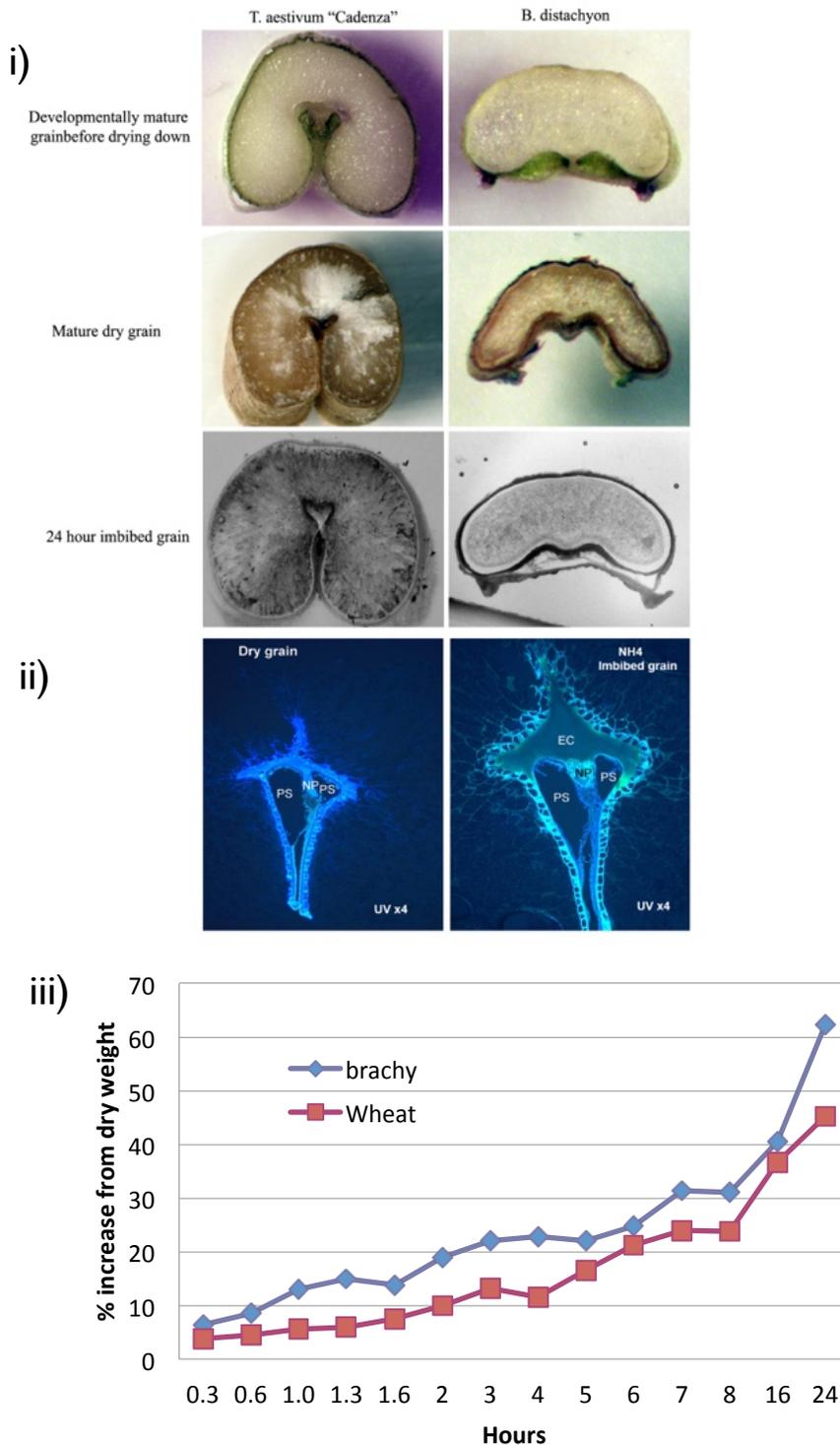
Figure 3.13: Water Uptake & Changes in Grain Shape Over Desiccation & Imbibition.
i) Transverse central sections of mature wheat and *Brachypodium* grains showing the
typical shape change observed between maturation, desiccation and imbibition. The
Brachypodium grain shows a notable change in shape at desiccation. ii) Florescence
microscopy of the wheat endosperm cavity region before and at imbibition, showing
significant expansion and water uptake. Image supplied by M. Parker (IFR). iii) Grain
weight percentage water uptake comparison between *Brachypodium* and wheat over
imbibition. A similar pattern and volume of water imbibition exists.

location and distribution may be associated with differences in function and endosperm storage reserves.

3.3 Chapter Conclusion

Results and observations presented in this chapter provide a comprehensive cellular and molecular description of grain development in the *Brachypodium distachyon*. Many of these observations have been published in a paper providing a reference map underpinning future work on genetic control of *Brachypodium* grain development (Opanowicz *et al*, 2011). *Brachypodium* has potential as a rapid and cost-effective model for dissecting numerous aspects of grass biology, providing a small rapid cycling model whose biology has not been influenced, at least directly, by humans[1]. This analysis of grain development indicates that, although somewhat more closely allied to the Triticeae, *Brachypodium* is intermediate in many respects between this group and rice, with features that may be unique to wild and forage grasses. We have identified significant differences in endosperm regional differentiation, notably an apparent lack of the modified aleurone region, poor definition of the peripheral aleurone layer and integration to the central endosperm, an important grain-processing trait. Low starch content is an atypical feature amongst the grasses and just one point of difference between

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[1] It is prudent to note here that whilst *Brachypodium* does represent a wild species this does not equate to complete freedom and isolation from domestication selection pressures. A cosmopolitan distribution and status as weed in some areas of crop production is indicative of *B. distachyon's* association with areas heavily influenced by human activity, and as a native to some of the areas where temperate cereal production is thought to have emerged it is likely to have felt some selection in relation to anthropised environments over a period of time that is potentially equal to that in which temperate cereal crops have been cultivated.

Brachypodium and wheat underlying a physiological distinction between these two species.

The *Brachypodium* grain is not only a good model for many aspects of cereal biology but it will also be informative in understanding the evolution of diversity in grain structure across the grasses. *Brachypodium* is recognized as an invasive weedy species in many parts of the world (Bakker et al, 2009) and so may provide a valuable model for the investigation of invasive traits and potential novel methods of control. Utilising *Brachypodium* and the growing collection of associated resources has potential to provide a great deal of valuable insight in a diverse range of research areas, notably the progression of the domestication process and endosperm biology, but in relation to grain biology and specifically the applicability to wheat functional research, care will need to be taken to ensure it is representative of the trait under investigation.

CHAPTER 4.

COMPARATIVE SURVEY OF GRAIN MORPHOLOGY AND ORGANISATION IN THE CORE POOIDS

4.1 INTRODUCTION

Members of the Core-Pooids represent the most important crops in temperate

zones including wheat, barley and oats. Their importance as crops is largely due to

the grain, particularly the storage capabilities of the endosperm. Grain shape and

structure has great significance relating to value and evolutionary history, but few

studies have compared features amongst the temperate cereals. In this study a

comprehensive survey of grain morphology and endosperm organisation, aspects

of ecology and physiology in representatives of wild and cultivated species

throughout the Core-Pooids was performed. Using macroscopic, histological and

molecular analyses distinct patterns of grain tissue organisation in a range of wild

and cultivated species, focusing on the peripheral and modified aleurone, are

described. As sister to the Core-Pooid tribes Poeae, Aveneae, Triticeae and

Bromeae the *Brachypodium* genus provides a taxonomically relevant reference

point. A great diversity in grain shape can be identified amongst the Pooid cereals

and our results indicate that endosperm organisation and differentiation is

correlated with conventional grain quality characters such as grain shape and

starch content. The evolutionary history of wheat offers an opportunity where high

levels of genetic similarity between modern hexaploid wheat and its progenitors

can be exploited. Grain domain specific molecular markers developed in hexaploid

wheat can be used to examine expression patterns in ancestral wheat species

revealing potential evolutionary patterns in grain structure and organisation within

the Pooideae. Features commonly associated with grains are largely defined by

analyses on lineages within the Triticeae and knowledge of grain structure may be

skewed as a result of focus on wheat and barley. This survey serves to

contextualize knowledge of cultivated temperate cereal grain morphology, to link it

to features of ancestral, wild grains and provide a backdrop for future research.

4.2 Results and Discussion

4.2.1 Distinct and Diverse Grain Shape Exists in the Pooideae

25 species spanning twelve Core-Pooid genera and neighbouring *Brachypodium*

were selected, providing good coverage and representation of important wild and

domesticated groups within the Pooideae. Figure 4.1 lists these species at the

tribal level within the Pooideae and larger grass family. The Pooideae is a large

subfamily occupying a position between the Ehrhartoideae and Panicoideae,

containing rice and maize respectively. The species examined here are focused on

the core Pooids, the Brachypoideae as a sister tribe to these groups forms an

outgroup and anchor to the range of genus examined.

A striking range of grain shape, size and colour can be seen amongst these

species, shown in figure 4.2. Initially we grouped grains according to shape

determined by ratios of size dimensions, length, width and depth (Fig 4.3). Whilst

a fairly continuous range could be seen in all dimensions it was possible to allocate

grains to three shape classes. These classes, defined by width and depth,

correlate to the grain cross-section profile. Allocation of these classes is shown on

the chart and also in table 4.2. Brachypoideae, Bromeae and the Poeae all show a

single shape class, flat. In contrast, the full range of shape classes can be seen

amongst the Triticeae with the rounded profile being dominant, particularly in

relation to the domesticated species. It should be borne in mind however that the
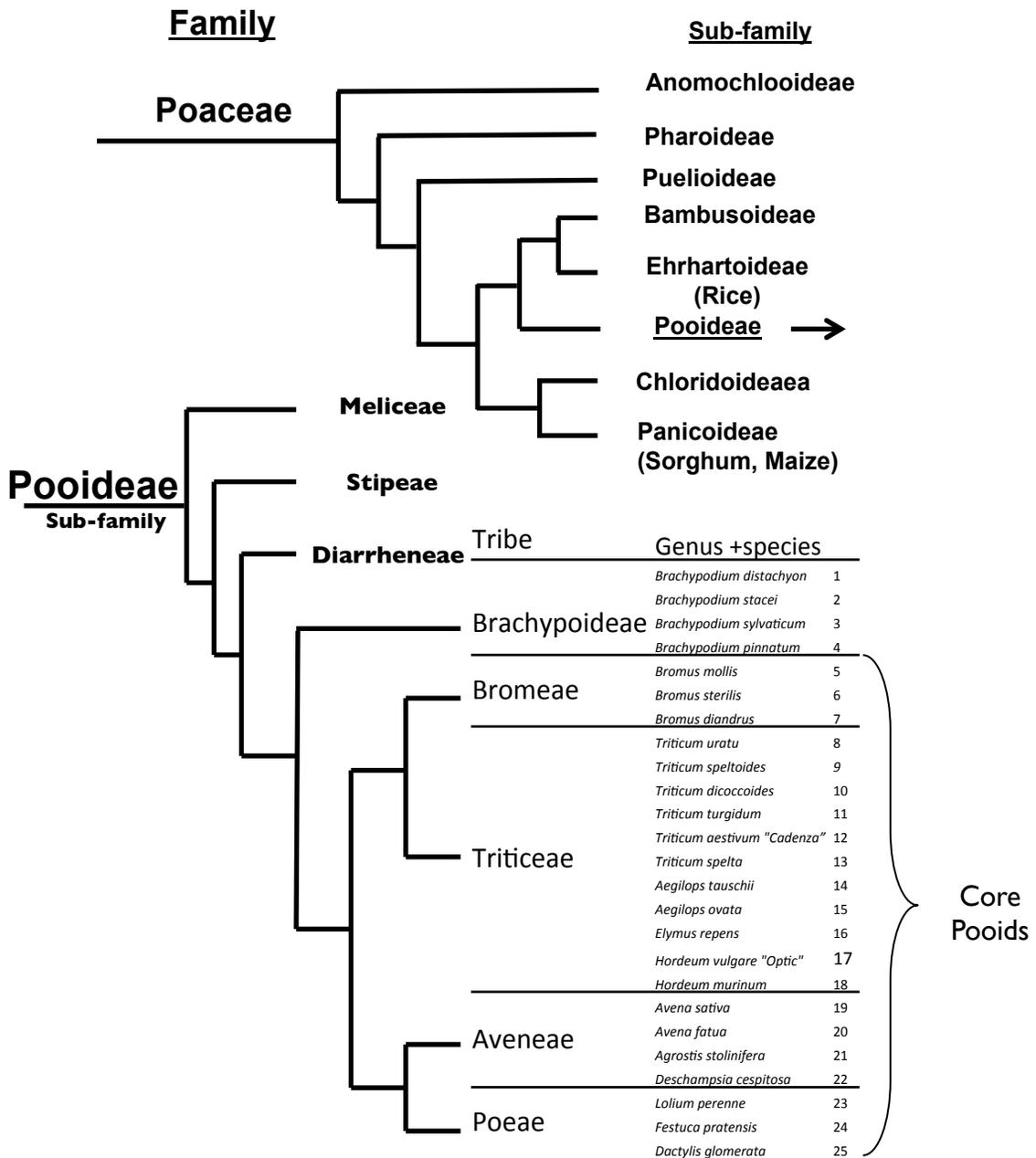
Figure 4.1: Identification & Phylogeny of Selected Taxa
Phylogeny for the species selected in this study showing the tribal groups of the
"core pooids". Pooideae sub-family can be seen to position intermediately to the
Panicoideae and Ehrahtoideae within the wider Poaceae. Based upon GPWG
phylogenies.
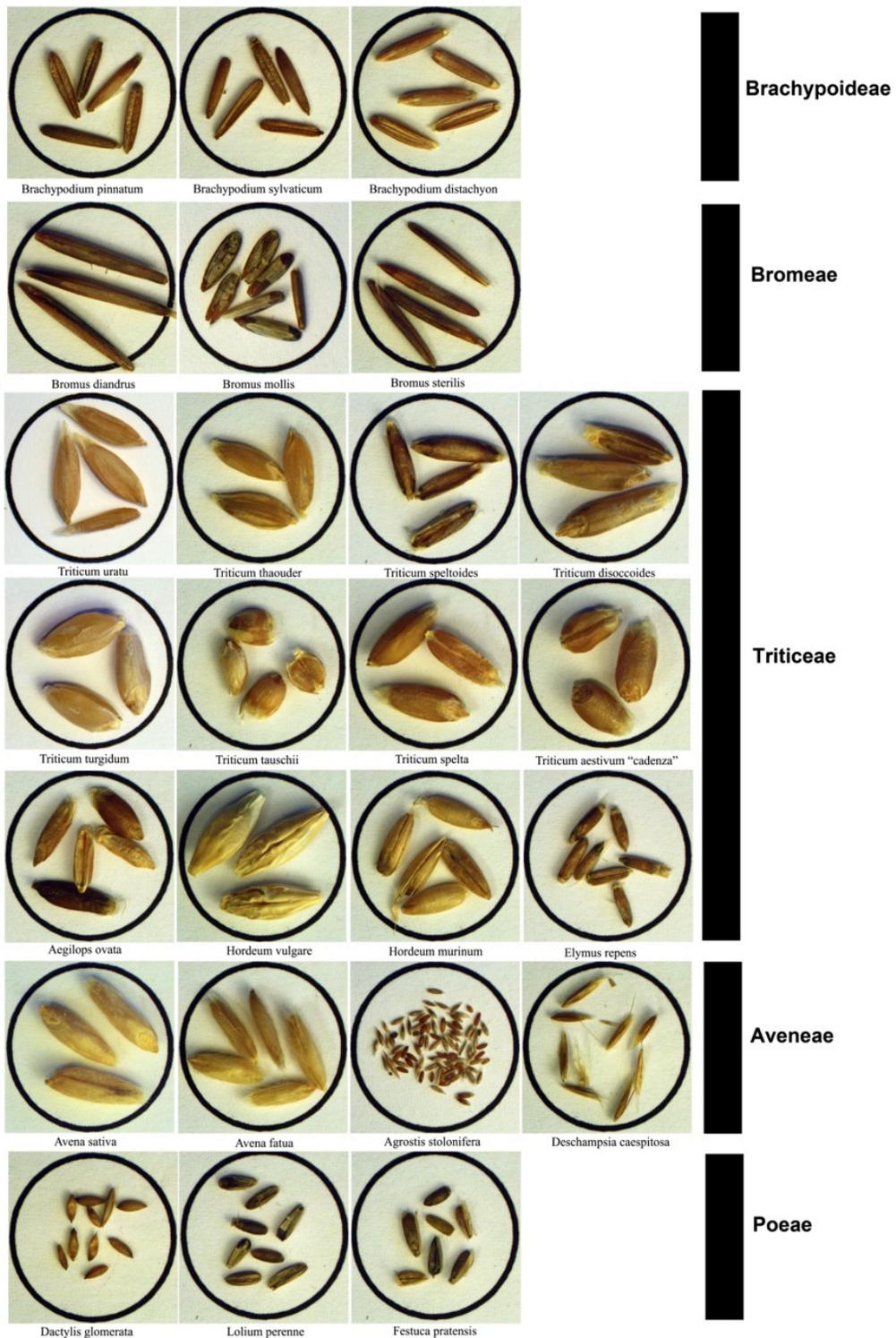
Figure 4.2: Selected Mature, Dry & Dehusked Grains.
Panel shows the contrasting and diverse range grain shapes seen various species examined here. Horizontal bars indicate organisation of species according to tribal groups and grains shown in circles 4cm diameter.

Triticeae is the largest tribal component and represents 52% of the overall

sampling, a factor that may account for some of the greater diversity seen. The

Bromeae tribe can be seen to show the greatest inter-genus size variability,

particularly in relation to grain length, such extreme variation not seen in any other

genus. The highest width and most of the highest depth measurements are shown

by domesticated species, whilst grain lengths are modest in these species,

indicating extreme length is perhaps not generally selected for under

domestication. Error bars in figure 4.3 indicate that the largest values are seen

mostly in wild species thus suggesting, not unexpectedly, that size variation is

more common amongst the wild species as compared to domesticates.

 To compare this grain size data more comprehensively we performed a PCA

(Principle Component Analysis) for grain dimensions. Figure 4.4 shows the plot for

components 1 and 2 which together encompass 96% of the variability and includes

the loadings to show correlation between variables. The broad distribution across

all areas of the plot reflects the diversity of grain sizes and coloured circles indicate

the tribal size ranges. The size range for Brachypoideae and Poeae are small, their

data points being very close, consistent to their similar sizes shown in figure 4.3.

Similarly, the larger elliptical range of Bromeae reflects the variation in length

shown. Notably, all of the domesticated species can be seen to fall into a fairly

discreet area on the left-hand side of the plot area distinct from the wild species.

Oat, barley and wheat differ significantly in floral morphology, usage and genetic

make-up and yet show a very similar grain size/profile under domestication. This

grouping for grain shape between the domesticated species suggests some

degree of uniform selection under domestication for this target grain size and
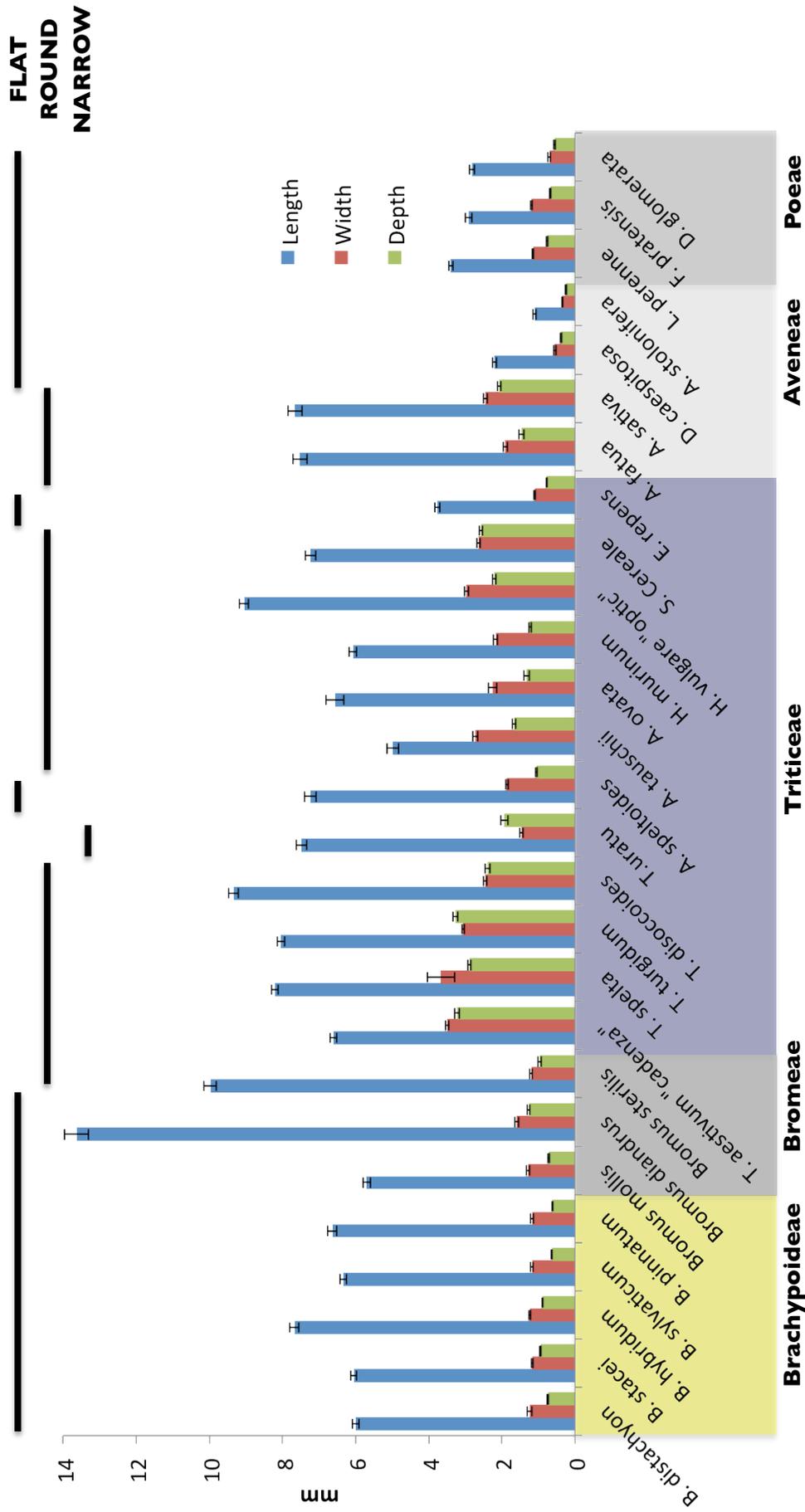
Figure 4.3: Mature Grain Dimensions (Length, width and depth) for Species Surveyed
Horizontal bars show grain profile classification. Tribal groups indicated by color shaded blocks below. n=20, error bars show ± 1 standard error

shape range. The size range of the Triticeae is a dominant feature, spanning the

majority of the plot area, although the large size of the Triticeae species sample

must again be taken into account.   However, it can be seen that the Triticeae

distribution is dominated by two clusters, with domesticates to the left hand side

and most wild species close to the centre of the plot area. The extreme range of

the Triticeae is largely a product of two species, *A. tauschii* and to an even greater

extent *E. repens*, located on the right-hand side of the plot area. These two grains

show some distinct and distinguishing features in relation to size and shape , *E.

repens* is particularly interesting in showing a profile quite different to most of the

other Triticeae members considered here. The Aveneae shows the next largest

size range and is the only other tribe to contain domesticated cereal crop species.

The range of the Aveneae is broad, *A. sativa* clusters with other domesticates, *A.

fatua* occupies a more central position but close to the domesticated area, whilst

other members are located on the far right.   Although not domesticated *A. fatua* is

frequently associated with anthropized environments and often recognised as a

weedy species (Hubbard, 1954), and so this position separate from the other wild

members and close to the domesticated species may be a reflection of these

weedy traits and influence over grain size.

4.2.2 Focus on Grain Shape Within the Brachypodium Genus

The recent reclassification of *B. distachyon* cytotypes has split the genus into 3

distinct species based on chromosome number and other significant molecular and

morphological differences (Catalan et al, 2012). *B. stacei* and *B. hybridum* were

described for the 2n=20 and 2n=30 cytotypes respectively, whilst *B. distachyon*
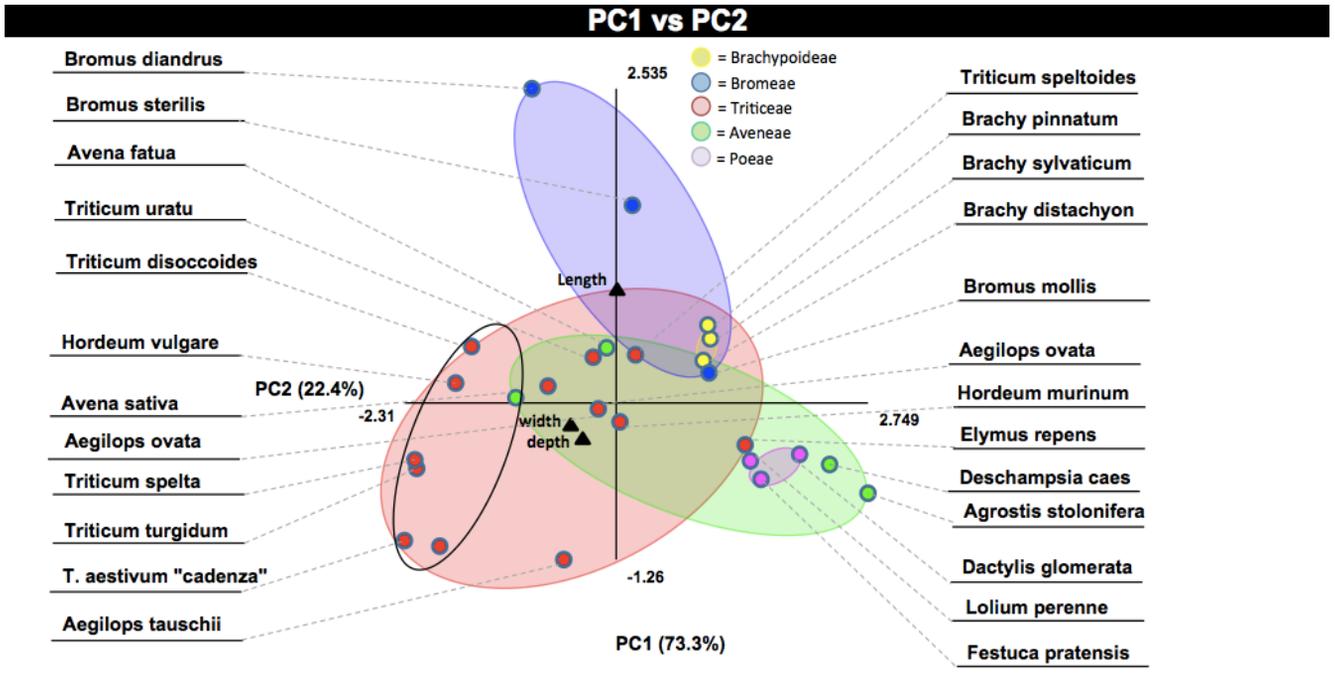
Figure 4.4: PCA for Grain Dimensions

Principle Component Analysis plot, including loadings, for average grain length, width and depth measurements of the grain transverse profile for selected species. Black triangles indicate scatterplot loadings, coloured circles indicate grain shape range of individual tribes and unshaded black circle highlights the grouping of all domesticated species.

	Genus species	Common name	habit	Domestic/ wild	Free threshing/ Hulled	C-S-R	Typical Habitat
Bromeae	*Bromus mollis*	Common soft brome	A	W	H	CS	Common. Moist and dry wasteland and grassland
	Bromus sterilis	Barren Brome	A	W	H	CS	Dry hedgebanks, roadsides and waste ground.
	Bromus diandrus	Great Brome	A	W	H	CS	Common. Waste ground and sandy shores
Triticeae	*Triticum uratu*	Two grain einkorn	A	D	H	n	Cultivation
	Triticum thaouder	Two grain einkorn	A	W	H	n	Cultivation
	Triticum speltoides	Goat grass	A	W	H	n	Dry regions, waste and grasslands
	Triticum aestivum "Cadenza"	bread wheat	A	D	F	n	Cultivation
	Triticum spelta	Spelt	A	D	F	n	Cultivation
	Triticum disoccoides	Emmer (wild)	A	W	H	n	Various but typically, dry open grasslands.
	Triticum turgidum	Durum	A	D	F	n	Cultivation
	Aegilops tauschii	Taush's/jointed Goat grass	A	W	H	n	Weed in wheat crops
	Aegilops ovata	Ovate goat grass	A	W	H	S	Roadsides, disturbed grass and agricultural land
	Elymus repens	common couch grass	P	W	H	C/CR*	Common. Grassland, roadsides, waste and cultivated ground. Bad weed.
	Hordeum vulgare "Optic"	cultivated barley	A	D	H	n	Cultivation
	Hordeum murinum	Wall barley	A	W	H	n	Annual, Lowland, wasteground and roadsides.
Aveneae	*Avena sativa hexaploid*	cultivated oat	A	D	H	n	Cultivation
	Avena fatua	wild oat	A	W	H	R/CR	Waste and arable ground.
	Agrostis stolinifera	Creeping bent grass	P	W	H	CR	Common. All grasslands except acidic. A weed in cultivated land
	Deschampsia cespitosa	Tufted hair grass	P	W	H	SC	Common, marshy grasslands and moors, damp woodlands.
Brachypoideae	*Brachypodium distachyon*	Purple false brome	A	W	H	n	Dry, open waste & grasslands
	Brachypodium sylvaticum	Wood False Brome	P	W	H	CS/CSR	Open woodland & scrub, hedgerows. Persists in originally wooded. alkaline ground.
	Brachypodium pinnatum	Chalk false brome/ Torgrass	P	W	H	C	Calcareous grassland, and coastal.
Poeae	*Lolium perenne*	Perennial ryegrass	P	W	H	CR/CSR	Common. Meadows & pastures, and often roadsides and waste ground
	Festuca pratensis	Meadow fescue	P	W	H	SC/CSR	Common & widespread. Low lying grasslands and meadows, often in water meadows
	Dactylis glomerata	Cocks foot	P	W	H	C/CSR	Common & abundant. Meadows, pastures, roadsides and roughgrasslands

Taken from Rose 1989, Hubbard 1992 and Kew grassbase. C-S-R score follows Grimes strategy, C = competitor, S = Stress tolerator & R = Ruderal. n= data not available. Scores collected from selected references detailed, and where *indicate scores allocated to genus level or similar species.

Table 4.1: Habit & Floral Characteristics

Details of selected species relating to habit, habitat, floral characteristics and and strategy. Species are arranged according to tribe, indicated in the left hand column. For some species the common name can yield information relating typical habitat or habit. The annual habit is dominant in the Triticeae examined here. D or W refers to species' status as either domesticated or wild. C-S-R shows published details of species primary strategy, were data is available.

remains for the 2n=10 cytotype. Some aspects of floral morphology can be seen

to reflect to this reclassification. In contrast to *B. distachyon,* flowers of *B. stacei*

show longer anther length and anthers are exerted at, or soon after, anthesis.

Grains in *B. stacei* and *B. hybridum* also appear to typically be larger than those of

B. distachyon and this may be a reflection of the overall larger plant size as

compared to *B. distachyon*. In order to investigate the possibility that differences in

grain size do exist between these three novel species we examined grain length,

width and depth at maturity. We included data from two other *Brachypodium*

species, *pinnatum* and *sylvaticum* and, after confirming the data was suited (Using

a F_{max} test), we performed a one-way parametric ANOVA for each. It was noted

that the F_{max} value for width was very slightly above the critical value, but being

very close we decided to proceed with the test. We found a highly significant

difference between these species in all dimensions (length; F=43.0, *p*>0.001,

width; F=5.29, *p*=0.001, depth; F=90.92, p>0.001). To further investigate we

repeated this test on just *B. distachyon*, *stacei* and *hybridum* and again found

highly significant differences in all grain dimensions (length; F=87.25, *p*>0.001,

width; F=12.33, *p*>0.001, depth; F=46.85, p>0.001). In both of these tests the

greatest differences exist in grain length and depth, where calculated values were

greatly higher than the critical (calculated values for this and subsequent tests can

be found in appendix figures A4b-c). Establishing that significant difference did

exist we went on to perform a Tukey's test in order to identify where the largest

differences could be seen. The results of this test are shown in figure 4.5, dotted

lines mark the $T_{critical}$ value whilst horizontal bars show the $T_{calculated}$ values for each

pair, with absolute values and detail included in appendix tables A4d. We found
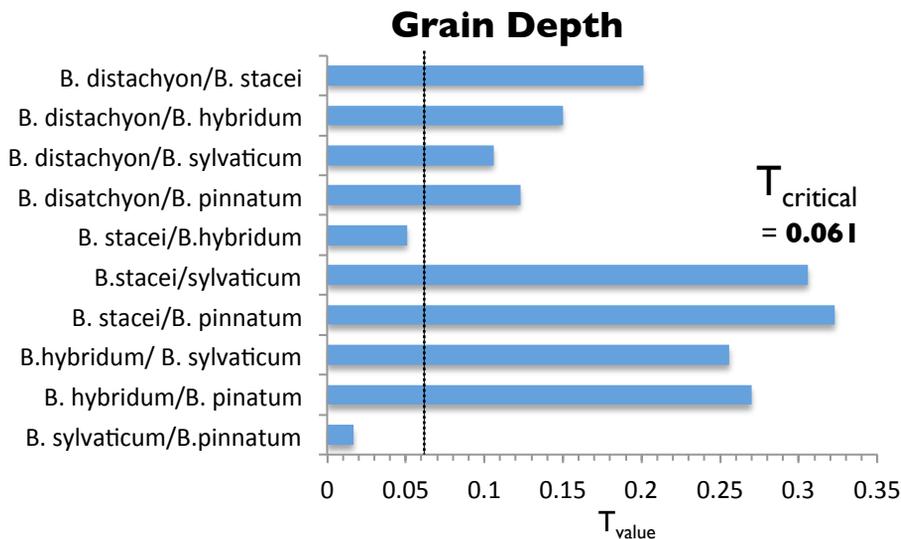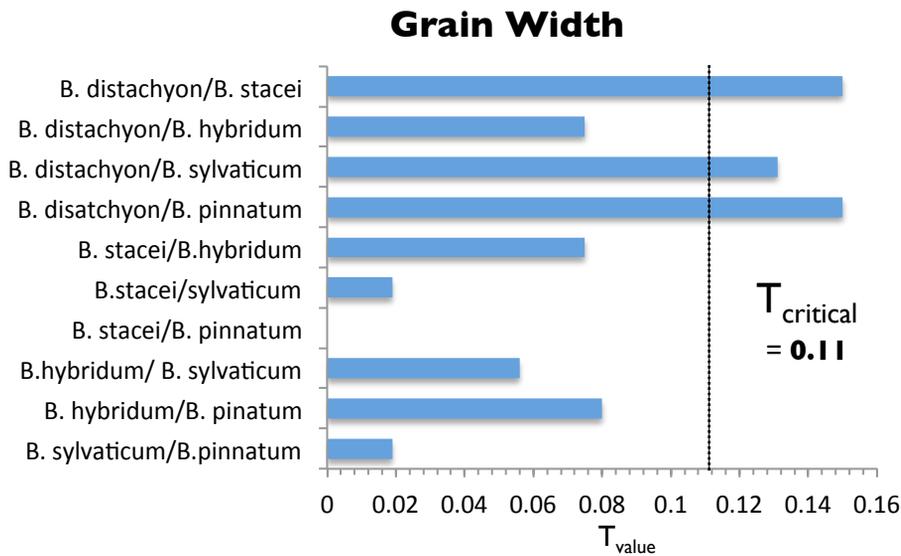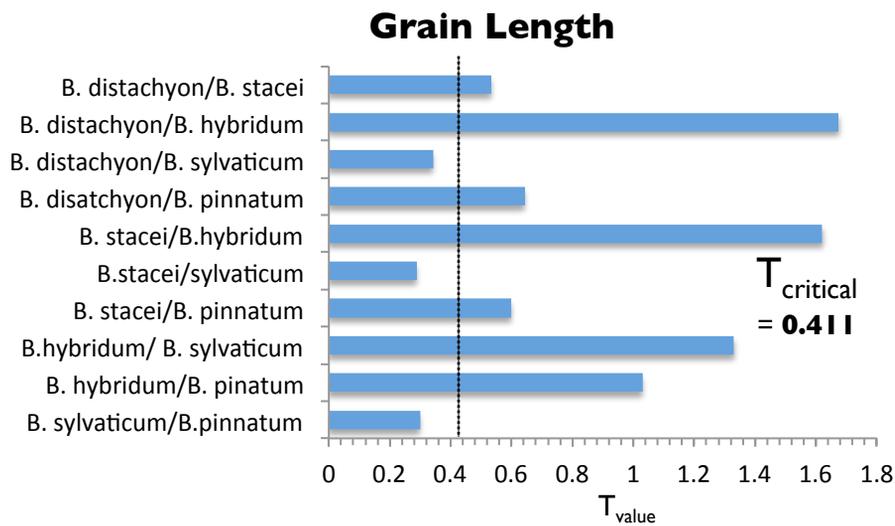
Figure 4.5: Graphical Illustration of Results of One-way Parametric ANOVA and Tukey's Test on Grain Length, Width and Depth in *Brachypodium* Species
Pairwise comparisons of grain dimensions between species are shown. Horizontal bars show $T_{calculated}$ values for all species and grain dimensions. Dotted line indicates $T_{critical}$ value for each data set.

significant and variable levels of difference to exist between both the novel and previously classified *Brachypodium* species, in all dimensions. Whilst varied, some notable features of this data were identified. Specifically, *B. stacei* and *B.hybridum* differ significantly only in length, in contrast to comparison of these species with *B. distachyon*, where highly significant difference can be seen in all dimensions with the exception of the grain width to *hybridum*. This inter-specific distinction can also be seen between *B. distachyon* and the two previously existing species, *sylvaticum* and *pinnatum*, where significant levels of difference are seen in all dimensions with the exception of length to *sylvaticum*. These results indicate that differences in grain size we have detected reflect this recent reclassification of the *B.distachyon* cytotypes and that highly significant grain size variation exists between both previously and newly classified species. Results also indicate that grain width for *B. distachyon* (2n=10) is a distinctive feature of this species, being the only one to show any significant difference. Failure to identify any significant difference between *pinnatum* and *sylvaticum*, and between *hybridum* and *stacei* and these species in some dimensions also suggests that grain shape may be a somewhat unique feature of the 2n=10 *B. distachyon* species.

Whilst the calculated values produced in this test often showed highly significant differences in grain size there is a great deal of variation and the relationship between these differences is difficult to describe and often somewhat unclear. Increasing the sample number to reduce some of the internal variation would be beneficial and may produce a clearer result. As a possibility for future research we would obtain a larger data set for grain size using instrumentation such as the MARVIN grain analyser (GTA; Sensorik). Repetition of this test on a much larger

data set could be used to confirm and more fully investigate these grain size differences.

4.2.3 Grain Ecology in the Core Pooids

Species' ecology, floral morphology and habit greatly influences grain characteristics and features of floral morphology are often critical to grain development, defining interaction with the environment and subsequent evolutionary adaptation (Elbaum et al, 2007; Primack, 1987). In comparative analysis examining just dried and dehusked grains it could be possible to overlook or misinterpret important features and characteristics. Table 4.1 provides key details for selected species' ecology and floral morphology.

The majority of the grains considered here are hulled, as is typical of all except the domesticated flour-crop species. The "hulled" state refers to grains shed from the plant in a protective structure comprised of a palea/lemma envelope, and often other floral parts. The "Grain Dispersal Unit" (GDU) can be used to describe the article, comprised of 1 or more grains, hull and other floral parts shed from the plant as the inflorescence reaches maturity, and can be distinctly variable between species for the level of floral organisation at which they remain attached and the point of disarticulation.

The outer layers comprising the hull not only have a great influence on grain morphology but also over factors such as dormancy, dispersal, germination and protection from predators (Finch-Savage & Leubner-Metzger, 2006). Figure 4.6 shows GDUs from some of the species examined here where some distinct tribal level differences can be seen, specifically relating to the level of floral organisation
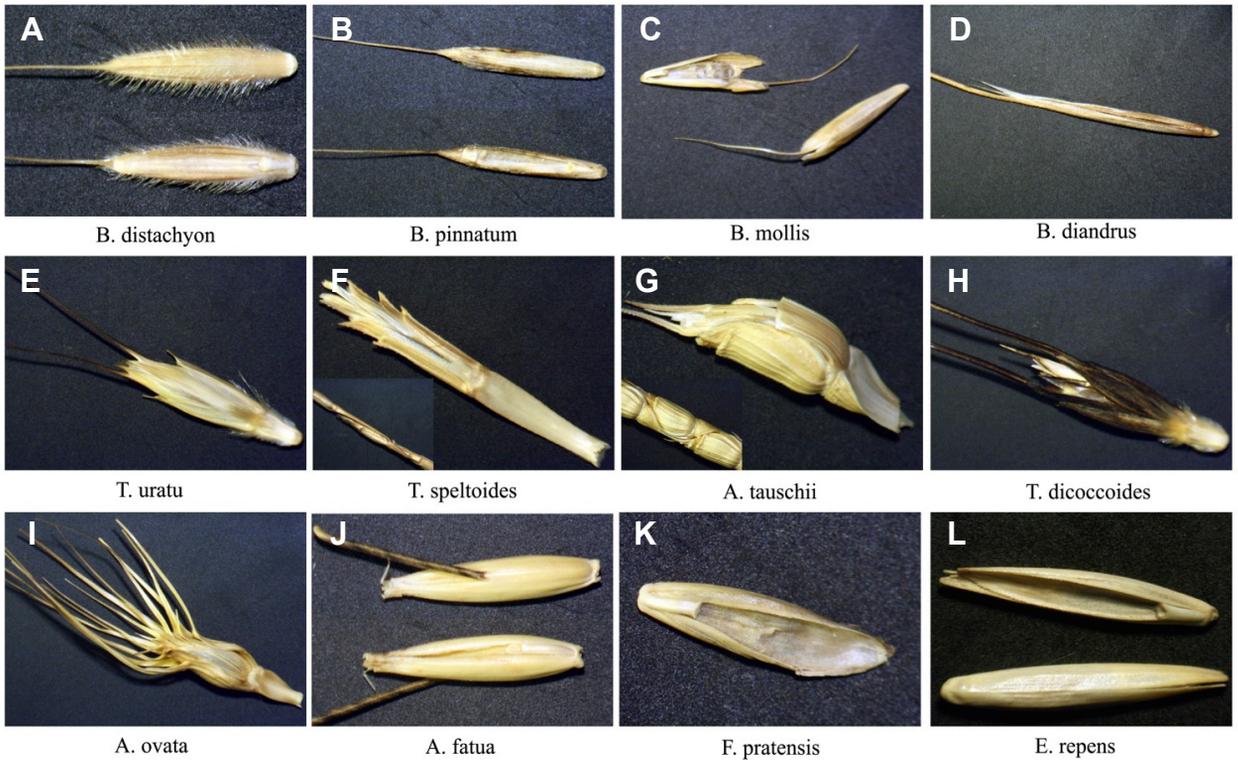
Figure 4.6: Grain Dispersal Units for Selected Species.
A highly varied shape and structure of grain dispersal units can be seen between selected species. Insets in F & G show similarity between inflorescence structure and the close fit of grain dispersal units into the spike structure.

at which disarticulation occurs. GDUs of all species outside of the Triticeae

examined here are comprised of the single seed with adherent and typically robust

lemma with associated features such as awns, hairs, barbs etc, a usually thinner

and sometimes transparent lemma, and a small section of rachilla. Disarticulation

occurs within the spikelet at the proximal end of the rachilla to give single-seed

grain dispersal units. The grains of *B. distachyon* and the *Bromus* species, along

with grains of *A. fatua*, *F. pratensis and E. repens* are all shed in units of this

composition (Fig 4.6, A-D, J-L). Distinctly different features and characteristics can

be observed between these species. *B. distachyon* shows a hairy and toughened

lemma in contrast to the papery, insignificant structure of *B. mollis*, *E. repens* and

F. pratensis, the latter two species do not posses awns. For the majority of

Triticeae members examined here however, the GDU correlates to the spikelet

level of floral organisation and disarticulation occurs at the proximal end of the

rachis. Here one or more grains and associated floret organs are shed inside

protective toughened glumes with an, often robust, rachilla attached as can be

seen in the *Triticum* and *Aegilops* species shown (fig 4.6 E-I). In particular in the

A. tauschii and *A. speltoides* GDU the glumes were found to be heavily toughened

and lignified, creating a very solid multi-grain dispersal unit. These GDUs can be

seen to fit together closely into a stout, robust spike structure (Insets, F-G).

Surfaces of these glumes are rough and minutely barbed, possibly to aid dispersal

via animal vectors. Certainly the numerous awns of the *A. ovata* seed dispersal

unit appear well suited to role in dispersal, most likely through attaching to sheep's

fleece, as its ovata name may suggest? As rich sources of nutrition it seems these

outer layers will also have some role in mechanical defence of the seed. The

glumes of the dispersal units in *A. tauschii* and *A. speltoides* are especially robust and grains would be well defended from all but the largest and most determined seed predators.

Seed-adherent floral organs have been shown to function in grain dormancy through the release of germination-inhibiting hormones, an important function likely favoring their retention in certain grasses (Finch-Savage & Leubner-Metzger, 2006). The robust glumes of Triticeae GDUs or lemma of *A. fatua* or *B. distachyon* would take time to become broken down sufficiently for germination-release, and so may provide a solid dormancy characteristic. *T. tauschii,* as an example, has an ancestral range spanning mountainous areas dominated by rocky habitat where robust dormancy characteristics in order to survive winter conditions at altitude may be favorable, and it is known that the D-genome possess some extreme cold hardiness traits (Limin & Fowler 1981; Valiellahi et al, 2010; Zohary & Hopf, 2001: Snyder et al, 2000).

A notable exception to the prevalence of spikelet-level GDUs amongst Triticeae species exists in *E. repens*, grain disarticulation occurring individually as it does in *Brachypodium*, *Bromus* and *Festuca*. As can be seen in figure 4.6 the *E. repens* GDU is in external appearance very dissimilar to that of the Triticeae species and is conspicuously smooth and awnless. The lack of these features indicates that in relation to grain dispersal *E. repens* may depend upon quite different mechanisms as compared to morphologically similar species such as *B. distachyon* or *B. mollis*, where hairs and papery lemma have function in insect or wind mediated dispersal respectively (Pulliam & Brand, 1975). This simple grain dispersal unit may relate to

a lesser significance of seed reproduction in *E. repens* and it's greater investment in vegetative reproduction (stoloniferous).

Images of naked grains, i.e. all outer layers manually removed, for all of the species examined here are shown in figure 4.2 providing an interesting comparison to the species included in figure 4.6. The difference and potential significance of these grain dispersal unit features is easily apparent and it is clear they will be major factor in any environmental adaptation of the grain. In most cases external features of the actual grain will have only minor role in their physical interaction with the environment.

Table 4.1 provides details of habit for selected species and where possible their primary strategy scores according to those defined by Grime (1977). Some of the variation seen amongst GDUs, and floral morphology, of these grasses is perhaps a reflection of different strategies and habits. Plant habit can be highly significant to reproductive strategy, the level of investment into and nature of seed production.

PCA for grain size and distributions shown in figure 4.4 can be seen to show some correlation to the differing species habit. All Triticeae species examined here are annuals, this habit typified by high investment into seed production (Grime, 1977). The only exception in this group is *E. repens*, which has a perennial habit and a notably a different grain morphology and size as compared to other Triticeae members. *Agrostis* and *Deschampsia* also both display a perennial habit and occupy a position at the opposite edge of the Aveneae's large grain size range to *A. fatua* and *A. sativa*, both with annual habits, and close to *E. repens* (Fig 4.4). Differences in habit are reflected in distribution according to grain size, with all of the perennial species grouping into the lower right-hand corner of the plot area.

This separation is as would be expected, as perennial plants will often tend to favour vegetative reproduction over seed production to some extent, with the frequent consequence of smaller seeds (Zhang, 1998). It is this cluster in the lower right corner of the plot area that is largely comprised the smallest seeds. Conversely, the large grained domesticated species are all located on the left-hand side of this plot area, which is again as would be expected as domesticated cereal crops are largely annual species.

Habit and strategy are largely defined by characteristics of a plant's typical environment, specifically conditions of high/low stress and disturbance define primary strategy (Grime, 1977), which in turn are greatly influential to grain characteristics. In relation to grain biology competitor and ruderal strategies are most relevant, these being most strongly linked to reproductive efforts, the stress-tolerance strategy being largely associated with vegetative physiology (Grime 1977). Table 4.1 lists the primary strategy for some species as identified through literature searches. The annual habit is strongly linked to the ruderal strategy and where information concerning strategy was available it appears that amongst the C-S-R scores obtained a competitor and ruderal element dominates. The domesticated environment primarily favours the ruderal strategy and to a lesser extent a competitor strategy with pressures like seedbed competition, selection for rapid maturity etc, where a larger seed size for rapid germination and growth will dominate in relation to domestication selection. The Bromeae share an annual habit and a grain size range that is somewhat distinct from the other species considered here, and also distinctive in their strategy, being CS (Competitor/stress tolerator strategy). A competitive-ruderal strategy can be seen in *A. fatua* and in *E.*

repens both of which are recognised as weedy and invasive species (Hubbard, 1954; Froud-Williams, 1984) a trait that is expected with this type of strategy.

The agricultural environment provides some of the highest disruption levels of any along with strong selection for rapid germination and growth. Data relating to the direct assessment of ecological strategy in domesticated species is limited, but knowledge of domestication process, the environment and habit of these species produces a strong expectation for the domesticated species to display dominantly ruderal traits. This contrasts to some extent with what is seen in the wild species' strategy listed here, where a significant competitor element is seen (Table 4.1). This suggests that amongst Pooid grasses ruderal and domestication traits are closely linked and are relevant to agriculturally important features of grain size/shape. *A. fatua* can again provide a good example here, as a semi-domesticate and weedy species it is located on the left-hand side of the PCA plot; its position is separate from those species with a strongly competitive strategy, focused on the right hand side, and close to the area populated by domesticates, and is also the only species that is identified as showing a dominantly ruderal strategy. An investigation of strategy and ecology between *H. murinum* and *H. vulgare* would provide an interesting discussion and test here. The position between these two *Hordeum* species in the PCA plot is similar to that of the two *Avena* species. A typical habitat of wasteland and roadsides, an often disturbed location, is suggestive *H. murinum* may follow an at least partially ruderal strategy, but we did not encounter any reports of weediness traits in this species. Examination of *H. murinum* to determine if greater competitive traits along with a

smaller grain size as compared to *H. vulgare* can be identified could then be used as further evidence for this link between ecology and grain size.

Detail of typical habitats included in table 4.1 indicate that species examined here exist across a great range of environments and conditions. This range spans xeric, mesic and hydric grasslands, wooded or scrub land and includes high stress environments such a shorelines, calcareous and extreme arid conditions. Within this wide range of ecological adaptation and strategy it is possible to question and compare what differences exist between grains from species adapted to these conditions.   Of the three *Brachypodium* species there is significant diversity in habitats. *B. distachyon* favours dry, poor conditions whilst *B. sylvaticum* is more suited to richer soils and lower light conditions. *B. pinnatum* is largely particular to calcareous coastal grassland and along with *B. sylvaticum* show a perennial habit, in contrast to the annual habit seen in *B. distachyon*. Grain size and shape amongst the Brachypoideae is very similar however. *B. distachyon* originates from an ancestral range similar to that of the primitive wheats, so will likely show adaptation to similar climatic conditions (Opanowicz et al, 2008). As an example *T. uratu* is highly aridity tolerant and typical of poor, rocky soils (Colledge, 2007), these traits are closely shared by *B. distachyon*. The small stature of *B. distachyon* particularly when grown under stressful, arid conditions and it's tendency towards such habitats suggests that it is unlikely to have felt the same pressures of anthropogenically altered environments or selection such as were in early wheats. However, grain size in relation to the plant is large in *Brachypodium* and figure 4.3 shows that grain length is very similar to that of the wheat species. This feature of grain size may be relevant in the dispersal and range of *B. distachyon*. The range

of *B. distachyon* has spread to become cosmopolitan and established in many grain producing areas across the world; this distribution may be attributable to some extent to this large grain size allowing them to become incorporated into harvests and equipment. If dispersal of this kind is responsible for the distribution of *B. distachyon* then it is also possible, and likely, that selection in relation to an anthropogenic environment has been felt in some populations.

 All of the Poeae species considered here are typical of moist environmental conditions quite different in character to those of *Brachypodium* and *Bromus*. The Bromeae and Poeae species are united in sharing a competitor and stress tolerator element to their strategies but all of the Poeae species show a perennial habit as opposed to annual. Poeae grains have more extreme lower right hand side distribution in the PCA plot suggesting that grain traits associated with a perennial habit influence grain shape in this way. The information produced here suggests an association between smaller seed size and perennial habit for those species favouring environments that are more amenable to plant growth, in contrast to specialisation of the *Bromus* species to more xeric habitats. The association between smaller seed size, occurrence in moist, amenable habitats and a perennial habit is plausibly explained by easier conditions for seedling establishment and a lower risk associated with higher investment in vegetative reproduction. The larger seed size of the Triticeae and *Avena* species however differs to that of the *Bromus* species and occurs where these species and underpins their adoption of the ruderal strategy, and so it is not unexpected that the majority of our cereal crop domesticates have arisen from this pool.

Consideration of species' ecology is a valuable aspect of a comparative study like this. Features of plant habit and environment can be seen to correlate to distinctive features of grain size and shape, and are important factors in specifying seed qualities. This comparison is fundamental but is relevant in the great many other factors and aspects of the environment defining grain biology and structure.

4.2.4 Distinct Grain Transverse Profiles and Features can be Defined

Our analysis of grain external dimensions reveals a great diversity of grain shape amongst these species but that the rounded grain profile (i.e. similar width and depth values) dominates amongst the domesticated species. Figure 4.7 shows the transverse section of dry, mature grains of several species at a central point in their length whilst table 4.2 summarises key features of the grain cross-section histological and structural organisations. Amongst these domesticated species a lobed endosperm gives a distinctive crease on the adaxial side of the grain. This shape, consistent amongst the high yielding Pooid crop species, is likely to be concomitant with maximising the accumulation of storage reserves originating from the central vascular bundle into the smallest space.   All species' grains are curved around a vascular strand to some extent, although only amongst the wheats, barley and oat (Both wild and cultivated) could a closed crease, where the lobes make contact on the adaxial side of the grain, be seen. The rounded, creased grain profile, in particular those of the Triticeae, is an indication of a high-yielding reserve-rich structure but the presence of a crease represents a barrier to efficient milling (Campbell, 2011; Evers & Millar, 2002). The closed-creased profile could be seen throughout almost all of Triticeae, always on the adaxial side of the grain
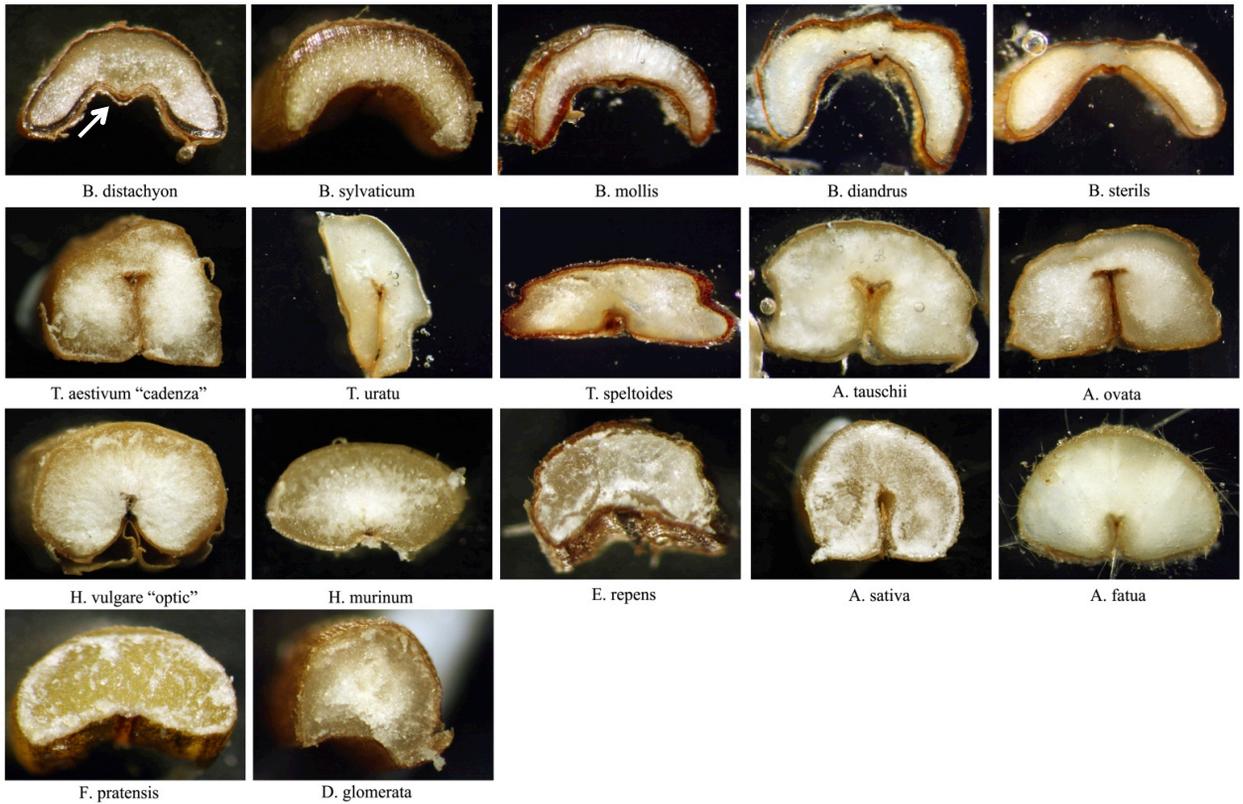
Figure 4.7: Selected Grain's Transverse Profile at Maturity.
Images show central cross-section of mature, dry grains. Not shown to scale. Variation in grain profile can be seen between species. Arrow highlights unique convexity in *Brachypodium* adaxial endosperm.

although depth was variable between species. A sharp contrast to the typical rounded profile of the hexaploid and tetraploid wheats could be seen in the diploid species *T. uratu* where the narrow profile results in a deep crease (fig. 4.7), and *A. speltoides* with a flat and only shallowly creased profile. The hexaploid spelt wheat, though rounded and with a closed crease, has a narrow abaxial endosperm bridge domain and therefore also a decidedly deep crease (Highlighted in later figure, 4.9. I). *E. repens*, though also in the Triticeae is exceptional in the openness of its adaxial side, and is the only member examined here that did not show the creased profile. With regard to this adaxial flatness it closely resembles the grains of *Lolium*, *Festuca* and *Dactylis* in the Poeae (see Fig. 4.7 and 4.9 for comparison). Grains of the Bromeae and Brachypoideae are distinct from the other tribes in showing a flat or crescent shaped profile. Within the Bromeae there was a noticeable difference between the *mollis* and *diandrus/sterilis* species. The latter species had a more obvious lobed profile reminiscent of the wheats, while *mollis* was starkly flat with barely any appearance of a crease (Fig. 4.7). Amongst those grains with a flat or crescent shaped profile *B. distachyon* is distinct in showing a prominent convexity in the crease region overlaying the vasculature not seen in other species (Fig. 4.7, arrowed).

An endosperm cavity is a feature visible in almost all of the grains with a rounded and deeply creased profile, notably being seen throughout the Triticeae with the exception of *Elymus* (Table 4.2). In the Aveneae, a cavity exits in *A. sativa* but was not obvious in *A. fatua*, nor in the uncreased grains of *D. glomerata* and *A. stolonifera*. The occurrence of an endosperm cavity can be seen to largely correlate with an obvious nucellar projection (Table 4.2). *B. mollis* and *E. repens*

	Genus species	Domesticated/ Wild	Grain profile	Crease	Nucellar projection	Cavity	Persistent nucellar epidermis
Brachypoideae	*Brachypodium distachyon*	W	Flat	**open**	-	-	+
	Brachypodium sylvaticum	W	Flat	**open**	-	-	+
	Brachypodium pinnatum	W	Flat	**open**	-	-	+
Bromeae	*Bromus mollis*	W	Flat	**open**	**+s**	-	+
	Bromus sterilis	W	Flat(crescent)	**open**	**+**	+	+
	Bromus diandrus	W	Flat(crescent)	**open**	**+s**	+	+
Triticeae	*Triticum uratu*	D	Round(Narrow)	**closed**	**+**	+	-
	Triticum speltoides	W	Flat	**closed**	**+**	+	-
	Triticum aestivum "Cadenza"	D	Round	**closed**	**+**	+	-
	Triticum spelta	D	Round	**closed**	**+**	+	-
	Triticum disoccoides	W	Round	**closed**	**+**	+	-
	Triticum turgidum	D	Round	**closed**	**+**	+	-
	Aegilops tauschii	W	Round(flat)	**closed**	**+**	+	-
	Aegilops ovata	W	Round	**closed**	**+**	+	-
	Elymus repens	W	Round(flat)	**open**	**+s**	-	-
	Hordeum vulgare "Optic"	D	Round	**closed**	**+**	+	-
	Hordeum murinum	W	Round	**closed**	**+s**	+	-
Aveneae	*Avena sativa hexaploid*	D	Round	**closed**	**-?**	+	-
	Avena fatua	W	Round	**closed**	**-?**	?	-
	Agrostis stolinifera	W	Round	**open**	-	-	-
	Deschampsia cespitosa	W	Round	**open**	-	-	-
Poeae	*Lolium perenne*	W	Round(flat)	**open**	-	-	+
	Festuca pratensis	W	Round(flat)	**open**	-	-	-
	Dactylis glomerata	W	Round	**open**	**nd**	-	?

Closed crease defined as maternal tissues on lobes being touching.   S= yes but only small or sometimes not apparent. nd = not determined; ? = status is unclear. D= domesticated. W= wild.

Table 4.2: Maternal Tissue & Grain Profile Characteristics

Summary of some maternal tissue and grain profile characters in mature grains of selected species.

represent exceptions to this general rule where a small pad of nucellar epidermis tissue can be identified, and does not correspond to an endosperm cavity. Figure 4.8 provides detail and comparison of the nucellar projection between species where it can be seen in the Bromeae species this structure is small and poorly defined as compared to what is typical in wheat species. The nucellar epidermis in wheat has been shown to be comprised of cells with transfer morphology and implicated as functioning in grain filling, facilitating the efficient flow of assimilates from vasculature to modified aleurone region transfer cells (Zheng & Wang, 2011; Bewley & Black, 1994). The route of assimilate flow into the rice endosperm however, has been seen to follow the path of the nucellar epidermis. A region analogous to the nucellar epidermis is absent and the nucellar epidermis is prominent across much of the rice grain's development (Oparka & Gates, 1981). The differences seen here suggest that grain filling mechanisms comparable to both rice and wheat can be identified in the species examined here and largely indicate that grain morphology favors just one of these routes.

The nucellar epidermis is persistent in *Brachypodium* and in *Bromus* genera only (Table 4.2) although there was also some evidence to suggest it could be seen in the Poeae, in *L. perenne* and possibly in *D. glomerata*. Images E-H figure 4.8 illustrate the range of nucellar epidermis thickness seen amongst wild species, with a prominent layer retained in *Brachypodium* and *Bromus* species, a reduced layer seen in *L. perenne* and the total obliteration of the layer by maturity in *E. repens* and other members of the Triticeae. In the majority of species outside of the Brachypoideae and Bromeae the nucellar epidermis was greatly reduced and compressed by maturity. These observations suggest that amongst the Pooideae

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there is potentially a distinct split in the mechanisms and route of endosperm filling where a survey at wider Poaceae sub-family level would be necessary to try and trace origins of these distinct morphologies.

4.2.5 Characteristics and Organisation of the Aleurone Layer Varies

As the most significant component of the grain we performed a focused analysis on endosperm organisation and structure between species, focusing on regional differentiation. Previous publications have shown high levels of similarity in endosperm organisation amongst grass species but that some key differences can be identified, such as differences in peripheral aleurone depth that can be seen in barley or rice as compared to wheat (Sabelli & Larkins, 2009; Costa et al, 2004). The presence or absence of a distinct modified aleurone functional domain is another significant area of difference, this region implicated as a major transfer tissue in the wheat grain. In the previous chapter we have detailed findings of a previously published analysis identifying distinct differences between *B. distachyon* and wheat endosperm. Amongst other differences, a key finding is that the *Brachypodium* aleurone layer is not regionally differentiated into distinct peripheral and modified aleurone regions and that this domain appears to be absent (Opanowicz et al, 2011).

We used vital staining as a simple and novel approach to identify and compare mature grain endosperm domains. Peripheral aleurone tissues are typically the only living cells in a mature cereal grain endosperm, other cells having undergone PCD at grain maturity (Young & Gallie, 2000; Consonni et al, 2005).

Again we used Tetrazolium chloride (TZ) staining to distinctively mark the actively respiring cells the peripheral aleurone domain in imbibed sections of mature grains (Peters, 2007). Figure 4.9 shows TZ stained sections of selected species and key details of this analysis are summarised in table 4.3. A larger panel of staining for the majority of species examined is included in the appendix figure A4e. All of the grains examined here show a living peripheral aleurone layer at maturity, as expected for grass caryopses, but variation in number of layers and regularity of organisation could be seen. A distinct single-cell layer was typical across the majority of species (see insets figure A4e for detail). Barley is the well-documented exception amongst the domesticated temperate cereal grains with a distinct 3-cell depth layer. However, a very similar 2-3 cell deep layer can be seen in *B. sterilis*, in contrast to the other members of the Bromeae. All of the *Brachypodium* species showed a highly irregular peripheral aleurone layer ranging from 1-4 cells in depth, very different from the aleurone of other species examined here. The *Brachypodium* peripheral aleurone layer appears somewhat disordered at the cellular level, this feature also being seen to some extent in *F. pratensis* (see A4e).

Across the Brachypoideae and certain other species a living aleurone could be seen to extend around the entire periphery of the grain. This continuation of the peripheral aleurone layer can be used as an indication of the absence of distinct modified aleurone region (summarised table 4.3). In a previous study we have shown that the modified aleurone region in wheat is typically dead in healthy mature grains (Opanowicz et al, 2011), this is also the case in cultivated barley grains (Fig. 4.9e). As a consequence, the living peripheral layer shows a distinct
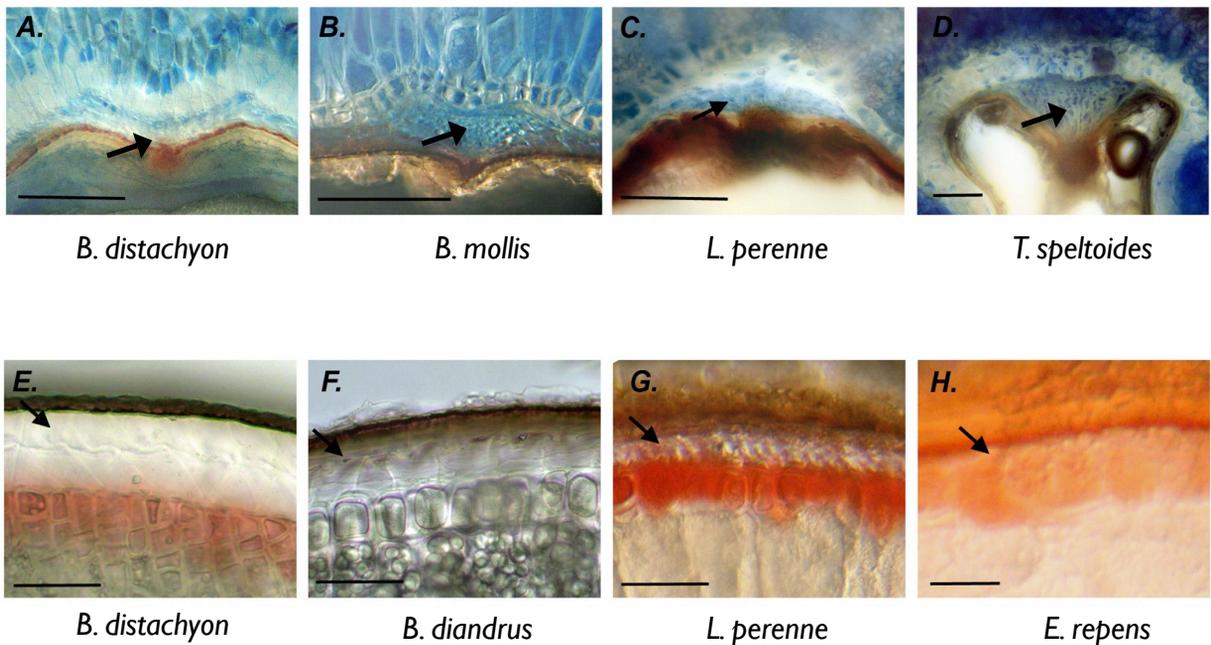
Figure 4.8: Differences in Nucellar Tissues Between Selected Species

Structural differences in nucellar projection and epidermis identified during examination of vital stained sections. A-D show differences in nucellar projection size and occurrence identified during Evan's blue vital staining. For *B. Distachyon* (A), almost no nucellar projection is visible, whereas in *B. mollis* (B) and *L. perenne* (C) a small and poorly defined structure exists in comparison to *T. speltoides* (D) where a prominent nucellar projection is visible. Black arrows highlight the nucellar projection region/tissues. Scale bars show 50 µm. E-F shows differences in nucellar epidermis thickness in the adaxial face of mature grains, the layer indicated by a black arrow in all images. *B. distachyon* (E) shows the thickest layer and *B. diandrus* (F) shows a nucellar epidermis of comparable thickness whilst *L. perenne* (G) shows a layer that is very much reduced and in *E. repens* (H) the nucellar epidermis is largely obliterated and barely visible, as is typical amongst the Triticeae. Scale bars show 40 µm.

Genus species	peripheral aleurone depth (Cells)	Modified aleurone vital stain	Cellularly differentiated MA region	central endosperm cell size	Central endosperm cell wall thickness	Starch granule morphology
Brachypodium distachyon	1-4 irregular	+	-	~ = al	> al	simple
Brachypodium sylvaticum	1-4 irregular	+	-	~ = al	> al	nt
Brachypodium pinnatum	1-4 irregular	+	-	~ = al	> al	simple
Brachypodium stacei	1-4 irregular	+	-	~ = al	~ = al	simple
Bromus mollis	1	?	?	> al	> al	simple
Bromus sterilis	2-3	-	+	> al	< al	simple
Bromus diandrus	nt	?	?	> al	< al	simple
Triticum uratu	1	-	+	> al	< al	bimodal
Triticum speltoides	1	+	+	> al	< al	bimodal
Triticum aestivum "Cadenza"	1	-	+	> al	< al	bimodal
Triticum spelta	1	+	+	> al	< al	bimodal
Triticum disoccoides	1	-	+	> al	< al	bimodal
Triticum turgidum	1	-	+	> al	< al	bimodal
Aegilops tauschii	1	+	+	> al	< al	bimodal
Aegilops ovata	1	?	?	> al	< al	bimodal
Elymus repens	1	-	+ s	> al	< al	bimodal
Hordeum vulgare "Optic"	3	-	+	> al	< al	bimodal
Hordeum murinum	1	?	?	> al	< al	bimodal
Avena sativa hexaploid	1	+	+ s	> al	< al	compound
Avena fatua	1	?	?	> al	< al	compound
Agrostis stolinifera	1	+	-	> al	< al	compound
Deschampsia cespitosa	1	+	-	> al	< al	compound
Lolium perenne	1	+	- s	> al	< al	compound
Festuca pratensis	1	-	- s	> al	< al	comp/simp
Dactylis glomerata	?	?	?	> al	< al	compound

nt = not tested; s = yes but slight; ? = status is unclear

Table 4.3: Mature Grain Endosperm Characteristics
Summary of endosperm histological and cytological characteristics in mature grains of selected species.

break across this central region overlaying the vasculature. Vital staining patterns in the adaxial domain where the modified aleurone would be expected varied across species but in all *Brachypodium* and *Bromus* this region consistently stained with TZ and, like the peripheral aleurone, is living tissue at maturity (Fig. 4.9a-c). Cultivated oat shows very similar shape and organisation as barley and wheat grains, but here we also consistently detected living tissues in the modified aleurone domain (Fig 4.9 h). A dead modified aleurone region could be seen in the majority of the Triticeae species but for some species variation in the consistency and intensity of staining in both the peripheral and presumptive modified aleurone made the results unclear. Specifically variation was seen in *T. spelta* and *A. tauschii* showing staining in this region. Variation was also seen in staining of the presumptive modified aleurone area in *E. repens* and *F. pratensis*, although for the latter variation was slight and the area more typically living. Evan's Blue staining provides a similar method of marking vital tissues but stains only dead tissues being excluded from living cells with an intact membrane, and has been used previously to show live and dead tissues in the wheat endosperm (Young & Gallie, 1999; Li et al, 2010; Opanowicz et al, 2011). We performed Evans blue staining as a complementary analysis to the TZ to confirm these results, shown in the appendix figures A4f and A4g. Results obtained very largely agreed with that of the TZ. In *T. speltoides* they are more indicative of a live modified aleurone region, but results remain unclear for *A. tauschii*. In *F. pratensis* results confirm a living continuous peripheral aleurone. Staining in *E. repens* was more consistent and reveals the presumptive modified aleurone region (i.e. that overlaying the vasculature) area was typically comprised of dead cells.
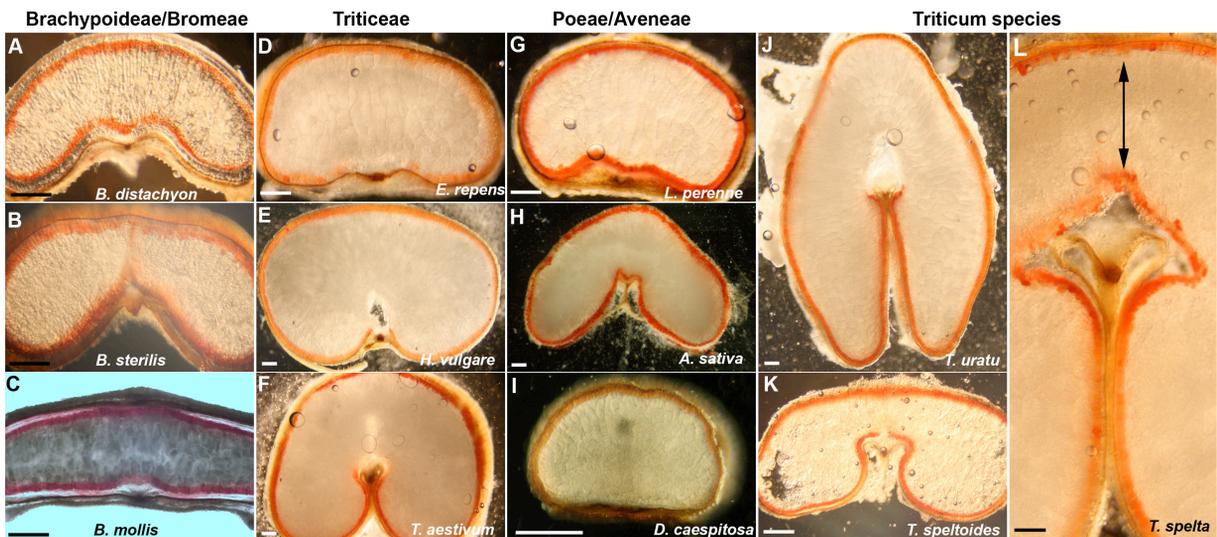
Figure 4.9: Vital Staining of Selected Mature Grains

Panel shows Tetrazolium Chloride (TZ) stained transverse central sections of mature grains from several genera revealing details of the extent of peripheral and modified aleurone domains. Variation in the status of the endosperm modified aleurone, i.e. that immediately overlaying the vascular tissue, as either live or dead can be seen. Dead modified aleurone tissues can be seen in some of the Triticeae (images E, F & J) whilst in others it is alive (K & I). Live modified aleurone region tissue can be seen in the Aveneae and Poeae species (G-I), and to some extent Bromeae (B & C). Living tissue in the presumptive modified aleurone region is visible in *B. distachyon* (A). Double ended arrow shows short bridge depth in Spelt wheat. Scale bars show 400 µm.

In microscopic analysis of sectioned cereal grains it was also possible to identify some physical differences in the modified aleurone region of cells in relation to both the peripheral aleurone and central endosperm region. These differences in many cases make the region visually distinct, and relate to combined features of cell size, shape and organisation as identified during extensive observations. These characteristics are most obvious when using toluidine blue or Evan's blue staining techniques. Observed differences of this kind are listed in table 4.3 as a cellularly differentiated region. In all except the Brachypoideae some degree of cellular differentiation in the endosperm region overlaying the crease was visible. In particular amongst the core Pooids, cellular differentiation was always associated with a dead modified aleurone region and was visible to some extent in the majority of species. Features of the *Brachypodium* peripheral aleurone layer distinguishing it from the central endosperm, which include viability status and features of cell size and wall thickness, were seen to extend around the entire periphery consistently and without interruption.

All of the sections examined here were taken from the central point in the length of the grain. To consider the possibility of variation in aleurone status along grain length longitudinal-sections and proximal-distal serial grain sections of some species were also examined using TZ staining. *Festuca*, *Lolium, T. aestivum* and *B. distachyon* were examined in this way with no difference in aleurone status identified, although some variation in staining pattern relating to imbibition was identified, detailed in appendix figure A4h. Another observation made during these analyses was the importance of using healthy and well-filled grains. In the wheat variety examined here, "cadenza", shriveled and poorly filled grains (typically

resulting from poor and stressful growing conditions) sometimes showed the occurrence of live modified aleurone cells, compared to well-filled grains where it is consistently dead. Evers and Millar (2002) state that endosperm shriveling is almost always a manifestation of poor nutrition or disease leading to poor endosperm development. We speculate that during grain filling a feed-back mechanism exists whereby as the central endosperm becomes filled it provokes PCD programs for the modified aleurone cells, and where assimilate flow to the endosperm is lower than usual these mechanisms are not triggered. It is possible that a similar explanation may also account for some of the variation in the vital staining results for *E. repens*, and *T. speltoides*.

4.2.6 Molecular Mapping of Wheat Endosperm

The previous histological analyses reveal distinctive aleurone layer characteristics amongst both wild and cultivated grains. We hypothesised that characteristics and level of aleurone organisation may correlate to species status as either wild or cultivated and influence grain traits desirable under domestication. A highly differentiated and regularly organised aleurone perhaps confers traits such as more efficient storage properties or enhanced seed germination. To test this hypothesis we investigated endosperm organisation in the primitive wheat species at a molecular level in an effort to trace and compare structure across both wild and domesticated species. The wheat species examined here offer a promising and valuable cohort for comparative analysis. Spanning both *Triticum* and *Aegilops* genera these species offer representation of different stages of the domestication process. They provide a useful system for exploring both

Central endosperm
6E2
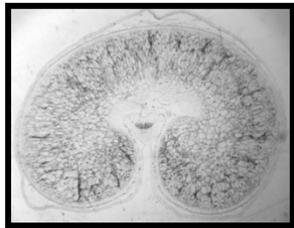
Aleurone
6B7

Modified aleurone
1G9

Storage protein

PPDK
(Photosynthesis)
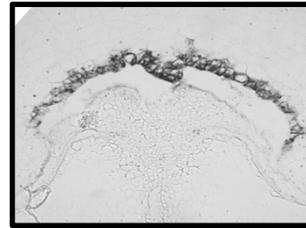
Ethylene forming
enzyme

**Figure 4.10: Domain Specific Expression Patterns of Wheat
Endosperm Molecular Mapping Probes in Hexaploid Wheat.**
Typical expression patterns for three ISH probes used for the mapping of
endosperm domains in primitive wheat species, shown in figure 4.11.
Each probe was shown to be very specifically expressed within their
respective endosperm domains (Drea et al, 2005b).
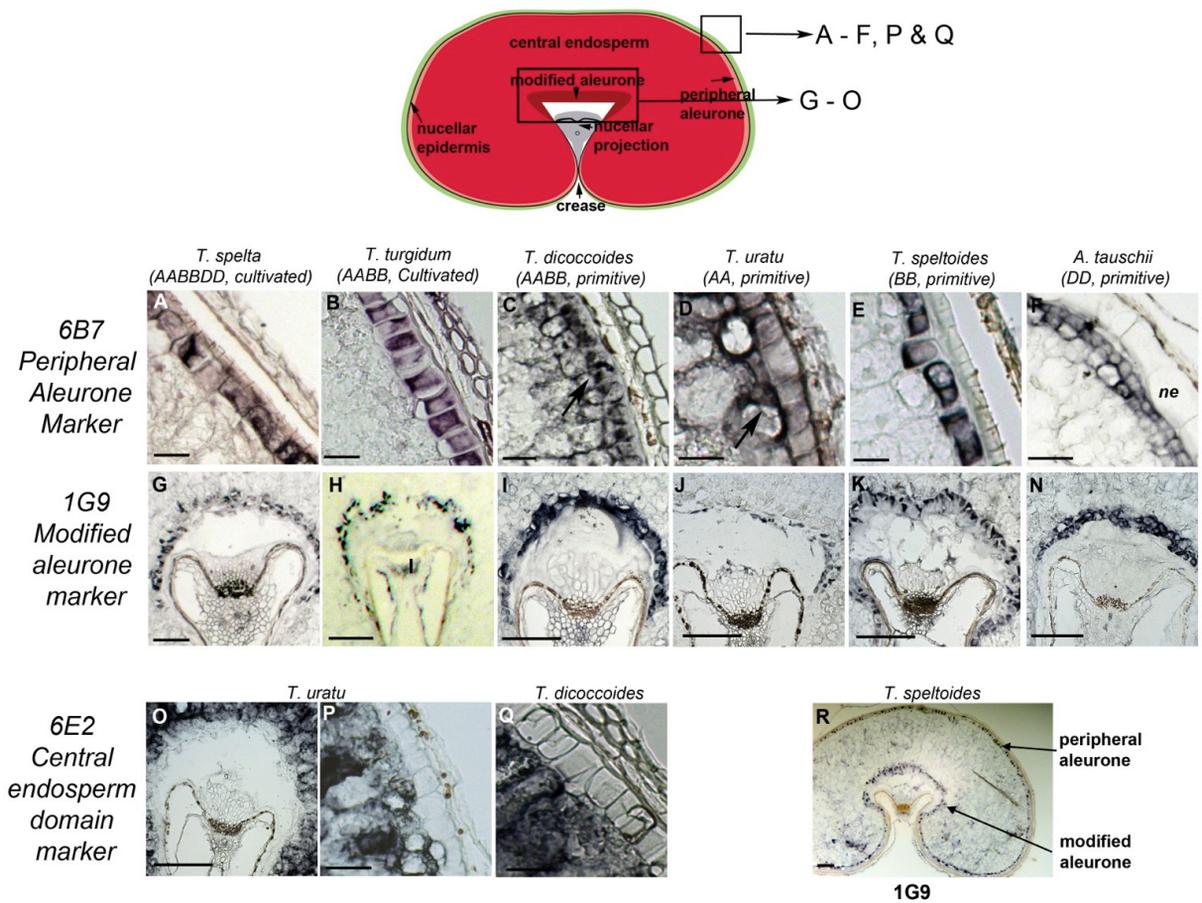
Figure 4.11: RNA ISH for Domain-specific Markers in Primitive and Cultivated Wheats
A-F show peripheral aleurone marker expression (6B7) in primitive and cultivated wheats. An
expression pattern restricted to a well defined aleurone is typical amongst the wheat species,
with the exception of *T. dicoccoides (C)* and *T. uratu (D)* with expression seen to extend into
the sub-aleurone layers, indicated with black arrows. G-N show modified aleurone marker
expression (1G9). Strong expression is detected for all species except *T. uratu*, where only
weak expression is seen giving a poorly defined modified aleurone region with this probe (J).
For *T. speltoides* 1G9 expression was not restricted to the modified aleurone domain and
could be seen throughout the peripheral aleurone, a larger image shown in R. O-Q show
central endosperm marker expression (6E2). O and P show expression in *T. uratu* with
exclusion from the modified and peripheral aleurone domains respectively, more clearly
defining these domains. Q shows expression in *T. dicoccoides* and again more clearly
defines an interface between central endosperm and peripheral aleurone domains. Scale
bars in A-F, P & Q show 50 μm, G-O show 200 μm and R shows 500 μm.

primitive/uncultivated and cultivated/domesticated species of the same tribe. A

modified aleurone region is identifiable in all mature grains but varies in its viability

status. In addition the primitive wheat species examined here represent all the

grain shape classifications we identified in the Pooideae, flat, round and narrow.

Previous work by Drea et al (2005b) identified a range of endosperm-domain

specific markers used in mRNA in-situ hybridisation (ISH) experiments to map

endosperm development and differentiation in hexaploid wheat. We selected

markers from this range showing strong and specific endosperm domain

expression and using a combination of PCR, comparative sequence analysis

where available, and speculative ISH established which of these probes show

homology and consistent, comparable expression across the primitive wheat

species (*T. uratu, A. speltoides, T. dicoccoides, A. tauschii*). Specifically we

isolated three probes; 6B7, a peripheral aleurone marker, encoding pyruvate

orthophosphate dikinase (*PPDK*), 1G9, specific to the modified aleurone region

encoding an iron/ascorbate oxidoreductase and 6E2 relating to a storage protein

gene with strong central endosperm specific expression. Localisation of these

three probes in hexaploid wheat is shown in figure 4.10.

mRNA ISH using DIG-labeled RNA probes was performed on mature grain

sections for primitive and uncultivated wheat species. The appendix figure A4i

shows the overall results of this analysis and figure 4.11 provides detail of

expression in peripheral and modified aleurone layers. Expression for each of the

three endosperm domain markers can be identified in all species with patterns

largely, and remarkably, similar to those seen in hexaploid wheat. However, small

but interesting variation in expression patterns can be identified, the greatest

disparity seen in some of the diploid species in relation to the distinction of the peripheral and modified aleurone regions. *A. speltoides* was interesting in that signal for the markers of the peripheral and modified aleurones could be seen in both domains simultaneously (Fig. 4.11 P). The modified aleurone region in *A. speltoides* is cellularly distinct, being several cells deep and irregular compared to the peripheral aleurone, but expression of 6B7, the peripheral aleurone marker could be seen to extend through this region, although expression was weaker as compared to that of the peripheral aleurone layer. The expression of 1G9 was detected in the modified aleurone region, where it is more than one cell deep, but was seen to extend around the endosperm in the peripheral aleurone at a consistent level (fig 4.11 P). In both *T. uratu* and *T. dicoccoides* the expression of 6B7 in the peripheral aleurone was comparatively weak and somewhat indistinct. Signal was detected into the subaleurone layers and give poor definition of the peripheral aleurone layer. In contrast the expression of 6E2, marking the central endosperm domain, could be seen to extend to a distinct and defined aleurone border (fig 4.11F & G). Together these results suggest a high level of similarity and some potential significant differences between endosperm functional domains in early wheat species and the more recent tetraploid and hexaploid domesticates.

4.2.7 Endosperm Cell Size and Cell Wall Thickness

Scanning electron microscopy (SEM) was performed on mature grains of representative species to examine endosperm cell size, organization and to determine cell wall thickness in the central endosperm compared to the aleurone layers. In this comparison members of the Brachypoideae were most distinct in

both their cell size and cell wall thickness comparison. Details of preliminary

examinations for all species are shown in table 4.3 whilst figure 4.12 provides

micrographs for selected species. A distinctive feature amongst *Brachypodium*

species was central endosperm cell size being similar to that of the aleurone (table

4.3). Within the *Brachypodium* genus *B. distachyon* showed consistently small

central endosperm cells, whilst cells were comparatively larger in other species, *B.*

stacei in particular showed some central cells larger than the peripheral aleurone.

Outside of the Brachypoideae endosperm central cells were several times larger

than the peripheral aleurone cells. To provide a more quantitative and specific

analysis we took precise cell length measurements of central endosperm and

aleurone cell size for selected species, listed in table 4.4. *B. mollis* was particularly

striking in relation to central cell length, where lengths of up to 80μm were

recorded, as compared to an aleurone cell length as small as 10μm. With such a

flat grain there may only be 4-6 cells spanning the abaxial-adaxial axis, this feature

is visible in the Evans blue stained section shown in appendix figure A4f. In

comparison the grains or *A. fatua* and the two Poeae species show an equally high

central endosperm to aleurone cell size ratio, however in these species it presents

a distinctly less visually imposing feature of the grain profiles as cell walls

thicknesses are notably lower.

  Brachypoideae species are the most distinctive in the thickness of their central

endosperm walls as compared to almost all other species, where cell walls of the

central endosperm are thicker than those of the aleurone (Table 4.4). *B. mollis*

provides the only comparable example amongst all other species examined here.

Along with the cell wall measurements included in table 4.4 figure 4.12 provides
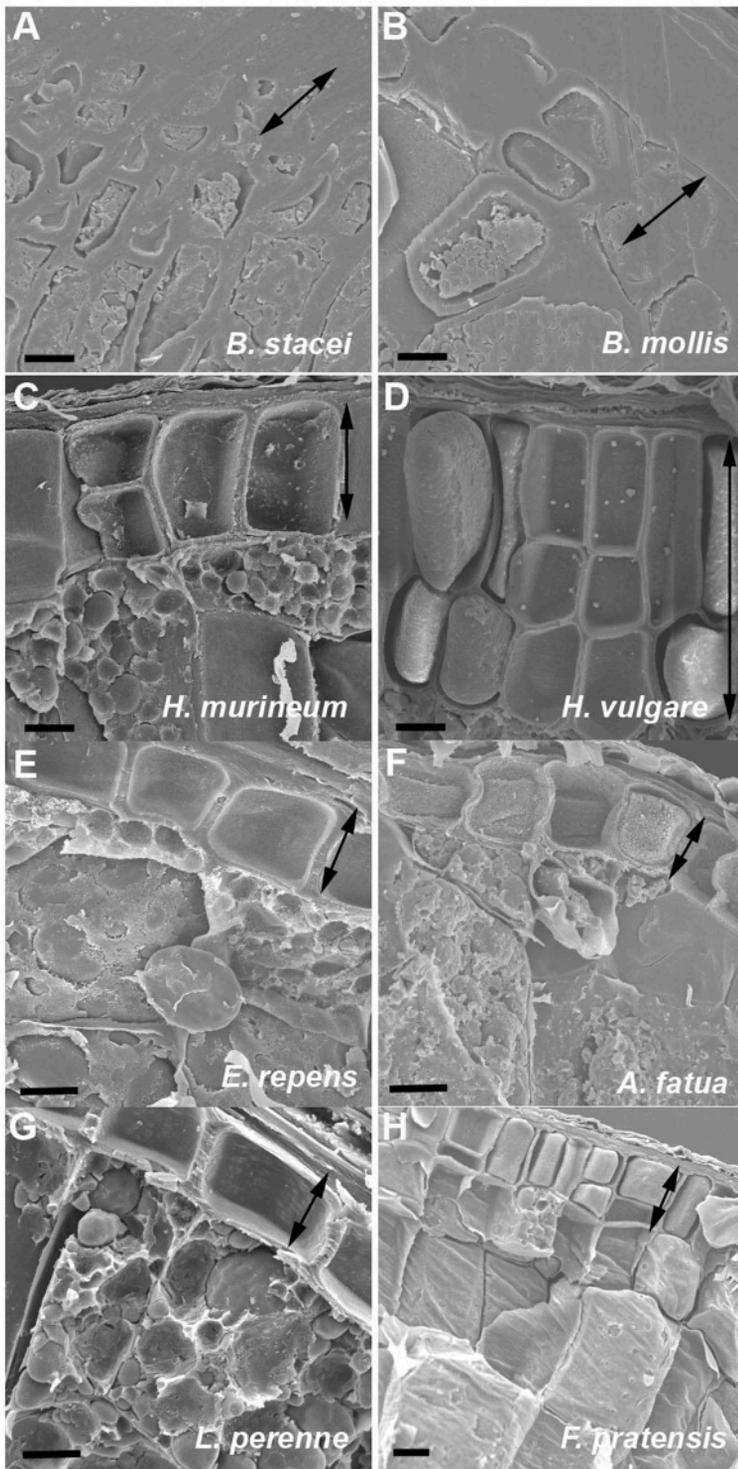
Figure 4.12: SEM of Peripheral Aleurone & Underlying Central Endosperm Cells in Selected Grains

Double ended arrows show peripheral aleurone thickness in each species, the irregular aleurone of *B. stacei,* and to a lesser extent *B. mollis,* contrasting sharply to other species. Central endosperm cell walls are very thin and barely visible for all species except *B. stacei* and *B. mollis*. Scale bars show 10 μm A, B, G, H; 20 μm C, D, E, F

Species	central wall	aleurone wall	central size	aleurone size
B. distachyon	2.73±0.42	2.07 ±0.51	27.5±4.68	18.23±4.29
B. mollis	2.41±0.67	3.06±1.04	59.22±6.23	12.59±1.65
B. diandrus	1.81±0.26	2.55±0.55	53.68±10.12	24.05±1.19
E. repens	0.41±0.14	1.08±0.20	45.31±9.53	17.43±4.45
T. uratu	0.39±0.07	0.79±0.14	nd	23.54±2.16
T. speltoides	0.31±0.04	1.31±0.11	78.15±9.27	19.89±2.62
L. perenne	0.37±0.17	1.42±0.34	79.81±33.56	16.59±2.64
F. pratensis	0.67±0.10	1.80±0.26	94.86±14.12	17.36±6.33
A. fatua	0.38±0.13	1.40±0.22	132.93±50.	25.51±3.02

Table 4.4: Endosperm Cell Wall Thickness and Cell Size for Selected Species Spanning the Range Examined.
Endosperm cell wall measurements taken from SEM images of mature grains reveal a typically a greater aleurone cell wall thickness as compared to central endosperm, and a larger central endosperm cell size as compared to the aleurone. *B. distachyon* is distinct in showing a central endosperm cell wall size greater than those of the aleurone layer, and a central endosperm cell size that is similar to that of the aleurone layer. Dimensions in µm showing ±1 standard deviation. n=10 nd= not determined

visual comparison of differences in endosperm cell wall thickness in selected species. The prominence of the cell walls (and cell size) of *Brachypodium* and *B. mollis* is also shown clearly with Evans blue staining where, with only cytoplasmic area being stained in the central endosperm, the walls are clearly visible (appendix fig A4f). *B. mollis* also provides the only exception, in relation to central endosperm cell wall thickness, to a trend for central endosperm cell size and cell wall thicknesses characteristics to be well conserved at the tribal level.

SEM was used to examine visual properties of cell walls. The irregular shape and size, particularly relative to the cuboidal peripheral aleurone cells, that defines the cellular differentiation of the modified aleurone region can be seen quite clearly in these SEMs. Cells of the modified aleurone have transfer function and classic features of transfer cells, including the BETL of maize, include invaginations in the walls serving to increase surface area for transport (Kovalchuk et al, 2009; Wang et al, 1994b; Becraft et al, 2001).   This feature was visible in SEM of cells walls of the modified aleurone of the wheat species observed, including those of primitive wheats (Fig. 4.13 a-c). These features were less obvious in grains with a flat profile such as *E. repens*, *Lolium* and *Festuca* (Fig. 4.13 d-f).

4.2.8 Differences in Endosperm Starch Accumulation Exist

The accumulation of storage reserves is a critical function of the cereal endosperm and typically occurs in the form of starch. The endosperm of cultivated cereal grains is especially starch rich but observations made in the previous chapter and published (Opanowicz et al, 2011; Guillon et al, 2011))
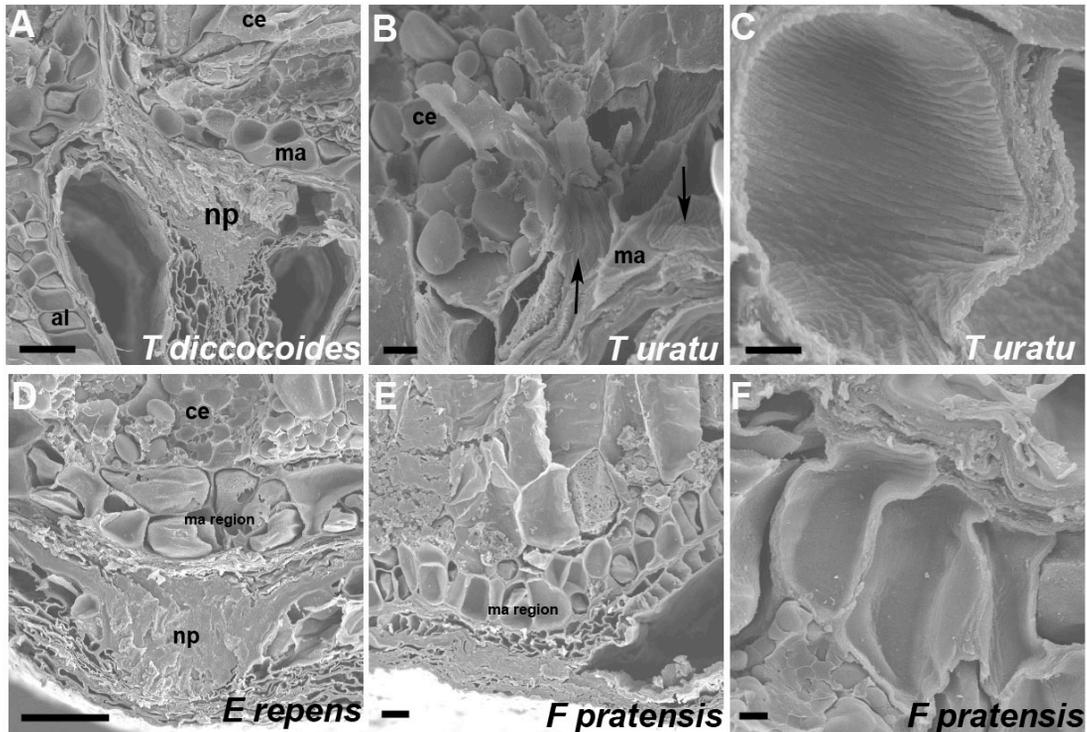
Figure 4.13: SEM of Modified Aleurone Region in Selected Grains.
Detailed analysis of cell morphology in the modified aleurone region reveals features associated with function and a role in grain filling in some species. A convoluted internal cell surface, suggesting transfer morphology, is visible in the modified aleurone region cells primitive wheat species, especially those with a heavily creased grain profile. Arrows in B indicate modified aleurone cells and C shows detail of internal cell wall convolutions. D-E show modified aleurone region cells in grains with a flatter, less creased grain profile where transfer cell features were less obvious. ce, central endosperm; ma, modified aleurone; al, aleurone; np, nucellar projection. Scale bars show 50 µm A, D, 10 µm B, E and 5µm C, F.

have reported significantly lower levels of starch in the *B. distachyon* grain, with mature grains comprised of ~10% starch. To test for differences in starch levels between the species considered here we initially examined Lugol's stained sections of selected mature grains, where intensity of staining and intracellular starch granule abundance can provide some measure of endosperm starch content. Stained sections, shown in figure 4.14, indicate that all species show a notably higher level of starch than *B. distachyon*, including other *Brachypodium* species. In the previous chapter we noted *B distachyon* to show a notably greater concentration of starch in the proximal endosperm (close to the embryo) as compared to distal, and that in distal half of the grain starch was largely absent from the lobes of the endosperm and confined to the central zone. For comparison we performed a similar analysis of starch distribution in *B. stacei* and found levels to be very obviously more abundant. A starch gradient, either transverse or longitudinal was not obvious in the *B. stacei* mature grain, shown in figure 4.14 C and also in *stacei* serial sections shown in the appendix A4j. Starch is excluded from the aleurone layer and the irregular edge between central endosperm and peripheral aleurone is clearly revealed (fig 4.9 K inset).

To further investigate and quantify potential differences we went on to perform a crude assay of mature grain starch content for the majority of species examined here. Results of this assay are shown in figure 4.15 and reveal a broad range of starch content. Previous, and potentially more sensitive, assessments of starch content in *Brachypodium* have detected total starch levels of ~10% (Guillon et al, 2012) whereas our test was able to detect only around 4%, suggesting our method of testing may not be fully detecting the starch in our samples. As a line of
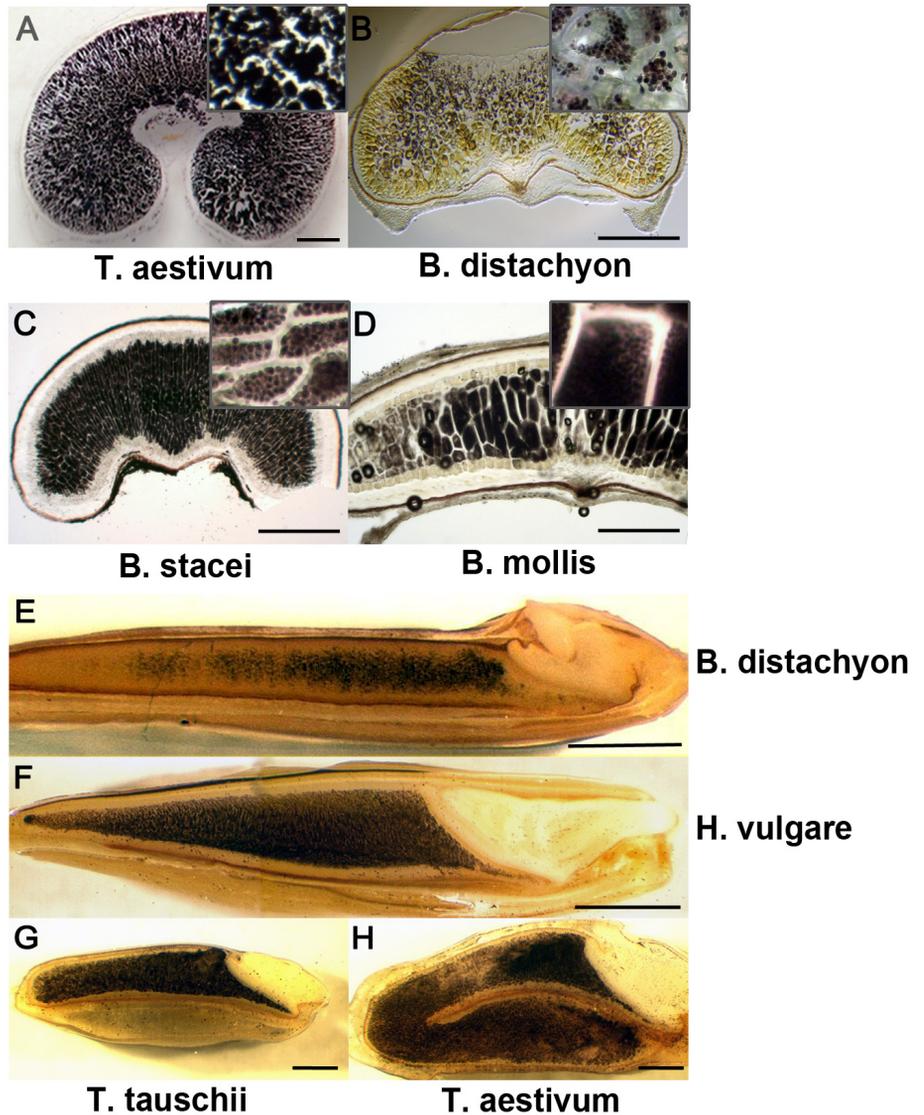
Figure 4.14: Preliminary Analysis of Starch Occurrence & Distribution in Selected Grains

A & B show comparative starch staining (Lugol's) intensity in 14 µm thick sections of *T. aestivum* and *B. distachyon* respectively. C & D show similarly stained free hand sections of *B. stacei* and *B. mollis* respectively, levels being notably higher than *B. distachyon*. Insets A – D detail intracellular starch granule abundance and distribution. E-H show starch distribution in longitudinal mature grain sections of *B. distachyon*, *H. vulgare, A. tauschii* and *T. aestivum* respectively. Consistent occurrence of starch throughout the entire endosperm area is apparent in all except *B. distachyon*. Scale bars in A-D show 0.5mm, E-H 1mm
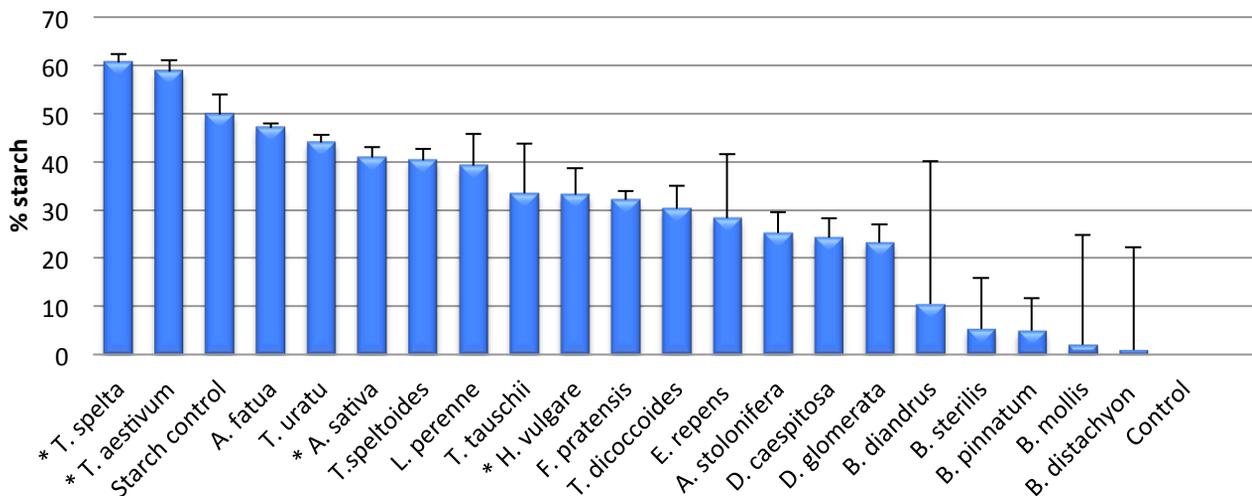
Figure 4.15: Analysis of Grain Starch Content

  Analysis of total grain starch levels reveals a broad range of starch contents across these Pooideae species. * indicates domesticated and cultivated cereal crop species, all of these species showing starch contents in the upper half of the range identified. Brachypoideae and Bromeae species all show a comparatively low starch content to the other tribal groups. Error bars show $\pm$ 1 standard error.

validation for this assay we included a sample of a commercially available strong white bread flour of known 67.1% starch content. Our assay detected 50% starch content for this sample suggesting an approximately 20% deficit in total starch detection that can be used to provide some degree of correction for our values. Whilst these results do not appear to be accurate for the identification of total starch levels they do provide valid comparative values for starch level between species. Raw data relating to this assay can be found in appendix figure A4k. These results show that high levels of starch exist in wild Triticeae and Poeae species as compared to *Brachypodium* or *Bromus* and that *B. distachyon* is exceptionally starch poor in comparison to all other species.

We used SEM to examine starch granule abundance in central endosperm cells, revealing differences in both number and packing of starch granules. The entire core Pooids seem to have the ability to generate large starch reserves and only *Brachypodium* and *Bromus* have significantly lower endosperm starch reserves. Lower starch levels appear to correlate a simple granule distribution, detected in *Brachypodium*, rather than the bimodal distribution typical of the Triticeae (Shapter et al., 2008), or compound granules observed in Aveneae. Species of the Poeae also have a compound starch granule morphology and in the case of *Festuca* we also observed simple granules in addition to the compound conformation (Table 4.3). These differences in starch packing suggest that differences in grain density may exist between species where those with the highest starch contents would be likely to show the highest density. Density is an important attribute, along with shape and size, for determining the market value of wheat grains (Gegas et al, 2010). We were initially able to test this hypothesis with

the observation that wheat grain (high starch content) would sink swiftly in water whilst other wild species' grains were either buoyant or would either sink more slowly. To test this observation more thoroughly we compared average grain mass against volume measurements (derived from length x width x depth). These results are shown in figure 4.16 and illustrate that domesticated wheat, barley and oat species show the highest mass as compared to volume, and this is likely attributable to their high endosperm starch levels. In contrast the majority of the wild species show a grain volume larger than mass. This result strongly suggests, in keeping with expectation, that increased density, likely through increased starch accumulation is strongly selected for under domestication.

4.2.8 Chapter Conclusion

This investigation provides detailed analysis of Pooid grain character and architecture at physical, histological, physiological and molecular levels. Our results describe a spectrum of character variation across different phylogenetic groups; the major trends and key features identified being summarised schematically in Fig. 4.17. Some of the greatest points of difference exist between the Brachypoideae and the other Core-Pooid tribes. *B. distachyon* has become an important model system for temperate grasses with an increasing array of molecular tools available (Mur et al., 2011). As a sister group and reference point for Core-Pooids group the comparison between the *Brachypodium* genus and neighboring taxonomic groups, both wild and cultivated is valuable and timely. Alongside these tribal level differences we identified significant intra-genus variation within the Brachypoideae relating to starch content and features of grain
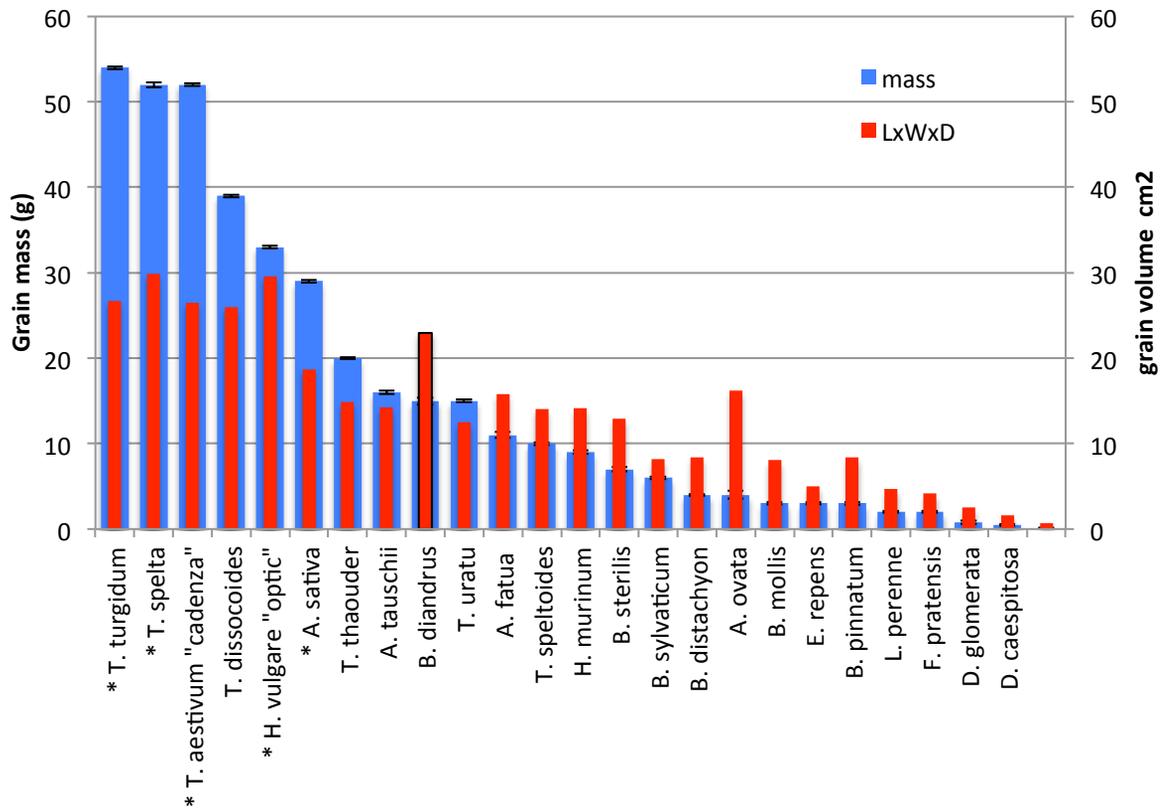
Figure 4.16: Grain Size & Mass Comparison for Selected Species.
Blue horizontal bars indicate grain mass n = 50 and error bars show ±
1 standard error. Overlaid red bars indicate grain volume as a cuboidal
value derived from length x width x depth. * indicates domesticated,
cultivated species. Domesticated species show a high mass to volume
ratio that is potentially attributable to increased starch accumulation.

morphology. In addition to its annual habit these results suggest *B. distachyon* is quite distinct amongst both the Brachypoideae and Pooideae as a whole in its grain morphology and physiology.

Within those tribes showing higher levels of similarity, specifically the Aveneae and the Poeae we describe significant points of difference between wild species whilst those under cultivation show higher similarity at multiple levels. This comparison suggests a distinct direction and influence of domestication selection towards a distinct grain shape and character. We have found that many features of grain architecture are conserved at the tribal level with the possible exception of the Bromeae where dramatic variations may reflect its complex and uncertain taxonomy (Smith, 1970: Oja & Jaaska, 1998). Within the Triticeae we detected high levels of similarity in many features of the species examined with the notable exception of *E. repens* where again a complex evolutionary history and allopolyploidisation may have contributed to taxonomic uncertainty (Mahelka & Kopecky, 2010).

A significant observation is the restriction of a distinctive modified aleurone and prominent crease to the Triticeae. Transfer cells have a unique architecture that perfectly mediate their central role in nutrient transfer (Offler et al, 2003). Transfer cells in the seeds and grains of cotton and *Sorghum* have been shown to have implications in grain size and yield (Pugh et al, 2010 ; Wang et al, 2012). The apparent lack of a typical modified aleurone and robust nucellar projection, the nucellar projection also being rich in transfer characteristics (Zheng & Wang, 2011), in genera such as *Brachypodium* may help explain its flat and starch-poor grains. Though the Triticeae species examined have aleurone layers that are
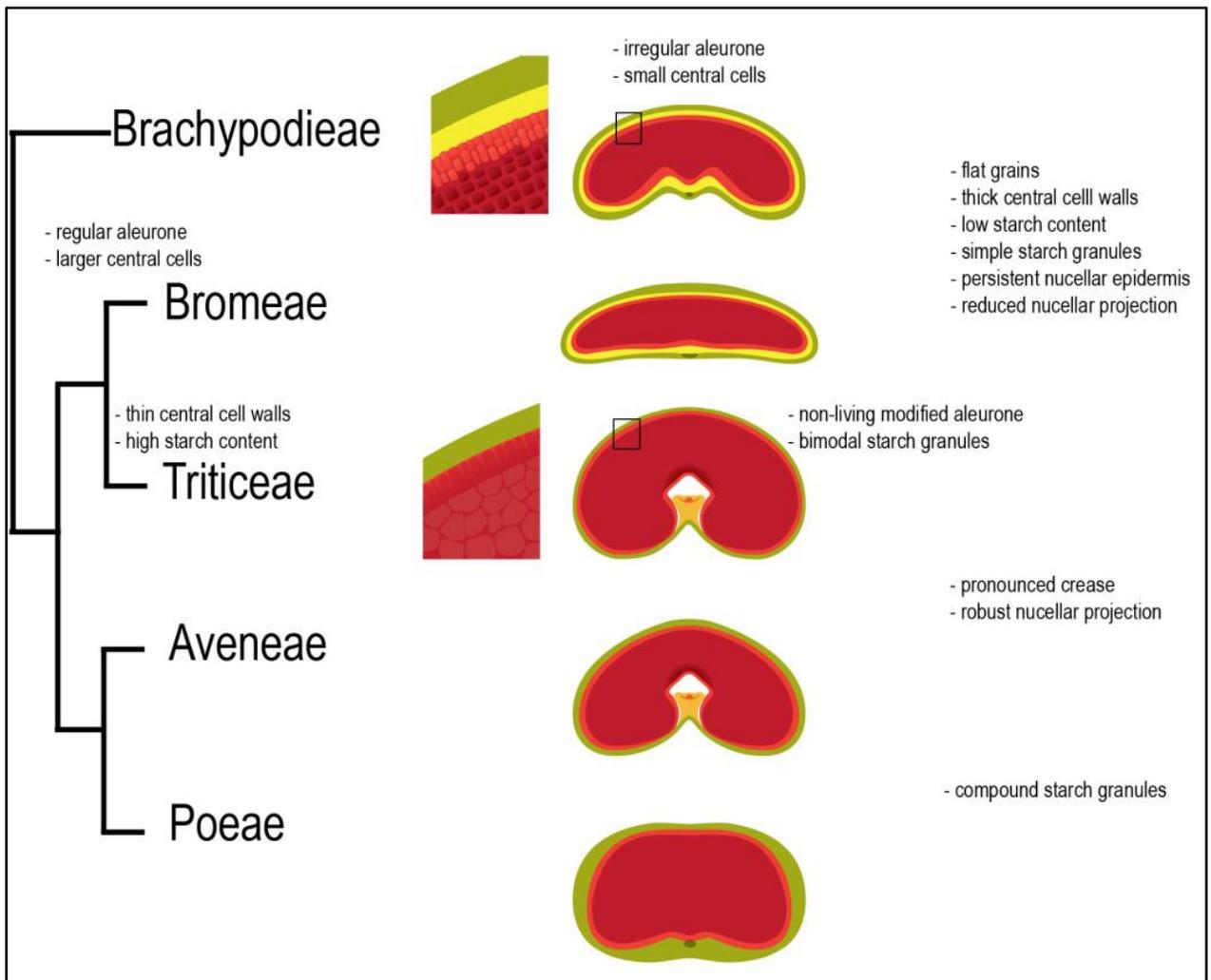
Figure 4.17: Schematic Summarising Trends in Grain Characteristics in Terms of Basic Phylogeny.
NOT to scale. Green, pericarp ; yellow, nucellar tissues ; light red, aleurone ; red ; central endosperm.

readily identified, spatial molecular analyses using mRNA ISH with markers previously shown to be layer-specific (Drea et al, 2005b) indicated that the aleurone layers of some species (*A. speltoides*, *T. uratu and T. diccoides)* may be less definitively differentiated, though central endosperm marker patterns were more tightly deliniated. Vital staining patterns in the adaxial domain where the modified aleurone would be expected to be located varied: in *B. distachyon* and *B. mollis* species this region consistently stained with TZ indicating a living modified aleurone at maturity, while in *B. sterilis* staining in this region was absent, similar to the pattern in cultivated wheat or barley. We aim to investigate this area further to assess the implications of this domain in relation to grain evolutionary history

 Observations relating to the nucellar tissues suggest that some polarisation may exist amongst the Pooideae in relation to grain filling pathways. Two distinct routes for assimilate transport are identified in small-grain cereals; a nucellar epidermis dominated route where no distinct region of endosperm transfer cells exists, as in rice, and a nucellar projection/modified aleurone region transfer-cell dominated route, as in wheat (Oparka & Gates 1981). Both *Brachypodium* and *Bromus* genera were seen to possess a persistent nucellar epidermis and central endosperm cell wall thickness comparable to the aleurone layer. This is in contrast to wheat (Opanowicz et al, 2011) and indeed to the Triticeae in general where the nucellar epidermis is largely obliterated early in grain development.

 Within the Bromeae noticeable difference exists between *mollis* and *diandrus/sterilis* species, the latter having a more lobed profile reminiscent of the wheats, while *mollis* was starkly flat with barely any appearance of a crease. All the Core-Pooids seem to have the ability to generate large starch reserves except for

the *Bromus* species examined – though *Bromus* starch reserves are generally greater than the *Brachypodium* species.   Analyses of grain composition in *Brachypodium distachyon* have reported a lipid and protein content and composition that is more similar to that of Avena species than any other (Guillon et al, 2012). In discussing differences such as these however, the selective forces of domestication need to be taken into account. Generally, this feature of low-starch reserves is linked to more irregular aleurone layers, a persistent nucellar epidermis and lack of a distinct crease.

 This cross-species comparative analysis provides a basis on which to interpret evolutionary and molecular data relating to grain form and evolutionary history. By aligning a grain survey focused on agriculturally relevant traits in the grain alongside the established phylogenies we reveal patterns of grain structure and organisation in the Pooideae and identify potential candidate species for informative genomic and transcriptomic comparisons.

CHAPTER **5.**

GRAIN DEVELOPMENT ASSOCIATED TRANSCRIPTION FACTOR IDENTIFICATION IN *BRACHYPODIUM DISTACHYON*

5.1 Introduction

Despite the focus of considerable research effort and numerous publications, many recent, the molecular regulation of grain development, particularly in relation to the temperate cereal species, remains poorly understood (Sabelli & Larkins, 2009). Until recently rice and maize have formed the primary resource for the investigation of grain development at a molecular level but these species show a grain morphology and physiology that is quite different and distinct from that of the temperate species. Transcription factors, both individually and at a familial level, have been shown to play critical roles in grass reproductive development but relatively few specific to grain and endosperm development have been identified (Sabelli & Larkins, 2009). In particular transcription factor function has been associated with the rapid selection for traits important in the domestication process (Purugganan & Fuller, 2009; Doebley et al, 1996), and this is likely a consequence of their high-level influence in gene networks controlling complex morphological traits (Century et al, 2008). *Brachypodium* offers a primary opportunity for the detailed and accessible investigation of molecular genetic regulation of grain development not only in wild grass species but also in a member of the as-yet understudied Pooideae. Such investigation offers possibility of further insight as to the mechanism and progression of the domestication process and forms an important basis of information for our future research.

We identified genes from several different regulatory gene groups including YABBY, MADS-box, MYB and bZIP factors known to be significant across grain development in a range of different grass species. Using BLAST alignment we

identified and confirmed *Brachypodium* orthologues for many of these genes and went on to examine expression and potential function across grain development. This survey provides candidate genes for ongoing research into grain development transcriptional control in *Brachypodium*. We aimed to collate this information in order to construct a preliminary picture and repository of information relating to genetic regulation of grain development in *Brachypodium* and to compare this to what is known for the other temperate grass and crop species.

5.2 Results and Discussion

5.2.1 Gene Identification And Analyses

To identify those genes known to have important roles in grain and seed development we began with a broad survey of literature relating to a range of cereal species. Transcription factors have prominent and critical roles in a great range of developmental process and we focused on their identification. Transcription factor function is well studied; very many genes and gene families of this functional class have been identified and their role in organ development characterized in detail. We identified several genes of interest and those we went on to examine here were selected based upon original function, characterization and orthology in *Brachypodium*. Tables 5.1, 5.3 and 5.5 lists these genes, identified against important orthologues and a brief description of function. Of these genes, most belong to major regulatory gene classes having important function across many areas of plant development. Genes are grouped according to the larger gene classes and to those with more grain specific expression. Many of the genes examined reflect the general bias of research to involvement in floral

Gene (Rice)	Other major orthologues		Class (MIKC)	Function and *expression patterns*	References
	Arabidopsis	monocots			
OsMADS14 AAf19047	FUL1 AP1	TaVRN-1/FUL1 AsFUL1	A-class. (AP1/FUL-like)	Spikelet meristem identitiy and general floral organ identity. *Immature spikelets and all floral organs*	Preston & Kellogg, 2007
OsMADs15 AAF19048	FUL2	AsFUL2	A-class. (AP1/FUL-like)	Spikelet meristem and outer floral whorl identity. *Immature spikelets and outer whorl floral organs.*	Preston & Kellogg, 2007
OsMADS3 L37528	AG	ZAG1/ZMM	C-class.	Stamen development and floral identity. *Early floral development*	Yamaguchi et al, 2006
OsMADS58 AB232157	AG	ZAG1/ZMM	C-class.	Carpel identity and floral meristem determinacy. *Early floral development*	Yamaguchi et al, 2006
OsMADS13 AF151693	STK/FBP7/11	ZAG2/ZMM	D-class.	Ovule identity. *Developing ovules and early seed development*	Lopez-Dee et al, 1999: Dreni et al, 2007
OsMADS21 Q8RU31	STK	--	D-class.	No obvious function. *Flower development (Ovules, carpels and stamens) and early seed development.*	Lee et al, 2003: Dreni et al, 2011
OsLHS1/MADS1 A2XDY1	SEP1/2/4	--	E-class.	Spikelet meristem determinacy and outer whorl floral organ identity. *Floral primordia and palea, lemma and pistil of mature flowers.*	Malcomber & Kellogg, 2004

Table 5.1: MADS-box Gene Identification

MIKC-type MADS box transcription factors from rice and other small grain cereals having significant function in grain development

Candidate name	Candidate gene Identifier	Protein Level Homology	BLASTP e-value	Sequences used in BLAST homology identification
BdFUL1	Bradi1g08340	87%	1e-129	**OsMADS14**
BdFUL2	Bradi1g59250	76%	1e-125	**OsMADS15**
BdMADS58	Bradi2g32910	76%	e-111	**OsMADS58**
BdMADS3	Bradi2g06330	88%	e-104	**OsMADS3**
BdMADS13	Bradi4g40350	75%	4e-98	**OsMADS13**
BdMADS21	Bradi2g25090	65%	2e-82	**OsMADS21**
BdLHS1	Bradi1g69890	75%	2e-99	**OsLHS1**

Table 5.2: *Brachypodium* MADS-box Candidate Genes

Brachypodium candidate genes identified by BLAST analysis potentially orthologous to the MADS-box genes shown in table 5.1

development and those genes with grain specific expression represent something of a minority class (table 5.5). Furthermore, of the five candidate genes listed as having an expected endosperm specific developmental function three were initially isolated only because they have a role in post germination development (Rubio-Somoza et al, 2006b, 2006a; Diaz et al, 2002, Mercy et al, 2003).

Using predominantly rice and temperate cereal species' gene sequence we performed BLAST analysis at nucleotide and protein levels against the *Brachypodium* genome. We were able to readily identify high-scoring candidate genes in *Brachypodium* for the majority of genes examined. High levels of synteny have been shown to exist between *Brachypodium*, wheat, barley and to a lesser extent rice, and ~77% of *Brachypodium* genes were found to have strong Triticeae EST matches (Huo et al, 2009). Genomic rearrangements represent the greatest source of diminishment in this sytenic relationship, most notably between *Brachypodium* and rice, but conservation at the nucleotide level is high (Gu et al, 2010; Faris et al, 2008). Tables 5.2, 5.3 and 5.5 list our *Brachypodium* candidate genes and their often-high protein level homology in relation to the identifying orthologues.

We went on to confirm the identity and homology of *Brachypodium* candidates through phylogenetic analysis and to provide expression analyses for selected genes, yielding information relating to function and role in grain development.

5.2.2 MADS-box Genes

Members of he MADS-box family of transcription factors have significant function in plant organ development, and are particularly well known for their role in floral

organ specification (Coen & Meyerowitz, 1991). We identified 7 genes (table 5.1) of most interest to us in relation to their effects upon grain development. MADS-box gene identity and function has been especially well studied in rice, and so rice sequence was primarily used in BLASTp searches with comparison to *Arabidopsis* and other temperate cereal species for corroboration. We were able to identify single sequences listed in table 5.2 as putative *Brachypodium* orthologues for these genes.

5.2.2.1 Phylogenetic Analyses

To confirm the identity of *Brachypodium* candidates we compiled amino acid alignments and derived phylogenetic trees for their functional groups. Figure 5.1 shows the amino acid alignment for C-and D-class MADS box genes from across the angiosperms. The high homology across the MADS box domain made it necessary to focus on the C-terminus to most clearly resolve groupings within these classes; a full length sequence alignment can be found in appendix figure A5a. The *AGAMOUS* or C-class genes were amongst the first MADS-box genes to be identified, having a critical role in carpel and stamen identity, and in floral meristem determinacy (Yanofsky et al, 1990). In *Arabidopsis* this functional class is represented by just *Ag* but a duplication and subsequent subfunctionalisation is thought to have occurred in rice, resulting in *MADS3* and *MADS58* (Yamaguchi et al, 2006; Lee et al, 2003) for which we were able to identify *Brachypodium* orthologues of both. *BdMADS3* and *BdMADS58* group closely with rice orthologues, and to other related sequences identified in wheat, barley and maize (Fig 5.1). The high protein level homology and position of *BdMADS3* and
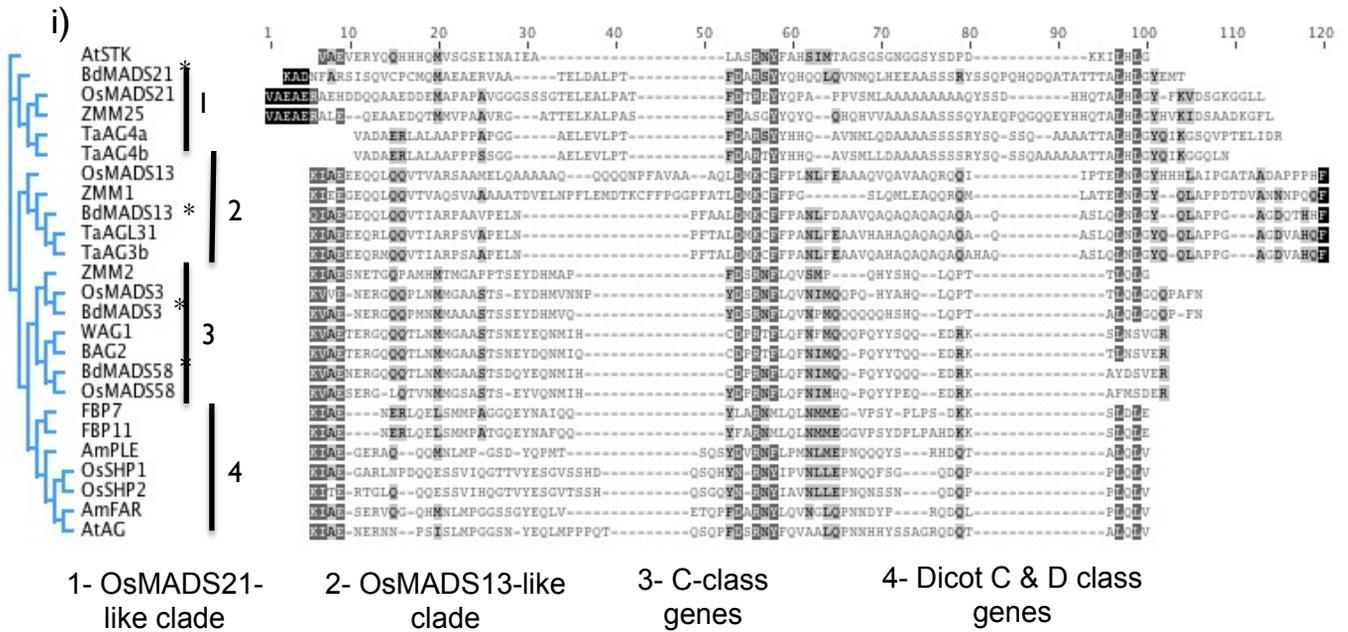
Figure 5.1: Phylogenetic Analysis of C & D class MADS-box Transcription Factors.

i) ClustalW alignment of amino acid sequences of variable c-terminal gene region from selected species across the angiosperms. Identical residues highlighted in black, similar residues highlighted in grey. Horizontal bars showing clades.

ii) Unrooted neighbour-joining tree derived from the above alignment. Branch lengths indicate genetic distance. *Brachypodium* orthologues throughout indicated with an *.
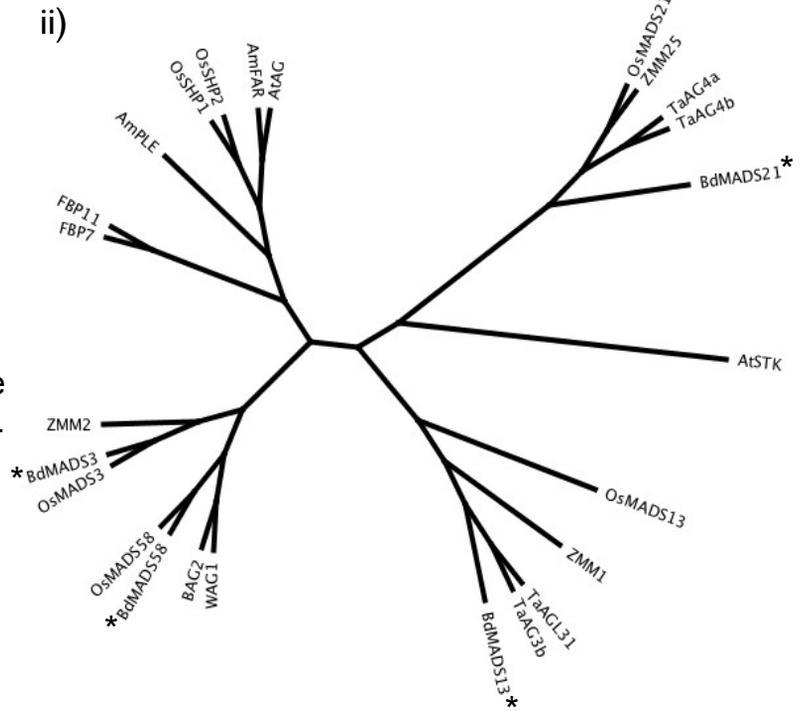
Abbreviations for species listed

At	*Arabidopsis thaliana*
Am	*Anthirrnum majus*
Bd	*Brachypodium distachyon*
Os	*Oryza sativa*
Ta	*Triticum aestivum*
Zm	*Zea mays*
B	Barley (*Hordeum vulgare*)

BdMADS58 in our phylogenetic analyses indicates these genes are homologous to those of rice.

A similar duplication also appears to affect rice D-class genes, resulting in *MADS13* and *MADS21* and again it was possible to identify candidates for both in *Brachypodium*. We initially used a wheat EST to identify the *BdMADS13* but this did not give a sufficient hit to identify the complete sequence. Using the full length *OsMADS13* sequence (Lopez-dee et al, 1999) we obtained a more complete and conclusive result. *OsMADS13* and *OsMADS21* both show a high degree of similarity to the D-class genes of the eudicots e.g. *AtSTK, FBP7, FBP11* (Yamaguchi & Hirano, 2006). Monocot D-class genes are divided into two distinct groups correlating to the *OsMADS13* like clade and the *OsMADS21* like clade (Dreni et al, 2007). These groupings are quite distinctly characterised by extreme c-terminus extensions, particularly apparent in the *OsMADS13* like clade (Fig 5.1). Homology levels between *BdMADS21* and *BdMADS13* to the rice orthologues was a little lower than those seen for the C-class genes, and both position within the two distinct D-class clades in the phylogeny. They do not cluster as distinctly with their rice counterparts here and in particular *BdMADS21* lies at the edge of the grouping, with sequence homology at the extreme c-terminal being low. *BdMADS13* groups most closely to wheat orthologues rather than rice, in contrast to the situation seen for C-class genes, although only few sequences are considered here. Again, phylogenetic analyses support our assertion that these two *Brachypodium* sequences are orthologous to the D-class genes identified in rice and other temperate cereals.

We were able to identify *Brachypodium* candidates for the two rice *FRUITFUL* (*FUL*) genes, both showing high levels of homology at the protein level. Amino acid alignment for cereal *FUL* orthologues is shown in figure 5.2. The MADS-box domain represents a large and well-conserved component of genes in this family and acts to elevate protein level homology. As expected, all genes show a high level of similarity across the MIKC-MADS box domain with much of the variation restricted to the c-terminal region. The two *Brachypodium* orthologues fall into the distinct groups correlating to the *FUL1* and *FUL2* clades with *BdFUL1* grouping most closely to the *AsFUL1* sequence whilst *BdFUL2* is closer to that of *OsFUL2* (*OsMADS15*). The lower protein level homology score observed for *BdFUL2* (Table 5.2) appears to relate to a lack of the distinct and highly conserved region at the extreme c-terminal, seen in all other genes. Preston and Kellogg (2007) report two sequences for *BdFUL2* splice variants designated *a* and *b*, where b represents a shorter transcript. Similar variants were also detected for the *Triticum FUL2* orthologue. However, both *Brachypodium* sequences are incomplete for the n-terminal region. We have used the REFSEQ sequence annotation (accession XM_003557635.1) as this appears to show greater coverage. Malcomber et al (2006) identify three grass *FUL* clades where a distinct *FUL3* clade exists in addition to those identified by Preston and Kellogg (2007). We have included *HvMADS3* as a member of this third clade and show it positioned distinctly outside of the *FUL1* and *FUL2* groupings. Showing they are not homologs to this clade this result supports our identification of sequences *BdFUL1* and *BdFUL2* as orthologues to members of the Poaceae *FUL1* and *FUL2* clades.

Figure 5.2: Phylogenetic Analysis for FRUITFUL Orthologues in *Brachypodium*.

i) ClustalW alignment of amino acid sequences of FRUITFUL orthologues from selected grass species. Identical residues highlighted in black, similar residues highlighted in grey. Horizontal bars indicate clades.

ii) Unrooted neighbour-joining tree constructed from the alignment. Branch lengths indicate genetic distance.

Brachypodium orthologues throughout indicated with an *.

1 = FUL-2 clade   2 = FUL-1 clade

Abbreviations for species listed

As	*Avena sativa*
Bd	*Brachypodium distachyon*
Hv	*Hordeum vulgare*
Os	*Oryza sativa*
Ta	*Triticum aestivum*

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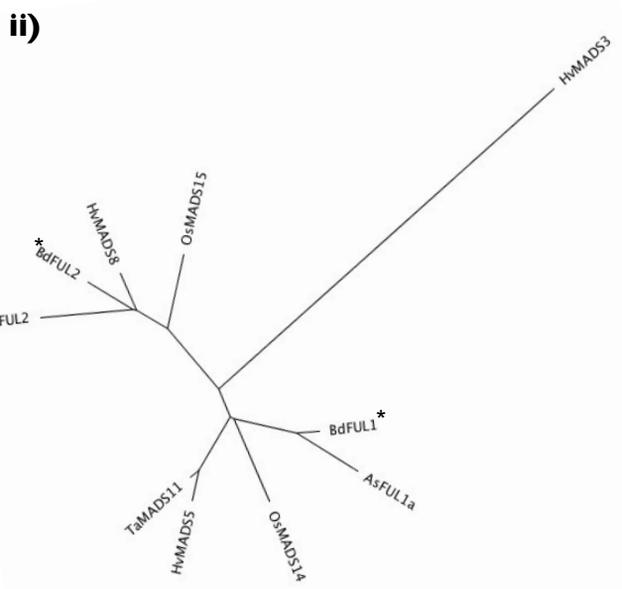
The final MADS-box gene examined here was a putative orthologue to the rice *LEAFY HULL STERILE1 (LHS1)*, an E-class function gene. A great many *SEPALLATA* genes have been identified and divided into two major clades, the SEP3 and LOFSEP clades (Malcomber & Kellogg, 2005). To confirm the orthology of our *Brachypodium LHS1* candidate we aligned amino acid sequences of *SEP* orthologues from cereal species spanning both clades. Several *Brachypodium* sequences with high homology, identified in the early BLAST analyses, were included along with prominent temperate cereal members of the two clades. This alignment, shown in figure 5.3, shows the *BdLHS* sequence clustering with *LHS* orthologues of rice, oat and barley, and distinct from the other genes, belonging to the *SEP3* clade. All members of the *LHS* subgroup of the LOFSEP clade are distinguished by a distinct c-terminal extension and conserved motif. The phylogenetic tree taken from this alignment also reveals *BdLHS1* groups most strongly with oat and barley orthologues. This result provides good evidence that *BdLHS1* is orthologous to its rice and temperate cereal counterparts.

5.2.2.2 Expression Analyses

To examination *Brachypodium* gene expression we adopted a two-stage approach, with an initial RT-PCR survey across late floral and grain development followed by more targeted mRNA in-situ hybridization (ISH) at various stages to provide spatial expression information and early indications of function. With comparison of these results to the published expression details for orthologs we
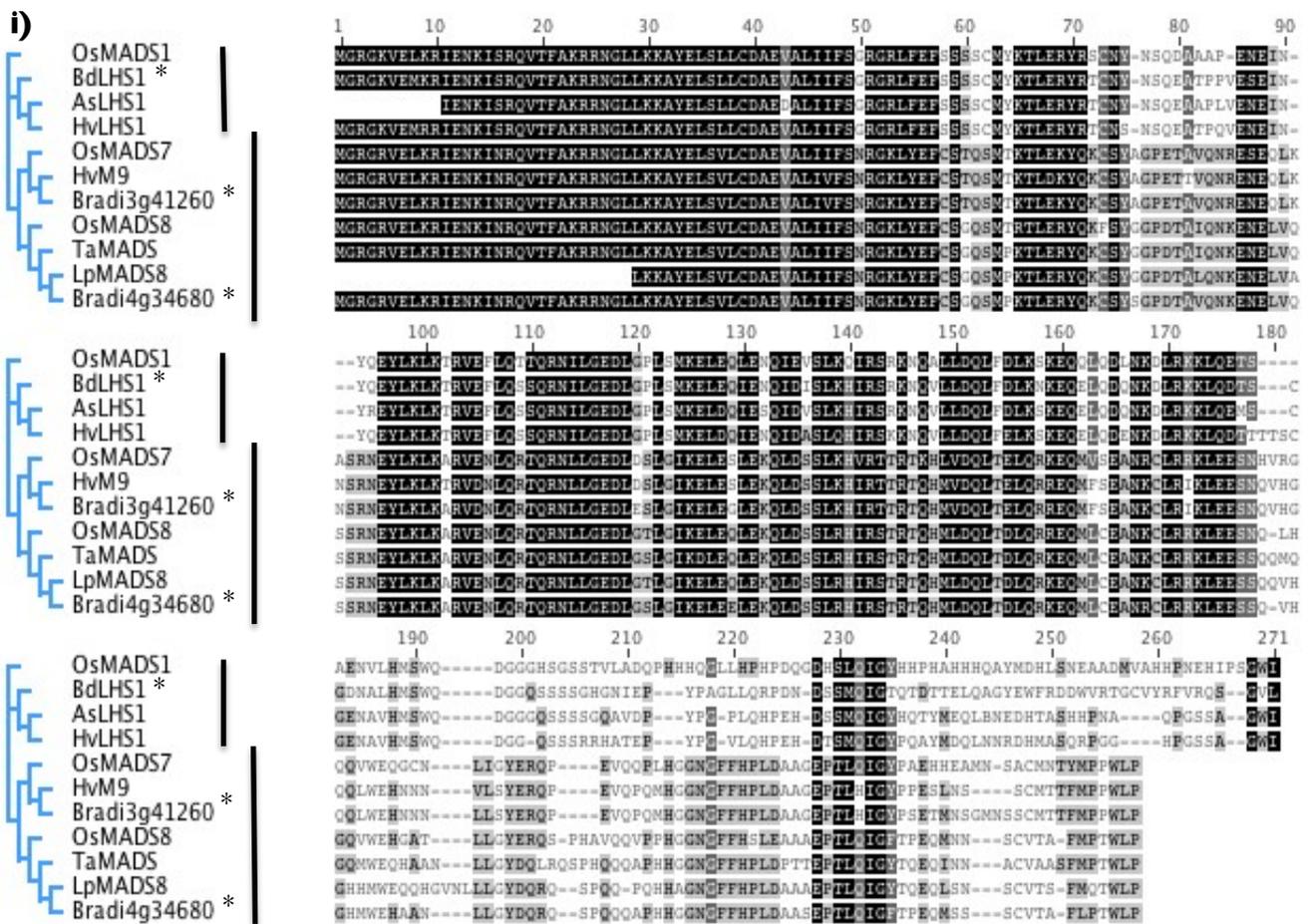
Figure 5.3: Phylogenetic Analysis for SEPALLATA Orthologues in *Brachypodium*.

i) ClustalW alignment of amino acid sequences of SEPALLATA orthologues from selected grass species. Identical residues highlighted in black, similar residues are highlighted in grey. Horizontal bars indicate major clades.

ii) Unrooted neighbour-joining tree constructed from the alignment. Branch lengths indicate genetic distance. *Brachypodium* orthologues throughout indicated with an *.
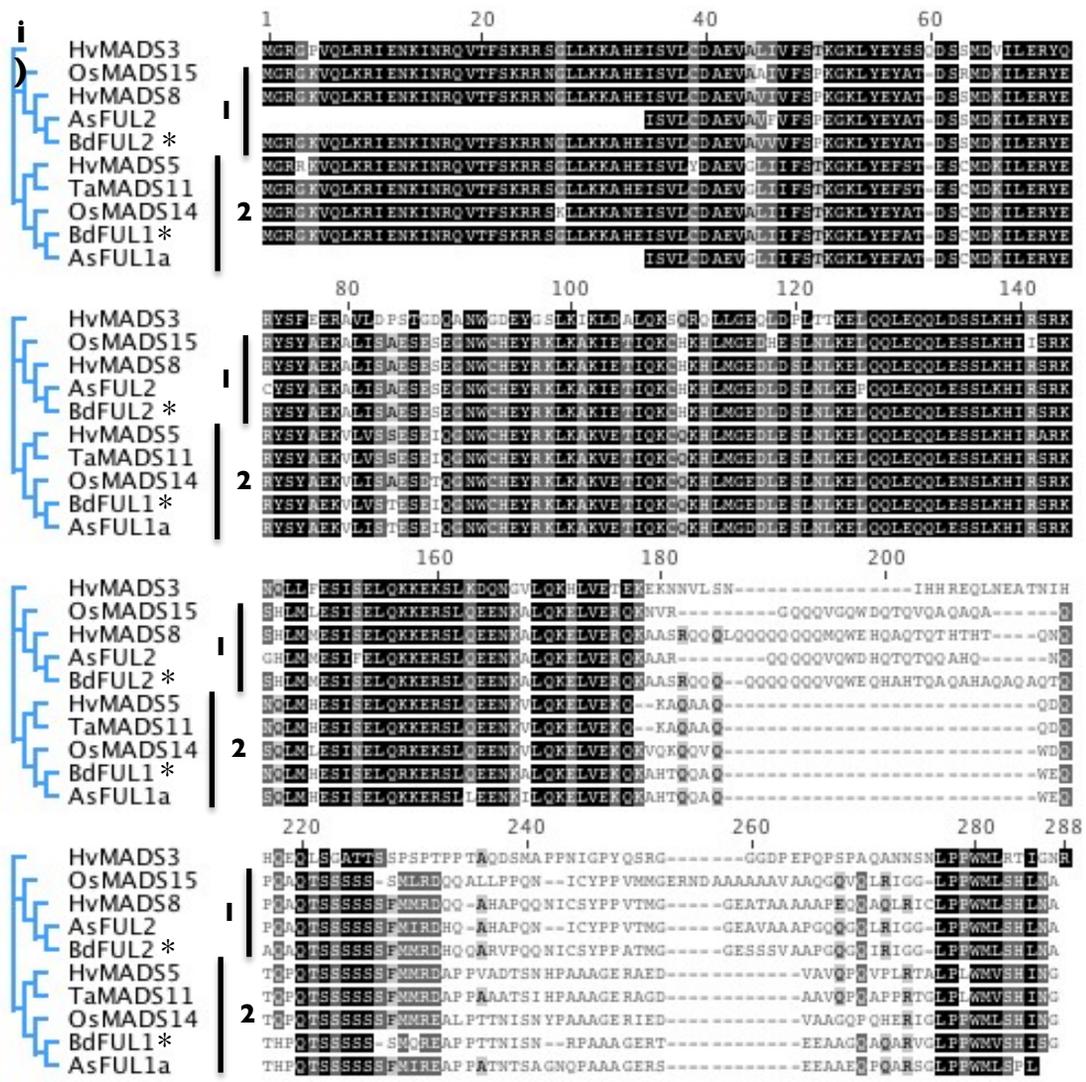
Abbreviations for species listed

As	*Avena sativa*
Bd	*Brachypodium distachyon*
Hv	*Hordeum vulgare*
Lp	*Lolium perenne*
Os	*Oryza sativa*
Ta	*Triticum aestivum*
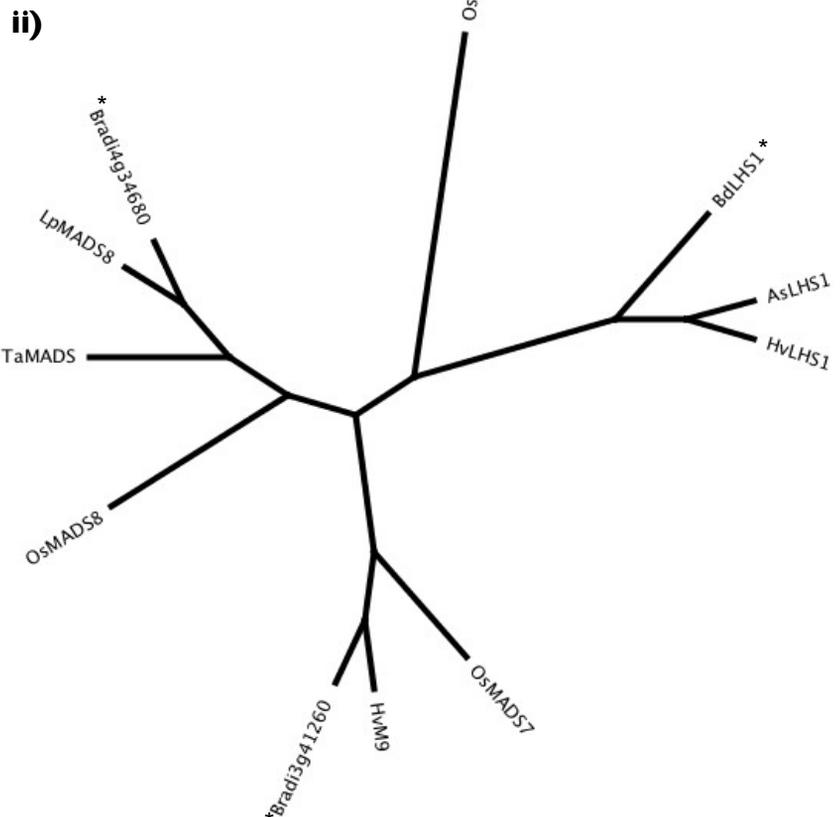
provided preliminary characterization and observations on function for these genes.

BdMADS58 & BdMADS3: Studies conducted by Yamaguchi et al (2006) and more recently Dreni et al (2011) have provided detailed analysis of expression and function of *MADS58* and *MADS3* in rice, although their findings are not in total agreement. Results of the earlier study indicate a strongly disrupted floral morphology using *MADS58* RNAi and temporally differing expression pattern for the two genes; the later study however finds no significant developmental defects in *MADS58* mutants and similar patterns of expression for the two genes. Results of RT-PCR profiling for *Brachypodium* orthologues of these genes are shown in figure 5.4 where we detect a similar expression profile for both genes. *BdMADS58* is expressed at fairly consistent level across grain development, with slightly higher expression during early stages, and similar pattern but lower overall level of expression for *BdMADS3*. This observation for similar expression between these two *Brachypodium* orthologues follows Dreni et al's (2011) findings. Expression for both genes in carpel and ovule primordia is also reported by Dreni et al (2011) and so the expression of both *Brachypodium* orthologues in our spikelet sample is not unexpected (Fig 5.4). The continued, strong expression of these genes across grain development raises questions regarding their function, as they are reported so far as functioning predominantly in floral organ development and meristem identity (Dreni et al 2011: Yamaguchi et al 2006). Expression for *BdMADS58* and *BdMADS3* is at its highest during mid grain development, correlating to the period of most intense maternal tissue growth and endosperm cellularisation, but
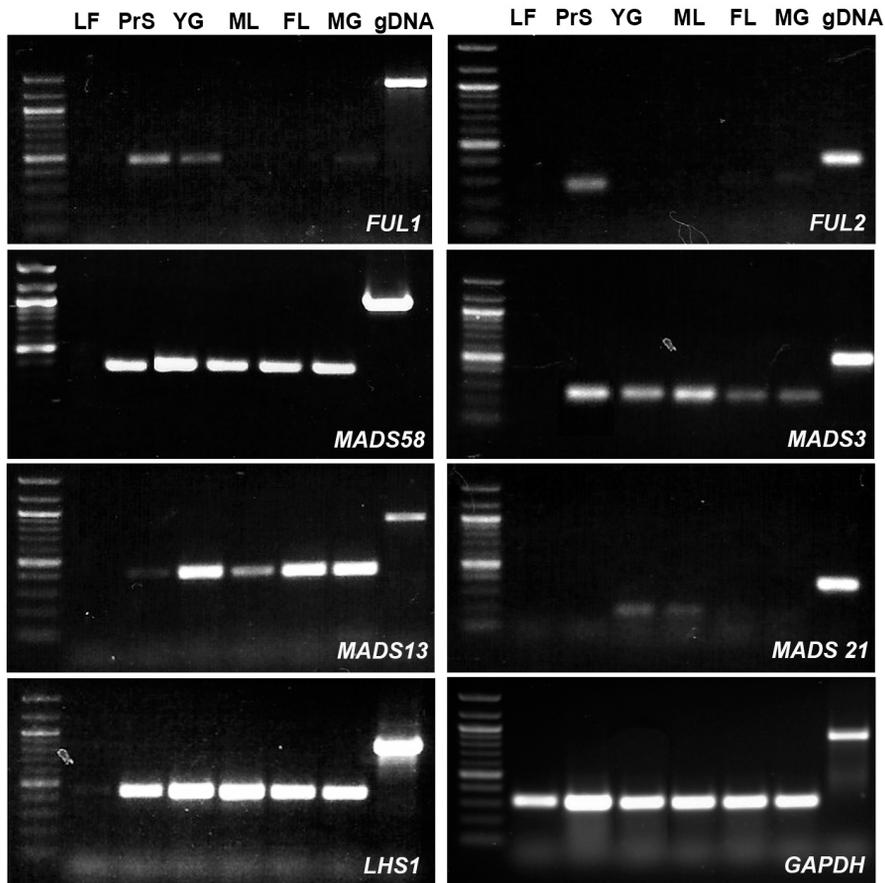
Figure 5.4: RT-PCR Expression Profiling for *Brachypodium* MADS- box Transcription Factors across Grain Development.

Expression analysis for *Brachypodium* MADS-box genes in leaf and developing grain tissues. Plant tissues and growth stages spanning grain development used for template cDNA production as follows, LF = Leaf, PrS = pre-anthesis spikelet, YG = Young Grain, ML = Mid Length Grain, FL = Full Length Grain, MG = Mature Grain, gDNA = genomic DNA sample.
Ladder shows 100bp intervals.

continues into later developmental stages when grain filling and dessication are the major processes occurring.

In mRNA ISH expression was detected in carpel tissues for both *BdMADS58* and *BdMADS3*, shown in figure 5.5 i) and ii) respectively. At an early developmental stage strong *MADS3* expression was detected in organ primordia regions in the developing inflorescence, shown in fig 5.5H. Expression can be seen in the ovule and adjacent central maternal tissues immediately prior to anthesis (Fig. 5.5 A & F). This stage is directly analogous to the PrS stage examined in the RT-PCR profiling. A very similar pattern of expression can be seen for *BdMADS58* at the same stage (5.5F), along with strong expression in the transmitting tissues. It proved difficult however to identify the precise location of expression for both genes in the developing grain. We were ultimately able to show expression appears to be restricted to maternal tissue layers across development. In the young grain (approx. 3-5 DAA) *BdMADS58* expression occurs in the outer integument extending into adjacent pericarp tissue and also vascular tissue. Beyond endosperm cellularisation expression could be detected for both genes only in, or just below, the inner integument (Fig 5.5 C, E & G). We later show expression of *OsMADS13* is also detectable in the integument layer of developing grains. Li et al (2011b) show genetic interaction between *MADS3* and *MADS13* in rice to regulate carpel and ovule development, raising the possibility that the same protein complexes functioning in floral and grain development are involved in later grain development function. Similarly *DL* is also found to be expressed in integument layers. *OsMADS6* has been shown to interact with *MADS3*, *MADS58*, *MADS13* and *DL* in rice (Li et al, 2011a). Localization of these C-class genes to the
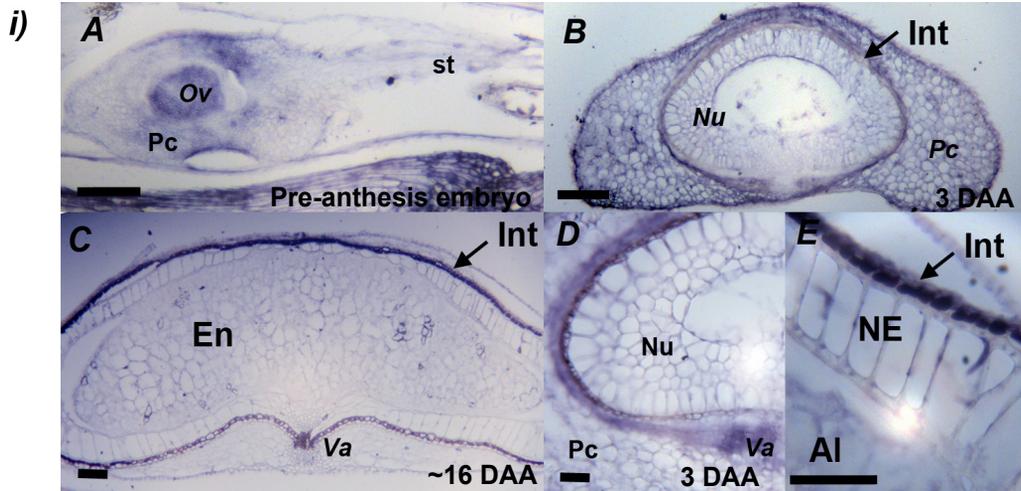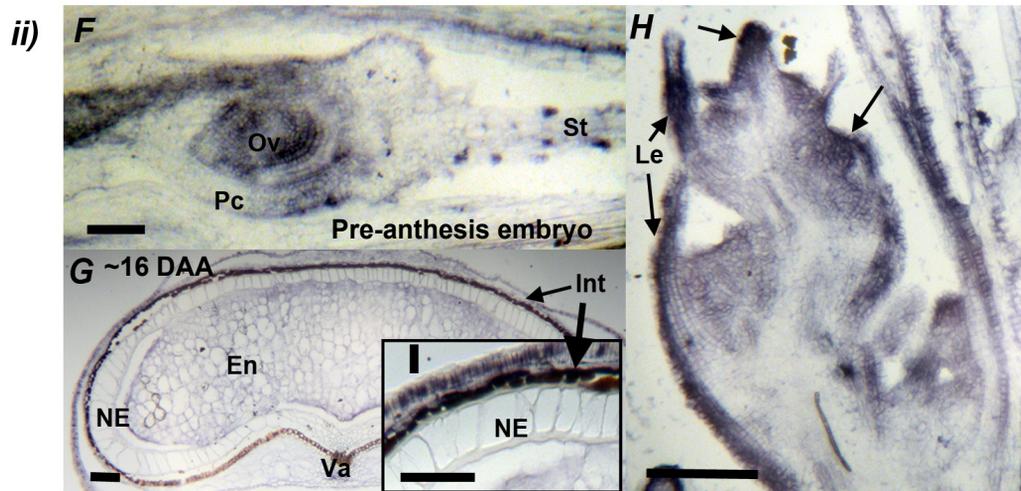
Figure 5.5: mRNA ISH for *BdMADS58* & *BdMADS3* Across Grain Development.

i) shows *BdMADS58* expression in (A) ovule and maternal tissue in an immediately pre-anthesis embryo, (B) shows expression in integument and maternal tissue in an approx. 3 DAA developing grain, detailed in (D). (C) shows integument expression in the mature grain (~16 DAA), detailed in (E) where expression can be seen as located in the inner integument.

ii) shows BdMADS3 expression in (F) a pre-anthesis embryo and (H) a developing spikelet showing expression in floret organ primordia. (G) shows expression potential expression in the mature grain inner integument (inset).

Ov, Ovule; St, stigma; En, Endosperm; Pc, pericarp; NE, Nucellar Epidermis; Nu, Nucellus; Int, integuments; Le, Lemma. Scale bars in A-C & F-H show 100 µm, D, E & I show 30µm.

integument layer suggests their involvement in defensive or cuticular-type functional roles. It is also interesting to note the translocation of expression for these C-class genes, being expressed in both filial and maternal tissues during early grain development, but becoming restricted to the maternal layer as the grain matures.

 BdFUL1 & BdFUL2: For both *BdFUL* genes expression was detected only during the early stages of grain development (fig. 5.4). *BdFUL1* was expressed most strongly in the pre-anthesis spikelet extending into the young grain, and weakly in the mature grain, potentially attributable to expression in the embryo. *BdFUL2* expression was detectable only in the pre-anthesis stage. *BdFUL1* expression was contrary to our expectations based upon published data. With presumed E-class analogous function (Preston & Kellogg 2007) we hypothesized *FUL1* expression may persist across grain development, similar to *LHS1,* potentially functioning in interactions between other grain development expressed MADS-box genes. We detected no expression beyond very early grain development indicating this was not the case. For *FUL2* we expected to see only early developmental expression, in keeping with previous reports for genes of A-class function (Kellogg & Preston, 2007). Here the expression of *BdFUL2* is in keeping with this expectation. Preston and Kellogg (2007) surveyed expression of *FUL1* and *FUL2* orthologues across temperate and tropical cereal species and our observations for *Brachypodium* agree largely with their findings for temperate cereal expression profiles. In two Pooideae species, *Avena sativa* and *Triticum monococcum*, in mature spikelets they found *FUL1* to be expressed in all floret organs whilst *FUL2* was expressed in all floret organs except carpels. In the *Brachypodium* PrS sample, which includes

all floret organs, we detect *FUL1* and *FUL2* expression whilst in the Young Grain

sample, comprised more specifically of isolated grains (carpels) soon after

anthesis, no *FUL2* expression is seen. These patterns of expression compare well

with what is seen for the rice orthologues of these genes, *OsMADS14* and

OsMADS15, initially expressed across the whole floral meristem region and

becoming restricted to outer whorl organ primordia, (Yamaguchi & Hirano, 2006).

These results do not indicate if any differences exist in the specific organs in which

FUL1 is expressed in the *Brachypodium* pre-anthesis spikelet however. An aim of

future research is to investigate, via tissue-specific PCR and in-situ hybridization, if

this pattern of expression is shared across the Core-Pooids.

BdMADS13 & BdMADS21: Studies in rice have shown *OsMADS13* to play an

important role in ovule identity (Dreni et al., 2007, Lopez-Dee et al., 1999) whilst

OsMADS21 was determined to have no obvious function (i.e., no phenotype

observed when the gene was silenced) independently or redundantly (Dreni et al.,

2007). The RT-PCR expression profile for the *Brachypodium* orthologues of these

genes is shown in figure 5.4. Consistent to expectations based upon rice

observations, *BdMADS21* expression is revealed to be very low across grain

development. Expression is at its highest, only just detectable, during the early

stages of grain development. In contrast, *BdMADS13* was strongly expressed

across grain development with a slight decline during mid-development and weak

expression in pre-anthesis spikelets. Again, previous analyses have focused

largely on *MADS13* function in floral development with expression during grain

development receiving little attention. Dreni et al (2007) saw *OsMADS13*

expression in developing rice grains rice at 5-10 days after pollination, providing

some support for a comparable function between *Brachypodium* and rice orthologues.

  To further investigate D-class gene expression throughout grain development we performed RNA ISH on grain stages from pre-anthesis through to maturity, shown in figure 5.6. *BdMADS13* expression is detectable in the inflorescence where ovule primordia have formed, discreetly located within the developing ovule (fig 5.6A). During the earliest stages of grain development expression appears to be confined to the endosperm syncytium and perhaps the integumentary layers (5.5B). Strong expression was seen in the early syncytial and cellularised endosperm, becoming restricted to the aleurone layer as development progresses (Fig 5.6 C-F). At maturity expression becomes confined to the aleurone layer along with some embryo expression (Fig 5.6 F-H). The strong RT-PCR expression detected during the two later stages of grain development can be attributed to this aleurone expression. In order to compare this previously unreported pattern of expression we performed ISH for the *MADS13* orthologue in wheat and compared these results to *Brachypodium*. A probe for the wheat *MADS13* was available through earlier characterisation of wheat grain expression domains (Drea et al, 2005b). During early wheat grain development, *MADS13* expression can be seen in the phloem and outer integument (See appendix A5d), a pattern very similar to what is seen in *Brachypodium*. In the mature grain *TaMADS13* expression is detected in the embryo and similarly to *Brachypodium* appears to be largely excluded form the scutellum, but the most notably is absent from the aleurone layer across grain development (Fig 5.5I).
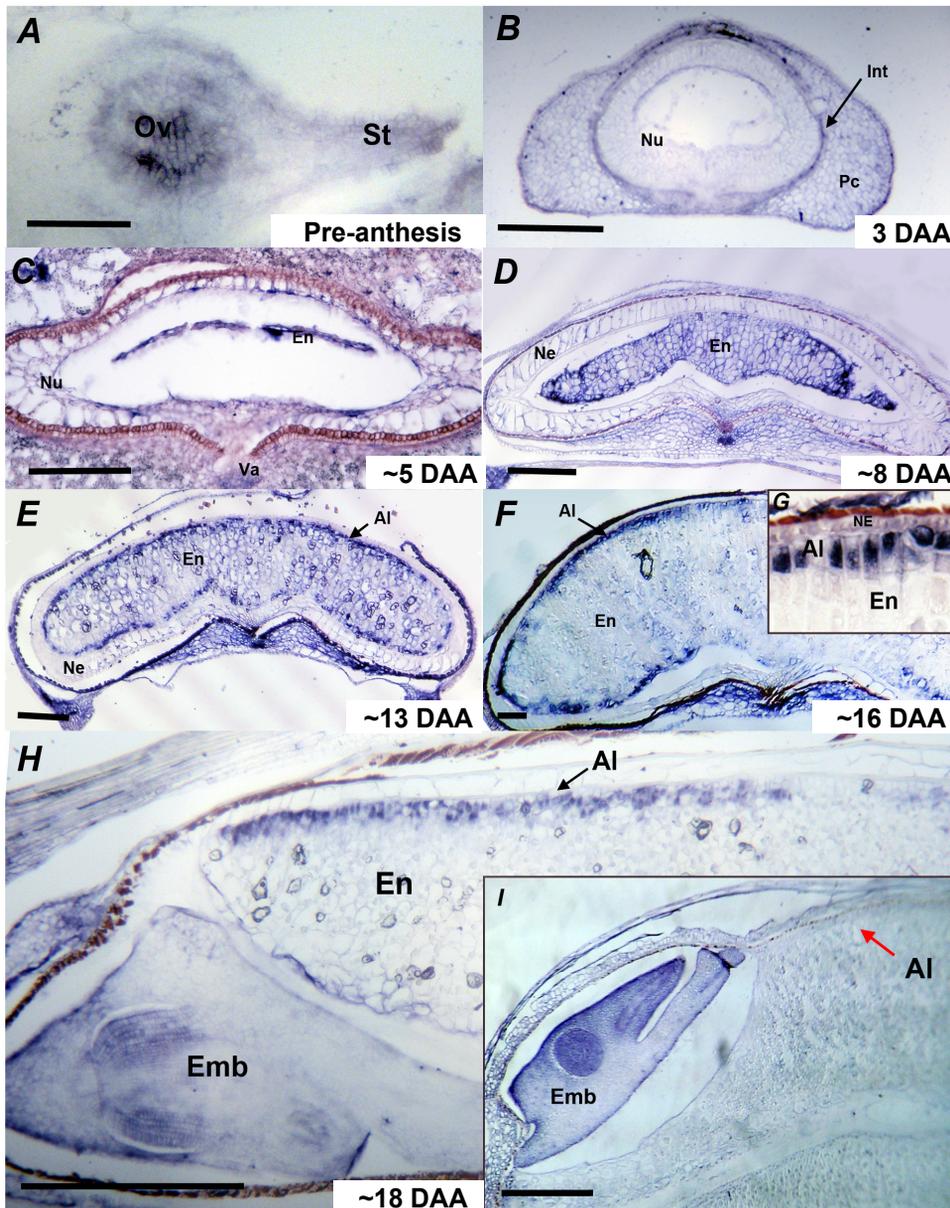
Figure 5.6: mRNA In-situ Hybridisation for *BdMADS13* across Grain Development.
(A) Shows *BdMADS13* expression in the developing ovule. (B) A 3DAA grain showing
expression in the integument layers and maternal tissues. (C & D) Expression in the
pre-cellular and throughout the early developing endosperm. (E & F) Shows
expression becoming restricted to the aleurone layer as development progresses and
(G) shows expression is tightly aleurone specific closer to maturity. MADS13
expression appears to be absent from the aleurone layer overlaying the crease in
(presumptive modified aleurone region; F). (H) Embryo and aleurone layer at maturity
in comparison to (I) showing *TaMADS13* in a mature wheat grain where embryo but no
aleurone expression is detectable. Ov, Ovule; St, stigma; Ne, Nucellar epidermis; En,
Endosperm; Al, Aleurone; Va, vasculature; Emb, Embryo. Scale bar in A shows 20 μm,
B-F 50μm, H & I 500μm.

Our findings indicate additional function in *Brachypodium* to that previously reported for this gene in other species. Ovule specific expression patterns of D-class genes in *Arabidopsis* and *Petunia* have been reported (Rounsley et al, 1995) and a similar pattern along with key function in ovule specification is reported for *OsMADS13* (Dreni et al, 2007). We have found expression in both embryonic and filial tissues, and the continued and domain specific expression in grain development observed for *BdMADS13* is indicative of additional function.   Over-expression of *OsMADS13* was not found to induce ectopic ovule formation, as it does in *Petunia* (Favaro et al, 2003), suggesting this gene may show differences in its role in ovule specification between monocots and dicot species. Differences in *MADS13* expression between wheat and *Brachypodium* identified here suggest functional divergence may also exist within the Pooideae. It is an ongoing aim to further characterize and investigate *BdMADS13* expression and we have begun work on RNAi constructs and insertional mutagenesis techniques to investigate this gene's role in *Brachypodium* grain development.

BdLHS1: We detected strong and consistent *BdLHS1* expression during inflorescence and grain development (Fig 5.4). Notably however, only very weak expression was seen in the leaf, in contrast to the findings of Malcomber and Kellogg (2004) where in several grass species examined, including barley and rice, leaf expression was seen. *LHS1* was identified here as an E-class gene with direct effects on grain development although is thought to be involved in multiple and diverse developmental roles (Malcomber & Kellogg, 2004). *LHS1* has been shown to be expressed, sometimes specifically, in different organs within mature florets, in oat *LHS1* is found to be specific for palea, lemma and pistil of mature florets, and in

some species is restricted to the ovule (Malcomber & Kellogg, 2004; Christensen & Malcomber, 2012).   These expression analyses do not extend across grain development however. *OsLHS1* is known to interact and form complexes with several other MADS-box genes including *OsMADS13*, interactions being identified for their role in floral development (Malcomber & Kellogg, 2005). Expression for *MADS13* is also seen to extend across grain development suggesting that any function during grain development is achieved via the same or similar protein complexes. Functional investigations suggest *LHS1* has a role in meristem determinacy and organ identity (Jeon et al, 2000), but its expression in developing grain tissues suggest it may have roles in other developmental processes. This function is likely associated with MADS-box protein complexes involved in grain development. Our ongoing research aims include RNA ISH to investigate expression patterns across grain development and we have initiated yeast two hybrid analyses for MADS-box genes including *LHS1* to identify interacting proteins.

5.2.3 *YABBY genes*

The YABBY gene family has been well studied in both *Arabidopsis* and rice. Its members have been shown to have particular significance in relation to floral, meristem and reproductive development. A comprehensive study of the family reports 8 YABBY genes in the rice genome (Toriba et al, 2007). We performed searches and comparison for these sequences against the *Brachypodium* genome and were able to identify candidate orthologues for all eight. We have focused our expression analyses on those members of the family shown to have the greatest

influence over aspects of grain development, the three members of the *FILAMENTOUS FLOWER*-like clade and *DROOPING LEAF*.

5.2.3.1 *YABBY Gene Classification and Nomenclature*

Inconsistent and conflicting naming has produced a confusing situation in relation to the identity of the monocot YABBY genes, largely in rice where they have been most well studied. To understand the situation we initially assembled all of the rice YABBY sequences detailed in three of the most recent and prominent papers examining either some or all members of this family where different naming conventions have been used. Using tBLAST and comparative approaches we positively identified and confirmed a single Rice Annotation Project (RAP) *Os* accessions for each of the 8 genes from the sequences listed in all three papers. Of these three papers Toriba et al (2007) is the only one detailing all members of the family, and the most recent, so we have adopted this labeling as definitive for the group and related other sequence listing to it. Differences identified in the naming between these three papers are listed across columns 2 – 4 of table 5.3 and genes are arranged according to the major subgroupings identified for the YABBY gene family in *Arabidopsis* (Bowman 2000). It can be seen that there is some overlap and agreement between the different authors but little consistency. It is likely that the high levels of homology amongst the family, annotation of the rice genome and multiple records across different databases have all contributed to these discrepancies.

YABBY gene classification (Arabidopsis)	Name (Dai)	Name (Jang)	Name (Toriba)	Os Accession	Rice expression pattern*	Brachy orthologue	Brachy annotation	Protein homology	BLASTP value
YAB2	--	OsYAB4 (NP_911282 BLASTP HITS Os07g0160100)	OsYABBY1 AB274013	Os07g0160100	Developing flowers and seeds	Bradi1g57070	--	86%	
	OsYAB1 (BAF12697) OsYAB2 (ABF97910)	OsYAB1 (Af098753) OsYAB2 (AAS07125)	OsYABBY2 AB274014	Os03g0650000	Developing flowers, seeds meristems and leaves	Bradi1g13570	--	85%	
	OsYAB5 (BAF30318)	--	OsYABBY6 AB274018	Os12g0621100		Bradi4g01300 Bradi1g13570	--	77% 65%	
FIL/YAB3	OsYAB4 (BAF26935)	OsYAB3 (NP_922256)	OsYABBY3 AB274015	Os10g0508300	Young inflorescence and shoot apices	Bradi3g30410 Bradi5g16910 Bradi3g50050	BdFIL-3	66% 57% 56%	
	OsYAB7 (BAF09473)	--	OsYABBY4 AB274016	Os02g06433200		Bradi3g50050	BdFIL-2	79%	
	OsYAB3 (BAF26935)	OsYAB5 (CAD41530)	OsYABBY5 AB274017	Os04g0536300		Bradi5g16910 Bradi3g50050	BdFIL-1	76% 70%	
INO	--	--	OsYABBY7 AB274019	(BAF45808) Os07g0571800	Reproductive organs at low levels	Bradi1g24050	--	59%	
CRC/DL	DL (ABF94636)	DL (BAD06552)	OsDL AB106553	(BAF11289) Os03g0215200	developing flowers, carpel/central leaf primordia	Bradi166990	BdDL	88%	

*Details of expression pattern taken from Toriba et al 2007

Table 5.3: YABBY Gene Family Identification and *Brachypodium* orthologues.
An identification rice YABBY sequences from 3 major publications and differing nomenclature relating to these sequences. *Brachypodium* candidate sequences identified by BLAST at the protein level.

5.2.3.2 *Phylogenetic Analyses*

BLAST analysis for all 8 members of the rice YABBY gene family against the *Brachypodium* genome produced highly similar candidate sequences for all eight genes. *Brachypodium* candidates and protein level homology scores are listed in table 5.3. The relationship between the rice YABBY genes and those identified in *Brachypodium* appears to be very close with frequently high levels of sequence homology. These results indicate the *Brachypodium* YABBY gene family is also comprised of at least eight members. We performed BLASTp analysis using the consensus sequence of the YABBY domain from these 8 genes against the *Brachypodium* genome but could identify no further members.

The amino acid alignment in figure 5.7 shows members of the YABBY gene family from all of major clades across the angiosperms. Throughout, *Brachypodium* candidate genes group closely and distinctly to their rice orthologues, and those of related temperate cereals. Conservation across conserved domains can be seen but some class-distinctive variation, relating to subgroupings of the family, can be seen in the central, n- and c-terminal domains. These discrete family level groupings and consistency provide a strong support for the identity of our *Brachypodium* candidates.

The phylogenetic tree shown in figure 5.8, derived from the previous alignment, makes more obvious the disparity between the monocot and dicotyledonous *FIL*-like sequences. All members of the YABBY gene family in *Arabidopsis*, rice and *Brachypodium* are included and monocot sequences are all accounted for in discreet groupings. In particular discrete groupings can be seen for all monocot
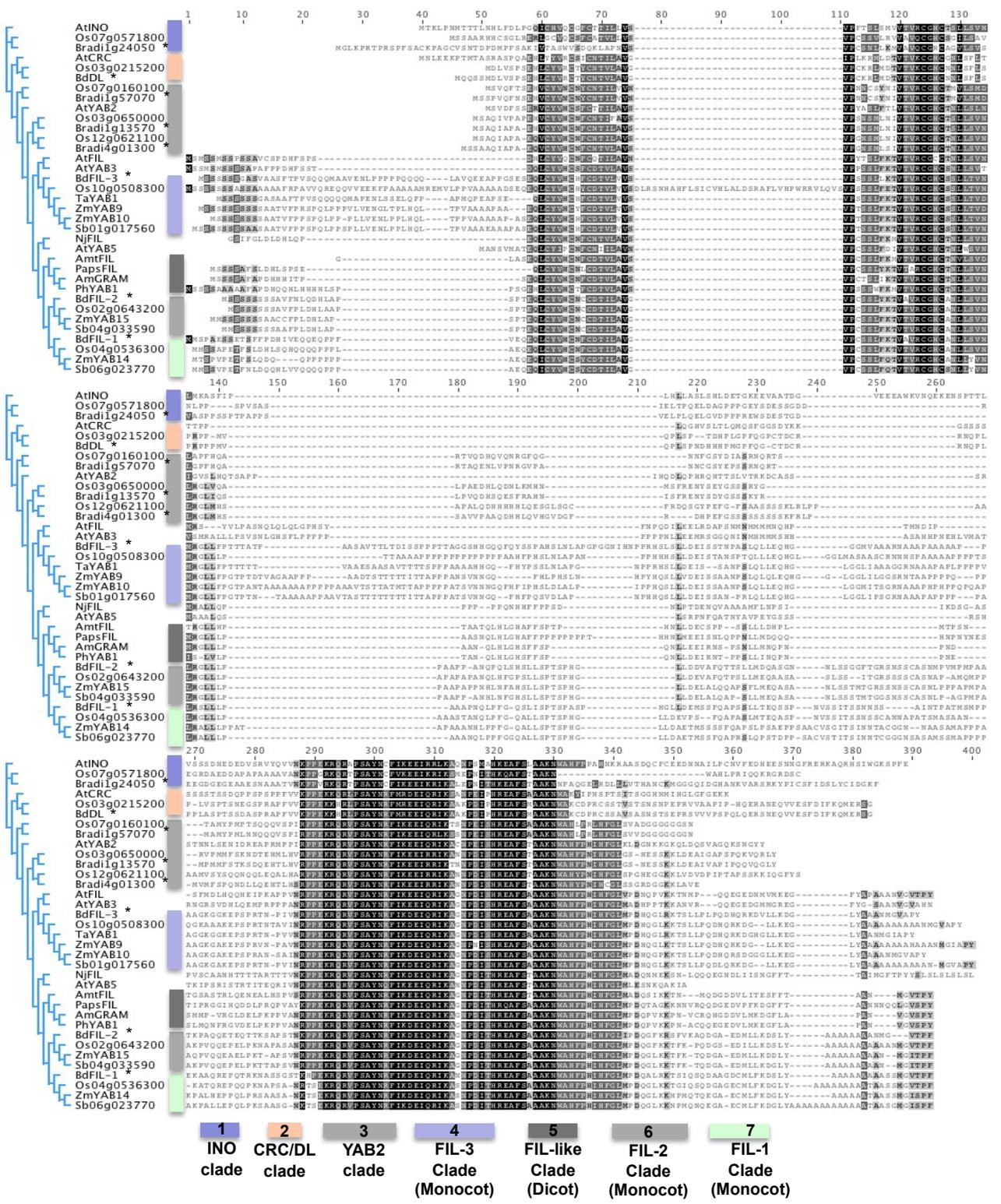
Figure 5.7: YABBY Gene Amino Acid Alignment.

ClustalW alignment of amino acid sequences of YABBY gene family orthologues from across the angiosperms. Identical residues highlighted in black, similar residues are highlighted in grey. Horizontal bars indicate major clades as shown in the phylogenetic tree, fig. 5.8. *Brachypodium* orthologues throughout indicated with an *.
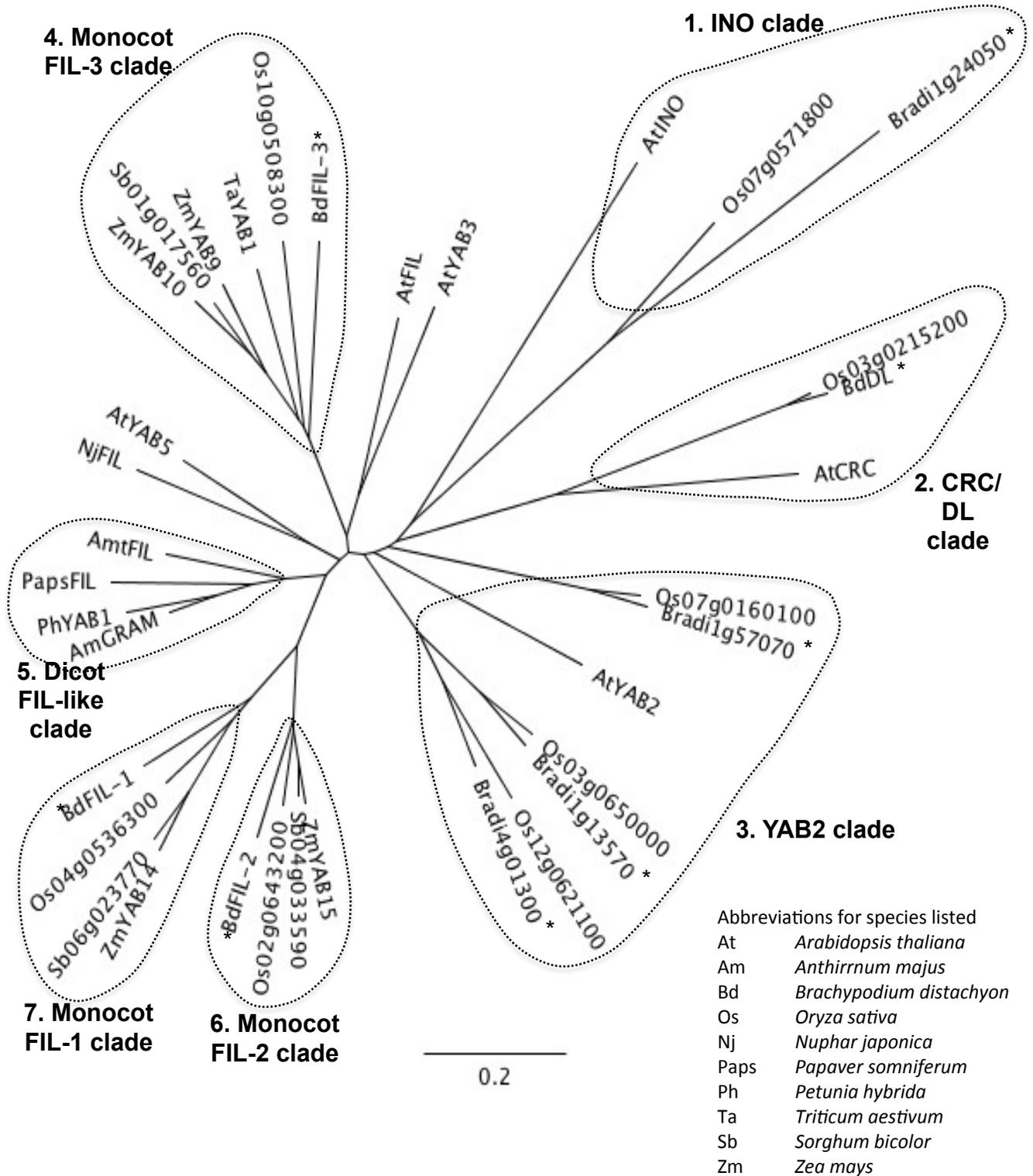
Figure 5.8: Phylogenetic Tree for the Angiosperm YABBY Gene Family
Unrooted neighbour joining tree for selected, prominent members of the monocot
and dicot YABBY gene family. All members for rice, *Brachypodium* and
Arabidopsis are included. Branch lengths indicate genetic distance.
Brachypodium sequences highlighted with *

and some dicot *FIL* clades. A notable feature is the isolation of *AtYAB5* in relation

to the monocot sequences examined. A recent investigation by Yamada et al

(2011) identifies 5 members of the ancestral YABBY gene family and found

orthologues of *AtYAB5*-like clade genes in *Cabomba* and other basal angiosperms.

In this family-wide survey we were able to identify no apparent *YAB5* equivalent in

Brachypodium or rice. Having allied the monocot YABBY clades to the *FIL*-like,

CRC and possibly even INO groupings this suggests the distinct eudicot *YAB5*

clade is possibly lacking in the Monocots? Our observations to this effect support

the earlier suggestion by Toriba et al (2007) who, when examining similarity across

the conserved YABBY domain in the angiosperms also identified the potential lack

of a monocot equivalent to the *YAB-5* clade. Previous findings and our own results

indicate that significant diversification and divergence has occurred in the YABBY

gene family, particularly in relation to what is seen between monocot and dicot

family members (Yamada et al, 2011). In relation to sub-grouping in this family

care should be taken in seeking to classify monocot family members according

dicot-derived classifications, which may be less meaningful. Monocot specific

classification will likely give a clearer picture of the evolutionary and possibly

functional groupings of this gene family.

5.2.3.3 Expression Analyses

 We selected members of the *FILAMENTOUS FLOWER/YAB3* clade and

DROOPING LEAF as being of greatest interest to us in relation to effects on

Brachypodium grain development. Based upon the high level of sequence

homology as compared to rice we hypothesised that similar expression patterns

may be seen in *Brachypodium*. An RT-PCR profile of expression over grain

development was produced (Fig. 5.9) and shows results that were largely in

keeping with expectations.

  Of the three *BdFIL* orthologues, all show expression in the pre-anthesis spikelet,

although it is typically weak. *BdFIL-1* and *BdFIL-2* also show weak expression in

the mature grain, likely attributable to expression in the embryo. *BdFIL-1*

expression alone can also be seen to extend into the young grain at low levels.

BdDL is expressed across floral and grain development, being at its strongest in

the inflorescence and young grain, diminishing and disappearing as the grain

matures (fig 5.9). This expression pattern is in keeping with a predicted role in

carpel development but suggests some ongoing function in grain development.

Weak expression is also seen in the leaf, in keeping with a role in leaf midrib

development seen in other grass species (Yamaguchi et al, 2004).

  Polar localization is an important feature of YABBY gene expression patterns in

the dicotelydonous species and also in maize. Expression is typically abaxially

located, although reversals have been noted in some species, such as maize

(Juarez et al., 2004). Polar localisation is however not a feature of YABBY gene

expression in rice (Dai et al, 2007a, 2007b; Jang et al, 2004). We performed RNA

ISH for *DL* in *Brachypodium* in order to further characterise and compare its

expression during grain development. Figure 5.10A shows strong *BdDL*

expression across the developing spikelet, with concentrations in developing floret

primordia. Images H and B show respectively more advanced stages of spikelet

development. Expression appears to be restricted to abaxial floret organ primordia

in H whilst expression can also be seen in glume and palea epidermal layers
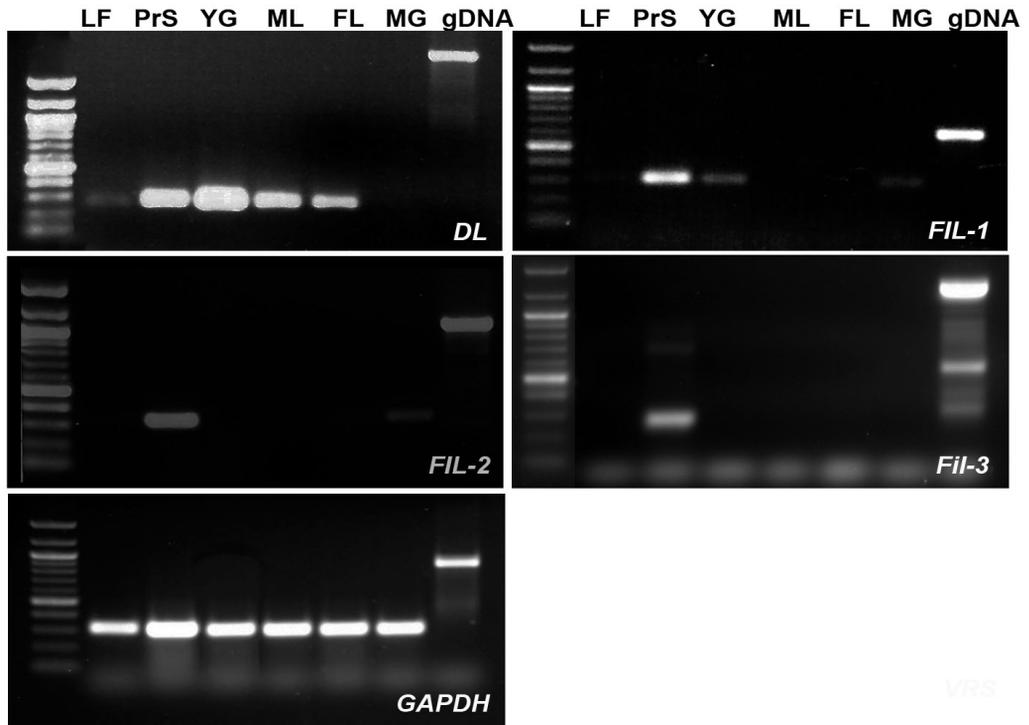
Figure 5.9: RT-PCR Expression Profiling for *Brachypodium* YABBY Genes

Expression analysis for YABBY family genes in *Brachypodium* leaf and developing grain tissues. Plant tissues and growth stages spanning grain development used for template cDNA production as follows, LF = Leaf, PrS = pre-anthesis spikelet, YG = Young Grain, ML = Mid Length Grain, FL = Full Length Grain, MG = Mature Grain, gDNA = genomic DNA. Ladder shows 100bp intervals.
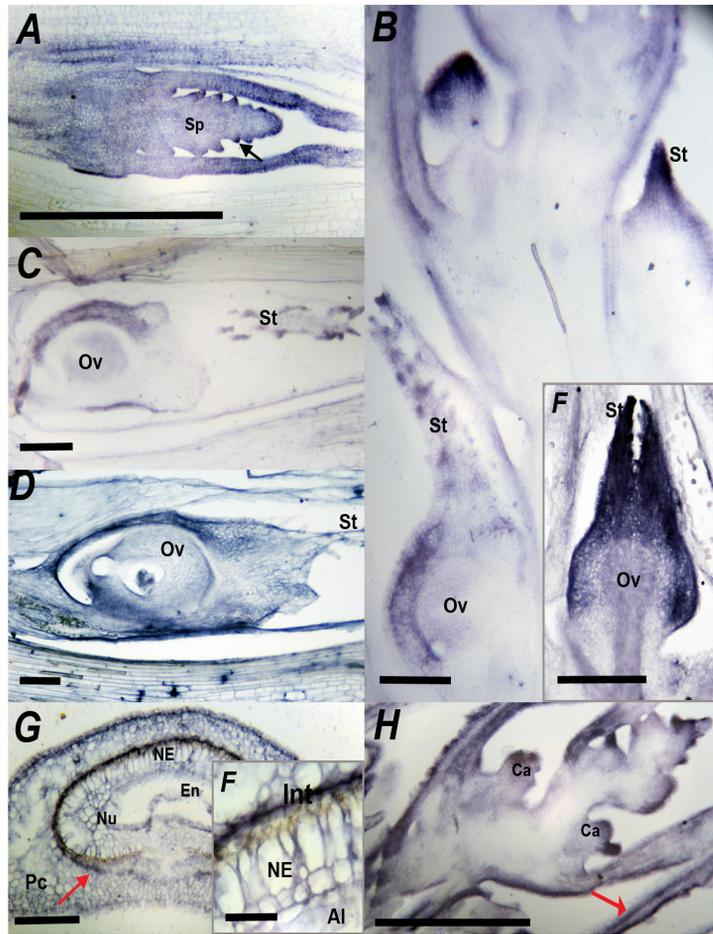
Figure 5.10: mRNA In-situ Hybridisation for *BdDL* during Inflorescence & Grain Development.

BdDL expression is shown in several tissues during grain development. (A) expression throughout a early developing spikelet with concentrations in floret primordia, arrowed. (H & B) later spikelet and carpel development showing expression now restricted to carpel primordia (H) and then to carpel abaxial and stigmatic region (B). Expression can also be seen in lemma and glume epidermal layers (arrowed). Inset (F) shows lateral section view of the carpel, expression extending to both sides and strong in the stigma. (C) shows abaxial carpel expression immediately pre-anthesis and (D) wider post anthesis expression. (G) shows integument expression in a developing grain (Approx. 3DAA), detailed in inset, and potential absence in grain adaxial region, arrowed. Sp, Spikelet; Ov, Ovule; St, Stigma; NE, Nucellar Epidermis; En, Endosperm; Nu, Nucellus; Pc, pericarp; Al, Aleurone; Ca, carpel. Scale bars show 100µm, except in F, showing 20µm.

(arrowed).   At a later stage in the developing spikelet strong expression is seen in carpels, discreet to the region of the stigma, distal and abaxial region of the developing carpel (5.10B). Inset (F) shows a lateral section through the carpel just before anthesis, where expression is particularly strong in the stigmatic region and extends to both sides of the carpel in this plane. This pattern of expression for *DL* in developing carpels appears well conserved between species. Yamaguchi et al (2004) show a very similar expression in the developing stigma region of rice flowers.   Ishikawa et al (2009) show RNA ISH for *DL* in wheat with expression restricted to the pre-anthesis stigma and carpal abaxial regions, an almost identical pattern to that we observe in *Brachypodium*. The epidermal lemma expression seen in *Brachypodium* appears absent in wheat but appears consistently in hybridisations performed here and would benefit from further investigation.

  Images C and D, Figure 5.10, show carpels just prior to and just after anthesis respectively. Pre-anthesis a dominantly abaxial pattern of expression is obvious whilst in the post-anthesis stages expression appears to have extended to both sides of the carpel to some extent. We were unable to conclusively locate expression in post-cellularisation stage grains but our observations suggest that *BdDL* remains restricted to the maternal tissues during development. We were able to identify some expression in the outer integument layer, shown in figure 5.5G and inset, and it is possible that the expression detected via RT-PCR during grain development is likely attributable to this integument expression. The expression detected in this region was however variable and sometimes weak. A further line of evidence in support of this site of expression can be seen in relation to known interactions between *DL* and MADS-box transcription factors. Li et al

(2011a) reports interactions between *OsMADS3* and *OsDL* in floral specification and organ identity, and co-localisation of their expression. We detected *BdMADS3* expression in the outer integument layer of developing grains and it is possible that similar interaction may be involved in the function of these genes during grain development. Yamaguchi et al (2004) reports the detection of *DL* transcripts in the embryo of developing rice grains at mid-developmental stages, indicating that the observations made here may be consistent to rice although minor differences exist. Our observations suggest that *BdDL* may maintain some weak abaxial localization in the developing carpel, possibly losing this distribution as it is expressed in the developing grain. This pattern of expression is not seen in rice although it does appear to be a feature shared with wheat. It is possible this similarity in expression between *Brachypodium* and wheat may be attributable to some shared features of grain morphology and developmental patterning in the early stages.

These findings provide a preliminary overview of the YABBY genes in *Brachypodium* as a basis for future investigation. We have identified high levels of sequence homology across the family and expression analyses indicate conserved functional homology for *DL*. Members of the YABBY gene family have increasingly been shown to have important functional roles both in fruit development and in morphological adaptations often associated with domestication. *FAS*, a YABBY gene in tomato has been seen to be important in the control of fruit size and recently a YABBY gene in sorghum has been identified as having a key role grain shattering, with functional orthologues identified in other cereals including rice (Lin et al, 2012). A TDNA insertional mutant for the *Brachypodium FIL-3* orthologue has been generated through the BrachyTag program. Our ongoing research aim is

to provide further functional analysis through the characterisation of this line, for which preliminary results are detailed in the following chapter.

5.2.4 *Grain Development Specific Transcription Factors*

A small number of genes have been shown to have important and notably specific roles in endosperm development. These genes, often members of the bZIP and MYB gene families have been found to function in the regulation of endosperm specifically expressed genes, such as storage proteins, and the specification of endosperm domains and transfer cells. Table 5.4 lists those genes identified with a significant role in grain development, their class and brief description of function. In contrast to genes examined so far, functional aspects have been best studied in barley and in maize rather than rice. BLAST analyses using predominantly barley sequences were used to identify candidate *Brachypodium* orthologues and these candidates are shown in table 5.5. Homology levels in relation to *Brachypodium* sequence varied significantly but using related sequences and analysis at both amino acid and nucleotide levels, we were able to identity putative, single candidate genes for all.

5.2.4.1 *Phylogenetic Analyses*

Phylogenetic analyses used to establish the identity of our *Brachypodium* candidates were broken down according to their smaller gene classes shown in column three of table 5.4. *ZmMRP-1* is significant in grain development as one of the few genes identified as responsible for the specification of particular tissues in

Gene	Other major cereal orthologues	Gene class	Function and expression patterns	References
ZmMRP-1	--	R1MYB	Transfer cell specific transcriptional activator. *Early grain/endosperm development*	Gomez et al., 2002; 2009
HvBLZ2	TaSPA/ZmO2	bZIP	Transcriptional regulation of endosperm specific genes e.g. storage proteins. *Predominantly mid development endosperm.*	Onate et al., 1999
HvBLZ1	OsREB	bZIP	Common pathway transcriptional regulator. *Developing endosperm,* also roots and leaves.	Vicente-Carbajosa et al., 1998
HvMCB1	ZmMCB1/TaMYB51	R1MYB	Transcriptional repressor/activator. *Developing endosperm and germinating aleurone.*	Rubio-Somoza et al., 2006b
HvMYBS3	ZmMybst	R1MYB	Aleurone specific Transcriptional repressor/activator. *Developing and germinating aleurone cells.*	Mercy et al., 2003; Rubio- Somoza et al., 2006a

Table 5.4: Endosperm Specifically Expressed Gene Identification
Transcription factors identified in maize (Zm) and Barley (Hv) showing expression patterns specific to the endosperm.

Candidate name	Candidate gene Identifier	Protein Level Homology	BLASTP e-value	Identification BLAST Sequence
BdMRP1	Bradi1g72300 (Closest hit)	37%	7e-28	ZmMRP-1
BdBLZ2	Bradi1g55450	41%	5e-58	HvBLZ2
BdBLZ1	Bradi1g05480	75%	e-143	HvBLZ1
BdMCB1	Bradi3g33440	59%	3e-95	HvMCB1
BdMCB3	Bradi3g33400	95%	5e-137	HvMYBS3

Table 5.5: *Brachypodium* Endosperm Specifically Expressed Candidate Genes.
Brachypodium candidate genes identified by BLAST analysis potentially orthologous to the endosperm specifically expressed genes shown in table 5.4.

the grain. A determinant of the maize BETL layer *MRP-1* encodes an atypical single repeat MYB protein and belongs to the SHAQKYF family of R1MYBs (Gomez et al, 2009). The *Brachypodium* genome contains several single- repeat MYB genes but we identified Bradi1g72300 as closest in sequence homology to *ZmMRP-1* focusing on the conserved VASHAQKYF domain. We were also unable to identify any orthologues in temperate cereals (no sequence homology extended beyond the conserved MYB- domain of the protein) or in rice, which lacks the BETL layer. Figure 5.11 shows an alignment of R1MYB genes from across the angiosperms. The *Brachypodium* orthologue groups with the maize and sorghum *MRP-1* genes, this similarity and grouping is visible in the phylogenetic tree, although it is clear that the majority of homology is confined to the conserved MYB domain.

  Other single repeat MYBs identified as having significant function in grain development are included in figure 5.13. The *Brachypodium MCB1* and *MCBS3* candidates group quite clearly with their barley and wheat orthologues and share several features in both the n- and c-terminal regions. *BdMCBS3* in particular shares a conserved region at the extreme c-terminus with both tropical and temperate cereal orthologues, although less features in the n-terminal region. Whilst we were able to identify the highest protein level homology hits in BLAST analysis these searches did produce two other highly scoring sequences as potential candidates, Bradi5g26110 and Bradi3g51757. We included these genes in the alignment to confirm the homology of our primary candidates and despite their similarity, it can be seen that these genes are unlikely to be orthologous.
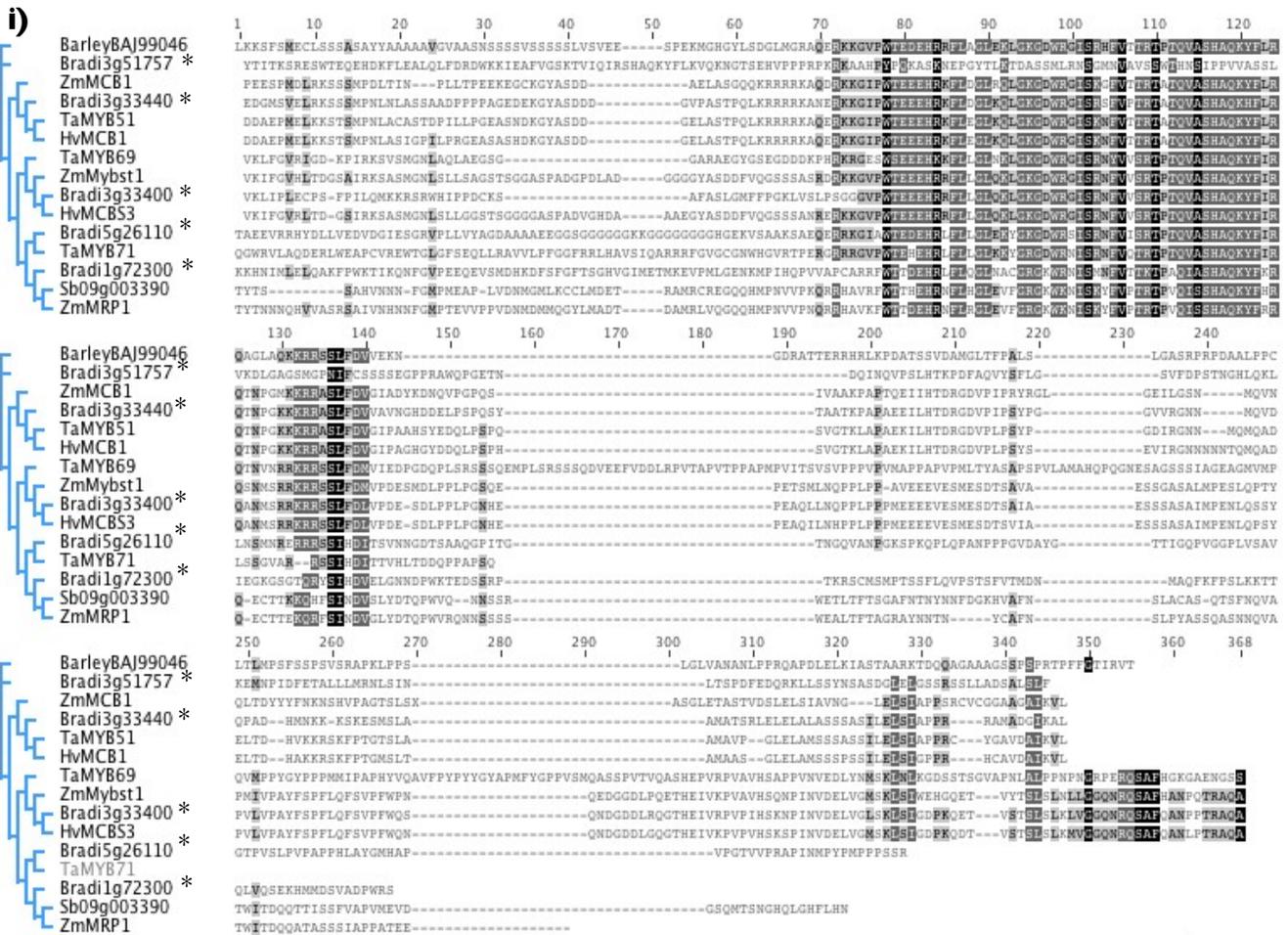
Figure 5.11: Sequence Analysis for the R1MYB Group Genes

i) ClustalW alignment of amino acid sequences from across the grasses. Identical residues highlighted in black, similar residues are highlighted in grey.
ii) Unrooted neighbour joining tree constructed form the alignment Phylogenetic tree constructed from the alignment. Distinct groupings containing the *Brachypodium* orthologues can be seen. *Brachypodium* orthologues throughout indicated with *.

Abbreviations for species listed
Bd   *Brachypodium distachyon*
Hv   *Hordeum vulgare*
Sb   *Sorghum bicolor*
Ta   *Triticum aestivum*
Zm   *Zea mays*
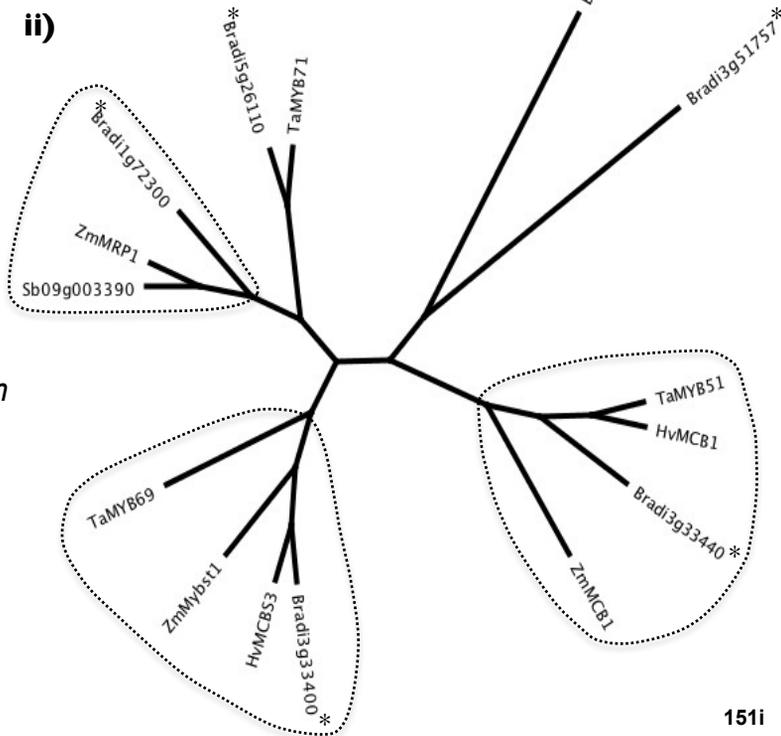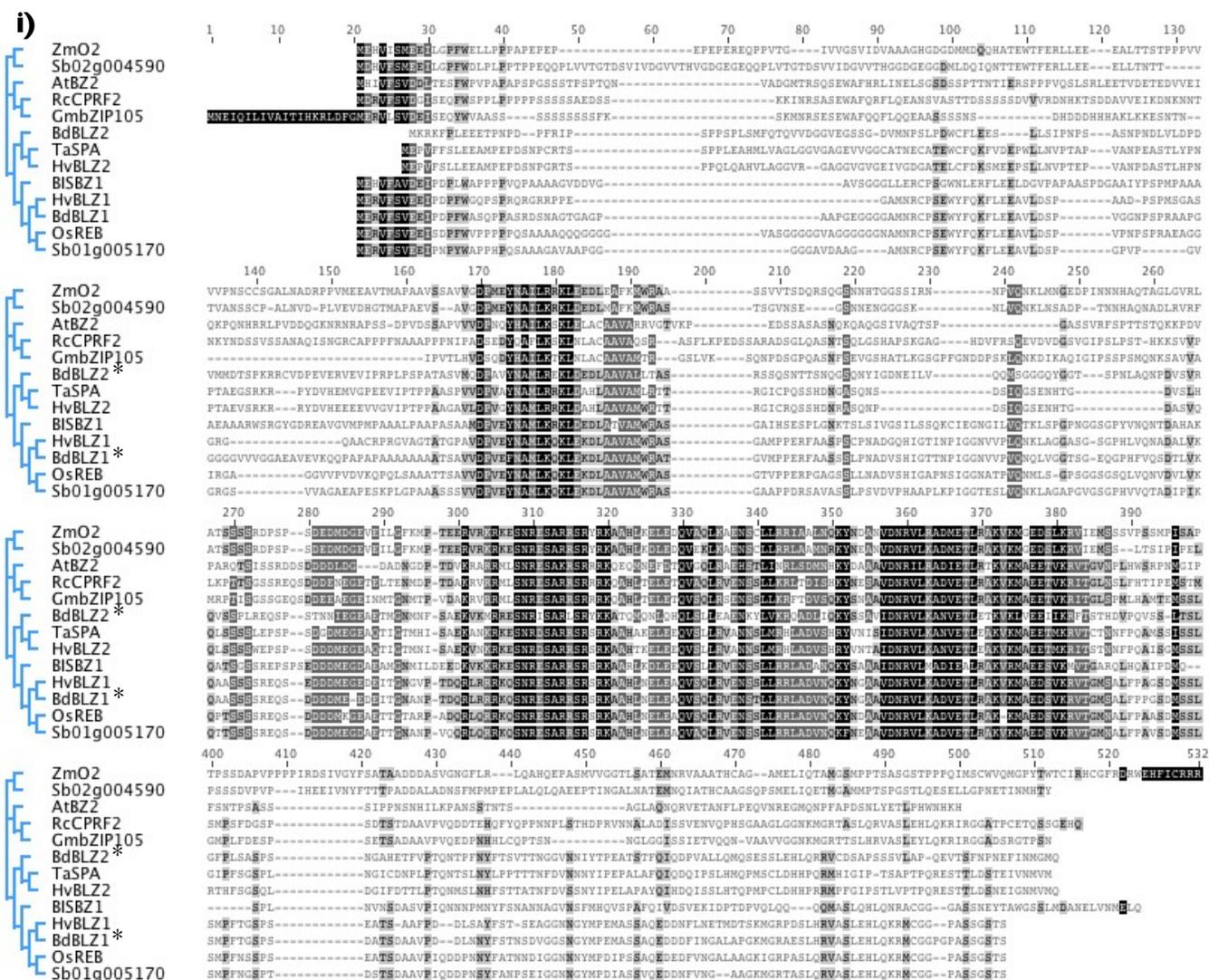
Figure 5.12: Phylogenetic Analysis for the OPAQUE Orthologue Genes.

i) ClustalW alignment of amino acid sequence of OPAQUE orthologues from across the angiosperms. Identical residues highlighted in black, similar residues are highlighted in grey.

ii) Unrooted neighbour joining tree constructed from the alignment. Branch lengths indicate genetic distance. *Brachypodium* orthologues throughout indicated with *.

Abbreviations for species listed

At	*Arabidopsis thaliana*
Bd	*Brachypodium distachyon*
Hv	*Hordeum vulgare*
Os	*Oryza sativa*
Sb	*Sorghum bicolor*
Ta	*Triticum aestivum*
Zm	*Zea mays*
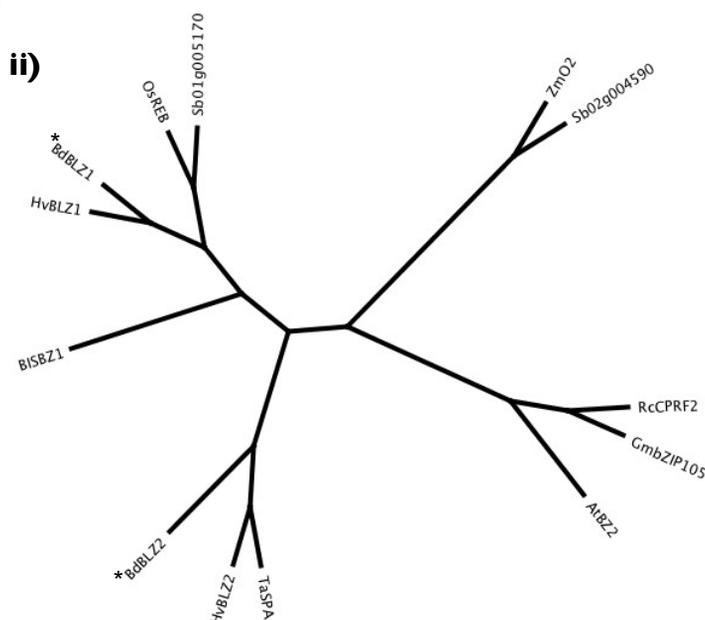
Orthologues of the key endosperm regulator genes *Opaque2/BLZ1/2* have been identified throughout the Poaceae. Using Barley *BLZ1* and *BLZ2* sequence we identified *Brachypodium* orthologues and found significantly greater protein level sequence similarity for *BLZ1* as compared to *BLZ2*. The amino acid alignment in figure 5.12 shows *Brachypodium* genes group closely to their temperate cereal orthologues rather than those taken from tropical species, in particular for *BLZ2*. Sequence similarity outside of the conserved domain is low, although the monocot contingent of this alignment does show some distinctive, conserved features in the c-terminal region. The differences between the monocot and dicot members of this group is perhaps most clearly shown by the length of the spurs leading to dicot members in the phylogenetic tree included in this figure. Vicente-Carbajosa et al (1998) reported a high level of sequence similarity between *OsREB* and *HvBLZ1* and the location of our *BdBLZ1* orthologue aligning tightly with these genes provides a good indication that they are orthologous.

5.2.4.2 Expression Analyses

RT-PCR expression profiling across grain development was performed for genes specifically expressed in the endosperm ,shown in figure 5.13. We were unable to detect any expression for Bradi1g72300, the *MRP-1* orthologue in developing grain tissues. This is in contrast to the high expression of *ZmMRP-1* described in developing maize endosperm. Specific expression patterns of *ZmMRP-1* in the basal pole region of the developing endosperm coenycyte, ectopic expression and other functional analyses have implicated this gene as a key regulator of endosperm transfer cell differentiation (Gomez et al, 2009, 2002). *ZmMRP-1*
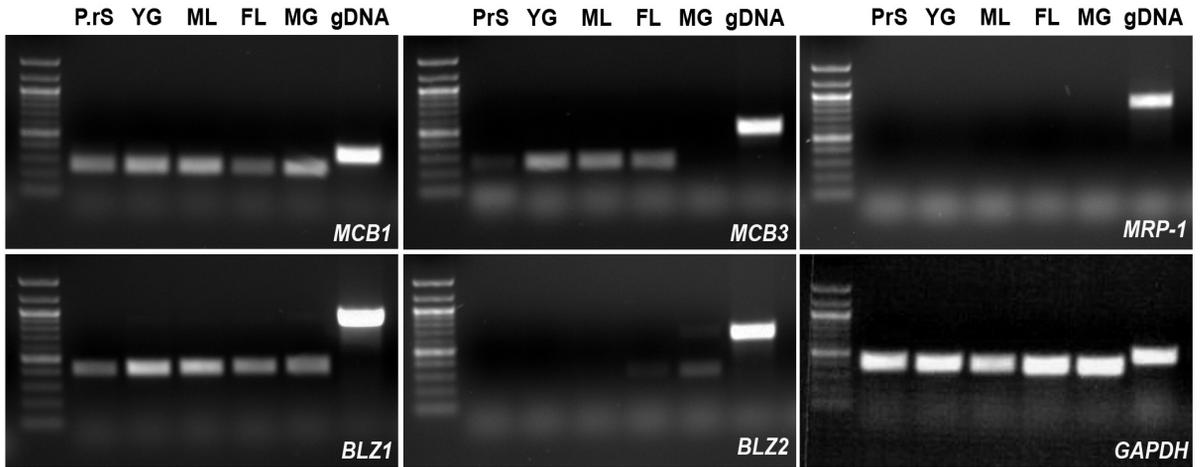
Figure 5.13: RT-PCR Expression Profiling for *Brachypodium* Endosperm Specifically Expressed Genes Across Grain Development.
An analysis of gene expression across grain development is shown. PrS sample comprises the entire spikelet whilst the following 4 stages represent just the isolated developing grain. Growth stages spanning grain development used for template cDNA production as follows, PrS = pre-anthesis spikelet, YG = Young Grain, ML = Mid Length Grain, FL = Full Length Grain, MG = Mature Grain, gDNA = genomic DNA. Ladder shows 100bp intervals.

strongly transactivates the promoters of two unrelated transfer cell-specific genes (Gomez et al, 2002). The maize BETL is functionally homologous the temperate cereal modified aleurone layer (Hueros et al, 1999). Based upon the apparent lack of an obvious modified aleurone region in *Brachypodium* and *BdMRP-1* expression, along with the inability to detect temperate cereal orthologues for this gene we propose that these regions are genetically distinct and have arisen independently as the products of parallel evolution.

The other genes specifically expressed in endosperm examined here were selected in order to make a comparison to published details of their expression in the barley grain. To fully compare and characterize the expression of the *Brachypodium* orthologues we prepared an RT-PCR panel for their counterparts in barley across grain development. Where available published barley primer sequences were used or primers were designed against accessions identified in the same publications. This expression profiling is shown in figure 5.14 where our results show a good match to what is seen in their original presentation (see table 5.4).

BdBLZ1 expression can be seen across grain development (Fig. 5.14), with a peak in expression level during the early stages. We were also able to detect expression in leaf tissues, as is found in barley and other species (data not shown: Vicente-Carbajosa et al, 1998). *BLZ1* has been reported as a potent activator of transcription for endosperm-specific gene promotors and shares DNA-binding and dimerization domains with members of the *O2* subfamily (Vicente-Carbajosa et al, 1998). The observed peak in *BdBLZ1* expression during early and mid grain development is consistent with this function and likely correlates to an increase in

153
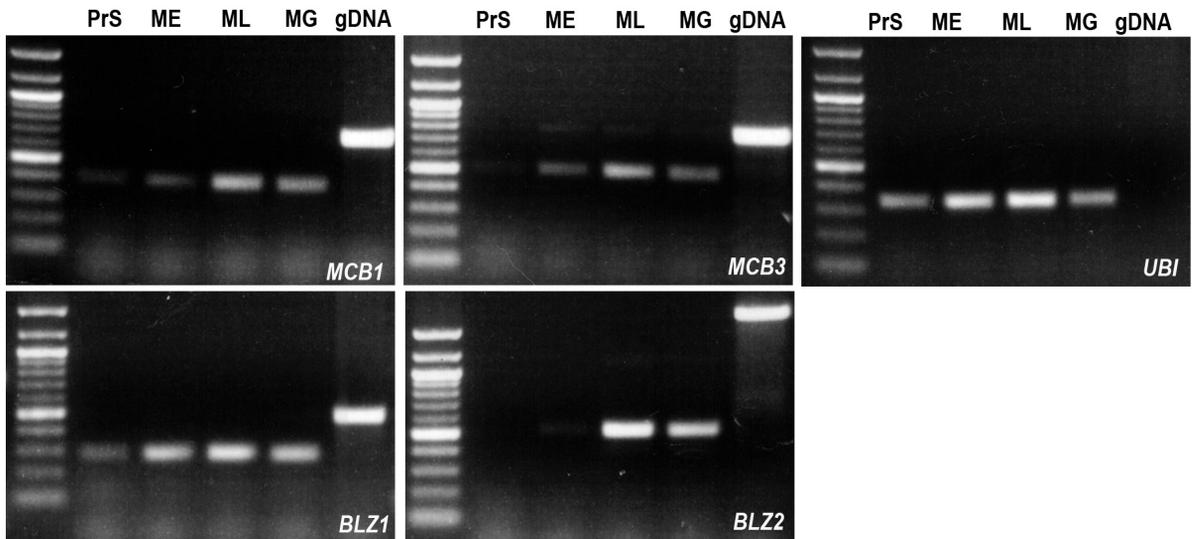
Figure 5.14: RT-PCR Expression Profiling for Endosperm Specific Transcription Factors Across Grain Development in Barley.
An analysis of expression across grain development for endosperm specifically expressed genes in Barley, providing a direct comparison to expression of their orthologues in *Brachypodium*. Expression at 4 stages spanning barley grain development was examined using samples as follows. PrS = Immediately pre-anthesis entire spikelet, ME = Mid Early Grains, post anthesis but not fully developed in length. ML = Mid Late Grain, fully developed in length but not fully filled. MG = Mature Grain, grains being filled but prior to the onset of desiccation. gDNA = genomic DNA. Ladder shows 100bp intervals.

expression of genes associated with grain filling etc. The peak in *Brachypodium*

expression occurs during early grain development, contrasting slightly to the later

peak in the developing barley grain (Fig 5.14 & 5.15), a difference in expression

that may relate to the differences in storage product profile between these species.

At the protein level *BLZ1* has a high sequence similarity to the rice *REB* and maize

OHP1 proteins, around 70%, more than to maize *O2* and wheat *SPA* where

similarity was closer to 35% (Vicente-Carbajosa et al, 1998). Similarly to *OHP1*,

BLZ1 is also expressed in roots and leaves raising the possibility that it plays

functional roles in these organs through interaction with different promoters and

that protein interaction is a potentially important aspect of its function (Vicente-

Carbajosa et al, 1998).   In endosperm tissues *BLZ1* has been seen to interact and

form heterodimers with other bZIP proteins, specifically *HvBLZ2*, orthologous to

wheat *SPA* and *ZmO2* (Onate et al, 1999). *BLZ2* shows a seed, and specifically

endosperm specific expression and is also shown to function as potent activator of

transcription in yeast systems in conjunction with *BLZ1* (Onate et al, 1999). Such

heterodimerization and protein interactions are not uncommon in the bZIP family of

transcriptional regulators and provide evidence that a network of complex protein

interactions and transcriptional regulation is involved in the synthesis of endosperm

storage proteins.

  For *BdBLZ2* we were able to detect only weak expression during late grain

development (Fig. 5.14). This pattern of expression has some similarity to that

observed in barley where expression was detected in mid and late grain

development in keeping with results of previous northern analysis (Onate et al,

1999). The level of expression observed in barley was however notably higher in

comparison to *Brachypodium*. An involvement in *Brachypodium* endosperm storage reserve accumulation would seem unlikely, as this process is largely complete in the mature grain stage. *BLZ* genes are regulators of prolamin storage protein genes in barley, rice and maize. Polymorphisms in maize and wheat have been shown to have huge implications in grain composition and quality (Onate et al, 1999; Onodera et al, 2001; Gibbon & Larkins, 2005; Ravel et al, 2009). Given the storage protein profile of *Brachypodium* is enriched for globulins rather than prolamins (Laudencia-Chingcuanco & Vensel, 2008; Larre et al, 2010) this variation and general reduction in the level of expression may not be unexpected, and again may have a relation to differences in grain storage product profile. *TaSPA* shows only partial sequence similarity but performs a similar function to *ZmO2* in regulating storage protein synthesis through recognition of the same endosperm motifs, and can activate maize α-zein expression to similar levels as the native gene (Albani et al, 1997). Ravel et al (2009) found a similar pattern of expression for three *TaSPA* homeologs to *ZmO2* subfamily genes, specific to developing endosperm, but detected significantly different levels of expression. Investigations of *TaSPA* function have gone on to suggest that in contrast to maize, a broader range of effect may be seen in wheat and that regulation is not restricted to a single class of storage proteins (Albani et al, 1997). Comparison of quantitatively different *SPA*-haplotypes show that in addition to the regulation of grain hardness, a typical *O2*-class function, there is also an influence on dough elasticity, a trait influenced by other storage protein classes (Glutelins) and of significant agronomic importance (Ravel et al, 2009).

The final two R1MYBs examined are also members of the SHAQKYF subfamily and are distinct in showing function in both developing and germinating grains. Expression analyses for both genes have found transcripts accumulating in endosperm tissues during grain development and in aleurone cells during germination (Rubio-Samoza et al, 2006b). These two genes function as both transcriptional activators and repressors of *ltr*, an endosperm specific defensive gene, and *amy6.4*, an amylase synthesis gene. *HvMCB1* has been seen to activate the *ltr* promoter in the developing endosperm and function as a transcriptional repressor for *amy6.4* in the germinating aleurone whilst *HvMYBS3* functions as a transcriptional activator in both the developing endosperm and germinating aleurone (Rubio-Samoza et al, 2006a).

The *Brachypodium* orthologues of *HvMCB1* and *HvMCBS3* were both found to be expressed in developing grain tissues (Fig. 5.14). Expression of the *MCB1* orthologue extends to both mature grain and inflorescence tissues, whilst *MCBS3* is weak during the inflorescence stage and absent in mature grain. These results compare with those previously reported in barley and maize (Rubio-Samoza et al, 2006b; Mercy et al, 2003), and our own analysis of expression in barley. We detected expression of *HvMCB1* and *HvMCB3* across all stages of grain development (Fig. 5.15), making the absence of *MCB3* expression in the *Brachypodium* mature grain the only notable discrepancy in the comparison of these expression profiles. The expression profile of their *Brachypodium* orthologues is consistent with their showing a similar function in aleurone development. Yeast three hybrid showed *HvMCBS3* interaction with complexes including *BLZ2* and function of R1MYBs in aleurone, and possibly wider epidermis,

specification and development seed regulatory networks is thought to be heavily influenced by protein interactions (Rubio-Samoza et al, 2006a).

5.2.4.3 Post Germination Expression

MCB1 and *MCBS3,* along with *BLZ1* have all been shown to have expression/function in germinating grains (Rubio-Samoza et al, 2006b; Mercy et al, 2003; Diaz et al, 2002; Vicente-Carbajosa et al, 1998). In order to compare whether this activity is shared in their *Brachypodium* orthologues we tested for expression in seedlings at 24 hours after germination. This data is shown in figure 5.15 where expression of the *Brachypodium* orthologues was detected. Speculatively we also included various genes examined so far in this survey and found weak expression for *MADS58* and *FIL-1*, although this is possibly attributable to some maintained or residual embryo expression. Excluding *BLZ1*, these endosperm specific genes all have a functional role in the germinating grain endosperm, with alternate roles as transcriptional repressors or promoters (Rubio-Samoza et al, 2006b; Gubler et al, 1995; Vicente Carbajosa et al, 1998). The expression of these genes in *Brachypodium* during the germination phase provides further evidence of likley functional homology between them and their barley/maize orthologues.

5.2.5 *HDZIP Transcription Factors in Grain Development*

We isolated *Brachypodium* orthologues for *ZmGT1* and *ZmVRS*, with 67 and 76% protein level homology respectively. These two highly similar HDZIP transcription factors, involved in floral morphology determination, have been shown to have
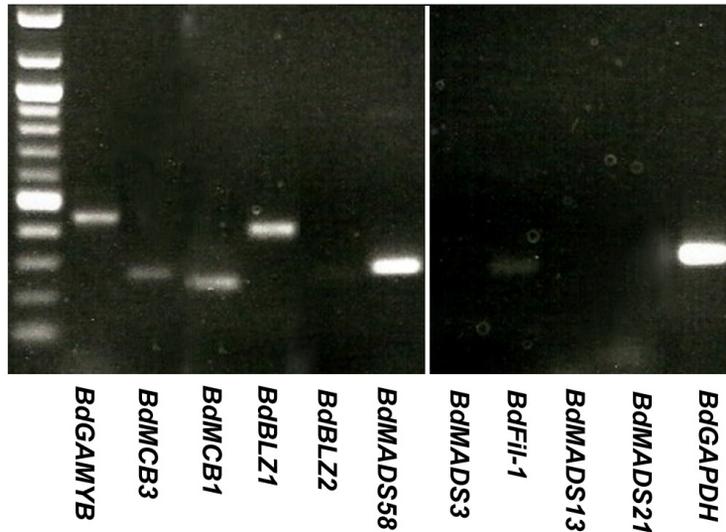
Figure 5.15: Gene Expression in Post-germination
***Brachypodium* Grains**

RT-PCR analysis for gene expression of selected genes of
known expression during grain development in germinating
Brachypodium grains. Tissue samples comprise the whole grain
at 24 hours after germination. Ladder shows 100bp intervals.

important roles in early domestication (Gross & Olsen, 2010). Although not intimately involved in grain development they are important in shaping the inflorescence and floral environment in which grain development takes place. Expression analyses for these two genes, shown in figure 5.16, reveals only weak expression during early grain development for *GT1* and an absence of expression for *VRS1*. In barley *VRS* has been shown to an important regulator of the six-rowed phenotype (Komatsuda et al, 2007). Here the loss of *VRS1* expression allows axilliary outgrowth in the barley spike and the resultant six-rowed phenotype. This compares interestingly to what is seen in *Brachypodium*, where no expression is seen yet the *Brachypodium* flower is very obviously restricted and at the spikelet level two-rowed (Distachyon = two-ranked). Germplasm collections (J. Doonan pers. Comm.) have identified *Brachypodium* ecotypes with high spikelet numbers, this increased axilliary outgrowth is potentially influenced by expression of this gene. Komatsuda et al (2007) found expression of *VRS1* in barley to be highly temporally specific to an early stage of floral development. A potential explanation for the failure to detect *BdVRS1* transcripts is for the tissue stages examined here falling outside of the likely range of expression. It will be necessary to examine earlier floral stages before any observations on the *Brachypodium VRS1* orthologue can be detailed.

Although weak, the expression of our potential *Brachypodium GT1* orthologue was detected, but only during early grain development. Whipple et al (2011) report expression in developing flowers and buds of maize where it promotes lateral bud dormancy. Expression in *Brachypodium* during early grain development, and an absence in the earlier spikelet sample, does not appear to reflect this function,
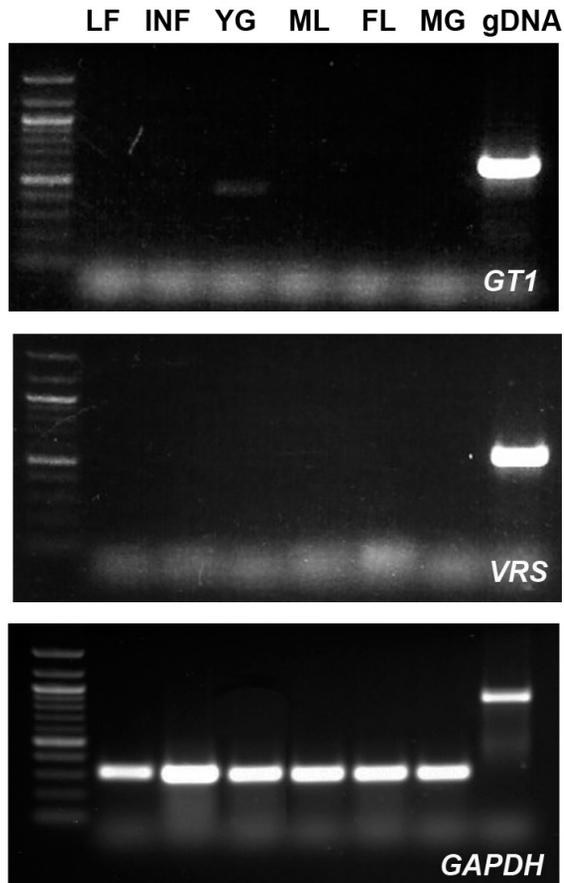
Figure 5.16: RT-PCR Expression Profiling for
Brachypodium GT1 and VRS1

RT-PCR expression analysis over grain development shows almost no expression for the putative *Brachypodium* orthologues of these genes. Ladder shows 100bp intervals. LF = Leaf, PrS = pre-anthesis spikelet, YG = Young Grain, ML = Mid Length Grain, FL = Full Length Grain, MG = Mature Grain, gDNA = genomic DNA.

although again it is likely that the developmental range examined here falls outside of the likely period of expression.

5.2.6 Chapter Conclusion.

The absence/presence of key genes (or paralogues) in genomes or variation in the expression pattern in one species compared to another, can be an indicator of the genetic basis for morphological and developmental differences. This analysis spans a range of functionally distinct and developmentally significant transcription factors associated with seed development. For the majority of genes investigated we provide an identification of *Brachypodium* orthologs and expression domain analyses reveal significant similarity and difference. Our findings for the YABBY gene family show high levels of sequence and expression domain similarity between *Brachypodium* and rice, suggesting a similarity potentially spanning the small grain cereals. We have also shown preliminary evidence that expression patterns may show some association with grain organisation and composition. The lack of expression of the *Brachypodium ZmMRP-1* and *BLZ2/Opaque2* orthologues (as determined by sequence analysis and RT-PCR) could be molecular genetic representations of the differences between *Brachypodium* and maize, and potentially representative of differences between Pooid and tropical cereals.

In relation to the MADS-Box genes we have been able to report expression of C- and D-class genes typically associated with floral development throughout grain development. The function of these genes in grain development is as yet unclear. Our analysis also provides previously unreported expression patterns for *MADS13* during grain development, and differences in expression pattern amongst the

Pooideae. These results are suggestive of grain developmental roles for both C- and D-class MADS box genes in *Brachypodium*, and for the YABBY gene *DL*, that have not been investigated previously. We aim to use these results as a basis for a wider and detailed characterization and investigation of the effects of transcription factor function in *Brachypodium* grain development. Future experiments will focus on distinction between the embryo and endosperm regions of the grain in order to determine if genes such as *DL* and *MADS58*, where we were unable to conclusively locate late developing grain expression, are present in the embryo. These analyses will initially use PCR of separated tissues and in-situ hybridisation of longitudinal sections. Hybridisations so far have focused on transverse central sections of the grain and this can be associated with the difficulty of obtaining good longitudinal sections and maintaining their integrity throughout hybridisations. This will entail establishing an altered protocol to take into account the fragility of this material. Functional investigation also forms a major part of our ongoing research effort; the following chapter details our initial investigations pursuing this aim with the characterisation of *Brachypodium* C-class and YABBY gene mutants, and attempts to establish a stable transformation protocol for future functional analysis of regulatory genes.

CHAPTER 6.

BRACHYPODIUM MUTANT CHARACTERISATION & TRANSFORMATION

6.1 Introduction

Characterization of transcription factor expression and function in *Brachypodium* grain development is an important aim in this project and part of a wider, ongoing program of research. We have initiated both forward and reverse genetic approaches in order to perform functional analyses of selected genes with scope for wider analysis. This chapter details efforts to implement and establish an *Agrobacterium* mediated transformation protocol for *Brachypodium* in the laboratory at Leicester university along with preliminary results for characterization of two TDNA insertion lines obtained through the BrachyTag project.

The BrachyTag TDNA mutagenesis project began in 2005 at the JIC as part of the international *Brachypodium* initiative (I.B.I.; http://www.brachytag.org). The project has produced over 5000 tagged Bd21 TDNA insertion lines many of which have been identified as affecting genes of known function, developmental and agricultural significance. These mutant lines are produced using an *Agrobacterium* mediated transformation protocol with the binary vector pVec-GFP, developed by Alves et al (2009). This vector contains two expression units within the TDNA, the first provides GFP (Green Fluorescent Protein) selection via the *sgfp*S65T reporter gene driven by the maize ubiquitin1 promoter, terminated by the nopaline synthase polyA sequence. The second expression unit contains a hygromycin selectable marker gene driven by the CaMV35s promoter, and a bacterial streptomycin resistance gene is also present in the vector backbone. Details of this vector are shown in figure 6.1.
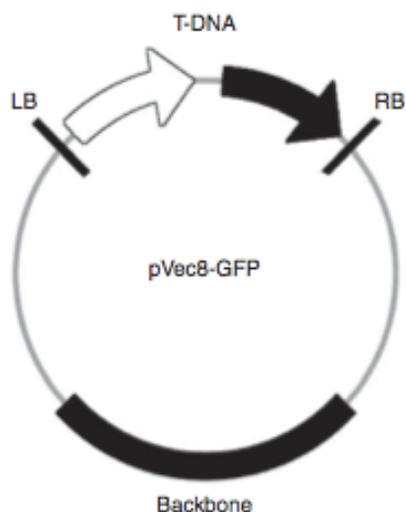
Figure 6.1: Binary Vector pVec8-GFP Used in the BrachyTag Transformations. Two universally expressed expression units for GFP and Hygromycin resistance shown by white and black arrows respectively. LB and RB indicate the left and right border extremity of the TDNA region. A bacterial streptomycin resistance gene is present in the vector backbone, shown with a black box. Reproduced from Alves et al, 2009.
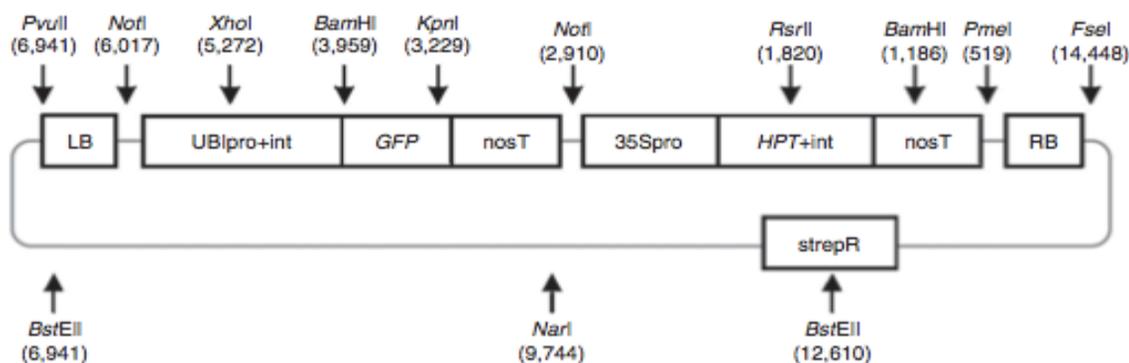
The BrachTag website lists the position of Flanking Sequence Tags (FSTs) for TDNA insertions in close proximity to known and putative orthologues of genes. These FSTs used to characterize the potentially disrupted genes are displayed in modelcrop.org and other genome browsers. Amongst those listed as containing an insertion, we identified genes corresponding to YABBY gene and *AGAMOUS* orthologues which, having already generated expression data, were of great interest to us. To date just two characterisations of *Brachypodium* insertion lines have been published, examining mutations to the eukaryotic initiation factor (*eIF4A*) and the *BRASSINOSTEROID INSENSITIVE* gene (*BRI1*) (Vain et al, 2011; Thole et al, 2012). Mutant analysis provides a rapid and accessible system in which to examine gene function, with complementation and additional transient gene knockout within the line via RNAi etc providing further information on gene function and interaction. Based upon their availability, and that of the increasing number of BrachyTag lines we decided to use mutant characterisation as our primary method of gene functional analysis. Alongside characterizing these lines we have also sought to establish, and describe, a simple, rapid and cost effective system by which we can test and confirm the insertion status and characterize mutant phenotypes. We hope to be able to use this system to characterize further lines of interest as they become available, and in characterization of our own transformants. Table 6.1 details the two insertion lines and material obtained examined here.

The past 4 years has seen the development of highly efficient *Agrobacterium*-mediated transformation strategies for several different *Brachypodium* genotypes, including the BD21 reference strain (Vogel & Hill, 2008; Pacurar et al, 2008; Alves

Identifying line (T$_0$ plant)	Insertion gene	JIC FST identifier	GFP status	Material obtained	Description
BdAA315	Bradi1g16910	JIC 00266_315	+ve	T$_1$ seeds	Putative YABBY domain containing protein mutant
BdAA382	Bradi2g32910	JIC 00316_382	+ve	T$_2$ seeds -A309 -A310 -A311 -A312 -A313 -A314 -A317	Putative AGAMOUS-like protein mutant
FST – Flanking Sequence Tag. GFP – Green Fluorescent Protein					

Table 6.1: Details of Insertion Lines

Identifying details as shown on the BrachyTag website for the two insertion lines examined here.

et al 2009). The BrachyTag project's success is largely attributable to the establishment of these techniques, in particular that of Alves et al (2009) who's protocol for the transformation of embryonic callus has been at the heart of the generation of very many lines (>5000) in work at the JIC and USDA-ARS Western Regional Research Center (Thole et al, 2012). This protocol, developed specifically for the transformation of the Bd21 strain offers the current most efficient protocol for this standard line and makes *Brachypodium* one of the most easily transformable grasses available (Mur et al, 2011).

6.2 *Characterization of Insertion line BdAA382 (AGAMOUS insertional mutant): Results, Methodology & Discussion*

Characterisation of the *AGAMOUS* T-DNA insertion line forms the major component for this part of the investigation. We first examined gene structure and insertion position. The insertion is characterized by the flanking sequence tag JIC00316_382 showing the insertion is located close to the 3' end of the 3rd intron of the gene (Fig. 6.2 B). A rice mutant carrying a *dSpm* insertion in the second intron shows a nearly 35-fold reduction in *MADS58* mRNA levels and has been used in previous functional analyses (Dreni et al, 2011). Our previous analyses have amassed considerable expression and localization data for mRNA of this gene in *Brachypodium*, and as a C-class gene it is one of our candidates likely to have significant impacts in grain development. Our aim for the primary generation was to grow plants to maturity and generate a stock of seed, identifying GFP+ve individuals (i.e. those expressing TDNA derived GFP) and to perform a preliminary survey for any obvious phenotype. Seeds of the T$_2$ generation mutagenized plants,

Figure 6.2: *BdAG* Gene Structure, Insertion and PCR Product Details.

A) Nucleotide sequence for the 5' region of Bradi2g32910 showing intron/exon structure, primer locations and insertion site.

B) Gene and insert structure showing insert located primers and exon1 gene primer. Insert diagram adapted from Thole et al, 2009.
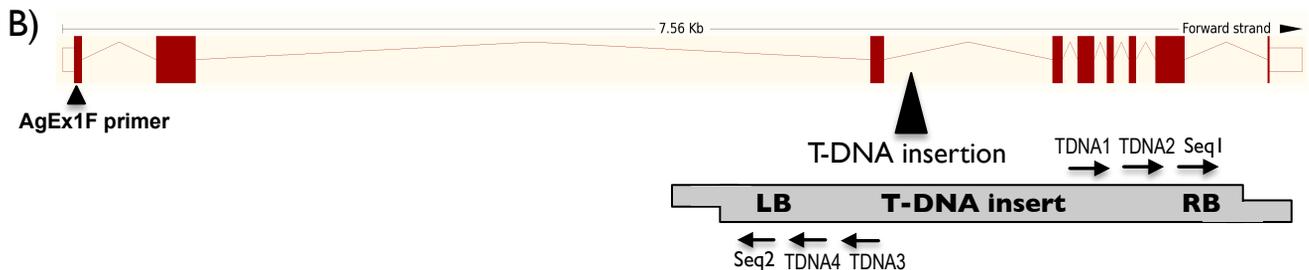
C) Approximate PCR product lengths for the various combinations used in this investigation.

PCR product lengths (~bp)			
Primer combinations		cDNA	gDNA
AgEx3F	- TDNA4	--	212
AgEx3F	- AgInt3R	--	649
AgEx3f	- AgInt3Rb	--	824
Seq1	- AgInt3R	--	601
Seq1	- AgInt3Rb	--	776
TDNA2	- AgInt3R	--	676
TDNA2	- AgInt3Rb	--	851
TDNA1	- AgInt3R	--	776
AgEx1F	- AgEx3R	320	>4KB
AgEx3F	- MADS58R	577	2493
MADS58F	- MADS58R	125	910

all of which appeared externally normal, were sown and grown under greenhouse conditions with supplemental heating and lighting to give 16hr minimum daylength and 20ºC.

6.2.1 GFP Screening Technique

An important objective during the early stages of the project was to establish a simple and cheap technique to screen seedlings for GFP. Selection for GFP+ve seedlings at an early stage offers a rapid way to reduce or exclude WT plants with significant advantage to both time and growth space requirements.

The initial GFP screening approach was to examine root fragments at 2-3 weeks growth when plants were sufficiently large that the removal of root tissue was unlikely to disrupt their growth. These root fragments were cleaned, mounted on slides and screened on a UV equipped compound microscope available as a departmental facility. A WT root fragment was used as a control. This technique was successful but several draw-backs to this system were immediately obvious. The dry, gritty compost used and limited root growth at this stage was insufficient to hold the root ball together when removed from the pot. Significant root disruption occurred whilst collecting the samples and would likely lead to a check in growth and altered development. Growing plants to a size where roots had branched and attained sufficient mass where sampling would not disrupt growth meant all seedlings germinated must be potted up, a time and growth space

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These experiments represent the first mutant screening involving GFP to be conducted in this lab. As a consequence there was no ready access to a mobile UV light source or similar equipment to screen for GFP in macro-scale tissues. At its inception, this mutant analysis represented just one avenue of investigation we were pursuing and as a consequence there was reluctance to commit funds to equipment purchase etc before establishing it as viable.

consuming exercise. Furthermore the preparation of slides suitable for screening on a compound microscope was also time consuming.

It was obvious that screening for GFP status *in-planta* at an early stage would be the more desirable approach. Typically a proprietary GFP lamp, either handheld or attached to a dissecting microscope, would be used to view GFP in seedling root tissues. Seedlings are germinated on moist filter paper before being transferred to compost once 2-3 cm root/shoot growth has developed. Being free from chlorophyll, GFP is easily detectable in root tissue against the white background of the filter paper. Screening at this stage is desirable as seedlings need not be disturbed and only GFP+ve seedlings need be potted on, but necessitates an external UV source. GFP excitation occurs at wavelengths around 400-500um. We identified a blue handheld laser, of the type used as a presentation aid, that emits light at a wavelength of 405nm. Using this type of laser and an orange transparent film to filter out blue light it is possible to visualize GFP fluorescence in root tissues. Figure 6.3 shows the set up used here, the laser costing around £10 (Maplin electronics) provides a cheap and simple system. Root tissues all appear green to some extent when viewed in this way and so a WT plant is used throughout for regular control comparison; GFP expressing tissues appear strikingly green and obviously luminous in comparison. The denser region of the root tip provides the best results, this region glowing most obviously bright and green under excitation.

This system was used to screen for GFP in seedlings and whilst not 100% accurate or conclusive we were able to largely exclude WT plants from our sample

Figure 6.3: Utilisation of Blue Laser Pointer to Visualise GFP Fluorescence.
Photo and illustration giving details of the set up and method used to screen seedlings for GFP.

Blue light filter

Seedlings in petri dish

Blue laser
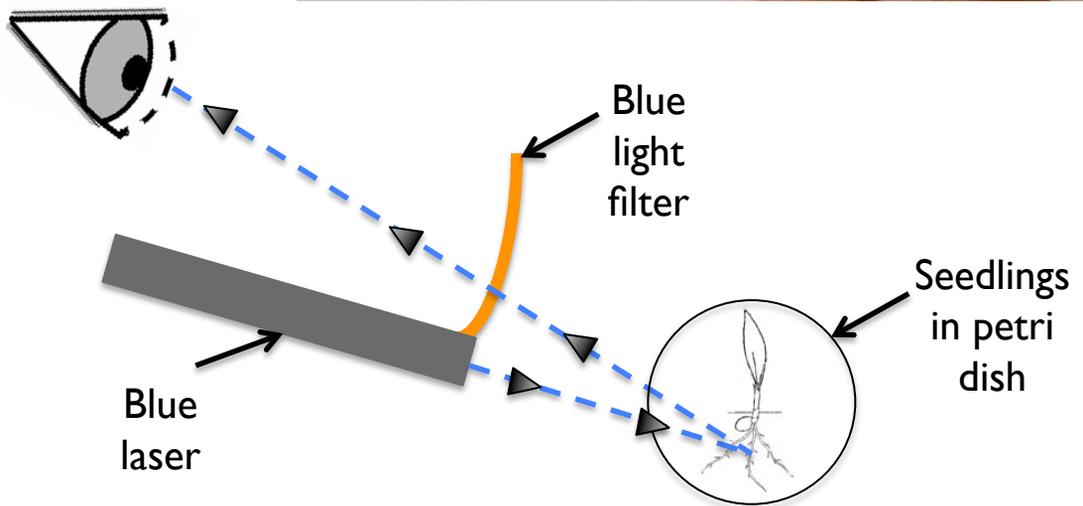
populations. PCR was used to confirm the presence of the insertion as well as to identify the insertion status of individuals as either hetero- or homozygous.

6.2.2 *BdAA382 First Generation*

We collected data for the number of spikelets, florets and grains produced by BdAA382 first generation plants, shown in table 6.2. A striking feature was the erratic spikelet, floret and grain numbers between plants. WT control plants grown alongside mutants were also found to be variable in their extent of flowering although a generally higher grain set was observed (Figures shown in appendix table A6b). This variation in both WT and mutant plants suggested some fluctuation and inconsistency in growing conditions, resulting in environmentally introduced variation. To avoid this problem, all future experiments were conducted in CER conditions where we have found that provided a high light intensity is maintained plants grow consistently and flower well.

Differences between WT and mutagenised plants may also have been related to the founder population. WT plants originated from a Bd21 stock obtained previously and grown in Leicester over many generations. It was noted that these WT plants were typically stocky in comparison to all of the mutagenized plants (Including mutant population GFP-ves; WT) which generally showed a thinner and more upright phenotype, less tillers and slightly longer internodes. It is possible that these differing characteristics may relate to some differences between the populations they are derived from. To exclude this possibility and to make future comparisons more meaningful we used only confirmed GFP-ve plants (i.e. non-GFP expressing individuals = WT) of mutagenized lines as controls.

T1 plant	Seedling (T2)	GFP	~ Spikelet no	~ Floret no	grains	Observations
A309	-1	-	5	35	nt	
	-2	-	13	100	nt	
	-3	-	4	30	10	
	-4	-	6	60	14	
A310	-1	+	15	100	0	
	-2	+	4	60	19	
	-3	-	18	66	10	
	-4	+	19	100	35	
	-5	-	10	50	12	
A311	-1*	+	8	50	14	Plants -2, -3 and -9 appear stunted. All slow to flower but otherwise appear normal
	-2	-	1	4	0	
	-3*	+	13	--	0	
	-4	+	11	68	12	
	-5	-	3	17	0	
	-6*	+	5	50	1	
	-7	+	9	40	0	
	-8	+	17	>100	2	
	-9	-	4	35	17	
A312	-1	-	8	62	40+	
	-2	+	12	73	23	
	-3	+	11	>100	31	
	-4	+	7	24	0	
	-5	-	4	45	29	
	-6	+	13	77	1	
	-7*	+	15	>100	8	
	-8	+	7	32	0	
	-9	+	4	24	0	
A313	-1*	+	7	47	2	All plants appear quite regular and showing very typical growth
	-2	+	6	68	0	
	-3	-	5	31	24	
	-4	+	4	28	0	
	-5	+	8	65	4	
	-6	-	--	--	--	
	-7	+	11	46	26	
	-8	+	5	42	35	
	-9	+	5	38	30	
A314	-1	+	4	28	21	Short spikelets and poor grain development
	-2	+	3	20	0	
	-3	+	7	50	0	
	-4	-	3	17	10	
	-5*	-	4	36	14	
	-6	+	4	40	0	
	-7	+	6	40	10	
	-8	+	2	30	0	
	-9	+	4	30	5	
	-10	+	5	35	0	
A317	-1	-	6	45	5	GFP+ves -5 to -7 showing stunted growth and poor floral development, -2, -8 and -9 in particular showing close to normal floral development but very low grain set.
	-2*	+	4	45	11	
	-3	-	8	65	0	
	-4	-	5	45	4	
	-5	+	3	19	0	
	-6	+	9	65	0	
	-7	+	2	8	0	
	-8	-	7	60	8	
	-9	-	13	80	5	
	-10	-	5	50	12	

Table 6.2: Results and Observations for 1st Generation *BdAG* Insertion Lines.

Recorded values for floral and grain production and GFP status for all T_2 generation plants . *= GFP status confirmed via PCR.

Screening for phenotype at this stage produced no obvious or consistent result, but allowed WT plants to be excluded. Screening for the first generation was performed via root fragments examined on a compound microscope, as described earlier. We selected 8 individuals from this analysis and confirmed results via PCR. These results, shown in figure 6.4, matched and confirmed that of the screening, giving the indication this method of testing was accurate. At this stage we did not attempt to directly confirm the heterozygous or homozygous status of individuals, but the presence of both GFP+ve and –ve progeny from the A317 and A313 identifies them as heterozygous for the insert. The variable levels of seed production and the observation that some GFP+ve individuals were producing no grain at all gave an initial suggestion that a grain development-lethal phenotype may exist in homozygotes, but it was unclear based upon the data accumulated so far.

6.2.3 *BdAA382 Second Generation*

As heterozygotes we focused on progeny of the A317 and A313 lines in the second sowing. GFP screening in second generation was performed using the laser technique as described earlier with only GFP+ve seedlings selected and grown on. Table 6.3 shows the numbers of seedlings germinated and numbers of GFP+ves and –ves amongst them. We examined plants for phenotype across development and identified a potential spikelet phenotype, observed most strongly in 317-2 and 317-8 line plants. This phenotype comprised an elongate spikelet with a high floret number, see figure 6.11 For detail (in later generation plants). Within elongate spikelets, few florets in the distal half of the spikelet were seen to
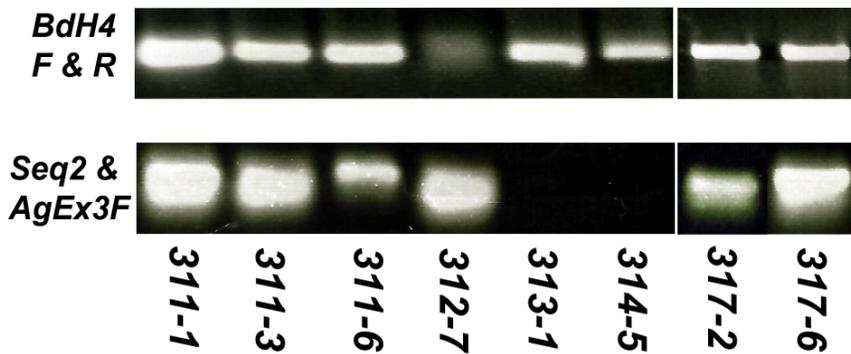
Figure 6.4: Insert Confirmation PCR for Selected 1st Generation Plants

Upper panel shows Bdhistone4 expression used as a control. Lower panel shows PCR results with TDNA junction spanning primers. The presence of TDNA:gene products confirm the results of the earlier GFP screening.

T2 plant	Seedling (T3)	% germinated seedlings GFP+ve	Spikelet no	~ Floret no	Grain number	Observations
313-2	-1	100%	9	>100	0	-1 shows very elongate spikelets.
	-2		4	30	0	
	-3		--	--	--	
	-4		9	>100	10	
313-7	-1 to -15	76%	Not recorded			
313-8	-1 to -9	69%	Not recorded			
317-2	-1	71%	32	>100	40	2 grains developed in -3 to full length, but aborted. Elongate spikelets in all, particularly -5
	-2		25	>100	92	
	-3		46	>150	0	
	-4		20	>100	86	
	-5		19	>100	11	
317-4	-1	66%	21	>100	94	
	-2 GFP-ve		22	>100	83	
	-3		6	35	0	
317-8	-1	100%	34	>100	25	Some elongate spikelets, very poor grain set in all.
	-2		29	>100	26	
	-3		34	>100	94	
	-4		32	>100	40	
	-5		33	>100	32	
317-9	-1	100%	21	>100	94	
	-2		34	>100	83	
	-3		15	87	0	
317-10	-1	100%	36	90	41	Very thin shriveled grains produced in -7. Some very short spikelets in -1 and -4
	-2		32	>100	82	
	-3		25	80	54	
	-4		36	90	80	
	-5		16	84	65	
	-6		41	>100	80	
	-7		19	>100	8	
	-8		18	85	48	

Table 6.3: Results and Observations for 2nd Generation *BdAG* Insertion Lines.

Recorded values for floral and grain production and GFP status for all T_3 generation plants.

develop grains and in comparison to first generation plants the overall number of spikelets and florets produced appeared higher. This maybe a reflection of difference between greenhouse and CER growing conditions however. A WT control population was included in this second generation comprised of 10 seedlings from A309, all 4 seedlings of which tested GFP-ve, along with GFP-ve individuals of 317-2 and 317-4. Ten grains from A309-3 were sown but were badly affected by a fungal infection soon after germination where the majority died and two remaining seedlings showed stunted growth and poor flowering. The three remaining WT plants, from the 317-2 and 317-4 lines were grown separately and so could still provide a limited control comparison. Grown alongside the mutant lines these plants did show a generally higher floral and grain production as compared to previous greenhouse grown plants, but based upon just three plants this observation is little more than speculation.

 The occurrence of GFP+ve seedlings in each line allows us to speculate that 317-8, -9 and -10, showing no GFP-ves, were potentially homozygous for the insert whilst 317-2, with a 5:2, ratio was heterozygous. Whilst this gives some indication as to zygosity status, numbers are low and direct assessment via PCR is necessary for meaningful result. PCR checks against gDNA were performed using gene specific primer pairs spanning the insert region and the junction between insert and genic flanking region. Details of primers combinations, locations and product lengths are shown in figure 6.2. The results of this analysis for the five GFP+ve 317-2 plants are shown in figure 6.5 and offer particularly well proportioned results for so small a sample number. A TDNA derived product was detected in all except 317-2-4, indicating this plant is WT whilst the others are
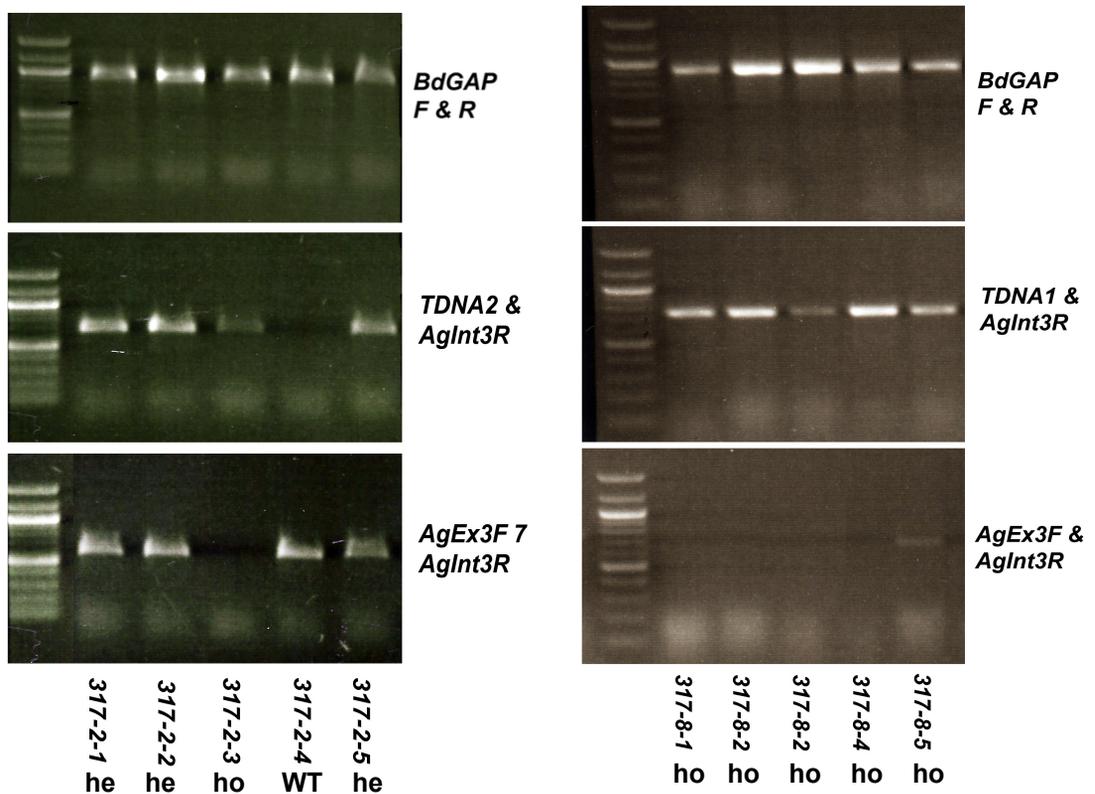
Figure 6.5: Insert Confirmation & Zygosity Status PCRs for Selected 2nd Generation Plants.

Insert junction-spanning primers (TDNA2/1 & AgInt3R) show the insert is present in all plants except 317-2-4, a WT. Insertion site spanning primers (AgEx3F & AgInt3R) show no product in homozygote plants, although some weak gene product expression can be seen in 317-8-5. BdGAP = primers for control housekeeping gene expression, BdGAPDH. he= hetorozgous, ho = homozygous, WT = Wild-Type. Ladder shows 100bp intervals.
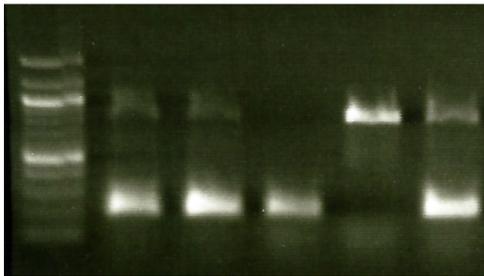
Figure 6.6: Insert & Zygosity Status Confirmation PCRS Using Three Primer Method. Repeat of results for 317-2 line second generation plants. Upper band shows insertion site spanning product (Ex3F & Int3R). Lower band shows TDNA junction spanning product (AgEx3F & TDNA4). Presence of both bands indicates heterozygosity, only a lower band indicates homozygosity. he= hetorozgous, ho = homozygous, WT = Wild-Type. Ladder shows 100bp intervals.

Figure 6.7: RT-PCR for *BdMADS58* Gene Expression on selected 2nd Generation Plants.

Upper panel shows *BdMADS58* F & R primers, located in the 3' transcript region and downstream to the insertion site, return no product in plants homozygous for TDNA insertion. Middle panel shows primers AgEX1f and AgEx3R, located at the 5' transcript region and upstream to the insertion site, return a product in some homozygous plants, but not all. A conflict exists between 317-8 and 317-2 line plants.(Primer locations shown in figure 6.2). Lower panels provide control reactions. *BdGAPDH* show housekeeping gene expression whilst *BdMADS3* primers provide a control of comparable expression pattern and level, and for cDNA quality. Examination of *MADS3* expression also investigates the possibility of cross-regulation between *MADS58* and *MADS3* in *Brachypodium* and consistent expression between both heterozygous and wild type plants is seen. he= heterozgous, ho = homozygous, WT = Wild-Type. Ladder shows 100bp intervals.

either heterozygous or homozygous. The primer pair spanning the insertion site however gave a product for all plants except 317-2-3. This result suggests that lines -1, -2 and -5 are heterozygous whilst -3 is homozygous for the insert. Compared to phenotype observations in table 6.3, a notable feature is the lack of grain production in 317-2-3, raising the suggestion that homozygotes may be lethal for grain development. However, this result appears to be confounded by the production of grain in the majority 317-8, -9 and -10 plants where the lack of GFP-ves suggests homozygosity for the insert.

Based upon phenotypic similarities we selected 317-8 plants for comparison. PCRs shown in figure 6.5 indicate that all are homozygous for the insert, although some weak signal for the insertion site spanning primers can be seen in in 317-8-5. Whilst all five putative homozygotes show the elongate spikelet, high floral production/low grain set phenotype these results demonstrate that homozygotes for the insertion are not sterile.

In order to further streamline zygosity status identification a colleague suggested using both left and right border flanking and insert-located primers in a single reaction, thus giving WT, homo or heterozygous determination in a single reaction. To confirm the validity of this approach we performed repeat PCRs on the second generation 317-2 plants (Fig. 6.5); a successful result shown in figure 6.6. This genotyping approach was used for all subsequent PCRs to determine insert zygosity status.

6.2.4 *Confirmation of Disruption of Gene Expression of BdMADS58 in Mutant*

    Lines

We used RT-PCR to check for *BdMADS58* expression in spikelet tissues just
after to the point of first anthesis in order to establish whether this line results in
complete gene knockout. Based upon the insert location within the gene our
expectation was for expression to be severely disrupted in plants homozygous for
the insert, and likely eliminated altogether, although the possibility that some
expression of a partial transcript for the 5' region of the gene, upstream of the
insertion, may continue was not discounted. Results of this expression analysis are
shown in figure 6.7 for selected 317-8 and 317-2 individuals. Using primer pair
BdMADS58F & R spanning the final three exons and UTR region no *BdMADS58*
transcript was detected in any of the 317-8 (all putative homozygote) plants
(although some faint genomic product is seen in 317-8-5). Similarly no product
was detected in 317-2-3, also shown to be homozygous, whilst a strong product is
seen in heterozygous and WT plants. These results give a strong indication that in
homozygotes no full-length transcript is produced.

To determine whether the insertion achieves a full-knockout of the gene, i.e. no
partial transcripts before the insertion, the primer pair AgEx1F and AgEx3R were
designed to amplify the region spanning the first three exons, the 5' region
upstream of the insertion site. Results obtained with these primers were unclear
and problems may relate to difficulties in the transcription of cDNA templates.
Amongst the 317-2 lines no expression was consistently detected in the
homozoygote, in keeping with a full knockout. In the three 317-8 lines examined

however, expression of a 5' transcript is detected (Fig 6.7), quite strongly, in two of the three lines.

We examined the expression of *BdMADS3* in order to investigate the possibility of cross-regulation between C-class genes, and also as a similarly expressed control with which to confirm the integrity of our cDNA templates. Figure 6.7 shows expression for GAPDH (A housekeeping gene) in all samples and *BdMADS3* expression detected in both homo- and heterozygous plants (Failure to detect expression in 317-8-3 being likely due to a failed cDNA transcription reaction).

6.2.5 BdAA382 Third Generation

Plants examined here comprised progeny of 317-8-1 to -5 and all grain-producing 317-2 lines with 20 seeds from each fertile parent sown. Again, all GFP+ve seedlings were selected and grown on, the ratio of GFP+ve plants shown in table 6.4. All six 317-2-4 germinating seedlings tested GFP-ve and were confirmed to be WT via PCR. These plants form the WT control for this generation. All 317-8 line seedlings examined here tested GFP+ve, in keeping with expectations of their being homozygous (based upon the results for the second generation). Zygosity status PCR checks for all of 317-8-1 and 317-8-3, and six 317-8-2 plants are shown in figure 6.8. The majority of plants show a positive result for the insert and all are negative for the gene product which is detected in WT control reactions. This result convincingly suggests that the 317-8-1, -3 and -2 lines are homozygous for the insert and, notably, confirms this line is not sterile, grain production being high in most of these plants (table 6.4).

T3 plant	Seedling (T4)	% germinated seedlings GFP+ve	~ Spikelet no	~ Floret no	grains	Elongate florets score
317-2-1		No germination				
317-2-2	-1	85%	12	136	43	Low
	-2		23	150	60	Low
	-3		20	192	111	Ab
	-4		16	140	37	Med
	-5		13	150	42	Low
	-6		--	--	--	--
	-7		26	274	107	Ab
	-8		--	--	--	--
	-9		22	180	40	Low
	-10		--	--	--	--
	-11		--	--	--	--
	-12		14	115	37	Med
317-2-4 (WT)	-1	0	31	170	30	low
	-2		18	140	53	Ab
	-3		33	120	40	Ab
	-4		15	150	70	--
	-5		13	80	44	--
	-6		9	60	45	Low
317-2-5	-1	66%	--	--	--	--
	-2		27	290	6	High
	-3		13	147	23	--
	-4		--	--	--	--
317-8-1	-1	100%	27	160	30	Low/Med
	-2		26	335	55	Low
	-3		29	311	26	Low
	-4		30	190	51	Low/Med
	-5		27	190	43	Low/Med
	-6		28	243	67	Ab
	-7		29	375	28	Med
	-8		24	130	42	Med
	-9		26	306	48	Low
	-10		22	160	27	Low
	-11		33	230	38	Low
	-12		27	190	30	Low
317-8-2	-1	100%	22	243	9	Low
	-2		27	260	57	--
	-3		23	150	55	Low
	-4		29	398	17	High
	-5		16	64	19	Low
	-6		23	242	36	Low
	-7		26	170	51	Low
	-8		14	133	18	Med
	-9		21	120	47	Low
317-8-3	-1	100%	13	251	4	High
	-2		20	120	66	Ab
	-3		16	172	26	Ab
	-4		23	140	38	Ab
	-5		19	100	38	Ab
	-6		25	150	53	Ab
	-7		35	140	45	Ab
	-8		24	130	44	Ab
	-9		37	160	62	Ab
	-10		25	145	28	Low
	-11		37	240	88	Ab
	-12		33	170	67	Ab

317-8-4	-1	100%	17	90	26	Low
	-2		20	130	47	Low
	-3		25	180	59	High
	-4		30	230	62	High
	-5		27	210	50	High
	-6		26	180	55	Low
	-7		28	160	49	Ab
	-8		25	150	63	Low
	-9		36	180	77	Ab
	-10		16	242	28	Med
	-11		20	250	34	Low
	-12		24	280	18	Low
	-13		14	294	14	High
	14		23	268	26	Med
	-15		24	384	22	High
	-16		20	303	15	High
	-17		29	120	54	--
317-8-5	-1	100%	28	250	10	High
	-2		28	170	63	Low
	-3		26	200	32	High
	-4		28	160	39	Low
	-5		26	294	13	Med
	-6		31	210	59	Low
	-7		25	230	51	Low
	-8		32	300	36	High
	-9		32	150	62	Med
	-10		36	360	74	High
	-11		36	235	64	Low
	-12		21	150	46	High
	-13		29	185	67	Low
	14		24	235	56	High
	-15		15	140	39	High
	-16		31	160	57	--
	-17		7	50	6	Med

Table 6.4: Results and Observations for 3rd Generation *BdAG* Insertion Lines.

Recorded values for floral and grain production and GFP status for all T_4 generation plants . Column 3 details the number of successfully germinated seedlings and the ratio of GFP+ve individuals. Some quantification for the level of potential phenotype is also given.
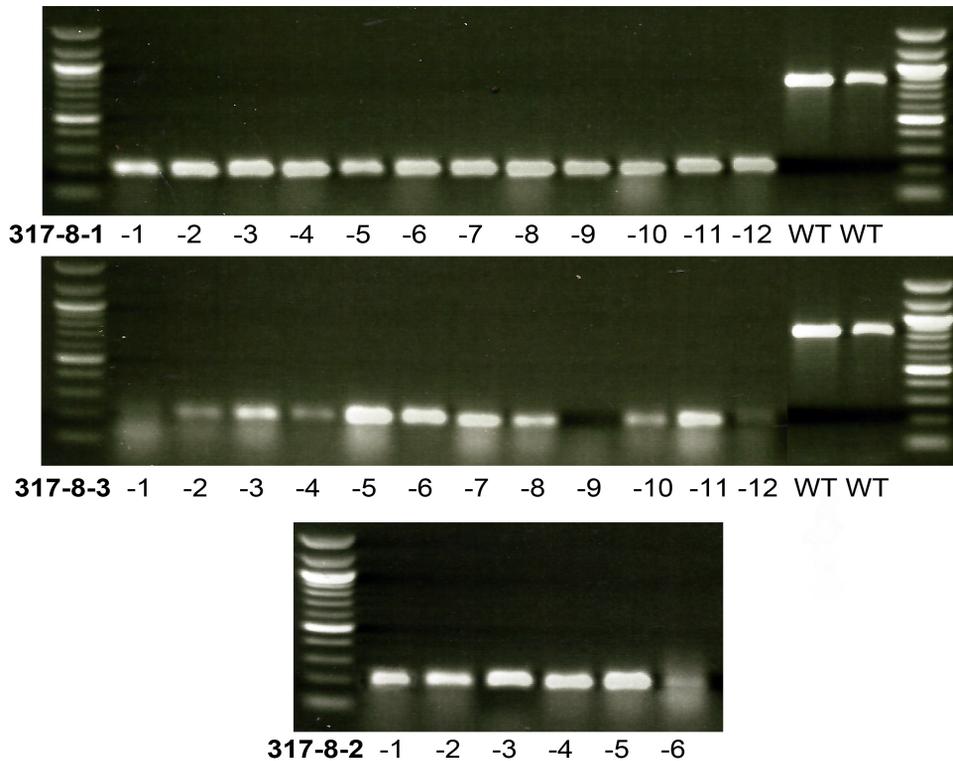
Figure 6.8: Insert Confirmation & Zygosity Status PCRs for 3rd Generation 317-8 lines.

Genotyping PCR reactions using three primers AgEx3F, AgInt3R and TDNA4 (Shown in figure 6.2) to give either insertion site or insertion junction spanning products for all 317-8-1 and 317-8-3 plants, and selected 317-8-2s. With the exception of some failed or weak reactions, Results show all plants as homozygous. Ladder shows 100bp intervals.

Results of zygosity status PCR confirmation for selected 317-2 line plants are shown in figure 6.9. We examined seven 317-2-2 plants and identified both heterozygous and homozygous individuals amongst them (fig 6.9). 317-2-5 line plants are also confirmed to be heterozygous with both homozygous and WT individuals identified here. 317-2-4-8 is included as a known WT control in this panel, with confirmation for three of the six 317-2-4 plants as WT shown in figure 6.12. These results provide a solid identification of additional lines of all zygosity status for comparison in relation to phenotypic differences etc.

Having made only preliminary observations in previous generations, we performed a more detailed survey of potential phenotype amongst the 3rd generation plants. Focusing on grain development as our primary interest and having identified that *MADS58* expression can be seen across development we examined grains at early, mid and mature developmental stages for any external differences. Figure 6.10 shows a panel of comparison between WT and mutant line developing grains. No differences in individual floral morphology, grain outward appearance or speed of development were identified in mutant lines. The elongate spikelet phenotype was again prominent amongst these plants, shown in figure 6.11, where again a low grain set was seen, almost always restricted to the proximal quarter of the spikelet. Distal florets were typically small and weakly developed. The occurrence of elongate spikelets appeared erratic with individual plants showing a range from just one or two to the majority of spikelets affected, some indication as to the extent of this phenotype for plants is detailed in table 6.4. Based upon this data it is difficult to conclusively link the observed spikelet phenotype to the effects of the insertion, homozygous plants did not consistently
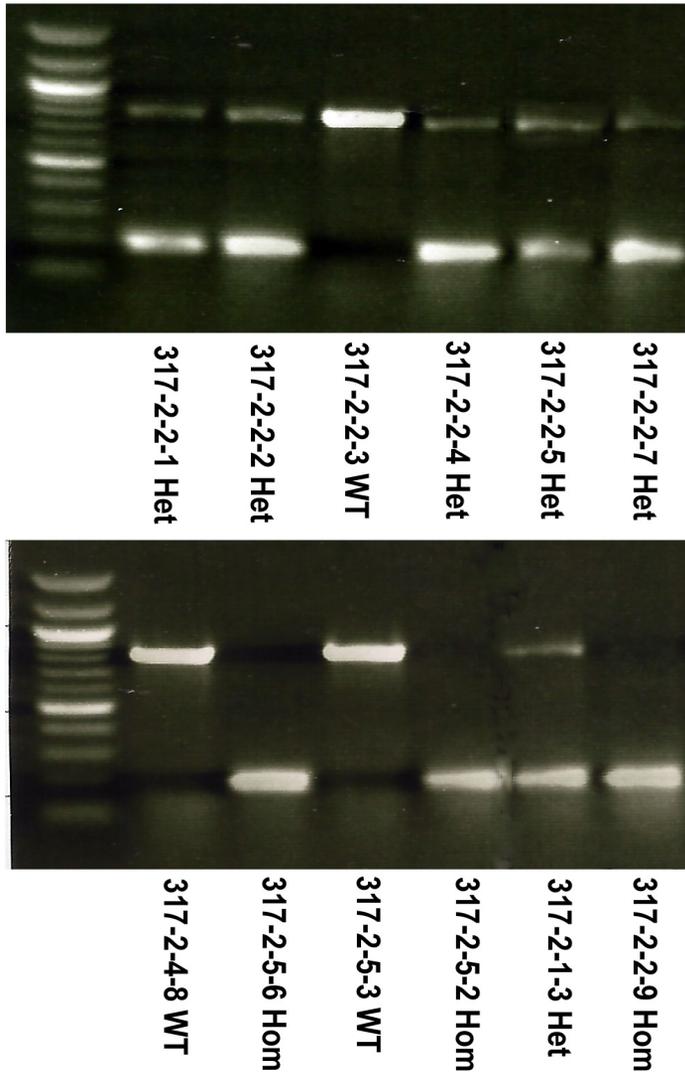
Figure 6.9: Insert Confirmation and Zygosity Status PCRs for Selected 3rd Generation 317-2 lines.

Genotyping PCR reactions using three primers AgEx3F, AgInt3R and TDNA4 (Shown in figure 6.2) to give either insertion site or insertion junction spanning products for several members of the 3rd generation 317-2 line plants. Heterozygous and homozygous individuals are identified in 317-2-2 and 317-2-5 lines. Ladder shows 100 bp intervals.

Figure labels (left to right):
- BdGAPDH F & R
- BdMADS 58 F & R
- BdMADS 3 F & R
- AgEx1F & AgInt3R

Lane labels (left gel): 317-2-2-1 Het, 317-2-2-5 Het, 317-2-5-6 Hom, 317-2-4-2 WT, 317-2-4-5 WT, 317-2-4-8 WT

Lane labels (right gel): 317-8-1-3-hom, 317-8-2-1 hom, 317-8-2-3 hom, 317-8-3-1 hom, 317-8-5-9 hom, WT
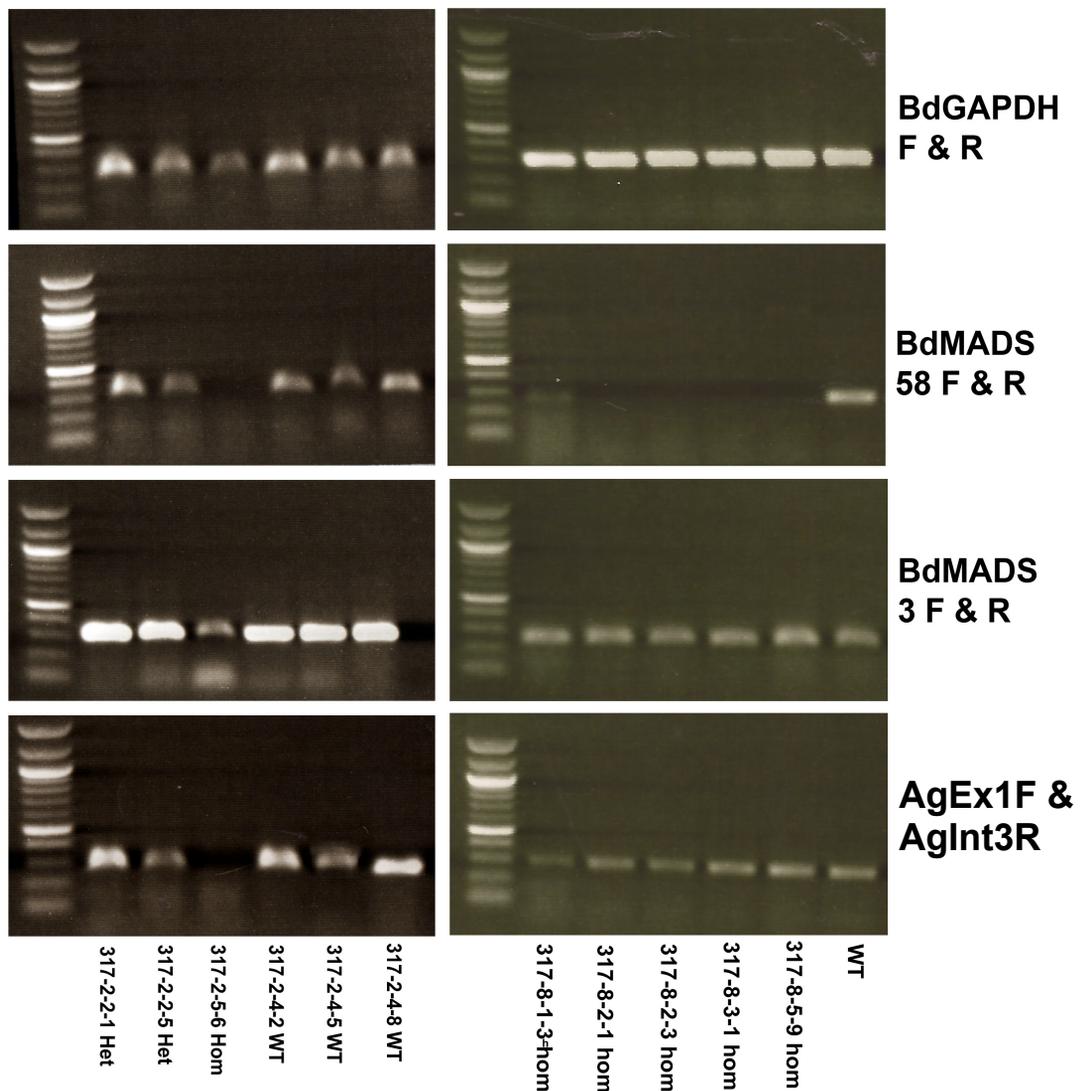
Figure 6.12: RT-PCR for *BdMADS58* Gene Expression in Selected 3rd Generation Plants.

Analysis of *BdMADS58* gene expression in mutant lines. *BdMADS58* F & R primers return a product for the majority 3' region of the transcript and results show expression of this part of the transcript is disrupted in all plants homozygous for the insertion. AgEx1F & AgInt3R primers return a product for 5' region of the transcript, upstream of the insertion site, and here a product is detected in 317-8 line homozygotes, but not 317-2-5-6. Expression of *BdMADS3* was found to be consistent in the homozygotes and heterozygotes, as compared to WT expression and *BdGAPDH* provides a control reaction. Ladder shows 100bp intervals.

demonstrate a phenotype (Table 6.4). This is evidenced most obviously in the inconsistency of the phenotype amongst the 317-8-1 and 317-8-3 plants.

It was noted that the affected spikelets with low grain set did not shatter as is typically seen. We hypothesize that the elongate spikelet may be a result of disruption to *MADS58* expression affecting spikelet meristem determinacy; *MADS58* has been shown to have effects in meristem determinacy (Yanofsky et al, 1990; Yamaguchi et al, 2006). Whilst the formation and function of a typical dehiscence zone may have some dependence on *MADS58* function it is perhaps more likely that disrupted fertility and failure of progression of typical grain development pathways to initiate and subsequent downstream disruption is the most direct cause of this reduced shattering. Disrupted early development or fertilization events regulated by *MADS58*, particularly amongst over-produced florets, may offer some explanation for this altered development. However it is possible that environmental factors influencing fertilisation will result in similar downstream results.

It was possible to identify a potential correlation between plants homozygous for the insert and elevated spikelet numbers. Table 6.5 gives average values for the numbers of spikelets, florets and grains in 3rd generation plants. In comparison to the 17.4 average spikelet number of WT plants, we see values of 27.3, 23.8 and 26.8 for the 317-8-1, 317-8-4 and 317-8-5 respectively. For the heterozygous plants we se an average spikelet number of 18.2, closer to that observed in the wild type plants.. Floret numbers are correspondingly higher in homozygotes also. These numbers do give some preliminary indication of a potential floral phenotype
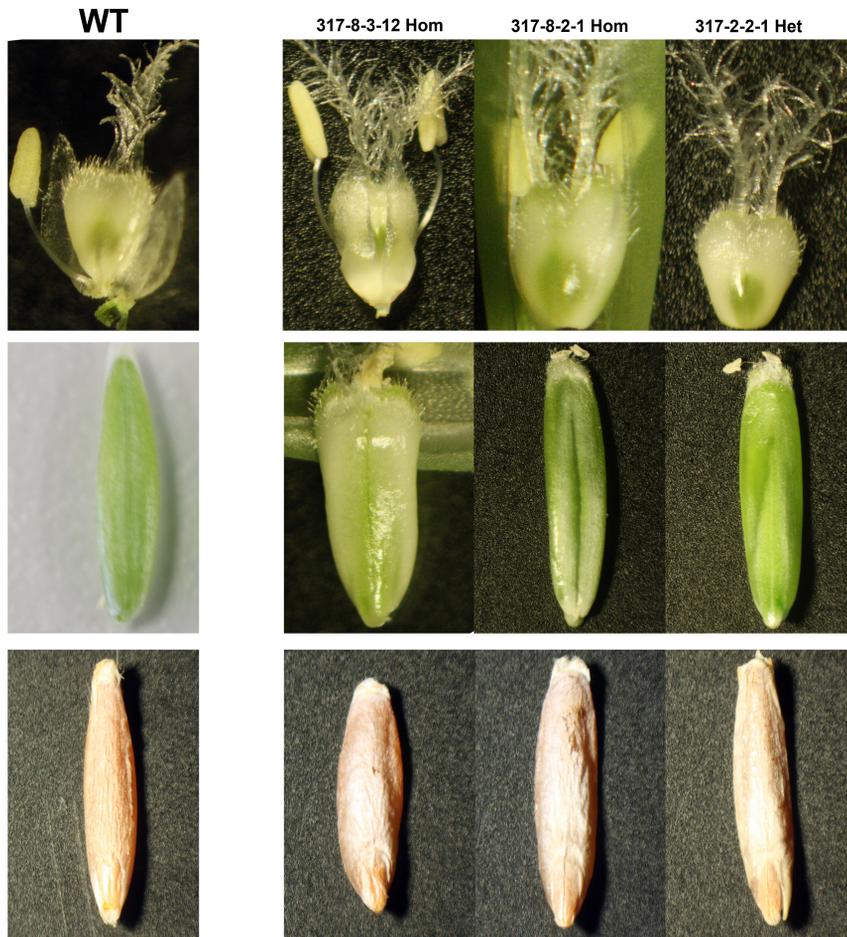
Figure 6.10: Homozygous & Heterozygous *Ag* Mutant Plants Show Normal Grain Development

Analysis of developing *Brachypodium* grains detected no external difference between wild type and mutant lines. Upper row shows extracted *Brachypodium* carpels at ~anthesis. The second row shows developing *Brachypodium* grains at ~8 days after anthesis where the grain has just attained its full proximal-distal development. Any slight difference visible in 317-8-3-12 at this stage can be attributed to this grain being at very slightly earlier developmental stage to its neighbors, normal development was observed in grains from this line. Lower panel shows grains at maturity where no external difference could be detected between grains of homozygous, heterozygous or wild type lines.
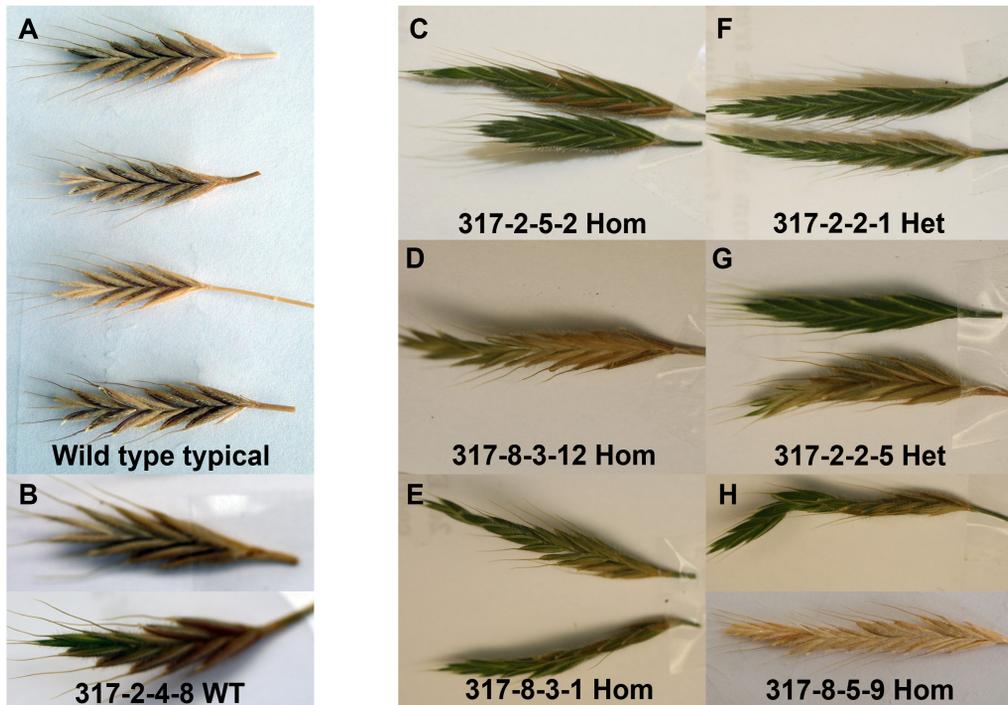
Figure 6.11: Spikelet Phenotype Observed in *Ag* Mutant Lines
  Panels A and B show the typical spikelet morphology in wild type plants. Panels C-H show spikelets of both confirmed heterozygous and homozygous mutant lines showing an aberrant elongate phenotype. These spikelets all showed a low grain set, with fertile florets being restricted to the lower quarter of the spikelet.

3rd Generation Plant line		Average ± 1 stdev			Average florets per spikelet	Average grains per spikelet
		spikelets	Florets	Grains		
WT	Wild Type (n=6)	17.38 ± 6.6	123.6 ± 53.3	54.8 ± 25.4	7.2	3.2
hetero	317-2-2 (n=8)	18.2 + 5.2	167.1 + 49.6	59.6 + 31.3	9.3	3.3
Homo	317-8-1 (n= 12)	27.3 ± 2.8	235 ± 79.1	40 ± 13	8.7	1.5
	317-8-3 (n=9)	24.89 ± 7	148.5 ± 39.7	47.4 ± 19.6	6.2	2.0
	317-8-4 (n=17)	23.8 ± 5.6	214 ± 76.9	41 ± 19.4	9.3	1.8
	317-8-5 (n=17)	26.7 ± 7.3	204.7 ± 73	45.5 ± 20.7	7.8	1.7

Table 6.5: Average Floral and Grain Production Values for 3rd Generation Plants

Table summarises data for confirmed homo or heterozygous plant lines. n= sample number relating to the data for individual plants detailed in table 6.4.

associated with this insertion although these traits may also be linked to

downstream developmental effects through altered fertility etc.

We repeated the earlier RT-PCR expression profiling on 3rd generation plants to

confirm the knockout of the gene and to determine if expression of the 5' region of

the transcript was detectable in homozygotes. The results are shown in figure

6.12. They are consistent to our earlier findings and show a different pattern of

expression between the 317-2 derived homozygotes and the 317-8s. In all plants

examined expression of the 5' region of the BdMADS58 gene is abolished in

homozygotes. In 317-2 lineage plants, using the AgEx1F and AgEx3R primers,

expression of the 5' transcript region is also not detectable in the homozygote 317-

2-5-6, but in the 317-8 lineage plants (Homozygotes) it is. All of these plants are

derived from a single insertion event and so, based upon the data gathered so far,

it is difficult to explain this confounding feature of our results. Resolution of this

conflict is to be a focus of future work. Again we included *MADS3* in this

expression profiling to investigate the possibility of cross-regulation, to examine if

difference in expression level could be seen between hetero- and homozygous

plants and as a control. Expression in the 317-8 lineage plants is consistent across all samples, including the WT control, suggesting there is no difference. In the 317-2 lineage a difference can be seen between the homozygous plant, 317-2-5-6 and the others, although we note that some decrease in *GAPDH* expression can also be seen for this sample.

6.2.6 Summary of BdAA382 Characterisation.

This work provides a genotyping and preliminary analysis of the BdAA382 TDNA insertion line. We were able to positively identify both hetero- and homozygous lines for the TDNA insertion in individuals and link this to disruption in *BdMADS58* expression. We were unable to detect expression of the majority of the 5' region of the *MADS58* gene transcript in plants homozygous for the insert, whilst heterozygotes show normal levels of gene expression.

The functional aspects of MADS-box genes are defined largely by the c-terminal region (Vandenbussche et al, 2003). Our results suggest that with expression of this region of *BdMADS58* eliminated in homozygotes, even where expression of the 5' region exists, it is likely that *MADS58* function is abolished. We were able to show that homozygous plants are not sterile, and their level of grain production appears similar to that of WT plants. This data provides an early indication that in *Brachypodium MADS58* expression is not critical in floral or grain development. This finding would be in keeping with observations of Dreni et al (2011) in rice where analysis of a rice *MADS58* insertional mutant showing highly reduced expression levels revealed a normal phenotype and fertility levels. Earlier observations of *MADS58* function in rice using RNAi have shown a severe loss of

determinacy phenotype in the florets (Yamaguchi et al, 2006).   A more recent investigation of the C-class genes in rice has produced some different findings to suggest that C-class gene function is more highly conserved (Dreni et al, 2011). Here a *MADS58* mutant is found to show no significant developmental defects and only in combined *MADS58/MADS3* mutants is reproductive organ identity disrupted.   Differences in expression profile were also observed with *MADS58* and *MADS3* showing similar expression during early floral development in contrast to Yamaguchi et al (2006) detecting only transient *MADS3* expression. Some similar observations have been made in maize where the *AG* orthologue *ZAG1* correlates to *OsMADS58* and two genes, *ZMM2* and *ZMM23* correlate to *OsMADS3* orthologues. *ZAG1* has been shows a greater expression in carpels compared to *ZMM2* which is more abundant in stamens and analyses of *ZMM23* have yet to be reported (Mena et al, 1996). These differences and contrasting findings raise a number of important questions regarding the exact role, function and conservation of the C-class genes in the grasses.

  A potential, mildly aberrant phenotype was detected in *Brachypodium*, although we were not able to conclusively link this phenotype to a homozygous state for the insert. The elongate spikelet and increased floret production may be attributable to some disruption of meristem determinacy action at the spikelet level for *MADS58*. An alternative possibility is that increased floret production occurs as a consequence of disruption to fertility and grain set, affecting subsequent normal developmental pathways. Our aspirations in relation to this part of the project are to further investigate the potential phenotype and to establish a more accurate method of quantification. Given the high level of variability seen in the flowering of

Brachypodium if attempting to assess the phenotype via floral numbers it would be necessary to include a much larger wild type control population. RNAi knockout of *BdMADS3* in this line will also provide valuable information. In addition to the value of the results produced here this investigation has also allowed the evolution of and optimization of the protocol that will be valuable in future investigations

6.3 Characterization of Insertion line BdAA315 (YABBY insertional mutant): Results & Discussion

In genotyping the BdAA315 insertion line our aim was to produce a preliminary characterisation, to confirm the insertion and determine if gene expression was affected. This insertion is characterized by the flanking sequence tag JIC00266_315 and is listed as affecting a putative YABBY domain containing protein. The insertion is located 156bp from the ATG inside the promoter region of the gene Bradi5g16910, the gene identified in our earlier analyses as a *FILAMENTOUS FLOWER* orthologue (*BdFIL-1*). Figure 6.13 Illustrates the TDNA insert position in the gene, along with primer positions and PCR product lengths. The location of the insert in the promoter region and the high levels of functional redundancy known to exist amongst the FIL-clade genes gave us a low expectation for an observable phenotype in this line, even if expression was found to be abolished.

We obtained seed from T_0 generation plants for this line but with the initial quota of seed being comprised of just ~20 grains our initial aim for the first generation was simply to grow plants on and generate a body of seed. The majority of the

A)

TDNA1 TDNA2 Seq1

LB   T-DNA insert   RB

Seq2 TDNA4 TDNA3
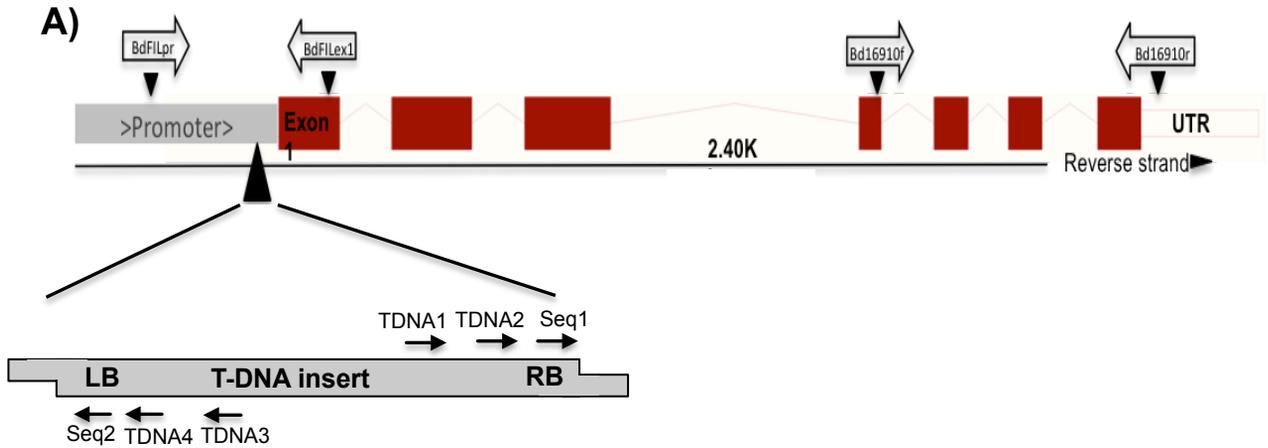
B)

Primers	
BdFILpr	TCAGAGGCAACAACAGATCC
BdFILex1	TTGCAGTGGACGTAGCACAG
Bd16910f	CGGCATACAACCGATT TATC
Bd16910r	AGAATGCAGCTCAAAG

Product lengths (bp)	gDNA	cDNA
BdFILpr & BdFILex1	585	
BdFILpr & TDNA4	380	
Bd16910f & Bd16910r	720	302

Figure 6.13: *BdFIL*-1 Gene Structure, Insertion and PCR Product Details

Figure provides detail of the primer locations and gene structure relevant in the analysis of the BdAA315 insertion line.

A.   Panel shows the structure of the Bradi3g16910 gene including the promoter and position of the TDNA insert. The positions of gene and TDNA primers are marked inside arrows indicating their direction.

B.   Table details primer sequence and expected product lengths

observations and data collected here are taken from the T_2 generation of plants. It is noted in observations made whilst characterizing the *Agamous* insertion line plants, that the early T_0 and T_1 populations often display abnormally weak growth, flowering and seed development and only basic observations relating to general flower and seed production were recorded for this initial population, shown in appendix table A6c. In keeping with expectations, flower and seed production in this generation was low, and it was also noted that the overall stature of plants was small as compared to WT controls under the same conditions. The onset of flowering was noted to be approximately 16% slower, also as compared to WT controls.

We germinated seeds for the second (T_2) generation from selected plants, selection correlating to the most obviously healthy, vigorous and seed producing members of the initial population. GFP screening was performed visually at the seedling stage, detailed in table 6.6. Both GFP+ve and –ve identified plants were grown on such that confirmed WT plants could be used as an internal control. We focused our analysis largely on plants belonging to the 315-3 and 315-13 lines where the ratio of GFP+ve seedlings suggested hetero- and homozygosity for the insert respectively. We performed PCR to confirm individual insertion status and found it necessary to experiment with both left and right border primer combinations to produce satisfactory results for genotyping. Ultimately we found combinations using the TDNA4 insert primer gave the best results. Using RB primers and exon 1 located gene specific primer (FILex1), SEQ1 and TDNA1 gave weak and erratic results whilst TDNA2 returned products longer than expected. Of the LB primers, SEQ2 also gave poor results and TDNA4 proved most effective,

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T1 parent plant	Grains germinated	GFP+ve	GFP-ve	Grown on
315-3	8	6	2	8
315-4	6	0	6	3
315-8	4	4	0	4
315-10	4	0	4	3
315-11	11	0	11	6
315-12	8	3 (weak)	5	3
315-13	29	29	0	29
315-14	15	0	15	5
315-17	7	0	7	3
315-19	12	6	5	9

Table 6.6: Results of GFP screening of 2nd Generation BdAA315 (YABBY) Insertion Line Seedlings

Table shows observations on Green Fluorescent Protein status in germinating YABBY insertion line seedlings and the numbers of seedlings subsequently grown on.

but does produce a double band for the insert product, clearly visible in figure 6.14. This double band is possibly be due to a double TDNA insertion in this line; the initial AA315 listing indicated two insertion loci, although the BrachTag website now lists just 1 TDNA loci for this line. Results for PCR reactions for the 315-3 line plants are shown in figure 6.14, where WT, hetero- and homozygotes can all be identified, indicating this line is heterozygous. The insertion-site spanning product (i.e. gene product; FILpr and FILex1) was found to be very weak in heterozygotes in three primer reactions. To positively confirm the result we performed separate reactions for the gene specific and insert junction spanning primer sets, these results being shown in the lower panel and giving clearer results. We optimized the PCR cycling conditions during these analyses with an increased annealing temperature achieving clearer results with these primers. Using this increased temperature in the three primer PCRs we were able to improve our results in subsequent reactions. Figure 6.15 shows the results of PCR checks on the 315-13 line plants where all are identified as homozygotes (Selected results here were also confirmed in separate two primer reactions, data not shown). In addition to our focus on these two lines the results of the GFP screening indicate the lines 315-11, 315-8 and 315-19 to be potential WT, homozygous and heterozygous respectively. We performed PCRs on selected members of these groups, results are shown in figure 6.16. This panel shows 315-11-4 and -5 to be WT, supporting the expectation that the parent was also WT. 315-8-1 we found to homozygous whilst 315-8-4 we found to be heterozygous, indicating the parent must also have been heterozygous rather than a homozygote as suspected from results shown in
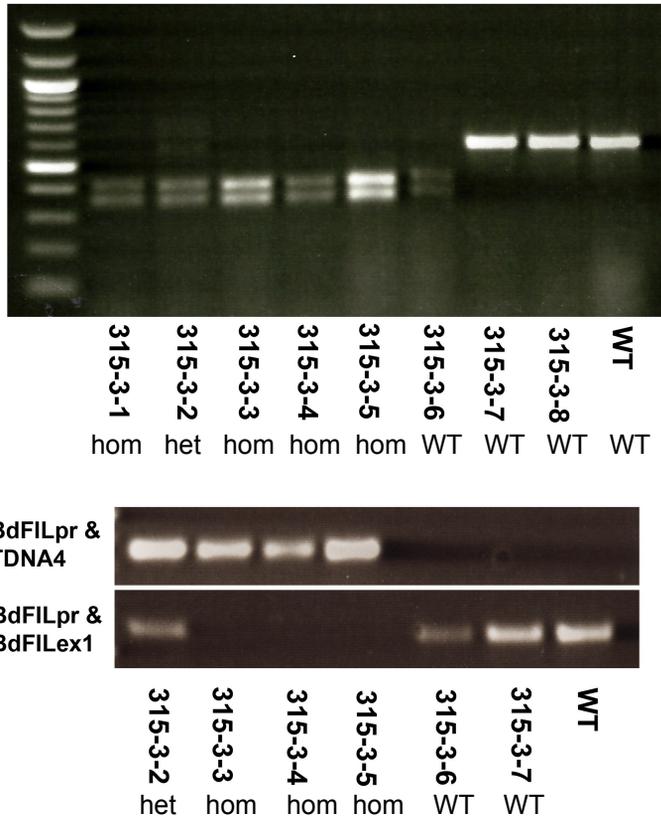
Figure 6.14: GFP Confirmation & Zygosity Check PCR for 315-3 Line Plants

Upper panel shows genotyping PCR reactions using three primers BdFILpr, BdFILex1 and TDNA4 to give insertion site or insertion junction spanning products (Shown in figure 6.13). Gene product (i.e. BdFILpr & BdFILex1 product) amplification is unclear. Lower panel shows a second optimized, analysis using separate primer pair reactions for the insertion junction and insertion site spanning PCRS. Heterozygous, and homozygous individuals are more clearly defined. Ladder shows 100bp intervals.

315-13-2
315-13-4
315-13-5
315-13-6
315-13-8
315-13-19
315-13-22
315-13-28
315-13-29
WT
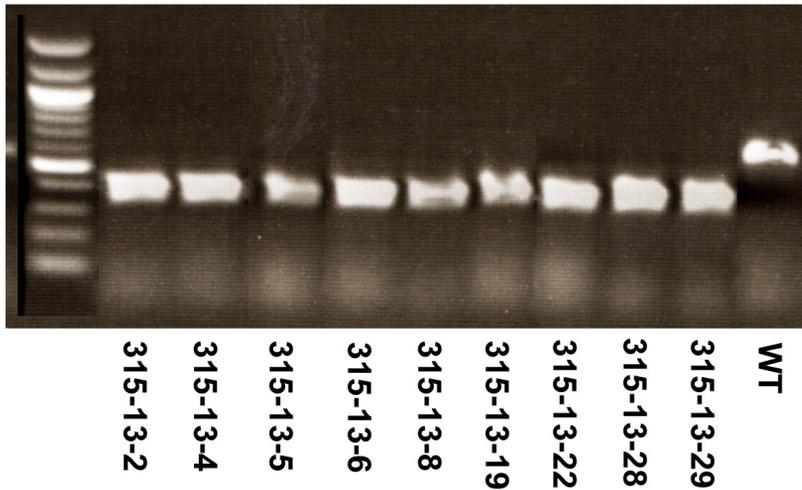
**Figure 6.15: GFP confirmation & Zygosity Check PCRs
for 315-13 Line Plants.**

  Genotyping PCR reactions using three primers BdFILpr,
BdFILex1 and TDNA4 to give insertion site or insertion
junction spanning products (Shown in figure 6.13). With
only TDNA derived product detected in all mutant plants
results indicate all plants to be homozygous for the
insertion.   Ladder shows 100bp intervals
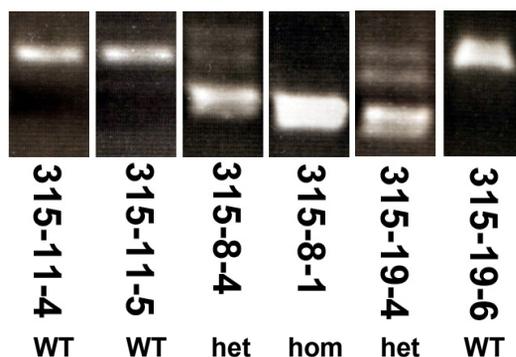
Figure 6.16: Zygosity Checking PCRs for Selected YABBY Insertion Line Individuals.

Genotyping PCR reactions using three primers BdFILpr, BdFILex1 and TDNA4 to give insertion site or insertion junction spanning products (Shown in figure 6.13). Upper band is the gene product (BdFILpr & BdFILEx1 product) and alone indicates wild type. Lower band is the TDNA product (BdFilpr & TDNA4) and alone indicates homozygosity for the TDNA insertion. Double bands from both products show heterozygosity for the TDNA insertion.

table 6.6. 315-19-4 and -6 were shown to be heterozygous and WT respectively, indicating the parent was a heterozygote.

To examine the effects of this TDNA insertion on *BdFIL-1* gene expression we performed RT-PCR using spikelet stage derived cDNA on confirmed WT, homo- and heterozygous plants. Our results, shown in figure 6.17, indicate that *BdFIL-1* expression is reduced but not abolished in homozygous mutants. In heterozygotes there is no reduction in expression as compared to WT plants of the same population. We were not able to detect any outward floral or vegetative phenotype associated with this mutation.

6.3.1 Summary of BdAA382 Characterisation.

We have compiled a valuable genotyping and useful preliminary analysis of this insertion line. Again we were able positively identify hetero- and homozygous individuals, and to associate a reduced level of *BdFIL* gene expression to a homozygous state for the TDNA insertion. As part of an ongoing program of research to investigate transcription factor function in grain development it is our intention to more fully characterize this line and to explore its potential for functional investigation. This line is anticipated to be useful in future reverse-genetic investigations (RNAi etc) targeting *FIL*-like gene function. Our goals also include more sensitive quantitative assay (Q-PCR) to determine the extent of expression reduction and to identify if evidence of any co-regulation and changes in the level of expression for other, related YABBY genes can be detected.
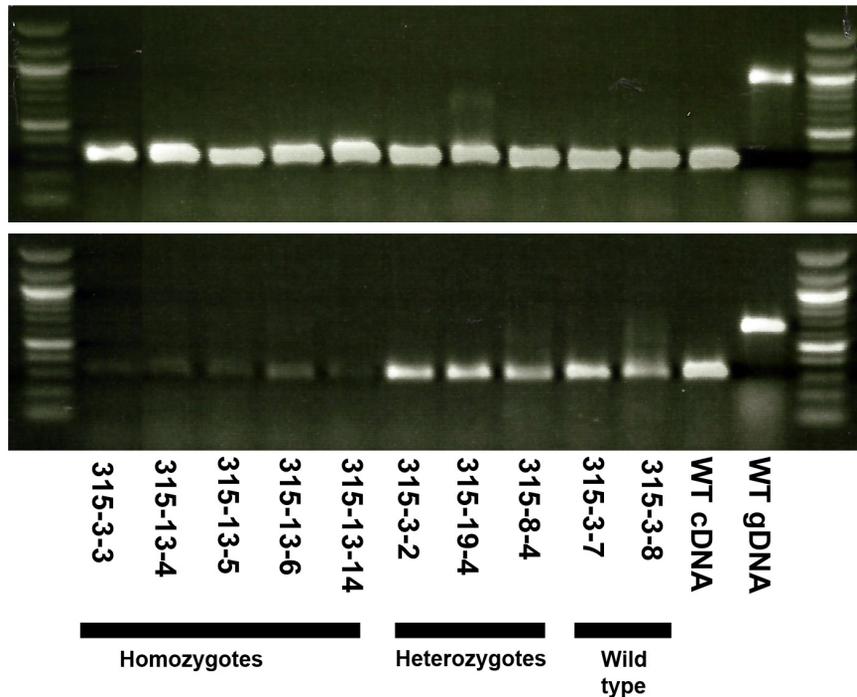
Figure 6.17: RT-PCR for *BdFIL*-1 Expression in Confirmed Hetero- and Homozygous Plants
An analysis of gene expression in plants of confirmed zygosity status for the TDNA insertion using Bd16910F & R primers (detailed in figure 6.13). Upper panel shows *BdGAPDH* expression as a control. Reduced gene expression can be seen in homozygotes as compared to heterozygous and WT expression levels. Ladder shows 100bp intervals.

6.4 Transformation.

Alves et al (2009) have published a detailed protocol (hereafter "the protocol") for the *Agrobacterium*-mediated transformation of Bd21 embryonic callus with high success rates. This technique has been used for the generation of thousands of TDNA insertion lines in the BrachyTag project. We have sought to implement and establish this protocol for the introduction of RNAi constructs in the laboratory with an aim to making stable transformation a key component of our genetic functional investigations. In previous work we have used and optimized transient transformation techniques (VIGS) and our aim was to create a comprehensive toolbox repertoire for gene functional analysis. What follows is an overview of our first attempt to establish this protocol. Unless detailed otherwise, all growth media, conditions and practice are as detailed in the protocol and our observations are related to the description provided in this publication.

Embryo isolation: It was clear in the earliest stages of reviewing this protocol that embryo isolation represented the most technically challenging and subjective aspect of this protocol. It is also pivotal to the smooth progression and success of the procedure. We germinated and grew 4 batches of wild type *Brachypodium* seedlings at one-week intervals under CER conditions detailed. This successive sowing provided a staggered supply of seed material for embryo isolation and repetition of collection. The protocol recommends embryo isolation at 7-9 weeks after germination, when embryos are < 0.3 mm in length. At 6 weeks growth we were able to isolate few embryos of an appropriate size and they were typically vanishingly small. Examining plants just a week older however yields several

appropriately sized embryos per plant, from the most proximal florets 3-4 florets of each spikelet in the first wave of tillers. In plants older than this, at 8 weeks growth, appropriately sized embryos could be isolated from only the uppermost fertile florets of the first spikelets, and yield was often low. Under the growth conditions available here, in nine-week old plants the embryos isolated were typically too large. Based upon our observation during their isolation, subsequent callus production and development we determined that embryos isolated from plants at between 7-7½ weeks growth under Leicester conditions gave the best feasibility of collection, CEC (Compact Embryonic Callus) production and likelihood of transformation efficiency.

CEC production: Across the three subcultures used in the generation of callus we saw high growth rates, with callus production being particularly prolific at the second subculture. As recommended we included $CuSO_4$ in the tissue culture medium which although not essential is stated in the protocol to promote growth and embryonic capacity. The extent of callus growth is shown in figure 6.18 where i) shows callus production during the first culture and ii) shows callus close to the end of the second culture period with abundant growth. Where such prolific callus growth was seen some incidence of browning and death was also observed. This is likely attributable to high callus densities causing an acidification of the growth media, as reported by Bosch et al (2010), and could be reduced by both increasing callus spacing on plates and not sealing plates with nescofilm, allowing some exchange of air within the plate. During these subcultures we were selective in the callus cultured on in order to reduce the overall volume. We selected the creamy yellow, tight CEC, fitting the description of the most desirable callus material for
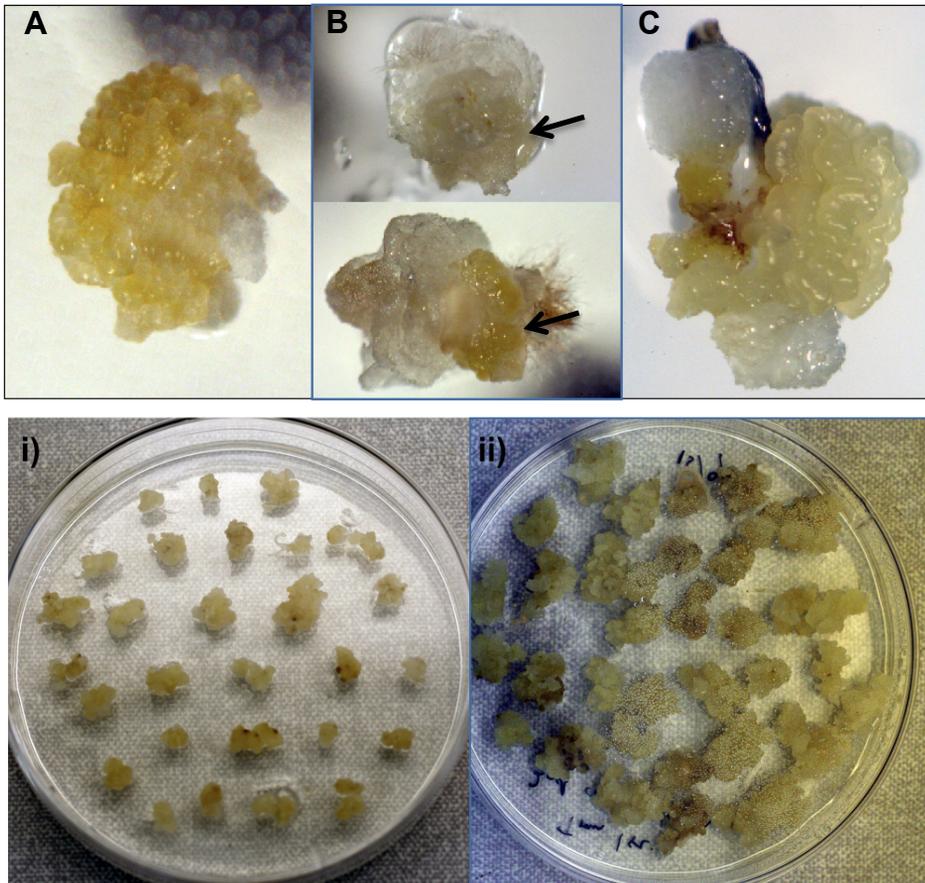
Figure 6.18: Compact Embryonic Callus Production

Upper panel shows the three different types of callus generated. A, shows the perceived ideal callus type; B, soft-wet callus with occasional creamy callus produced from root regions (arrowed); C, shows a embryo developing both types of callus, where creamy callus was selected at subculture points.
Lower panel shows callus growth during the first culture (i) and abundant growth in (ii), pictured close to the end of the second subculture period.

transformation. From initial cultures of between 20-30 embryos some 6-8 plates of callus were generated. It was also noted that we were able to identify all callus types described as being produced in the protocol. In keeping with the description provided, larger embryos typically gave rise to soft-wet callus whilst smaller embryos produce the creamy yellow, dry callus. However we found a fairly even mix of both compact callus and the loose, friable callus without any obvious reason for the difference in callus type produced. Figure 6.18 panel shows these different callus types. Embryos producing the soft-wet callus type are shown and it can be seen that even where this type of production is dominant some small production of the creamy, compact callus may occur, and was typically seen to originate from root/scutellum tissue. Image C shows the production of mixed callus types from a single embryo. Here we isolated only the creamy CEC for growth when subculturing. The callus produced in A is a good representation of that we identified as most ideal for transformation based upon description given. It was this callus type we typically favored during subcultures although care was taken to continue a subculture with some soft-wet and other callus types to see if any change in growth or type would be observed across the culture period, but none was seen. It was also noted that smaller isolated embryos gave a lower incidence of shoot/root formation in explants, a desirable feature necessitating less risk of contamination whilst these structures are excised. Having germinated several *Brachypodium* generations we performed repeats of callus generation over which we found our ability to isolate embryos at the correct stage, callus division and aseptic culture techniques improved successively and gave increased CEC production.

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Transformation: From previous work we had available the pCambia binary vector containing a *PapsFIL* (*Papaver somniferum* FILAMENTOUS FLOWER) gene under the camv35s promoter, illustrated in figure 6.18. We decided to attempt the transformation using this general purpose vector due to its availability and as a test for suitability in *Brachypodium* (Whilst recognizing pVec8 represents the current standard for *Brachypodium* transformation). As no visual selection component is included in this construct our aim was to rely upon chemical selection (hygromycin) and to confirm the insert in callus/plant material via PCR. The vector was transformed into *Agrobacterium* strain GV3101 with all aspects of the culture and preparation performed as described in the protocol. For comparison we performed parallel co-cultures on both jellified growth medium and filter paper according to the two alternative methods detailed in the protocol in three independent transformation attempts.

  We detected no signs of *Agrobacterium* overgrowth on any of these co-cultures, as was suggested may be observed. Only minor signs of *Agrobacterium* growth was seen where callus made contact with jellified medium and we could detect no signs of growth on those callus cultured on filter paper. Following transformation callus was transferred to hygromycin selective medium. At this point callus showed no further growth, became pale, frequently brown and quickly died. These results indicate that transformation was not successful and callus died under selection, and is likely attributable to incompatibility of our transformation vector. Monocots are typically recalcitrant to transformation and pVec8 was designed as monocot vector, and has shown good results for transformation in barley and now in *Brachypodium*. More recently and specifically developed vectors such as
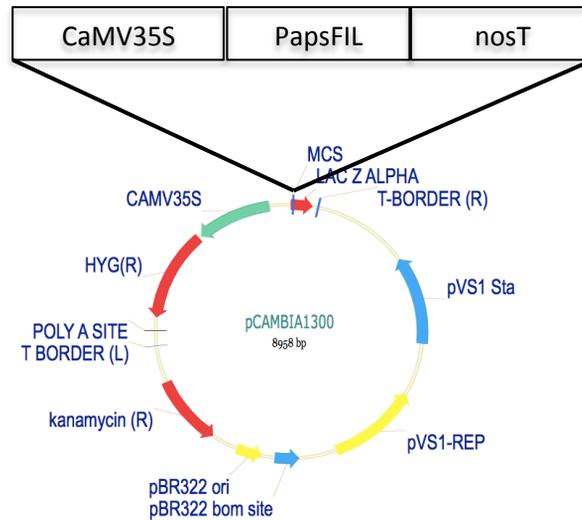
Figure 6.19: Transformation

i) pCAMBIA vector map showing the PapsFIL insert used in this transformation.

ii) Transformed callus at three days on selection media, where all callus has died. Transformed callus fragments showed some growth before uniform browning and death.

pBrachyTAG may see even greater levels of efficiency (Thole et al, 2010). We are in the process of obtaining the pVec8-GFP vector in order to repeat and establish this part of the protocol for future work.

Plant regeneration: Whilst our transformation attempts were not successful we decided to attempt plant regeneration using untransformed calli. We initially transferred callus to MSB3+As60 plates for 2 days and then performed another subculture onto MSB+Cu0.6 plates for 3 weeks to at least partially simulate the effects of transformation and to ensure callus was of the same age as that which had undergone transformation. After this we followed the protocol for plant regeneration with the exception of including antibiotic selection in the growth media. Repeated subculturing involved in regeneration did result in some heavy fungal and bacterial growth; this was removed or callus/plantlets were re-cultured where necessary. Despite some losses to fungal etc growth we were able to successfully regenerate plants, shown in figure 6.20 Regenerated plants were small but did produce seed, albeit at low levels.

Having completed all components of this protocol we demonstrate that with use of a more appropriate vectors we expect to achieve successful transformation and regeneration with the facilities available.

6.5 Chapter Conclusion

Mutagenesis offers a valuable and highly effective way to investigate plant gene function. The study of gene function in *Brachypodium* as the first temperate grass model, in particular regulatory and other genes of known significance to domestication traits, is a novel and auspicious area of both applied and
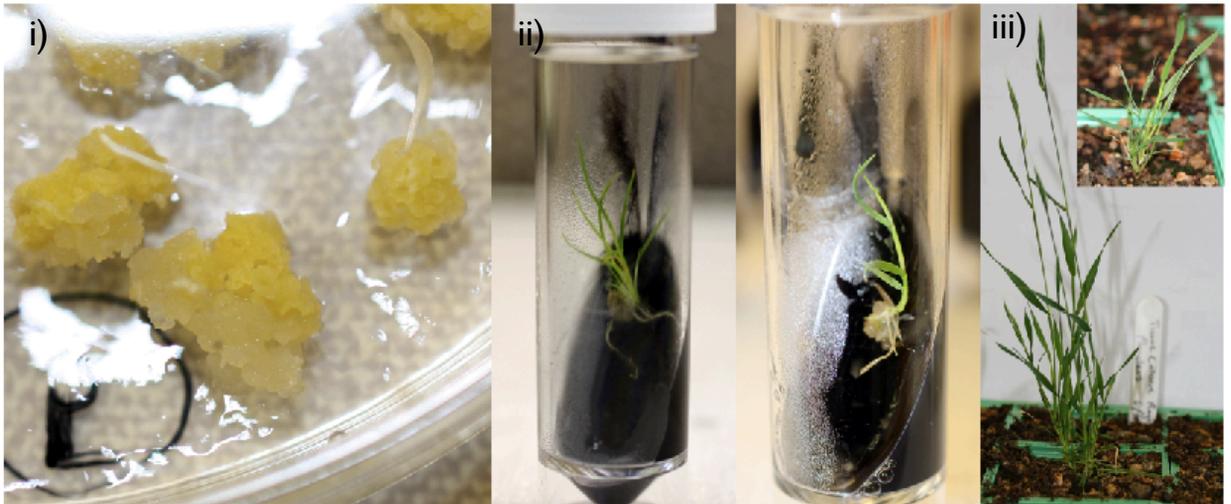
Figure 6.20: Explant Regeneration

i) Regenerating callus on MSR26 media. Small, weakly photosynthetic, shoot growth can be seen.

ii) Plantlets on germination medium showing strong growth.

iii) Regenerated plants at flowering. Image shows one of the largest and most vigorous plants, which had a close to normal morphology. Inset shows one of the smallest, but still fertile plants. A range plant size between these two extremes was observed.

fundamental research. The BrachyTag mutant collection represents an important component of such research and a promising resource for which published descriptions have only just become started to become available. We have contributed to this emerging area of research with preliminary characterisation for two of these lines. Our results show that disruption of the *Brachypodium AGAMOUS* orthologue produces no detectable effects to carpel development or fertility, and that function of this gene in *Brachypodium* is similar to that recently reported for rice (Dreni et al 2011). We have established a rapid and effective protocol for the analysis of *Brachypodium* insertional mutants, demonstrated with the characterisation of a second BrachyTag insertion for the FIL orthologue. Using this system we were able to identify and confirm homozygotes and record a reduced gene expression within a single generation of plants. Recognising the value of stable transformation as an incipient tool for functional gene analysis in *Brachypodium* we have also made good progress in implementing a industry standard *Agrobacterium* mediated transformation protocol for use in the laboratory at Leicester university. Together the components of this chapter demonstrate an effective array and implementation of resources that form the basis of an ongoing program of genetic functional analyses in *Brachypodium*.

CHAPTER 7.

FINAL DISCUSSION

FINAL DISCUSSION

A broad range of character variation and interrelated features relating to significant aspects of physiology and economic value of temperate cereal grains is described. Chapters 1 and 2 characterise grain development and morphology focusing on *B.distachyon* and a wider sample of the major Pooid species. Features of *B. distachyon* grain morphology distinguish it from wheat and the rest of the Pooideae whilst reflecting a position outside of the core-pooids and intermediate to rice and the Triticeae (Kellogg, 2001). Although some developmental patterns are shared, organisational differences in the maternal tissues and aleurone layer, the absence of the modified aleurone region and differences in endosperm storage reserve reveal a distinctly different grain physiology as compared to wheat. Although posited as an important model for wheat these findings suggest *Brachypodium* may not offer as ideal a model for grain development as first anticipated. Never the less, the gene family similarities demonstrated in chapter 5 along with both the shared and contrasting attributes of grain morphology and physiology revealed in chapter 4, indicate *Brachypodium* has great value in the investigation, modeling and origins of various aspects of grain developmental biology for Pooideae species. Specifically it is possible to envisage a role for *Brachypodium* in modeling aspects of oat physiology and as an important species for tracing the origins of cereal grain diversity. As a functional model *Brachypodium* will be especially valuable but care will need to be taken to ensure it is representative for the trait under investigation.

Chapter 4's comparison of Brachypodieae to Core-Pooid characters revealed a range of character variation but high similarity amongst tribally distinct

domesticated species. Shared features suggest domestication selection towards a distinct target of grain size and morphology while differences in floret structure and grain morphology between wild and cultivated species reflect ecological adaptation and habit. Molecular and cellular mapping reveals patterns of aleurone organisation at both tribal and family levels and may correlate to grain quality characters such as shape or starch content. Morphological differences suggest 2 distinct modes of grain filling exist in the Pooideae with a nucellar-projection/modified-aleurone region transfer cell mediated route being dominant. Data suggests that the highly distinct modified aleurone, dead at maturity, is restricted to the Triticeae. Differences in central endosperm cell walls between wild and domesticated species have been identified in previous studies e.g. Shapter et al (2008) report a greater retention of cell wall material in wild species as compared to domesticates. We report thin central endosperm cell walls compared to thicker aleurone walls as a consistent feature of both wild and cultivated core-pooids, but not shared by the Brachypoideae. This morphological analysis provides a basic picture of Pooid grain morphology and details to its evolution, and a basis on which to interpret current and future molecular data relating to grain developmental regulation.

 Chapter 6 details work forming the practical basis of ongoing transcription factor functional investigation in *Brachypodium*. As a monocot model system *Brachypodium* has many advantages along with a developing array of tools and technologies to facilitate analysis (Vogel et al, 2010; Bevan et al, 2010; Mur et al, 2011). We described preliminary results for characterisation of BrachyTag TDNA mutant lines for a C-class MADS-box and a YABBY gene along with the

establishment of simple, rapid system for future characterisations. Mutant

investigation is a significant aim for future work where, from the existing and

upcoming collection of BrachyTag lines, we will select for those affecting

transcription factors involved in fruit development based upon previous results.

RNAi approaches will be significant in on-going research. In the event that TDNA

lines for genes of interest are not available we will design and build constructs for

transformation; this approach will also be used for complementing existing mutant

lines. We have already designed constructs for a *BdMADS3* knockout to

complement the *mads58* mutant line for further examination of C-class function.

Brachypodium transformation is well described and is now a routine procedure,

chapter 6 describing our progress in establishing a protocol. It is important and

pressing a goal of future work to implement and optimise this protocol in order to

investigate gene function.

  We also plan further exploration of MADS-box gene function in *Brachypodium*

through investigation of protein interaction. Studies have shown protein-protein

interaction is critical in MADS-box gene DNA-binding activity (Favaro et al, 2003).

Yeast hybrid studies of a B-class protein heterodimer was instrumental in the

proposal of the "quartet model" for MADS-box transcription factor function (Egea-

Cortines et al, 1999; Theissen 2001; Ng & Yanofsky, 2001). This model integrates

the ABC genetic model with the known protein-protein interactions the MADS-box

gene families. Based upon expression patterns data generated we have designed

hybrid experiments and have already initiated yeast two hybrid assays for *BdLHS1*

and C-class genes.

A final key advantage to exploiting *Brachypodium* as the basis for study is the recent release of microarray chips for transcriptomic analyses through NASC (Nottingham Arabidopsis Stock Centre). We aim to use our knowledge of grain development in *Brachypodium* to select 5 key stages for a developmental time-course microarray analysis that will compare to previous expression analyses of wheat of milestone stages (Drea et al, 2005b). We will use this data to identify large-scale gene expression patterns coincidental to key developmental events such as endosperm proliferation, cellularisation and differentiation. This data will feed in to our program of characterisation and functional analyses.

Surveying and characterizing a range of cytological and molecular characters of temperate cereal grain development we have identified a broad similarity and a number of specific and significant points of difference. These findings provide novel insight into key features of grain biology achieving an important aim of this thesis, that being the establishment of a comprehensive footing for further investigation with *Brachypodium* at its core. Over the past 4 years Research involving *Brachypodium* has increased at a massive rate. A Pubmed search in 2008 for papers relating to *Brachypodium* as a model system returned just 15 significant hits whilst the equivalent search today produces more than 250. Providing some basis for a specialised repository of information specific to cereal grain development this thesis has attempted to contextualize grain development in *Brachypodium* as it becomes established as a successful and important model integral to future research of cereal grain biology and domestication.
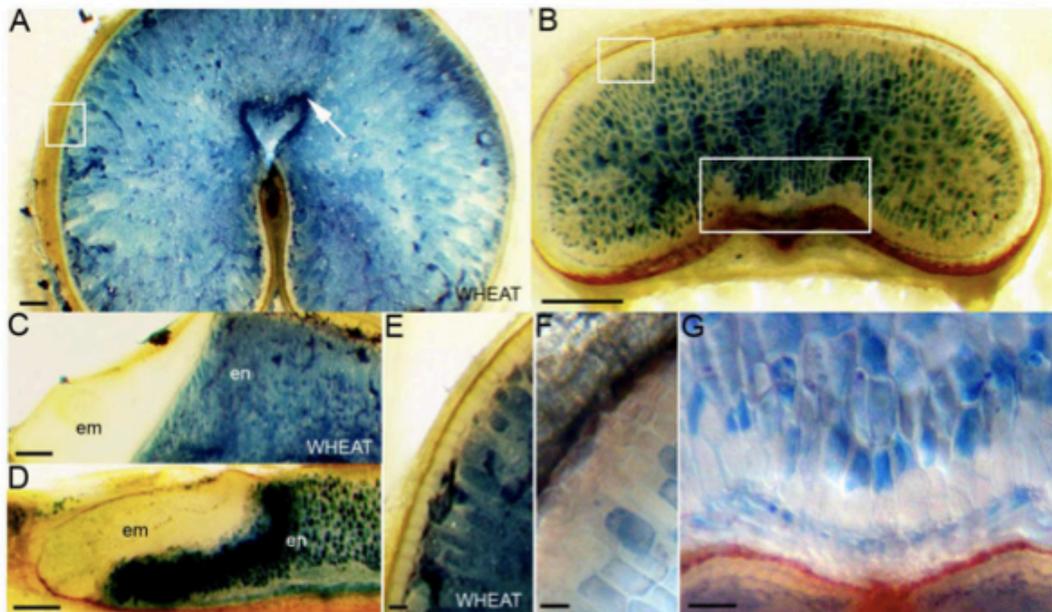
A3c: Evans blue vital staining on mature *Brachypodium* and wheat endosperm.
A) Cellular domains in the Brachypodium endosperm. (A) Mature wheat and (B) Brachypodium grain cross sections stained with Evans Blue. Boxed regions are shown in more detail in E, F and G. Scale bars, 200µm (C) Wheat and (D) Brachypodium embryo are clear of staining. em, embryo; en; endosperm. Scale bars, 500µm need annotation (E) Wheat and (F) Brachypodium peripheral aleurone layers are clear of staining. Scale bars, 10 and 50µm (G) Brachypodium endosperm cells above the vascular/crease region are unstained. Scale bar, 50µm

	Brachypodium shoot length (mm)		
Hours growth	No endosperm	Half endosperm	Full endosperm
48hrs	3.56	4.79	4.65
72hrs	5.99	11.97	11.26
96hrs	8.5	18.04	16.04
120hrs	10.81	21.77	24.19
144hrs	11.24	25.08	27.63
192hrs	14.62	26.52	29.82

A3d: Raw data for chart included in figure 3.12

ESR non-specific staining
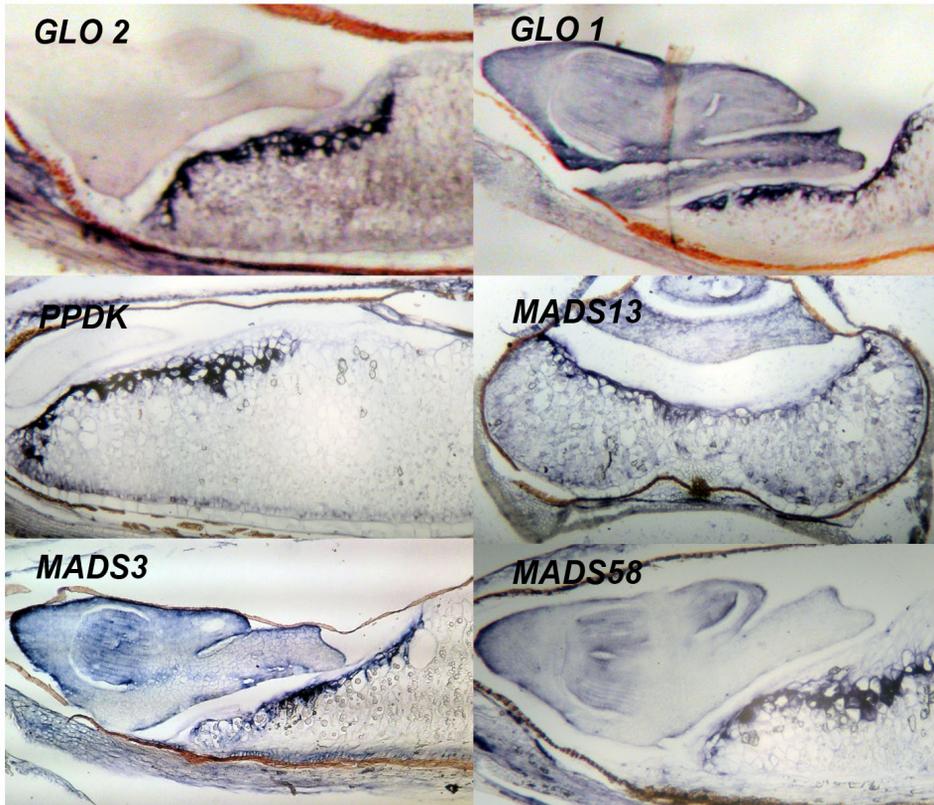
A3e: Collected examples of non-specific hybridisation in the ESR region of the endosperm in *Brachypodium*. The occurrence of staining in this area with many different probes is indicative that some feature of the tissue in this area binds probe of developing solution. This type of staining is typically only seen in mature grain sections suggesting it may be feature of some aspects of early breakdown of the endosperm by scutellum-originating products.

Species	Measurements (mm)								
	Length	St Dev	St error	Width	St Dev	St error	Depth	St Dev	St error
B. distachyon	6.000	0.439	0.098	1.250	0.301	0.067	0.750	0.074	0.016
B. stacei	6.050	0.360	0.081	1.170	0.080	0.018	0.950	0.063	0.014
B. hybridum	7.670	0.548	0.122	1.240	0.111	0.025	0.900	0.067	0.015
B. sylvaticum	6.340	0.400	0.089	1.180	0.166	0.037	0.640	0.070	0.016
B. pinnatum	6.640	0.549	0.123	1.170	0.153	0.034	0.620	0.069	0.015
Bromus mollis	5.700	0.466	0.104	1.280	0.177	0.040	0.720	0.091	0.020
Bromus diandrus	13.640	1.469	0.329	1.590	0.208	0.046	1.270	0.167	0.037
Bromus sterilis	9.980	0.743	0.166	1.200	0.151	0.034	0.960	0.187	0.042
T. aestivum "cadenza"	6.610	0.389	0.087	3.500	0.214	0.048	3.220	0.267	0.060
T. spelta	8.200	0.406	0.091	3.670	1.670	0.373	2.890	0.174	0.039
T. turgidum	8.050	0.462	0.103	3.060	0.153	0.034	3.270	0.293	0.066
T. disoccoides	9.340	0.578	0.129	2.460	0.206	0.046	2.400	0.310	0.069
T.uratu	7.490	0.655	0.147	1.460	0.182	0.041	1.940	0.458	0.102
A. speltoides	7.240	0.702	0.157	1.860	0.169	0.038	1.060	0.130	0.029
A. tauschii	4.980	0.709	0.159	2.720	0.291	0.065	1.670	0.216	0.048
A. ovata	6.570	1.106	0.247	2.260	0.520	0.116	1.320	0.314	0.070
H. murinum	6.080	0.458	0.102	2.170	0.235	0.053	1.230	0.161	0.036
H. vulgare "optic"	9.050	0.547	0.122	2.970	0.256	0.057	2.210	0.235	0.052
S. Cereale	7.240	0.646	0.144	2.640	0.204	0.046	2.580	0.204	0.046
E. repens	3.770	0.323	0.072	1.110	0.102	0.023	0.780	0.089	0.020
A. fatua	7.520	0.884	0.198	1.910	0.257	0.058	1.460	0.324	0.073
A. sativa	7.660	0.869	0.194	2.450	0.232	0.052	2.070	0.200	0.045
D. caespitosa	2.210	0.256	0.057	0.550	0.151	0.034	0.390	0.089	0.020
A. stolonifera	1.110	0.190	0.042	0.350	0.049	0.011	0.250	0.065	0.015
L. perenne	3.400	0.278	0.062	1.150	0.072	0.016	0.770	0.089	0.020
F. pratensis	2.910	0.380	0.085	1.190	0.114	0.025	0.680	0.085	0.019
D. glomerata	2.810	0.297	0.066	0.710	0.166	0.037	0.570	0.112	0.025

A4a: Raw data for chart comprising figure 4.3

Analysis of Variance (One-Way) LENGTH

Summary

Groups	Sample size	Sum	Mean	Variance
B. distachyon	20	119.98	5.999	0.19282
B. stacei	20	121.05	6.0525	0.1298
B. hybridum	20	153.46	7.673	0.29984
B. sylvaticum	20	126.84	6.342	0.1599
B. pinnatum	20	132.86	6.643	0.301

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	37.27642	4	9.31911	43.01004	0.E+0	3.06984
Within Groups	20.58392	95	0.21667			
Total	57.86034	99				

Analysis of Variance (One-Way) WIDTH

Summary

Groups	Sample size	Sum	Mean	Variance
B. distachyon	20	26.26	1.313	0.00737
B. stacei	20	23.26	1.163	0.00766
B. hybridum	20	24.76	1.238	0.01233
B. sylvaticum	20	23.64	1.182	0.02743
B. pinnatum	20	23.26	1.163	0.02345

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	0.33158	4	0.08289	5.29692	0.00068	3.06984
Within Groups	1.4867	95	0.01565			
Total	1.81828	99				

Analysis of Variance (One-Way) DEPTH

Summary

Groups	Sample size	Sum	Mean	Variance
B. distachyon	20	14.94	0.747	0.00543
B. stacei	20	18.95	0.9475	0.00401
B. hybridum	20	17.94	0.897	0.00449
B. sylvaticum	20	12.82	0.641	0.00496
B. pinnatum	20	12.49	0.6245	0.00475

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	1.71931	4	0.42983	90.92544	0.E+0	3.06984
Within Groups	0.44909	95	0.00473			
Total	2.1684	99				

A4b : Calculated values for grain size one-way parametric ANOVA for all *Brachypodium* species.

Analysis of Variance (One-Way) LENGTH

Summary

Groups	Sample size	Sum	Mean	Variance
B. distachyon	20	119.98	5.999	0.19282
B. stacei	20	121.05	6.0525	0.1298
B. hybridum	20	153.46	7.673	0.29984

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	36.20772	2	18.10386	87.25287	0.E+0	4.19323
Within Groups	11.82678	57	0.20749			
Total	48.0345	59				

Analysis of Variance (One-Way)

Summary

Groups	Sample size	Sum	Mean	Variance
B. distachyon	20	26.26	1.313	0.00737
B. stacei	20	23.26	1.163	0.00766
B. hybridum	20	24.76	1.238	0.01233

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	0.225	2	0.1125	12.33268	0.00004	4.19323
Within Groups	0.51996	57	0.00912			
Total	0.74496	59				

Analysis of Variance (One-Way)

Summary

Groups	Sample size	Sum	Mean	Variance
B. distachyon	20	14.94	0.747	0.00543
B. stacei	20	18.95	0.9475	0.00401
B. hybridum	20	17.94	0.897	0.00449

ANOVA

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	0.435	2	0.2175	46.85144	9.25E-13	4.19323
Within Groups	0.26462	57	0.00464			
Total	0.69962	59				

A4c: Calculated values for grain size one-way parametric ANOVA for three recently reclassified *B. distachyon* cytotypes.

		LENGTH			
	B. distachyon	B. stacei	B. hybridum	B. sylvaticum	B. pinnatum
B. distachyon		0.053500000	1.674	0.343	0.644
B. stacei			1.6205	0.28950000	0.5905
B. hybridum				1.331	1.03
B. sylvaticum					0.301
B. pinnatum		T critical	0.411134844		
B. sylvaticum/B.pinnatum			0.301		
B. hybridum/B. pinatum			1.03		
B.hybridum/ B. sylvaticum			1.331		
B. stacei/B. pinnatum			0.6		
B.stacei/sylvaticum			0.29		
B. stacei/B.hybridum			1.621		
B. disatchyon/B. pinnatum			0.644		
B. distachyon/B. sylvaticum			0.343		
B. distachyon/B. hybridum			1.674		
B. distachyon/B. stacei			0.535		

		WIDTH			
	B. distachyon	B. stacei	B. hybridum	B. sylvaticum	B. pinnatum
B. distachyon		0.1500000	0.075	0.131	0.150
B. stacei			0.0750	0.01900000	0.0000
B. hybridum				0.056	0.08
B. sylvaticum					0.019
B. pinnatum		T critical	0.110494146		
B. sylvaticum/B.pinnatum			0.019		
B. hybridum/B. pinatum			0.08		
B.hybridum/ B. sylvaticum			0.056		
B. stacei/B. pinnatum			0		
B.stacei/sylvaticum			0.019		
B. stacei/B.hybridum			0.075		
B. disatchyon/B. pinnatum			0.15		
B. distachyon/B. sylvaticum			0.131		
B. distachyon/B. hybridum			0.075		
B. distachyon/B. stacei			0.15		

		DEPTH			
	B. distachyon	B. stacei	B. hybridum	B. sylvaticum	B. pinnatum
B. distachyon		0.2005000	0.150	0.106	0.123
B. stacei			0.0505	0.306500	0.3230
B. hybridum				0.256	0.27
B. sylvaticum					0.017
B. pinnatum		T critical	0.060745298		
B. sylvaticum/B.pinnatum			0.017		
B. hybridum/B. pinatum			0.27		
B.hybridum/ B. sylvaticum			0.256		
B. stacei/B. pinnatum			0.323		
B.stacei/sylvaticum			0.306		
B. stacei/B.hybridum			0.051		
B. disatchyon/B. pinnatum			0.123		
B. distachyon/B. sylvaticum			0.106		
B. distachyon/B. hybridum			0.15		
B. distachyon/B. stacei			0.201		

A4d: Raw data for Tukey's test shown in figure 4.5.
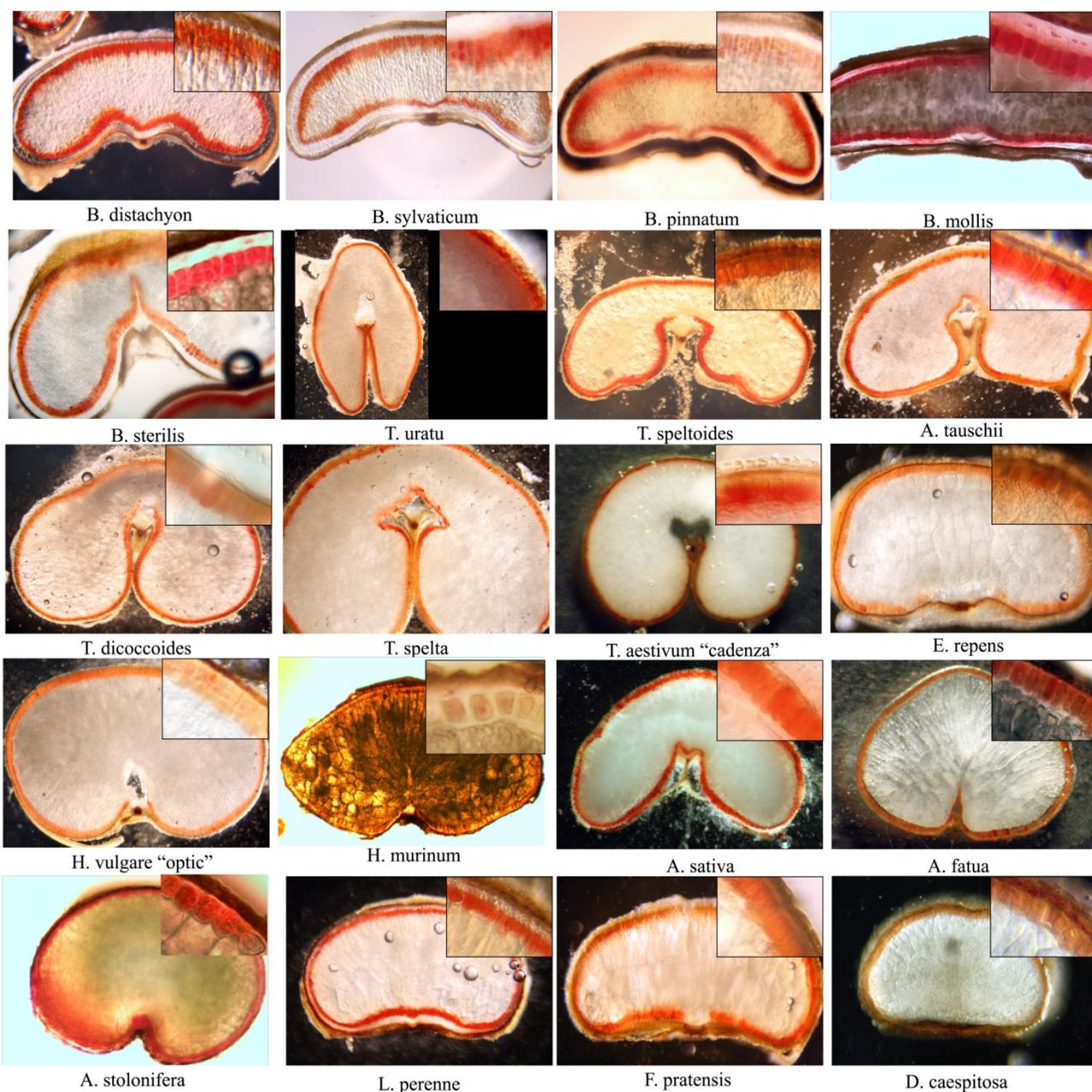
A4e: TZ staining of mature imbibed grain central cross sections.
Insets show magnification of adaxial aleurone region in each species
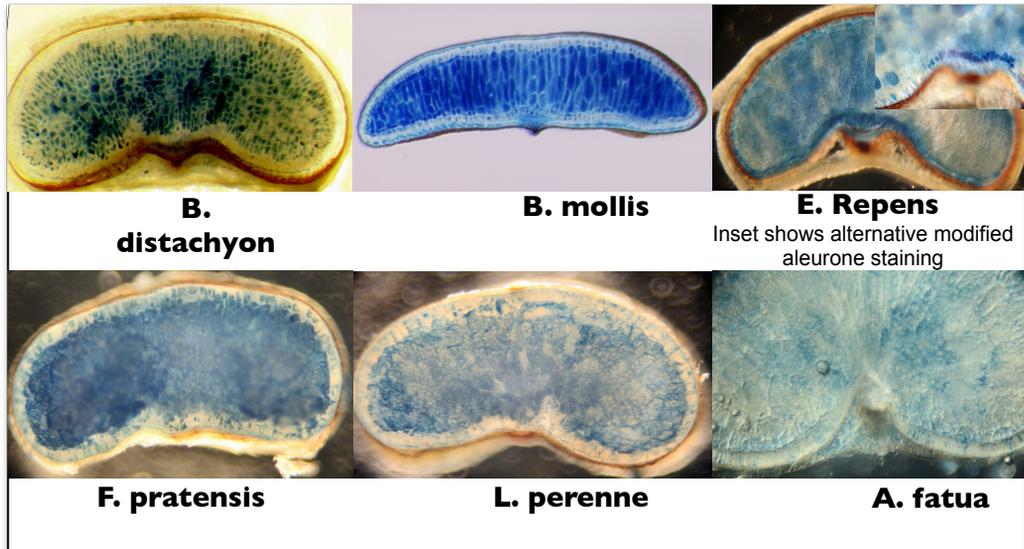
A4f : Evans blue vital staining for selected wild species to confirm TZ results and provide additional evidence for live/dead modified aleurone status
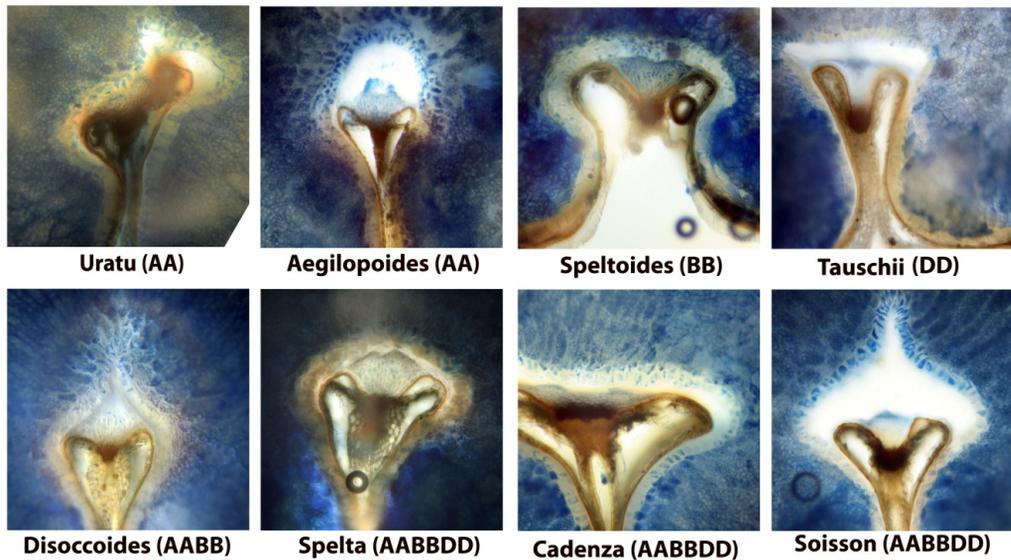
A4g: Evans blue vital staining for the modified aleurone region of primitive and cultivated wheats. *T. aegilopoides* is closely related to *T. uratu* and a another potential AA genome donor for modern wheats. "Soissons" is a another elite cultivar of T. aestivum, along with "cadenza". Result for *A. tauschii* is not confirmed and inconsistent staining was observed for the modified aleurone region.

Extreme proximal section

Extreme distal section

B. distachyon   F. pratensis   L. perenne
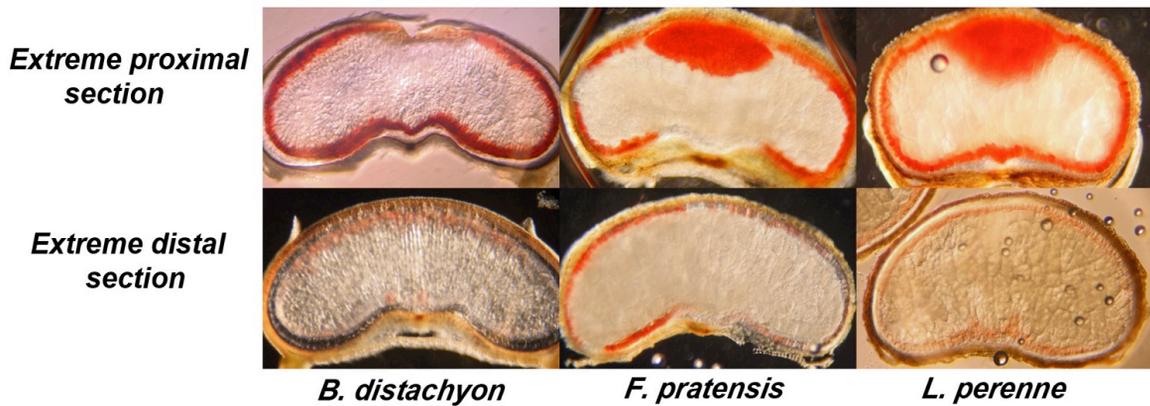
A4h: Differences in Proximal Distal TZ staining at 12 hours imbibition

Grains were examined at differing periods of imbibition to assess the consistency of TZ staining. In relation to the modified aleurone region no difference in staining was seen, this region being consistently dead in wheat. However, some minor differences in the extent of peripheral aleurone staining in the distal half of the grain could be seen in some species. We identified the possibility that these differences relate the full uptake (Imbibition) and movement of water along the grain and subsequent increase in cellular activity (respiration) in the aleurone influencing the extent of staining. To investigate this possibility we examined the pattern and extent of TZ staining in grains subject to differing imbibition periods, 0 and 12 hours. In dry (i.e. un-imbibed grains) sections, TZ staining aleurone is not visible or is just barely detectable. Staining can be detected strongly in the aleurone at any point along the length of grain for all species at > 24 hours imbibition, and at 12 hours imbibition only proximal grain-half staining is detectable. This would be in keeping with the movement of water into the grain from the micropyle, its progression along the grain, and the commencement of increased cellular activity in the aleurone cells with hydration. Importantly, at 24 hours imbibition, the period typically used here, no variation was seen at the central point of the grain in the species examined here. In order to consider status along the full length of the grain a 24hr imbibition period should be used as a minimum and ideally longer in conjunction with maintaining a cooler temperature to delay germination.
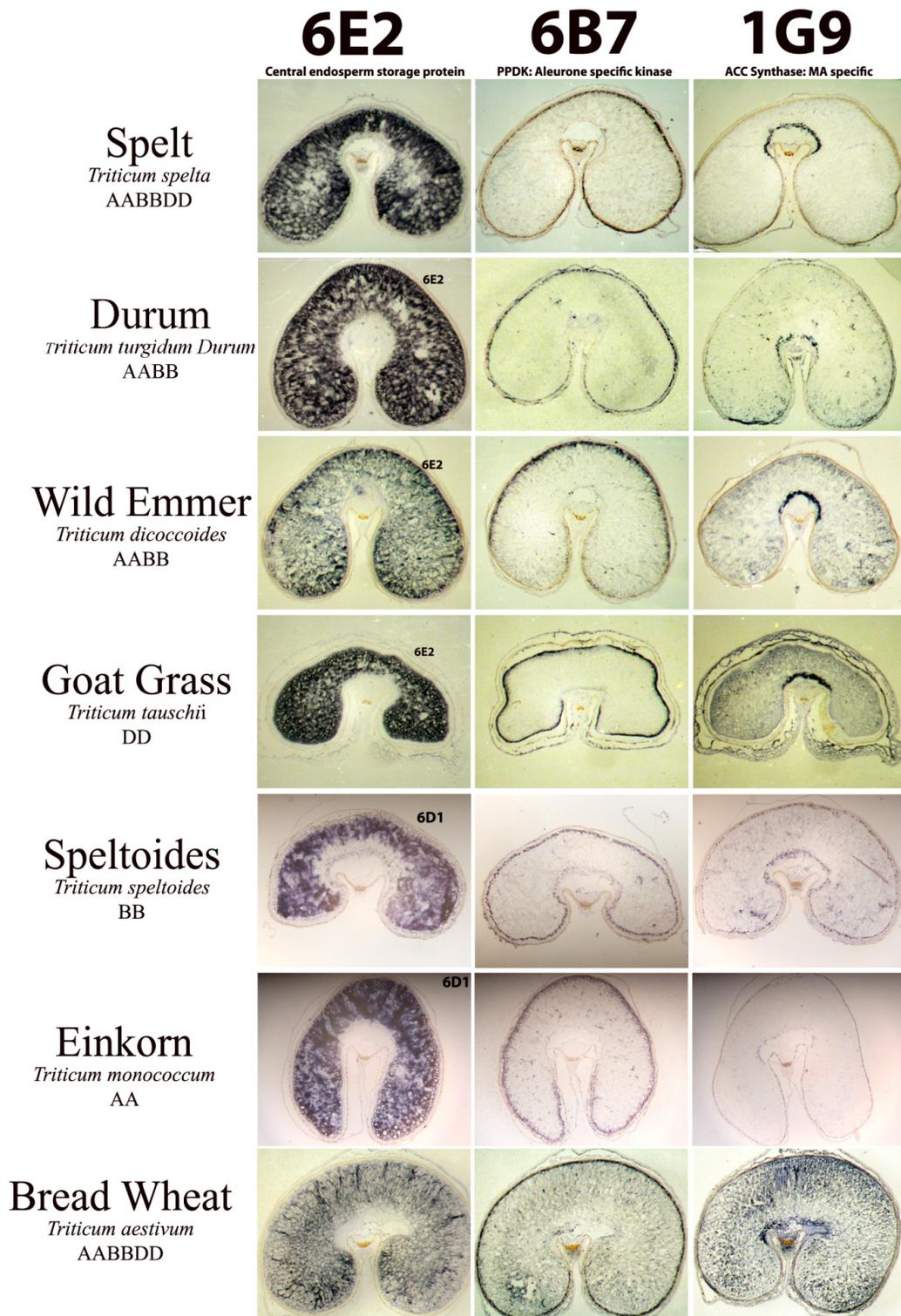
A4i: mRNA in situ hybridisation of endosperm domain specific markers on cultivated and primitive wheats.
Molecular mapping of endosperm domains across hexaploid, tetraploid and diploid cultivated wheat species and progenitors. Specific expression for each probe can be identified in al species but some variation can be seen in expression domains of the peripheral and modified aleurone markers in the diploid species.
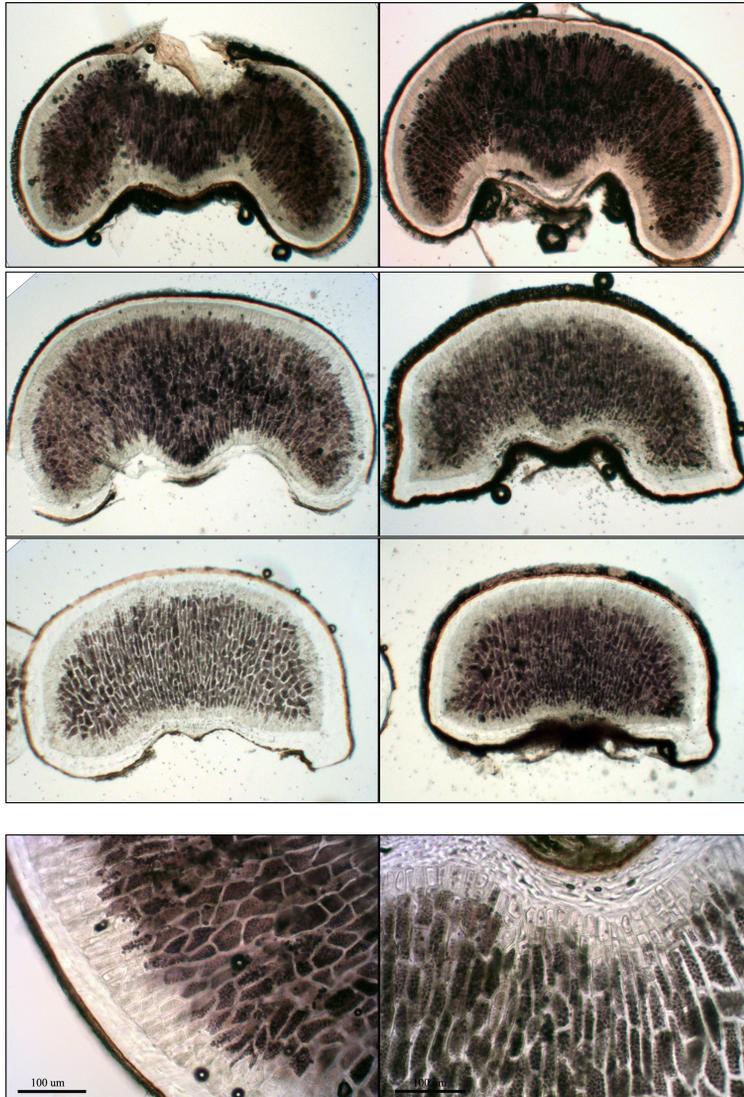
A4j: Lugols staining on serially sectioned mature *B. stacei* grain. Staining shows high starch leves along the grain length. No transverse gradient is visible. Lower two magnified images show starch exclusion from the peripheral aleurone layer where aleurone visible on the grain adaxial side is more discreet as compared to the abaxial layer, similar to what is seen in *B. distachyon.*

620 nm curve

y = 0.0779x
R² = 0.96437

Absorbance (y-axis, 0 to 2)
mg starch (x-axis, 0 to 25)

◆ 620 nm curve
— Linear (620 nm curve)
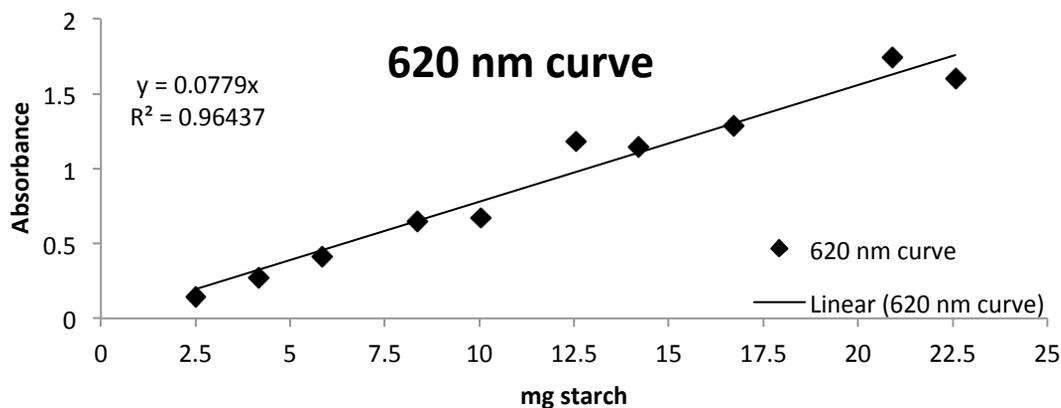
Blank lugols extract OD = 0.16 ; Abs at 620 nm

sample weight (mg)	%	mg starch	xtrct	plus lugols	minus control abs
0.003	3	83.33333333	2.5	0.003	**0.142**
0.005	5	83.6	4.18	0.007	**0.272**
0.007	7	83.57142857	5.85	0.004	**0.413**
0.01	10	83.6	8.36	0.017	**0.649**
0.012	12	83.58333333	10.03	0.002	**0.672**
0.015	15	83.6	12.54	0.011	**1.182**
0.017	17	83.58823529	14.21	0.001	**1.145**
0.02	20	83.6	16.72	0.003	**1.288**
0.025	25	83.6	20.9	0.004	**1.742**
0.027	27	83.59259259	22.57	0.009	**1.603**

| | 620 nm (Amylose) | | | | | | | | | | | | |
| | H2O abs | | | | Lugols abs | | | | | | | | |
	R1	R2	R3	mean	R1	R2	R3	mean	minus control	stdev	mg starch	% starch	% stdev	% SE
Spelt	0.025	0.033	0.023	0.03	1.234	1.198	1.269	1.23	**1.18**	0.04	15.16	**60.62**	3.0	1.7
Cadenza	0.029	0.012	0.024	0.02	1.15	1.18	1.242	1.19	**1.14**	0.05	14.67	**58.69**	4.1	2.4
Flour control	0.55	0.017	0.014	0.19	1.24	1.22	1.11	1.19	**0.97**	0.07	12.46	**49.82**	7.2	4.2
A. fatua	0.057	0.037	0.043	0.05	0.971	1.002	0.991	0.99	**0.92**	0.02	11.76	**47.05**	1.7	1.0
T. uratu	0.024	0.032	0.026	0.03	0.936	0.902	0.893	0.91	**0.86**	0.02	11.00	**44.01**	2.6	1.5
Oat (H)	0.03	0.028	0.035	0.03	0.855	0.883	0.823	0.85	**0.80**	0.03	10.23	**40.91**	3.8	2.2
T.speltoides	0.02	0.02	0.018	0.02	0.832	0.86	0.796	0.83	**0.78**	0.03	10.06	**40.26**	4.1	2.4
L. perenne	0.017	0.01	0.019	0.02	0.709	0.821	0.881	0.80	**0.76**	0.09	9.79	**39.14**	11.5	6.6
T. tauschii	0.047	0.019	0.048	0.04	0.826	0.729	0.593	0.72	**0.65**	0.12	8.37	**33.48**	18.0	10.4
H. vulgare	0.039	0.025	0.028	0.03	0.67	0.773	0.663	0.70	**0.65**	0.06	8.28	**33.14**	9.5	5.5
F. pratensis	0.027	0.012	0.016	0.02	0.685	0.675	0.646	0.67	**0.62**	0.02	8.01	**32.06**	3.2	1.9
T. dicoccoides	0.018	0.016	0.021	0.02	0.661	0.661	0.576	0.63	**0.59**	0.05	7.55	**30.21**	8.3	4.8
E. repens	0.02	0.012	0.036	0.02	0.635	0.707	0.459	0.60	**0.55**	0.13	7.08	**28.33**	23.1	13.4
A. stolonifera	0.027	0.022	0.01	0.02	0.554	0.562	0.495	0.54	**0.49**	0.04	6.31	**25.23**	7.4	4.3
D. caespitosa	0.045	0.02	0.073	0.05	0.535	0.519	0.582	0.55	**0.47**	0.03	6.08	**24.30**	6.9	4.0
D. glomerata	0.032	0.02	0.042	0.03	0.537	0.513	0.478	0.51	**0.45**	0.03	5.80	**23.21**	6.6	3.8
B. diandrus	0.016	0.006	0.01	0.01	0.362	0.189	0.172	0.24	**0.20**	0.11	2.62	**10.49**	51.5	29.7
B. sterilis	0.019	0.002	0.013	0.01	0.157	0.144	0.12	0.14	**0.10**	0.02	1.32	**5.29**	18.2	10.5
B. pinnatum	0.022	0.01	0.021	0.02	0.147	0.126	0.143	0.14	**0.10**	0.01	1.22	**4.88**	11.7	6.8
B. mollis	0.019	0.004	0.014	0.01	0.06	0.089	0.084	0.08	**0.04**	0.02	0.50	**2.02**	39.4	22.8
B. distachyon	0.012	0.01	0.015	0.01	0.048	0.037	0.048	0.06	**0.02**	0.01	0.22	**0.88**	37.0	21.4
Control	0.008	0.001	0.003	0.00	0.034	0.027	0.029	0.03	**0.00**	0.00	0.00		#DIV/0!	#DIV/0!

A4k: Calibration curve for grain starch assay and raw data for chart comprising figure 4.15.

Species	Mass (g)	LxWxD (mm)
Triticum turgidum	54	26.718
Triticum spelta	52	29.852
Triticum aestivum "cadenza"	52	26.439
Triticum dissocoides	39	25.935
Hordeum vulgare "optic"	33	29.530
Avena sativa	29	18.720
Triticum thaouder	20	14.819
Aegilops tauschii	16	14.217
Bromus diandrus	15	22.952
Triticum uratu	15	12.539
Avena fatua	11	15.809
Triticum speltoides	10	14.007
Hordeum murinum	9	14.114
Bromus sterilis	7	12.928
Brachypodium sylvaticum	6	8.137
Brachypodium distachyon	4	8.404
Aegilops ovata	4	16.163
Bromus mollis	3	8.045
Elymus repens	3	4.985
Brachypodium pinnatum	3.0	8.350
Lolium perenne	2.0	4.686
Festuca pratensis	2.0	4.143
Dactylis glomerata	0.8	2.550
Deschampsia caespitosa	0.5	1.607
Agrostis stolonifera	0.1	0.634

A4I: Raw data for chart comprising figure 4.16
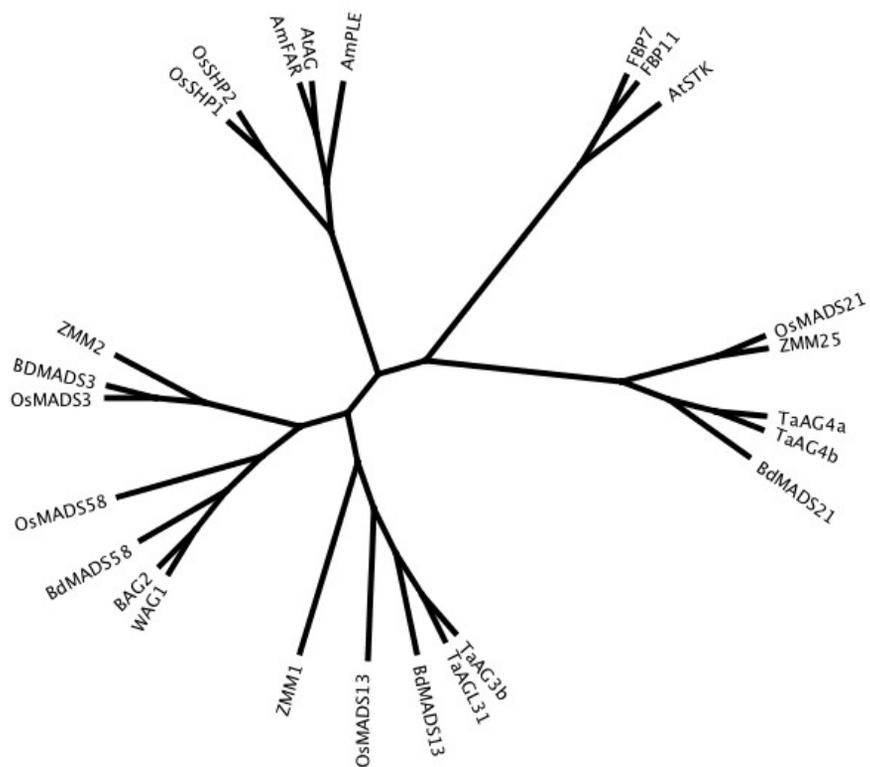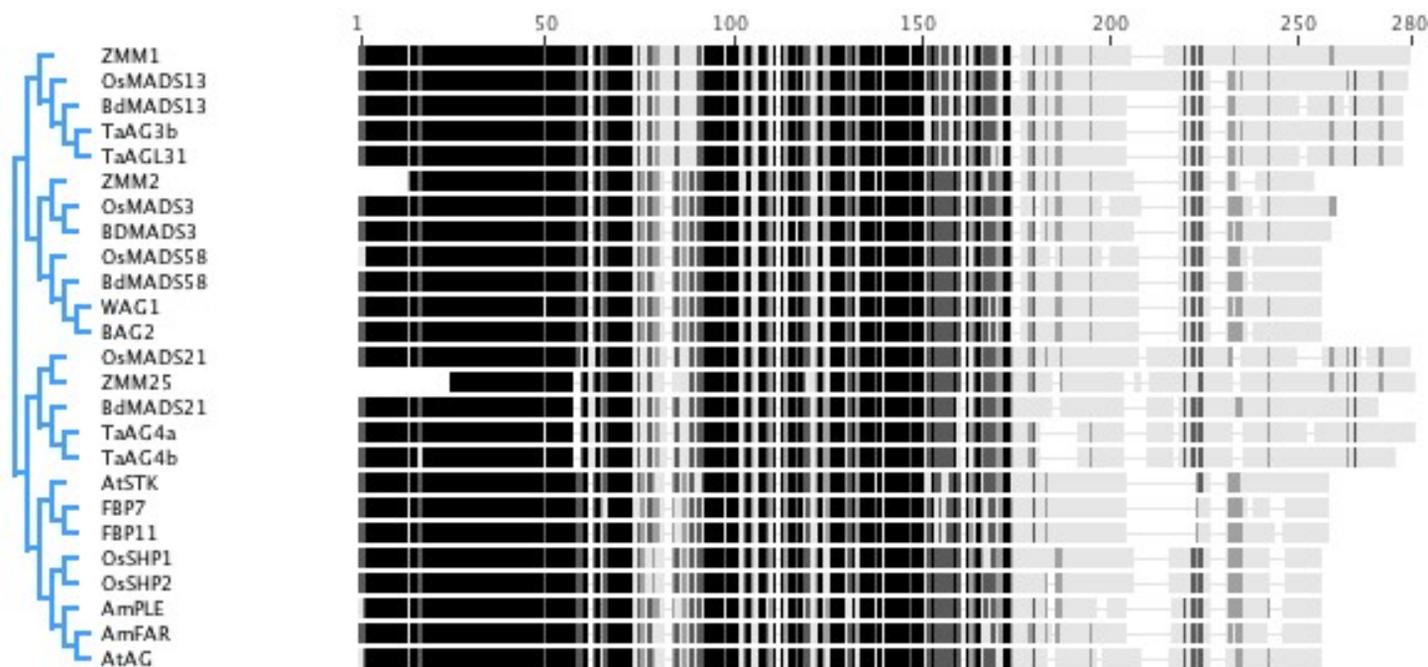
A5a: C & D class MADS-box transcription factors full sequence alignment (Block representation) and associated unrooted neighbor-joining tree

Gene ID	Accession	Gene ID	Accession
Opaque orthologues		Bradi3g33400	XP_003572075
HvBLZ1	CAA56374	TaMYB40 (speltoides)	AEV91163
TaSPA	Y09013	TaMYB41 (speltoides)	AEV91164
ZmO2	NM_001111951	TaMYB51 (speltoides)	AEV91174
OsREB	AAL10017	TaMYB53 (T. aestivum)	AEV91176
HvBLZ2	CAA71795	TaMYB54 (T. aestivum)	AEV91177
AtBZ2	CAC79658	TaMYB65 (T. aestivum)	AEV91188
RcCPRF2	XP_002531296	TaMYB69 (T. aestivum)	AEV91192.1
GmbZIP105	NP_001237113	TaMYB71 (T. aestivum)	ABK79908
BlSBZ1	NP_001059048	HvMCBS3	CAJ53899
Sb01g005170	XP_002463740	Bradi2g05590	XP_003564624
Sb02g004590	XP_002459432	Bradi3g17170	
GAMYB orthologues		Bradi2g42870	XP_003569313
AsMYB	CAB40189	Bradi2g23710	XP_003568340
Bradi2g53010	XP_003564452	Bradi3g33440	XP_003572080
ZmLOC100191731	NP_001130632	Bradi2g02810	XP_003568449
Sb03g037680	XP_002456516	Bradi3g14830	XP_003573373
Os01g0812000	NP_001044592	HvMCB1	AJ303354
TaMYB3	AAT37169	ZmMCB1	ACG33343
HvGAMYB	AY008692	Bradi2g13860	XP_003565794
LtGAMYB	AAD31395	Bradi2g55797	XP_003567296
RcMYB	XP_002525574	ScR1MYB	ACI12883
AtMYB33	NP_850779	Bradi5g26110	XP_003580818
SlGAMYB-like1	NP_001234357	Bradi2g55217	XP_003564630
MRP orthologues		Bradi3g51757	XP_003570106
Sb09g003390	XP_002440574	Bradi1g32330	XP_003563391
BarleyBAJ99046	BAJ99046	BarleyBAJ92648	BAJ92648
Bradi1g72300	XP_003558648	BarleyBAJ97156	BAJ97156
Bradi2g05760	XP_003564752	BarleyBAJ91904	BAJ91904
ZmMRP1	AJ318518	BarleyBAJ87437	BAJ87437
ZmMybst1	ACF85648	HvMYBSt1	AJ965496

A5b: Accession numbers for genes examined in chapter 5

Gene	Bd orthologue	Brachypodium Primers	
		Forward	Reverse
FUL1	Bradi1g08340	CTGGTGTCACGAATATAGGA	TTAGCCGCTGATGTGGCTCACCAT
FUL2	Bradi1g59250	ATGCTCAAGCTCAAGCCCAA	TCTTCGATACACCTCATCGA
MADS58	Bradi2g32910	AGCTGCAGAATGACAACTTG	TTCAGTAGCTTCAGTCTCAC
MADS3	Bradi2g06330	TCCCATCAGCTGCAGCCAACT	CTAGCGGTAGCACTTAGTAC
MADS13	Bradi4g40350	AGCTGCCAAGTTGCGCCATCA	GCTCAGGCGCAGGCATCGTT
MADS21	Bradi2g25090	GGAGATCGAGTACATGCAG	TCGTGCAGCTGCATGTTCAC
LHS	Bradi1g69890	GCTTGACCAGCTATTTGACC	CCAAGAGCTAGCAGTCTCAA (LHS1RB)
DL	Bradi1g6990	TTCCATCTGCTTACAATCGC	TGATCCCGGCATCATGAATG
FIL-1	Bradi5g16910	CGGCCATACAACCGATTTATC	AGCTAGAATGCACCTCAAAG
FIL-2	Bradi3g50050	ATCCGCGTACAACCGCTTCA	GACATTGGCAAAGAACTTG
FIL-3	Bradi3g30410	TCGGCATACAACCGCTTCAT	GAAGCTCGGCGATGATGTAG
GT1	Bradi1g71280	GCCGTGTGGT TCCAGAACCG	CATCATCCCTCCAGATGTAC
VRS	Bradi1g23460	GCCGTGTGGT TCCAGAACCG	TCTGCATGCAGTTGGTGTCC
ZmMRP-1	Bradi1g72300	TTGCACCATGTGCCAGAAG	AGCTCTACATCATGGATGC
BLZ1	Bradi1g05480	GAACTCCACACTGTTAAGGC	AGATGCTCCAGACTTGCCAC
BLZ2 /Opaque2/TaSPA	Bradi1g55450	ATAGCAGTGCTGTCATTGAC	TTGATTCAGACTGCATCTGC
GAMYB	Bradi2g53010	TGTCAGTACACCATGTGACAC	TGACAAGCATGTGGCATGTTG
ZmMybst1/ *HvMCBS3*	Bradi3g33400	ACTCAAGTAGCAAGTCATGC	ATTCTCTGGCATGATAGCAG
HvMCB1	Bradi3g33440	TCGTCAGAGGCAATAACATG	AAGCTGGATGGAGGTTGTGG
Cr4	Bradi1g29720	AGTGTGACCTCATCGCAGAG	ATAGGAACTACCAACACTGC
Dek1	Bradi3g53020	CATAAGCTCAAGCATGTCCC	TCGAGTTCACATAATCTGTC
Sal1	Bradi1g30430	AAGTCCATGGGCAACATCGT	TCGTCGACCTTCTCCTTGTC
HMG	Bradi3g09690	AAGGCTCCCT ATGAGGAGGT	GTGTGAGCGTTGGTGTATTCC
ACTIN	Bradi4g41850	AAGCTGCTGGAATCCATGAGAC	GACCAGACTCATCATACTCACC
GAPDH	Bradi3g14120	ACATCATTCCTAGCAGCACTGG	TGACGAAATGGTCGTTCAGAGC

A5c: *Brachypodium* primer sequences for chapter 5.

A5d: *TaMADS13* expression in the outer integument pre-endosperm cellularisation wheat grain.

Primer sequences	
AgEx3F	GTGAAGGCAACCATTGAGAG
AgEx3R	CTCTCAATGGTTGCCTTCAC
AgInt3R	TCAAGTGAAGCTAGCTAGC
AgInt3Rb	ACCTCAAAGTGCAGTTATGC
BdMADS58F	AGCTGCAGAATGACAACTTG
BdMADS58R	TTCAGTAGCTTCAGTCTCAC
AgEx1F	ACTCAAAGAGCAGCTGGCTAC
TDNA1	CTGATAGTGACCTTAGGCGA
Seq2	CGATGATAAGCTGTCAAA
Seq1	GGTCATAACGTGACTCCCTTA
TDNA4	CGGCCGCATGCATAAGCTTA
TDNA2	GGCTGAGTGGCTCCTTCAA
Table A6a: Primer sequences used for Genotyping and RT-PCR in mutant analyses	

Gene annotation	identifier	Class	Sequence similarity	Homology (Protein)	BLASTP E-value	Function & expected expression pattern (in the original species)	reference
BdHMG	Bradi3g09690	HMG – cell cycle	HMG (At4G23800)	47%	4e-77	Nuclear protein associated transcriptional regulator. *Meristematic regions, in a cell cycle (m-phase) specific manner*	Derbyshire et al, 2008
BdFUL1	Bradi1g08340	MIKC type MADS box A-class. AP1/FUL-like	OsMADS14 AAf19047	87%	1e-129	Spikelet meristem identity and general floral organ identity. *Immature spikelets and all floral organs*	Preston & Kellogg, 2007
BdFUL2	Bradi1g59250	MIKC type MADS box A-class. AP1/FUL-like	OsMADS15 AAF19048	76%	1e-125	Spikelet meristem and outer floral whorl identity. *Immature spikelets and outer whorl floral organs.*	Preston & Kellogg, 2007
BdGT1	Bradi1g71280.1	class I HD leucine Zip	ZmGT1	76%	2e-40	Floral morphology determinant, specifically controlling lateral branching. *Leaf primordia and early developing leaves of lateral and apical meristems.*	Whipple et al, 2011
BdVRS	Bradi1g23460	HD leucine Zip	HvVRS1	67%	4e-83	Floral morphology determinant, specifically regulating 2 or 6 rowed flower phenotype. lateral spikelet primordial of immature spikes	Komatsuda et al, 2006
BdLHS1	BRADI1G69890	MIKC type MADS box E-class	OsLHS1 (OsMADS1)	75%	2e-99	Spikelet meristem determinacy and outer whorl floral organ identity. *Floral primordia and palea, lemma and pistil of mature flowers.*	Malcomber & Kellogg, 2004
BdFIL-1	Bradi5g16910	YABBY	OsYAB3 (**Os04g0536300**)	76%	e-102	Meristem function, leaf and floral organ development. *Floret meristems, leaf and floral organ primordia, becoming restricted to stamens and carpel. Excluded from SAM and non-polar in floral organs*	Dai et al, 2007; Jang et al, 2004
BdFIL-2	Bradi3g50050	YABBY	OsYAB7 (Os02g0643200)	79%	7e-99		Dai et al, 2007; Vosnakis et al, 2011
BdFIL-3	Bradi3g30410	YABBY	OsYAB4 (Os10g0508300)	55%			Dai et al, 2007; Vosnakis et al, 2011
BdMADS58	Bradi2g32910	MIKC type MADS box C-Class	OsMADS58 (Agamous)	76%	e-111	Carpel identity and floral meristem determinacy. *Early floral development*	Yamaguchi et al, 2006
BdMADS3	**Bradi2g06330**	MIKC type MADS box C-Class	OsMADS3 (Agamous)	88%	e-104	Stamen development and floral identity. *Early floral development*	Yamaguchi et al, 2006
BdDL	**Bradi1g69900**	YABBY	OsDL/AtCRC	88%	1e-98	Carpel specification. *Flower meristem and Carpel primordia*	Yamaguchi et al, 2004
BdMADS13	Bradi4g40350	MIKC type MADS box D-Class	OsMADS13 (AGL11)	75%	4e-98	Ovule identity. *Developing ovules and early seed development*	Lopez-Dee et al, 1999; Dreni et al, 2007
BdMADS21	Bradi2g25090	MIKC type MADS box D-class	OsMADS21 (AGL11)	65%	2e-82	No obvious function. *Flower development (Ovules, carpels and stamens) and early seed development.*	Lee et al, 2003: Dreni et al 2011
BdMRP1	Bradi1g72300 (Closest hit)	R1MYB	ZmMRP-1	37%	7e-28	Transfer cell specific transcriptional activator. *Early grain/endosperm development*	Gomez et al., 2002; 2009
BdBLZ2	Bradi1g55450	bZIP	HvBLZ2 TaSPA/ZmO2	41%		Transcriptional regulation of endosperm specific genes e.g. storage proteins. *Predominantly mid development endosperm.*	Onate et al., 1999
BdBLZ1	Bradi1g05480	bZIP	HvBLZ1	75%		Common pathway transcriptional regulator. *Developing endosperm, also roots and leaves.*	Vicente-Carbajosa et al., 1998
BdMCB1	Bradi3g33440	R1MYB	HvMCB1	59%	3e-95	Transcriptional repressor/activator. *Developing endosperm and germinating aleurone.*	Rubio-Somoza et al., 2006b
BdGAMYB	Bradi2g53010	MYB	HvGAMYB	89%	0.0	Aleurone specific Transcriptional repressor/activator. *Developing and germinating aleurone cells.*	Diaz et al., 2002
BdMCB3	Bradi3g33400	R1MYB	HvMYBS3/ ZmMybst	95%	5e-137	Aleurone specific Transcriptional repressor/activator. *Developing and germinating aleurone cells.*	Mercy et al., 2003; Rubio-Somoza et al., 2006a

A5e: Combined chapter 5 genes and orthologues table

T0 parent plant	spiklets	~florets	~grains	
315-1	5	25	17	
315-2	--	--	--	
315-3*	10	40	30	
315-4*	8	29	26	
315-5	4	21	15	
315-6	2	9	7	
315-7	6	24	19	
315-8*	7	32	24	
315-9	5	23	17	
315-10*	6	30	25	
315-11*	12	47	31	
315-12*	6	32	28	
315-13*	13	75	50	
315-14*	8	45	35	
315-15	5	27	23	
315-16	4	17	14	
315-17*	7	32	27	
315-18*	4	21	20	
315-19	7	50	35	
* indicates lines grown on for analysis				

Table A6c: Observations for initial AA315 line plants

Wild Type plants			
	Spikelet no	~Floret no	Grains
WT1	7	55	59
WT2	8	27	31
WT3	16	65	48
WT4	17	83	88
WT5	9	60	21

Table A6b: Floral and grain production values for 1st generation Wild Type control plants

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