Control of the size and composition of the embryo in cereals

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I. Abstract

The embryo is an important source of lipid, protein, minerals and vitamins in the cereal caryopsis, and modification of embryo size and/or composition has the potential to improve the nutritional value of the grain. In this study, two approaches were taken to investigate the control of embryo size and composition.

To investigate whether lesions affecting grain development also have effects on the embryo, a collection of shrunken-grain mutants of barley were studied. Embryo weight was found to be unaffected or to be reduced concomitantly with grain weight, with the exception of mutants with lesions at the *Lys3* locus. Four *lys3* mutants were shown to have greater embryo weight (as previously described in *lys3* mutant Risø1508 [Tallberg, 1977]) and *lys3* embryos contained greater amounts of protein and micronutrients. An attempt was made to identify the gene underlying the *Lys3* locus by characterising the mutant phenotype. The rice *giant embryo* (*ge*) mutant was identified as having a similar phenotype to *lys3*. The barley orthologue of the rice *Ge* gene was located on chromosome 2H and it therefore cannot underlie *Lys3* on 5H. The possibility that *Lys3* and *Ge* act in the same metabolic pathway is discussed.

The role of starch in the embryo was investigated by the identification and characterisation of an ADP-glucose pyrophosphorylase large-subunit mutant of rice (*apl3-2*), which had a 35 percent reduction in starch in the embryo. There was no discernable difference in embryo size or lipid content in the mature embryo. As such, there is no evidence that the quantity of starch accumulated during development is important for determining final embryo composition or for normal embryo development. The *apl3-2* mutant was shown to have greatly reduced starch in the culms which adds to our understanding of the function of this gene.

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IV. Abbreviations

ABA	abscisic acid
ADP	adenosine 5'-diphosphate
AGPase	ADP-glucose pyrophosphorylase
Arabidopsis	*A. thaliana*
ATP	adenosine 5'-triphosphate
BAC	bacterial artificial chromosome
bc	uncalibrated ^{14}C years
BLAST	basic local alignment search tool
bp	base pair
bpm	beats per minute
Brachypodium	*B. distachyon*
BSA	bovine serum albumin
cDNA	complementary DNA
CER	controlled environment room
cM	centiMorgans
cv	cultivar
DAF	days after flowering
d.f.	degrees of freedom
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
DW	dry weight
EDTA	ethylenediaminetetra acetic acid
EST	expressed sequence tag
EMS	ethyl methane-sulphonate
FW	fresh weight
FPLC	fast protein liquid chromatography
GFP	green fluorescent protein
GLM	general linear model
h	hours
HEPES	N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulphonic acid)
IHO	Illinois high oil
ILO	Illinois low oil
JIC	John Innes Centre
kb	kilobase

kD	kilodalton
L	litre
LSU	large subunit
min	minutes
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
QTL	quantitative trait locus
RNA	ribonucleic acid
RSE	relative standard error
RT-PCR	reverse transcription PCR
s	seconds
SDS	sodium dodecyl sulphate
SE	standard error
SSU	small subunit
TILLING	targeting induced local lesions in genomes
UV	ultra-violet
v	version

1. General introduction

Cereals are the single most important source of food for human nutrition, contributing on average 47 percent of dietary energy (FAO, 2009). Cereals are annual grasses within the family Poaceae and the grain or kernel forms the primary component of the harvest, for food, feed and a variety of other end-uses. The grain or kernel is composed of the seed with the pericarp fused to the outside of the seed forming a fruit, known as a caryopsis (Figure 1.1). The seed comprises the endosperm, the embryo, the nucellus and the testa (seedcoat). The major grain/kernel component, by weight, is the starch-rich endosperm. Although the embryo is of minor importance by weight (Table 1.1), it contributes significantly to the nutritional value of the grain (Poey *et al.*, 1979; Gabert *et al.*, 1995; Zhang *et al.*, 2005). Starch, a carbohydrate, is the major constituent of the grain, the vast majority of which is in the endosperm. In contrast, the mature embryo contains little starch but is a rich source of lipid, protein and micronutrients. As such, understanding the control of embryo size and composition has the potential to allow the manipulation of cereal grain composition for a variety of end uses including improving nutritional value.

Table 1.1 – Typical constituents of the mature cereal caryopsis by percentage of total grain weight.

Cereal	Pericarp and testa	Endosperm	Embryo
Wheat	8.5	82	2.8
Maize	6.0	77.8	13.5
Rye	10.0	86.5	3.8
Sorghum	7.9	82.3	9.8
Rice	6.0	90.9	3.1
Barley (hulled)	2.9	87.0	3.0
Oats	12.0	84.0	2.8

Modified from Black *et al.* (2006).

The aim of the work described in this thesis was to understand the control of embryo size and composition as a means of altering end-use quality. Two approaches were taken: firstly, a collection of shrunken-grain barley mutants was interrogated for clues as to whether decreased starch in the endosperm (of which shrunken grain is a typical consequence) leads to alterations in carbohydrate metabolism, size and/or composition in the embryo. One such barley line (Risø1508), containing a lesion in an unknown gene, has previously been described as a large-embryo mutant (Tallberg, 1977). This work set out to determine whether other shrunken barley mutants had similar or different effects on embryo size and whether reduced starch in the endosperm was associated with altered embryo composition in the mutants. This work also sought to provide information about the genes underlying the mutant loci in large-embryo lines, especially Risø 1508, to

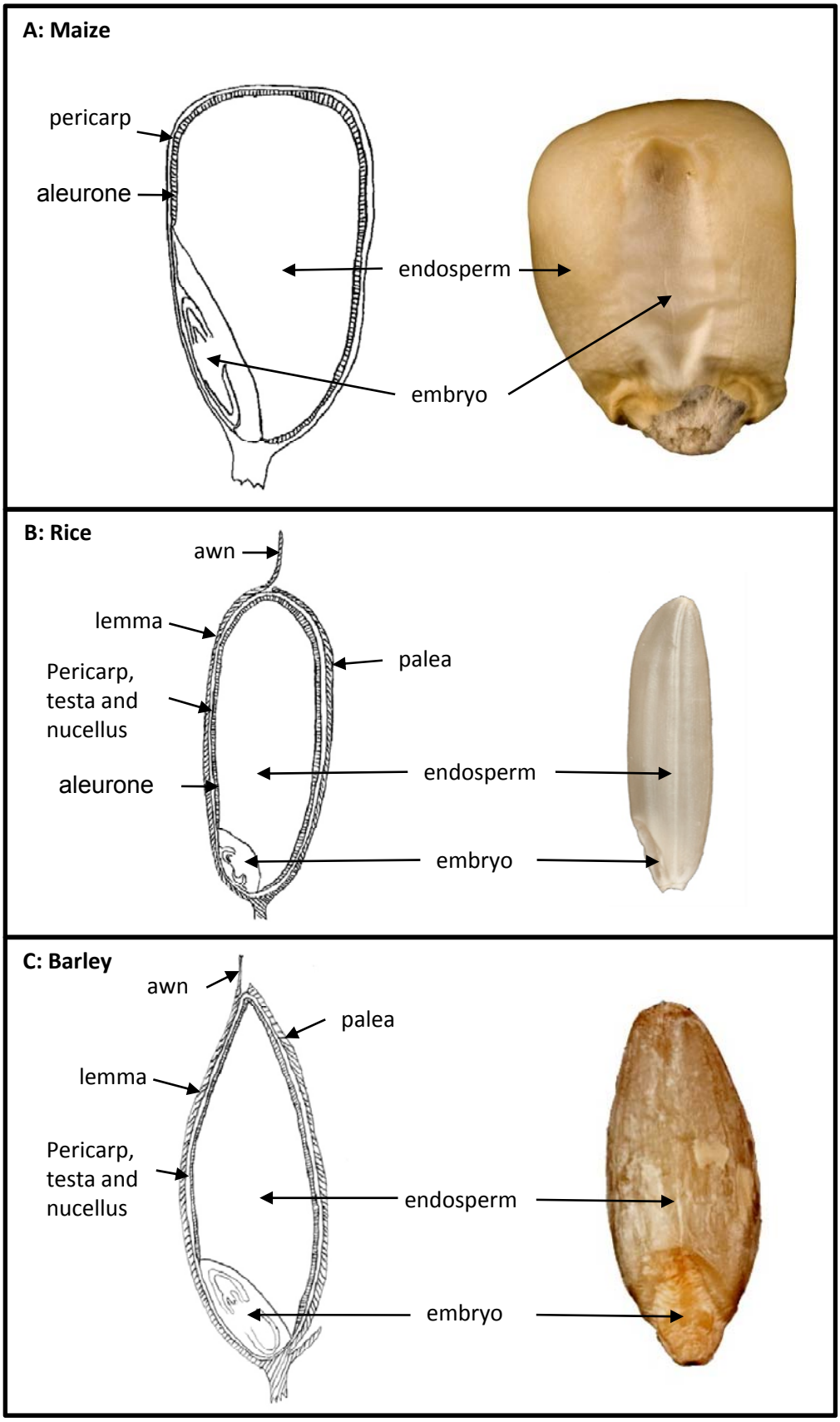

Figure 1.1 – Caryopses of maize (A), rice (B) and barley (C).
Not to scale.

underpin identification of the genetic determinants of embryo size. To do this, homologues were sought in barley of genes known to condition large embryo size in other species of cereals. The second approach taken to understanding the control of embryo size and composition in cereals was to investigate the role of embryo starch content. This approach involved the identification and characterisation of starch biosynthetic mutants, defective in embryo starch synthesis. This work also allowed the elucidation of the relative importance of different starch biosynthetic enzymes in grain development and composition as a whole.

The following introduction provides background information on the importance of the embryo in determining end-use quality of the grain and describes current understanding of the formation, nutrition and developmental control of size and composition of the embryo in cereals.

1.1. The importance of cereals

As stated previously, cereals contribute on average 47 percent of dietary energy (FAO, 2009). This figure does not include the use of cereals either for fodder to feed livestock for dairy and meat products or for brewing or other non-food end-uses. Cereals are grown in a wide variety of environments and for a wide variety of end uses. In 2007, cereals were grown on an estimated 700,000 km^2 of land worldwide, producing 2.4 billion tonnes of grain (FAO, 2009). The major cereals, by global area harvested, are wheat, rice, maize and barley. In addition to these widely grown species, there are many other cereals of regional importance including sorghum, millets and oats (Table 1.2) (FAO, 2009). In the developed world, cereals generally contribute less than 47 percent of dietary energy by direct consumption whereas in the developing world, cereals can directly contribute as much as 80 percent (e.g. rice in the average Bangladeshi diet; FAO, 2009). In many regions of the world, a single cereal species is the primary staple food. Therefore, cereals are both a major source of nutrition around the world, particularly in the developing world, and of economic importance for regional and international trade.

Cereals have a long history of domestication and are thought to have played a crucial role in the transition of early humans from hunter-gathers to agriculturalists. The modern cereal caryopsis is the product of a long history of domestication beginning with barley and wheat in the Pre-Pottery Neolithic (7900-7500 bc) in the Near East (Zohary and Hopf, 2000). Rice and maize were domesticated entirely independently and in different climatic regions to barley and wheat. Rice is thought to have been first domesticated from around 6400-5800 bc in the Yangtze Valley in China (Zohary and Hopf, 2000) and maize is

thought to have been domesticated from around 6700 bc in the Balsas Valley in Mexico (Piperno *et al.*, 2009).

Table 1.2 – The major cereals by subfamily (Family Poaceae).

Subfamily	Tribe	Cereal	Domestication centre
Chloridoideae	Eragrostideae	Tef (*Eragrostis tef*)	Ethiopia
		Finger millet (*Eleusine coracana*)	East Africa
Panicoideae	Andropogoneae	Maize (*Zea mays*)	South Mexico
		Sorghum (*Sorghum bicolor*)	West Africa
		Job's tears (*Coix lacryma-jobi*)	East Asia
	Paniceae	Foxtail millet (*Setaria italica*)	Central Asia
		Proso millet (*Panicum milieaceum*)	East Asia
		Pearl millet (*Pennisetum glaucum*)	West Africa
		Fonio (*Digitaria* spp.)	West Africa
Ehrartoideae	Oryzeae	Rice (*Oryza sativa*)	South China
		African rice (*Oryza glaberrima*)	West Africa
Pooideae	Aveneae	Oats (*Avena sativa*)	Near East
	Triticeae	Wheat (*Triticum* spp.)	Near East
		Barley (*Hordeum vulgare*)	Near East
		Rye (*Secale cereale*)	Near East
		Triticale (x *Triticosecale*)	N/A

Modified from Black *et al.* (2006) and Glemin and Bataillon (2009).

Cereals are derived from just four subfamilies within the Poaceae (Table 1.2) and these subfamilies fall into two clades; the PACCMAD clade, which contains domesticated millets, maize and sorghum and the BEP clade which contains domesticated rice, wheat, barley and oats (Figure 1.2) (GPWG, 2001; Bouchenak-Khelladi *et al.*, 2008). Domesticated cereals produce seeds in which starch-rich endosperm is the dominant tissue, as do the majority of grass species (Glemin and Bataillion, 2009). In modern cereal breeding, high yield (grain weight per harvest area) and resistance to pests and disease are the primary traits under selection. Quality traits are generally of secondary importance, with a few exceptions such as bread-making quality in breeding programmes for bread wheat (*T. aestivum*). Grain size is a central determinant of yield (along with grain number and planting density) and is largely determined by endosperm size. During early domestication, ancient humans are thought to have inadvertently selected for large grain size due to large grains being more likely to germinate successfully as they tolerate deeper burial and produce seedlings with increased vigour (Harlan *et al.*, 1973; Purugganan and Fuller, 2009). Domesticated cereal varieties such as wheat, barley, maize and rice produce larger grains than their respective progenitor species suggesting that larger grain size is a domestication-associated trait. Harlan *et al.* (1973) attributed the increase in grain size to the endosperm alone. However, to my knowledge, no

investigation of embryo size in domesticated varieties compared to wild progenitors has been carried out. Therefore, the increase in grain weight may be attributable to the endosperm alone or there may have been a concomitant increase in embryo size. However, if historical increases in grain size are attributable to the endosperm alone, this represents a decrease of the proportion of the grain contributed by the embryo and therefore a reduction in the contribution of the embryo to the nutritional value of the grain as a whole.

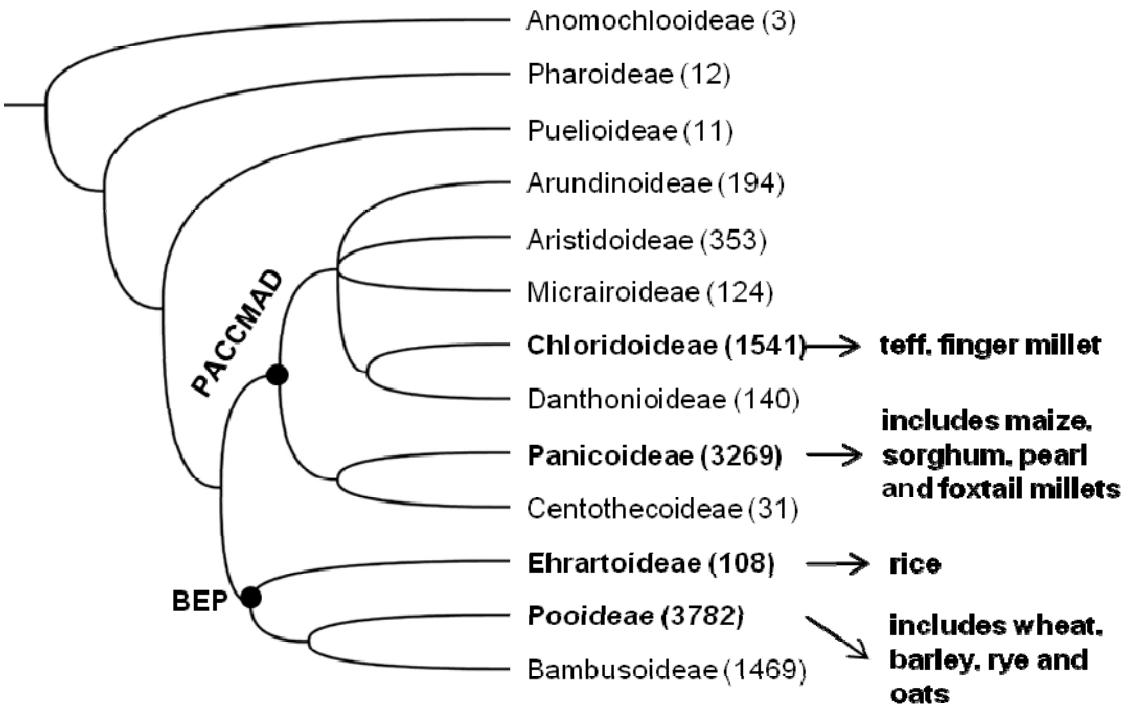

Figure 1.2 – Phylogeny of the subfamilies within the grasses (*Poaceae*) after Bouchenak-Khelladi *et al.,* (2008).

Diagram was modified from Glemin and Bataillon (2009). BEP: Bambusoideae, Ehrartoideae, Pooideae; PACCMAD: Panicoideae, Aristidoideae, Centothecoideae, Chloridoideae, Micrairoideae, Arundinoideae, Danthonioideae. The number of species in each subfamily, given in brackets, is taken from Glemin and Bataillon (2009).

Although the majority of grass species produce seed in which the endosperm forms the largest component, there are exceptions. For example, three distinct genera within the Bambusoideae (sister group to the Pooideae; Figure 1.2) produce bacoid, fleshy or berry-like, fruit (*Melocanna*, *Dinochloa* and *Melocalamus*) (Yang *et al.*, 2008). The seed comprises a large, starch-rich scutellum and a reduced endosperm, and is coated in a fleshy pericarp (Rudall and Dransfield, 1989). The embryo size is comparable to that seen in cereals, with *Dinochloa* species producing caryopses between 7 mm and 30 mm in length (depending on species) (Rudall and Dransfield, 1989). In the bacoid fruits of

Ochlandra and *Dinochloa* species, the endosperm expands and accumulates starch early in seed development, as is typical of seeds in other grasses (Rudall and Dransfield, 1989). However, later in development, the endosperm becomes compressed, the starch is degraded and the scutellum expands (Rudall and Dransfield, 1989). This suggests that mechanisms for producing radically altered caryopses exist within the Poaceae.

1.2. Formation of the cereal caryopsis

1.2.1. Fertilisation

Seed development in angiosperms is initiated by the double fertilisation of the embryo sac. During fertilisation, the pollen tube delivers two nuclei to the embryo sac. The first nucleus fuses with the egg cell to form the diploid zygote and the second nucleus fuses with the two nuclei of the central cell to form the triploid endosperm. The zygote develops into the embryo, which becomes the vegetative plant, and the endosperm develops into a starch-rich tissue providing resources for the developing embryo and/or post-germinative growth (Sabelli and Larkins, 2009). The developing seed is surrounded by the nucellus, testa and pericarp, all of maternal origin, which become fused to the exterior of the seed forming a specialised fruit structure known as a caryopsis (Black *et al.*, 2006). Fertilisation of the egg cell and the central cell occur simultaneously in barley (Pope, 1937), wheat (Bennett *et al.*, 1975) and maize (Kiesselbach, 1949) as well as a number of other cereals (Sabelli and Larkins, 2009). Following fertilisation, the embryo and endosperm undergo cell division, differentiation and growth.

1.2.2. Embryo development

Post-fertilisation embryogenesis can be divided into two developmental phases, morphogenesis and maturation (Ohto *et al.*, 2007; De Smet *et al.*, 2010). During the morphogenesis phase, the zygote divides and differentiates to establish the basic embryo form (Figure 1.3). This also includes the establishment of the shoot and root meristems. In cereals, the embryo form is radically different from that of the relatively well-studied dicotyledonous plants *Arabidopsis* and *Vicia faba*. In these dicotyledonous model species, the bulk of the mature seed is mainly comprised of embryonic tissue and the endosperm is highly reduced. The embryo in these species is composed of the root-shoot axis and two cotyledons. In *Arabidopsis* and other oilseeds, oil in the cotyledons is the main storage compound for post-germinative growth. In *Vicia* spp. and other legumes, starch and proteins in the cotyledons are the main storage compounds. In cereals, the root-shoot axis is embedded within the scutellum. The scutellum is considered to be an enlarged single cotyledon and forms a shield-like structure in contact with the endosperm

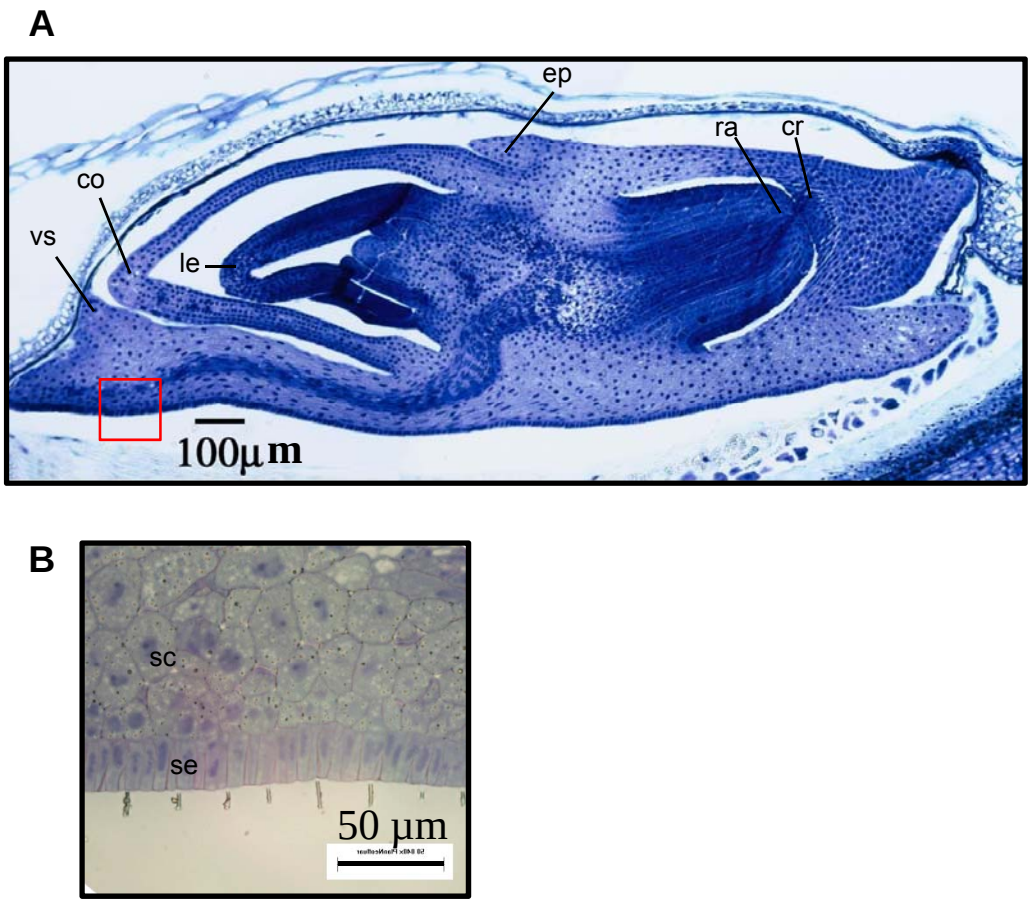

Figure 1.3 – A wheat embryo in longitudinal cross-section (A) at 26 days after flowering and a region of the scutellum (B).

The region shown in B is a region of the barley embryo corresponding to the region enclosed in the red box in A. Features are labelled as follows: vs – ventral scale, co – coleoptile, le – leaf primordia, ep – epiblast (not present in all cereal embryos), ra – radicle, cr – coleorhiza, sc – scutellum and se – scutellar epithelium. Both images were stained with Toluidine Blue. Image A is courtesy of: 'WHEAT:THE BIG PICTURE', www.wheatbp.net.

(Nardmann and Werr, 2009). The scutellar epithelium (Figure 1.3B) has been implicated in the uptake of nutrients during embryogenesis (Matthys-Rochon *et al.*, 1998) and the secretion of phytohormones and enzymes during germination (Briggs, 1972; Negbi, 1984).

During the maturation phase, the embryo generally accumulates storage products. In cereals, these are mainly lipids and proteins with relatively little starch (Black *et al.*, 2006; Neuberger *et al.*, 2008). Also associated with embryo maturation is the accumulation of non-storage proteins known as late-embryogenesis-abundant (LEA) proteins, thought to be required for primary dormancy and tolerance of desiccation. These have been identified in a variety of species, including wheat, barley and rice (Dure *et al.*, 1989). The final phase the embryo enters is the quiescent or dormant state, in which the seed can remain for many years until conditions are favourable for germination.

The control of embryogenesis is not well understood and is likely to require the concerted action of a large number of genes including those involved in the cell cycle, cellular metabolism and cell to cell communication. In *Arabidopsis*, genetic screens have identified a large number of genes involved in embryogenesis (Meinke, 1991; Tzafrir *et al.*, 2004). Current estimates suggest that between 1 and 2 percent of the genes in the *Arabidopsis* genome are required for embryogenesis (De Smet *et al.*, 2010). In cereals, a number of embryo-defective mutants have been described in rice (Hong *et al.*, 1995a) and maize (Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980; Clark and Sheridan, 1991). These are discussed later in this chapter.

1.2.3. Endosperm development

Endosperm formation in cereals (as in angiosperms generally) is characterised by four phases: (1) syncytial, (2) cellularisation, (3) differentiation and (4) maturation (Bosnes *et al.*, 1992). The syncytial phase is characterised by nuclear divisions in the absence of cell divisions (Lopes and Larkins, 1993; Olsen, 2004; Sabelli and Larkins, 2009). These nuclei align around the periphery of the vacuolated central cell. Cellularisation occurs moving from the periphery towards the centre of the cell. During differentiation, specialised domains of the endosperm become apparent. At the chalazal end of the endosperm is the specialised basal transfer layer. This region is located adjacent to maternal vasculature and is characterised by wall protuberances and labyrinthine structures (Sabelli and Larkins, 2009). This region is considered to be the main conduit for assimilates entering the endosperm. At the micropylar end of the endosperm is the embryo surrounding region (ESR). This region is characterised in maize by small cells with dense cytoplasm. The expression of a number of genes is limited to this region (Opsahl-Ferstad *et al.*, 1997; Bonello *et al.*, 2000; Bate *et al.*, 2004). The function of this region is not known but roles in

protection and nutrition of the embryo have been proposed (Cossegal *et al.*, 2007). The central or starchy endosperm forms the majority of the endosperm in cereals and is the main location for starch and protein deposition during grain filling. Around the periphery of the starchy endosperm is the aleurone, typically a single cell layer in cereals although there is some variation between species and cultivars (Black *et al.*, 2006). The aleurone plays a role in regulation of dormancy and germination (Jacobsen *et al.*, 1995; Gubler *et al.*, 2005). The final phase in endosperm development is maturation, during which the storage products are accumulated, mainly in the form of starch (Lopes and Larkins, 1993; Sabelli and Larkins, 2009).

Traditionally, the endosperm is assigned a role in embryo nutrition during both development (see Section 1.2.4) and germination (Maheshwari, 1950; Lopes and Larkins, 1993; Olsen, 2004). In cereals, the endosperm contains the bulk of the carbon for post-germinative growth and its role in the nutrition of the germinating embryo is well established. During germination, enzymes in the endosperm and those released by the aleurone, break down cell walls, starch and lipid to supply the embryo with metabolites for growth (Nonogaki *et al.*, 2007). In non-endospermic seeds, the endosperm does not contain the bulk of carbon for post-germinative growth. However, there is evidence that it is physiologically important for dormancy and germination (Penfield *et al.*, 2006). In *Arabidopsis*, the endosperm forms a thin cell layer surrounding the embryo, it is rich in triacylglycerols and has been shown to be required for normal post-germinative growth (Penfield *et al.*, 2004).

1.2.4. Assimilate supply to the developing embryo

The developing seed must obtain carbon, nitrogen and mineral ions from the maternal tissues to sustain its development. In cereals, the grain is a powerful sink for photoassimilates and nitrogen. Carbon fixed in leaves or stored in other vegetative structures is mobilised in the form of sucrose to supply the developing seed (Schnyder, 1993). Sucrose is unloaded from the phloem into the apoplast between the maternal and filial tissues (Weber *et al.*, 1997; Sabelli and Larkins, 2009). Cell wall invertases, localised in the apoplast, cleave the sucrose into hexose thus maintaining a sucrose gradient and high osmotic potential in the developing caryopsis (Hirose *et al.*, 2002). Sugars in the apoplast are taken up by the endosperm by sucrose and hexose transporters localised predominantly in the basal endosperm transfer layer (Aoki *et al.*, 1999; Weschke *et al.*, 2000; Aoki *et al.*, 2002; Chourey *et al.*, 2006). The activities of cell wall invertases have been shown to exert a high degree of control over the flux of carbon to the developing grain in maize (Miller and Chourey, 1992), barley (Weschke *et al.*, 2003), rice (Hirose *et*

al., 2002) and *Vicia faba* (Weber *et al.*, 1996a). For example, large-seeded genotypes of *V. faba* have longer periods of high invertase activity in the seed coat than small seeded varieties (Weber *et al.*, 1996a). In maize *miniature1* mutants (which carry a lesion in an endosperm-specific cell-wall invertase gene), there is a dramatic decrease in invertase activity in the developing grain and final kernel weight is reduced to 20 to 30 percent of normal weight (Miller and Chourey, 1992; Cheng and Chourey, 1999). A shift from hexose sugars to sucrose in the cotyledons of *V.faba* has been found to correlate with the induction of storage product accumulation (Weber *et al.*, 1997).

There is some information in cereals to indicate the route by which photoassimilates subsequently reach the embryo, however the story may not be complete. Indeed, there may be more than a single route and routes may vary or alter in their relative importance at different stages of development.

Early in development, the nutrition of the embryo is likely to be based on the structures already in place for the development of the embryo sac prior to fertilisation. The embryo sac is enveloped by the nucellus which degenerates soon after fertilisation in wheat (Morrison *et al.*, 1978), barley (Norstog, 1974) and maize (Russell, 1979). Nucellar wall projections, possibly specialised for nutrient transfer, are visible at the micropylar end of the embryo sac in cotton (Schulz and Jensen, 1977), soybean (Chamberlin *et al.*, 1993) and *Arabidopsis* (Mansfield and Briarty, 1991). ^{14}C-labelled photoassimilates are concentrated in this micropylar region of the soybean embryo sac (Chamberlin *et al.*, 1993). This suggests that sucrose moves to both the chalazal endosperm region (via the well-documented basal endosperm transfer layer) and to the micropylar region at the base of the embryo/zygote.

As previously stated, the endosperm has traditionally been assigned a nutritive role during embryo development (Maheshwari, 1950; Olsen, 2004). Evidence for this in barley has largely come from the existence of a distinct embryo surrounding region and a starch-depleted ‘crushed layer’, both adjacent to the embryo (Smart and O'Brien, 1983; MacGregor and Dushnicky, 1989). Support for a role for the endosperm in embryo nutrition has more recently come from studies of maize. Mattys-Rochon *et al.* (1998) and Griffith *et al.* (1987) both found that embryos in culture *in vitro* performed best when the scutellum was in contact with the medium and that sucrose, glucose and maltose were readily taken up. Rolletschek *et al.* (2005) found that radio-labelled sucrose microinjected into endosperm was taken up by the embryo and incorporated into lipids. This work demonstrates that the embryo is capable of importing sugars from the developing endosperm.

It may be the case that embryo and endosperm import sugar independently during early development and there may be a temporal or developmental shift to the embryo utilising nutrients from the endosperm. *In vitro* culturing of cereal embryos invariably leads to altered embryo development and, provided the embryo has reached the requisite stage in development, precocious germination (Kent and Brink, 1947; Cameron-Mills and Duffus, 1977; Matthys-Rochon *et al.*, 1998). This demonstrates that endosperm and embryo interact during development and that the endosperm plays an important role in the suppression of germination.

1.3. Grain composition

The three main storage components of the cereal grain are carbohydrates (mostly in the form of starch), proteins and lipids (Table 1.3). These compounds are not distributed evenly throughout the grain (Tables 1.4 and 1.5)

Table 1.3 – Typical composition of cereal grains.

Crop	Starch (%)	Protein (%)	Lipid (%)
Maize[1,4]	73.5	9	4.3
Rice[2,5]	77.2	8.3-9.6	2.1-3.3
Barley[1,6]	63-65	9.5-11.5	2-3
Wheat[3,7]	58.5	12	1.8
Oats[1,8]	-	13.8-22.5	9.6

[1]dry weight basis, [2]14 percent moisture basis, [3]15 percent moisture basis, [4](Earle *et al.*, 1946 cited in Glover and Mertz, 1987), [5](Juliano, 1972, 1980 cited in Coffman and Juliano, 1987), [6](Harris, 1962 cited in Foster and Prentice, 1987), [7](Inglett, 1974 cited in Johnson and Mattern, 1987), [8](Youngs, 1972 and Youngs *et al.*, 1977 cited in Youngs and Forsberg 1987).

Table 1.4 – Composition of parts of the wheat grain (by percentage of weight).

	Protein	Lipid	Starch
Whole grain	12.0	1.8	58.5
Aleurone	24.0	8.0	0.0
Outer endosperm	16.0	2.2	62.7
Inner endosperm	7.9	1.6	71.7
Embryo	26.0	10.0	0.0

Determined at 15 percent moisture content. Modified from Inglett (1974).

Table 1.5 – Composition of parts of the maize kernel (by percentage of weight).

	Protein	Lipid	Starch
Whole kernel	9.0	4.3	73.5
Pericarp	3.6	1.0	7.2
Embryo	18.3	33.5	8.0

Modified from Earle *et al.* (1946).

Grain composition is determined by both carbon and nitrogen metabolism, both of which are subject to environmental and genetic variations. Protein content is frequently

expressed as concentration (percentage of dry weight) and is therefore a function of both the total nitrogen made available to the developing grain (as nitrogen is limiting) and the total dry weight of the grain. There is a well established inverse relationship between yield and protein concentration in the grain of cereals (Bhatia and Rabson, 1987; Simmonds, 1995; Shewry, 2007). Two hypotheses for this relationship have been suggested: that there is competition between carbon and nitrogen metabolism for energy (Munier-Jolain and Salon, 2005) or there is dilution of nitrogen by other carbon-based compounds (e.g. starch) (Acreche and Slafer, 2009). The first hypothesis suggests that mutations affecting starch metabolism in the grain will lead to the accumulation of more protein. There is some evidence that this might be the case. For example, antisense-mediated suppression of the synthesis of the major storage protein C-hordein in barley resulted in four out of five transgenic lines showing increases in mean grain weight compared to the control lines (Lange *et al.*, 2007). This suggests that flux into starch may be increased in these lines. However, reductions in starch do not necessarily lead to absolute increases in the protein content of the grain. For example, barley line Risø86 which contains a lesion in the nucleotide sugar transporter *Nst1* (required for normal starch biosynthesis) has reduced starch per endosperm (Patron *et al.*, 2004) but there is no increase in the total amount of protein per grain (Doll *et al.*, 1974). If plants are grown under nitrogen-limiting conditions they will be unable to utilise any additional energy made available from reduced starch biosynthesis for protein synthesis.

Although less well studied, there is also evidence of a negative correlation between grain starch and grain lipid in oats and maize. In the Illinois Long-Term Selection Experiment, maize kernel oil content was found to be negatively correlated with grain starch (Moose *et al.*, 2004) and high oil genotypes of oats were found to accumulate less starch than low oil genotypes (Banaś *et al.*, 2007). In oats, where most of the oil is stored in the endosperm (Youngs and Forsberg, 1987), high oil genotypes appear to partition radio-labelled carbon into lipids, specifically triacylglycerols, at the expense of partitioning into non-lipids such as starch (Ekman *et al.*, 2008). However, in maize, where most of the lipid is in the embryo, there is also a positive correlation between lipid content and embryo size (Hopkins *et al.*, 1903; Moose *et al.*, 2004). As such, the high oil content of Illinois High Oil maize is likely to be largely due to a larger embryo (Hopkins *et al.*, 1903) rather than due a trade-off between starch and lipid accumulation.

Control of flux into starch, protein and lipid is under complex genetic and environmental regulation. The production of each storage compound has different requirements for energy and for precursors and cofactors (Schwender, 2008). As well as genetic factors,

oxygen and ATP concentrations in the developing grain have been implicated in controlling the balance of flux into these different pathways (Emes *et al.*, 2003; Rolletschek *et al.*, 2004; Rolletschek *et al.*, 2005). The nature and biosynthesis of the three main storage compounds are described below.

1.3.1. Starch

Starch is the major storage product in the grain (Table 1.3) and is composed of glucose chains linked by linear α-(1→4) glucosidic bonds and branched with α-(1→6) glucosidic bonds. Cereal starches are typically made up of around 25 percent amylose, comprising linear glucans, and around 75 percent amylopectin, containing a backbone of linear glucans with non-random branch-points comprising around 5 percent of the glucosidic bonds (Hannah and James, 2008). These polymers form insoluble starch granules with complex ultrastructures.

In non-photosynthetic starch-storing organs, such as the cereal grain, starch is synthesised from imported sugars. Imported sucrose is catabolised in the cytosol to hexose phosphate for starch biosynthesis. Sucrose is also used for respiration to provide ATP for anabolic processes. Sucrose is catabolised to glucose 6-phosphate either via the action of sucrose synthase as shown in Figure 1.4 or via the action of invertase to produce glucose and fructose which can be phosphorylated to glucose 6-phosphate and fructose 6-phosphate by hexokinase. Fructose 6-phosphate can be converted to glucose 6-phosphate by the action of glucose 6-phosphate isomerase. Glucose 6-phosphate and ATP are transported across the plastid envelop via specific translocators (Neuhaus and Wagner, 2000). Within the plastid, glucose 6-phosphate is converted to glucose 1-phospate by the action of phosphoglucomutase.

The first committed step in starch biosynthesis is the formation of the activated hexose ADP-glucose from glucose 1-phosphate in a reaction catalysed by ADP-glucose pyrophosphorylase (AGPase) (Figure 1.4). AGPase is a heterotetrameric enzyme composed of two large subunits (LSU) and two small subunits (SSU). In plants, AGPase is encoded by multiple genes and activity is plastidial in all species and tissues examined with the exception of the endosperm in cereals. In addition to plastidial isoforms of AGPase, there is a cytosolic AGPase isoform present in the endosperm of maize (Denyer *et al.*, 1996), barley (Thorbjornsen *et al.*, 1996a), rice (Sikka *et al.*, 2001), wheat (Tetlow *et al.*, 2003b) and *Brachypodium* (Haleux *et al.*, in preparation) (Figure 1.4). In fact, in these species cytosolic AGPase activity accounts for most of the AGPase activity in the endosperm, for example, 70 percent of the activity in wheat (Tetlow *et al.*, 2003b) and 95

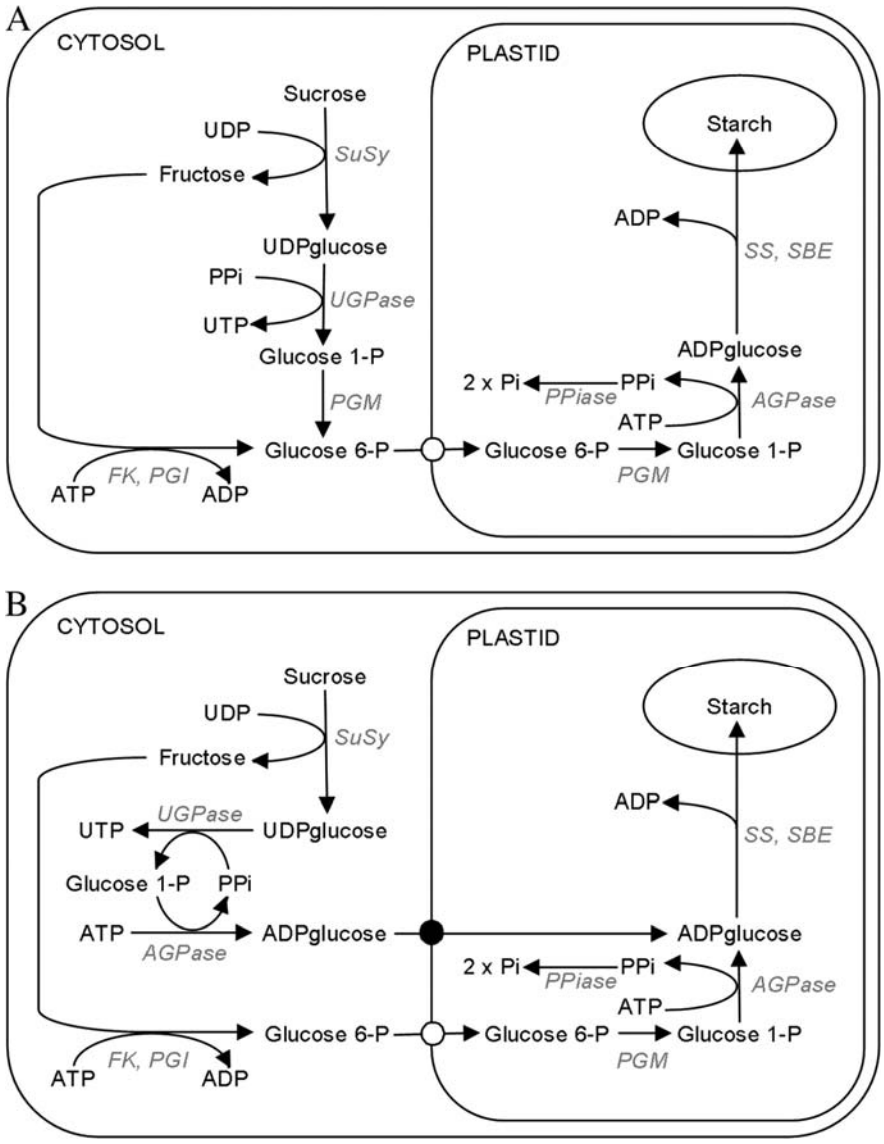

Figure 1.4 – The pathways of starch biosynthesis in a typical starch storing tissue (A) and in a cereal endosperm cell (B) are shown.

The cytosolic and plastidial compartments are indicated. Abbreviations for enzymes (italic, grey) are SuSy, sucrose synthase; UGPase, UDPglucose pyrophosphorylase; PGM, phosphoglucomutase; FK, fructokinase; PGI, phosphoglucose isomerase; PPiase, pyrophosphatase; AGPase, ADPglucose pyrophosphorylase; SS, starch synthase; SBE, starch-branching enzyme. The transporters in the plastidial inner membrane are shown as circles: black circle, ADPglucose/ADP transporter; white circle, glucose 6-phosphate/phosphate transporter. Other abbreviations are PPi, pyrophosphate, Pi, phosphate.

Reproduced from Comparot-Moss and Denyer (2009) with permission.

percent in maize (Denyer *et al.*, 1996). The number of genes encoding AGPase subunits in cereals and their subcellular localisation is examined in greater detail in Chapter 5.

As a consequence of the cytosolic production of ADP-glucose in the cereal endosperm, ADP-glucose must be transported across the plastid envelope for starch synthesis. This transport is carried out by a nucleotide sugar transporter in exchange for ADP (Möhlmann *et al.*, 1997; Tetlow *et al.*, 2003a; Bowsher *et al.*, 2007). Lesions in the gene encoding the nucleotide sugar transporter of barley and maize cause reduced starch content and ADP-glucose accumulation in the grain or kernel (Shannon *et al.*, 1998; Patron *et al.*, 2004). The presence of cytosolic AGPase activity has been described only in the endosperm of species in the Poaceae (Beckles *et al.*, 2001; Comparot-Moss and Denyer, 2009). There is no evidence of cytosolic AGPase activity in the embryos of cereals. In barley, the cytosolic AGPase small subunit protein (encoded by *Agps1*) is apparently absent from the embryo (Rösti *et al.*, 2006).

Plastidial ADP-glucose is the substrate for glucan polymer elongation. The glucosyl moiety of ADP-glucose is added to the non-reducing end of pre-existing α-glucan chains by starch synthases to form the linear α-(1-4)-glucan polymers. Cereals have multiple and distinct isoforms of starch synthase and these show preferences for elongating glucan chains of differing structures and lengths (Jeon *et al.*, 2010; Tetlow, 2011). The glucan polymers are modified by starch-branching enzymes and starch debranching enzymes. Starch-branching enzymes catalyse the cleavage of internal α-(1→4) glucosidic bonds and transfer the released glucan chain to an α-(1→6) linkage, thereby introducing a branch point. Starch de-branching enzymes cleave α-(1→6) thereby removing branch points. Normal starch biosynthesis in cereals requires the concerted action of starch synthases, starch-branching enzymes and starch de-branching enzymes (Jeon *et al.*, 2010; Tetlow, 2011).

1.3.2. Protein

Cereals contain a variety of proteins and their distribution through the grain is not uniform (Tables 1.4 and 1.5). Traditionally, cereal proteins have been characterised based on their solubility (Osborne, 1924). Osborne fractions are based on the sequential extraction of plant proteins in water (albumins), dilute saline (globulins), alcohol/water mixtures (prolamins) and dilute alkali or acid solutions (glutelins). If the function of the protein is known, they can be classified according to function rather than solubility. Cereal proteins can be classified into three broad functional groups: structural and metabolic proteins, defensive proteins, and storage proteins (Shewry and Morell, 2001; Shewry and Halford, 2002). Structural and metabolic proteins include, for example, enzymes for routine cell

functions and structural proteins in cell walls and membranes. Defensive proteins function to protect the seed, in various ways, from pests and pathogens. Examples of defensive proteins include inhibitors of hydrolytic enzymes (such as protease inhibitors), endochitinases and ribosome-inactivating proteins (Shewry, 1999). Storage proteins are defined as those whose major function is to supply nitrogen, carbon and sulphur for growth at a later date, for example during germination (Shewry and Morell, 2001).

Storage proteins, the majority of which are in the endosperm, account for the bulk of the proteins in the cereal caryopsis (Table 1.6). In the Triticeae and the Panicoideae, prolamins are the major storage proteins accounting for as much as 60 percent of total grain protein whereas in Oryzeae and Aveneae, the major storage proteins are globulins (Shewry and Morell, 2001).

Table 1.6 – Storage proteins of cereals grains.

Storage tissue	Cereal	Major storage protein group
Starchy endosperm	Wheat (*T. aestivum*)	Prolamin (gliadin and glutelin)
	Barley (*H. vulgare*)	Prolamin (hordein)
	Maize (*Z. mays*)	Prolamin (zein)
	Oat (*A. sativa*)	11S globulin and prolamin (avenin)
	Rice (*O. sativa*)	11S globulin and prolamin (oryzenin)
Embryo/aleurone	Wheat (*T. aestivum*)	7S globulin
	Barley (*H. vulgare*)	7S globulin
	Oat (*A. sativa*)	7S globulin
	Rice (*O. sativa*)	7S globulin
	Maize (*Z. mays*)	7S globulin

Modified from Shewry, (2000).

Prolamins are a diverse super family of proteins characterised by multiple domains differing in structure and in the presence of repetitive amino acid sequences (Shewry and Halford, 2002). Prolamins contain a high proportion of proline and glutamate residues but are relatively poor in the essential amino acids lysine and threonine. Whilst oats and rice contain prolamin storage proteins, these are quantitatively less abundant in the endosperm than the globulin fraction which accounts for 70 to 80 percent of total grain nitrogen (Shewry and Morell, 2001; Shewry and Halford, 2002). The 11S and 7S globulins share homology with dicotyledonous seed storage proteins whereas prolamins are cereal-specific. Globulins are the predominant storage proteins in the protein-rich embryo (Table 1.6).

Nitrogen is supplied to the developing grain in the form of amino acids. Commonly transported amino acids include glutamine, asparagine, serine, alanine and glycine (Tabe *et al.*, 2002). These then undergo extensive metabolism in the developing seed to form

other amino acids (Macnicol, 1977). Amino acids are synthesised from a range of intermediates of central carbon metabolism. Many are formed from intermediates in the tricarboxylic acid (TCA) cycle (Slocum, 2005; Azevedo *et al.*, 2006). Storage proteins are synthesised on the rough endoplasmic reticulum and co-translationally transported into the lumen of the endoplasmic reticulum. These proteins are then either stored in endoplasmic reticulum-derived vesicles or they pass via the Golgi apparatus into protein storage vacuoles (Shewry and Halford, 2002; Kawakatsu and Takaiwa, 2010).

The quantity of storage protein accumulated in the grain is dependent on both genetic and environmental factors. More specifically, grain protein composition is determined by both the supply of nitrogen to the grain and the ability of the grain to assimilate the nitrogen into protein (Simmonds, 1995; Bogard *et al.*, 2010; Seebauer *et al.*, 2010). The application of nitrogen-containing fertiliser will increase yield, percentage protein composition and total protein yield, up to a point (Bhatia and Rabson, 1987; Simmonds, 1995).

1.3.3. Lipid

As is the case with protein, cereals do not generally contain high levels of lipids (Table 1.3) and their distribution through the grain is not uniform (Tables 1.4 and 1.5). Cereals contain a range of lipids, the most abundant being neutral lipids (60 to 90 percent), glycolipids (2 to 20 percent) and phospholipids (6 to 20 percent) (Price and Parsons, 1975). The neutral lipids are mostly triacylglycerols which are derived from glycerol 3-phosphate and fatty acyl-CoA (fatty acids with coenzyme A in the place of the terminal carboxyl group). Much of current knowledge of lipid biosynthesis comes from work in *Brassica napus* embryos, for a recent review see Baud and Lepiniec (2010). Fatty acid biosynthesis is plastidial and the first committed step is considered to be the conversion of acetyl-CoA to malonyl-CoA by the action of acetyl-CoA carboxylase. Triacylglycerols are assembled in the endoplasmic reticulum and stored in membrane-bound oil bodies in the cytosol. By far the most abundant fatty acid in cereals is the essential long-chain polyunsaturated fatty acid linoleic acid (18:2) (Price and Parsons, 1975). Other polyunsaturated fatty acids found in cereals include the essential α-linolenic acid (18:3) and oleic acid (18:1), and also some saturated fatty acids including palmitic acid (16:0) and stearic acid (18:0) (Price and Parsons, 1975).

In all cereals, the embryo is rich in lipid (Tables 1.4 and 1.5). However, there is variation between cereal species in the contribution of embryo lipid to total grain/kernel lipid. For example, in maize, 85 percent of the total kernel lipid is located in the embryo (Glover and Mertz, 1987) whereas in wheat and barley, only around 20 percent of the total grain lipid is located in the embryo (Tables 1.1 and 1.5) (Price and Parsons, 1979).

1.3.4. Minerals and vitamins

The cereal grain contains a variety of minerals and vitamins and many of these are concentrated in the embryo (Liu *et al.*, 1974; Stewart *et al.*, 1988; Black *et al.*, 2006). Mineral content of the grain is controlled by the availability of minerals in the soil or from foliar applications and the effective uptake and redistribution of minerals to the developing grain (White and Broadley, 2009; Cakmak *et al.*, 2010; Kutman *et al.*, 2010). The embryo contributes between 3.2 and 18.5 percent of the total iron and between 10.6 and 26.9 percent of the zinc in wheat (Lyons *et al.*, 2005). In addition to being rich in iron and zinc, the embryos of barley and wheat are also rich in a range of other micronutrients including phosphorous, potassium, magnesium and calcium (Liu *et al.*, 1974; Lyons *et al.*, 2005). Commercial germ preparations, from a range of cereals, have shown that the embryo is also a rich source of thiamine (vitamin B1) and riboflavin (vitamin B2) (Barnes, 1982).

Taken together, the embryo is an important source of protein, oil, vitamins and minerals. The following section describes how manipulation of the size and/or composition of the embryo has the potential to improve the nutritional value of cereals.

1.4. Cereals for food and feed

Where cereals are the primary staple in the diet, the carbohydrates provide the bulk of the dietary calories. Calories alone are not sufficient for a healthy diet. In addition, a healthy diet must also contain several essential amino acids, some fatty acids and micronutrients in the form of dietary minerals and vitamins (White and Broadley, 2009; Deckelbaum and Calder, 2010; Hawkesworth *et al.*, 2010). Undernutrition is a significant global health burden and is the result of an inadequate diet or due to disease leading to the excessive loss or inability to absorb nutrients in the gut (WHO/NHD, 2006). The causes of undernutrition are many and complex but certain forms of undernutrition are caused by an overdependence on a few, nutrient-poor staple foods (WHO/NHD, 2006). For example, a diet rich in cereals may not provide sufficient protein, lipid, or micronutrients for optimal health. Sub-optimal diet can be addressed by providing dietary supplements, fortifying existing foods with nutrients (especially micronutrients) during processing, promoting dietary diversity, or biofortification. Fortification of foods with micronutrients (e.g. commercially-available breakfast cereals) is commonplace in the developed world. Supplementation programmes, usually involving pharmaceutical-grade doses requiring medical administration, are effective, particularly in cases of acute malnutrition but require repeat dosages. A diverse diet is widely regarded to be the preferred approach to a balanced diet (The Royal Society, 2009). However, biofortification has the potential to be a cost-effective measure for controlling undernutrition in areas where dietary diversity is

unavailable or unaffordable (Stein *et al.*, 2008; Meenakshi *et al.*, 2010). Biofortification, plant breeding to produce more nutritious crop varieties, aims to improve health by modifying the intrinsic nutritional value of staple foods (POST, 2010). Although undernutrition is most prevalent in the developing world, it is a global health problem also affecting developed counties. As such, increasing the nutritional value of staple foods is a worthwhile goal.

The nutritional value of cereals varies between and within the different cereal species and is also influenced by growing conditions and post-harvest storage, processing and cooking methods. As previously stated, the mature cereal caryopsis (the primary harvestable component) is composed of the endosperm, the embryo (sometimes referred to as the germ), and the bran (nucellus, testa and pericarp) (Figure 1.1). The grain or kernel is typically processed in some manner post-harvest prior to use as food, feed or other end-use. The post-harvest processing varies widely depending on the species and/or end-use. The manner of post-harvest processing also dictates whether the embryo portion of the grain is maintained in the final food/feed product. Where cereals are consumed directly as food, they are generally processed by some form of threshing and/or milling prior to cooking, although there are exceptions (e.g. sweet corn can be eaten directly off the cob after cooking) (Johnson and Mattern, 1987; Baik and Ullrich, 2008; Nuss and Tanumihardjo, 2010). For example, although the vast majority of rice grown each year is destined for direct consumption as food, it is most often milled (to remove the pericarp and embryo by abrasion) to produce white rice for reasons of cultural preference and to enable long-term storage. The degree of milling dictates the quantity of pericarp, endosperm and embryo removed in rice, but typically 6 to 10 percent of total grain weight is removed (Coffman and Juliano, 1987). Thus, although rice can be consumed as whole-grain brown rice, often most of the embryo and bran is removed and therefore the nutritional value is somewhat diminished. That said, where whole grains are consumed, whether as food or feed, the nutritional value of the embryo is important.

1.4.1. Starch

As previously stated, starch is the largest component of the grain (Table 1.3) and provides the majority of the calories. Much research has been carried out on manipulating starch quantity and quality in cereals, for a recent review see Tetlow (2006). As the embryo does not contribute a significant amount of starch to the mature grain (Tables 1.4 and 1.5), improving the quality and/or quantity of starch in the grain is beyond the scope of this thesis.

1.4.2. Protein

Protein is required for normal growth and development. Protein deficiencies exist when there is adequate protein quantity and/or protein of inadequate quality in the diet and leads to stunting and wasting (WHO/NHD, 2006). Different cereals contain different amounts of protein (Table 1.3) of differing quality from a nutritional perspective. Protein nutritional quality is governed by the amino acid composition, especially amounts of essential amino acids, and the protein digestibility. As cereals are a major source of protein, particularly in diets where animal protein sources are scarce, there has been considerable effort to improve the nutritional value of cereal proteins (Nelson, 1969; Munck *et al.*, 1970; Doll and Welch, 1984; Kumamaru *et al.*, 1988; Prasanna *et al.*, 2001; Shewry, 2007). Similarly, where cereals are used as animal feed, increasing the amount and quality of the protein in cereals has also been a target for breeders (Munck *et al.*, 1970; Gabert *et al.*, 1996; Hastad *et al.*, 2005).

Increasing embryo size has the potential to increase the amounts of non-prolamin proteins in the grains of the Triticeae and maize and to increase the diversity of proteins in diets of those who rely on these species for dietary protein. Globulin storage proteins are predominant in the embryo (Table 1.6) and these have a better balance of amino acids, from a nutritional perspective, than prolamins (Shewry, 2007). Therefore, the embryo is not only a protein-rich tissue (Tables 1.4 and 1.5) contributing to total protein, but embryo proteins also provide protein diversity.

Altering protein quality in maize, although not via an increase in embryo size, has been shown to have a positive impact on health. Quality protein maize (QPM) was bred at CIMMYT using the high-lysine *opaque2* mutant of maize (Prasanna *et al.*, 2001; Gibbon and Larkins, 2005). QPM maize kernels contain higher levels of essential amino acids lysine and tryptophan than ordinary maize although there is no difference in total protein content. Meta-analysis of community-based intervention studies in undernourished communities in several countries around the world found that QPM increased growth weight by 12 percent and height by 9 percent in children in these communities (Gunaratna *et al.*, 2010).

1.4.3. Lipid

Lipids are required as part of a healthy diet, however not all are of equal nutritional value. In human and animal nutrition, it is the long-chain polyunsaturated fatty acids, linoleic (18:2) and α-linolenic (18:3) acid, which are essential (Deckelbaum and Calder, 2010). In the past, breeding for increased lipid content of cereals has mainly been in an effort to

increase the energy density of cereals for animal feed purposes (Hopkins, 1899; Welch, 1978; Dudley and Lambert, 1992). The embryo is a lipid-rich tissue (Tables 1.4 and 1.5) such that the Illinois Long-Term Selection Experiment for increasing maize kernel oil content inadvertently selected for a large embryo (Hopkins *et al.*, 1903; Lambert *et al.*, 1997; Moose *et al.*, 2004). Illinois High Oil maize varieties have around 20 percent of kernel weight as lipid.

It should be noted that the embryo and aleurone are the tissues with the highest concentrations of oil and it is these tissues which are often removed during processing. These tissues, once removed, can be used for feed or for oil production. Corn germ oil and rice bran/germ oil are currently available as industrial/commercial cooking oils. It has also been suggested that a high-lipid cereal crop (e.g. 25 percent lipid in wheat) could be an economically-viable alternative to oil seed rape (*Brassica napus* L.) in northern Europe (Ekman *et al.*, 2008). However, to my knowledge, such a variety will not be available in the foreseeable future.

1.4.4. Minerals and vitamins

Micronutrient malnutrition has been identified as a major form of undernutrition leading to a range of negative health outcomes from reduced life expectancy to reduced cognitive ability (WHO/NHD, 2006). Biofortification of maize, wheat and rice with zinc and iron is being explored by the HarvestPlus initiative (www.harvestplus.org). Feeding trials of an iron-rich rice variety, bred by the International Rice Research Institute, found that the degree to which the grain was milled was crucial for ensuring iron reached the food on the plate (Haas *et al.*, 2005). This suggests that the embryo is also an important source of iron in rice.

1.5. Control of the size of the embryo and endosperm

Control of organ size is complex, involving intricate coordination and feedback between the cell cycle, differentiation and cell growth (Figure 1.5). To complicate matters further, a large number of factors (genetic, physiological and environmental) have been shown to influence these dependencies and feedbacks. However, ultimately organ size is determined by cell number and cell size.

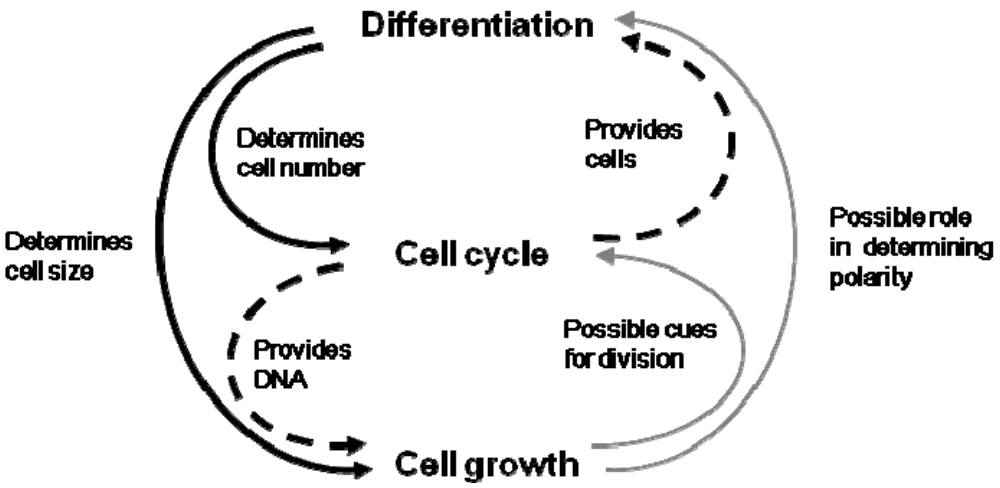

Figure 1.5 – Relationship and feedbacks between differentiation, cell cycle and cell growth.

Solid lines represent established relationships, dashed lines represent loose relationships and grey lines possible relationships. Modified from Harashima and Schnittger (2010).

1.5.1. Endosperm size

Grain size in cereals (a component of yield) is largely determined by the size of the endosperm. Consequently, this aspect of grain development has attracted most research attention. Current understanding of the control of endosperm size and composition is summarised here. Endosperm development is known to be under strong maternal control, both pre- and post-fertilisation (Gambin *et al.*, 2006; Sadras and Denison, 2009). Pre-fertilisation conditions, such as temperature, affect grain size in wheat, barley and triticale (Calderini *et al.*, 1999b; Ugarte *et al.*, 2007). This may be mediated, at least in part, by maternal control of carpel size (Calderini *et al.*, 1999a). Post-anthesis there is maternal control of resource allocation to the developing grain as well as control of other aspects of grain development. A strong determinant of endosperm size is the number of cells. Endosperm cell number is largely determined during the syncytial endosperm development phase (Brocklehurst, 1977; Cochrane and Duffus, 1983). Once grain filling begins, both rate and, to a lesser extent, duration of grain filling determines final endosperm size (Egli, 2006; Sadras and Egli, 2008). Thus, endosperm size is determined by both genetic potential (which is under strong maternal control) and the supply of photoassimilates during grain filling.

1.5.2. Embryo size

As discussed in the previous sections, controlling embryo size and composition has the potential to allow the manipulation of grain composition. Embryo size is determined by cell

number and cell size. Cell number is determined during the proliferation phase of development, whereas cell size is determined during the differentiation and expansion phase later in development (Figure 1.6). The timing of the switch between cell proliferation and maturation affects the duration of the proliferation phase and is therefore likely to influence embryo size.

Sugar metabolism has been implicated as having a role in determining the timing of the transition between cell proliferation and differentiation/maturation phases in both the endosperm and the embryo of the legume *V. faba* (Wobus and Weber, 1999). There is a correlation between a shift in endogenous sugar concentrations from hexose-rich to sucrose-rich and the transition from cell proliferation to storage product accumulation (Weber *et al.*, 1996a; Weber *et al.*, 1996b). Exogenous hexose and sucrose have been shown to influence embryo development of *Vicia* spp. both by transgenic manipulation *in vivo* and in tissue culture *in vitro* (Weber *et al.*, 1996a; Weber *et al.*, 1998). Hexose promoted cell division while sucrose promoted starch accumulation in developing *Vicia* spp. embryos. This work led to the theory that sugar metabolism might play a central role in determining seed size. Sugars, including sucrose and glucose, are known to modulate the expression of genes involved in storage product synthesis so this is a credible theory (Koch, 2004; Ramon *et al.*, 2006). The effect of exogenous sugar on the development of the embryo in *Vicia* spp. also led to the suggestion that control of sugar metabolism in the endosperm was of importance to the development of the embryo (Wobus and Weber, 1999). Initial work in *B. napus* suggested that a similar sugar switch controlling embryo development might also exist (Hill and Rawsthorne, 2000). However, recent work in *B. napus* has demonstrated that the hexose sugars of the endosperm are not in contact with the embryo and therefore cannot modulate embryo development in the way postulated by the sugar switch hypothesis (Morley-Smith *et al.*, 2008). Morley-Smith *et al.* (2008) were not able to determine the endogenous level of hexose and sucrose in the *B. napus* embryos at the transition from cell proliferation to lipid accumulation. Therefore the possibility remains that endogenous sugar concentrations in the embryo modulated this switch. The key mediators of the regulation of sugar metabolism are likely invertases and sucrose synthases which degrade sucrose to hexose sugars, but the mechanisms of regulation are not known.

Genetic screens of mutants of rice, maize and barley have uncovered some genes involved in embryo development. These are described in the following paragraphs. A large number of maize defective kernel (*dek*) mutants have been described (Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980). Mutants in this class have lesions affecting

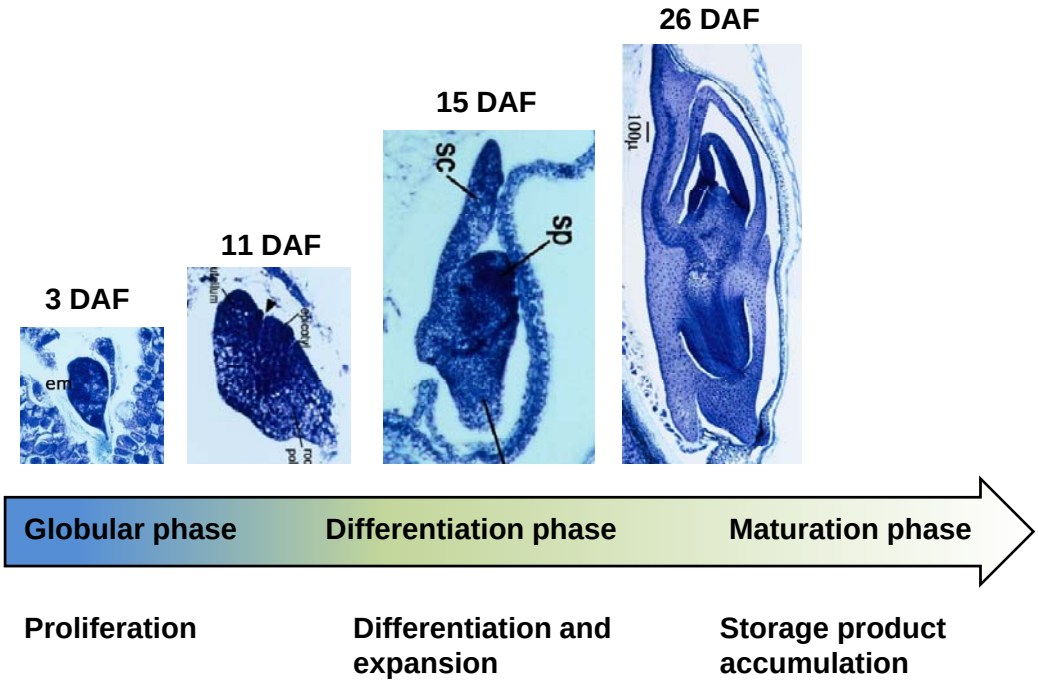

Figure 1.6 – Stages of embryo development.

Images courtesy of: 'WHEAT:THE BIG PICTURE', www.wheatbp.net.

development of both the embryo and the endosperm. Typically, embryo development is arrested at some stage during development in *dek* mutants. Mutants with embryos that are still capable of germinating or growing in some form when cultured *in vitro* are classified as having 'nutrition-type' mutations on the assumption that their phenotype is the result of an inadequate embryo nutrition (Sheridan and Neuffer, 1981). The second class of *dek* mutants, where embryo development is blocked prior to embryo axis formation, is defined as having 'developmental-type' mutations (Sheridan and Neuffer, 1981). A subset of *dek* mutants are designated *emp* (*em*pty *p*ericarp) mutants based on their radical effect on the endosperm. The affected genes have been cloned in just four *dek* mutants. These encode: a novel transmembrane protein possibly involved in auxin transport (Stiefel *et al.*, 1999), a member of the calpain protein family possibly involved in the transduction of an unknown signal (Lid *et al.*, 2002), a mitochondrion-targeted pentatricopeptide repeat (PPR) protein possibly involved in post-transcriptional processes in organelles and a heat shock protein which has a possible role in protecting cellular process during heat stress (Fu *et al.*, 2002). All these mutations are embryo-lethal due to early abortion of embryogenesis. None of these mutants, nor any of the other *dek* mutants, appear to have aberrations specifically in the determination of embryo size.

A second class of maize kernel mutant, namely *emb*ryo-specific (*emb)* mutants, were isolated from genetic screens (Clark and Sheridan, 1991; Heckel *et al.*, 1999). These mutants were isolated based on the normal appearance of the endosperm but a non-viable embryo. As with *dek* mutants, this screen produced mutants with arrested or aborted embryos rather than mutants with altered embryo size. The affected gene has been cloned in one *emb* mutant and found to be a plastid ribosomal protein constitutively expressed in all major tissues of wild-type maize plants (Magnard *et al.*, 2004).

There are several rice mutants which provide potentially interesting insight into the control of embryo size (Hong *et al.*, 1996). Particularly of interest in this study are those mutations which lead to an increase in embryo size. There are a number of mutations in rice which lead to larger embryos and these are briefly summarised in the next paragraphs.

The *giant embryo* (*ge*) mutant has an enlarged embryo compared to wild type due to an increase in scutellum size (Hong *et al.*, 1996). The increase in embryo size in the mutant is accompanied by a concomitant reduction in endosperm size in the mutant such that total grain volume is unchanged when compared to wild type (Nagasawa *et al.*, 2003). The increase in scutellum size in *ge* compared to wild type is a result of larger cells rather than more cells (Hong *et al.*, 1995a; Nagasawa *et al.*, 2003). Interestingly, the cells of the root-shoot axis are also enlarged in the *ge* mutants compared to wild type however there

is no overall increase in root-shoot axis size (Nagasawa *et al.*, 2003). The gene at the *ge* locus is a cytochrome P450 of unknown function (Singletary *et al.*, 2001). The rice *ge* phenotype and possible functions of the *Ge* gene are elaborated on in Chapter 4.

The *embryoless1* (*eml1*) mutant predominantly produces seeds with either absent or greatly reduced embryo. Under normal growing conditions (25 to 30 ºC), *eml1* plants also produce a small percentage (~1 percent) of normal seeds and seeds with a large embryo and reduced/absent endosperms (Hong *et al.*, 1995b). However, when *eml1* rice plants are grown under cold conditions (18 ºC), the number of normal seed and large-embryo seeds increases to 17 percent and 32 percent, respectively (Hong *et al.*, 1995b). In these large-embryo variants, endosperm development aborts at three days after pollination. The enlargement of the embryo is due to a greatly over-proliferated scutellum but the root-shoot axis appears unaffected (Hong *et al.*, 1995b). A second rice mutant, *endospermless1-2 (enl1-2)*, exhibits a similar phenotype to *eml1* (Kageyama *et al.*, 1991; Hong *et al.*, 1996). As with *eml1*, only a small percentage of mutant seeds exhibit abnormal development (10 to 20 percent) but the effect of temperature on the phenotype was not investigated (Hong *et al.*, 1996). Abnormal seed development results in early endosperm abortion and enlargement of the embryo due to overproliferation of the scutellum (Hong *et al.*, 1996). The low penetrance of the phenotype suggests that environmental factors determine whether the aberrant phenotype is shown. The similarity of phenotypes between *eml1* and *enl1-2* suggest that they may be allelic but to my knowledge this has not been tested. Both these mutants suggest that there may be a mechanical and/or physiological constraint on embryo size which is removed when the endosperm aborts.

A recent study of the role of B-type cyclins in seed development in rice found that reduced expression of *Orysa;CycB1;1* results in abnormal grain development in 29 to 51 percent of the seed (Guo *et al.*, 2010). Cyclins bind to cyclin-dependent kinases (CDKs) and these complexes regulate the transitions between different phases of the cell cycle (Harashima and Schnittger, 2010). In the abnormal grains produced when *Orysa;CycB1;1* expression is repressed, the endosperm aborts by three days after pollination and the embryo expands such that the mature grain is composed of only a large embryo. The large embryos have larger cells in the scutellum and the root-shoot axis than wild-type embryos but no difference in the number of cells (Guo *et al.*, 2010). GUS-reporter fusions located the expression of rice *Orysa;CycB1;1* to both the embryo and the endosperm although endosperm expression, strong initially, diminishes between five and 12 days after pollination (Guo *et al.*, 2010).

A glutamate carboxypeptidase mutant of rice (*plastochron3/goliath; pla3/go*) exhibits a large-embryo phenotype as well as a range of phenotypes associated with the shoot apical meristem (Kawakatsu *et al.*, 2009). *Pla3* mutants have a shortened plastochron (the period between leaf initiations), accelerated leaf maturation and an early transition to flowering compared to wild type (Kawakatsu *et al.*, 2009). Embryos of *pla3* mutants are larger than those of wild type due to the enlargement of both the scutellum and the embryonic root-shoot axis. Mature *pla3* grains are viviparous and show reduced sensitivity to ABA during germination (Kawakatsu *et al.*, 2009). Glutamate carboxypeptidases are thought to catabolise small peptide molecules (Helliwell *et al.*, 2001). It is postulated that they are involved in the production of a mobile signal molecule although their substrate and potential products are unknown (Helliwell *et al.*, 2001; Kawakatsu *et al.*, 2009).

To my knowledge, no mutant screen has been carried out for embryo defective mutants in the Triticeae. However, a screen of mutagenised barley lines for high lysine (using a dye-binding method) (Mossberg, 1969) identified, amongst other high-lysine mutants, a barley mutant with larger than normal embryos (Tallberg, 1977). This line was named Risø1508 and it contains a lesion at the *Lys3* locus. Risø1508 had the largest change in lysine composition (44 percent higher) of all the high lysine barley mutants identified at the Risø Institute (Doll *et al.*, 1974). For this reason, it has been the subject of considerable interest (Munck, 1992). Attempts to use Risø1508 in breeding programmes has been hampered by a shrunken-grain phenotype which negatively impacts on yield (Bang-Olsen *et al.*, 1991). Subsequently, three allelic mutants of Risø1508 were identified (Aastrup, 1983; Munck, 1992). The *Lys3* mutants are included in this study and a more thorough description of their phenotypes follows in the introductions to Chapters 3 and 4.

1.6. Embryo composition

In the previous section, the role of sucrose and hexose in the determination of the developmental switch between cell proliferation and storage product accumulation was described. Embryo composition is likely to be determined by the duration and rate of storage product synthesis during embryo maturation and is hence also likely to be affected by the timing of the switch between proliferation and differentiation/maturation phases. As with embryo size, most of the experimental work has been carried out in oilseeds and legumes (*Arabidopsis*, *B. napus* and *Vicia* spp.) rather than in cereals.

As previously noted, there are indications from the literature that altering the accumulation of one storage product can promote the accumulation of another. Recent work has focussed on the genetic manipulation of carbon partitioning from starch into protein. For

example, work has investigated reducing flux into starch by disrupting AGPase activity and the transport of glucose 6-phosphate into the plastid. In *Vicia narbonensis*, anti-sense inhibition of the plastidial glucose 6-phosphate/phosphate translocator or the starch biosynthetic gene AGPase reduces starch synthesis and consequently promotes protein synthesis and storage (Rolletschek *et al.*, 2002; Rolletschek *et al.*, 2007). As starch synthesis in *V. narbonensis* is strictly plastidial, reducing glucose 6-phospate flux into the plastids reduces the concentration of glucose 6-phosphate for starch biosynthesis. A similar result was obtained when AGPase was repressed in pea embryos (*Pisum sativum*) (Weigelt *et al.*, 2009).

In cereals embryos, as in *B. napus* and *Arabidopsis* (Norton and Harris, 1975; Andriotis *et al.*, 2010), starch is accumulated during development but it is broken down during maturation such that there is little or no starch at maturity (Black *et al.*, 1996). There is speculation that starch acts as a temporary carbon store prior to the accumulation of lipids and/or protein. This theory and others surrounding the role of starch in the developing embryo are elaborated upon in Chapter 5. If starch does play a role in determining the amount of lipid or protein accumulated in the embryo, then manipulation of the starch biosynthetic pathway may provide new tools for improving the nutritional value of cereals.

1.7. Research aims

- To interrogate a collection of shrunken-grain barley mutants to determine whether decreased starch in the endosperm leads to alterations in carbohydrate metabolism, size and/or composition of the embryo.

- To understand the genetic control of embryo size by seeking to identify the genes underlying loci shown to alter embryo size and/or composition in barley.

- To investigate the role of starch in the cereal embryo and its importance for determining embryo composition and/or size, by identifying and characterising a mutant line containing a lesion in a starch biosynthetic gene expressed in the embryo.

2. Materials and methods

2.1. Plant materials

2.1.1. Barley

The barley lines used in this study are listed below, ordered by source.

BBSRC Cereals Collection, JIC: Bomi, Glacier, Carlsberg II, Ingrid, Maythorpe, Golden Promise, Risø8, Risø1508, Pentlandfield Glacier, Risø13, Risø527, Notch2 and Risø16.

USDA-ARS National Small Grains Collection, Idaho, USA: Bowman, NP113, Seg6, Sex8, Sex7, Risø86, Seg7 and Sex6.

Nordic Gene Bank, Alnarp, Norway: Risø29 and Risø17.

Tom Blake, Montana State University, USA: Nubet and Franubet.

Birthe Møller Jespersen, University of Copenhagen, Denmark: Minerva, Risø18, Risø19, and M1460.

Alan Schulman, University of Helsinki, Finland: Shx

More information about these lines and their pedigrees are given in Table 3.1.

2.1.2. Rice

Rice (*Oryza sativa*) insertion lines NE1391 and NF3982, with insertions in *Os05g0580000* (also known as *Os05g50380*), were requested from the *Tos17* insertion database held at the National Institute of Agrobiological Sciences, Japan (NIAS; http://tos.nias.affrc.go.jp). Wild-type Nipponbare was a gift from Barbara Worland, JIC. The *apl1* mutant seed was previously obtained from NIAS (Rösti *et al.*, 2007). The mutants *osagpl2-1* and *osagpl2-2* and their wild type, Dongjin, were obtained from Jong-Seong Jeon, Kyung Hee University, Korea (Lee *et al.*, 2007).

2.1.3. Maize

Maize (*Zea mays*) lines Illinois Low Oil (accession NSL 20624) and Illinois High Oil (accession NSL 20626) were obtained from the USDA germplasm collection at Illinois State University.

2.1.4. Wheat-barley chromosome addition lines

Wheat-barley chromosome addition lines (Islam *et al.*, 1981) were obtained from the Germplasm Collection at the John Innes Centre, Norwich.

2.2. Plant growth

All plants were grown at the John Innes Centre, Norwich.

2.2.1. Growth conditions and soils

Barley

Barley was germinated by placing the seeds directly into a peat and sand mix with an individual seed in each well of a nursery tray (24-well tray, each well with a volume of ~10 cm^3). Once the seeds had germinated and the seedlings were established (two to three weeks), each seedling was transplanted into an individual 1 L pot containing barley mix (375 L Levington M3 compost [Scotts Professional, Ipswich, UK], 100 L perlite, 200 L 4-mm grit, 1.6 kg Osmocote® [Scotts Professional, Ipswich, UK]).

Barley was grown in the glasshouse either during the summer or the winter season. During winter, additional lighting was provided by sodium lamps for 16 h per day and temperatures were maintained between 15 °C (night) and 20 °C (day). Barley grown in a controlled environment room (CER) had conditions of 16 h light (15 °C) and 8 h (12 °C) darkness and 80 percent humidity. Light-cycle quantum irradiance was 300 to 350 µmol s^{-1} m^{-2} at pot height and 400 to 450 µmol s^{-1} m^{-2} at ear height.

Rice

Rice seeds were surface sterilised prior to germination (see Section 2.2.2). Seeds were then placed in Petri dishes lined with sterile filter paper and half submerged in sterile water. Petri dishes were sealed using Micropore™ tape (R & L Slaughter Ltd., Upminster, Essex). Germination took place over three to five days at 28 °C in the dark. Seedlings were transferred from Petri dishes to nursery trays (24-well trays, each well with a volume of ~10 cm^3) containing rice mix (100 L John Innes No.1 [William Sinclair Holdings, Lincoln, UK], 75 L horticultural silver sand, 500 g Osmocote® [Scotts Professional, Ipswich, UK]) and placed on flooded benches in a CER. CER conditions were 12 h light (28 °C) and 12 h (24 °C) darkness, 90 percent humidity. Light-cycle quantum irradiance was 300 to 350 µmol s^{-1} m^{-2} at pot height and 400 to 450 µmol s^{-1} m^{-2} at panicle height. After two to three weeks the seedlings were transplanted into individual 1 L pots containing rice mix.

2.2.2. Seed sterilisation

De-hulled seeds were submerged in 70 percent ethanol for three min, followed by three rinses in sterile water. Seeds were then submerged for 10 min with continuous shaking in bleach solution (a commercial bleach containing six percent w/v hypochlorite diluted 1:1 with sterile water and with one drop of 20 percent SDS per 100 mL). This was followed by three rinses in sterile water.

2.2.3. Embryo weights

Embryos dissected from developing grains were weighed immediately. Mature seeds were imbibed overnight in sterile water at 4 °C in the dark before the embryos were removed and weighed.

2.2.4. Judging the developmental stage of grains

Barley

Time after flowering was used to determine the age of the grains for the metabolite assays in Chapters 3 and 4. Anthesis occurs while the ear is enveloped in the flag leaf so exact day of anthesis was difficult to determine routinely. Instead, the time after the day on which the awns of the developing ear were visible more than 1 cm above the leaf sheath was used. This is referred to as days after flowering (DAF).

Rice

Time after flowering was used to determine the age of the panicle for the stem starch assay in Chapter 5. The panicle was judged to have flowered when anthers were first visible outside the floret and developmental stage will be referred to as days after flowering (DAF). As individual florets on a developing panicle flower asynchronously, for metabolite and enzyme assays, the developmental age of the embryos and endosperms was based on endosperm fresh weight. Assays were carried out on endosperm and embryo tissue during grain filling (10 to 15 days after flowering) and during this time endosperm fresh weight increases as the grain is filling (Ishimaru *et al.*, 2005).

2.2.5. Crossing

Individual florets of both barley and rice were emasculated by hand prior to anther dehiscence. In barley, this was carried by making an incision in the side of the floret and removing the three anthers with fine forceps. In rice, this method was not possible due to the rigid palea and lemma so the top of the floret was removed using scissors and the six anthers removed. After emasculation of an entire barley ear or rice panicle, the

ear/panicle was bagged to prevent cross-pollination and allowed to mature for three to five days. Following this period, the florets were pollinated by applying pollen from the relevant pollen donor to the stigmas. The ear/panicle was then bagged again until maturity.

2.3. Biochemical methods

2.3.1. Carbohydrate extraction

To minimise turnover of carbohydrate in tissues during harvesting, samples were harvested into tubes on ice, weighed and frozen in liquid nitrogen prior to extraction. Harvesting was carried out as fast as possible to minimise the time samples were on ice prior to freezing.

All samples were ground in 0.77 M ice-cold perchloric acid and allowed to fully acidify for 30 min on ice.

- Barley embryo samples of 2 to 70 mg (20 to 45 DAF) were harvested and freeze-dried to obtain dry weight. Frozen tissue was ground thoroughly, for 90 s at 1500 bpm, in 600 µL of perchloric acid in the Genogrinder® (SPEX CertiPrep, New Jersey, USA, using 1.5 mL polyvinyl tubes and stainless steel balls 3/8” in diameter).
- Developing barley endosperm samples of 10 to 65 mg (20 to 45 DAF) were harvested and freeze-dried. Frozen tissue was ground in the Genogrinder® as above but in 500 µL of perchloric acid. Following grinding, a further 1 mL of perchloric acid was added to the homogenate.
- Mature barley grains were imbibed overnight at 4 °C in the dark and were ground in the Genogrinder® as above but in 500 µL of perchloric acid. Following grinding, a further 1 mL of perchloric acid was added to the homogenate.
- Developing rice embryo samples of 2.5 to 5 mg (~10 to 15 DAF), from rice grains during grain filling, were harvested on ice and frozen. Frozen tissue was ground by hand in 600 µL of perchloric acid using a micropestle.
- Rice culm/peduncle samples (the entire stem from just below the panicle to just above the first node) weighing between 170 and 530 mg were harvested on ice and frozen. Frozen tissue was ground in a pestle and mortar, in the presence of silica sand, in 5 mL of acid.

The insoluble material, including starch, was separated from the soluble metabolites, including sugars, by centrifugation of the extract at 20 000 *g* for 10 min at 4 °C.

In embryo carbohydrate extraction, the supernatant was removed and the pellet re-suspended in 100 µL of sterile water. Following another centrifugation as above, the second supernatant was added to the first.

The supernatant was used to measure glucose, fructose and sucrose. The pellet was further extracted prior to assay for starch as follows:

Embryo samples: the pellet was re-suspended in 250 µL of sterile water from which four representative aliquots were taken for starch assay (two were digested and two were undigested controls).

Barley endosperm samples: to remove excess supernatant and oligosaccharide contaminants from the insoluble pellet, the pellet was washed by re-suspension in 1 mL of sterile water, followed by two re-suspensions in 1 mL of 80 percent ethanol solution. Finally the pellet was re-suspended in 1 mL of sterile water from which four representative aliquots were taken for starch assay (two were digested and two were undigested controls).

Rice culm/peduncle starch samples: to remove excess supernatant and oligosaccharide contaminants from the insoluble pellet was washed by re-suspension in 5 mL of ice-cold 100 percent ethanol solution, centrifuged and the supernatant removed, and three re-suspensions in 5 mL of 80 percent ethanol solution, followed by centrifugation and supernatant removal as before. Finally the pellet was re-suspended in 4 mL of sterile water from which four representative aliquots were taken for starch assay (two were digested and two were undigested controls).

2.3.2. Carbohydrate assay

Starch

The samples were autoclaved for 20 min at 120 °C to solubilise the starch. Starch was digested to glucose using α-amylase and amyloglucosidase (both from *Aspergillus oryzae,* manufactured by Megazyme Ltd., Co. Wicklow, Ireland) and the glucose measured enzymatically as described in Rösti *et al.* (2006). Coupling enzymes in the glucose assay, hexokinase and (NADP-dependant) glucose-6-phosphate dehydrogenase were manufactured by Roche Diagnostics (Basel, Switzerland). The reaction was monitored at 340 nm on either a spectrophotometer or a microtitre plate reader. Initial absorbance was recorded before the reaction was started by the addition of (NADP-dependant) glucose-6-phosphate dehydrogenase. The reaction was monitored until steady absorbance was reached. The difference between initial absorbance and final absorbance was used to

calculate glucose in the samples. The mass of starch (g) was calculated as moles of glucose x162. Undigested controls were included in the analysis to account for glucose in the pellet not from starch.

Glucose, fructose and sucrose

The supernatant from carbohydrate extraction was neutralised to pH 7.0 by addition of KOH/MES/KCl (2 M KOH, 0.4 M MES, 0.4 M KCl), centrifuged at 12 000 *g* to remove the insoluble potassium perchlorate and glucose, fructose and sucrose were measured enzymatically as described in Rösti *et al.* (2007). The sucrose was digested using invertase (β-fructosidase) manufactured by Megazyme (Co. Wicklow, Ireland). Fructose-6-phosphate was converted to glucose-6-phosphate using phosphoglucose isomerase (Roche Diagnostics, Basel, Switzerland). Free glucose and fructose contents were measured in undigested controls. Sucrose content was calculated by subtracting free glucose and fructose contents from those in the digested controls.

2.3.3. Protein extraction and assay

Mature seeds were imbibed overnight in sterile water at 4 °C in the dark before the embryos were removed and freeze-dried. A single embryo (1.0 – 6.0 mg) was thoroughly ground, for 90 s at 1500 bpm, in 500 µL of sterile water in the Genogrinder® (SPEX CertiPrep, New Jersey, USA, using 1.5 mL polyvinyl tubes and stainless steel balls 3/8" in diameter). Then 500 µL of protein extraction buffer (0.2 M MOPS pH 7.2, 4 percent SDS, 2 mM DTT) was added and proteins were allowed to dissolve for 30 min at room temperature with continuous rocking. Samples were then centrifuged for 5 min at 12 000 g before the supernatant was assayed using a BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific, Rockford Illinois, USA) according to the manufacturers instructions and using BSA standard solutions.

2.3.4. Lipid extraction and measurement

Total lipid in rice embryos was determined gravimetrically using a method based on Coxon and Wright (1985). Samples of 20 embryos (each of 12 to 15 mg DW) were dissected from mature rice grains after they had been imbibed, in the presence of sterile water, overnight at 4 °C. These were then freeze-dried and ground thoroughly in 1 mL of chloroform/methanol (2:1) using a ceramic pestle and mortar. The homogenate was removed to a 1.5 mL tube and warmed to 60 °C for 1 min before being left at room temperature overnight. Next, the homogenate was centrifuged at 12 000 *g* for 5 min and the supernatant removed to a new Eppendorf tube. The pellet was twice re-suspended in 250 µL of chloroform/methanol (2:1), allowed to stand for 30 min at room temperature,

and centrifuged. Following each centrifugation the supernatant was added to the first. Total supernatant was made up to 1.5 mL and than a 1.2 mL aliquot was subjected to three washes with 0.8 mL of a 1:1 mixture of saturated NaCl solution and 1 M HCl to remove non-lipid contaminants (Folch *et al.*, 1957). During each wash the sample was mixed vigorously before allowing phase-separation and each time the top, aqueous phase was removed. The remaining volume of solvent (lower phase) was made up to 1 mL. Duplicate aliquots of 0.4 mL were evaporated overnight in pre-weighed 0.5 mL Eppendorf tubes which were then reweighed. Significant residues were measurable from solvent evaporations alone so six control extractions, following all steps above but in the absence of tissue, were carried out and the average control residue was subtracted from each sample residue.

2.3.5. ADP-glucose pyrophosphorylase extraction

To preserve enzyme activity, samples were harvested into tubes on ice and extracted immediately or harvested and frozen immediately on liquid nitrogen and stored at -80 °C before extraction.

All steps were performed at 4 °C or on ice. Endosperm or embryo tissue (10-100 mg tissue per mL of buffer) was homogenised in extraction buffer (50 mM HEPES pH7.4, 5 mM KCl, 2 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 5 percent (v/v) ethanediol [embryo extraction buffer also contained 1 percent (w/v) BSA and 1percent (w/v) PVP]) using either a pestle and mortar and all-glass homogeniser for endosperm samples or a micropestle for embryo samples. After centrifugation for 15 min at 20 000 *g*, the supernatant was assayed for enzyme activity.

2.3.6. ADP-glucose pyrophosphorylase assay

AGPase was assayed in the pyrophosphorolytic direction by monitoring of the rate of production of NADH at 340 nm using a spectrophotometer as described by Smith *et al.* (1989) and modified by Burton *et al.* (2002a).

2.3.7. Separation of plastidial and cytosolic AGPase isoforms using fast protein liquid chromatography (FPLC)

All steps were performed at 4 °C or on ice. Samples of 150 to 400 mg of endosperm were homogenised in 5 mL of AGPase extraction buffer (Section 2.3.5). The homogenate was centrifuged for 15 min at 20 000 *g* before 2 ml of supernatant was loaded onto a 1 mL HiTrap Q HP column (GE Healthcare, Little Chalfont, Bucks, UK), pre-equilibrated with 50 mM Bis-tris propane pH 7.4, 5 mM KCl, 1 mM DTT and 5 percent (v/v) ethanediol. Protein was eluted from the column by applying a linear salt gradient (KCl) from 5 mM to 700 mM

at a flow rate of 0.5 mL min^{-1}. Fractions of 0.5 mL were collected into tubes containing 10 µL of 20 percent (w/v) BSA and assayed immediately for AGPase activity.

2.3.8. SDS-PAGE and immunoblotting

Samples were homogenised in 1x Laemmli sample buffer containing 70 mM DTT, boiled for 5 min and centrifuged at >12 000 *g* for 5 min (Laemmli, 1970). Samples were loaded on SDS polyacrylamide gels and voltage was applied using a vertical electrophoresis cell (Mini-Protean II, BioRad). SDS-PAGE gels were then blotted onto PVDF (polyvinylidene difluoride) transfer membrane (Thermo Scientific) by Western blotting. Probing of the blot was carried out according to Blake *et al.* (1984) except that the initial blocking step contained 30 g L^{-1} BSA and 20 g L^{-1} dried milk powder.

Antisera raised against BRITTLE2 and SHRUNKEN2 from maize (gifted by Curt Hannah, University of Florida, USA) were used at a dilution of 1/5000. Antiserum raised against AGPase LSU from pea (gifted by Alison Smith, JIC) was used at a dilution of 1/750. Antiserum raised against AGPase LSU from spinach leaf (gifted by Jack Preiss, Michigan State University, USA) was used at a dilution of 1/5000. Antisera raised against BEPL and BEPS from barley (a gift from Tine Thorbjornsen, formerly in laboratory of Odd-Arne Olsen, Agricultural University of Norway, Ås) were used together with a combined dilution of 1/1000.

2.4. Molecular methods

2.4.1. Isolation of RNA from plant tissue

Total RNA was isolated using the Trizol® reagent (Invitrogen) according to the manufacturer's instructions as follows: tissue was homogenised in 1 mL Trizol® reagent per 50 to 100 mg tissue in a sterilised, pestle and mortar, transferred to a 1.5 mL tube and centrifuged at 12 000 *g* for 10 min at 4 °C. Phase separation was carried out on the supernatant using 0.2 mL chloroform per 1 mL of supernatant, to remove contaminating proteins. After mixing the aqueous and organic phases by inverting, the aqueous upper phase containing the RNA, was transferred to a clean 1.5 mL tube. The RNA was precipitated by the addition of an equal volume of isopropanol. After incubation for 10 min at room temperature, the RNA precipitate was collected by centrifugation at 12 000 *g* for 10 min at 4 °C. The RNA pellet was washed using 75 percent (v/v) aqueous ethanol, briefly air-dried, and then re-suspended in 30 µL sterile water.

2.4.2. Isolation of genomic DNA from plant tissue

Two methods were used for isolating DNA from plant leaf tissue. Both are rapid methods for the isolation of DNA suitable for genotyping. Method A was used when only a small number of extractions were required. Method B was for high-throughput.

Method A: leaf fragments were ground using a micropestle in DNA extraction buffer (0.2 M Tris HCl pH7.5, 25 mM EDTA, 0.5 percent (v/v) SDS, 250 mM NaCl). After centrifugation for 3 min at 20 000 *g*, one volume of isopropanol was added to the supernatant to precipitate the DNA. The precipitate was collected by centrifugation at 20 000 *g* for 7 min. The pellet was air-dried and re-suspended in sterile water.

Method B: DNA extraction using the REDExtract-N-Amp™ Plant PCR Kit (Sigma Aldrich) according to the manufacturer's instructions. This kit has a single-step genomic DNA extraction reagent and is supplied with specially-formulated PCR ready-mix.

2.4.3. First strand cDNA synthesis

First strand cDNA was synthesised using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. RNA, extracted as described in section 2.4.1., was DNase-treated (Promega) prior to incubation at 65 °C for 10 min in the presence of oligo-$(dT)_{17}$ primer and dNTPs. The reaction was briefly cooled to 4 °C on ice before RT buffer (Invitrogen), DTT and reverse transcriptase were added. First strand synthesis was carried out for 50 min at 42 °C before the reaction was stopped by incubation for 15 min at 70 °C. The cDNA was treated with RNaseH (5 U μL^{-1}, Amersham Pharmacia) to remove the RNA prior to use in PCR.

2.4.4. Polymerase chain reaction (PCR)

All PCRs were performed using *Taq* (Qiagen) or Phusion® High Fidelity (Finnzymes) DNA polymerase. Reaction mixes (total volume 50 µL) were made up according to the DNA polymerase manufacturer's instructions using the buffers supplied and 4 µL dNTPs (10 mM total, 2.5 mM of each), 1 µL primers (10 mM), 1 U DNA polymerase and genomic DNA or cDNA. PCR cycler was programmed as follows: initial denaturing step of 2 min at 94 °C; 35 cycles of 30 s at 94 °C, annealing for 30 s at 45-60 °C and extension at 72 °C for 1 min kb^{-1} product (standard) or 30 s kb^{-1} (Phusion®); and final extension step of 72 °C for 10 min.

2.4.5. Primers

Primers used in this study are shown in Table 2.1 below.

Table 2.1 - Primers for DNA amplification

Design sequence	Use	Primer sequence (5' to 3')
OsApl3	Genotyping NE1391 (P1)	ACTTTGCGGATCCAAATGAG
	Genotyping NE1391 (P2)	GGCCAGGAATTTCAAGATGA
	Genotyping NC7135 (P3)	TTAGTATTTGGCTGTGGCCC
	Genotyping NC7135 (P4)	CAACAACCACGCAAATCATC
	Genotyping NF3982 (P5)	ATGATTTGCGTGGTTGTTGA
	Genotyping NF3982 (P6)	GGGAAATTTAGTCGGGTCGT
	cDNA amplification forward	TGCAGTTCAGCAGTGTGTTTC
	cDNA amplification reverse	CACGATTCCCGACCTTATGT
OsApl1[a]	Genotyping *osapl1* forward (P7)	GAGATGGATTCGTCGAGGTG
	Genotyping *osapl1* reverse (P8)	TCCATGTAGTCCATGCGGTA
Tos17	Genotyping 3' internal	AGGTTGCAAGTTAGTTAAGA[b]
Actin	Control forward	CATGCTATCCCTCGTCTCG
	Control reverse	CGCACTTCCATGATGGAGTTG
HvGe	*HvGe* forward	AGGCGTACGTCGGTAACATC
	HvGe barley-specific reverse	TCGGTATGCTTGTAGAATGTAGAG

[a] Rösti *et al.* (2007). [b] NIAS; http://tos.nias.affrc.go.jp.

2.4.6. Visualisation of PCR products

PCR product was mixed with 4x loading buffer (Bioline), loaded onto agarose gel (typically 1.5 percent w/v agarose) and separated by applying a voltage of 90-120 V. The PCR products were visualised by ethidium bromide staining under ultra-violet light (typically 4 µL of ethidium bromide was added to 100 ml of agarose).

2.4.7. DNA sequencing

PCR products were sequenced using product-specific primers and the Big Dye terminator kit v3.1 (Perkin Elmer) according to the manufacturer's instructions. Analysis of reaction products was performed by TGAC (Norwich Research Park).

2.5. Light microscopy

2.5.1. Fixation

Barley embryos were fixed for light microscopy by Sue Bunnewell and Kim Findlay at Bioimaging facility at JIC using the following method:

Embryos were harvested directly from the ear into a fixative solution (2.5 percent [v/v] glutaraldehyde in 0.05 M sodium cacodylate, pH 7.3) and vacuum-infiltrated until they sank. Fresh fixative was added and embryos were left overnight to adequately fix all the cells. The fixative was washed out by three successive 10-min washes in 0.05 M sodium cacodylate and then post-fixed in 1 percent (w/v) OsO_4 in 50 mM sodium cacodylate for one hour at room temperature. The osmium fixation was followed by three 15-min washes in sterile, distilled water before passing through an ethanol-dehydration series (10 percent,

20 percent, 30 percent, 50 percent, 70 percent, and 95 percent each for a minimum of 30 min and then two 100 percent ethanol solutions each for an hour). Following dehydration, samples were gradually infiltrated with LR White resin (London Resin Company, Reading, Berkshire) by successive changes of resin:ethanol mixes over three days at room temperature (1:1 for 16 h, 2:1 for 8 h, 3:1 for 16 h, 100 percent resin for 8 h then 16 h and again 8 h with fresh resin at each stage). Samples were then transferred into gelatine capsules filled with fresh LR White and placed at 60 °C for 16 h to polymerise. The material was sectioned with a glass knife using a Leica® UC6 ultramicrotome (Leica, Milton Keynes).

Sections of 0.5 µm were dried onto glass slides and stained with 0.5 percent (w/v) Toluidine blue “O” in 0.5 percent (w/v) borax.

2.5.2. Visualisation and analysis

Sections were examined using a Zeiss Axiophot® light microscope (Carl Zeiss Ltd). Images were captured using a Pixera Penguin® 600CL digital camera. Image capture and subsequent analysis was carried out using Image Pro Plus v4 (Media Cybernetics UK).

2.6. Bioinformatic methods

2.6.1. Sequence alignment

Sequences were handled and aligned using AlignX (part of the VectorNTI® Suite v11, Invitrogen). Alignments were visually inspected and hand-edited. Intron/exon structure was deduced by aligning cDNA sequence with genomic DNA sequence using Sim4 online tool (http://pbil.univ-lyon1.fr/sim4.php).

2.7. Statistical methods

Statistical analysis was carried out using Genstat v11 (VSN International). Regression analysis was carried out by general linear model. Pair-wise comparisons were by Student’s T-test (*t*) or Mann-Whitney (*U*) and subscript numbers denote the degrees of freedom.

3. Endosperm-embryo interactions: embryo size and composition in mutant barley lines with altered starch accumulation in the endosperm

3.1. Aim

To investigate how perturbations in endosperm development - due either to lesions in genes encoding enzymes in the starch synthesis pathway or to lesions in unknown genes - influence the development of the embryo.

3.2. Introduction

The embryo and endosperm develop from different cells in the embryo sac and follow different developmental patterns, yet both rely on the sugars from the same maternal tissue for their growth and development. The primary route for entry of assimilate into the developing caryopsis is via the endosperm transfer cells into the endosperm (Lim *et al.*, 2006; Zheng and Wang, 2010). The route by which carbon reaches the embryo in cereals is not fully understood but a nutritive role has been assigned to the endosperm. If the primary route of carbon from the maternal tissue to the embryo is via the endosperm, perturbations in sugar supply or sugar metabolism in the endosperm may directly affect sugar supply to the embryo. Furthermore, the identity of the sugars being supplied to the embryo by the endosperm may modulate its development under the 'sugar switch' hypothesis discussed in Chapter 1. Thus perturbations in endosperm sugar metabolism are also likely to affect the development of the embryo.

In this study, a collection of barley mutants with shrunken grain were used to try to elucidate the extent to which embryo development is influenced by endosperm development, and whether the embryo responds to changes in endosperm development by changing its size and/or composition.

The shrunken phenotype is a good indication that starch biosynthesis is reduced during grain development in this collection of lines. Indeed, mutations in three shrunken barley mutants have been found to be located in genes directly involved in starch biosynthesis (Burton *et al.*, 2002a; Johnson *et al.*, 2003; Patron *et al.*, 2004). A reduction in starch biosynthesis in the endosperm in these lines leads to changes in sugar metabolism in the endosperm, notably to a general increase in sugar levels. The shrunken phenotype of the

mature grain is likely to be a direct result of altered osmotic potential in the developing endosperm as a consequence of increased sugar concentration.

The collection of shrunken barley mutants was assembled from publically-available germplasm collections (Table 3.1). The collection includes lines where the lesion is in a yet-to-be-identified gene which may or may not be involved directly in starch biosynthesis. Previously-published work on lines in the collection has indicated that shrunken mutant Risø1508 has larger embryos and the embryos accumulate starch to higher levels than the parental wild-type line Bomi (Tallberg, 1977; Deggerdal *et al.*, 1986). To my knowledge, embryo size has not been measured in the three lines reported to be allelic to Risø1508 (Table 3.1) nor has embryo size been measured in any of the other lines. Ultrastructural studies on developing embryos of a number of the shrunken Risø mutants reported that the scutellum accumulated higher levels of starch (Olsen *et al.*, 1984). This suggests that reducing flux into starch in the endosperm may make more sugar available for embryo metabolism. This additional supply of carbon may be used for starch synthesis. Starch is stored in the developing embryos of wheat (Black *et al.*, 1996) and is therefore likely to be stored in the developing embryos of barley. However, starch is not stored in the embryo at maturity (Black *et al.*, 1996). It is conceivable that starch stored at higher levels during development is then converted to other storage compounds as the grain matures leading. Therefore, higher levels of starch may lead to higher levels of other storage compounds, such as protein, in the mature embryo.

Biochemical measurements of starch content in the developing embryos of one of the low starch lines (Risø16, AGPase SSU mutant) found starch content to be no different between mutant and parental wild-type line (Rösti *et al.*, 2006). Therefore, questions remain surrounding the possible effects of reduced flux into starch in the endosperm on composition of the embryo.

The barley lines in the collection are shown in Table 3.1. Seven lines have lesions in known genes. Risø17 and Notch2 are null mutants of *Isoamylase1* (*Isa1*) encoding a starch debranching enzyme (Burton *et al.*, 2002a). *Isa1* mutants have radically altered endosperm starch granule morphology and accumulate high levels of the highly-branched, water-soluble glucan, phytoglycogen in the grain (Burton *et al.*, 2002a). *Isa1* is required for normal starch biosynthesis in the endosperm and possibly for normal starch biosynthesis in the embryo. Risø16 contains a large deletion in the AGPase small subunit gene (*Agps1*) required for the majority of AGPase activity in the endosperm (Johnson *et al.*, 2003). *Agps1* mutants have reduced AGPase activity in the endosperm and therefore reduced starch content in the endosperm (Johnson *et al.*, 2003). The effects of a lesion in

Table 3.1 – Mutant barley lines used in this study.

Protein	Line	Gene/ Locus	Parent line	Mutagen	Original selection criterion	References
ADP-glucose transporter	Risø13	*Nst1*	Bomi	EMS	High lysine	(Doll, 1976; Patron *et al.*, 2004)
	Risø29		Carlsberg II	EMS	High lysine	(Doll, 1972; Patron *et al.*, 2004)
	Risø86		Carlsberg II	EMS	High lysine	(Doll, 1972; Patron *et al.*, 2004)
	Sex7*		Bowman	Induced	Shrunken grain	(Franckowiak, 1994)
AGPase SSU	Risø16	*Agps1*	Bomi	Fast neutrons	High lysine	(Doll, 1976; Johnson *et al.*, 2003)
Isoamylase1	Risø17	*Isa1*	Bomi	Fast neutrons	High lysine	(Doll, 1976; Burton *et al.*, 2002a)
	Notch2		NP113	EMS	High lysine	(Sekhara *et al.*, 1976; Burton *et al.*, 2002a)
Unknown	Risø8	*Lys4*	Bomi	EMS	High lysine	(Doll, 1976)
Unknown	Pentlandfield Glacier	*Amo1*	Glacier	Spontaneous	High amylose	(Merritt, 1967)
Unknown	Seg6	*Seg6*	Ingrid	Spontaneous	Shrunken grain	(Ullrich and Eslick, 1978c)
Unknown	Seg7	*Seg7*	Ingrid	Spontaneous	Shrunken grain	(Ullrich and Eslick, 1978c)
Unknown	Sex8	*Sex8*	Bowman	Induced	Shrunken grain	(Franckowiak, 1994)
Unknown	Franubet		Nubet	Chemical	Aberrant starch	(DeHass *et al.*, 1983)
Unknown	Risø1508	*Lys3a*	Bomi	Ethylenimine	High lysine	(Tallberg, 1973)
Unknown	Risø18	*Lys3b*	Bomi	Na azide	Unknown	(Munck, 1992)
Unknown	Risø19	*Lys3c*	Bomi	Na azide	Unknown	(Munck, 1992)
Unknown	M1460	*Lys3m*	Minerva	Na azide	Low β-glucan	(Aastrup, 1983)
Unknown	Risø527	*Lys6*	Bomi	γ-rays	High-lysine	(Doll, 1976)
Unknown	Shx	*Shx*	Bomi	Spontaneous	Shrunken grain	(Schulman and Ahokas, 1990)

*Sex7 was found to be carrying a lesion in the *Nst1* gene (Dr Kay Trafford, personal communication).

Agps1 are confined to the endosperm as AGPS1 is absent from the embryo (Rösti *et al.*, 2006). There are four lines carrying lesions in the nucleotide sugar transporter (*Nst1*). *Nst1* is required for the transport of cytosolic ADP-glucose across the plastid envelope (Patron *et al.*, 2004). *Nst1* mutants accumulate ADP-glucose in endosperm as a consequence of the inability to utilise cytosolic ADP-glucose for starch biosynthesis. As current evidence suggests that AGPase activity in the embryo is exclusively plastidial (Rösti *et al.*, 2006), a lesion in *Nst1* is unlikely to affect starch biosynthesis in the embryo. In conclusion, the lines with lesions in characterised genes can be divided into two groups: the first group carry mutations that are likely to perturb starch biosynthesis in both embryo and endosperm (*isa1* mutants) and the second group carry mutations in genes required specifically in endosperm cells for the synthesis of ADP-glucose in the cytosol and its transport across the plastid membrane (*agps1* and *nst1* mutants).

All the mutants in the collection show seed-shrivelling under the control of a single recessive locus. Seg (shrunken endosperm genetic) mutants show shrivelling dependent on maternal genotype whereas all the other lines show shrivelling dependent on the filial genotype. In both Seg6 and Seg7 a premature termination of endosperm filling coincided with the necrosis and crushing of the chalazal and nucellar projections of the pericarp (Felker *et al.*, 1985). This evidence suggests that the mutations in these lines cause a disruption in the functioning of nutrient transport from the maternal tissue to the developing grain rather than a defect of metabolism or development of the endosperm or embryo. The shrunken phenotypes in the other lines are dependent on the genotype of the seed, irrespective of maternal phenotype.

This work investigated changes in embryo weight and metabolism in shrunken barley mutants. Embryo size was measured at maturity to test whether mutations leading to a shrunken-grain phenotype were associated with alterations in embryo size. Three possible scenarios were considered in Figure 3.1: 1) the embryo may develop to normal size regardless of endosperm development, 2) the embryo may grow larger due to increased sugar availability, or 3) the embryo may be smaller if, for example, normal starch metabolism in the endosperm is required to establish grain sink strength and thus the supply of assimilate to the caryopsis as a whole.

This work also investigated whether a reduction in starch accumulation in the endosperm led to shifts in the accumulation of starch, sugars and/or protein in the embryo. As discussed above, decreased starch biosynthesis in the endosperm may lead to increased sugar availability to the embryo which may be stored by the embryo as starch or another storage compound such as protein. Conversely, altered sugar metabolism in the

endosperm may alter the developmental timing of embryo development and reduce the accumulation of embryo starch and/or other storage products.

Finally, this chapter investigated the nutritional improvement afforded by a large embryo. Risø1508 is known to have high lysine content (percent of total protein) (Ingversen *et al.*, 1973) and accumulate more oil (percent weight) than wild-type cultivar Bomi (Munck, 1976) and here the effect of a large-embryo phenotype on micronutrient content is assessed.

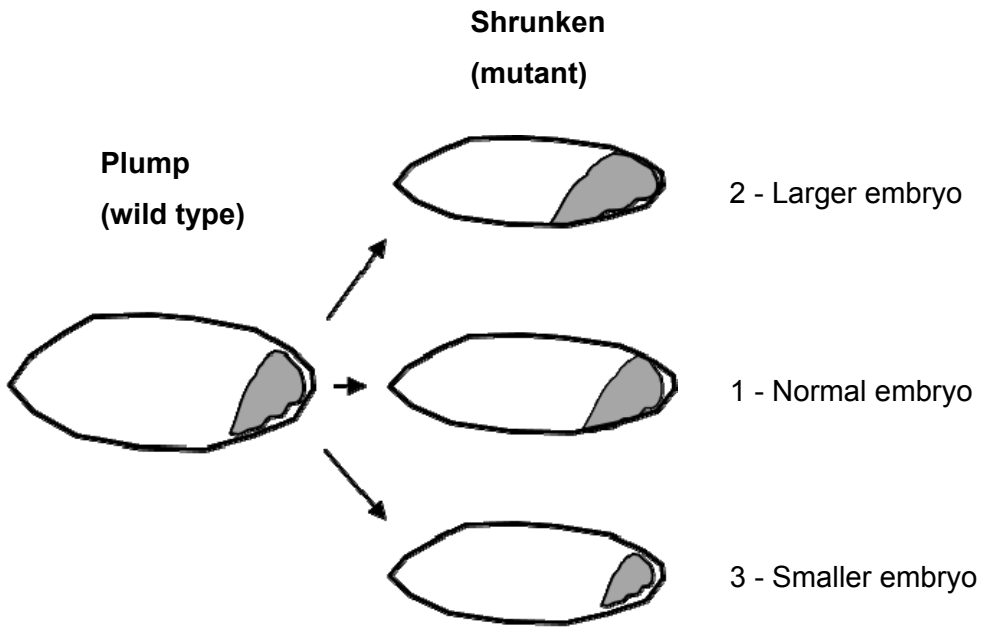

Figure 3.1 – Possible effects of mutations causing shrivelling of the endosperm on embryo size.

The possible effects on embryo size in barley shrunken grain mutants are numbered as referred to in the text.

3.3. Results

3.3.1. Mature grain weight and embryo weight in mutant barley lines

In an initial check of the phenotypes of the grain, it was noted that the Risø19 seed supplied to us contained a mixture of genotypes. Risø19 has been described previously as being allelic to Risø1508 (Munck, 1992) and hence was expected to have a large-embryo phenotype. Two morphologically-distinct grain types were identified within the Risø19 supplied, both with a shrunken-grain phenotype but with either large or normal embryos. Those with a shrunken grain and a large embryo were tested for allelism to Risø1508 by crossing (see Chapter 4) and, following allelism confirmation, designated Risø19LE (large embryo). The second shrunken grain type, of unknown origin, had normal embryos and was designated Risø19NE (normal embryo).

To identify lines with altered embryo size, embryo weight was measured at maturity in each of the mutant lines and the wild-type parent lines. All the shrunken lines had lower grain weight than their respective wild types, but to varying extents (Figure 3.2A; Appendix 7.1). In half the lines examined (10 of 20) the reduction in grain weight was accompanied by a concomitant, statistically significant reduction in embryo weight compared to wild type (Figure 3.2B). In five lines there was no statistically significant effect on embryo weight and in five others lines there was a statistically significant increase in embryo weight. Of those lines with an increase in embryo weight, four are allelic and have lesions at the *Lys3* locus (Tallberg, 1973; Aastrup, 1983; Munck, 1992). The increase in embryo weight relative to wild-type parents was dramatic, increases ranging from 60 percent to 147 percent. Notch2 also showed a small but statistically significant increase in embryo weight. However, there was no significant difference in embryo weight between Risø17, which also carries a mutation in the *Isoamylase1 (Isa1)* gene, and the wild-type parent (Burton *et al.*, 2002a). This suggests that the increase in embryo weight in Notch2 is due to an effect other than that of the *Isa1* lesion alone.

Of the five lines in which there was no difference in embryo weight compared to wild type, four carry known lesions in starch biosynthetic enzymes (Table 3.1). These are Risø17 (lacking *isa1*) (Burton *et al.*, 2002a), Risø16 (lacking AGPase SSU) (Rösti *et al.*, 2006) and two allelic mutants Risø13 and Risø29 (both lacking the nucleotide sugar transporter *Nst1*) (Johnson *et al.*, 2003). The effect of a lesion in *Nst1* is equivocal as allelic lines Risø86 and Sex7 show a reduction in embryo weight concomitant with the effect on the endosperm. As with the *isa1* mutants, the differences in the effect on embryo weight between the *nst1* mutant lines cannot reliably be attributed to the lesion in *nst1* alone, as

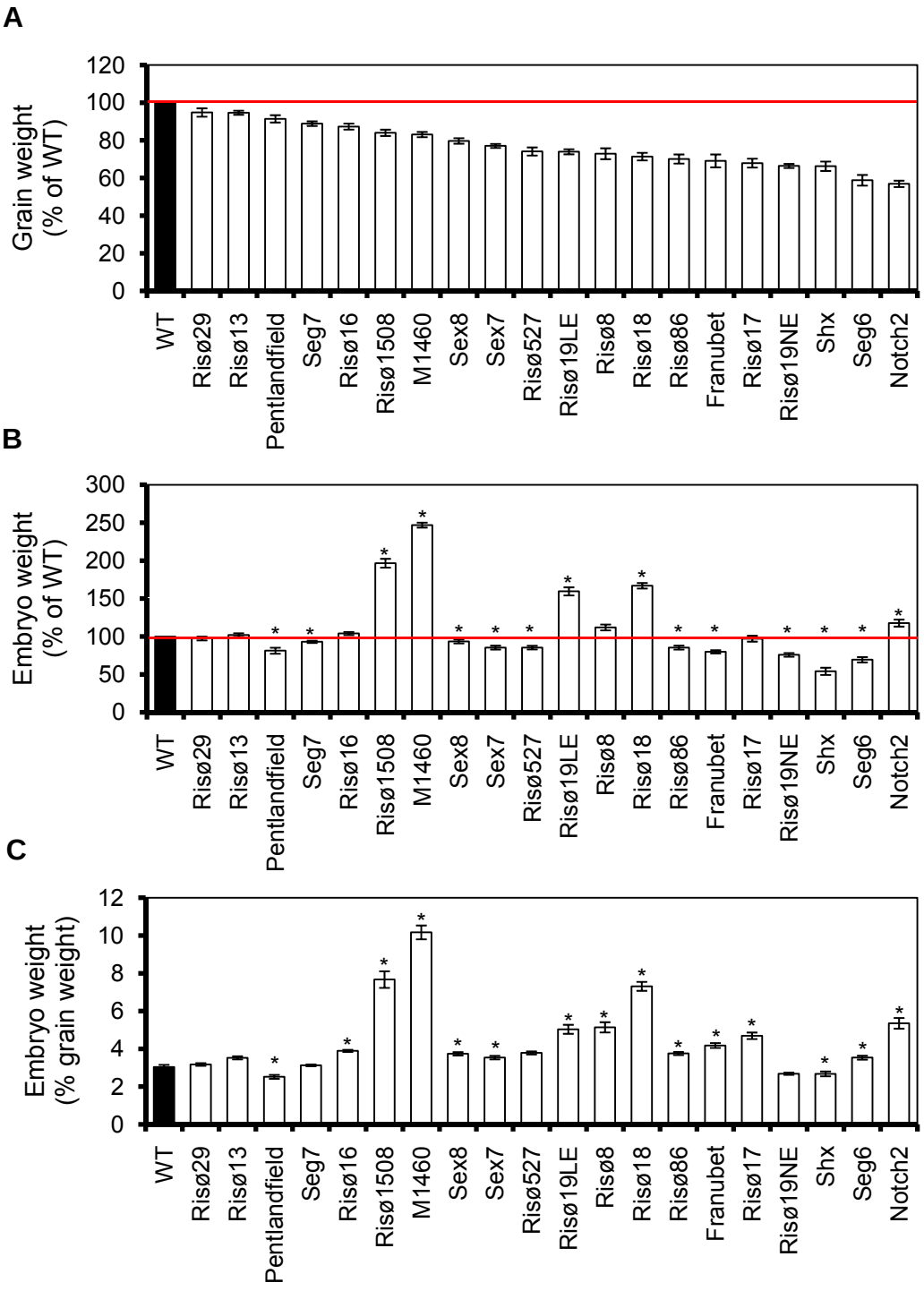

Figure 3.2 – Grain and embryo weight in barley mutants.

Mature grains and embryos (dissected from grains and freeze dried) were weighed. Values are means ± RSE (A and B) or ± SE (C) of measurements of 10 to 25 grains from a minimum of two separate plants per genotype (RSE, relative standard error = SE as percentage of the mean). Each mutant line and corresponding wild type were grown simultaneously under either glasshouse or CER conditions. Significant differences (Student's t-test, $p < 0.05$) from wild type are indicated by an asterisk. For full data see Appendix 7.1. Lines are ordered along the x-axis according to their reduction in grain weight.

there may be genetic difference in the background. The fifth line where there is no difference in embryo weight compared to wild type is the mutant Risø8 which carries a lesion in an unknown gene at the *Lys4* locus.

Of the 10 lines with lower embryo weight than wild type, only two (Risø86 and Sex7) have lesions in identified genes. Reductions in embryo weight are not limited to lines with severe effects on grain weight, suggesting that the effects on the embryo not are directly caused by endosperm shrivelling. For example, Pentlandfield Glacier has only a 9 percent reduction in grain weight and has a smaller than wild type embryos.

To further examine the relationship between grain weight and embryo weight, the embryo weight was expressed as a percentage of grain weight (Figure 3.2C; Appendix 7.1). This allows a direct comparison between the reduction in grain weight in each mutant line and the size of the embryo, thus allowing identification of lines in which the embryo weight is disproportionately affected compared to endosperm weight. In wild-type barley line, embryo weight accounted for between 2.4 percent and 3.5 percent of mature grain weight. Two mutant lines, Pentlandfield Glacier (2.5 percent of mature grain) and Shx (2.7 percent of mature grain), had significantly reduced proportional embryo weight compared to their respective wild types (Figure 3.2C). Five mutant lines had no significant difference in proportional embryo weight compared to wild-type and the remaining 13 lines had a significant increase in proportional embryo weight compared to wild type. Unsurprisingly, given the increase in absolute embryo weight, the *lys3* mutants had the most dramatic effect; the embryo was 10 percent of the grain weight in the case of M1460. There were a further nine lines showing proportional increases in embryo weight to varying degrees; those lines with the greatest increase in proportional embryo weight were allelic mutants Risø17 (4.7 percent of mature grain) and Notch2 (5.4 percent of mature grain), and mutant Risø8 (5.1 percent of mature grain). Five lines had no significant difference in proportional embryo weight (Risø29, Risø13, Seg7, Risø527 and Risø19NE).

In conclusion, the *lys3* mutants are unique among the shrunken-grain mutants as they have enlarged embryos compared to wild type. The increase in embryo weight of Notch2 was not attributable to the lesion in *isa1* as the allelic Risø17 did not show a similar increase. In all the other lines, the mutations had either no effect on absolute embryo weight (five lines) or a reduction in absolute embryo weight compared to wild type (10 lines). As well as in the *lys3* mutants, proportional embryo weight was higher than wild type in nine other lines. In these lines, there was either no decrease in absolute embryo weight or the decrease in grain weight was greater than the decrease in absolute embryo weight. The lines with the largest increases in proportional embryo weight were *isa1*

mutants (Notch2 and Risø17) and Risø8. In just two lines (Shx and Pentlandfield Glacier) there was a decrease in proportional embryo weight.

3.3.2. Embryo starch content in wild-type cultivar Bomi

The pattern of starch accumulation in wild-type embryos was determined in order to identify an appropriate time during embryo development to make comparisons between wild type and mutant embryos. Embryo starch content was measured from 20 days after flowering (DAF) until 45 DAF (Figure 3.3). The pattern of starch accumulation was the same as that previously described for wheat embryos (Black *et al.*, 1996). The starch content of the barley embryo was highest at 30 DAF. Therefore, 30 DAF is ideal for determining if there are differences in starch content between the embryos of wild type and shrunken mutant barleys.

3.3.3. Starch content of mutant embryos compared to wild type

To investigate whether starch accumulation is increased in the embryo when starch accumulation in the endosperm is reduced, the starch content of the embryos of four low endosperm starch lines was examined (Risø16 [*Agps1*], Risø17 [*Isoamylase1*], Risø1508 and Risø8). Risø16 was chosen as it carries a lesion in a gene that is expressed only in the endosperm (Rösti *et al.*, 2006). It has previously been shown to accumulate starch to normal levels in developing embryos (Rösti *et al.*, 2006). Risø17 was chosen as it carries a lesion in a gene that is likely to be expressed constitutively in both endosperm and embryo and because the embryos were normal in weight despite a reduced grain weight. Risø1508 and Risø8 were chosen because they have larger than normal embryos. Starch and soluble sugars were measured in developing embryos of mutant lines and wild-type Bomi (Figure 3.4). Embryo starch content was significantly lower than wild type in Risø16 (28 percent lower), Risø17 (29 percent lower) and Risø8 (13 percent lower) compared to wild type but not significantly different in Risø1508 compared to wild type (Figure 3.3A). The reduction in starch content in Risø17 may be due to the mutant making some water-soluble phytoglycogen in the embryo which is not included in the starch assay.

For comparison, the starch content of mature whole grains was measured (Table 3.3). In all three mutants with reduced starch content in the embryo, the proportional reduction in starch content in the embryo was smaller than the proportional reduction in the grain compared to wild type. Of the three lines with reduced embryo starch content, only Risø16 had an increase in soluble sugars compared to wild type. Risø16 embryos had 28 percent more glucose and 38 percent more fructose but not significantly more sucrose than wild type (Figures 3.4B and 3.4C). The sugar contents of Risø17, Risø1508 and Risø8

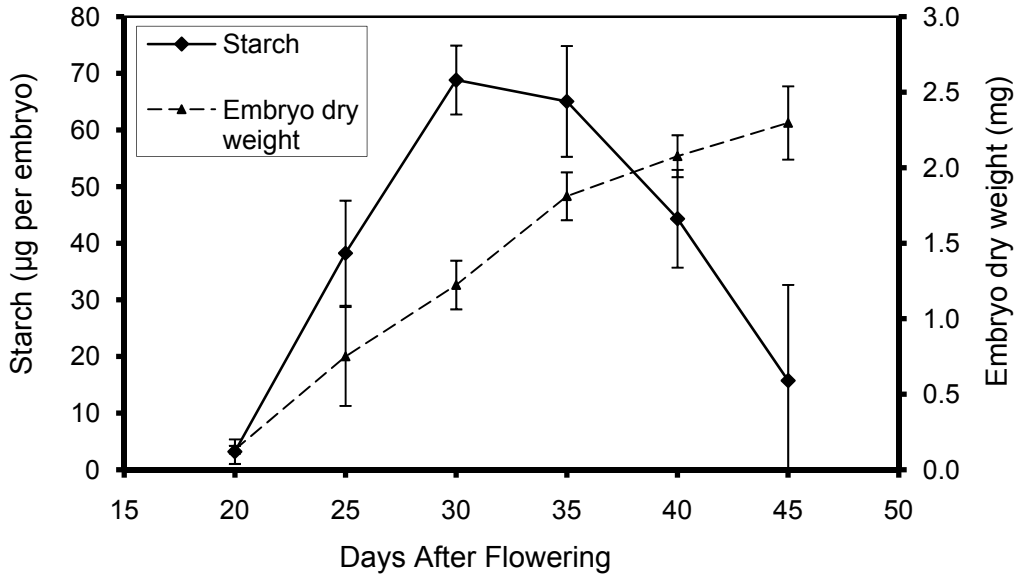

Figure 3.3 – Starch accumulation in developing barley embryos.

Embryos were dissected from developing grains of variety Bomi, freeze-dried, weighed and analysed for starch content. Values are means ± SD of samples from five plants, each consisting of six to eight embryos. Plants were grown under glasshouse conditions.

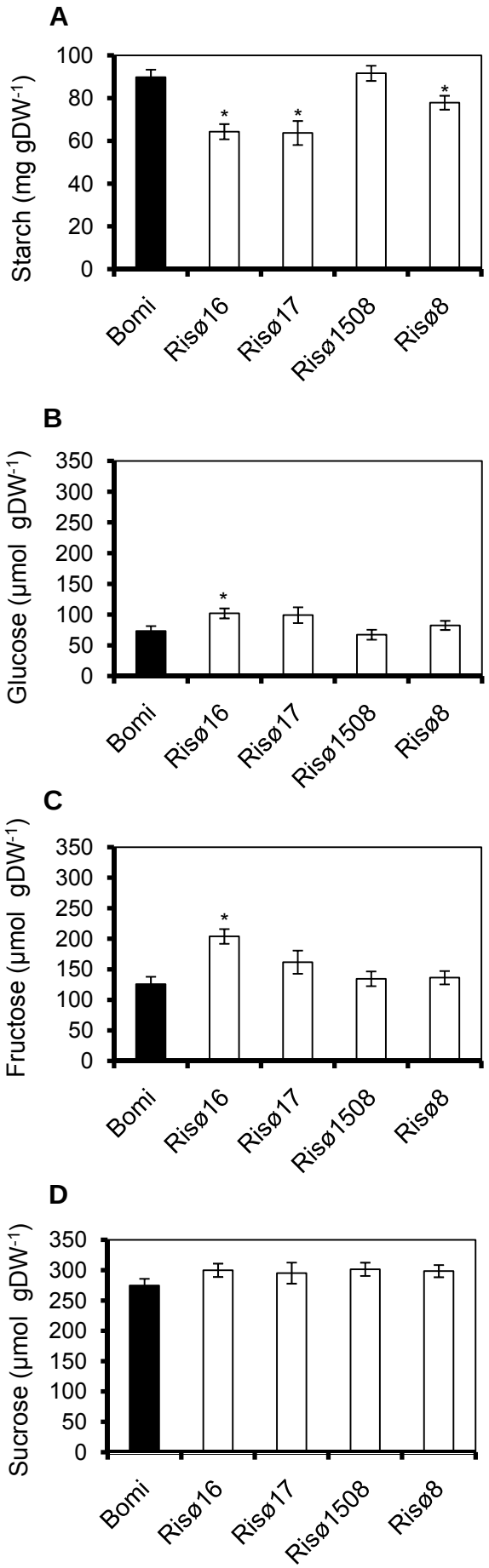

Figure 3.4 – Starch and sugar contents of developing barley embryos.

Embryos were dissected from developing grains at 30 DAF, weighed, freeze-dried and analysed for starch and sugar content. Values are means ± SE of measurements of between two and six samples, each of six embryos (Bomi n = 5, Risø16 n = 5, Risø17 n = 2, Risø1508 n = 5, Risø8 n=6). Each sample was from a separate plant grown under glasshouse conditions. Statistical analysis was carried out using GLM (model fitted = constant + line) with pair-wise comparisons by t-test. Statistical difference at $p < 0.05$ from wild type Bomi are indicated by an asterisk.

embryos were not significantly different from those of the wild-type Bomi. The results for Risø17 should be regarded with some caution as the means are only two values in Figure 3.4. This may be insufficient for statistical detection of small difference in means. However, as each value is itself the mean of six embryos measured in a single sample, the values themselves are likely to be accurate reflections of the population.

Table 3.3 – Starch content of mature grains.

Line	Grain weight (mg)	Starch (mg g^{-1})	Starch content (% of parental line)
Bomi	**51.4 ± 3.4**	**454 ± 15**	**100**
Risø16	47.2 ± 2.8	292 ± 8	64
Risø17	38.6 ± 2.4	134 ± 18	30
Risø1508	47.0 ± 2.3	398 ± 8	88
Risø8	37.8 ± 2.4	365 ± 29	80

Whole grains were weighed and their starch content determined as described in Materials and Methods. Bomi is the parental control line. Values are means ± SE of measurements of five individual grains, each from a separate plant.

Together these data show that, in the shrunken mutants examined, there was no evidence of higher embryo starch contents compared to wild type, as was reported by Olsen *et al.* (1984). In fact, a decrease in starch content was observed in Risø16, Risø17 and Risø8. Previous measurements of Risø16 embryo starch content did not reveal a difference compared to wild-type (Rösti *et al.*, 2006). This discrepancy may be due to difference in age of the developing embryos or variations in growing conditions. Decreases in embryo starch were minor in comparison to those in the whole grain/endosperm. There is no evidence of altered sugar metabolism in the embryos of Risø17, Risø1508 or Risø8, whereas the embryo of Risø16 has higher sugar content.

3.3.4. Protein content of mutant embryos compared to wild type

To investigate whether the protein content in the embryos of the shrunken mutants is altered, the protein content of the embryos was measured at maturity. In the majority of lines, there was no statistically significant difference in the amount of protein in the embryo compared to wild type (Figure 3.5A; Appendix 7.2). None of the mutants examined accumulated more protein in the embryo than wild type (Figure 3.5A). Protein content was statistically significantly lower than normal in the embryos of three of the *lys3* mutants Risø1508 (28 percent lower), Risø19 (50 percent lower) and M1460 (36 percent lower) although not statistically significantly lower in Risø18. This discrepancy between Risø18 and the other *lys3* mutants may be because replication was insufficient to detect small differences between means, although differences in the genetic background between the lines cannot be ruled out. Protein content was also statistically significantly

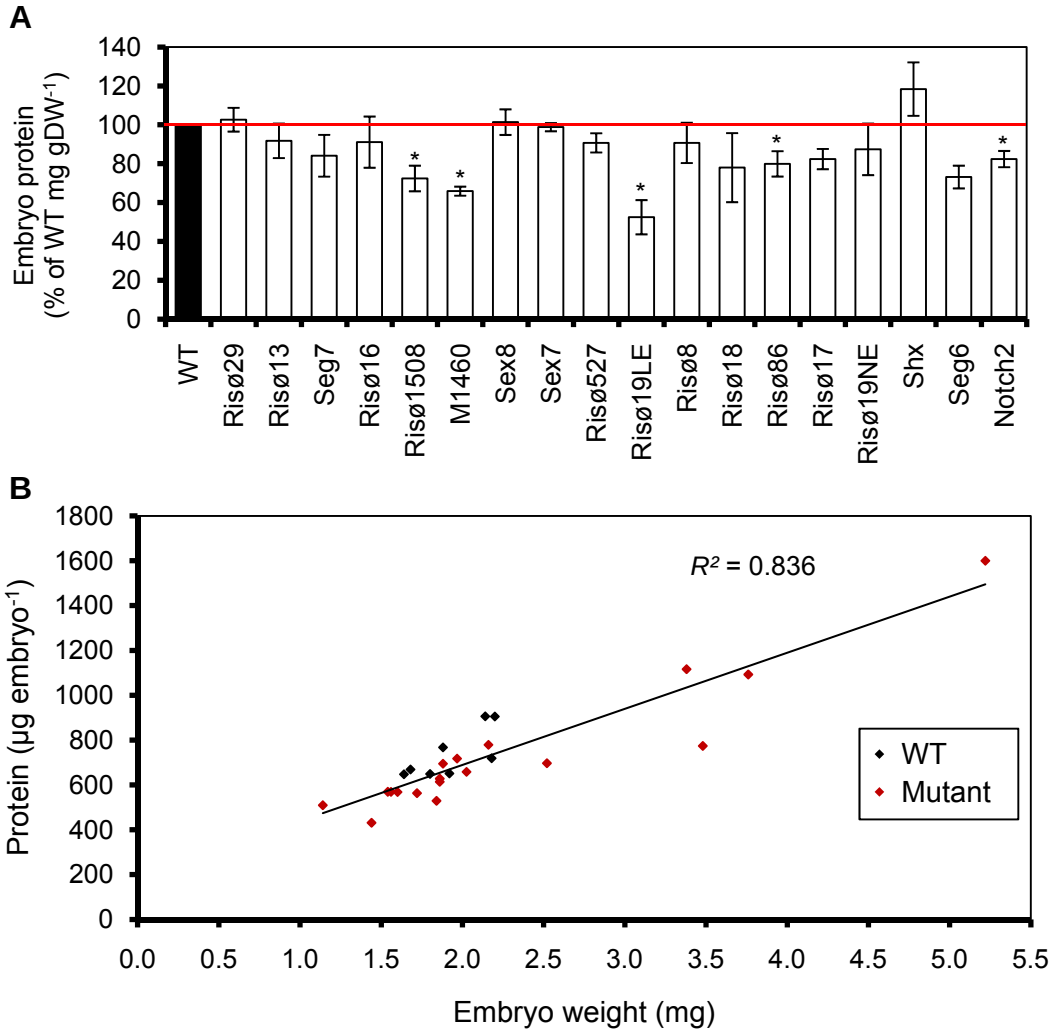

Figure 3.5 – Protein content of barley embryos.

Embryos were dissected from mature grains, freeze-dried, weighed and analysed for protein content. Values are means (B) or means ± RSE (A) of measurements of three to five individual embryos (RSE, relative standard error = SE as percentage of the mean). Each mutant line and its corresponding wild type was grown simultaneously under the same conditions. Lines are ordered along the x-axis (A) according the their grain weight in Figure 3.2A. Significant differences (Student's t-test, $p < 0.05$) from wild type are indicated by an asterisk. For full data see Appendix 7.2.

reduced in the embryos of Notch2 (*isa1*; 18 percent lower) and Risø86 (*nst1*; 20 percent lower).

Across the wild-type cultivars and mutant lines, embryo protein content (µg per embryo) was positively correlated with embryo weight (Figure 3.5B; R^2 = 0.836). This correlation shows that embryo size largely determines the total amount of protein in the embryo. However, there was no relationship between either mean grain weight (Figure 3.2A) and total embryo protein (µg per embryo) or mean grain weight and protein concentration in the embryo (mg gDW^{-1}).

3.3.5. Micronutrient content of *lys3* mutants compared to wild type

The embryo is relatively rich in micronutrients (see Section 1.3.4) (Lyons *et al.*, 2005). To determine whether the large-embryo phenotype in *lys3* mutants makes a positive contribution to total grain micronutrient content, micronutrient content was measured in M1460 and wild-type cultivar Minerva. Although not tested statistically due the the small number of biological replicates (n = 3), M1460 flour was found to have higher mean concentrations of five micronutrients: potassium, phosphorus, magnesium, sulphur and iron than parental wild-type Minerva (Figure 3.6). In absolute terms, there was a reduction in the total amount, per grain, of all the micronutrients examined in M1460 compared to wild type. This was due to the reduction in grain weight in M1460 compared to wild type (Figure 3.7). However, in many cases because the embryos are larger, the effect of a reduction in grain weight on total micronutrient content is ameliorated by an increase in micronutrients contributed by the embryo. Of the micronutrients examined, with the exception of iron, sodium and copper, all were more abundant in the embryos of M1460 than wild-type embryos.

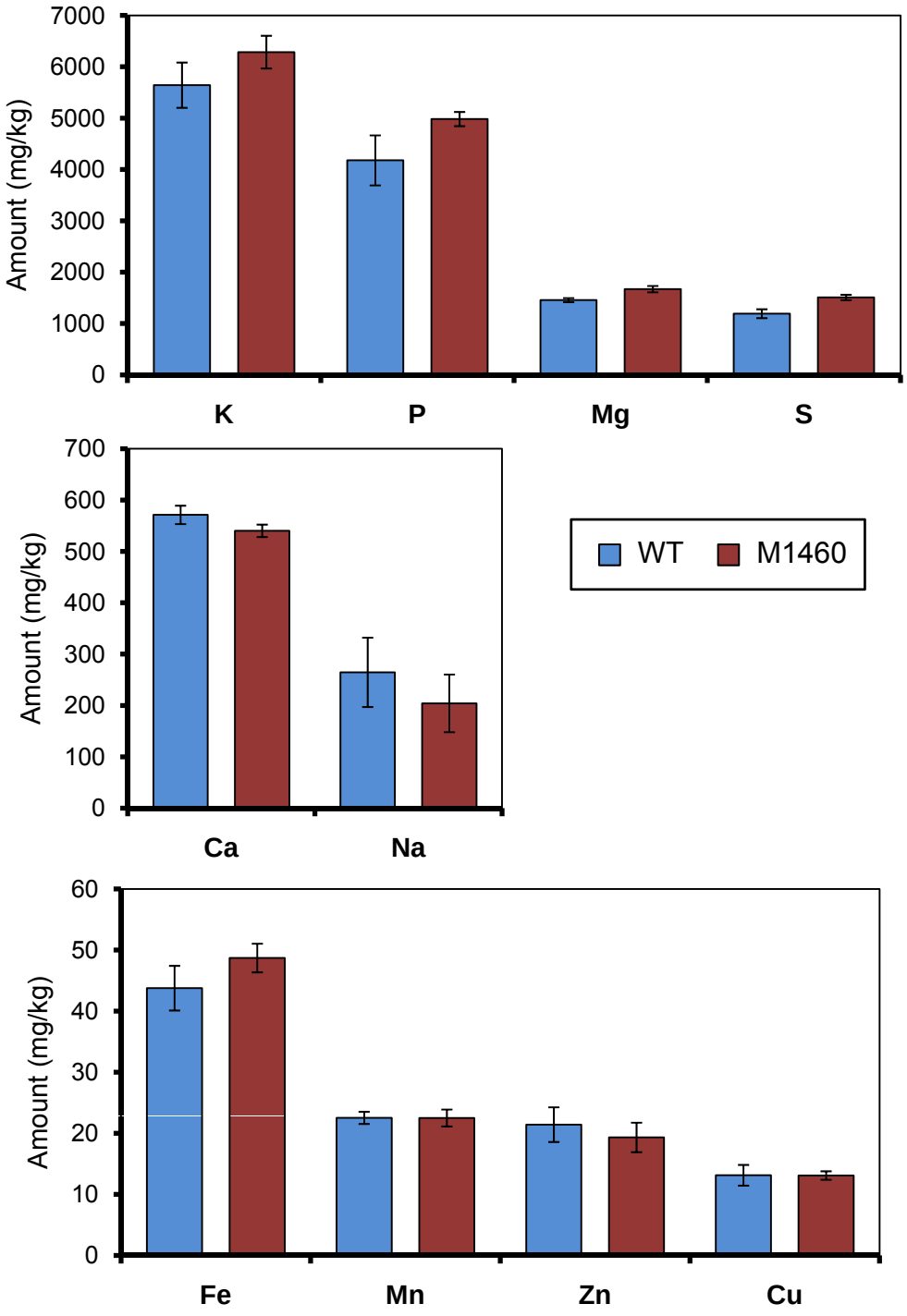

Figure 3.6 – Micronutrient concentration of M1460 flour compared to wild-type Minerva flour.

Samples of whole grains were milled to flour (Tecator Cyclotec) and micronutrient content was determined by inductively coupled plasma mass spectrometry (ICP-MS) by Dr Fangjie Zhao at Rothamsted Research. Values are means ± SD of measurements of three samples.

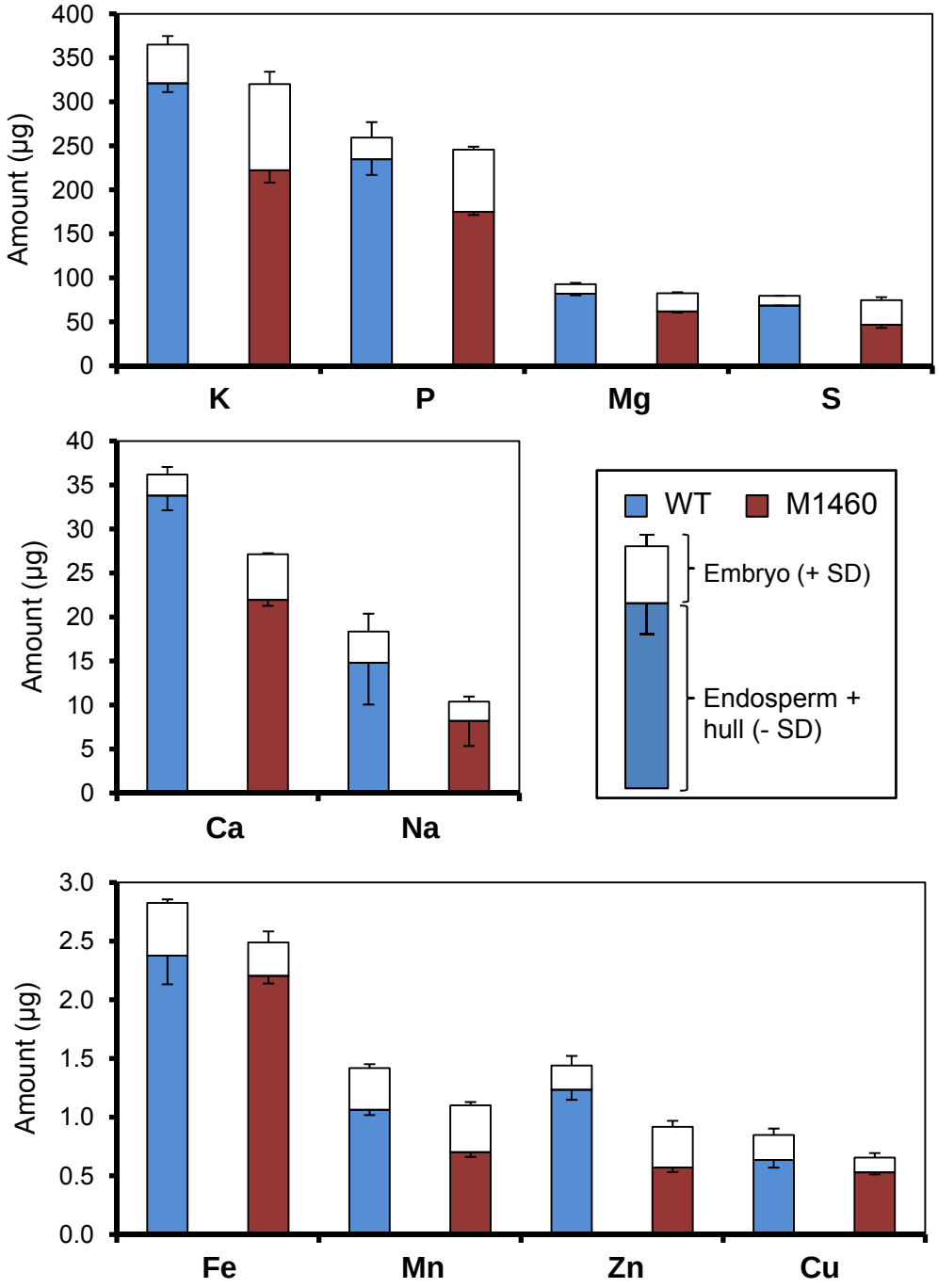

Figure 3.7 – Relative micronutrient contents of embryo and endosperm of M1460 grain compared to wild-type Minerva.

Embryo-depleted samples (with the embryos removed by hand) of Minerva and M1460 grain were milled to flour (Tecator Cyclotec) and micronutrient content of the flour was determined by inductively coupled plasma mass spectrometry (ICP-MS) by Dr Fangjie Zhao at Rothamsted Research. The micronutrient content of the embryo was calculated using the difference between measurements of whole grain (Figure 3.6) and embryo-depleted samples. Values are means - SD (endosperm + hull) or + SD (embryo) of measurements of three samples.

3.4. Discussion

The work in this chapter has shown that a lesion at the *Lys3* locus causes an increase in embryo weight compared to wild-type parental lines, in agreement with Tallberg (1977). As other shrunken lines do not have larger embryos compared to wild type, it is possible to conclude that the increase in embryo size in *Lys3* mutants is not a direct result of reduced starch biosynthesis in the endosperm. Therefore *Lys3* mutants aside, there is no evidence that a reduction in starch biosynthesis in the grain leads to an increase in embryo weight. In several lines, there was no difference in embryo weight compared to wild type. However, the majority of mutant lines showed a concomitant reduction in both grain weight and embryo weight. In lines where embryo weight was unaffected in the mutant, the reduced endosperm resulted in an increase in proportional embryo weight. There were four lines which can be classified as large-embryo mutants on such a proportional basis namely: Risø17, Notch2, Risø16 and Risø8. Of these lines, Risø17, Notch2 and Risø16 contain lesions in starch biosynthetic genes. Starch biosynthesis is integral to determining endosperm filling and therefore endosperm weight, but is of little quantitative importance in the embryo at maturity. This suggests that the proportional increase in embryo weight in lines with lesions in starch biosynthetic genes is likely to be due to endosperm-specific reductions in weight rather than an effect directly on the determination of embryo and/or endosperm weight. Risø8, also has proportionally large embryos, and contains a lesion in an unknown gene therefore the mechanism of gene action is also unknown.

Of the lines with reduced embryo weight compared to wild type, there was a tendency towards a small but statistically significant increase in proportional embryo weight compared to wild type. This suggests that the lesions in these lines have a greater effect on endosperm development than embryo development. There were two notable exceptions, Shx and Pentlandfield Glacier, which showed a greater decrease in embryo weight than in grain weight. These lines can therefore be classified as small-embryo mutants on a proportional basis. Both lines contain lesions in unknown genes.

There is no evidence that a reduction in starch biosynthesis in the endosperm leads to an increase in starch or protein biosynthesis in the embryo (Figures 3.3 and 3.4). In the starch mutants Risø16 and Risø17, there were small but significant reductions in the starch content of the developing embryos compared to wild type, but this was not accompanied by either a difference in weight or a difference in protein content at maturity. Risø16 carries a lesion in the gene encoding the cytosolic AGPase small subunit which is not present in the embryo (Rösti *et al.*, 2006). The reduction in embryo starch content is

therefore likely to be an indirect effect of perturbations in endosperm metabolism. Risø17 carries a lesion in a gene encoding starch debranching enzyme isoamylase1 and is likely to be constitutively expressed in both embryo and endosperm. Therefore, the decrease in granular starch content of Risø17 embryos compared to wild type may be due to either direct effects of the lack of isoamylase1 activity in the embryo, or an effect of perturbations in the endosperm, or both. However, it should be noted that the decrease in the starch content of the embryo in *isa1* mutants is minor compared to the decrease in starch content of the endosperm (Burton *et al.*, 2002a). The way in which *Isa1* functions in barley grains is not fully understood. The endosperms of *isa1* mutants have abnormal starch-granule morphology and contain much higher levels of phytoglycogen than wild type (Burton *et al.*, 2002a). The way in which *Isa1* acts on starch in the embryo was not determined. Risø8 was also found had moderately reduced starch in the embryo compared to wild type but, as with Risø16 and Risø17, there was no effect on protein accumulation in the embryo or lead to differences in embryo weight.

Lesions at the *Lys3* locus result in greater embryo weight, greater total embryo protein (despite a reduction in protein per gram dry weight) and greater amounts of a number of micronutrients in the embryo (although micronutrient data was not tested for statistical significance). This indicates that *Lys3* could be useful locus for increasing the nutritional value of barley. However, the negative effects of mutations at *Lys3* on endosperm weight and therefore on yield (Bang-Olsen *et al.*, 1991) must be overcome to fully realise this potential.

To understand the function of the gene underlying the *Lys3* locus, it is necessary to first identify the gene. A map-based (positional) cloning approach to identifying the gene is not a trivial undertaking in barley. Another approach is to identify orthologous mutations in cereal species for which there is a complete genome sequence. These approaches are investigated and discussed in the following chapter.

4. Identification of genes responsible for controlling embryo size in barley

4.1. Aim

The aim of the work in this chapter is to identify the genes responsible for controlling embryo size in barley.

4.2. Introduction

In the previous chapter, it was confirmed that *lys3* mutants have larger embryos than wild type. The previous chapter also showed that reductions in grain weight and embryo weight were often concomitant in barley lines with shrunken grain. Lesions in starch biosynthetic genes led to increases in embryo weight as a proportion of grain weight due to reductions in endosperm weight. Risø8, containing a lesion in an unknown gene at the *Lys4* locus, also lead to an increase in proportional embryo weight. As *lys3* and *lys4* mutant lines do not follow the pattern of concomitant reduction in endosperm and embryo weight described in Chapter 3, they may therefore provide useful insight into the developmental interactions between embryo and endosperm. Identification of the underlying genes is essential for the understanding of these traits at a molecular level. *Lys3* mutants are the main focus of this work as they have large-embryos in both absolute and relative terms. Risø8 (*lys4*) is included for comparison.

Currently, *Lys3* and *Lys4* have been located within genetic maps of the barley genome based on morphological markers (Franckowiak, 1997). A promising approach to identify the genes at these loci, in the absence of detailed physical maps or genome sequences, is map-based (positional) cloning. In the Triticeae, this approach has been successfully used to identify a growing number of genes underlying loci controlling a range of traits of interest (Krattinger *et al.*, 2009). This approach requires a fine-mapping population, segregating for the trait of interest, and a high density of molecular markers with which to refine the genetic map of the locus before turning to a BAC library to identify candidate genes within the fine-mapped region. This process is hampered by the large genome size of barley and the requirement that there are sufficient molecular markers in the region of interest. However, conservation of gene order (colinearity) between the barley genome and the fully-sequenced genomes of rice and *Brachypodium* (Moore *et al.*, 1995; International Rice Genome Sequencing Project, 2005; The International Brachypodium Initiative, 2010) provides the possibility of inferring the genes present in region of interest in the barley genome. While this approach has its limitations, as not all regions of the

barley genome are colinear with regions of rice and/or *Brachypodium*, it has the potential to accelerate the identification of candidate genes.

The fine-mapping of *Lys3* and *Lys4* is taking place in our laboratory. The primary aim of the work described in this chapter is to inform the identification of candidate genes by characterising the phenotypes of these mutants. Secondly, I aimed to identify from the literature similar (orthologous) mutants in other grass species, to provide a list of gene classes that may have a role in controlling embryo size in grasses.

Risø1508 (*lys3a*) was the first of *lys3* mutant alleles to be described and therefore most of the previously published work on *lys3* mutants includes only work on Risø1508. Risø1508 (*lys3a*) has attracted attention for its improved nutritional value (for review see Munck 1992). Originally selected for high lysine content (Mossberg, 1969; Ingversen *et al.*, 1973), grains of Risø1508 have altered protein composition such that there is an increase in essential amino acids such as lysine and threonine, a decrease in non-essential amino acids such as proline and glutamate, and more oil by percentage weight (Munck, 1976). In addition, the larger-than-normal embryo of M1460 (*lys3m*) was shown in the previous chapter to contain more micronutrients than the embryo of wild-type Bomi. As noted in Chapter 3 and widely reported in the literature, Risø1508 also has a shrunken-grain phenotype and a reduction in starch content, which negatively impacts on yield. The negative impact on yield can be reduced by modifying the genetic background (Munck, 1972; Bang-Olsen *et al.*, 1991). By breeding Risø1508 with a high yielding cultivar, it was possible to narrow the yield gap but not to close it completely (Bang-Olsen *et al.*, 1991; Munck, 1992). Animal feeding trials, using Risø1508-derived lines Piggy and Lysimax, have shown that barley lines containing the *lys3a* mutant allele are a more effective source of protein (i.e. for maximum growth rate) than the wild-type barley varieties from which they are derived (Munck, 1972; Mortensen *et al.*, 1988; Gabert *et al.*, 1995; Gabert *et al.*, 1996). It has also been suggested that the improved amino acid profile of *lys3* barley will reduce the required protein concentration in animal feed and therefore reduce the amount of nitrogen entering the environment from animal faeces (Munck and Jespersen, 2009). Identification of the gene lying at the *Lys3* locus may allow separation of the favourable (nutritional enhancements) and unfavourable (yield depression) traits. This would potentially allow more effective utilisation of the *lys3* trait in barley and in other cereals.

Work in the past 40 years has described the diverse effects of lesions at *Lys3,* in particular in line Risø1508, on protein, carbohydrate and lipid accumulation in the developing grain. This information is presented in the following paragraphs. The increase

in lysine in the grain is mainly due to alterations in endosperm protein composition rather than to the larger embryo (Tallberg, 1977). In the endosperm, there is a reduction in the lysine-poor prolamin (hordein) proteins and an increase in free lysine and other more lysine-rich protein fractions (Ingversen *et al.*, 1973; Brandt, 1976). The reduction in total prolamin content is due to reductions in the major prolamins (by weight), B- and C-hordein (Kreis *et al.*, 1984). Although there is a increase in minor prolamin (by weight), D-hordein, this is insufficient to compensate for the decrease in B and C hordein (Kreis *et al.*, 1984). The reduction in B- and C-hordein is correlated with a decrease in expression of the genes encoding these proteins (Kreis *et al.*, 1984; Sørensen *et al.*, 1989). The reduced expression is correlated with alterations in the methylation state of the promoter regions of these genes (Sørensen, 1992). The correlation between methylation of the promoter and repression of gene expression has also been observed for zein genes in maize endosperm (Bianchi and Viotti, 1988). In wild-type Bomi, the promoter of B-hordein was methylated in leaf tissue and demethylated in endosperm tissue, reflecting expression of B-hordein in the endosperm and repression of B-hordein the leaves. In Risø1508, the promoter was found to be methylated in both leaf and endosperm tissue (Sørensen, 1992). *In vitro* methylation of B- and D-hordein promoters fused to GUS demonstrated that methylation was capable of inhibiting gene expression when transiently expressed in Bomi and Risø1508 endosperm (Sørensen *et al.*, 1996). Transient expression of unmethylated B- and C-hordein promoters fused to GUS and expressed in Bomi and Risø1508 endosperm tissue suggested that C-hordein-promoter-driven expression in Risø1508 was recovered to that of the expression in Bomi endosperm whereas B-hordein-promoter-driven expression was not (Sørensen *et al.*, 1996). This suggests that the action of *Lys3* is required for demethylation of B- and C-hordein promoters but its actions are not on hordein gene methylation state alone. The level of methylation of the D-hordein promoter is unaffected in Risø1508 (Sørensen *et al.*, 1996).

The reduction in starch content in mature grains of Risø1508 is responsible for most of the reduction in grain weight in this mutant compared to wild-type Bomi (Kreis, 1978, 1979; Salomonsson *et al.*, 1983). The rate of starch accumulation during development is lower in Risø1508 than in wild type grains; this is not due to sugar limitation as Risø1508 grains accumulate higher levels of soluble sugars (Kreis, 1978). AGPase activity in the developing grain is unaltered in Risø1508 compared to wild-type Bomi (Kreis, 1979). The reason for reduced starch biosynthesis in the endosperm is not currently understood.

In addition to the altered starch and protein levels of Risø1508 grains, a number of other phenotypes have been described. Risø1508 has higher amounts of lysine-rich

chymotrypsin inhibitor protein (CI-1) in the mature grain and increased expression of chymotrypsin inhibitor genes CI-1 and CI-2 during endosperm development (Williamson *et al.*, 1988). Germinating Risø1508 grains have reduced diastatic power, of which β-amylase is a major constituent, and reduced β-amylase gene expression in developing endosperm (Allison, 1978; Kreis *et al.*, 1987). Independent *lys3* mutants Risø1508 (*lys3a*), Risø18 (*lys3b*) and M1460 (*lys3m*) have less than half the β-glucan in the mature grain than their respective wild-type lines (Aastrup, 1979, 1983; Munck *et al.*, 2004). However, Risø19 (*lys3c*) has near-normal levels of β-glucan. This discrepancy may be due to differences between the mutant alleles themselves or due to differences between the genetic backgrounds of the lines. There is also evidence of altered phytohormone levels in the developing grain of Risø1508. Mounla *et al.* (1980) found lower levels of auxin in the developing grain and higher levels of gibberellin-like activity. In addition, lipid metabolism is altered in Risø1508 grain compared to wild-type. ^{14}C-feeding experiments showed that Risø1508 accumulated greater quantities of both polar and non-polar lipids during endosperm development (Shewry *et al.,* 1979). The increase in embryo size in Risø1508 grains also contributes to the higher oil content (by percentage weight) (Welch, 1978; Bhatty and Rossnagel, 1979, 1980).

As well as the wide range of metabolic changes outlined above, perturbations to cell expansion and cell division in the developing embryo and endosperm of Risø1508 have been described. The central endosperm cells stop expanding earlier in Risø1508 than in wild type Bomi (Olsen and Krekling, 1980). Risø1508 embryos are enlarged due to scutellar parenchyma cells having a greater cross-sectional area than wild type (Olsen *et al.*, 1984; Deggerdal *et al.*, 1986). The adaxial epithelial cells of the scutellum were found to have an abnormal shape (Deggerdal *et al.*, 1986). The complex effects of a lesion at the *Lys3* locus make it impossible to distinguish whether the primary effect of the mutations are at the metabolic or the developmental level. Indeed, these options may not be mutually exclusive.

In contrast to Risø1508, Risø8 (*lys4*) has received little research attention since its first description by Doll *et al.* (1974) as a high-lysine line. The *Lys4* locus is on chromosome 1H and the mutant allele shows incomplete dominance with respect to the shrunken-grain phenotype (Ullrich and Eslick, 1978a; Franckowiak and Lundqvist, 1997). Like Risø1508, Risø8 grains have reduced mature grain weight, increased protein as a percentage of grain weight and altered protein composition compared to wild type Bomi (Olsen *et al.*, 1984). Germinating Risø8 grains, like Risø1508 grains, also have lower diastatic power than wild type (Allison, 1978). The limited ultrastructural data available show that

endosperm cells are smaller in Risø8 than wild type at 33 days after flowering, and that there is no difference in cell size in the scutella of Risø8 compared to the wild type (Olsen *et al.*, 1984).

To identify possible interactions between the phenotypes of *lys3* mutants and other lines, with mutations at independent loci, homozygous double mutant lines of Risø1508/Risø8, Risø1508/Risø16 and Risø1508/Risø17 were analysed. Previous studies have shown that the Risø1508/Risø527 double mutant has a greater reduction in grain weight than either of the respective single mutants (Kreis and Doll, 1980; Klemsdal *et al.*, 1986). This suggests that the mutations in Risø1508 (*lys3*a) and Risø527 (*lys6*) are acting independently of one another. The mutations in Risø1508 and Risø527 may interact synergistically with respect to the embryo phenotype. The effect of both mutations (in the Risø1508/Risø527 double mutant) on embryo weight at 24 and 33 days after flowering is greater than the sum of the effects of the two mutations independently. Deggerdal *et al.* (1986) reported that Risø527 embryos were 13 percent heavier than wild type and Risø1508 embryos were 56 percent heavier than wild type at 33 days after flowering, whereas the embryos of the double mutant were 122 percent heavier at the same time point.

As noted previously, an aim of this work was to identify possible orthologous mutations in other grass species. The gene responsible for the *giant embryo* (*ge*) mutant of rice was identified as a possible orthologue because the phenotype of the *ge* mutant grain is similar to that of *lys3* mutants (Satoh and Omura, 1981). Grains of *ge* rice have a larger embryo then wild type rice due to an increase in the size of the scutellum (Figure 4.1) (Hong *et al.*, 1996; Nagasawa *et al.*, 2003). The increased embryo size is also accompanied by a reduction in endosperm size (Nagasawa *et al.*, 2003). Grains of *ge* rice also have nutritional enhancements similar to those of the *lys3* mutants including high lipid, high protein and high lysine (Zhang *et al.*, 2005). The rice *Ge* gene encodes a CYP78A13 protein (Cahoon *et al*., 2006) that belongs to the same family of cytochrome P450s as the *Arabidopsis* KLUH/CYP78A5 protein (Anastasiou *et al.*, 2007). *Arabidopsis kluh* mutants have reduced leaf, flower and seed size, and over-expression of *KLUH/CYP78A5* leads to larger leaves, flowers and seeds (Anastasiou *et al.*, 2007; Adamski *et al.*, 2009). *KLUH/CYP78A5* regulates organ size through non-cell-autonomous control of cell proliferation and it has therefore been suggested to be in involved in the production or transduction of a signal molecule (Adamski *et al.*, 2009; Mizutani and Ohta, 2010). The identity of the signal is not known but it appears to be distinct from known phytohormones: *KLUH* regulates genes that are different from known hormone-responsive

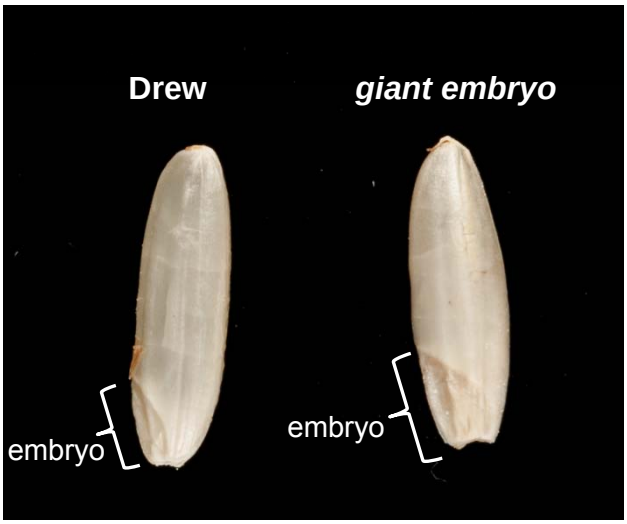

Figure 4.1 – Mature rice grain of *giant embryo* (*ge*) rice compared to parental wild-type cultivar Drew.

genes in *Arabidopsis* (Anastasiou *et al.*, 2007). CYP78A cytochrome P450s thus represent a class of genes already known to have a role in regulation of organ size. It is therefore possible that the gene underlying *Lys3* is in this class.

Cytochrome P450s are a large family of proteins which carry out oxygenation/hydroxylation reactions in a wide range of pathways (for recent review see Mizutani and Ohta, 2010). CYP78A proteins are found widely in the embryophytes (land plants; Nelson *et al.*, 2008). In a comparison of six plant genomes (*Vitis vinifera, Carica papaya, Populus trichocarpa, O. sativa, A. thaliana* and *Physcomitrella patens*), there were between three and ten putative CYP78 gene sequences (Nelson *et al.*, 2008).

The only cereal CYP78A for which there is functional information is CYP78A1 in maize. *In vitro* expression of the maize CYP78A1 in *Saccharomyces cerevisiae* demonstrated that it catalyses the mono-oxygenation of lauric acid (Imaishi *et al.*, 2000) and the gene was found to be preferentially expressed in the developing inflorescence (Larkin, 1994). However, to my knowledge, no CYP78A1 mutant of maize exists and so its precise biological function is unknown. In addition to rice *ge*, one other CYP78 mutant exists in cereals, namely the rice *plastochron1* (*pla1*) mutant. *Pla1* encodes a CYP78A11 protein which is required for the normal timing of leaf initiation (Miyoshi *et al.*, 2004). Rice *pla1* mutants have rapid leaf initiation, but also have ectopic leaf formation during panicle development (Itoh *et al.*, 1998; Miyoshi *et al.*, 2004). There is no mention of abnormal seed production in *pla1* mutants in Miyoshi *et al.* (2004) which suggests that PLA1 may be of minor importance in the grain. However, to my knowledge, this has not been tested.

Cahoon *et al.* (2006) identified a barley sequence and three maize sequences with similarity to the rice *Ge* at the deduced amino acid level. These sequences were designated *HvGe* and *ZmGe1*, *ZmGe2* and *ZmGe3,* respectively. Illinois High Oil (IHO) maize, from the Illinois Long-Term Selection Experiment, has elevated oil contents in the grain, at least in part due to a large embryo size (Figure 4.2) (Moose *et al.*, 2004). Cahoon *et al.* (2006) mapped *ZmGe1* to a region on maize chromosome 7, where there is also a high-oil QTL. This high-oil QTL was identified using high-oil inbred lines developed from the Illinois High Oil (IHO) lines. *ZmGe2* was found to have reduced expression, relative to a maize cultivar with normal oil content, in the developing embryos of three high-oil maize lines including Illinois High Oil (IHO; Cahoon *et al.*, 2006). Together this suggests that *ZmGe1* and *ZmGe2* may have some functional similarity to rice *Ge*. If so, there may be a *Ge*-like gene in all cereals, including barley.

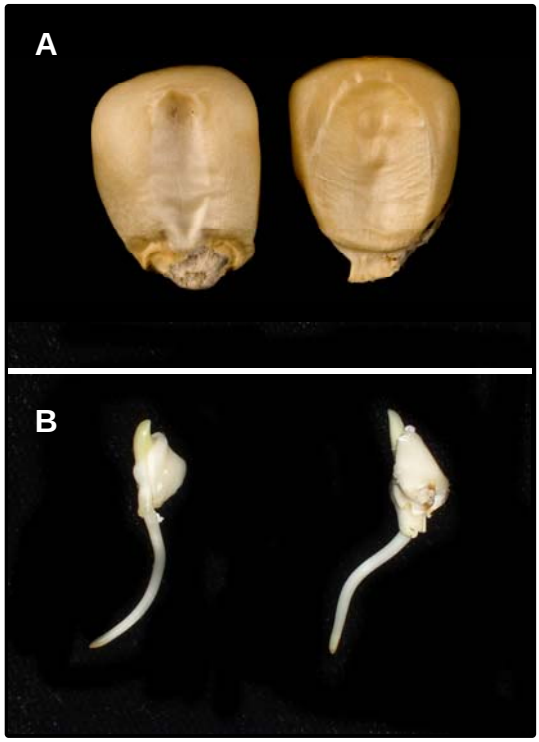

Figure 4.2 – Illinois Low Oil maize kernel (left) compared to Illinois High Oil maize kernel (right).

A – mature maize kernels
B – developing seedlings excised from the germinating kernel (~ 3 days after imbibing)

In this chapter, the roles of *Lys3* and *Lys4* in grain development were further characterised through study of *lys3* and *lys4* mutants, and the effects of the *lys3* mutations on germination and whole-plant development were investigated. The possibility that *Lys3* encodes a CYP78 with functional similarity to rice *Ge* is explored.

4.3. Results

4.3.1. Embryo and grain weight in *lys3* mutants

There are four independent lines with mutations at the *Lys3* locus. Of these, only Risø1508 (*lys3a*) had been described previously as a large-embryo mutant (Tallberg, 1977). In the previous chapter, it was shown that all four lines have large embryos compared to their wild-type parents Bomi and Minerva. The grain weight and embryo weight data for the *lys3* lines, previously presented in Chapter 3, is summarised in Table 4.1. To test whether the lesions at the *Lys3* locus is responsible for the large-embryo phenotype in all the *lys3* lines, Risø18, Risø19 and M1460 were crossed with Risø1508. F1 seed from these crosses was visually inspected and all F1 seed were found to have a large-embryo phenotype. As the mutations in these lines are known to be recessive, this indicates that the lesions giving rise to the large-embryo phenotype are in the same gene.

Table 4.1 – Grain weight and embryo weight of the four lys3 mutant lines.

Line (*allele*[d])	Number of grains	Grain weight (mg)	Difference (%)	Embryo weight (mg)	Difference (%)
Bomi[a]	10	68.2 ± 1.2		2.1 ± 0.1	
Risø1508 (*lys3a*)[a]	25	45.1 ± 0.8	-34	3.5 ± 0.2	+62
Risø18 (*lys3b*)[a]	20	48.7 ± 1.0	-29	3.6 ± 0.1	+67
Bomi[b]	25	76.6 ± 0.7		1.8 ± 0.1	
Risø19 (*lys3c*) [b]	25	56.7 ± 0.8	-26	2.9 ± 0.2	+60
Minerva[c]	25	62.0 ± 1.2		2.1 ± 0.1	
M1460(*lys3m*)[c]	25	51.5 ± 0.7	-17	5.2 ± 0.2	+147

Five grains from each of two to five plants were weighed, and the embryos from these grains were excised, freeze-dried and weighed. Values shown are means ± SE. [a]Winter glasshouse grown. [b]CER grown. [c]Summer glasshouse grown. [d]Mutant allele nomenclature used here is after Jensen and Doll (Franckowiak, 1979; Jensen and Doll, 1979) and Munck *et al.* (2004).

To determine the developmental time point at which the *Lys3* allele is required for normal grain development, two of the *lys3* mutant lines were studied in greater detail, namely M1460 (the line with the greatest effect on embryo weight) and Risø1508 (the line for which there is the most published information). The fresh weight of Risø1508 and M1460 grains differed from those of their respective wild-type controls from 20 days after flowering (the earliest time point examined here; Figure 4.3A). Between 20 and 30 days after flowering, grain fresh weight was higher than wild type in M1460 but lower than wild-type in Risø1508. Both *lys3* mutants had reduced fresh weights from 40 days after flowering, the time in development at which the grain was beginning to desiccate. In M1460, embryo fresh weight was statistically significantly higher than in Minerva at the earliest time-point examined here (Figure 4.3B; 20 DAF; $t = 5.13$, $p < 0.001$). Risø1508 embryo fresh weight was statistically significantly higher than in Bomi five days later, at 25 days after flowering ($t = 3.87$, $p < 0.001$). The increase in embryo fresh weight in the

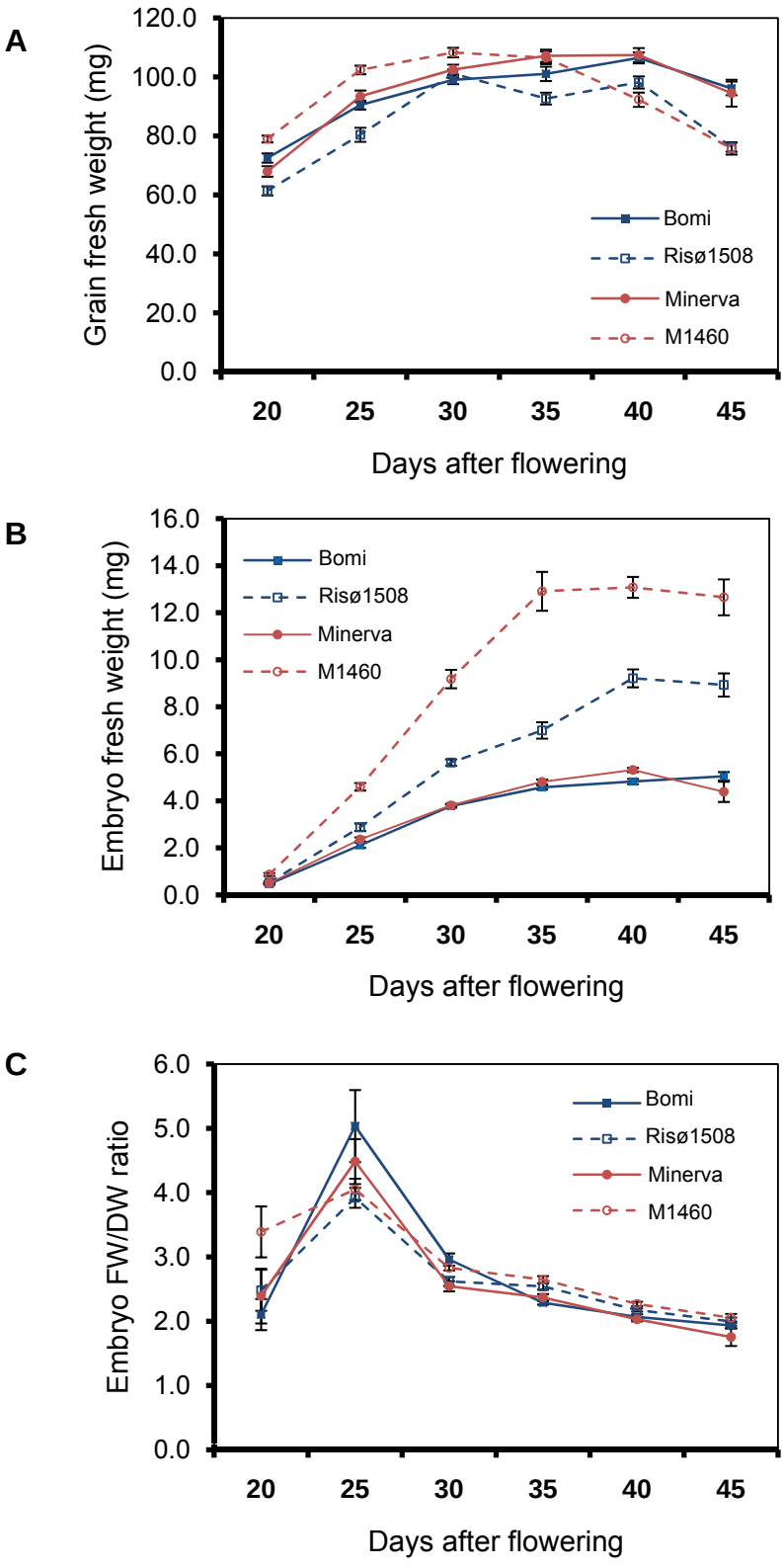

Figure 4.3 – Developing grain and embryo weights of the two *lys3* mutants compared to wild type.

Five grains from each of four to five plants (n = 20-25) were taken at each time point and weighed. The embryos from these grains were excised, weighed, dried and reweighed. Values are means ± SE.

mutants is accompanied by a proportional increase in embryo dry weight such that embryo fresh weight to dry weight ratio is unchanged (Figure 4.3C).

Taken together, these data show that *Lys3* is required for normal grain development from the earliest time point examined here (20 days after flowering). In M1460, the embryo is affected earlier and the effect is greater than in Risø1508. As Risø1508 and M1460 have differing genetic backgrounds, it is not possible to determine whether this is due to a difference between the mutant alleles or due to an effect of genetic background.

4.3.2. Embryo and grain weight in a *lys4* mutant

To my knowledge, Risø8 is the only line with a lesion at the *Lys4* locus thus far described. In the previous chapter, Risø8 was shown to have larger embryos as a proportion of grain weight than normal. However, the absolute weights of embryos of Risø8 were not statistically significantly different from those of the parental line Bomi. The data presented in Chapter 3 and additional data from a second growing season are summarised in Table 4.2. The proportional increase in embryo weight was observed in both growing seasons despite variations between the experiments in absolute grain weight and absolute embryo weight.

Table 4.2 – Grain weight and embryo weight of Risø8 (lys4) in two different growing seasons in the glasshouse.

Season	Line	Number of grains	Grain weight (mg)	Embryo dry weight (mg)	Embryo dry weight (% mature grain weight)
Winter	Bomi	25	53.7 ± 1.7	1.8 ± 0.2	3.3 ± 0.1
	Risø8 (*Lys4*)	25	39.1 ± 1.1	2.0 ± 0.4	5.1 ± 0.3
Summer	Bomi	9	69.4 ± 2.5	2.3 ± 0.1	3.3 ± 0.1
	Risø8 (*Lys4*)	12	57.8 ± 2.4	2.2 ± 0.1	3.9 ± 0.1

Five grains from each of five plants (winter) and three grains from each of three to four plants (summer) were weighed, and the embryos from these grains were excised, freeze-dried and weighed. Values shown are means ± SE.

To determine the developmental time point at which *Lys4* is required for normal grain development, embryo and grain weight at three stages in development and at maturity was investigated (Figure 4.4). Both grain fresh weight and embryo dry weight were lower in Risø8 compared to wild type at the earliest developmental stage examined (Grain FW, $t = 2.57$, $p = 0.015$; Embryo DW, $t = 2.50$, $p = 0.017$; Figure 4.4A and 4.4B). Grain fresh weight was consistently lower in Risø8 compared with wild type throughout development. Embryo dry weight was lower in Risø8 compared to wild type during development but at maturity there was no statistically significant difference ($t = 0.72$, $p = 0.483$). Embryo fresh weight to dry weight ratio was not statistically significantly different in Risø8 compared to wild type at weeks one and three and slightly higher in Risø8 at week two ($t = 4.00$, $p <$

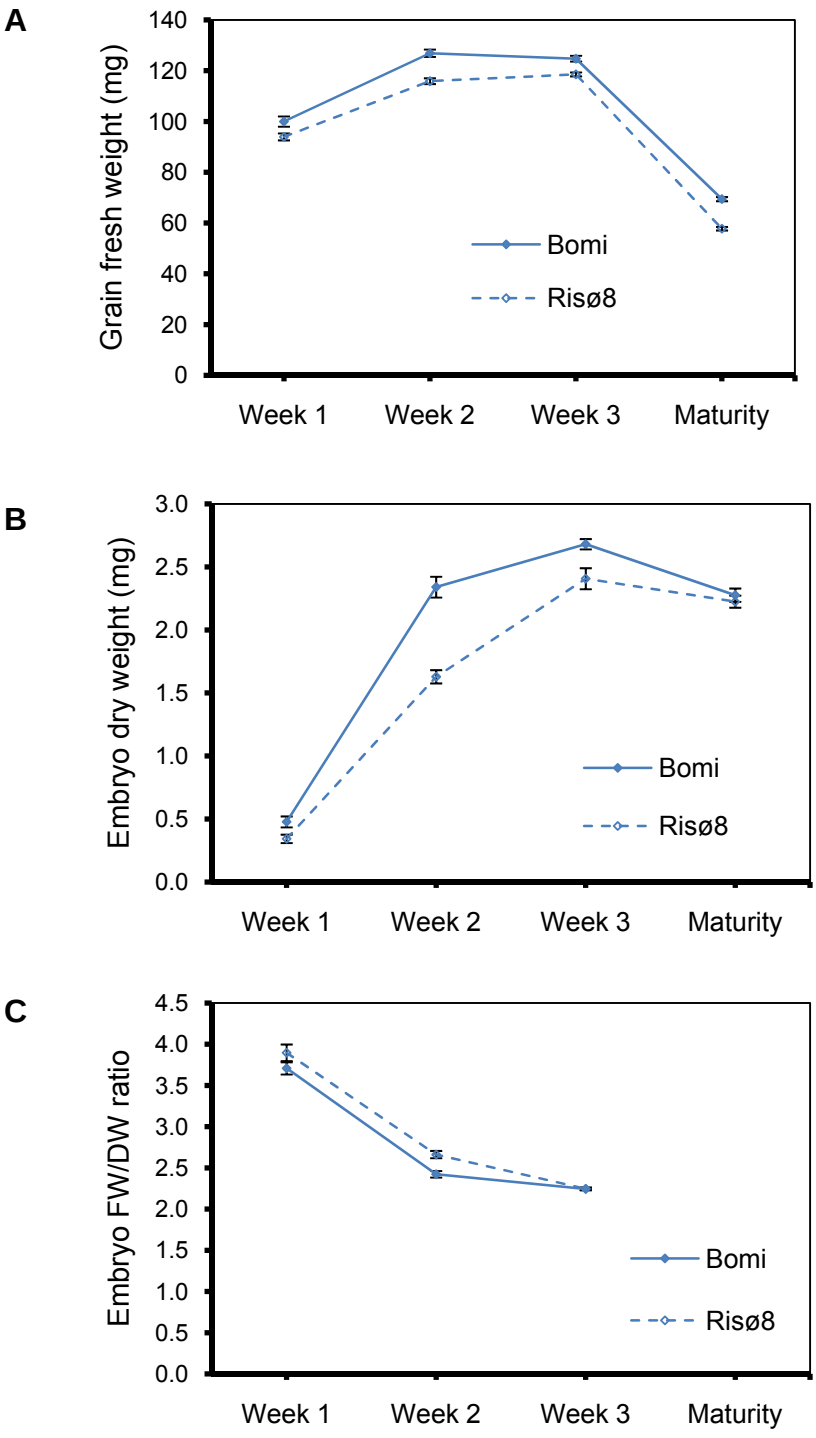

Figure 4.4 – Developing grain and embryo weights of Riso8 (*lys4*) compared to wild type.

Three to five grains from each of three to four plants (n = 9-20) were taken at each time point and weighed. The embryos from these grains were excised, weighed, dried and reweighed. Sampling was started at Week 1 an arbitrary time designated as flowering date was not determined in this experiment. In a previous experiment where Risø8 and Bomi plants were tagged at anthesis, the first plants for each genotype flowered within 1 day of each other (data not shown). Values are means ± SE.

0.001). Interestingly, the embryos of Risø8 have lower dry weight than wild type initially but this difference disappears later in development such that there is no difference in absolute embryo dry weight at maturity. Taken together, these data suggest that *Lys4* is required for normal grain development from the earliest time-point examined.

4.3.3. Morphology of *lys3* and *lys4* embryos

An enlarged and deformed scutellum has previously been described in Risø1508 (Deggerdal *et al.*, 1986). Examination of embryos from all four lines carrying a lesion at the *Lys3* locus (Risø1508, Risø18, Risø19 and M1460) established that they all have an enlarged scutellum (Figure 4.5). In M1460, the enlargement of the scutellum is accompanied by a conspicuous deformation of the adaxial surface. In Risø1508, Risø18 and Risø19, the roughening of the adaxial scutellum is less or pronounced or possible absent. The embryo of Risø8 does not appear altered in size or scutellar morphology.

Anatomical investigation of the large-embryo phenotype in lines M1460, Risø1508 and Risø18 was carried out using thin sections to further examine the differences in anatomy of the developing embryos (Figure 4.6A-D). Risø8 and wild-type controls were also included for comparison. The enlarged and convoluted scutellum is clearly visible in Risø1508 and M1460 (Figure 4.6A). The root-shoot axis is not enlarged in Risø1508 but in M1460, the coleoptile is extended beyond the ventral scale (Figure 4.6A-B). The embryos of Risø18 appear largely normal in shape and size. The immature embryos of Risø1508 and M1460 not only have enlarged scutella, particularly visible in the area subtending the ventral scale (Figure 4.6C), but also exhibit changes to the structure of the scutellar epithelium clearly visible under increased magnification in Figure 4.6D. In the wild type, the epithelium is composed of a single layer of columnar epithelial cells. In the Risø1508 and M1460, there appears to be over-proliferation of epithelial cells leading to the formation of an epithelium composed of multiple and disorganised layers of cells. The embryos of Risø8 and Risø18 do not appear enlarged or deformed (Figure 4.6A-B) and the scutellar epithelium consists of a single, ordered row of cells, as seen in the wild type.

To investigate whether the large-embryo phenotype in M1460 is due to a greater number of cells, larger cells or both of these, the number of cells in a particular area of the embryo and their cross-sectional area was determined for M1460 and wild type at two different stages in development. The cells that were sampled were those along a line running perpendicular to the adaxial surface of the scutellum to the ventral scale (Figure 4.7). At 25 days after flowering, M1460 embryos had greater scutellum width, greater scutellar epithelium width and a greater number of cells along the sample line than wild-type Minerva (Table 4.3). There was no statistically significant difference in average cellular

Figure 4.5 – Mature grain (A), dehulled mature grain (B) and adaxial view of embryos 24 hours after imbibition (C) of *lys3* and *lys4* mutants compared to wild type. The red lines delineate the embryo (B).

Figure 4.6 – Longitudinal sections through the developing *lys3* and *lys4* embryos.

Embryos were excised from developing grains, fixed and infiltrated with resin. Longitudinal sections of 0.5 µm in thickness were dried onto glass slides and stained with 0.5 % (w/v) Toluidine blue. Scale bar is 100 µm in Figures 6A and 6B, and 50 µm in Figures 6C and 6D.

Embryo structures indicated are: sc – scutellum, se – scutellar epithelium, co – coleoptile, cr – coleorhiza, ra – radicle, vs – ventral scale.

A – the apical scutellum and coleoptile
B – the basal scutellum and coleorhiza
C – the apical scutellum in the region subtending the ventral scale
D – the scutellar epithelium in the region subtending the ventral scale

Figure 4.6A

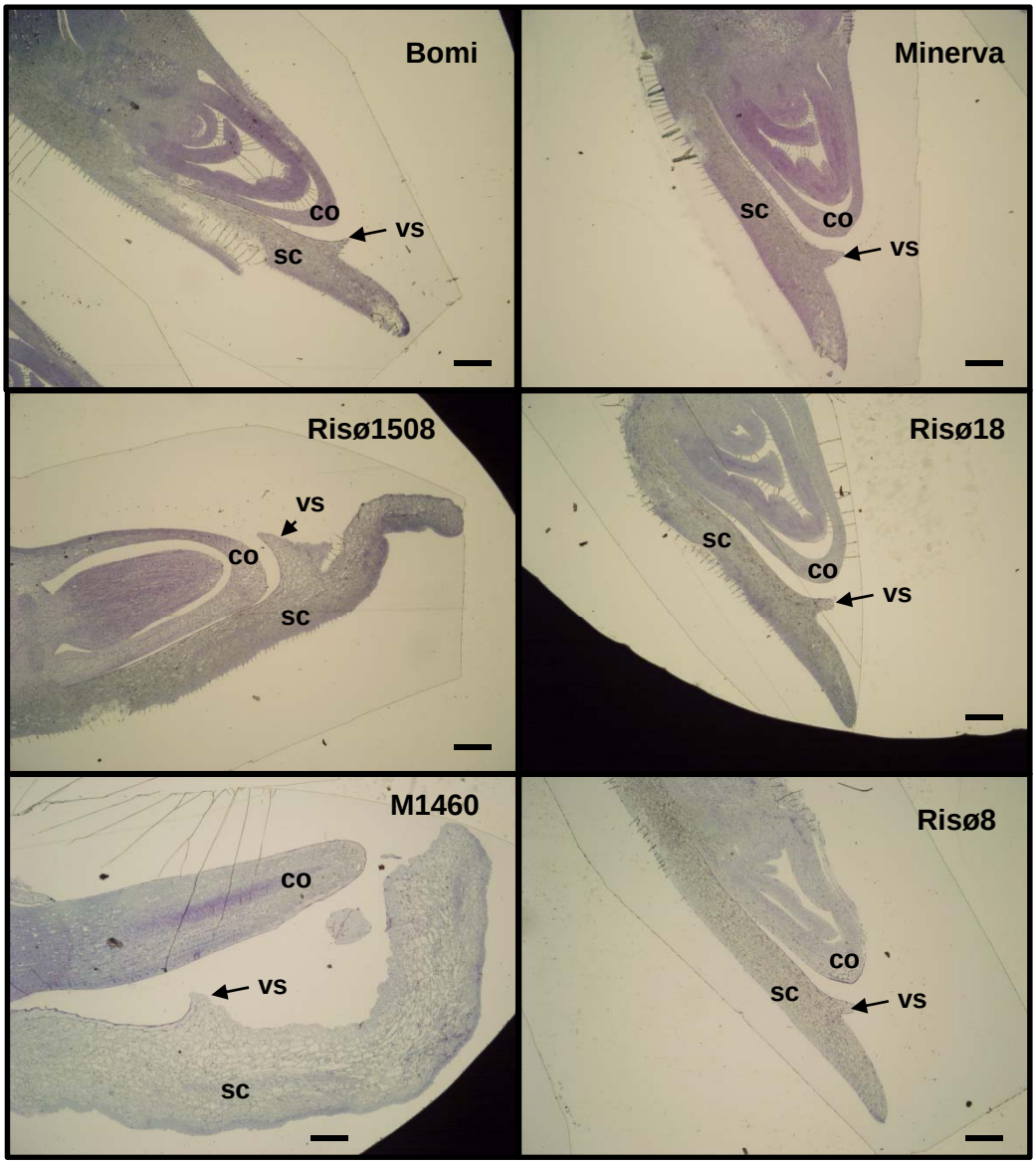
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Figure 4.6B

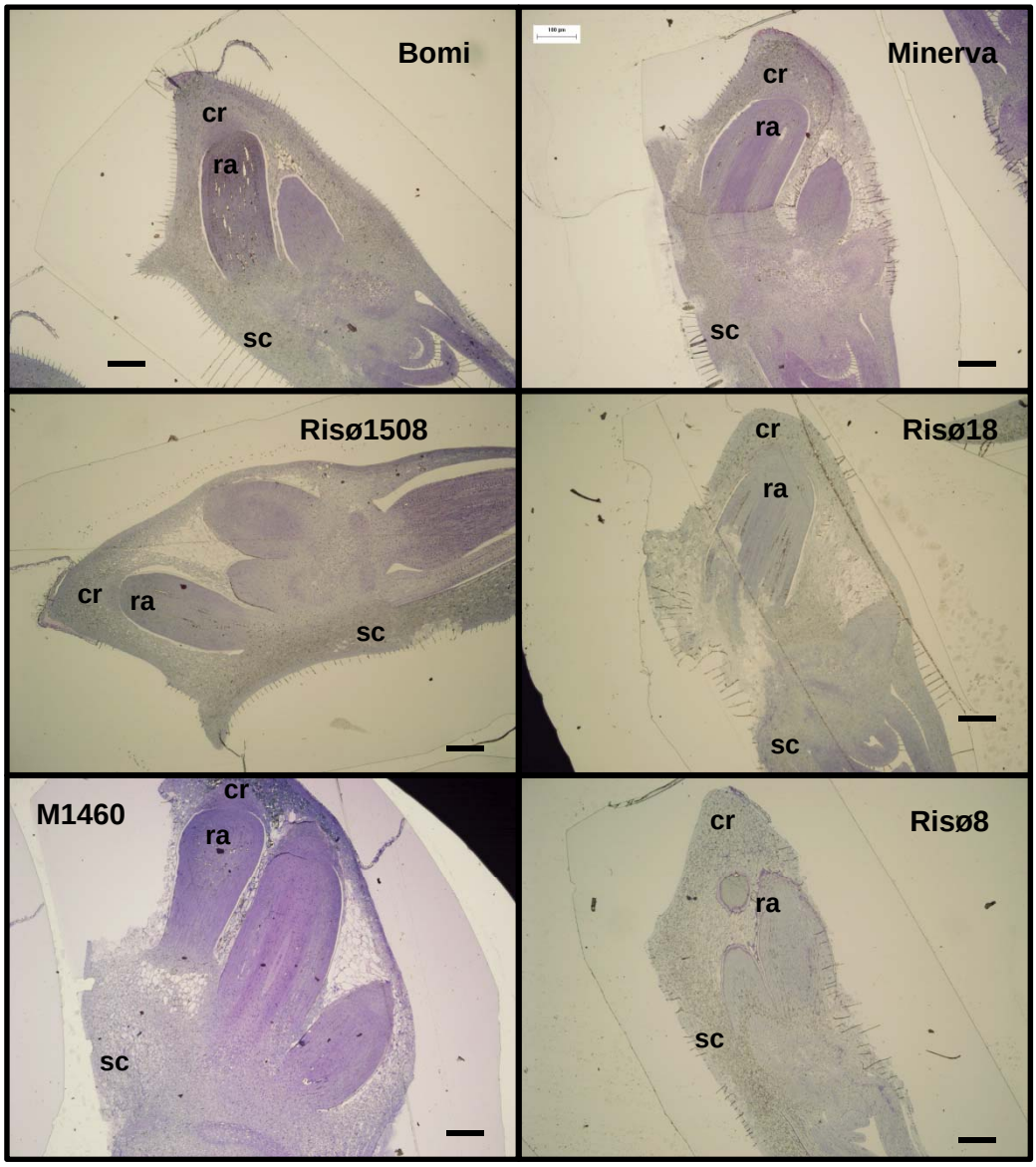
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Figure 4.6C

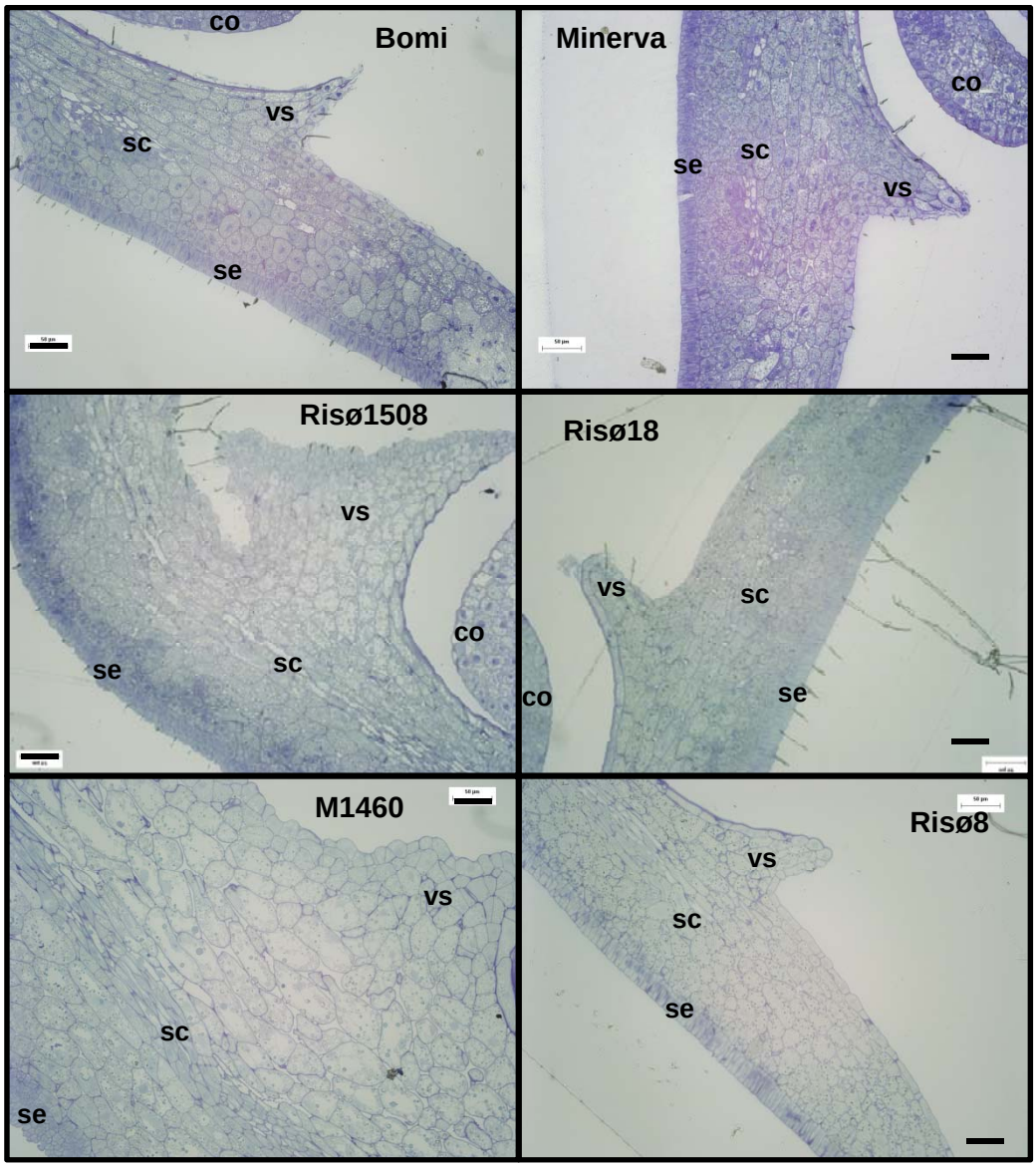
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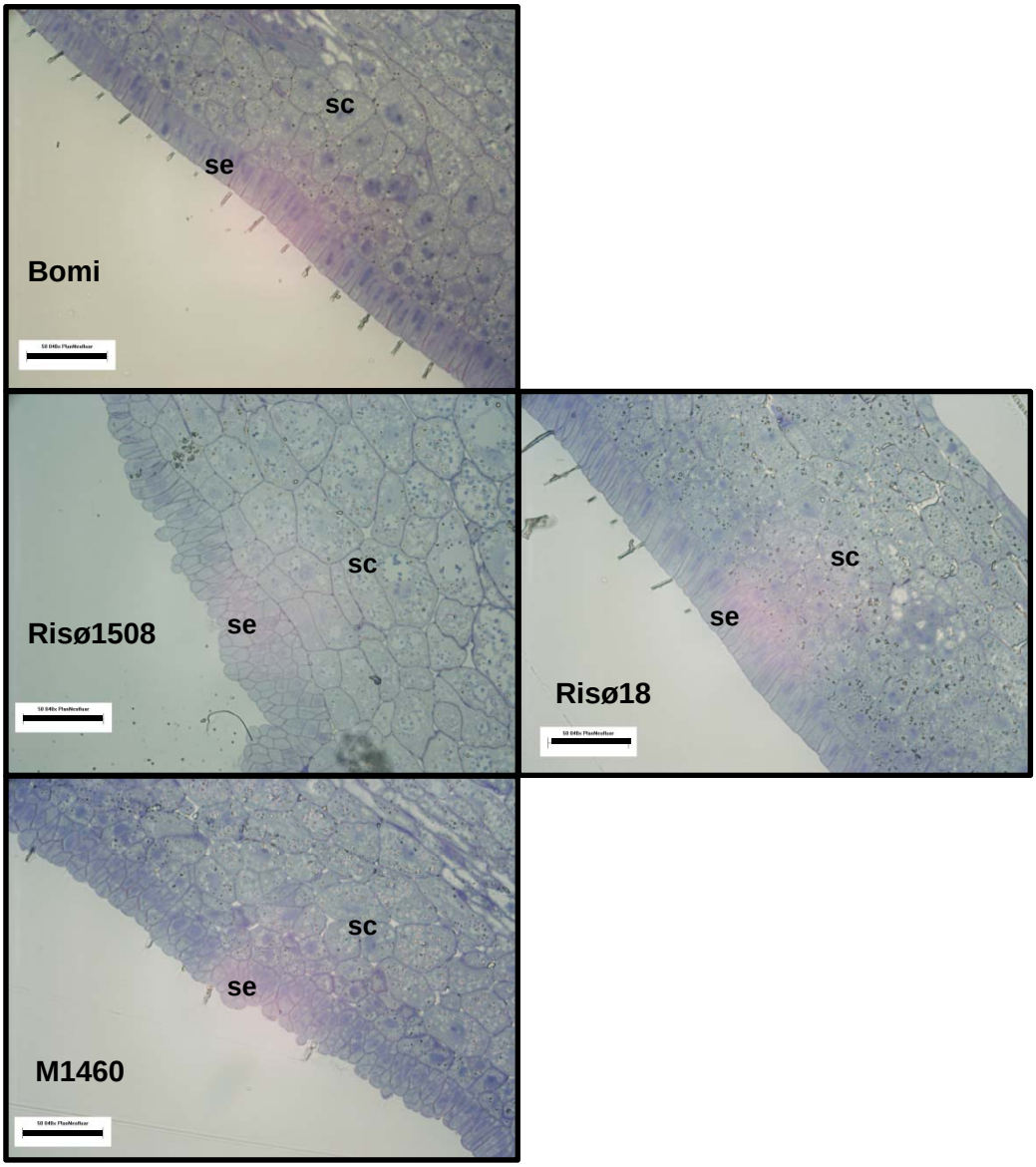
Figure 4.6D



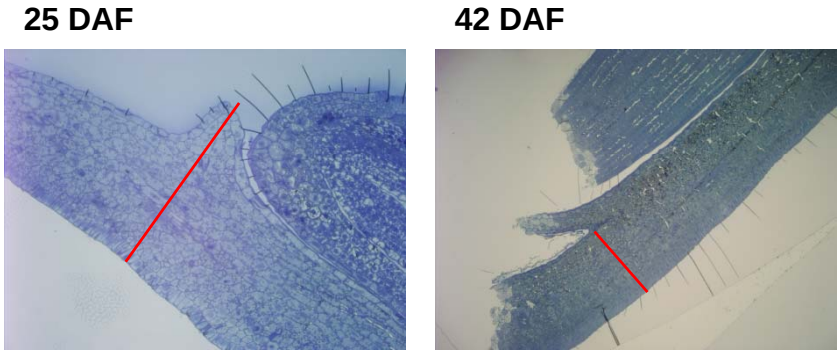


Figure 4.7 – Longitudinal sections through the apical scutellum.

The red line indicates the transect along which total scutellar width, epithelium width, cell number and cell area was analysed (see Figure 4.8).

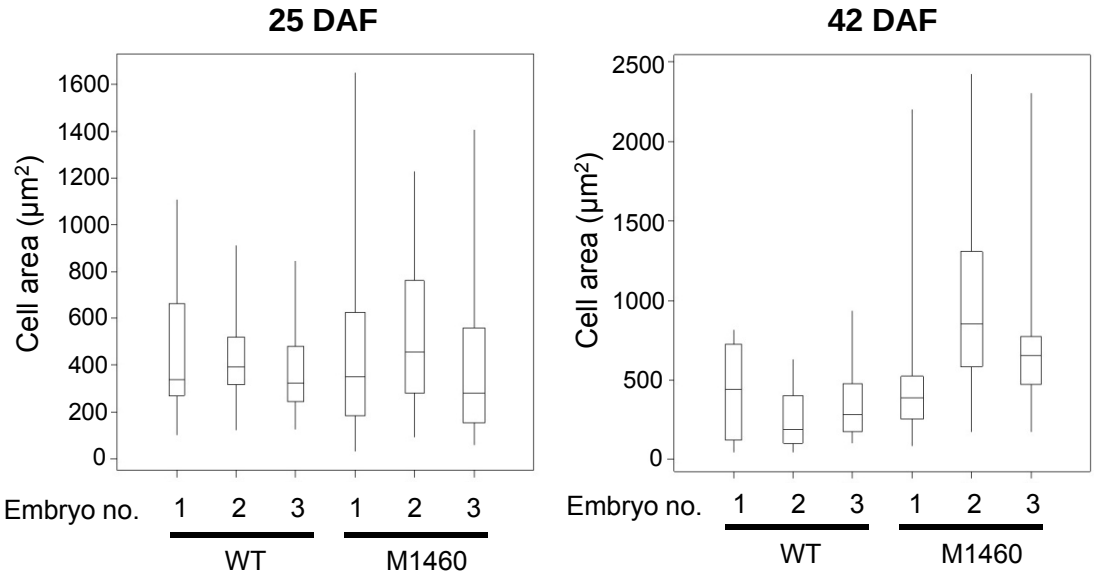


Figure 4.8 – Cross-sectional area of the scutellum cells of M1460 and wild-type Minerva measured at two different time points in development.

Cell area was measured along the line shown in Figure 4.7. Boxes denote the interquartile range and are bisected at the median. Whiskers denote the range.

cross-sectional area between M1460 and wild type at 25 days after flowering (U = 2124.5, p = 0.827). However, cell area was more variable in M1460 (Figure 4.8). The total scutellar width in M1460 relative to Minerva increased between 25 days after flowering and 42 days after flowering (the M1460 scutellum was 41 percent wider than that of Minerva at 25 days and 95 percent wider at 42 days), and so too did relative epithelium width (28 percent wider at 25 days; 73 percent wider at 42 days). The number of cells in M1460 relative to the wild type decreased slightly between 25 days after flowering and 42 days after flowering (from 54 percent more cells to 39 percent more cells). However, as it was not possible to count the number of cells in the exactly the same location across the scutellum between time points (see Figure 4.7), this difference may not be meaningful. Cell cross-sectional area was statistically significantly higher in M1460 compared to wild type at 45 days after flowering (U = 903.0, p < 0.001) but not at 25 days after flowering. At both time points, M1460 also had a few much larger cells leading to a wider range of cell areas (Figure 4.8).

Table 4.3 – Comparison of total scutellar width, epithelium width, cell number and cell area in embryo sections of M1460 and wild-type Minerva.

Measurement	DAF	N (Minerva, M1460)	Minerva	M1460	M1460 (% of WT)
Total scutellum width	25	3,3	310.5 ± 8.3	437.8 ± 22.8	141
(µm)	42	3,3	264.7 ± 2.8	517.4 ± 80.1	195
Epithelium width	25	3,3	23.3 ± 1.0	29.8 ± 2.6	128
(µm)	42	3,3	34.7 ± 1.0	59.9 ± 9.9	173
Number of cells	25	3,3	17.7 ± 0.9	27.3 ± 2.4	154
	42	3,3	17.0 ± 1.5	23.7 ± 0.9	139
Cell area (μm^2)	25	53,82	353.7	342.7	97
	42	51,71	233.1	562.4	241

Measurements were made along a line through the apical scutellum shown in Figure 4.7. Values are means ± SE except cell area where the values are medians.

In conclusion, the *lys3* lines all have enlarged embryos however there are varying effects on the morphology of the embryo. As with the variations in the effect on embryo weight between the *lys3* lines, the variation in embryo morphology may be due to a difference between mutant alleles or due to an effect of genetic background.

4.3.4. Carbohydrate metabolism in the endosperm and embryo of Risø1508 during development

To determine whether differences in carbohydrate metabolism are associated with the developmental alterations in *lys3* mutant embryos, starch and soluble sugar contents were determined in the embryo of Risø1508 (*lys3a*) during development. For comparison,

starch content in the developing endosperm was also determined (Figure 4.9). In accordance with previous data (Doll *et al.*, 1974; Tallberg, 1977; Shewry *et al.*, 1979), endosperm weight in Risø1508 was lower than that in the wild-type Bomi (Figure 4.9A). This difference was correlated with lower starch content per endosperm (Figure 4.9B) and per gram dry weight (Figure 4.9C).

In Chapter 3, there was found to be no statistically significant difference in starch content of Risø1508 embryos and wild-type embryos. Here, embryo starch content of Risø1508 embryos was compared to wild-type embryo between 20 and 45 days after flowering (Figure 4.10A). Embryo starch content was higher in Risø1508 embryos compared to wild type from 25 DAF to 45 DAF. Sugar content was also generally higher in Risø1508 embryos compared to wild type (Figure 4.10B-D). The differences between mutant and wild type sugar content were greatest early in development and by 45 days after flowering, developmentally close to grain maturity, there was no longer a difference.

Taken together, these results indicate that Risø1508 embryos not only contain higher levels of starch and sugars per embryo (as the embryos are larger) but also contain higher levels of starch and sugars per gram dry weight than wild-type embryos.

4.3.5. Germination and dormancy in Risø1508

Although effects on germination and dormancy in *Lys3* mutants are likely to be secondary to the effects of the lesion on grain development, reduced dormancy during development often leads to viviparity (precocious germination on the ear or cob) (Koornneef *et al.*, 2002). Viviparity is a conspicuous phenotype and often scored in published cereal grain mutants. To identify similarities between Risø1508 and other published cereals mutants, germination, seedling growth rate and dormancy were compared in Risø1508 and wild type. Trials carried out on grain that had been stored for more than three months showed that there was no difference in the time taken to germinate or in total viability between Risø1508 and wild type (Figure 4.11). Both mutant and wild type showed 100 percent germination within 24 h. Measurements of seedling growth (root and shoot length) at five days after imbibition showed that there was no statistically significant difference in root length between mutant and wild type (Figure 4.12; $t = 1.3$, $p = 0.2$). However, in 11 of the 20 Risø1508 seedlings, shoot emergence was impeded due to the shoot apex becoming lodged within the enlarged scutellum. In the wild type, the elongating shoot travels between the hull and the endosperm and emerges at the distal end of the grain (Figure 4.13). In Risø1508, emerging shoots often formed a ring, with the shoot apex lodged within the grain. In some instances, the shoot apex would turn through 180 degrees and emerge from the proximal end of the grain (Figure 4.13). The Risø1508 shoots that

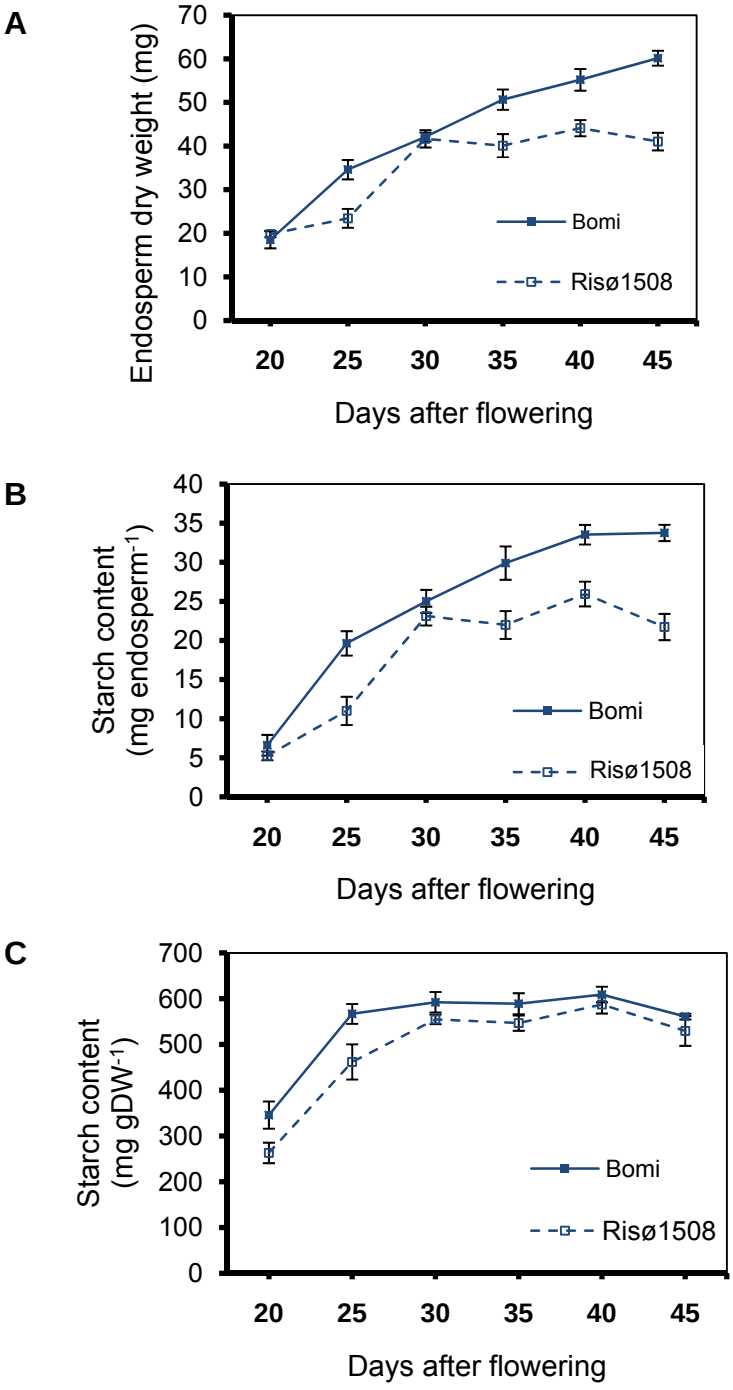


Figure 4.9 – Endosperm weight and starch content of developing grains of Risø1508 and wild type.

Grains (with the embryo removed), each from an separate plant, were freeze-dried, weighed and extracted for starch. Values are means ± SE of four to five grains. Plants were grown under summer glasshouse conditions.

Regression analysis of starch content (mg gDW^{-1}): Model = DAF+genotype, variation accounted for 81.1 %, DAF variance ratio = 42.6, d.f. = 5, $p < 0.001$, genotype variance ratio = 14.8, d.f. = 1, $p < 0.001$

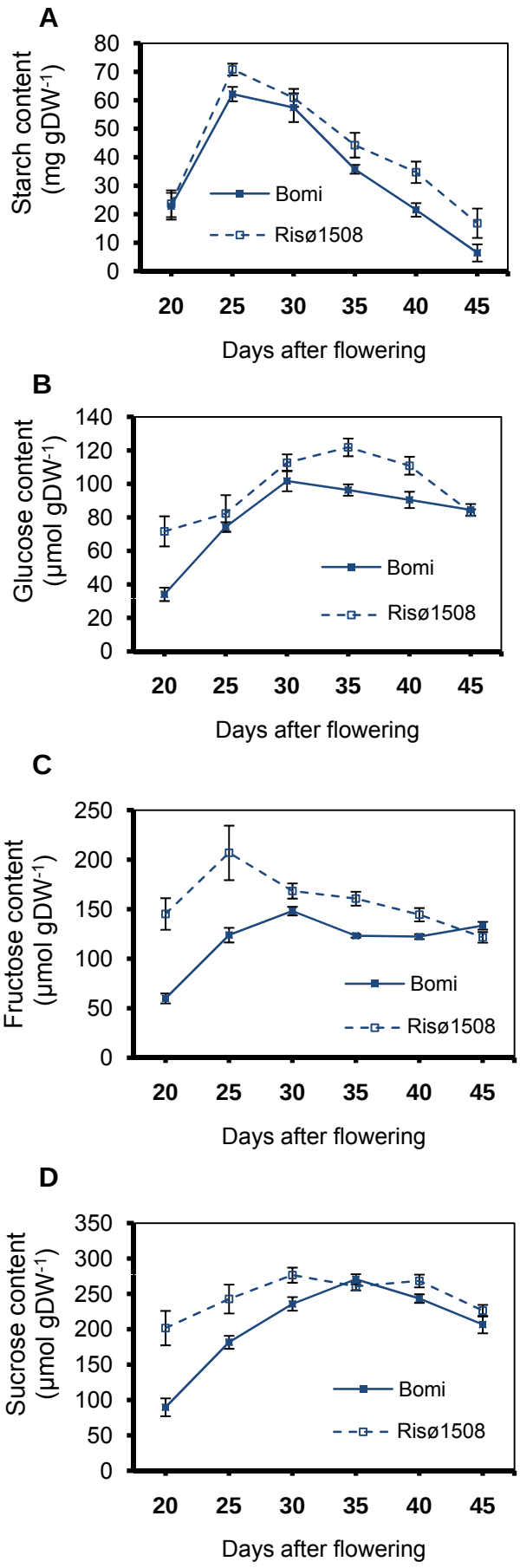


Figure 4.10 – The accumulation of starch and sugars during development in Risø1508 and wild-type embryos.

Embryo samples, each consisting of six to eight embryos, were freeze-dried, extracted and assayed for starch and soluble sugars. Each sample was from a separate plant. Values are means ± SE for between three and five samples. Plants were grown under summer glasshouse conditions.

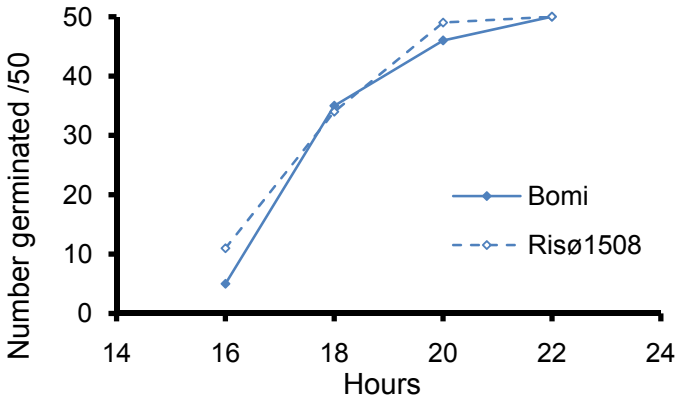


Figure 4.11 – Germination rate of Risø1508 and wild type grain.

Grains were germinated on moist filter paper at 17 ºC in the dark. Germination was judged to have taken place when the radicle was visible outside the hull.

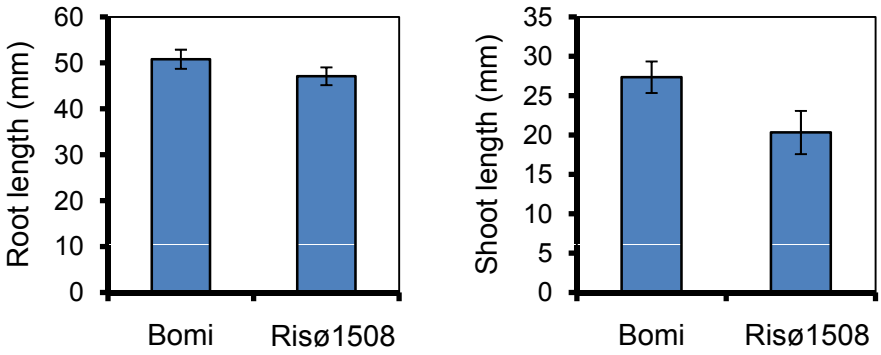


Figure 4.12 – Root and shoot length five days after imbibition of Risø1508 and wild type grains.

Grains were germinated on moist filter paper at 17 ºC in the dark. Values are means ± SE for 20 grains.

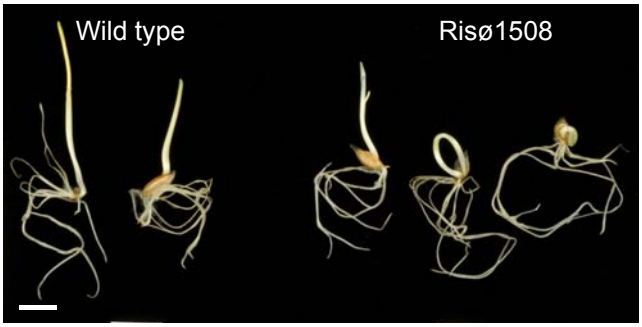


Figure 4.13 – Risø1508 and wild-type seedlings.

Two examples of wild-type seedlings, both showing normal shoot emergence, and three examples of Risø1508 seedlings, one showing normal shoot emergence and two showing impeded shoot emergence. Scale bar is 1 cm.

emerged normally were not statistically significantly different in length from wild-type shoots (t = 2.0, p = 0.056).

Dormancy can be defined as 'a seed characteristic, the degree of which defines what conditions should be met to make the seed germinate' (Vleeshouwers *et al.*, 1995). In practical terms, during maturation of the barley grain so-called primary dormancy is imposed on the embryo such that at maturity the grain will not germination under conditions that are usually favourable to germination (Black *et al.*, 2006). During dry storage, the process of after-ripening (through largely unknown physiological changes in the grain) leads to dormancy being released, such that the grain will germinate under favourable conditions. In temperate cereals such as barley, moisture and temperatures around 15 to 20 ºC are favourable to germination (Leymarie *et al.*, 2009). Post-harvest dormancy in barley cultivars tends to be low (particularly in malting varieties) and is influenced by environmental conditions during grain development (Black *et al.*, 2006). To compare dormancy between Risø1508 and wild-type Bomi, germination trials were carried out on mature grain post-harvest (Figure 4.14). Risø1508 grain was significantly less dormant than wild type grain during the first two weeks post harvest (Figure 4.14A). At four weeks post harvest, dormancy had been broken in both mutant and wild type. Moisture content can modify the imposition of dormancy during grain maturation and the breaking of dormancy during dry after-ripening. However, reduced dormancy in Risø1508 was not associated with altered moisture content (Figure 4.14B).

These results indicate that the *lys3a* mutant Risø1508 has lower dormancy than the wild type, Bomi. Once dormancy is broken, there is no effect of the mutation on grain viability or seedling growth.

4.3.6. Plant growth and development in *lys3*

To identify effects of a mutation at the *Lys3* locus on plant growth and development, a range of whole-plant metrics and yield components were measured. Growth rate (measured as plant height) in the mutant Risø1508 was indistinguishable from that in the wild type, Bomi (Figure 4.15). Growth rate decreased markedly around anthesis which occurred at 72 days after germination in both Risø1508 and wild-type plants.

At maturity, a range of spike and whole plant features were analysed (Table 4.4). In Risø1508, spikes were eight percent shorter than those of the wild type but there was no statistically significant reduction in the number of grains per spike. Mean grain weight was 10 percent lower in Risø1508 than in the wild type but total grain yield per plant was not significantly different. This indicated there must be more grains per plant in Risø1508 than

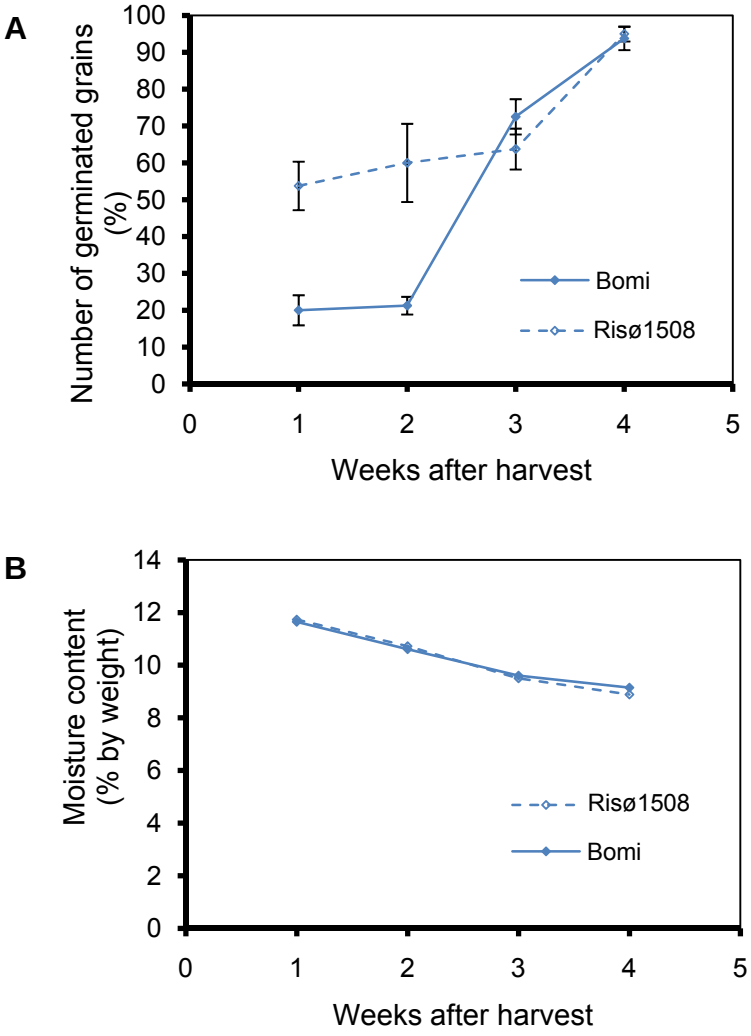


Figure 4.14 – Dormancy and the effects of after-ripening in Risø1508 and wild type grains.

Six plants per genotype were grown in the CER. Plants were dried down for several weeks without watering prior to harvesting. Grain from the primary tillers was stored at room temperature in the dark prior to germination trials.

A – Total germination
Samples of 20 grains were incubated on moist filter paper at 17 ºC in the dark at one-week intervals after harvest. Germination was scored at five days after imbibition and was judged to have occurred when the radicle was first visible outside the hull. Values are the mean ± SE for four replicate samples.

B – Moisture content of samples
Samples of 20 grains were weighed, incubated at 80 ºC for 48+ hours and reweighed. The loss of weight during incubation was taken to indicate moisture content.

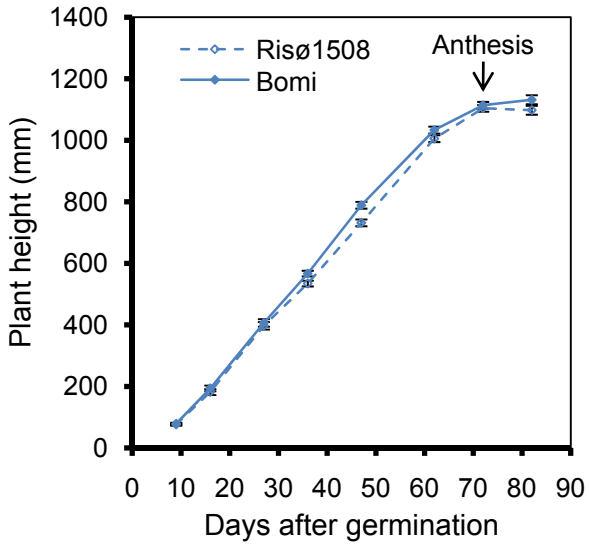


Figure 4.15 – Plant growth in Risø1508 and wild type, Bomi.

Plant height was measured from the base of the stem to the tip of the longest leaf. Values are means ± SE for 10 plants. Plants were grown under CER conditions.

Table 4.4 – Growth metrics and yield components in Risø1508 and wild type, Bomi.

Values are mean ± SE of measurements made on 10 plants grown under CER conditions. Spike length (not including awns) and number of grains per spike were measured in 10 spikes from each plant. Average grain weight was calculated from the weight of 100 grains per plant. Straw height was measured from the base of the stem to the top of the tallest peduncle. Peduncle length was measured from the first node below spike to the base of the spike in three tillers per plant. Probability (*p*) values were generated using Student's t-tests.

	Feature	Bomi	Risø1508	*p* - value
Spike				
	Spike length (cm)	10.3 ± 0.2	9.5 ± 0.2	0.007
	Number of grains per spike	26.4 ± 0.5	26.9 ± 0.3	0.46
	Grain weight (mg grain^{-1})	66.5 ± 1.3	59.8 ± 1.1	<0.001
Whole plant				
	Number of tillers per plant	22.8 ± 1.6	25.5 ± 2.2	0.34
	Total weight of spikes (g plant^{-1})	40.2 ± 2.4	39.7 ± 2.7	0.90
	Straw height (cm)	92.2 ± 1.6	88.3 ± 1.1	0.059
	Peduncle length(cm)	33.6 ± 0.4	30.9 ± 0.4	<0.001
	Total grain yield (g plant^{-1})	32.7 ± 2.0	32.6 ± 2.1	0.97

in wild-type. Neither the number of grains per spike nor the number of tillers per plant was statistically significantly higher in Risø1508 so it was not possible to determine which of these traits (or possibly both) contributed to the higher number of grains per plant. The peduncle (the stem section subtending the spike) was 2.7 cm shorter in Risø1508 than the wild type. However, there was no significant reduction in straw height.

Thus, in addition to the previously discussed effects on the grain, Risø1508 plants (compared to wild type) have, on average: shorter spikes (without an effect on the number of grains per spike), shorter peduncles, and more grains per plant. The increase in grain number is such that the reduction in average grain weight in Risø1508 does not lead to a reduction in total grain yield per plant in these experiments.

4.3.7. Phylogenetic analysis of CYP78s in grasses

To identify and compare CYP78s in grasses (Poaceae), *OsGe* was used to identify similar grass sequences using Phytozome (www.phytozome.net). Phytozome searches for homologous genes in species with sequenced genomes (*Brachypodium*, rice, maize, foxtail millet and sorghum). Phylogenetic analysis of the sequences identified by Phytozome together with the *Ge*-like sequences identified by Cahoon *et al.* (2006) was carried out by Dr Kay Trafford and Dr Sylviane Comparot-Moss (Figure 4.16) (unpublished). Seven full length CYP78 genes were identified in the rice genome (Table 4.5; two partial rice sequences *Os03g40600* and *Os03g40610* were omitted from the study).

Table 4.5 – CYP78 genes in the rice genome.

NCBI (RAP)	MSU (TIGR)	Name* (previously)	Mutants
Os11g0489250	Loc_Os11g29720	CYP78D1	
Os10g0403000	Loc_Os10g26340	CYP78A11	*plastochron1 (pla1)*
Os03g0417700	Loc_Os03g30420	CYP78A12 (B4)	
Os07g0603700	Loc_Os07g41240	CYP78A13 (B5)	*giant embryo (ge)*
Os08g0547300	Loc_Os08g43390	CYP78A15 (C5)	
Os09g0528700	Loc_Os09g35940	CYP78A16 (C6)	
Os03g0134500	Loc_Os03g04190	CYP78A17 (C7)	

* Nomenclature is after http://drnelson.uthsc.edu/.

Eight genes were identified from the maize genome. Three of these had been previously identified by Cahoon *et al*. (2006) and are labelled ZmGE1, ZmGE2 and ZmGE3 in Figure

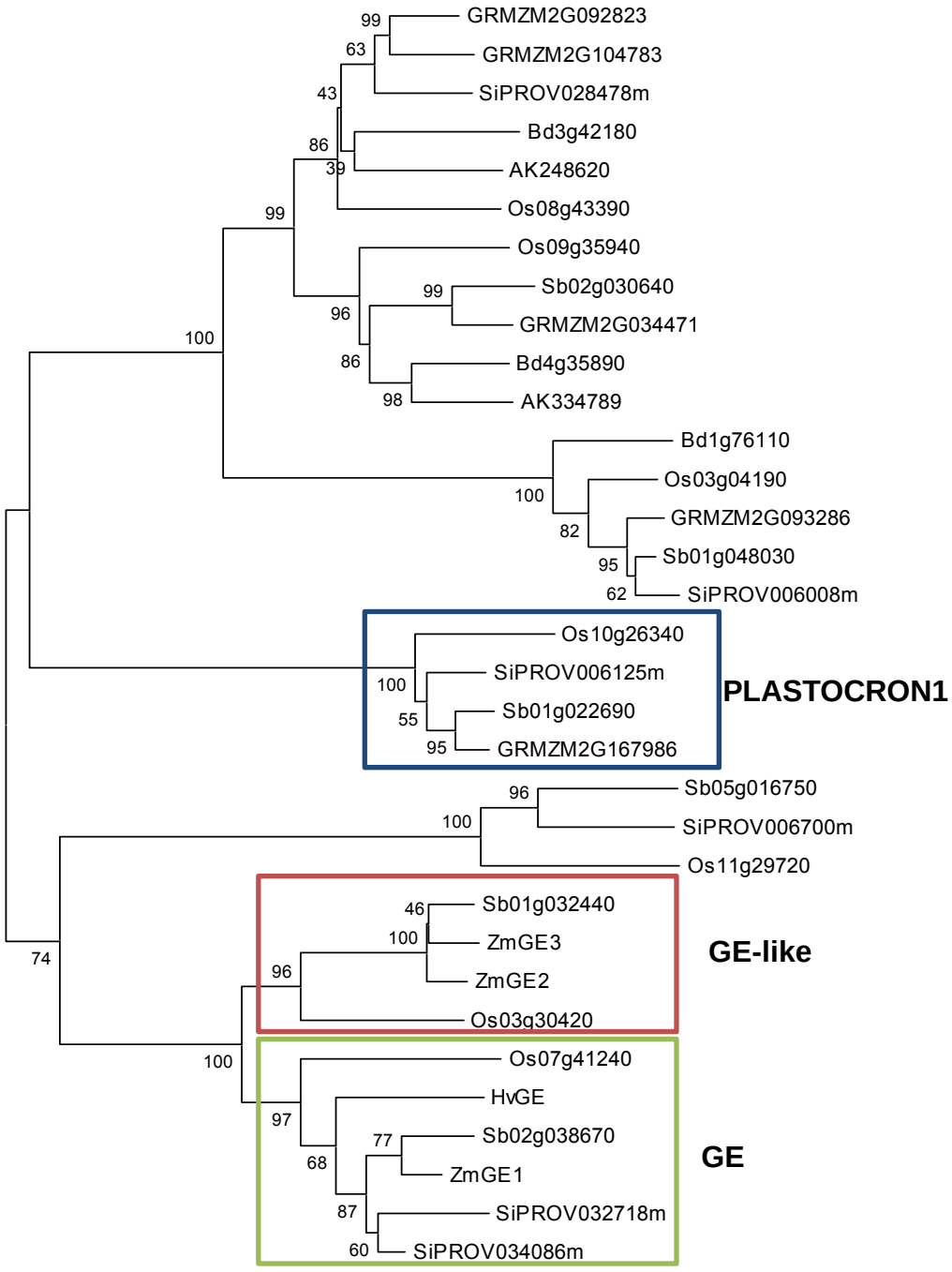


Figure 4.16 – Phylogenetic analysis of CYP78 proteins in grasses.

Sequences related to rice CYP78s were mined using Phytozome (phytozome.net). In addition to barley (Hv) and maize (Zm) sequences from Cahoon et al. (2006), sequences from rice (Os), maize (GRMZM), sorghum (Sb), millet (Si) and brachypodium (Bd) were identified . Where maize seqences from the patent matched seqences in the databases, only the name of patent sequence is shown. AK248620 (barley) and AK334789 (wheat) sequences were deduced from cDNA. Sequences were aligned in ClustalW and trimmed. Phylogenetic tree was inferred from 1000 replicates using the Neighbor-Joining method (Saitou and Nei,1987). The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) are shown (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992) and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Alignment and tree constructed by Dr Kay Trafford

4.16. Six genes were identified in both the millet and sorghum genomes and three genes were identified in the *Brachypodium* genome. The barley gene identified by Cahoon *et al.* (2006), referred to as HvGE in Figure 4.16, was found to align with two barley ESTs (CA009374 and BG416626). A partial wheat EST also aligns with *HvGe* but is not included in the Figure 4.16. Other full-length cDNAs from barley and wheat are also included (Figure 4.16).

Phylogenetic analysis of the CYP78 proteins in the grasses (Figure 4.16) showed that rice GE (*Os07g41240*), HvGE, ZmGE1, *Sb02g038670* and two sequences from millet (*SiPROV032718* and *SiPROV034086*) form a clade. This is referred to as the 'GE' clade. Brachypodium does not have a related sequence in the GE clade despite its relatively close evolutionary relationship to barley.

ZmGE2 is in a separate clade to OsGE and ZmGE1. The *ZmGe2* gene is also associated with the high-oil/large embryo trait suggesting that ZmGE2 may also have a role in determining embryo size in maize (Cahoon *et al.*, 2006). For this reason, the clade containing ZmGE2 is referred to as GE-like. Also in this clade are ZmGE3 and representatives from sorghum (*Sb01g032440*) and rice (*Os03g30420*; Figure 4.16). Again, as with the GE clade, not all grasses have a representative in this clade.

The third gene for which there is some functional information is *Os10g26340 (Plastochron1)*. PLASTOCHRON1 (PLA1) has putative orthologues in maize (*GRMZM2G167986*), sorghum (*Sb01g033690*) and millet (*SiPROV006125*) but not in *Brachypodium*. As outline in the introduction, the available data suggest that GE and PLASTOCHRON1 have divergent functions.

4.3.8. Identifying the chromosomal location of *HvGe*

Phylogenetic analysis has determined that *HvGe* is an orthologue of *OsGe* (*Os07g41240*). Next, I investigated whether *HvGe* could lie at the *Lys3* locus. The *Lys3* locus has been mapped to the centromeric region of chromosome 5H in Risø1508 using phenotypic markers (Ullrich and Eslick, 1978b; Jensen, 1979). This location was confirmed in our laboratory by bulk segregant analysis, with Illumina™ sequencing, of *lys*3 x Morex F_2 populations of three *lys3* mutants (Risø1508, Risø18 and Risø19) (Dr Sylviane Comparot-Moss and Dr Kay Trafford, unpublished).

To establish whether *HvGe*, the barley orthologue of *OsGe*, lies on the same chromosome (5H) as *Lys3*, I used wheat-barley chromosome addition lines to locate *HvGe*. The chromosome addition lines are wheat-barley hybrids of Chinese Spring (wheat) and Betzes (barley). A set of addition lines, each with a single barley chromosome (disomic) or

chromosome arm (ditelosomic) in a wheat background, is available for barley chromosomes 1H to 7H (Islam *et al.*, 1981). Using the partial wheat EST sequence (AK334789.1), which aligns with *HvGe*, it was possible to design *HvGe*-specific primers to a region of the *HvGe* gene (Figure 4.17A). In this case, primers were designed to a polymorphic region that was identified in an intron. Using these primers, a PCR product matching the specific region of *HvGe* was obtained with the 2H addition line DNA (Figure 4.17B). No products were obtained with the DNA from the other addition lines (the 1H addition was not included in this study as the DNA was not available at the time). PCR was carried out using three independent wheat-barley chromosome addition line DNA preparations. This demonstrates that the othologue *HvGe* is located on chromosome 2H. Ditelosomic lines of 2H were used to locate *HvGe* to the short of arm of this chromosome (Figure 4.17B).

This work has demonstrated that a barley orthologue of the rice *GIANT EMBRYO* gene (*HvGe*) identified by Cahoon *et al.* (2006), lies on a different chromosome to the *Lys3* locus and therefore cannot underlie the *Lys3* locus. Although unlikely, the possibility that another barley *Ge* homologue underlies the *Lys3* locus cannot be definitively ruled out.

4.3.9. Genetic interactions between *lys3* and other shrunken-grain loci

To investigate whether there is an interaction between the *Lys3* and *Lys4* loci, Risø1508 (*lys3a*) was crossed with Risø8 (*lys4*). From the F_2, which was segregating for both *lys3* and *lys4*, eight putative double mutant grains were selected based on their having a large embryo and very shrunken grain. These were grown alongside Risø1508 and Risø8 and their relative grain weight and embryo weights were compared (Figure 4.18). Comparison of grain weight and embryo weight showed that these varied significantly between individual putative double mutant plants. This suggests that they are not all of the same genotype and backcrossing to each parental mutant is required to discover which plant, if any of these, is a *lys3/lys4* double mutant.

Dr Kay Trafford had previously crossed each of the endosperm-specific starch mutants, Risø16 (*agps1*) and starch mutant Risø17 (*isa1*) with Risø1508 (*lys3a*). Dr Trafford had selected individual F_2 plants homozygous for either *agps1* (confirmed by the absence of the protein encode by the affected gene) or *isa1* (confirmed by visualisation of starch granule morphology which is characteristically altered in this mutant). From this material, I selected those plants with large embryos as putative *lys3* mutants. These putative double mutants were grown alongside the wild type Bomi and parental single mutants and their grain weight and embryo weights were compared (Figure 4.19). These data must be

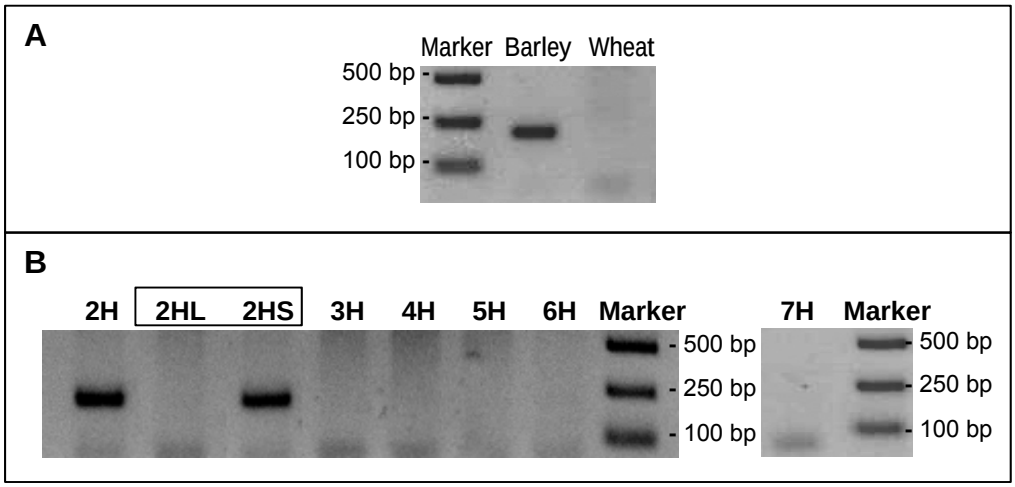


Figure 4.17 – Identifying the chromosomal location of a putative barley orthologue of *OsGe* using wheat-barley chromosome addition lines.

A: Confirmation of primer specificity for *HvGe* during PCR amplification.
Specificity was confirmed using barley (Betzes) and wheat (Mardler) genomic DNA as template. For primers see Chapter 2.4.2.

B: *HvGe*-specific primers used to amplify gDNA from wheat-barley chromosome addition lines.
Shown are PCR products obtained when using disomic wheat-barley chromosome addition lines 2H to 7H and ditelosomic lines containing only the long arm (2HL) or the short arm (2HS) of 2H.

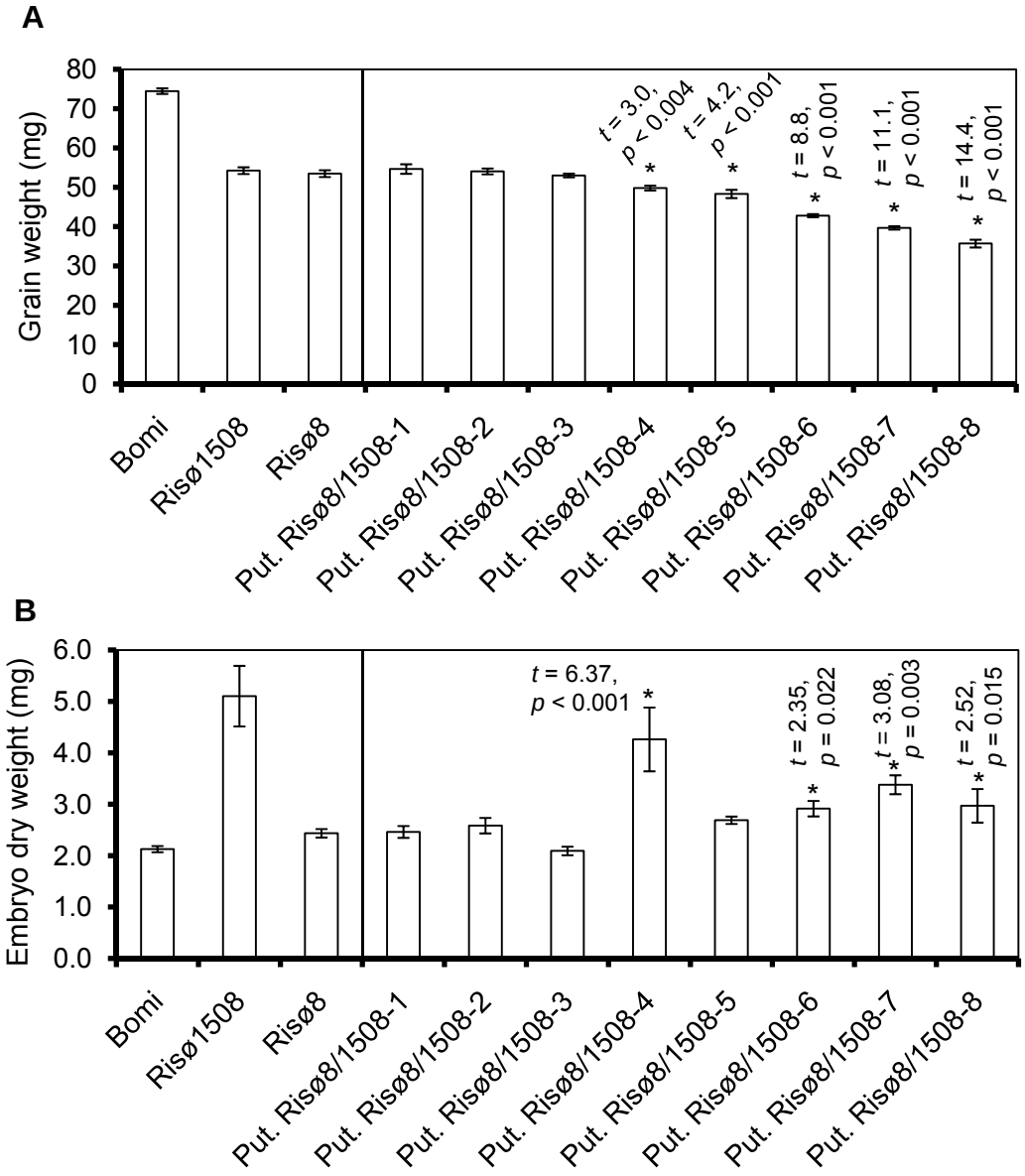


Figure 4.18 – Grain weight (A) and embryo weight (B) in the putative (Put.) Risø1508/Risø8 double mutants.

Three to five grains from each of two to five plants (n=6-15) were taken at maturity from Bomi, Risø1508 and Risø8 and weighed. The embryos from these grains were excised, freeze-dried and re-weighed. Five grains from each individual putative Risø8/Risø1508 double mutant plant were weighed as above. Values are means ± SE. The asterisk indicates (A) a statistically significant reduction in grain weight compared to both Risø1508 and Risø8 (t-test comparison with Risø8 only are shown) or (B) a statistically significant increase in embryo weight compared to wild type (Bomi).

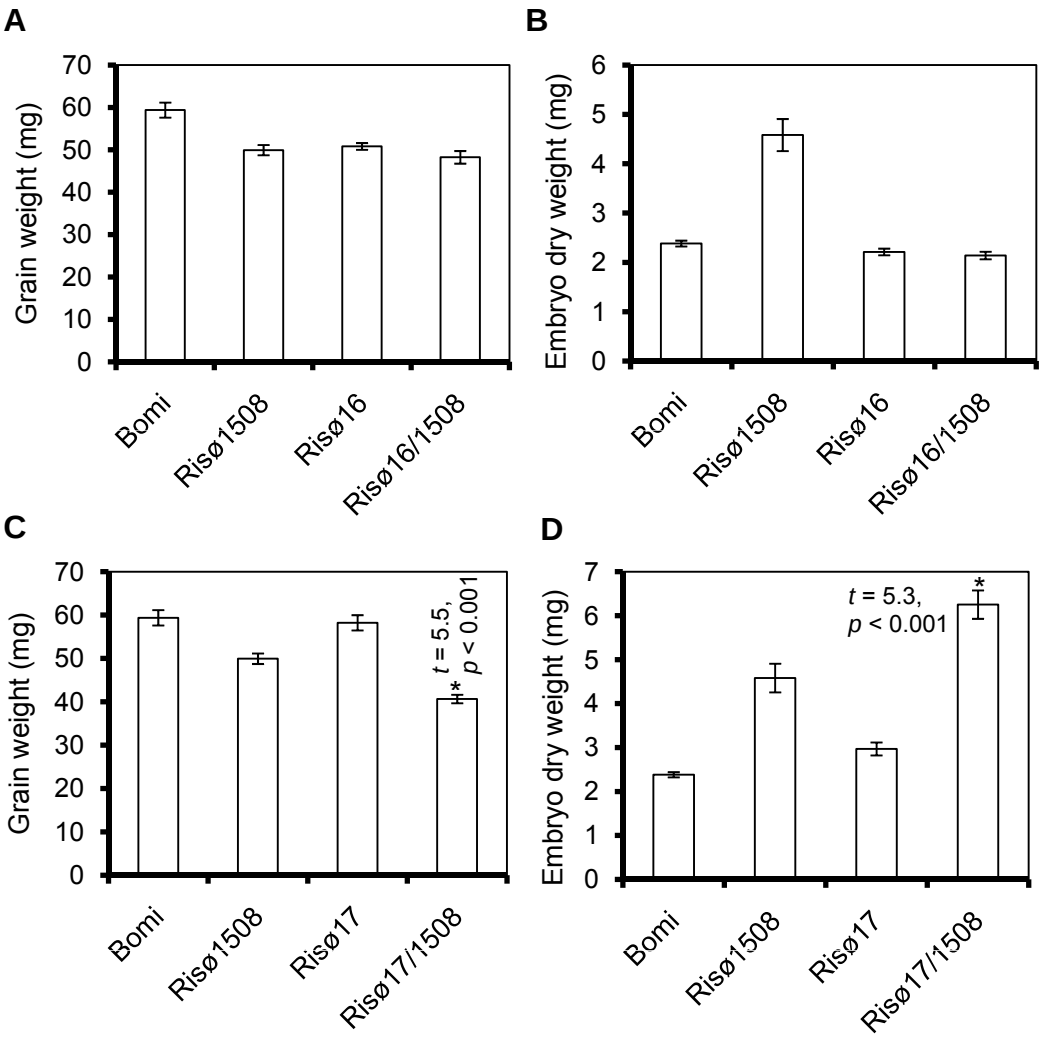


Figure 4.19 – Grain weight and embryo weight in putative double mutants Risø1508/Risø16 (A and B) and Risø1508/Risø17 (C and D).

Three to five grains from each of three to 10 plants (n=15-30) were taken at maturity and weighed. The embryos from these grains were excised, freeze-dried weighed. Values are means ± SE. The asterisk indicates (C) a statistically significant reduction in grain weight compared to both Risø1508 and Risø17 (t-test comparison with Risø1508 only is shown) or (D) a statistically significant increase in embryo weight compared to both Risø1508 and Risø17 (t-test comparison with Risø1508 only is shown).

treated as preliminary as back-crossing to parental mutants to confirm the genotypes has not yet been carried out.

The putative *agps1/lys3a* double mutant was not statistically significant different in grain weight from either the Risø1508 or Risø16 single mutants (Figure 4.19A) and its embryo weight was significantly different from that of Risø1508 but not from that of Risø16 (Figure 4.19B). This suggests that the line may not be homozygous mutant at the *lys3* locus even though the putative mutant grain was selected visually as having a large embryo. Further work is required to confirm this.

The putative Risø1508/Risø17 double mutant had statistically significantly lower grain weight than either Risø1508 or Risø17 single mutants (Figure 4.19C). Embryo weight in the putative double mutant was higher than that of either of the single mutants (Figure 4.19D). This suggests that the effects of the mutations in Risø1508 and Risø17 may be additive with regard to both effects on grain weight and effects on embryo weight. As with the previous double mutants, these results should be interpreted with caution as further work is required to confirmed the *lys3* genotype.

4.4. Discussion

The phenotype of *lys3* mutants indicates that the gene responsible is highly pleiotropic and it is therefore difficult to determine the primary effect (or effects) of the lesion in these lines. However, phenotypic data provide useful insights into the possible pathways in which *Lys3* is required for normal development which may eventually help to identify the gene responsible. The next section summarises the effects of a lesion at the *Lys3* locus, temporally and spatially, on development in barley.

The effects of a mutation at the *Lys3* locus appear to be spatially confined to the inflorescence and the reproductive tissues suggesting that *Lys3* expression is required during the development of these tissues. *Lys3* has been shown here to be required for normal stem (peduncle) elongation, normal spike elongation as well as normal grain development. *Lys3* appears not to be required for normal vegetative growth. However, I was unable to determine whether there was more grain per plant in Risø1508 due to an increase in number of tillers or number of grains per tiller. A subtle role for *Lys3* in the determination the number of tillers per plant therefore cannot be ruled out. Grain development is altered in *lys3* mutants in a wide variety of ways, as described in the introduction. Here, it was demonstrated that *Lys3* is required for normal development of both embryo and endosperm from the earliest time point examined. Although total germination and germination rate were the same in *Lys3* mutant grain and wild type in non-dormant grain, *lys3* mutant Risø1508 showed lower post-harvest dormancy than wild-type. In this study, it has not been possible to determine the primary effect a lesion at *Lys3* nor whether the effects are primarily on embryo development, endosperm development or on the development of both.

The effect of a lesion at the *Lys3* locus on post-harvest dormancy suggests that there are defects in the imposition or breaking of dormancy. The imposition of so-called primary dormancy (dormancy imposed prior to seed dispersal) on the grain takes place during grain/seed maturation and is controlled by both endogenous and exogenous cues (Koornneef *et al.*, 2002; Nambara *et al.*, 2010). ABA metabolism (ABA synthesis and ABA sensitivity) in the developing grain has a central role in the imposition of dormancy and the intensity of primary dormancy in the mature grain varies greatly according to genotype and environmental growing conditions during development (Koornneef *et al.*, 2002; Kermode, 2005; Black *et al.*, 2006; Nambara *et al.*, 2010). At maturity, the desiccated grain often displays dormancy, the lack of germination under conditions which would normally be favourable to germination. During dry after-ripening, dormancy is released such that the grain will germinate in a widening window of temperature conditions (Gubler

et al., 2005; Iglesias-Fernández *et al.*, 2011). The mechanisms of dormancy breaking are largely unknown however roles for phytohormones (gibberellins, ABA metabolism and sensitivity, ethylene) and hormone-independent signals (such as reactive oxygen species, nitrate and NAD) have been suggested (Iglesias-Fernández *et al.*, 2011). Hormonal changes have previously been described in Risø1508; Risø1508 exhibited a peak in gibberellin-like activity higher and later in development than wild-type Bomi and Risø1508 auxin content was low throughout development, one tenth of the maximum content in Bomi (Mounla *et al.*, 1980). As gibberellins and ABA act antagonistically in a range of developmental processes including the imposition of primary dormancy in maize (White *et al.*, 2000), the increase gibberellin-like activity in Risø1508 may be a consequence of altered ABA metabolism. Reduced ABA activity during grain development leads to preharvest sprouting or viviparity (premature germination during grain maturation which is negatively associated with dormancy [Lin *et al.*, 2009]). Preharvest sprouting was not observed in any of the *Lys3* mutants during this study. However, recent work by Dr Thomas Howard (unpublished) has shown that Risø1508 and many other low-starch barley mutants are prone to preharvest sprouting under certain growing conditions. This suggests that reduced post-harvest dormancy observed in Risø1508 may be, at least in part, due to reduced induction of primary dormancy as a result of reduced starch accumulation in *lys3* mutants.

The alterations to embryo anatomy in the *lys3* mutants are highly distinctive. To my knowledge no other mutant, in any plant species, has been found to cause similar changes to the scutellar epithelium. Interestingly, this aspect of the *lys3* phenotype appears to be very variable; M1460 embryo scutellar epithelium was highly deformed (almost tumourous), whereas the Risø18 embryo appeared normal during development. Given that the scutellar epithelium is in close contact with the endosperm and likely to be important for embryo:endosperm interactions, it is possible that the primary function of the gene underlying *Lys3* is in embryo development and the effects on the endosperm are secondary. Cell number and cell size are both altered in the embryo of *lys3* mutants, whereas in the endosperm, only cell size and not cell number was found to be reduced in Risø1508 (Olsen and Krekling, 1980). The reduced cell size in the endosperm may be due to reduced starch biosynthesis. However, the possibility that the primary function of *Lys3* is in the endosperm or in both the embryo and the endosperm cannot be ruled out.

As outlined in the introduction to this chapter, the rice *giant embryo* (*ge*) mutant shares a number of phenotypic similarities with *lys3* mutants. The GE protein belongs the CYP78A class of cytochrome P450s and an *Arabidopsis* CYP78A (*KLUH/CYP78A5*) has been previously shown to regulate organ size through a non-cell-autonomous, novel signal

molecule (Anastasiou *et al.*, 2007; Adamski *et al.*, 2009). An analogous pathway may exist in cereals. Both *ge* and *lys3* have large embryos and reductions in endosperm size, however I have shown that a closely related barley orthologue of *Ge* (*HvGe*) does not lie on the same chromosome as *Lys3*. Therefore, this homologue cannot be the gene underlying the *Lys3* locus. However, another homologue or paralogue of *Ge* may exist in the barley genome.

Cahoon *et al.* (2006) suggest that two of the maize *Ge* homologues (*ZmGe1* and *ZmGe2*) were associated with the high-oil trait in Illinois High Oil maize and therefore, by inference, with embryo size. Although this information is reported in a patent rather than published in a peer-reviewed publication, this suggests that in maize there is more than one CYP78A with a role in determining embryo size. Phylogenetic analysis (Figure 4.16) has shown *ZmGe1* to be the maize orthologue of rice *Ge*. Therefore, the gene underlying *Lys3* may be a yet-to-be-discovered barley orthologue of the maize *ZmGe2*. However, there is a lack of conservation of CYP78As between the different grass species. For example, the *Brachypodium* genome does not appear to have orthologues of either rice *Ge* or *Pla1* nor does it contain an orthologue in the *Ge*-like clade. For this reason, it is impossible to infer how many CYP78As there are in barley or what their possible functions might be. The gene lying at the *Lys3* locus may not be a CYP78A but may be a factor in the same pathway. For example, *HvGe* may be involved in the production of a mobile signal and *Lys3* may have a role in signal transduction or recognition. Further work is required to determine whether this is the case.

Alternatively, the gene underlying *Lys3* may not be involved in a CYP78A mediated pathway and it may be involved in another signalling pathway. In rice, the *Plastochron3* (*Pla3*) mutant has larger embryos than wild-type, as well as an increased rate of leaf initiation during vegetative growth (Kawakatsu *et al.*, 2009). *Pla3* encodes a glutamate carboxypeptidase which is thought to catabolise small acidic peptides, possibly releasing signal molecules (Helliwell *et al.*, 2001). There is an indication that *Pla3* and the *Pla1/CYP78A11* may operate in the same pathway as they share a number of phenotypes and both PLA1 and PLA3 are required for maintenance of the shoot apical meristem. The gene underlying the *Lys3* locus may be an orthologue of the rice *Pla3* glutamate carboxypeptidase gene. However, *lys3* mutants do not share all the features of the rice pla3 mutant, for example, altered timing of leaf initiation.

A recently published paper has shown that a reduction in expression of the rice cyclin gene *Orysa;CycB1;1* results in some grains developing with greatly enlarged embryos and endosperms which aborted around six days after pollination (Guo *et al.*, 2010). The

enlargement of the embryo was due to an increase in cell size but not cell number, particularly in the scutellum (Guo *et al.*, 2010). Cyclins bind to cyclin-dependent kinases (CDKs) and these complexes regulate the transitions between different phases of the cell cycle (Harashima and Schnittger, 2010). Cyclins are a family of proteins involved in the regulation of cell division which is a central determinant of organ size (Mizukami, 2001; Harashima and Schnittger, 2010). There are 49 predicted cyclin genes in the rice genome (La *et al.*, 2006). Analysis of expression patterns of 25 rice cyclin genes showed temporal and spatial variation in gene expression (Guo *et al.*, 2007). While some cyclin genes were expressed constitutively in all tissues examined, others were preferentially expressed at certain development stages and in certain organs (Guo *et al.*, 2007). As cell division is a central determinant of organ size, genes involved in the regulation of core cell cycle, such as a cyclin, may be underlying the *Lys3* locus.

5. Probing the role of starch in the cereal embryo

5.1. Aim

The primary aim was to investigate the role of starch metabolism in the developing cereal embryo by searching for a mutation leading to a reduction in starch biosynthesis in that tissue. A rice mutant with reduced AGPase activity in the embryo was identified and characterised.

5.2. Introduction

In the mature cereal caryopsis, starch is the major storage compound. The vast majority of this starch is stored in the endosperm; in contrast, starch is of little quantitative importance in the mature embryo. However, the embryo is relatively rich in lipid and protein and as such, it is a potential target for manipulating the nutritional value of cereals. A patent filed in 1996 claims that reduced starch biosynthesis in the developing embryos of maize increases the amount of lipid in the mature embryo without altering embryo size (Singletary *et al.*, 2001). This suggests that there is a trade-off between the accumulation of starch and accumulation of lipid in the embryo. The patent, however, does not elaborate on the extent to which lipid is accumulated and does not investigate the mechanisms. To my knowledge, no variety has been commercialised under this piece of intellectual property and no further publications have elaborated on this finding.

The role of starch metabolism in the development of the cereal embryo is not well understood. However, the pattern of starch accumulation has been described in wheat and barley. Starch accumulates in the embryo during development and is then broken down towards maturity (Figure 3.3) (Black *et al.*, 1996). At its peak, around 25-30 days after flowering, embryos of wheat contain around 25 mg gFW^{-1} starch (Black *et al.*, 1996) and embryos of barley contain around 20 mg gFW^{-1} or 60 mg gDW^{-1} starch (Figure 3.3). This pattern of starch accumulation has also been observed in embryos of the Brassicaceae species *Arabidopsis* (Baud *et al.*, 2002) and *B. napus* (da Silva *et al.*, 1997; Eastmond and Rawsthorne, 2000) and attempts have been made to elucidate the relationship between starch and lipid in the embryos of these species. From this work three main hypotheses have emerged, as outlined below.

Firstly, *B. napus* embryos have been shown to degrade their starch at around the time at which rapid lipid accumulation begins. This has led to the idea that starch serves as a temporary carbon store for lipid synthesis (Norton and Harris, 1975; Eastmond and

Rawsthorne, 2000). There is little or no experimental evidence to support this hypothesis. The work of Vigeolas *et al.* (2004) is equivocal. Embryo-specific reduction in AGPase activity in *B. napus* embryos led to a reduction in embryo starch accumulation and a reduction in the rate of lipid biosynthesis early in development but there was no reduction in total lipid at maturity. This suggested that a reduced ability to sequester starch does not impact on the overall ability to synthesise lipid, which is clearly contrary to data for maize embryos as reported in Singletary *et al.* (2001).

The second hypothesis concerning the relationship between starch and oil accumulation in the embryo suggests that early accumulation of starch may contribute to establishing the embryo as a sink organ (da Silva *et al.*, 1997; Vigeolas *et al.*, 2004). Converting sucrose into starch will decrease sucrose concentration and this may increase sucrose import, thus contributing to sink strength. This idea is supported by the observation that the rate of oil synthesis is reduced early in the development of *B. napus* embryos with reduced AGPase activity (Vigeolas *et al.*, 2004). This reduction in synthesis rate may be an indication of reduced sucrose flux into the embryo.

Based on the complex spatial and temporal accumulation of starch in the embryos of *Arabidopsis*, it has been speculated that starch accumulates and turns over in cells that are dividing or have recently divided (Borisjuk *et al.*, 1995; Andriotis *et al.*, 2010). Androitis *et al.* (2010) cite a number of examples of starch being found to accumulate around meristematic tissues. This suggests that starch may be a temporary carbon store, providing energy for mitosis and cell expansion. If this hypothesis were true, it would be anticipated that individual cells within a tissue would be accumulating and degrading their starch according to developmental stage, i.e. when cells move from division to differentiation phase. Evidence for this possibility comes from work on *B. napus* plastid preparations, which showed that starch synthesis and starch degradation are occurring simultaneously in developing embryos (da Silva *et al.*, 1997). In addition, *A. thaliana* mutants unable to degrade their starch, accumulate starch to higher levels in the embryo from early in their development. This suggest that under normal circumstances starch is turned over from early in development (Andriotis *et al.*, 2010).

While cereals and oil seeds share a similar pattern of embryo starch accumulation, the mature seeds are quite different. *Arabidopsis* and *B. napus* have a much reduced endosperm and rely on embryo oil as the major carbon store for post-germinative growth, whereas cereals rely on the starchy endosperm for post-germinative growth. Therefore it is not clear to what extent work on Brassica embryo development is applicable to cereals. To further investigate the role of starch in the embryos of cereals, I aimed to identify

cereal mutants with reduced starch content in the embryo. To do this, a search for mutants with reduced ability to synthesise starch in the embryo was carried out. Furthermore, in order to elucidate the role of starch in the embryo specifically, I aimed to identify a mutation that would reduce embryo starch biosynthesis without effect, or with little effect, on endosperm starch biosynthesis. Effects on starch biosynthesis in the endosperm can have radical effects on grain morphology, such as grain shrivelling. As such, it was important to select mutants with minimal or reduced endosperm phenotype. With this in mind, I searched for mutants of AGPase. AGPase in cereals is a heterotetrameric enzyme comprising two large subunits (LSU) and two small subunits (SSU). Both subunits are required for normal activity *in vivo* (Giroux and Hannah, 1994; Lee *et al.*, 2007). AGPase catalyses the conversion of glucose-1-phosphate to ADP-glucose and is the first committed step in the pathway. In cereals, there are multiple genes which encode the different subunits of AGPase. Although knowledge is incomplete, it is known that different subunits combine to form different isoforms of AGPase. These different isoforms are responsible for AGPase activity in different tissues and also in different compartments of the cell. In the endosperm of grasses, the major AGPase isoform is cytosolic (Denyer *et al.*, 1996; Thorbjornsen *et al.*, 1996a; James *et al.*, 2003). However, there is no evidence of cytosolic AGPase activity in the embryo and AGPase in this organ is therefore likely to be entirely plastidial. I hypothesise that reducing or entirely abolishing plastidial AGPase activity in the grain, will reduce starch biosynthesis in the embryo with only a small effect on AGPase activity, and therefore starch content, in the endosperm.

In barley, wheat and rice, there are two genes encoding SSUs and these fall into two classes, Type 1 and Type 2 (Table 5.1). Maize has at least one additional Type 1 SSU gene (*Aps3*), likely due to a whole genome duplication which occurred after maize had diverged from the Triticeae (for review see Comparot-Moss and Denyer 2009). In the Triticeae, the Type 1 SSU gene is responsible for both the plastidial AGPase activity in the leaf and the cytosolic AGPase activity in the grain by alternate splicing of the first exon (Thorbjornsen *et al.*, 1996b; Burton *et al.*, 2002b; Rösti *et al.*, 2006). The Type 2 SSU gene is thought to be responsible for the plastidial activity in both the embryo and the endosperm, as plastidial AGPase activity in these organs is not entirely abolished in Type 1 gene null mutants. Therefore, Type 2 SSU mutants would be expected to have reduced or absent AGPase activity in the embryo and relatively normal (cytosolic) AGPase activity in the endosperm. Thus, a Type 2 SSU mutant should have reduced starch content specifically in the embryo.

Table 5.1 – Summary of AGPase small subunit (SSU) genes in cereals (rice, barley, wheat and maize) and their expression.

Gene class	Species	Gene name[2]	Previous names	Expression pattern (a & b denote alternative transcripts)	Protein	
					Subcellular location	Tissue location
Type 1	Rice	*OsAps1*	*Aps2, Agps2*	a – strong in the grain, absent in leaves[3]	Cytosol[1]	Endosperm[1]
				b - strong in leaves, weak in grain[3]	Plastid[1]	Leaf[1]
	Maize	*ZmAps1*	*Brittle2, Agp2*	a – strong in endosperm, weak in leaves, roots and embryo[7,8]	Cytosol[5,6]	Endosperm[5,6]
				b – Some expression in leaf, stem, root, embryo and endosperm[7,8]	Plastid*[8]	Unknown
		ZmAps3	*L2, agpslzm*	a – unknown[a]		Unknown
				b – strong in the leaf, weak in the endosperm, present in the stem, roots and embryo[7,11]	Plastid*[8]	Unknown
	Barley	*HvAgps1*	*Agps1, beps, blps*	a – strong in the endosperm, present in the embryo, absent in leaf[9]	Cytosol[9,10]	Endosperm[9,10]
				b – strong in the leaf and embryo, weak in the endosperm[9]	Plastid[9]	Leaf[9]
	Wheat	*TaAgps1*		a - strong in the endosperm, weak in the leaf[8]	Cytosol*[12]	Unknown
				b - strong in leaf and endosperm[8]	Plastid*[12]	Unknown
Type 2	Rice	*OsAps2*	*Aps1, Agps1*	Present in the grain, leaves and leaf sheath[2,4,8]	Plastid[1]	Unknown
	Maize	*ZmAps2*	*agpsemzm*	Present in the endosperm, embryo, stem and roots, and also weak in leaves[7,8]	Plastid*[13]	Unknown
	Barley	*HvAgps2*		Present in endosperm and embryo and weak in leaves[8,9]	Plastid*[13]	Unknown
	Wheat	*TaAgps2*		Present in the endosperm and weak in leaves[8]	Plastid*[13]	Unknown

* predicted subcellular location. [a] exon 1a present but unlikely to be functional (Rösti and Denyer, 2007).

[1]Lee *et al.* (2007), [2]Akihiro *et al.* (2005), [3]Ohdan *et al.* (2005), [4]Hirose *et al.* (2006), [5]Denyer *et al.* (1996), [6]Tsai and Nelson (1966), [7]Cossegal *et al.* (2008), [8]Rösti and Denyer (2007), [9]Rösti *et al.* (2006), [10]Thorbjorgsen *et al.* (1996b), [11]Prioul *et al.* (1994), [12]Burton *et al.* (2002b), [13]Comparot-Moss and Denyer (2009).

A second mutation likely to reduce starch content in the embryo specifically would be one affecting the LSU responsible for the plastidial AGPase activity in the grain. As with the SSU, there are multiple LSU genes in cereals (Table 5.2). Knowledge of LSU number and function is most complete in rice. There are four LSU genes in the rice genome. *OsApl1* is required for almost all the AGPase activity in the leaves (Rösti *et al.*, 2007). *OsApl2* is required for normal grain filling and APL2::GFP fusions were shown to localise to the cytosol (Lee *et al.*, 2007). *OsApl3* and *OsApl4* mutants have not yet been identified but GFP-fusions of both genes localise to the plastid (Lee *et al.*, 2007) and are expressed in the developing grain (Akihiro *et al.*, 2005; Ohdan *et al.*, 2005). Taken together, this suggests that both *OsApl3* and *OsApl4* are good candidates for encoding the LSU responsible for plastidial AGPase activity in the grain. In barley, the knowledge of the LSU genes is incomplete. Two barley LSU genes have been identified: *HvApl1* (*blpl*) which is expressed mostly in leaves (Eimert *et al.*, 1997) and *HvApl3* (*bepl*) which is strongly expressed in the endosperm (Villand *et al.*, 1992). It is not clear whether *HvApl3* (*bepl*) is responsible for cytosolic activity, plastidial activity or both in the barley endosperm. This uncertainty about the role of *HvApl3* (*bepl*) made it an unsuitable candidate for this study.

Our first approach to obtaining a mutant with reduced starch content in the embryo was to use the Scottish Crop Research Institute's barley TILLING platform to identify null mutations in *HvAps2.* From primers designed to a partial cDNA sequence, mutations were identified in the DNA of 27 plants. Only three of these were subsequently confirmed as mutants. Unfortunately, all three mutant lines contained mutations within an intron. Since mutations in non-coding sequence are highly unlikely to affect expression or translation, they were of no use in this study. This lack of success with the barley TILLING approach led me to try to identify AGPase mutations in publicly-available rice mutant collections.

I investigated the genetic resources available in rice including T-DNA-insertion lines and *Tos17*-insertion lines. The NIAS database was searched for insertions in *OsAps2*, *OsApl3* and *OsApl4* (Table 5.3) (National Institute of Agrobiological Sciences, Japan; http://tos.nias.affrc.go.jp). Three lines were identified with *Tos17* insertions in *OsApl3*. There were lines NC7135, NE1391 and NF3982. The characterisation of these mutant lines is described in this chapter. No insertions were found in either *OsAps2* or *OsApl4*.

Tos17 is an endogenous retrotransposon, found at low frequency in the rice genome, which has been used for large-scale *Tos17*-mediated mutagenesis. The *Tos17* transposon is 4114 bp long and induces transformations in the genome. These insertions are considered stable as the transposable element is not active under normal growing

Table 5.2 - Summary of AGPase large subunit (LSU) genes in cereals and their expression.

Gene class	Species	Gene name[2]	Previous names	Expression pattern	Protein	
					Subcellular location	Tissue location
Type 1	Rice	*OsApl1*	*Agpl3*	Strong in leaves and stem, weak in the grain and absent from roots[2,3]	plastid[5]	leaf[5]
	Maize	*ZmApl1*		Unknown	plastid*[9]	Unknown
	Barley	*HvApl1*	*Blpl*	Strong in leaves, weak in endosperm, embryo and roots[6,7,8]	plastid*[9]	Unknown
	Wheat	*TaApl1*	*Aga1*	leaf[13]	plastid*[9]	Unknown
Type 2	Rice	*OsApl2*	*Shrunken2*	Strong in the grain, weak in stem and roots absent from leaves[2,3]	cytosol[1]	endosperm[1]
	Maize	*ZmApl2*	*Shrunken2*	endosperm[11]	cytosol[12]	endosperm[12]
Type 3	Rice	*OsApl3*	*Agpl1*	Present in the grain, stems, weak in leaves and absent from roots[2,3,4]	plastid[1]	Unknown
	Maize	*ZmApl3*	*Agp1*	Strong in the embryo, weak in the endsperm[10]	plastid*[9]	Unknown
	Barley	*HvApl3*	*Bepl*	Present in endosperm and stem and weak in leaves and roots[7,8,9]	plastid*[9,14]	Unknown
	Wheat	*TaApl3*	*Agp2, Aga7*	Present in endosperm and embryo and 1 homoeoallele in the leaves, weak in roots[13,15,16]	plastid*[9]	Unknown
Type 4	Rice	*OsApl4*		Weak in the grain, stem and leaves, absent from roots[2,3]	plastid[1]	Unknown

* predicted subcellular location.

[1]Lee *et al.* (2007), [2]Akihiro *et al.* (2005), [3]Ohdan *et al.* (2005), [4]Hirose *et al.* (2006), [5]Rösti *et al.* (2007), [6]Doan *et al.* (1999), [7]Eimert *et al.* (1997), [8]Villand *et al.* (1992), [9]Comparot-Moss and Denyer (2009), [10]Giroux and Hannah (1994), [11]Prioul *et al.* (1994), [12]Tsai and Nelson (1966), [13]Olive *et al.* (1989), [14]Rösti (2006), [15]Thorneycroft *et al.* (2003), [16]Ainsworth *et al.* (1995).

conditions. Transposon activation takes place during tissue culturing, by reverse transcription of an RNA intermediate, and is inactivated in regenerated plants. This allows the production of large insertion-mutant libraries, similar to the T-DNA libraries available for *Arabidopsis thaliana*. There are typically between five and 30 transposon copies per haploid genome after regeneration (Hirochika, 2001; Miyao *et al.*, 2003). Large populations have been screened, using *Tos17*-flanking sequence, to locate the position of individual inserts in the rice genome. This information and seed from these insertion lines are publicly available at NIAS (http://tos.nias.affrc.go.jp).

Table 5.3 – Genes encoding large and small subunits of AGPase in the rice genome.

Subunit	Gene name	NCBI	TIGR
LSU	*OsApl1*	*Os03g0735000*	*Os03g52460*
	OsApl2	*Os01g0633100*	*Os01g44220*
	OsApl3	*Os05g0580000*	*Os05g50380*
	OsApl4	*Os07g0243200*	*Os07g13980*
SSU	*OsAps1*	Os08g0345800	Os08g25734
	OsAps2	*Os09g0298200*	*Os09g12660*

5.3. Results

5.3.1. Confirmation of insert position and genotype of the mutations

T2 seed from each of the three Apl3 insertion lines was obtained. Lines NC7135 and NE1391 were reported to have *Tos17* inserts in the intron between exons two and three. Line NF3982 was reported to have a Tos17 insert in exon 6 (Figure 5.1A). Primers were designed to identify plants carrying only the wild-type *Apl3* gene, only the mutant *apl3* gene or carrying both (Figure 5.1B and Figure 5.1C). Twelve T2 seeds from each line were germinated and the seedlings genotyped.

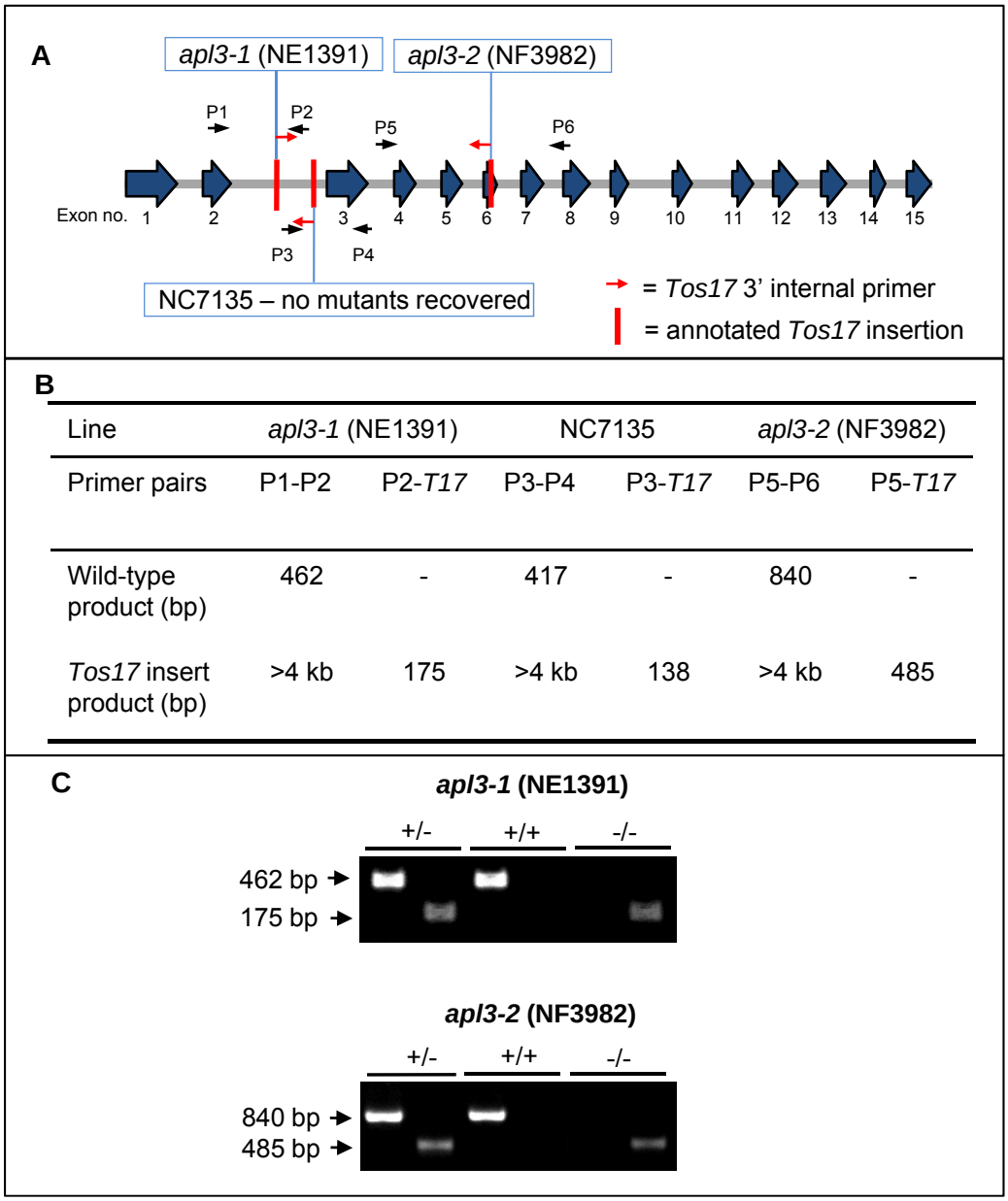
All NC7135 seedlings were found to have only the wild-type *Apl3* gene. Subsequently, the remaining eight T2 seeds of the 20 seeds supplied by the NIAS were germinated and genotyped but were also found to contain only the wild-type gene. At this point NC7135 was discarded from the study as none of the seed supplied contained an insertion in *Apl3*.

NE1391 seedlings were found to be segregating for the *Tos17* insert. Two wild-type plants, four plants heterozygous for the insert and six homozygous mutants were identified. Sequencing of the PCR products confirmed the insert position to be between exons two and three. The progeny from homozygous mutant plants and homozygous wild-type plants were used for subsequent analysis and are referred to as *apl3-1* (homozygous mutant) and wild-type segregant (homozygous wild type).

NF3982 seedlings were also found to be segregating for the *Tos17* insert. Nine heterozygous plants and three homozygous mutants but no homozygous wild types were identified. Sequence of the PCR products confirmed that the *Tos17* insert was within exon six. The progeny from homozygous mutant plants were used for subsequent analysis and are referred to as *apl3-2*. The wild-type segregant was subsequently selected from the T3 progeny of a heterozygous T2 plant.

5.3.2. Amplification and sequencing of the mutant transcript

To establish the effects of the mutations in *apl3-1* and *apl3-2* on transcription, reverse transcriptase (RT) PCR was carried out on RNA prepared from the developing grain of plants homozygous for the mutant gene or homozygous for the wild-type gene. First strand cDNA was used as a template to amplify and sequence the *apl3-1* and *apl3-2* transcripts. The *apl3-1* PCR product was indistinguishable in size from that of the wild type but the *apl3-2* product was slightly (~100 bp) smaller than that of the wild type (Figure 5.2A). Sequence analysis of these PCR products revealed that the *apl3-1* product was indistinguishable from wild type. Presumably the *Tos17* insertion is spliced out, together



Line	*apl3-1* (NE1391)		NC7135		*apl3-2* (NF3982)	
Primer pairs	P1-P2	P2-*T17*	P3-P4	P3-*T17*	P5-P6	P5-*T17*
Wild-type product (bp)	462	-	417	-	840	-
Tos17 insert product (bp)	>4 kb	175	>4 kb	138	>4 kb	485

Figure 5.1 – Genotyping of *Tos17* segregating plants.

A – Diagrammatic representation of *OsApl3* showing the annotated *Tos17* retrotransposon insertion positions. Blue arrows represent exons and grey lines represent introns. Exons are numbered 1 to 15. Red and black arrows indicate the position of primers used for PCR-based genotyping.

B – Primer pairs used for genotyping segregating plants by PCR.
For each of the mutant lines primers were designed to amplifying genomic DNA across the annotated *Tos17* insertion site. PCR was used to determine presence or absence of the *Tos17* insertion. Expected fragment sizes of >4 kb were too large to amplify under the PCR conditions used for genotyping.

C – Example of PCR analysis to distinguish between heterozygous (+/-), homozygous wild type (+/+) and homozygous mutants (-/-). PCR was carried out using the primer pairs shown in section B and approximate sizes determined by separation in agarose. The sizes of the sequenced products are indicated.

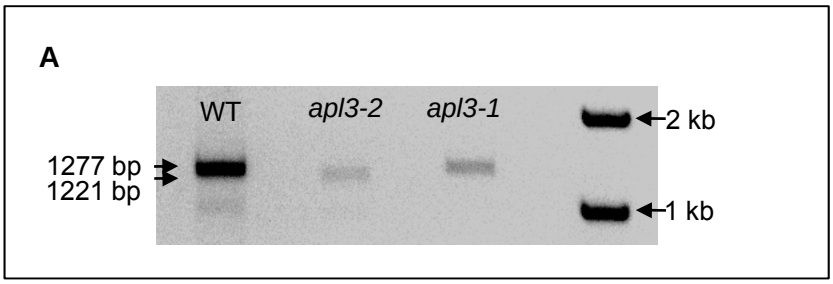


Figure 5.2 – RT-PCR analysis of *Apl3* transcripts in wild type and homozygous *apl3-1* and *apl3-2* mutants.

Primers were designed amplify a 1277 bp region of the *Apl3* transcript between exon 2 and exon 15. cDNA was prepared from RNA extracted from developing grain.

A – Example of PCR products separated in 1.2 % agarose. The sizes of the sequenced products are indicated. Wild type (WT) shown here is *Apl3-2* wild-type segregant.

B

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OsApl3 coding sequence  (1)    ATGCAGTTCAGCAGTGTGTTTCCCTTAGAGGGTAAAGCATGCGTGAGCCCAATAAGGAGAGGTGGTGAGGGCTCA

OsApl3 coding sequence  (76)   GCTAGTGATAGATTGAAGATCGGGGACAGTAGCAGCATCAAGCATGATAGAGCAGTGAGGAGGATGTGTCTTGGT

OsApl3 coding sequence  (151)  TATAGGGGCACCAAAAACGGTGCACAATGTGTGCTCACCTCAGATGCTGGCCCAGACACTCTTCATGTCCGAACA

OsApl3 coding sequence  (226)  TCATTCCGGAGGAACTTTGCGGATCCAAATGAGGTTGCTGCTGTTATATTGGGTGGTGGCACCGGGACTCAACTT
apl3-1 consensus               -------------ACTTTGCGGATCCAAATGAGGTTGCTGCTGTTATATTGGGTGGTGGCACCGGGACTCAACTT
apl3-2 consensus               -------------ACTTTGCGGATCCAAATGAGGTTGCTGCTGTTATATTGGGTGGTGGCACCGGGACTCAACTT

OsApl3 coding sequence  (301)  TTTCCTCTCACAAGCACAAGGGCCACGCCTGCTGTTCCTATTGGAGGATGCTATAGGCTTATCGATATCCCCATG
apl3-1 consensus               TTTCCTCTCACAAGCACAAGGGCCACGCCTGCTGTTCCTATTGGAGGATGCTATAGGCTTATCGATATCCCCATG
apl3-2 consensus               TTTCCTCTCACAAGCACAAGGGCCACGCCTGCTGTTCCTATTGGAGGATGCTATAGGCTTATCGATATCCCCATG

OsApl3 coding sequence  (376)  AGCAACTGTTTCAACAGTGGCATAAACAAGATATTCATAATGACTCAATTCAACTCAGCATCTCTTAATCGTCAC
apl3-1 consensus               AGCAACTGTTTCAACAGTGGCATAAACAAGATATTCATAATGACTCAATTCAACTCAGCATCTCTTAATCGTCAC
apl3-1 consensus               AGCAACTGTTTCAACAGTGGCATAAACAAGATATTCATAATGACTCAATTCAACTCAGCATCTCTTAATCGTCAC

OsApl3 coding sequence  (451)  ATTCATCGTACGTACCTTGGTGGTGGAATCAACTTTACTGATGGATCTGTTGAGGTATTAGCCGCTACACAAATG
apl3-1 consensus               ATTCATCGTACGTACCTTGGTGGTGGAATCAACTTTACTGATGGATCTGTTGAGGTATTAGCCGCTACACAAATG
apl3-2 consensus               ATTCATCGTACGTACCTTGGTGGTGGAATCAACTTTACTGATGGATCTGTTGAGGTATTAGCCGCTACACAAATG

OsApl3 coding sequence  (526)  CCTGGGGAGGCTGCTGGTTGGTTCCAGGGTACAGCAGATGCAGTTAGAAAATTTATCTGGGTTCTTGAGGACTAT
apl3-1 consensus               CCTGGGGAGGCTGCTGGTTGGTTCCAGGGTACAGCAGATGCAGTTAGAAAATTTATCTGGGTTCTTGAGGACTAT
apl3-2 consensus               CCTGGGGAGGCTGCTGGTTGGTTCCAGGGTACAGCAGATGCAGTTAGAAAATTTATCTGGGTTCTTGAGGACTAT

OsApl3 coding sequence  (601)  TACAAGCATAAAGCTATAGAACACATTTTAATCTTGTCAGGAGATCAGCTTTATCGTATGGACTACATGGAGCTT
apl3-1 consensus               TACAAGCATAAAGCTATAGAACACATTTTAATCTTGTCAGGAGATCAGCTTTATCGTATGGACTACATGGAGCTT
apl3-2 consensus               TACAAGCATAAAGCTATAGAACACATTTTAATCTTGTCAGGAGATCAGCTTTATCGTATGGACTACATGGAGCTT

OsApl3 coding sequence  (676)  GTGCAGAAACATGTTGATGACAATGCTGACATTACTTTATCATGTGCTCCTGTTGGAGAGAGTCGAGCATCTGAC
apl3-1 consensus               GTGCAGAAACATGTTGATGACAATGCTGACATTACTTTATCATGTGCTCCTGTTGGAGAGAGTCGAGCATCTGAC
apl3-2 consensus               GTGCAG----------------------------------------------------------TCGAGCATCTGAC

OsApl3 coding sequence  (751)  TATGGACTAGTGAAGTTCGACAGTTCAGGCCGTGTAATTCAATTTTCTGAAAAACCCAAGGGCACTGACTTGGAA
apl3-1 consensus               TATGGACTAGTGAAGTTCGACAGTTCAGGCCGTGTAATTCAATTTTCTGAAAAACCCAAGGGCACTGACTTGGAA
apl3-1 consensus               TATGGACTAGTGAAGTTCGACAGTTCAGGCCGTGTAATTCAATTTTCTGAAAAACCCAAGGGCACTGACTTGGAA

OsApl3 coding sequence  (826)  GCAATGAAAGTGGATACCAGCTTTCTCAATTTTGCCATAGACGACCCGACTAAATTTCCCTACATTGCTTCGATG
apl3-1 consensus               GCAATGAAAGTGGATACCAGCTTTCTCAATTTTGCCATAGACGACCCGACTAAATTTCCCTACATTGCTTCGATG
apl3-2 consensus               GCAATGAAAGTGGATACCAGCTTTCTCAATTTTGCCATAGACGACCCGACTAAATTTCCCTACATTGCTTCGATG

OsApl3 coding sequence  (901)  GGAGTTTATGTCTTCAAAAGAGATGTTCTTTTAAACCTTCTAAAGTCAAGATATGCAGAACTACATGACTTTGGG
apl3-1 consensus               GGAGTTTATGTCTTCAAAAGAGATGTTCTTTTAAACCTTCTAAAGTCAAGATATGCAGAACTACATGACTTTGGG
apl3-2 consensus               GGAGTTTATGTCTTCAAAAGAGATGTTCTTTTAAACCTTCTAAAGTCAAGATATGCAGAACTACATGACTTTGGG

OsApl3 coding sequence  (976)  TCTGAAATCCTCCCAAGAGCTTTACATGAGCACAATGTACAGGCATATGTCTTCGCTGACTACTGGGAAGACATT
apl3-1 consensus               TCTGAAATCCTCCCAAGAGCTTTACATGAGCACAATGTACAGGCATATGTCTTCGCTGACTACTGGGAAGACATT
apl3-2 consensus               TCTGAAATCCTCCCAAGAGCTTTACATGAGCACAATGTACAGGCATATGTCTTCGCTGACTACTGGGAAGACATT

OsApl3 coding sequence (1051)  GGAACGATCAGATCGTTCTTTGATGCAAACATGGCCCTTTGCGAGCAGCCTCCCAAGTTTGAGTTTTATGATCCA
apl3-1 consensus               GGAACGATCAGATCGTTCTTTGATGCAAACATGGCCCTTTGCGAGCAGCCTCCCAAGTTTGAGTTTTATGATCCA
apl3-2 consensus               GGAACGATCAGATCGTTCTTTGATGCAAACATGGCCCTTTGCGAGCAGCCTCCCAAGTTTGAGTTTTATGATCCA

OsApl3 coding sequence (1126)  AAAACTCCCTTCTTCACTTCACCTCGATATTTGCCGCCAACAAAGTCAGATAAATGCAGGATTAAAGATGCGATA
apl3-1 consensus               AAAACTCCCTTCTTCACTTCACCTCGATATTTGCCGCCAACAAAGTCAGATAAATGCAGGATTAAAGATGCGATA
apl3-2 consensus               AAAACTCCCTTCTTCACTTCACCTCGATATTTGCCGCCAACAAAGTCAGATAAATGCAGGATTAAAGATGCGATA

OsApl3 coding sequence (1201)  ATTTCCCATGGCTGTTTCTTGCGTGAATGTACCATCGAGCATTCGATAGTTGGAGTCCGCTCACGCCTTAACTCT
apl3-1 consensus               ATTTCCCATGGCTGTTTCTTGCGTGAATGTACCATCGAGCATTCGATAGTTGGAGTCCGCTCACGCCTTAACTCT
apl3-1 consensus               ATTTCCCATGGCTGTTTCTTGCGTGAATGTACCATCGAGCATTCGATAGTTGGAGTCCGCTCACGCCTTAACTCT

OsApl3 coding sequence (1276)  GCATGTGAGCTCAAGAATACCATGATGATGGGTGCGGATTTGTACGAAACTGAAGATGAAATTTCAAGACTACTG
apl3-1 consensus               GCATGTGAGCTCAAGAATACCATGATGATGGGTGCGGATTTGTACGAAACTGAAGATGAAATTTCAAGACTACTG
apl3-2 consensus               GCATGTGAGCTCAAGAATACCATGATGATGGGTGCGGATTTGTACGAAACTGAAGATGAAATTTCAAGACTACTG

OsApl3 coding sequence (1351)  TCAGAAGGCAAGGTCCCCATTGGTGTAGGGGAAAACACAAAGATAAACAACTGCATCATCGACATGAACGCGAGG
apl3-1 consensus               TCAGAAGGCAAGGTCCCCATTGGTGTAGGGGAAAACACAAAGATAAACAACTGCATCATCGACATGAACGCGAGG
apl3-2 consensus               TCAGAAGGCAAGGTCCCCATTGGTGTAGGGGAAAACACAAAGATAAACAACTGCATCATCGACATGAACGCGAGG

OsApl3 coding sequence (1426)  GTTGGAAGGAACGTGGTCATCACAAACAGCGAGGGTGTCCAAGAAAGTGATCGGCCTGAGGAAGGGTACTACATA
apl3-1 consensus               GTTGGAAGGAACGTGGTCATCACAAACAGCGAGGGTGTCCAAGAAAGTGATCGGCCTGAGGAAGGGTACTACATA
apl3-2 consensus               GTTGGAAGGAACGTGGTCATCACAAACAGCGAGGGTGTCCAAGAAAGTGATCGGCCTGAGGAAGGGTACTACATA

OsApl3 coding sequence (1501)  AGGTCGGGAATCGTGGTGATCCTGAAGAACGCGACCATCAAGGACGGGAAGGTCATATAG
apl3-1 consensus               AGGTCGGGAATCGTG-----------------------------------------------
apl3-2 consensus               AGGTCGGGAATCGTG-----------------------------------------------
```

5.2B – Consensus sequence of cDNA from homozygous *apl3-1* and homozygous *apl3-2* mutant plants. The sequence used to design primers to amplify *OsApl3* are underlined. The box indicates a 56 bp region absent from the *apl3-2* sequence.

with the intron sequence, and does not induce a mutation in the transcript (Figure 5.2B). Analysis of the *apl3-2* product revealed that it lacked a 56-bp region.

Alignment of genomic DNA and coding sequence showed that the 56-bp region absent from the *apl2-3* transcript corresponded exactly to the sequence encoded by exon six (Figure 5.3A). The absence of this sequence leads to a frame-shift mutation and premature stop codon. The absence of exon six from the mature transcript is likely to be a result of mis-recognition of splice junctions (Figure 5.3B). This phenomenon has been previously observed in *Tos17* mutants, for example see Rösti *et al.* (2007). In *apl3-1*, the *Tos17* insertion has had no effect on exon-intron splicing resulting in a wild type transcript (Figure 5.3B). The normal removal of an intron containing a *Tos17* insert has previously been described, for example see Chen *et al.* (2007).

5.3.3. Analysis of APL3 proteins

To test for presence or absence of APL3 protein (or truncated APL3 protein) in *apl3-2*, endosperm and embryo extracts were probed with antisera raised to AGPase subunits from a variety of sources. To my knowledge, there are no antisera raised against rice LSUs. Rice embryo extract was probed with antisera raised to maize endosperm LSU and SSU (Shunken2 and Brittle2, respectively) (Greene and Hannah, 1998), spinach leaf LSU, barley endosperm LSU and SSU (Bepl and Beps, respectively) and pea embryo LSU. Only the pea LSU antiserum produced bands around the predicted size of the LSU (~54 kD) but this antibody produced multiple bands (Figure 5.4C). In an effort to identify the different bands recognised by the pea LSU antiserum, endosperm and embryo extracts from previously described *apl1* and *apl2* mutants were included (Lee *et al.*, 2007; Rösti *et al.*, 2007) and blots were carried out with Brittle2 and Shunken2 antisera (Figure 5.4A-C).

The maize endosperm SSU antiserum (Brittle2) recognised a single band in both the endosperm and the embryo extracts of *apl1, apl2, apl3-2* and wild type. The maize endosperm LSU antiserum (Shunken2) recognised a single band in the endosperm extracts of *apl1, apl3-2* and wild type but not in the extract of *apl2*. The Shrunken2 antiserum failed to give any bands in the embryo extract. The pea embryo LSU antiserum produced multiple bands in both endosperm and embryo extracts.

The single band recognised by the Brittle2 antiserum at 45-50 kD is consistent with the size of the AGPase SSU (Greene and Hannah, 1998; Rösti *et al.*, 2007). Therefore, the band in the endosperm extracts is likely to be the rice cytosolic SSU (cS) and the band in the embryo extracts is likely to be the rice plastidial SSU (pS). The band in the embryo extract appears to be somewhat smaller than the band in the endosperm extracts (cS).

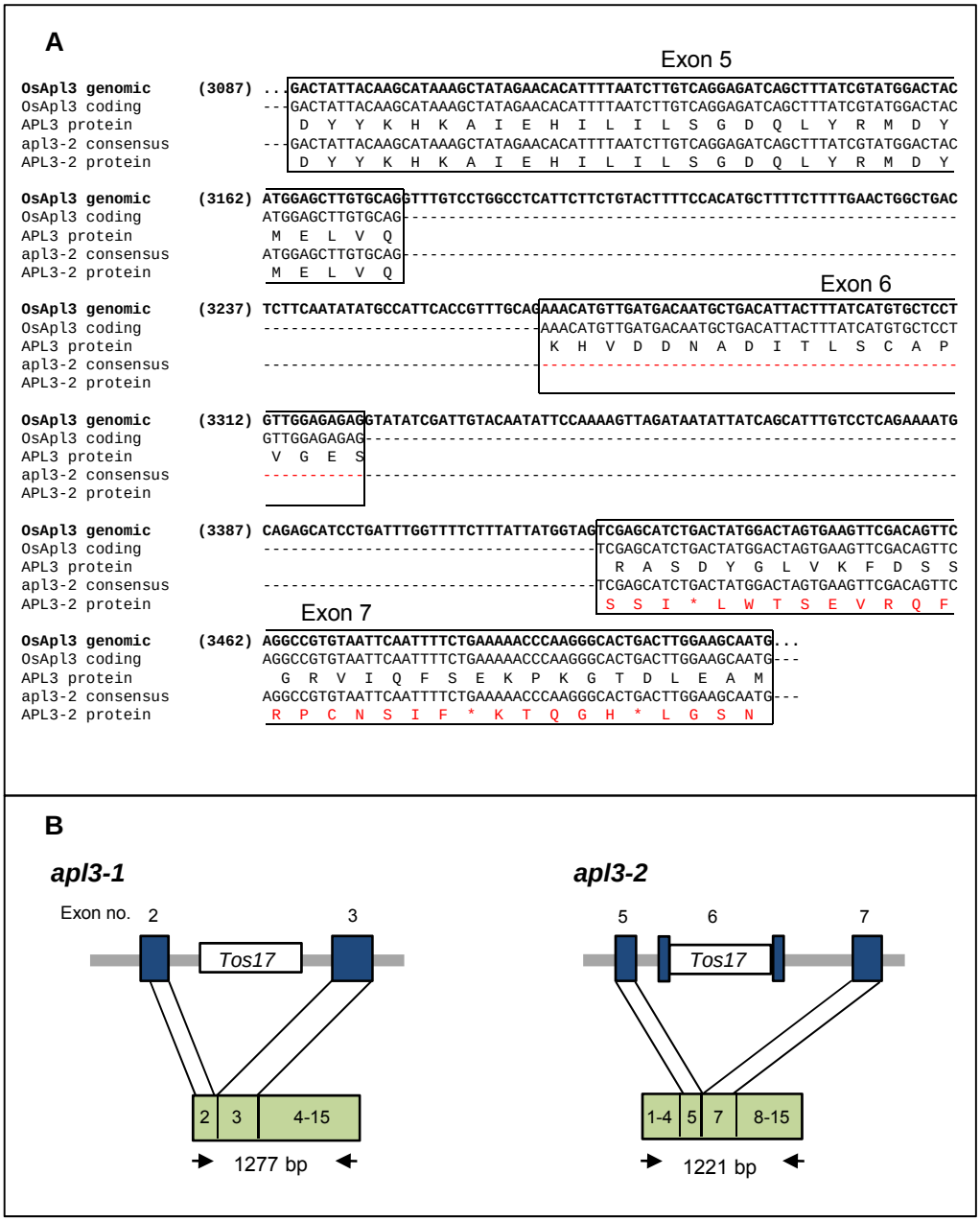

Figure 5.3 – The effects of the *Tos17* insertions on transcripts and predicted protein sequences.

A – Alignment of gDNA, cDNA and predicted amino acid sequences. Amino acid sequence was predicted from cDNA sequence. Exons are boxed. Deviations in the sequence found in *apl3-2* when compared to *Apl3* are shown in red.

B – A diagrammatic representation of the exon splicing. The blue boxes denote exons, the grey lines introns and the resulting transcript is shown by the green boxes below. PCR priming positions are indicated by the arrows.

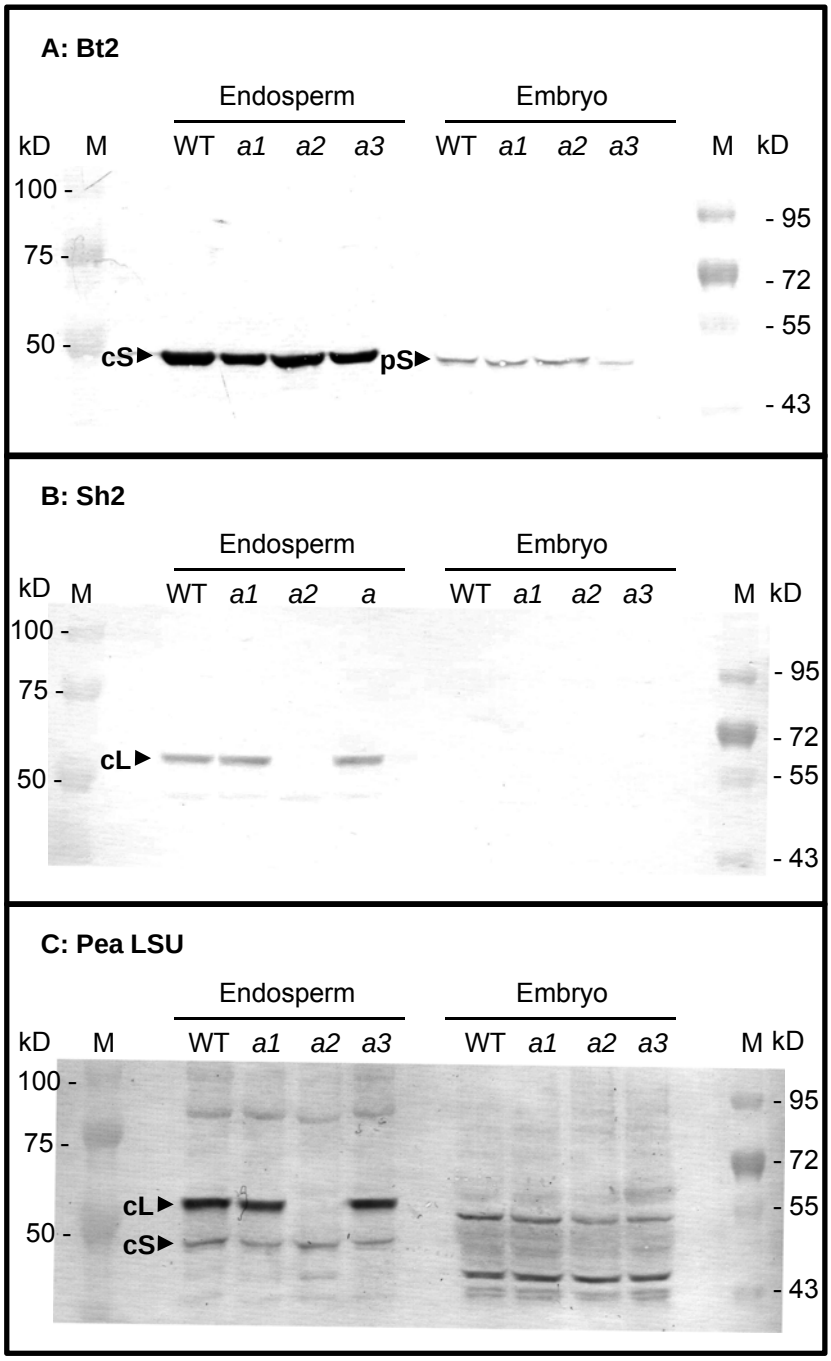

Figure 5.4 – Immunoblot analysis of endosperm and embryo extracts from wild type (WT) and *apl1 (a1)*, *apl2 (a2)* and *apl3-2 (a3)* mutants using antibodies raised to AGPase subunits.

Extracts of developing endosperm and developing embryo were fractionated by SDS-PAGE, transferred to PVDF membrane and probed with antisera. One endosperm of approximately 18 mg was extracted and samples equivalent to 0.25 mg were loaded onto the gel. Embryo samples of approximately 5 mg (7-10 embryos) were extracted and samples equivalent to 0.5 mg were loaded on to the gel. Antisera used were: A: Brittle2, raised against maize cytosolic SSU, B – Shunken2, raised against maize cytosolic LSU (both used at 1/5000 dilution) and C: Pea embryo LSU, raised against a pea LSU (used at 1/750 dilution). The molecular weights (kD) of marker proteins (M) are shown. Putative AGPase subunits are labelled: cytosolic LSU = cL, cytosolic SSU = cS and plastidial SSU = pS.

This is consistent with the plastidial SSU lacking the functional transit peptide cleaved off during translocation into the plastid. The plastidial SSU (pS) identified by the Brittle2 antiserum appeared to be less abundant in the *apl3-2* embryo extract (Figure 5.4A). The reduced size of band in the *apl3-2* embryo extract was seen in two independent experiments. This suggests that in the embryos of the *apl3-2* mutant, there is a reduction in the amount of SSU protein. The single band recognised by the Shrunken2 in endosperm extracts at ~55 kD, and its absence from the *apl2* mutant extract, demonstrates that this band corresponds to the rice endosperm cytosolic LSU protein (cL, Figure 5.4B). Of the multiple bands seem in the blot using the pea embryo LSU, it is possible to identify the cytosolic large and small subunits in the endosperm extracts based on their correspondence to Brittle2 and Shrunken2 blots. In the embryo extracts probed with the pea LSU, it is not possible to identify which band corresponds to either the LSU or the SSU. However, there is no band, present in the wild type embryo extract, that appears to be absent in the *apl3-2* mutant. Therefore, an *OsApl3*-specific antibody is required to determine whether APL3 is present or absent in the *apl3-2* mutant.

5.3.4. AGPase activity in the embryos of *apl3-1* and *apl3-2* mutants

To discover whether *Apl3* is required for normal AGPase activity in the embryo, AGPase activity was measured in the embryos of *apl3-1* and *apl3-2* mutants compared to wild-type segregants (Figure 5.5). Although *apl3-1* was shown to produce transcript identical to wild type, it was included here to test whether there was an effect on AGPase enzyme activity in the embryo as a result of quantitative changes in gene expression. In the *apl3-1* mutant, there was no statistically significant difference in embryo AGPase activity compared to wild type ($t_{(8)}$ = 0.48, p = 0.643). At this point *apl3-1* was discarded from the study as it does not affect *Apl3* transcript sequence or AGPase activity in the embryo. In the *apl3-2* mutants, there was a statistically significant reduction in embryo AGPase activity compared to wild type ($t_{(8)}$ = 8.72, p < 0.001). The mutant had 67 percent less AGPase activity than the wild-type on a fresh weight basis (Figure 5.5). This suggests that *Apl3* is required for most but not all of the AGPase activity in the embryo.

To test whether the remaining AGPase activity in embryo samples of *apl3-2* was due to small amounts of endosperm contamination of the samples, the amount of endosperm contamination required to give the observed AGPase activity was calculated. In the *apl3-2* mutant, the activity was ~0.5 μmol min^{-1} gFW^{-1} whereas endosperm AGPase activity is typically 20 times higher, in the region of 10 μmol min^{-1} gFW^{-1} (see Figure 5.9). The ability of the Brittle2 antibody to detect cytosolic SSU (cS) in endosperm samples at 20-fold dilution was tested (Figure 5.6). At a 20-fold dilution of endosperm tissue, the Brittle2

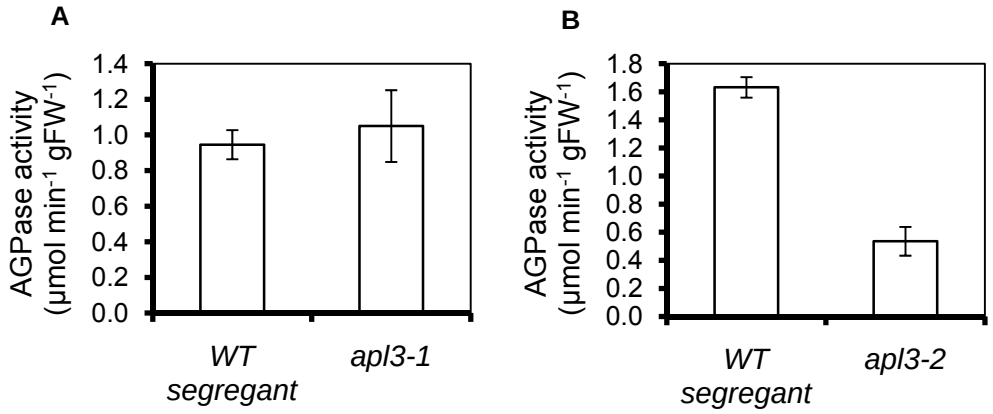

Figure 5.5 – AGPase activity in the embryos of *apl3-1* and *apl3-2* compared to wild-type (WT) segregants.

Embryos were dissected from developing grains approximately 10-15 days after flowering and towards the end of the light period. Samples of six to 11 embryos, each sample from a separate plant, were assayed for AGPase activity. Values are means (± SE) of measurements of five extracts, each assayed in duplicate. Mutant and respective wild-type segregant were grown simultaneously under controlled environment conditions. There was no significant difference in mean embryo weight between mutant and respective wild-type segregant. Mean embryo weight for experiment A was 0.79 mg whereas mean embryo weight for experiment B was 0.63 mg.

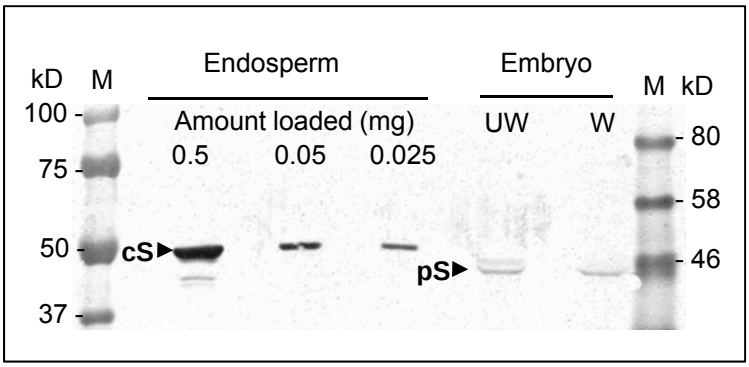

Figure 5.6 – Immunoblot analysis of wild-type endosperm and embryo extracts using Brittle2 antiserum.

Extracts of developing endosperm and developing embryo were fractionated by SDS-PAGE, transferred to PVDF membrane and probed with Brittle2 antiserum (used at 1/500- dilution). Five endosperms of approximately 80 mg were extracted and samples equivalent to 0.5 mg, 0.05 mg and 0.025 mg were loaded on to the gel. Embryo samples of approximately 8 mg (10 embryos) were extracted and samples equivalent to 0.5 mg were loaded on to the gel. Embryo samples were either excised and extract un-washed (UW) or washed in 1 mL cold extraction buffer (W) prior to extraction. The molecular weights (kD) of marker proteins (M) are shown. Putative AGPase subunits are labelled: cytosolic SSU = cS and plastidial SSU = pS.

antiserum is still clearly able to identify the cytosolic AGPase SSU in endosperm samples. This means that, if contamination of embryo samples with endosperm was responsible for the remaining activity in *apl3-2,* we would expect to find a corresponding band in the embryo samples. This band is absent in both the washed and unwashed embryo samples. Only a smaller band is detectable (pS). This band is likely to correspond to the plastidial AGPase SSU. This is good evidence that the activity in the *apl3-2* mutant embryos is not due to contamination with endosperm AGPase activity, at least not in its entirety.

5.3.5. Segregation analysis

To establish whether the *Tos17* insertion in the *apl3-2* mutant is responsible for the low embryo AGPase activity or whether it is due to mutation in another unlinked gene, segregation analysis was performed. A segregating population (n=60) of T3 plants was genotyped and AGPase activity measured in developing embryos (Figure 5.7). The population contained 16 wild type, 32 heterozygous and 12 mutant plants. Regression analysis showed that genotype was a significant factor explaining variation in AGPase activity ($F_{(2,57)}$ = 27.81, p < 0.001, genotype explaining 47.6 % of variation in the regression model). The mean AGPase activity was 1.05 µmol min^{-1} $embryo^{-1}$ in the wild type and 0.47 µmol min^{-1} $embryo^{-1}$ in the mutant. A semi-dominant allele would dictate that the heterozygous AGPase activity would fall equidistant between these values. The theoretical heterozygote activity is therefore 0.76 µmol min^{-1} $embryo^{-1}$. The measured activity in heterozygous plants was 0.77 µmol min^{-1} $embryo^{-1}$ which does not differ statistically significantly from that of the theoretical heterozygote ($t_{(31)}$ = 0.21, p = 0.381). Although, it should be noted that individual embryos in the samples from heterozygous plants will be homozygous wild type, heterozygous and homozygous mutant in the ratio of 1:2:1, the mean value will still be that of heterozygous embryos. Taken together, these results demonstrate that the AGPase activity co-segregates with the *Tos17* insertion in *Apl3* and that the *Apl3-2* allele is semi-dominant compared to the *apl3-2* mutant allele, with respect to AGPase activity in the embryo.

5.3.6. Starch content of *apl3-2* mutant embryos

To test whether decreased AGPase activity in the embryos of the *apl3-2* mutant had an impact on embryo starch content, starch in the embryos of developing grains of the *apl3-2* mutant was measured and compared to wild type (Figure 5.8). Regression analysis was carried out (Table 5.4). Mean embryo fresh weight did not differ statistically significantly between *apl3-2* and wild type. Mean embryo fresh weight was a statistically significant covariate with starch content for both *apl3-2* and wild type. Embryo starch content was significantly lower in *apl3-2* embryos than in wild type embryos. Mean starch content per

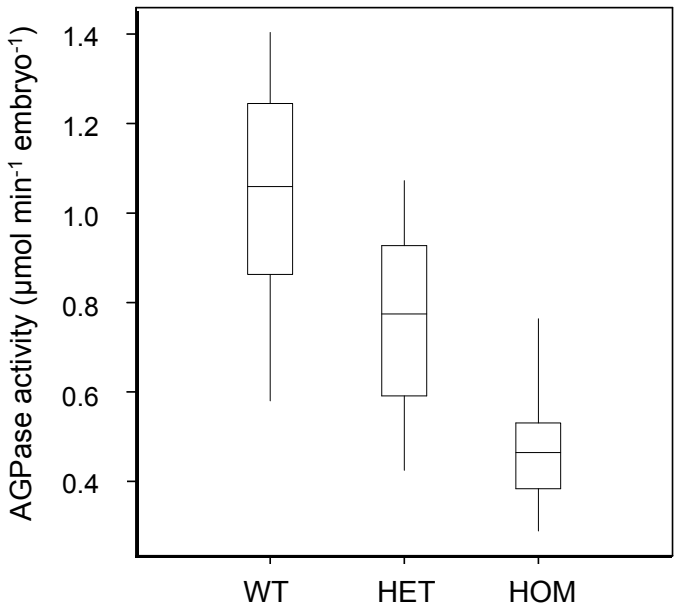

Figure 5.7 – AGPase activity in the embryos of a population segregating for *apl3-2*.

A population of segregating T3 plants (n = 60) were genotyped for *apl3-2* and embryo AGPase activity measured. AGPase activity for each genotype is represented by boxes with whiskers. Boxes denote the interquartile range of the data (median value is shown by the bisecting line) and whiskers denote the range. The genotypes are: WT - homozygous wild type, HET – heterzygous, and HOM - homozygous *apl3-2* mutant.

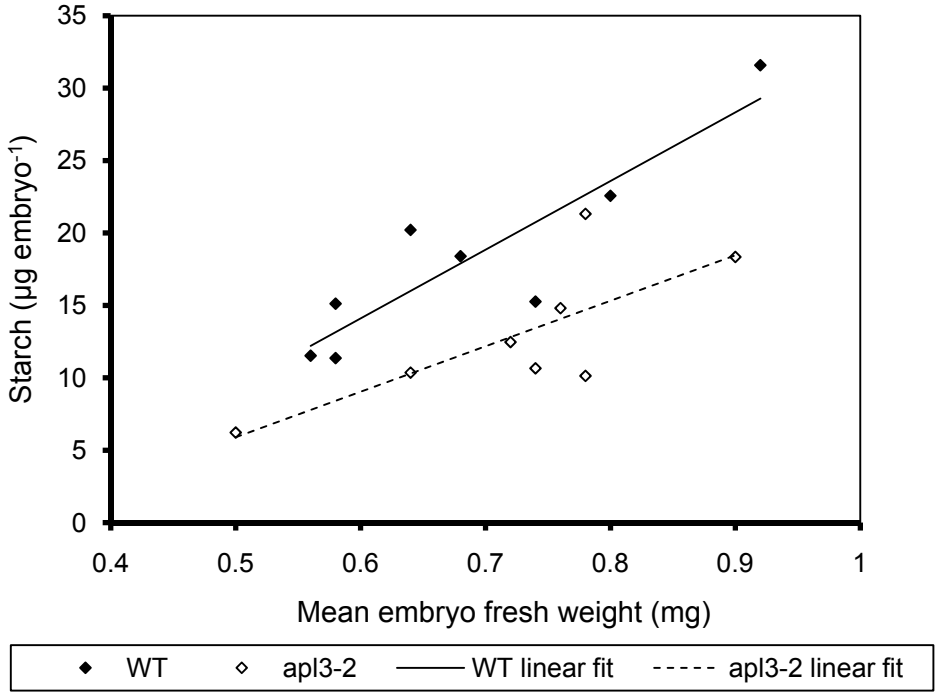

Figure 5.8 – Starch content in the embryos of *apl3-2* compared to wild-type (WT) segregant.

Embryos were dissected from developing grains approximately 10 to 15 days after flowering and towards the end of the light period. Samples of five embryos, eight samples from four plants, were weighed, extracted and assayed for starch. Each sample was assayed in triplicate. Plants were grown simultaneously under controlled environment conditions.

Table 5.4 – Accumulated analysis of variance for starch per embryo. The regression model accounted for 71.3 % of total variance.

Source of variation	Degrees of freedom	Mean square	Variance ratio	*p* - value
+ Mean embryo weight	1	260.1	22.8	< 0.001
+ Genotype	1	180.0	15.8	0.002
+ Mean embryo weight * genotype	1	13.2	1.2	0.300
Error	12	11.3		

embryo in *apl3-2* (12.4 µg embryo^{-1}, SE 1.2, n = 8) was 35 percent lower than that in the wild type (19.2 µg embryo^{-1}, SE 1.2, n = 8). The reduction in AGPase activity, shown in the previous section to co-segregate with the *Apl3* mutation in *apl3-2*, is likely to be responsible for the concomitant reduction in embryo starch content.

5.3.7. Mature embryo dry weight and lipid content of *apl3-2* mutant embryos

To investigate whether a reduction in starch content in the embryo during development leads to an increase in lipid accumulation or an alteration in the dry weight of the mature embryo, embryos were dissected from mature grains after imbibition overnight at 4 °C in the dark and freeze-dried prior to weighing and lipid extraction. Five samples, each of 20 embryos and each from a separate plant, were analysed. There was no statistically significant difference in mean embryo dry weight between *apl3-2* and wild type (0.66 mg and 0.68 mg respectively; $t_{(8)}$= 0.97, p = 0.361) or in lipid content (85.25 µg embryo^{-1} SE 10.4 and 84.23 µg embryo^{-1} SE 11.0, respectively; $t_{(8)}$ = 0.28, p = 0.789).

5.3.8. AGPase activity in the endosperm of *apl3-2* mutants

To investigate the role of *Apl3* in the endosperm, endosperms collected at 10 to 15 days after flowering were assayed for AGPase activity (Figure 5.9). This time corresponds to a period in grain filling where AGPase activity is high (Nakamura and Yuki, 1992). Regression analysis showed that endosperm weight of the sample was a significant covariate and that endosperm AGPase activity was significantly lower in the *apl3-2* mutant than in wild type (Table 5.5). Mean AGPase activity in *apl3-2* (11.2 µmol min^{-1} gFW^{-1}, SE 0.6) was 23 percent lower than wild type (14.4 µmol min^{-1} gFW^{-1}, SE 0.6). This reduction in AGPase activity in the endosperm is likely to be due to the *Apl3* mutation in *apl3-2*.

The major form of AGPase activity in the endosperm is cytosolic and is encoded by *Apl2* and *Aps1* (Lee *et al.*, 2007). Plastidial activity is thought to contribute around 10 percent of the total endosperm activity (Sikka *et al.*, 2001). Data presented here (Figure 5.9) corroborate the idea that plastidial AGPase activity is of minor quantitative importance in the endosperm.

To test the hypothesis that the reduction in endosperm AGPase activity is due to a reduction in the plastidial activity, ion-exchange chromatography was carried out to separate cytosolic and plastidial activities (Figure 5.10A). This method of separation has previously been demonstrated in barley by Johnson *et al.* (2003) and in wheat by Burton *et al.* (2002b). Recovery of AGPase in the samples after fractionation was low for both wild type and mutant (on average 26 % for the wild type and 31 % for the mutant). Fractions were loaded onto a SDS-PAGE gel for separation and immunoblotting with

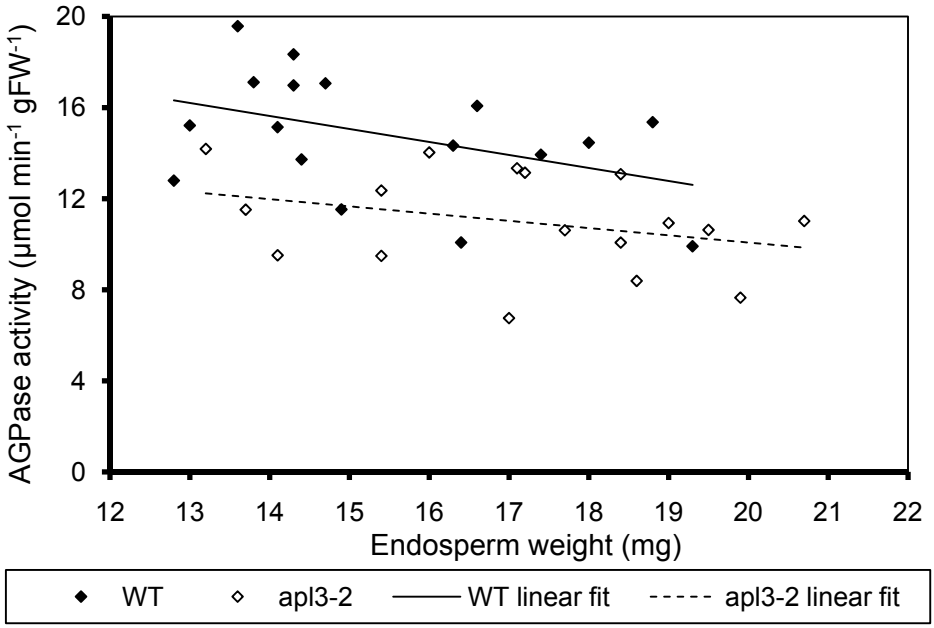

Figure 5.9 – AGPase activity in the endosperm of *apl3-2* compared to wild-type (WT) segregant.

Endosperms were harvested from developing grains approximately 10 to 15 days after flowering and towards the end of the light period. Individual endosperms, 17 per genotype from three to four separate plants, were assayed for AGPase activity. Each samples was assayed in duplicate. Plants were grown simultaneously under controlled environment conditions.

Table 5.5 – Accumulated analysis of variance for AGPase activity in the endosperm. The regression model accounted for 44.1 % of total variance.

Source of variation	Degrees of freedom	Mean square	Variance ratio	*p* - value
+ Endosperm weight	1	81.3	15.1	< 0.001
+ Genotype	1	69.6	13.0	0.001
Error	31	5.4		

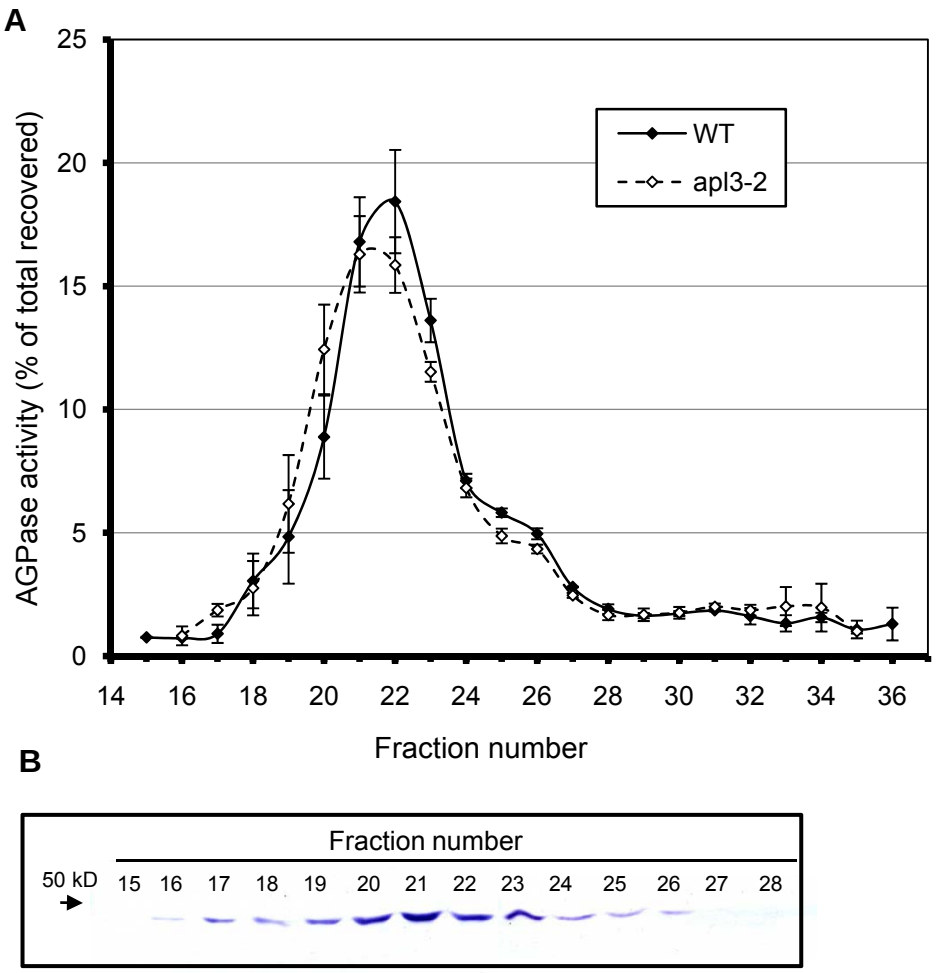

Figure 5.10 – Separation of AGPase isoforms using ion-exchange chromatography in *apl3-2* and WT endosperm extract.

Endosperms were harvested from developing grains approximately 10 to 15 days after flowering and towards the end of the light period. Values are means (± SE) of three independent experiments. Samples of 10 to 19 endosperms were extracted and the extract loaded onto the ion-exchange column (HiTrap MonoQ). After washing, a sodium chloride gradient was applied to the column and fractions (0.5 mL) were collected.

A – Amount of AGPase activity in each fraction

B – Immunoblot of fractions with Bt2 antiserum
Three volumes of each fraction were mixed with one volume of four times SDS-PAGE sample buffer and boiled for five minutes. Fifteen microlitres of each fraction was loaded onto a SDS-PAGE gel, fractionated, transferred to PVDF membrane and probed with Brittle2 antiserum (used at 1/500- dilution)

Brittle2 (SSU) antiserum (Figure 5.10B). Brittle2 antiserum clearly identified the cytosolic SSU and the pattern of its abundance corresponded to the large peak in activity between fractions 17 and 25. This provides evidence that the large peak in activity in the both the wild type and the mutant between fractions 17 and 25 is likely to correspond to the cytosolic AGPase activity. As the Brittle2 antiserum did not detect the plastidial SSU, it was not possible to identify the location of the peak containing the plastidial SSU. In previous studies, the peak in activity containing the plastidial activity eluted from the column after the cytosolic activity (Johnson *et al.*, 2003). In Figure 5.10A, I hypothesise that the plastidial activity is either poorly separated from the larger cytosolic peak in fractions 24 to 28 or distributed through fractions 28 to 35. In addition, there is no clear deviation of the *apl3-2* mutant profile from that of the wild type. Therefore, further work is required to resolve the hypothesis and to determine whether the reduction in total endosperm AGPase activity is due to a reduction in plastidial or cytosolic activity or both.

5.3.9. The effect of the *apl3-2* mutation on starch accumulation in various tissues of the plant

Apl3 has been shown to be expressed in the developing grain and stems, and weakly in leaves but transcript is absent from roots (Table 5.2). To investigate whether *Apl3* is required for normal starch biosynthesis in the various tissues of the plant, different tissues were collected at the end of the light period and stained with Lugol's solution (Figure 5.11). There was no discernable difference in the intensity of the staining of the leaves (base of flag leaf shown in A and G) or of the leaf sheath (top shown in A and G and base shown in D and J). Developing panicles (B and H) appeared to stain slightly darker in the wild type than the *apl3-2* mutant. Closer examination of the florets (F and L) suggested that the palea and lemma of the wild type stained slightly more than those of the mutant. In the culms of developing panicles, staining was strongest just above the node. In the *apl3-2* mutant, there was a clear reduction in staining of the culms compared to those of the wild type (C and I). This difference was also apparent in the staining of the first node below the panicle (E and K). There was no apparent difference in the staining of the pollen from *apl3-2* mutant and wild type (Figure 5.12).

Culm starch accumulation was quantified in the *apl3-2* mutant and the wild type (Figure 5.13). The wild type had a low level of starch in the culms at flowering (3.4 mg gFW^{-1}) but starch accumulated to 54.6 and 59.3 mg gFW^{-1} at 5 and 10 DAF, respectively. In the mutant there were lower levels of starch in the culms at flowering (mean 0.7 mg gFW^{-1}) and starch levels remained low with mean values of 3.1 and 1.6 mg gFW^{-1} at 5 and 10 DAF, respectively. Thus, in the *apl3-2* mutant, there was 97 percent less starch in the culm at 10 DAF compared to wild type.

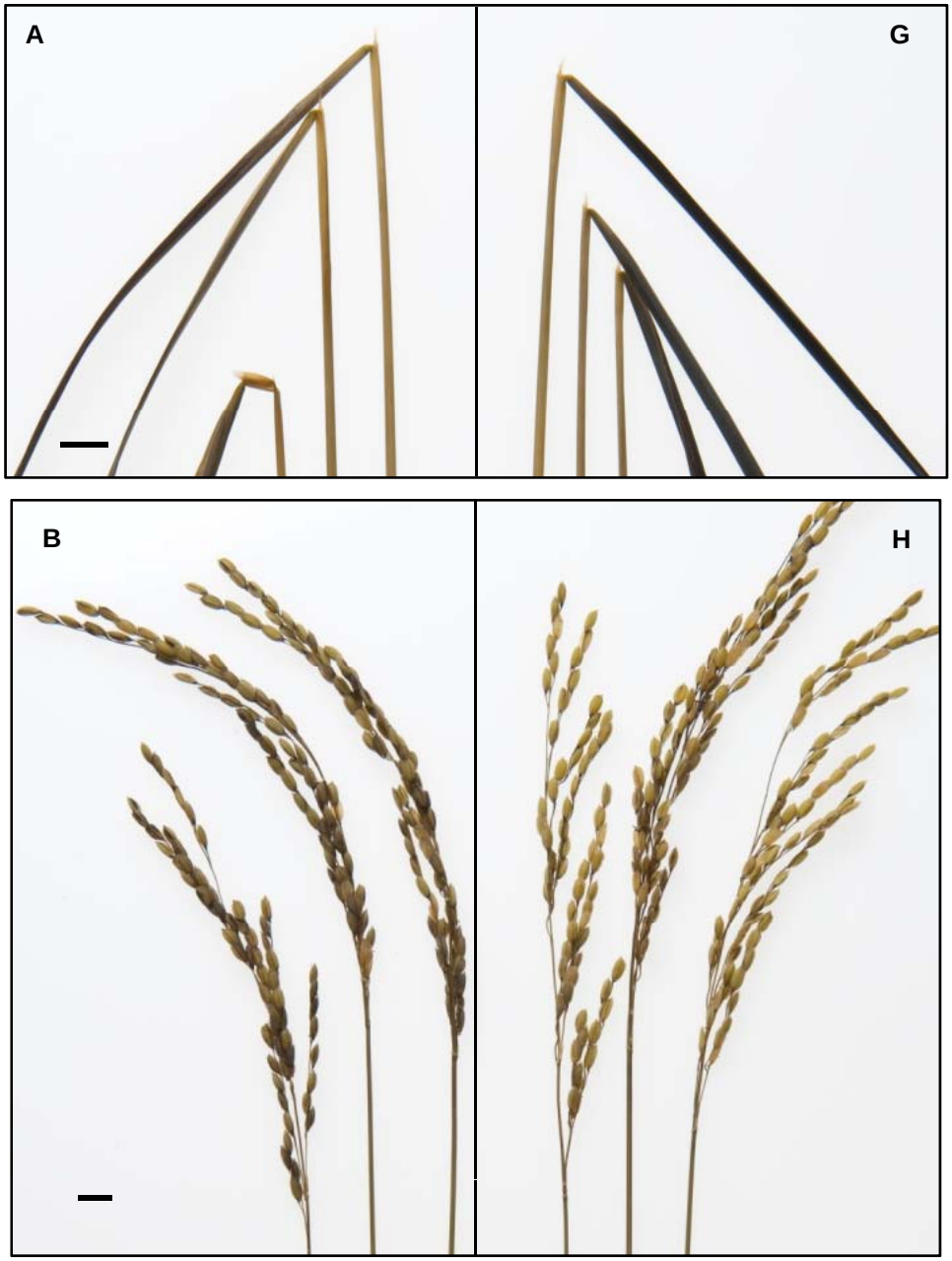

Figure 5.11 – Photographs of different parts of wild type and *apl3-2* mutant rice plants at 10 days after anthesis, stained with dilute Lugol's solution.

Panicles and flag leaf were harvested towards the end of the light phase from each of three separate plants, destained by boiling 70 % ethanol, and stained with dilute Lugol's solution.

Images are as follows (this page): A and G – top of flag leaf sheath and base of flag leaf blade, B and H – panicles, (over leaf) C and I – base of culm just above the first node, D and J – base of flag leaf sheath, E and K – first node, F and L – florets. A to F are wild type and G to L are *apl3-2* mutant. Scale bar = 1 cm.

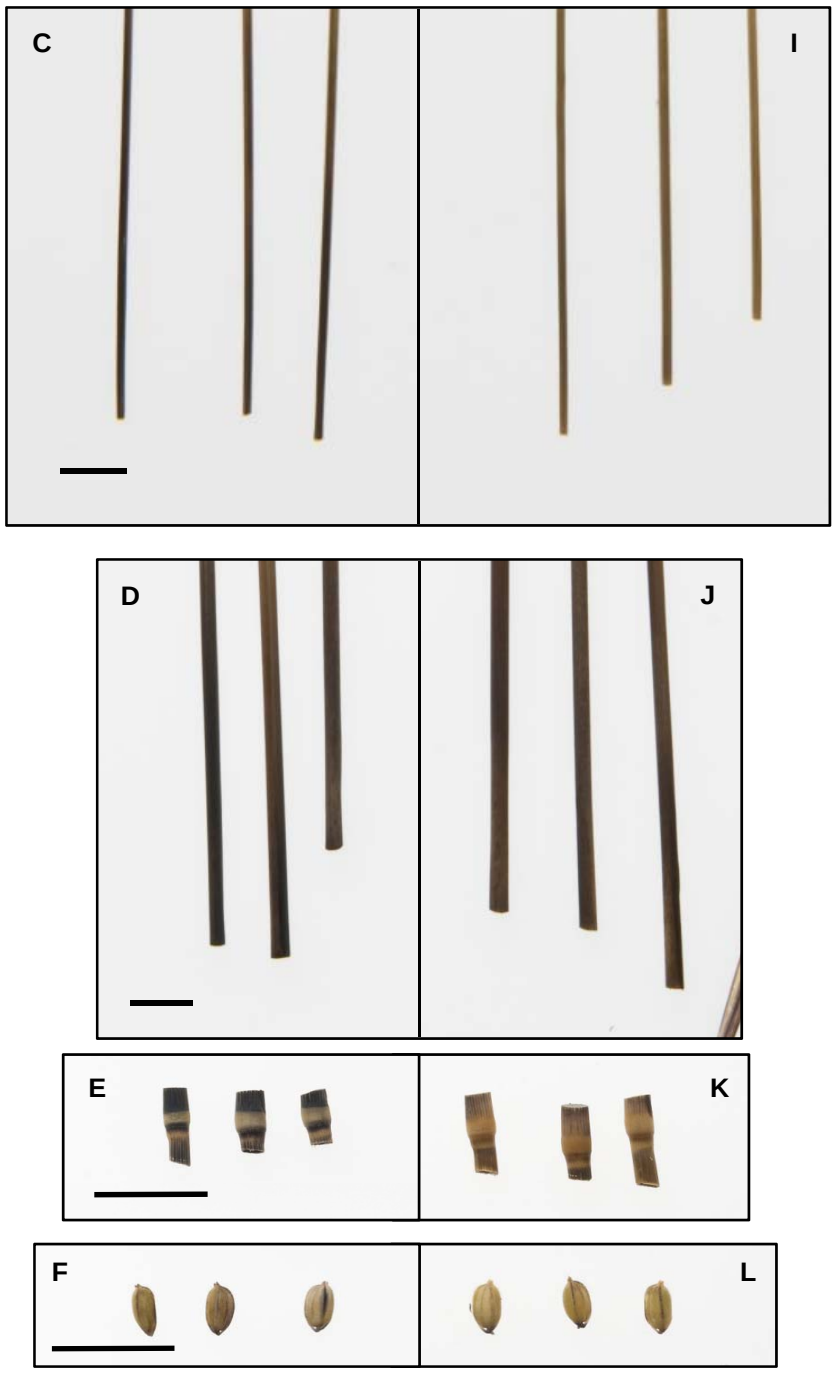

Figure 5.11 continued...

C and I – base of culm just above the first node, D and J – base of flag leaf sheath, E and K – first node, F and L – florets. A to F are wild type and G to L are *apl3-2* mutant. Scale bar = 1 cm.

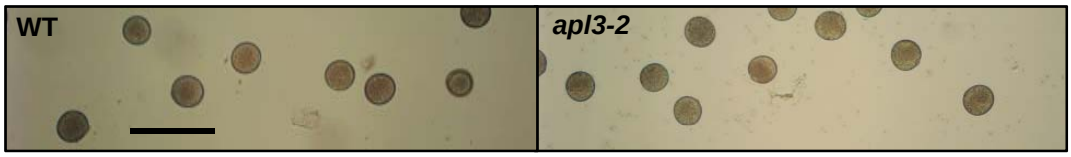

Figure 5.12 – Light micrographs of pollen from wild type and apl3-2 mutant stained with dilute Lugol's solution.

Anthers from flowering panicles were removed and the pollen applied to a microscope slide. Pollen was stained with dilute Lugol's solution and visualised under a light microscope. Scale bar = 100 µm.

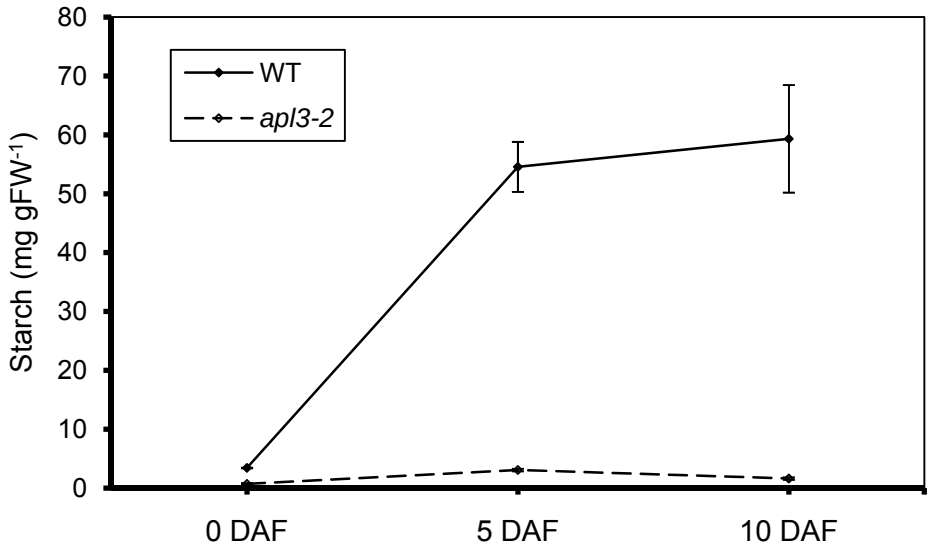

Figure 5.13 – Starch content in the culm of *apl3-2* compared to wild-type (WT) segregant.

Culms were harvested from developing panicles at anthesis (0 DAF), five days after flowering (5 DAF) and 10 days after flowering (10 DAF). Culms, from below the panicle to just above the first node, were harvested at the end of the light period, weighed and immediately frozen in liquid nitrogen. After extraction in perchloric acid, samples were assayed for starch content. Values are means (± SE) of measurements of three extracts, each from a separate plant. Each extract was assayed in triplicate.

5.3.10. The effect of the apl3-2 mutation on plant development and yield components

To evaluate the effects of the *apl3-2* mutation on plant growth and yield components, five *apl3-2* mutant plants were compared to five wild-type plants (Table 5.6). The date of flowering of the first panicle was recorded and statistical analysis showed that there was no significant difference in flowering time (Mann-Whitney test, $U_{5,5}$ = 11.5 p = 1.0). At maturity, a range of whole-plant metrics were measured (Table 5.6A). There was no statistically significant difference between wild type and mutant for any of the metrics measured. There was also no statistically significant difference in grain weight between the wild type and mutant (Table 5.6B).

5.3.11. The effect of *apl1* on embryo AGPase activity

In *apl3-2*, AGPase activity is not abolished in the embryo. To test whether *OsApl1* is responsible for the remaining activity in the embryo, AGPase activity was measured in the developing embryos of *apl1* mutants (Figure 5.14). *Apl1* has previously been shown to be required for all the AGPase activity in the rice leaf (Rösti *et al.*, 2007) and it is expressed at relatively low levels, compared to *Apl2* and *Apl3*, in the developing rice caryopsis (Ohdan *et al.*, 2005). In the homozygous *apl1* mutant, there was no significant reduction in embryo AGPase activity compared wild type ($t_{(10)}$ = 0.18, p = 0.857).

To eliminate the possibility that *Apl1* contributes to AGPase activity in the absence of *Apl3*, *apl3-2* and *apl1* homozygous mutants were cross-pollinated. The F1 seed was planted and allowed to self-pollinate. The resulting F2 seed was planted and the *apl3-2/apl1* double mutant was selected. The homozygous *apl3-2/apl1* appeared to grow normally and seed was set. The double mutant has yet to be analysed for embryo AGPase activity.

Table 5.6 – Analysis of growth metrics and yield components of apl3-2 compared to wild-type (WT) segregant.

Mature plants were allowed to dry down for several weeks prior to harvest. Values are means ± SE.

A – Whole plant metrics

	Wild type	*apl3-2* mutant	t-test *p*-value
Panicles /plant	7.4 ± 0.7	9.6 ± 1.3	0.178
Grain weight (hulled) /plant (g)	9.4 ± 1.2	11.4 ± 1.8	0.377
Straw weight /plant (g)	9.0 ± 1.1	9.9 ± 1.4	0.643
Total aerial weight /plant (g)	19.1 ± 2.3	22.2 ± 3.3	0.452
Total number of grains /plant	354.0 ± 42.0	435.2 ± 62.0	0.310
No of florets /panicle	56.7 ± 2.8	56.1 ± 2.7	0.921
No of florets unfilled /panicle	8.8 ± 0.7	10.8 ± 0.8	0.110
No of florets filled /panicle	47.8 ± 2.5	45.3 ± 2.4	0.525
Grain weight (hulled) /panicle (g)	1.3 ± 0.1	1.2 ± 0.1	0.423
Mean grain weight (mg)	26.5 ± 0.2	26.2 ± 0.2	0.416

B – Grain weight

Ten grains were removed from the first flowering panicle from each of the five plants and the 50 grains were individually weighed with and without the hull. Values are means ± SE.

	Wild type	*apl3-2* mutant	t-test *p*-value
Hulled grain weight (mg)	26.8 ± 0.26	26.3 ± 0.26	0.140
Naked grain weight (mg)	22.6 ± 0.22	22.2 ± 0.21	0.117
Calculated hull weight (mg)	4.2 ± 0.06	4.1 ± 0.05	0.476

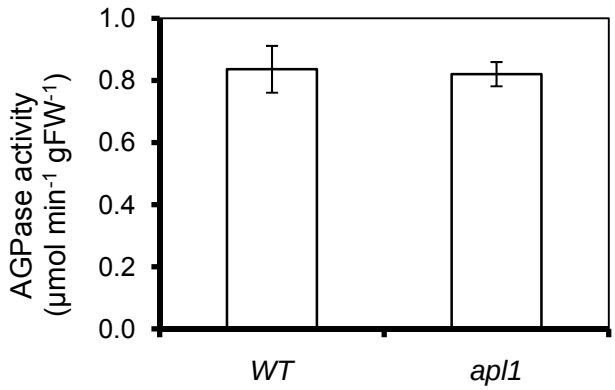

Figure 5.14 – AGPase activity in the embryos of *apl1* compared to wild-type (WT) segregants.

Embryos were dissected from developing grains approximately 10 to15 days after flowering and towards the end of the light period. Samples of 10 embryos, each sample from an individual plant, were assayed for AGPase activity. Values are means (± SE) of measurements of six extracts, each assayed in duplicate. There was no significant difference in mean embryo weight between mutant and respective wild-type segregant.

5.4. Discussion

In this chapter, the effects of two independent *Tos17* insertions in the rice AGPase LSU gene *OsApl3* have been characterised. These two lines, homozygous for the insert, were designated *apl3-1* and *apl3-2*. In *apl3-1*, the *Tos17* insert was located within an intron and no effect was observed on the mature RNA transcript or AGPase activity in the embryo. In *apl3-2*, the *Tos17* insert was located within an exon and led to a premature stop codon in the mature RNA transcript. In *apl3-2*, there was a large reduction in AGPase activity in the embryo and a small reduction in AGPase activity in the endosperm of developing rice caryopses. These reductions in AGPase activity led to reduced embryo starch content in the embryo but had no effect on mature grain weight. It was also shown that in *apl3-2* there is a drastic reduction in the ability to accumulate starch in the culms.

The Tos17 insertion in *OsApl3* was shown to be the likely cause of the reduction in AGPase activity in the embryo. A disadvantage of the *Tos17* system is that it is not easy to complement the mutant line with a functional *Apl3* gene, as the *Tos17* transposon is activated under the tissue culture conditions necessary for stable rice transformation. Independent mutant lines of *OsApl3* displaying the same phenotype would also provide evidence for a causal relationship between mutant gene and phenotype. However, as *apl3-1* had no effect on the mature RNA, this line could not be used to confirm a causal link. Here, I have shown that the reduction in AGPase activity co-segregated with the insertion in *OsApl3*. This provides good evidence of a causal link between AGPase phenotype and *Tos17* insertion. Segregation analysis does not rule out the possibility, although improbable, that the phenotype is due to a second mutation in a closely-linked gene.

Analysis of the mature transcript has shown that there is highly unlikely to be a functional APL3 protein in *apl3-2*. However, without an antibody to recognise APL3, it has not been possible to confirm the presence or absence of APL3 in any tissues. Should an antibody become available, this would be a useful validation.

Reducing AGPase activity in the developing embryos of rice was shown here to reduce embryo starch content. This reduction does not lead to any change in oil content or embryo dry weight. This is not consistent with the evidence from maize that reducing starch accumulation in the embryo leads to an increase in lipid accumulation (Singletary *et al.*, 2001). However, it is possible that with only a 35 percent reduction in embryo starch content, any change in embryo lipid accumulation would be smaller than could be reliably measured using our method. Here, lipid content was calculated to be 12 to 13 percent of

embryo dry weight. This figure is somewhat lower than published figures, for example *japonica* cultivar Kinmaze has been shown to have ~ 30 % oil in mature embryos (Matsuo *et al.*, 1987). However, varietal and environmental differences are likely to influence embryo oil content.

The reduced ability to accumulate starch in the embryo does not appear to have any effect on embryo development or grain filling as both embryos and mature grain were of normal weight. This suggests that, if starch is required in the embryo to establish sink strength or to provide a temporary carbon store during development, the quantity of starch is not critical to these functions. A greater decrease in starch accumulation is required to further investigate this question.

AGPase activity in the embryo was not to be abolished in *apl3-2* mutants. There are two possible scenarios to explain this: either there are multiple LSU genes responsible for embryo AGPase activity or the SSU is active in the absence of a LSU. To investigate the first scenario, I have shown that in the *apl1* mutant there is no effect on embryo AGPase activity. This shows that that APL1 is not required for embryo AGPase activity. However, APL1 may play a role in the absence of APL3. For example, mutations in *Aps1* caused multiple changes in expression of genes encoding other starch synthesising enzymes, including AGPase subunits. In *OsAps1* mutants, all other AGPase genes were up-regulated in the seed (Ohdan *et al.*, 2005) and in maize *ZmAps1* (*Bt2*) mutants, there were wide-spread changes in expression of genes involved in carbohydrate metabolism (Cossegal *et al.*, 2008). Although transcriptional up-regulation is not necessarily reflected in protein level or enzyme activity, it cannot be ruled out that APL1 plays a role in embryo AGPase activity in the absence of APL3. In order to examine this possibility, an *apl1/apl3-2* double mutant has been selected. This line is awaiting analysis of embryo AGPase activity. A second possibility is that APL4 is responsible for the remaining AGPase activity. OsAPL4 is predicted to be localised to the plastid and *OsApl4* is expressed at low levels in the leaves, stem and grain (Akihiro *et al.*, 2005; Ohdan *et al.*, 2005). To my knowledge, no APL4 mutant with which to test this hypothesis exists.

The second scenario is that the SSU of AGPase is active in the embryo in the absence of the LSU. There is some evidence, *in vitro,* that this may be possible. *Escherichia coli* expression of a recombinant potato tuber AGPase SSU showed that the SSU was active in the absence of a LSU (Ballicora *et al.*, 1995; Salamone *et al.*, 2000). Expression of recombinant barley *HvAps1* (*Beps*) in insect cells (*Spodoptera frugiperda*) also showed that the SSU is active in the absence of the LSU (Doan *et al.*, 1999). It is not clear if this in the case *in vivo*. In maize, the SSU was detectable in the endosperm of the LSU mutant of

Apl2 (*Sh2*)(Giroux *et al.*, 1994). This was not the case in barley leaves where *OsApl1* mutants did not have detectable levels of SSU in the absence of the LSU (Rösti *et al.*, 2007). This suggests that the SSU is unstable in the absence of the LSU. Indeed, although the SSU protein was detectable in maize endosperm early in development, it disappeared later in development (Giroux *et al.*, 1994). Further work is needed to discover if the SSU is present and active in the absence of the LSU in *apl3-2* mutant rice.

This work has shown that *Apl3* is required for normal AGPase activity in the embryo and also for normal AGPase activity in the developing endosperm. Although AGPase activity in the endosperm is reduced, there does not appear to be an effect on grain filling as no difference in mature grain weight was found. The reduction in AGPase activity in the endosperm is likely to be due to reduced plastidial activity. In fact, Lee *et al.*, (2007) found that the cytosolic AGPase activity, encoded by rice *apl2,* is responsible for 77 to 80 percent of the total AGPase activity in the endosperm. This is exactly the reciprocal of the 23 percent reduction in AGPase activity found in the *apl3-2* mutant.

I have attempted to confirm this hypothesis experimentally by ion-exchange FPLC. Good separation of peaks corresponding to cytosolic and plastidial AGPase, as previously validated in barley and wheat (Burton *et al.*, 2002b; Johnson *et al.*, 2003), has proven impossible and total AGPase activity recovered, after FPLC fractionation, was low (~ 30 %). As a result, it has not been possible to determine whether the reduction in endosperm AGPase activity is due to a reduction in plastidial activity and, if so, what proportion of the total plastidial AGPase is remaining.

In addition to the effects on grain AGPase activity, it was shown here that *Apl3* is required for almost all the starch accumulation in the culm. This is in contrast to *Apl1* which had no effect on starch accumulation in the culms at anthesis (Rösti *et al.*, 2007). Cereals are monocarpic and remobilise carbon from around the plant to the grain during senescence. Carbon remobilised from vegetative parts of the plant is estimated to contribute between 0 and 40 percent (depending on conditions) of the carbon for grain filling in rice (Yoshida, 1972). Starch and water soluble carbohydrates (including fructans, sucrose, glucose and fructose) are accumulated in the stem as reserves (Pollock, 1986; Xue *et al.*, 2008). Starch accumulates in the stems of rice around anthesis (Perez *et al.*, 1971; Watanabe *et al.*, 1997) and is remobilised during grain filling and senescence (Yang *et al.*, 2001). Stem carbon stores are an important source of carbon for grain filling particularly under water stress conditions (Blum, 1998). For example, stem starch is remobilised faster under water stressed conditions (Yang *et al.*, 2001). Starch accumulation in the stem is also associated with resistance to lodging (Kashiwagi *et al.*, 2006; Ishimaru *et al.*, 2008).

Taken together, this suggests that *apl3-2* mutant rice should be more susceptible to drought stress and to lodging. It is interesting to note, that under our growing conditions a marked decrease in starch accumulation in the culm had no effect on mature grain weight.

6. General discussion

The work presented in the previous chapters provides important insights into the determination of the size and composition of the embryo in cereals and the interaction between embryo and endosperm. A larger embryo, either in absolute terms or in proportion to endosperm, has the potential to increase the amount of lipid, protein and micronutrients in the grain. Manipulating grain composition may be of interest for a range of end-uses, including increasing the nutritional value of cereals.

6.1. Barley mutants for elucidating embryo-endosperm interactions

Shrunken-grain mutants have previously been used to identify genes required for normal starch biosynthesis. This has led to the identification and characterisation of the genes encoding: endosperm cytosolic AGPase SSU (Johnson *et al.*, 2003), starch synthase IIa (Morell *et al.*, 2003), a starch de-branching enzyme isoamylase1 (Burton *et al.*, 2002a), and a nucleotide sugar transporter NST1 (Patron *et al.*, 2004). In this study (Chapter 3), the majority of shrunken-grain mutants were shown to have a concomitant reductions in both endosperm and embryo weight. A lesion at the *Lys3* locus was confirmed to lead to larger-than-normal embryos, as previously described for Risø1508 (Tallberg, 1977). Mutant Risø8 was identified as having embryos that are proportionally larger than normal (Chapter3). This suggests that the lesion (at the *Lys4* locus) in this line may be in a gene involved in the determination of embryo size. However, as a gene specifically affecting endosperm size (*Agps1,* via its effects on endosperm starch biosynthesis; Rösti *et al.*, 2006) also had large embryos on a proportional basis, the *Lys4* locus may also have an endosperm-specific action rather than an effect on embryo size directly. Two mutants, Pentlandfield Glacier (*amo1*) and Shx (*shx*), had embryos that were smaller than normal as a proportion of grain weight. This suggests that the mutations in these lines may also be in genes involved in determining embryo size. With all these loci, identifying the underlying gene is crucial for understanding their function. The work in Chapter 4 describes my approach towards identifying the gene at the *Lys3* locus. Similar approaches could be taken to identify the genes underlying the mutant loci in Risø8 (*lys4*), Pentlandfield Glacier (*amo1*) and Shx (*shx*). Indeed, some fine mapping of these loci has already been carried by Dr Sylviane Comparot-Moss and others in the John Innes Centre.

6.2. Identifying large-embryo genes

Lines with lesions at *Lys3* were selected for further study due to the dramatic increase in embryo size in these lines compared to those of the other shunken-grain mutants (Chapter 4). As described in Chapter 4, a lesion at the *Lys3* locus has a wide range of

effects including altered starch and protein accumulation in both the embryo and the endosperm (Chapters 3 and 4) (Kreis and Doll, 1980; Shewry *et al.*, 1980); altered phytohormone levels in the developing grain (Mounla *et al.*, 1980); reduced primary dormancy of the grain (Chapter 4); reduced cell size in the endosperm (Klemsdal *et al.*, 1986); increased cell size and cell number in the embryo (Chapter 4) (Deggerdal *et al.*, 1986); and reduced peduncle and spike length (Chapter 4). The phenotypic data presented in Chapter 4, as well as previously published data, was used to identify known mutants in other species with similar phenotypes, with the aim of identifying candidate genes for the *Lys3* locus. The pleiotropic nature of the mutation does make it difficult to speculate as to the primary function of the gene at *Lys3.* However, a possible orthologue was identified in the rice *giant embryo* (*ge*) mutant (Satoh and Omura, 1981; Hong *et al.*, 1996). This mutant has larger than normal embryos due to a larger scutellum and also has altered scutellar epithelial cells (Hong *et al.*, 1996; Nagasawa *et al.*, 2003). However, in Chapter 4, it was demonstrated that a closely related barley orthologue of the rice *Ge* gene does not lie on the same chromosome as the *Lys3* locus.

More recently, the rice B cyclin gene *Orysa;CycB1;1* has been shown to control embryo size (Guo *et al.*, 2010). Reduced *Orysa;CycB1;1* expression (using RNAi) leads to rice grains with reduced endosperm, large embryos (due enlarged cells in the scutellum), more starch granules in the scutellum and evidence of embryo viviparity (Guo *et al.*, 2010). The possibility of using a similar approach to *Orysa;CycB1;1,* as was taken for the rice *Ge* gene in Chapter 4, was recently investigated. A BLAST search of nucleotide sequences using the *Orysa;CycB1;1* mRNA (AY647458.1) failed to find a strong barley homologue. This suggests that a putative barley orthologue of *Orysa;CycB1;1* is not currently known. Future work could include attempting to identify barley cyclin B genes using primers designed to conserved cyclin B sequence regions to amplify cyclin B genes in the barley genome.

As noted in Chapter 4, my approach to identifying the gene underlying *Lys3* was complementary to the direct approach of mapping of the *Lys3* locus that was being carried out at the John Innes Centre. The genetic distance within which *Lys3* lies has been narrowed to within 1 cM (Dr Sylviane Comparot-Moss, personal communication). The relationship between genetic distances and physical distances varies. In the Triticeae, a region of <0.5 cM is generally required to identify a BAC contig likely to contain the gene of interest (Krattinger *et al.*, 2009). Therefore, more mapping work is required. The synteny of the region in barley containing the *Lys3* locus with those of rice and *Brachypodium* is poor and it has not yet been possible to identify any promising candidate genes from these genomes by the mapping approach.

Lesions at the *Lys3* locus alter the characteristics of the grain in ways that may be interest for a number of end uses, including improving the nutritional value of feed and food. However, the effect on grain filling which results in a reduction in yield in field trials (Bang-Olsen *et al.*, 1991) has limited the use of this locus in breeding programmes. Should the gene underlying *Lys3* be identified, it is possible that further understanding of its function will lead to new ways of exploiting the positive effects of a lesion and reducing or eliminating the negative effects. For example, expression of the gene underlying *Lys3* using an endosperm-specific promoter in a *lys3* mutant background might restore normal endosperm phenotype but maintain the large-embryo phenotype. However, if the effect on the embryo is secondary to an effect on endosperm development, it may not be possible to separate the two phenotypes. Either way, discovery of the gene responsible for *lys3* is likely to lead to fundamental insights into grain development in cereals.

6.3. The role of starch in the cereal embryo

This work has identified four cereal mutants with reduced starch content in the developing embryo: three barley mutants (Risø16 [*agps1*], Risø17 [*isa1*] and Risø8 [*lys4*]) and one rice mutant (*apl3-2*). In none of these mutants did the reduction in starch content during development, ranging from 13 percent lower in Risø8 barley to 35 percent lower in *apl3-2* rice, lead to changes in embryo size or changes in the amount of protein (barley mutants) or lipid (*apl3-2*) in the mature embryo (Chapters 3 and 5). Therefore, there is no evidence that the quantity of starch accumulated during development is important for determining final embryo composition nor is there any evidence that normal starch metabolism is required for normal embryo development. However, a total knockout of starch biosynthesis in the embryo, rather than a moderate knock down as achieved here, would more rigorously test whether starch plays a role in embryo development.

As discussed in Chapter 5, one approach to knocking out starch biosynthesis completely in the embryo is to identify and knockout the AGPase LSU gene or genes responsible for the remaining AGPase activity in *apl3-2* mutants. As noted in Chapter 5, there are four AGPase LSU genes in the rice genome (Table 5.2). In addition to the mutant described in this work, there are mutants available in two of the other rice LSU genes (*apl1* and *apl2*) (Lee *et al.*, 2007; Rösti *et al.*, 2007). I have shown that *apl1* (Rösti *et al.*, 2007) mutants have normal AGPase activity in the developing embryo and I have selected the *apl1/apl3-2* double mutant in order to test in the future the possibility that APL1 is expressed in the developing embryo in the absence of APL3. A further cross between *apl3-2* and *apl2* (*osagpl2-1* and *osagpl2-2* [Lee *et al.*, 2007] with seed kindly donated by Professor Jong-Seong Jeon of Kyung Hee University) was attempted, but without a success due to poor

germination and growth of the *apl2* grain. This meant that measurement of AGPase activity developing embryos was also not possible. To my knowledge, no mutant with a lesion in the fourth rice AGPase LSU gene (*Apl4*) is currently available.

An altogether more direct approach, which was discussed in the introduction to Chapter 5, is to identify a mutant with a lesion in the AGPase SSU required for AGPase activity in the embryo. There are only two AGPase SSU genes in barley and rice (Table 5.1). Mutants have been identified in the SSU gene responsible for the most of the endosperm (cytosolic) activity of these species (Johnson *et al.*, 2003; Lee *et al.*, 2007). Therefore, the remaining activity in the grain (and all of the activity in the embryo) is presumably due to the second SSU gene in these species. Although, to my knowledge, no mutant is currently available is affected in this SSU gene in either rice or barley, as more genetic resources become available (e.g. TILLING) such a mutant may become available in the future.

7. Appendices

Table 7.1 – Mature grain weight and embryo weight of shrunken barley mutants.

Mature grains and embryos (dissected from grains) were freeze-dried and weighed. Values are means ± SD of measurements of 10 to 25 individual grains. A minimum of two separate plants (grown simultaneously) were sampled per genotype. Wild-type lines are shown in bold. Statistical difference was determined by Student's t-test.

7.1A. Barley grown under glasshouse conditions in winter.

Line	Gene responsible	Number	Mature grain weight (mg)	Statistical difference from parent line	
NP113	-	**25**	**67.3 ± 4.5**		
Notch 2	*Isoamylase1*	25	38.3 ± 3.2	$t_{48} = 26.3$	$p < 0.001$
Glacier	-	**25**	**67.2 ± 7.9**		
Pentlandfield Glacier	*Amo1*	20	61.4 ± 5.3	$t_{43} = 2.8$	$p = 0.008$
Bowman	-	**25**	**63.5 ± 4.0**		
Sex7	*Nst1*	25	49.0 ± 2.6	$t_{72} = 14.6$	$p < 0.001$
Sex8	*Unknown*	25	50.6 ± 3.7	$t_{72} = 13.0$	$p < 0.001$
Carlsberg II	-	**25**	**54.7 ± 5.4**		
Risø86	*Nst1*	25	38.4 ± 4.6	$t_{72} = 11.0$	$p < 0.001$
Risø29	*Nst1*	25	51.9 ± 5.8	$t_{72} = 1.91$	$p = 0.061$
Ingrid	-	**25**	**53.7 ± 2.9**		
Seg6	*Unknown*	25	31.6 ± 4.5	$t_{72} = 22.3$	$p < 0.001$
Seg7	*Unknown*	25	47.7 ± 2.9	$t_{72} = 6.0$	$p < 0.001$
Bomi	-	**25**	**53.7 ± 8.7**		
Risø13	*Nst1*	25	50.8 ± 2.7	$t_{182} = 2.0$	$p = 0.045$
Risø1508	*Unknown*	25	45.1 ± 3.8	$t_{182} = 6.1$	$p < 0.001$
Risø16	*Agps1*	25	46.9 ± 3.7	$t_{182} = 4.8$	$p < 0.001$
Risø17	*Isoamylase1*	15	36.4 ± 3.3	$t_{182} = 10.6$	$p < 0.001$
Risø527	*Unknown*	25	39.8 ± 4.3	$t_{182} = 9.9$	$p < 0.001$
Risø8	*Unknown*	25	39.1 ± 5.7	$t_{182} = 10.3$	$p < 0.001$
Shx	*Unknown*	25	35.6 ± 4.4	$t_{182} = 12.9$	$p < 0.001$
Nubet	-	**25**	**50.0 ± 3.0**		
Franubet	*Unknown*	25	34.6 ± 5.9	$t_{48} = 11.7$	$p < 0.001$

7.1A continued.

Line	Gene responsible	Embryo dry weight (mg)	Statistical difference from parent line		Embryo dry weight (% of mature grain weight)	Statistical difference from parent line	
NP113	**-**	**1.72 ± 0.20**			**2.56 ± 0.22**		
Notch 2	*Isoamylase1*	2.03 ± 0.47	$t_{72} = 3.39$	$p < 0.001$	5.35 ± 1.41	$t_{72} = 3.39$	$p < 0.001$
Glacier	**-**	**1.89 ± 0.24**			**2.84 ± 0.43**		
Pentlandfield Glacier	*Amo1*	1.54 ± 0.27	$t_{43} = 4.61$	$p < 0.001$	2.52 ± 0.47	$t_{43} = 2.40$	$p = 0.021$
Bowman	**-**	**2.02 ± 0.19**			**3.18 ± 0.24**		
Sex7	*Nst1*	1.73 ± 0.22	$t_{72} = 4.77$	$p < 0.001$	3.54 ± 0.48	$t_{72} = 3.10$	$p = 0.003$
Sex8	*Unknown*	1.89 ± 0.25	$t_{72} = 2.14$	$p = 0.036$	3.74 ± 0.44	$t_{72} = 4.89$	$p < 0.001$
Carlsberg II	**-**	**1.68 ± 0.23**			**3.08 ± 0.42**		
Risø86	*Nst1*	1.44 ± 0.19	$t_{72} = 4.19$	$p < 0.001$	3.76 ± 0.41	$t_{72} = 6.02$	$p < 0.001$
Risø29	*Nst1*	1.64 ± 0.20	$t_{72} = 0.74$	$p = 0.464$	3.17 ± 0.36	$t_{72} = 0.78$	$p = 0.436$
Ingrid	**-**	**1.60 ± 0.15**			**2.99 ± 0.32**		
Seg6	*Unknown*	1.11 ± 0.19	$t_{72} = 11.10$	$p < 0.001$	3.54 ± 0.51	$t_{72} = 5.07$	$p < 0.001$
Seg7	*Unknown*	1.49 ± 0.11	$t_{72} = 2.52$	$p = 0.014$	3.13 ± 0.25	$t_{72} = 1.29$	$p = 0.201$
Bomi	**-**	**1.76 ± 0.22**			**3.28 ± 0.41**		
Risø13	*Nst1*	1.79 ± 0.21	$t_{180} = 0.29$	$p = 0.781$	3.53 ± 0.41	$t_{180} = 0.83$	$p = 0.408$
Risø1508	*Unknown*	3.45 ± 1.00	$t_{180} = 13.76$	$p < 0.001$	7.67 ± 2.20	$t_{180} = 14.96$	$p < 0.001$
Risø16	*Agps1*	1.83 ± 0.18	$t_{180} = 0.57$	$p = 0.572$	3.90 ± 0.28	$t_{180} = 2.10$	$p = 0.037$
Risø17	*Isoamylase1*	1.71 ± 0.26	$t_{180} = 0.35$	$p = 0.724$	4.69 ± 0.67	$t_{180} = 4.18$	$p < 0.001$
Risø527	*Unknown*	1.50 ± 0.18	$t_{180} = 2.08$	$p = 0.039$	3.79 ± 0.42	$t_{180} = 1.72$	$p = 0.088$
Risø8	*Unknown*	1.97 ± 0.38	$t_{180} = 1.71$	$p = 0.089$	5.14 ± 1.36	$t_{180} = 6.34$	$p < 0.001$
Shx	*Unknown*	0.95 ± 0.22	$t_{180} = 6.46$	$p < 0.001$	2.68 ± 0.63	$t_{180} = 2.05$	$p < 0.042$
Nubet	**-**	**1.77 ± 0.19**			**3.54 ± 0.33**		
Franubet	*Unknown*	1.41 ± 0.15	$t_{48} = 7.48$	$p < 0.001$	4.18 ± 0.64	$t_{48} = 4.39$	$p < 0.001$

7.1B – Barley grown under glasshouse conditions in winter.

Line	Gene responsible	Number	Mature grain weight (mg)	Statistical difference from parent line	
Bomi	-	**10**	**68.2 ± 3.9**		
Risø18	*Unknown*	20	38.3 ± 4.4	$t_{28} = 11.9$	$p < 0.001$

7.1B continued.

Line	Gene responsible	Embryo dry weight (mg)	Statistical difference from parent line		Embryo dry weight (% of mature grain weight)	Statistical difference from parent line	
Bomi	-	**2.13 ± 0.23**			**3.14 ± 0.40**		
Risø18	*Unknown*	3.56 ± 0.58	$t_{28} = 7.41$	$p < 0.001$	7.31 ± 1.06	$t_{28} = 11.99$	$p < 0.001$

7.1C – Barley grown under glasshouse conditions in summer

Line	Gene responsible	Number	Mature grain weight (mg)	Statistical difference from parent line	
Minerva	-	**25**	**62.0 ± 6.2**		
M1460	*Unknown*	25	51.5 ± 3.6	$t_{48} = 7.20$	$p < 0.001$

7.1C continued.

Line	Gene responsible	Embryo dry weight (mg)	Statistical difference from parent line		Embryo dry weight (% of mature grain weight)	Statistical difference from parent line	
Minerva	-	**2.11 ± 0.27**			**3.41 ± 0.34**		
M1460	*Unknown*	5.21 ± 0.85	$t_{48} = 20.9$	$p < 0.001$	10.17 ± 1.83	$t_{48} = 25.27$	$p < 0.001$

7.1D – Barley grown under CER conditions.

Line	Gene responsible	Number	Mature grain weight (mg)	Statistical difference from parent line	
Bomi	-	**25**	**76.6 ± 3.4**		
Risø19LE	*Unknown*	25	56.7 ± 3.8	$t_{72} = 20.84$	$p < 0.001$
Risø19NE	*Unknown*	25	50.9 ± 2.8	$t_{72} = 26.87$	$p < 0.001$

7.1D continued.

Line	Gene responsible	Embryo dry weight (mg)	Statistical difference from parent line		Embryo dry weight (% of mature grain weight)	Statistical difference from parent line	
Bomi	-	**1.80 ± 0.26**			**2.35 ± 0.29**		
Risø19LE	*Unknown*	2.88 ± 0.76	$t_{71} = 8.06$	$p < 0.001$	5.04 ± 1.18	$t_{71} = 16.57$	$p < 0.001$
Risø19NE	*Unknown*	1.37 ± 0.17	$t_{71} = 3.30$	$p = 0.002$	2.68 ± 0.28	$t_{71} = 1.67$	$p = 0.099$

Table 7.2 - Embryo protein content of shrunken barley mutants at maturity

Embryos were dissected from mature grains, freeze-dried, weighed and analysed for protein content. Values are means ± SD of measurements of three to five individual embryos. Each mutant line and corresponding wild type was grown simultaneously. Wild-type lines are shown in bold. Statistical difference was determined by Student's t-test or Mann-Whitney (U) when samples had unequal variance.

7.2A - Grown under glasshouse conditions in winter

Line	Gene responsible	Embryo protein					
		µg per embryo	Statistical difference from parent line		mg gDW^{-1}	Statistical difference from parent line	
NP113	**-**	**651.2 ± 27.6**			**339.7 ± 17.1**		
Notch 2	*Isoamylase1*	696.7 ± 126.2	$t_8 = 0.79$	$p = 0.471$	279.9 ± 26.2	$t_8 = 4.27$	$p = 0.003$
Bowman	**-**	**718.8 ± 50.4**			**334.5 ± 42.2**		
Sex7	*Nst1*	613.9 ± 17.7	$t_{12} = 2.55$	$p = 0.025$	330.6 ± 15.7	$t_{11} = 0.17$	$p = 0.866$
Sex8	*Unknown*	658.2 ± 99.0	$t_{12} = 1.47$	$p = 0.167$	339.1 ± 44.7	$t_{11} = 0.19$	$p = 0.849$
Carlsberg II	**-**	**648.8 ± 83.5**			**360.9 ± 35.0**		
Risø86	*Nst1*	529.1 ± 68.6	$t_{12} = 2.71$	$p = 0.019$	288.5 ± 42.2	$t_{12} = 2.66$	$p = 0.021$
Risø29	*Nst1*	569.2 ± 54.6	$t_{12} = 1.80$	$p = 0.097$	370.5 ± 50.7	$t_{12} = 0.35$	$p = 0.732$
Ingrid	**-**	**647.9 ± 40.2**			**406.2 ± 99.2**		
Seg6	*Unknown*	431.3 ± 98.5	$t_{11} = 4.86$	$p < 0.001$	297.1 ± 38.8	$t_{11} = 2.16$	$p = 0.054$
Seg7	*Unknown*	563.3 ± 35.0	$t_{11} = 1.90$	$p = 0.084$	341.6 ± 82.0	$t_{11} = 1.28$	$p = 0.228$
Bomi	**-**	**668.9 ± 72.1**			**410.7 ± 69.2**		
Risø13	*Nst1*	694.5 ± 62.4	$t_{32} = 0.48$	$p = 0.634$	377.0 ± 75.1	$t_{30} = 0.66$	$p = 0.515$
Risø1508	*Unknown*	1092.6 ±138.7	$t_{32} = 7.96$	$p < 0.001$	297.4 ± 43.7	$t_{30} = 2.22$	$p = 0.034$
Risø16	*Agps1*	717.5 ± 44.8	$t_{32} = 0.91$	$p = 0.368$	374.2 ± 85.5	$t_{30} = 0.62$	$p = 0.541$
Risø17	*Isoamylase1*	627.9 ± 74.6	$t_{32} = 0.77$	$p = 0.446$	338.4 ± 39.5	$t_{30} = 1.41$	$p = 0.168$
Risø527	*Unknown*	569.4 ± 41.4	$t_{32} = 1.87$	$p = 0.071$	371.6 ± 40.9	$t_{30} = 0.76$	$p = 0.451$
Risø8	*Unknown*	779.0 ± 135.5	$t_{32} = 2.07$	$p = 0.047$	372.6 ± 86.5	$t_{30} = 0.74$	$p = 0.462$
Shx	*Unknown*	509.6 ± 25.2	$t_{32} = 2.99$	$p = 0.005$	486.2 ± 149.4	$t_{30} = 1.48$	$p = 0.150$

7.2B – Grown under glasshouse conditions in summer

Line	Gene responsible	Embryo protein					
		µg per embryo	Statistical difference from parent line		mg gDW^{-1}	Statistical difference from parent line	
Bomi		**905.4 ± 116.7**			**447.4 ± 131.5**		
Risø18		1116.3 ± 241.7	$t_8 = 1.76$	$p = 0.117$	349.0 ± 138.6	$t_8 = 0.283$	$p = 0.283$

7.2C – Grown under glasshouse conditions in summer

Line	Gene responsible	Embryo protein					
		µg per embryo	Statistical difference from parent line		mg gDW^{-1}	Statistical difference from parent line	
Minerva		**906.0 ± 27.0**			**465.7 ± 167.7**		
M1460		1599.9± 150.4	$U_{5,5} = 0.0$	$p = 0.008$	306.9 ± 15.9	$U_{5,5} = 1.0$	$p = 0.016$

7.2D – Grown under CER conditions

Line	Gene responsible	Embryo protein					
		µg per embryo	Statistical difference from parent line		mg gDW^{-1}	Statistical difference from parent line	
Bomi		**767.7 ± 159.8**			**447.4 ± 131.5**		
Risø19LE		774.1 ± 203.4	$t_{12} = 0.07$	$p = 0.949$	222.1 ± 43.8	$t_{12} = 3.38$	$p = 0.005$
Risø19NE		568.2 ± 69.8	$t_{12} = 2.04$	$p = 0.064$	369.7 ± 109.6	$t_{12} = 0.90$	$p = 0.387$

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