INVITED REVIEW

Physiology of the aleurone layer and starchy endosperm during grain development and early seedling growth: new insights from cell and molecular biology

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Abstract

Cereal grain germination and early seedling growth involve the co-ordinated action of endosperm and embryo tissues to mobilize the storage reserves of the starchy endosperm. This mobilization is accomplished by hydrolases secreted from the aleurone and scutellar tissues. The breakdown products are then transported to the growing seedling by the scutellum. This resource-harvesting system is regulated at multiple levels. One well-defined aspect of control is brought about by the hormone gibberellin (GA). Gibberellin is released from the embryo upon imbibition and activates the aleurone cells. The secretory apparatus of the aleurone then proliferates, supporting increased hydrolase synthesis and secretion to degrade the starchy endosperm. The molecules that regulate this response to GA are now being increasingly characterized. Elements such as cGMP, calcium, calmodulin and protein kinases are well known as regulators in other eukaryotic cell types and are emerging as key control factors in the aleurone hormone response. However, superimposed upon this molecular regulatory system is another level of control, the structural pattern of tissues and stored macromolecules that was laid down during grain development. It is the interaction of these structural motifs combined with the molecular regulatory mechanisms that ensure the appropriate timing and positioning of hydrolase production and endosperm reserve mobilization. This integrated control system ensures an extended release of nutrients to fuel early seedling growth.

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Abbreviations: ABA, abscisic acid; CaM, calmodulin; CDPK, calmodulin-like domain protein kinase; cGMP, cyclic guanosine monophosphate; GA, gibberellin; *O2*, *Opaque2*

Keywords: abscisic acid, aleurone, amylase, gibberellin, scutellum, signal transduction

Introduction

Seed germination and early seedling growth are highly vulnerable stages in the life history of a plant. The embryo is heterotrophic and requires a sufficient support system to sustain growth until the seedling reaches photosynthetic, autotrophic status. In cereals, this support system is mainly the starchy endosperm, and its activities spell success or failure for the plant's future (Lopes and Larkins, 1993). In addition, cereals form the vast majority of the agriculturally important crops, and their major nutritional value is generally derived from the endosperm. A failure in molecular cues at the time of activation of endosperm mobilization can lead to premature germination and cause not only decreased seedling production but also major crop losses via pre-harvest sprouting (Auranen, 1995). Conversely, extended dormancy leads to a failure to germinate in the optimal season for growth, with consequent failure of seedling establishment (Auranen, 1995; Bewley, 1997).

Evolution has, therefore, shaped monocot germination and endosperm mobilization into a highly regulated process. This system involves control at all levels – from subcellular processes, such as the co-ordinated expression of suites of genes, to the interactions of cells within entire tissues and organs. Hormones, such as GA, mediate much of this co-ordinated behaviour, but such molecular regulation is superimposed on a structural design that promotes the correct patterning of endosperm processes. Thus, an explanation of how the endosperm is mobilized during germination and early seedling growth involves not only understanding the molecular aspects of the process, but

also how seed development has laid down the structural blueprint upon which this molecular regulation is expressed (Fincher, 1989; Olsen *et al*., 1992, 1995, 1998). Therefore, in this review we will outline the developmental progression that generates the characteristic structural elements of the endosperm. The story that emerges is one of developmental patterning that results in precise tissue and nutrient placements prior to desiccation and quiescence/dormancy of the mature seed. This is the setting in which the germination machinery will be activated. The interactions between the structural features of the endosperm and the hormone-regulated molecular events of germination (Jones and Jacobsen, 1991; Bethke *et al*., 1997; Ritchie and Gilroy, 1998c) then lead to the extended release of storage reserves that fuel germination and early seedling growth.

Endosperm development and maturation

At maturity the grain is almost dry and has a very low metabolic activity. Thus, it can survive extended periods of harsh environmental insults. The mature endosperm is packed with reserves of carbohydrate (principally starch), protein, lipid, and minerals that provide a sustained reserve to nourish the developing seedling for several days. As shown in Fig. 1, the nutrients contained in the starchy endosperm confer an advantage during early seedling growth. Seedlings grown from grains with most of the starchy endosperm and aleurone removed (Fig. 1, bottom) are much smaller than those grown from intact grain (Fig. 1, top).

The endosperm is, however, a much more complex structure than merely a bag of nutrients. Surrounding the starchy endosperm is a highly specialized layer of endosperm tissue that remains cellular and viable at grain maturity. This is the aleurone, a highly differentiated, specialized tissue. During grain development the aleurone is a protein- and lipid-rich storage tissue. Upon imbibition the aleurone layer uses these stored reserves to synthesize and secrete many of the digestive enzymes that mobilize the insoluble reserves in the starchy endosperm.

The second structure intimately involved in regulating endosperm reserve mobilization is the scutellum. This is the highly modified cotyledon of monocots that transmits hormones, produces digestive enzymes, and transports nutrients from the endosperm to the embryo. Thus, grain development has laid down a co-ordinated pattern of highly differentiated tissues derived from the embryo (scutellum) and endosperm (aleurone) oriented towards storage reserve mobilization and transport during germination and initial growth. Figure 2 shows the location of these tissues in a longitudinal section of a barley grain and how their placement and co-ordinated activities contribute to some of the processes that occur during germination.

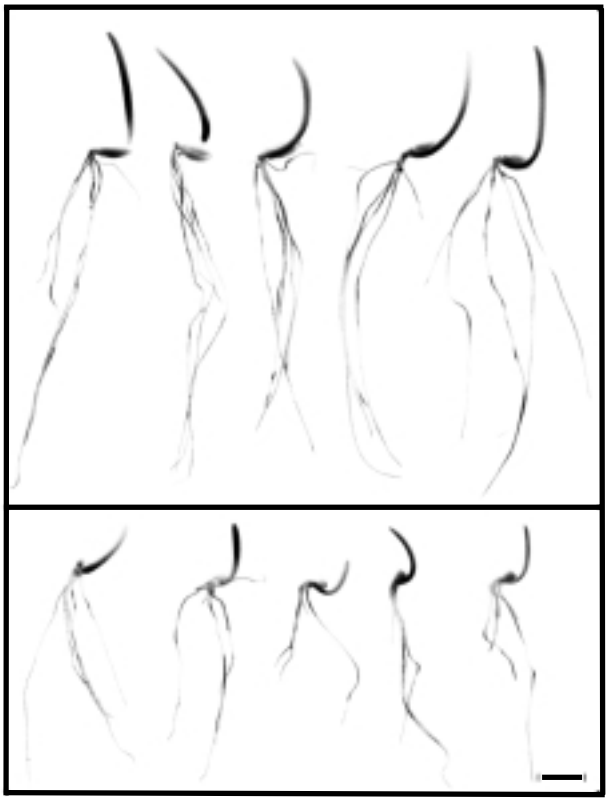

Figure 1. Barley seedlings 3 d after the start of germination. Seedlings germinated from intact grains (top) and seedlings germinated from grains with most of the starchy endosperm and aleurone removed prior to imbibition (bottom). Scale bar = 1 cm.

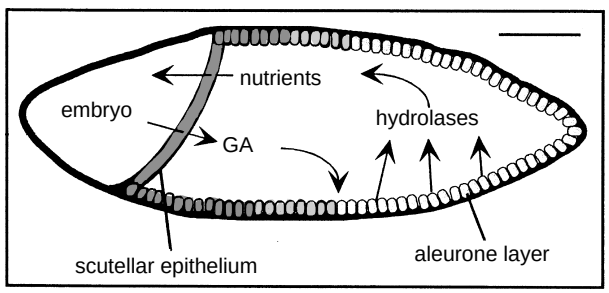

Figure 2. Diagram of a longitudinal section through a barley grain. Indicated are structures in the grain and processes during and after germination. The shading of the aleurone cells represents the wave of activation during and after germination, i.e. the darker cells are activated earliest. Scale bar = 1 mm.

Laying down patterns in the seed: endosperm development

The patterns underlying endosperm development are very similar in all cereals (reviewed in Bosnes *et al*., 1992; Olsen *et al*., 1992, 1995, 1998). A few days after fertilization, the endosperm consists of a large central "vacuole" surrounded by a syncytium and endosperm nuclei (Fig. 3A). True smaller vacuoles appear within the syncytial cytoplasm, and then cell wall formation and new cell division are initiated. Before cell walls are laid down, the planes of division are predicted by highly organized changes in the microtubule cytoskeleton (Brown *et al*., 1996). The new cells formed by this concerted, oriented division gradually push into the central "vacuole", until the endosperm region is filled and the central vacuolar space disappears.

Cellular differentiation of endosperm into aleurone and starchy endosperm then commences. Aleurone cells first become apparent around the ventral groove of the endosperm. As cell division progresses, aleurone cells also start to form along the dorsal side of the grain, and these divisions continue until the starchy cells are completely encased by aleurone cells. The wave of aleurone development not only occurs from the ventral groove to the dorsal side, but also from the region proximal to the embryo to the distal end. Analysis of aleurone development in wild-type barley and a range of endosperm differentiation mutants revealed that the endosperm left and right "halves" represent separate cell lineages derived from the daughter nuclei of the primary endosperm nucleus (Bosnes *et al*., 1992).

Three classes of aleurone cells are distinct from the typical aleurone surrounding most of the starchy endosperm. The aleurone cells around the ventral groove are termed modified aleurone; there is evidence that they are involved in nutrient transfer from the maternal tissue during endosperm development, in addition to having the functions of nutrient storage and hydrolase synthesis (Cochrane and Duffus, 1980). The second class of modified aleurone cells surrounds the embryo. They have many morphological similarities to the aleurone cells around the starchy endosperm. However, histochemical staining suggests these cells have more lipid and protein, but less polysaccharide than their starchy endosperm counterparts (Raju and Walther, 1988). These differences probably reflect specialized functions for this class of aleurone cell. Since they do not surround starchy tissue which requires hydrolysis during germination, it is unlikely that this aleurone produces large quantities of α-amylases. Instead, these cells may serve to protect the embryo against pathogens and control H_2O passage to the embryo (Raju and Walther, 1988). The third class of modified aleurone is the portion directly in contact with the starch of the starchy endosperm. Early in differentiation these subaleurone cells look much like the aleurone cells but, when the tissues are fully differentiated, the subaleurone contains starch granules and storage protein more characteristic of starchy endosperm cells. In addition, similar to the starchy endosperm, the subaleurone cells die upon grain maturation while the rest of the aleurone cells remain viable (Young *et al*., 1997).

The starchy endosperm also has a heterogeneous developmental origin. It is divided into two zones that result from two different waves of differentiation. After cellularization, the meristematic cells in the ventral groove area (Fig. 3B) start to divide and differentiate toward the dorsal region, forming a central zone of prismatic starchy cells, as well as the dorsal aleurone. Whereas, starting later, the cells in the "wings" of the seed develop into irregular starchy cells as depicted in Fig. 3 (Olsen *et al*., 1992).

Defining the aleurone cell: differentiation and GA responsiveness

What signals occur during grain development to give aleurone cells their unique characteristics? As described above, the aleurone is derived from the

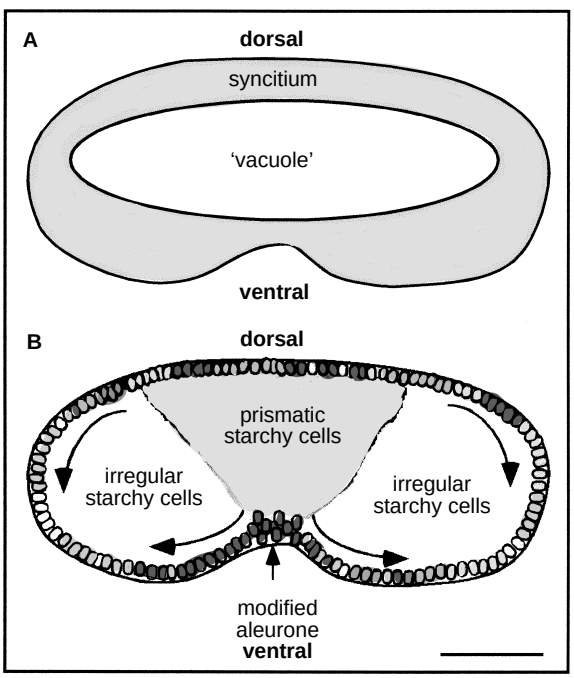

Figure 3. Cross-section of a barley grain (A) prior to cellularization and (B) after cellularization during endosperm differentiation. The darker shading of the aleurone cells represents regions in which cells differentiate earliest. Scale bar = 0.5 mm.

same cells as the starchy endosperm, yet its final developmental fate is drastically different. Aleurone cells are a GA-responsive, living secretory tissue at grain maturity, whereas the starchy endosperm cells are dead (Young *et al.*, 1997; Young and Gallie, 1999). This divergence of cell fate is determined during early endosperm differentiation. It is only later during seed maturation, however, that the aleurone develops its other essential characteristics of GA sensitivity and the desiccation tolerance needed to survive until the dry, mature grain imbibes.

Although the molecular regulators responsible for these developmental phases of the aleurone remain enigmatic, clues have emerged from studies of developmental mutants, differentiation-specific gene expression, and from treatments promoting aleurone GA sensitivity in the developing seed. The maize mutant, *crinkly4* (*cr4*, Becraft *et al.*, 1996) exhibits impaired aleurone development, resulting in regions of the caryopsis that completely lack aleurone cells, while other regions appear normal. In *cr4*, the end of the caryopsis opposite the embryo is more likely to contain areas lacking aleurone cells. In maize grain development, the regions of aleurone around the embryo develop first. Therefore, the effects of the *cr4* mutation may be partially dependent on the timing of aleurone cell differentiation, being most pronounced in the latter stages of aleurone formation. The gene responsible for the *cr4* mutation has been identified and has homology to receptor-like protein kinases (Becraft *et al.*, 1996). Since the aleurone layer first develops at the edge of the endosperm closest to the maternal tissue, it is probable that the ligand for the extracellular portion of the kinase comes from this direction. Extending this model, the binding of the maternally derived ligand to the receptor portion of this protein would then activate a signal transduction cascade leading to aleurone differentiation. Genetic analysis of barley grain developmental mutants also suggests that maternal gene products are important in specifying the development of the endosperm (Felker *et al.*, 1985). It is highly likely that other molecules work in concert with the Cr4 receptor kinase, since *cr4* mutants only have a small percentage of mutant grain with an altered aleurone phenotype. Interestingly, *cr4* is the only gene identified to date as a control element in determining aleurone cell fate.

A number of other genes, however, have been identified which are likely to represent later points in the hierarchy of transcriptional regulation of grain development. For example, two maize genes, *Viviparous1* (*Vp1*) and *Opaque2* (*O2*), play roles in the behaviour of the embryo and endosperm during seed maturation. *Vp1* is involved in preventing premature germination, and, in the aleurone, it may act as a repressor of GA-regulated gene expression (Hoecker *et al.*, 1995). In maize it can play a role in regulating anthocyanin synthesis through interaction with the maize Myb-like transcription factor *C1* (Hattori *et al.*, 1992). The other well-characterized endosperm-related gene, *O2*, is a transcription factor of the bZIP class (Schmidt *et al.*, 1990). It controls the expression of specific sets of storage proteins in maize grains and has been implicated in other maturation processes (discussed in Vettore *et al.*, 1998). Homologues to maize *O2* have been identified in other cereals, and these are also specifically expressed in the endosperm (reviewed in Vettore *et al.*, 1998). In turn, *O2* appears to act in concert with another bZIP transcription factor, *OHP1* (Pysh *et al.*, 1993).

Several endosperm-specific genes encoding proteins other than transcription factors have been identified from developing grain (Jakobsen *et al.*, 1989; Olsen *et al.*, 1990). These include genes for the puroindolines, proteins with a possible anti-pathogenic role (Jakobsen *et al.*, 1989; Dubreil *et al.*, 1998), and the lipid transfer proteins, whose actual *in vivo* function is still uncertain (Olsen *et al.*, 1990; Kalla *et al.*, 1994; Dubreil *et al.*, 1998; Digeon *et al.*, 1999). Truncations of the promoter of one wheat puroindoline gene showed that different regions of the promoter are responsible for localization to either endosperm, scutellar epithelium or pericarp (Digeon *et al.*, 1999). Similarly, the promoters of the *LTP1* and *LTP2* (lipid transfer protein 1 and 2) genes contain motifs that confer endosperm-specific expression in a host of other genes such as storage proteins and α-amylase (Kalla *et al.*, 1994). Not surprisingly, however, control is more complex than driving expression by a promoter that is simply active in endosperm tissue. Evidence is accumulating for some control by other regions of the grain. Thus, when aleurone layers of developing barley grains are isolated free from starchy endosperm and embryo, the mRNA for *LTP2* declined rapidly compared with that in the intact grain (after 1 h; Olsen *et al.*, 1990). This suggests that not only is the mRNA of *LTP2* highly labile, but also that a factor coming from the embryo or starchy endosperm is regulating its expression. There are also Myb transcription factor binding motifs within this *LTP2* promoter. In maize Myb-based regulators are involved in expression of genes regulated during seed development by ABA and the transcription factor Vp1 (McCarty, 1995), suggesting *LTP2* might likewise be responsive to a range of signals.

Thus, common themes are emerging in these molecular studies of how endosperm-specific functions are established. One obvious feature is that information exchange and interaction between

tissues underlies the co-ordinated regulation of grain development. This integration is seen at the level of cell fate specification (the Cr4 receptor and its putative ligand from maternal tissue) down to tissue-specific gene expression (maintenance of aleurone *LTP2* expression by embryo/starchy endosperm signals). Similarly, each molecular regulator is part of an interacting control complex (e.g. *Vp1* and its Myb interactions).

The second picture that emerges from these studies of endosperm development is of a cascade of signals and molecular regulators involved in laying down the precise cellular and subcellular patterning that is the hallmark of cereal endosperm formation. Genes such as *LTP2*, whose expression is regulated during endosperm development, must themselves be under control of elements such as Cr4 involved in the earliest stages of cell fate specification.

Insights from these studies of endosperm development may also have important implications for other developmental processes in plants. Indeed, the *cr4* mutant is named not after its endosperm phenotype, but for its effects on leaf development, where the mutation leads to defective leaf morphogenesis and a crinkled leaf phenotype. Thus, the signals and/or regulators involved in endosperm formation may be of much more widespread significance than simply seed-specific developmental regulators.

Endosperm reserve deposition and subcellular development

Cell division and cell fate determination in the grain are followed by cell expansion and reserve deposition (reviewed by Bewley and Black, 1994). The starchy endosperm comprises the greatest portion of the grain, and its role is to store not only carbohydrates and proteins but also other macromolecules and minerals important to the functioning of other tissues in the grain following germination. During grain formation the cellular endosperm lays down storage polymers that are largely insoluble and have a low osmotic activity. These central endosperm cells then die in a programmed manner which may be mediated by the transient increase in ethylene detected in developing grain (Young *et al.*, 1997; Young and Gallie, 1999). This developmental programme yields the polymer-rich storage tissue of the starchy endosperm evident at grain maturity. The primary stored polymer is starch, which accumulates in endosperm amyloplasts during grain filling. However, not all of this starch will remain in the mature grain to fuel seedling growth. Some is broken down by amylases and other enzymes that function during grain development. These development-related hydrolases degrade areas of the endosperm near the growing embryo and scutellum to allow for their expansion (MacGregor and Dushinsky, 1989).

During starch deposition, some hydrolase expression occurs that will subsequently contribute to starch breakdown following germination (Shewry *et al.*, 1988). The amylases that degrade the starchy endosperm after germination are largely secreted by the aleurone layer after imbibition (see below). However, barley β-amylase progressively associates with starch grains during their deposition and during seed desiccation. This enzyme is, therefore, perfectly positioned to degrade these same starch grains upon imbibition and germination (Hara-Nishimura *et al.*, 1986). This is another example of structural patterning laid down during endosperm development that will reinforce the molecular activities resulting in an efficient reserve mobilization system upon germination.

Storage proteins accumulate during grain development and serve as a reserve of nitrogen and sulphur for the seedling. The composition of these proteins greatly affects the usefulness of grains in the nutrition of humans and other animals. For example, the major storage proteins in the starchy endosperm of barley grains are hordeins, classified as an alcohol-soluble prolamin. The equivalent primary maize storage proteins are the zeins. Both hordeins and zeins are deficient in the essential amino acid lysine. Some progress has been made in altering grain nutritional value by identifying mutants with improved protein composition or via inserting genes that result in a nutritionally superior grain (Carneiro *et al.*, 1999; Ye *et al.*, 2000).

While most cereals store one type of seed storage protein, rice accumulates both prolamins and another class of storage proteins, the globulins (salt-soluble storage protein). Interestingly, each of these occupies different subcellular compartments in the same cell. During rice endosperm development, prolamins are directly sequestered within the ER. However, the globulins are transported from the ER lumen to the Golgi and then deposited in the vacuole (Kim *et al.*, 1988). The subcellular targeting and retention mechanisms differ for each of these types of proteins (Robinson and Hinz, 1999). Ultimately, during seed germination and post-germinative growth, endosperm storage proteins are digested by proteases either secreted by the aleurone tissue or that were preformed in the starchy endosperm during grain development.

In addition to starch and protein, the starchy endosperm is a repository for nucleic acids and micronutrients. Nucleic acids are not deposited

specifically as a storage polymer but rather are present during the development of the starchy cells and remain in the dead cells to be mobilized as a phosphate source after grain germination (Hall and Hodges, 1966). Nucleases secreted by the aleurone after imbibition degrade starchy endosperm DNA and RNA (Brown and Ho, 1986). The starchy endosperm nucleic acids provide a fraction of the phosphate needed by the growing embryo; most phosphate in the grain is stored as phytin in the aleurone layer (see below). In addition to phosphate, endosperm calcium is another nutrient important to the functioning of the grain. Levels of calcium in the starchy endosperm drop concomitant with calcium accumulation in the embryo during the first 5 d of growth (Stewart *et al.*, 1988). Starchy endosperm calcium is also important in the functioning of the aleurone layer, as aleurone layers with their associated starch require less calcium than do isolated aleurone layers for GA responses (Deikman and Jones, 1985).

Along with providing hydrolases for the breakdown of the reserves in the starchy endosperm, the aleurone is also a storage tissue in its own right. At grain maturity, an aleurone cell is filled with hundreds of protein storage vacuoles, each surrounded by numerous oleosomes that are possibly linked to the vacuole via a phospholipid layer (Fernandez and Staehelin, 1985). Inside the vacuoles are storage proteins (salt-soluble 7S globulins; Yupsanis *et al.*, 1990), phytin (a potassium, magnesium, and calcium salt of *myo*-inositol hexakisphosphate; Stewart *et al.*, 1988), and carbohydrate (Jacobsen *et al.*, 1971). The phytin in aleurone protein storage vacuoles is the major phosphate store in the grain. At least two types of dense inclusions are formed inside the protein storage vacuoles as well as some of the enzymes required to mobilize them (Bethke *et al.*, 1998).

Yoshida *et al.* (1999) isolated a clone from rice encoding a *myo*-inositol-1-phosphate synthase, which catalyses the first step in the synthesis of inositol hexaphosphate, and hence phytin synthesis. *In situ* hybridization revealed that the gene is expressed early in seed development in the embryo and scutellum, though not in the epithelial cells of the scutellum. In the aleurone, expression followed the pattern of aleurone cell differentiation; the *in situ* signal first appears in the ventral groove region, and then rapidly appears throughout the aleurone. In the rice grain, phytin is deposited in globoids that develop earlier than the accumulation of storage proteins. Importantly, the timing and pattern of *myo*-inositol-1-phosphate synthase expression corresponded closely to the development of phytin-containing globoids.

Acquisition of grain desiccation tolerance and aleurone hormone sensitivity

The final steps in endosperm development are desiccation and developmental arrest. These processes can permit seed dispersal and tolerance to harsh environments until favourable growth conditions occur. The initiation of desiccation, developmental arrest and dormancy is associated with increased abscisic acid (ABA) levels, which may also be involved in the induction of desiccation tolerance in the aleurone.

ABA is thought to be involved in the regulation of the expression of a host of proteins that function to allow seed desiccation and perhaps prevent pathogen attack. Many genes have been identified that are expressed late in seed development (Chandler and Robertson, 1994), and many have been shown to be up-regulated in other plant tissues in response to ABA and other dehydration-related stresses, such as extreme temperatures or salt treatment. Some of these proteins have possible roles in the acquisition of aleurone desiccation tolerance. For example, Hong *et al.* (1988, 1992) reported that the *HVA1* gene is rapidly induced by ABA in barley aleurone layers. Its message accumulates in the aleurone and embryo (but not the starchy endosperm) from 25 d post-anthesis and stays high in dry grains. The peak of *HVA1* expression coincides with the peak ABA accumulation. *HVA1* is a lea (late embryo abundant) protein thought to protect the cytoplasm during dehydration by binding water and ions. However, despite such potential roles in the desiccation programme of the aleurone, a direct role for ABA and ABA-regulated proteins in endosperm development has not been thoroughly investigated.

Dehydration not only leads to a grain that can tolerate harsh environments and has low metabolic activity, but also seems to be important for the subsequent ability of the aleurone to respond to GA and produce normal levels of amylase (Evans *et al.*, 1975). Aleurone cells of cereal grains that have developed to the stage of complete cellularization are not yet competent to respond to GA. Desiccation of the grain during maturation is thought to be the main event that induces the ability of aleurone tissue to respond to GA and so becomes competent to produce the hydrolytic enzymes required during germination. In barley and wheat grains prematurely removed from the mother plant, the aleurone layer is not able to produce amylase in response to GA. However, if these grains are artificially dried, the behaviour of the aleurone in response to GA is similar to the behaviour of those grains matured on the plant (Armstrong *et al.*, 1982; Cornford *et al.*, 1986; Jiang *et al.*, 1996). Additionally, treating the wheat grain with elevated temperatures, specifically above 27°C, can substitute

for the dehydration treatment in conferring GA sensitivity on the aleurone cells of the grains prematurely harvested (Norman *et al*., 1982). It seems that a transition of grain drying to below 30% H_2O promotes GA responsiveness.

The endosperm during grain germination and early seedling growth

The preceding sections described how endosperm development and desiccation have generated a protective support system for the embryo packaged within the dry grain. The stage is now set for the events of germination to unfold. The initial event triggering germination is imbibition of water. The storage polymers in the starchy endosperm must then be mobilized and transported to the embryo to fuel its subsequent heterotrophic growth. Such mobilization and transport are accomplished by co-ordinating activities of the aleurone and scutellum. Imbibition triggers the embryo to release GA. This GA then diffuses to the aleurone layer where it induces this tissue to synthesize and secrete a spectrum of hydrolases that degrade the starchy endosperm. The products are then transported via the scutellum to the embryo. GA biosynthetic inhibitors do not inhibit the increase in amylase production during germination, suggesting the embryo is mobilizing stored GA precursors rather than synthesizing GA *de novo* (Grosselindemann *et al*., 1991).

In addition to the aleurone, the scutellum also secretes hydrolytic enzymes to aid in starchy endosperm mobilization (Fincher, 1989). In a barley mutant with reduced levels of GA, mRNA for a high pI amylase is expressed at very low levels in the scutellum, but can be restored to higher levels by the application of GA, implying this gene is normally regulated by GA levels (Chandler and Mosleth, 1990). Interestingly, amylase genes that are GA regulated in the aleurone are also expressed in the scutellum (Sugimoto *et al*., 1998). However, whether α-amylase expression in the scutellum is regulated by GA in wild-type barley remains unknown.

ABA antagonizes the events induced by GA in the aleurone at many levels. For example, the promoters of the high pI amylase genes have regulatory regions that respond to GA by enhancing transcription (e.g. the GA response element, GARE) as well as repressor elements that inhibit this transcriptional activation (reviewed in Ritchie and Gilroy, 1998c). Napier *et al*. (1989) reported undetectable ABA levels in the aleurone of the mature grain, but recent measurements (J.V. Jacobsen, personal communication) indicate that the ABA levels (<10 nM) in the aleurone are still within the range that could alter GA responses (Ritchie *et al*., 1999). Additional evidence for ABA effects during grain germination is the observation that ABA also up-regulates a series of genes in the mature aleurone (such as the amylase–subtilisin inhibitor; Mundy *et al*., 1984), and sensitivity to ABA may show developmental regulation analogous to that seen for GA (Rogers and Rogers, 1992). However, the physiological role of these ABA responses during grain germination remains less clear than for GA. For example, the conditions under which the embryo might release ABA to slow endosperm mobilization already underway remain poorly characterized.

The physiological context for GA-modulated events is much better defined. As described above, the endosperm of the mature grain is packed with storage polymers rich in carbohydrate, protein and lipid. However, these reserves are unavailable to the embryo until processed through the action of hydrolytic enzymes. The breakdown products from the hydrolytic activity, such as glucose, maltose and peptides, are transported into the scutellum where they may be further modified prior to transport to the growing embryo. The source of hydrolytic enzymes that digest starchy endosperm reserves is largely from both the scutellum and the aleurone. Indeed, aleurone development has produced a tissue specialized to synthesize and secrete hydrolases. This it does for at least 4 d, until the storage reserves of the endosperm are depleted and the aleurone cells die. Thus, the aleurone has a highly determined developmental programme that starts with imbibition and activation by GA, proceeds through a phase of extensive synthesis and secretion of hydrolases, and ends with a controlled programme of cell death (reviewed by Fath *et al*., 1999).

Detailed studies by many groups have revealed the changes in fine structure of the aleurone cell as it progresses through these stages, summarized in Fig. 4 (Jones and Jacobsen, 1991). In the freshly imbibed grain, aleurone cells have a dense cytoplasm filled with many small vacuoles packed with storage polymers including proteins and phytic acid. Upon activation by GA, the vacuolar contents are hydrolysed to produce raw materials such as amino acids and inorganic ions used by the synthetic machinery of the cell to produce secreted hydrolases. Concomitantly, hydrolase gene expression, including high and low pI α-amylases, is increased (Fig. 4B) and the secretory apparatus (Golgi and ER) proliferates (Fig. 4A). This is the phase of maximal enzyme secretory activity in the cell and is sustained for several days. The vacuoles coalesce as their contents are used up, until the cell appears to contain a thin layer of cytoplasm surrounding one or two large vacuoles. At this point, the programme of cell death

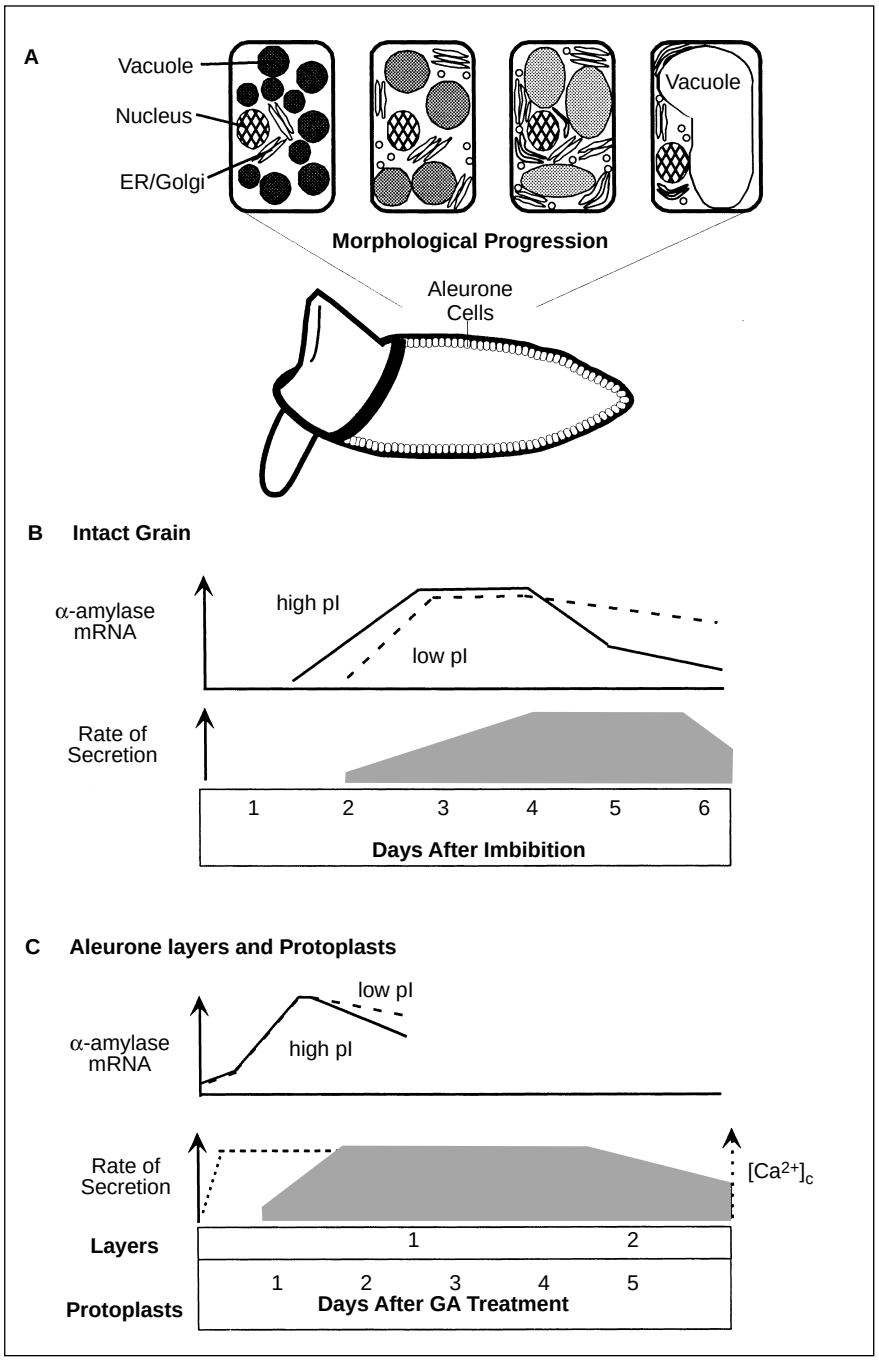

Figure 4. Progression of aleurone cell responses after imbibition or after GA treatment. (A) Morphological changes in an aleurone cell after GA perception. (B) Relative levels of α-amylase mRNA and relative rate of hydrolase secretion by the aleurone layer in intact grain from the start of imbibition. (C) Relative levels of α-amylase mRNA, hydrolase secretion, and increase in cytoplasmic Ca^{2+} after GA treatment of aleurone layers or protoplasts. Layers and protoplasts were prepared from de-embryonated, imbibed grain (Deikman and Jones, 1985; Karrer *et al*., 1991; Bush, 1996; Gilroy, 1996; Appleford and Lenton, 1997; Sugimoto *et al*., 1998). $[Ca^{2+}]_c$ = cytoplasmic calcium level.

becomes evident in the tissue, and aleurone cells die over the next 1 or 2 d (Wang *et al*., 1996; Bethke *et al*., 1999; Lonsdale *et al*., 1999). The molecular mechanisms leading to the death of aleurone cells are unknown but are likely to involve increased production of non-secreted hydrolases (Holstein *et al*., 1991; Bethke *et al*., 1999).

Many of the techniques used to understand molecular and cellular aspects of signalling and regulation in the aleurone cannot be readily applied to intact grain. Therefore, most work has been performed on isolated aleurone layers (in which the starch is removed) and protoplasts (aleurone cells lacking the cell wall). However, the same cellular events that occur in the aleurone of whole imbibed grain also occur in GA-treated, de-embryonated half grain (including the starchy endosperm, aleurone, and testa-pericarp), isolated aleurone layers and protoplasts. As illustrated in Figs 4B and 4C, the timing of events is faster in isolated aleurone layers than in the whole grain. The speed of the response likely differs because the tissue is pre-imbibed before being exposed to GA in the isolated layer, whereas in the intact grain imbibition must occur prior to developing the full GA response from the system.

GA and aleurone function

Perception of GA by aleurone tissue

A plant GA or ABA receptor has yet to be identified. However, as a first step towards understanding hormone responses, the sites of GA and ABA perception have been localized in oat and barley aleurone cells. GA and ABA can readily cross cellular membranes, suggesting that they could enter the cell and act through a cytoplasmic "steroid-like" receptor system. Alternatively, the receptor could lie on the external face of the plasma membrane. Defining this perception site has been a major goal of aleurone researchers, as knowledge of where these hormones are perceived is central to developing models of subsequent regulatory events.

Recent evidence strongly supports an external GA perception site. GA_4 has been chemically altered to be cell impermeant (Hooley *et al*., 1991; Beale *et al*., 1992), and these analogues still elicit amylase production from oat aleurone. Likewise, an anti-idiotypic antibody to GA_4 was found to antagonize GA action in oat aleurone protoplasts (Hooley *et al*., 1992), again consistent with a surface receptor system for GA. Lastly, microinjection of GA into barley aleurone protoplasts was ineffective in eliciting the GA response, whereas externally applied GA induced the GA response systems (Gilroy and Jones, 1994).

For ABA signal transduction, the genetics of the ABA response suggests multiple receptor sites (McCarty, 1995), and in barley aleurone cells internal and external receptor systems are indicated (Ritchie and Gilroy, 1998c). A receptor on the plasma membrane is important in inhibiting the GA response whereas an internal receptor appears to modulate other ABA responses (Ritchie and Gilroy, 1998c). Thus, one point of flexibility in the control systems in this tissue may lie in a multiplicity of receptors, each tailoring the extent of a particular aspect of the response to the ambient hormone level. The critical, but unresolved, question that remains is the molecular identity of the receptors for these hormones.

Signal transduction after GA perception by aleurone

The receptor systems for GA and ABA trigger a network of molecules that co-ordinate the diverse features of the aleurone response. These signal transduction elements can be broadly classed as those altering cytoplasmic responses, such as the activity of the secretory apparatus and membrane transporters, and those involved in changes in gene expression. A suite of these signal transduction elements amplifies and integrates the hormone signal. The varied cellular activities of the aleurone need to be highly co-ordinated, and many regulatory molecules act to control several different aspects of the response system. The regulators that have emerged in the aleurone signalling are also well-known controllers of cell function in animals and yeast. The unique aspect of their action is how these elements interact to facilitate co-ordination of aleurone secretion.

G-proteins

In animal and yeast cells, GTP-binding proteins (G-proteins) are ubiquitous intermediates passing signals from a receptor to downstream regulatory elements. In plants the role of G-proteins is less clear, but circumstantial evidence points to their role in signalling processes as diverse as response to pathogen attack, light and hormones (Millner and Causier, 1996). Regulatory G-proteins can be subdivided into small monomeric or heterotrimeric forms, with the heterotrimeric G-proteins consisting of α, β and γ subunits. A putative Gα and two Gβ subunits have been cloned from GA-treated oat aleurone cells (Jones *et al*., 1998). These transcripts were low in abundance and potentially part of a multigene family. Although the functional role of the G-proteins in most plants remains elusive, compounds that alter G-protein function, such as bacterial toxins, peptides and GTP analogues, all

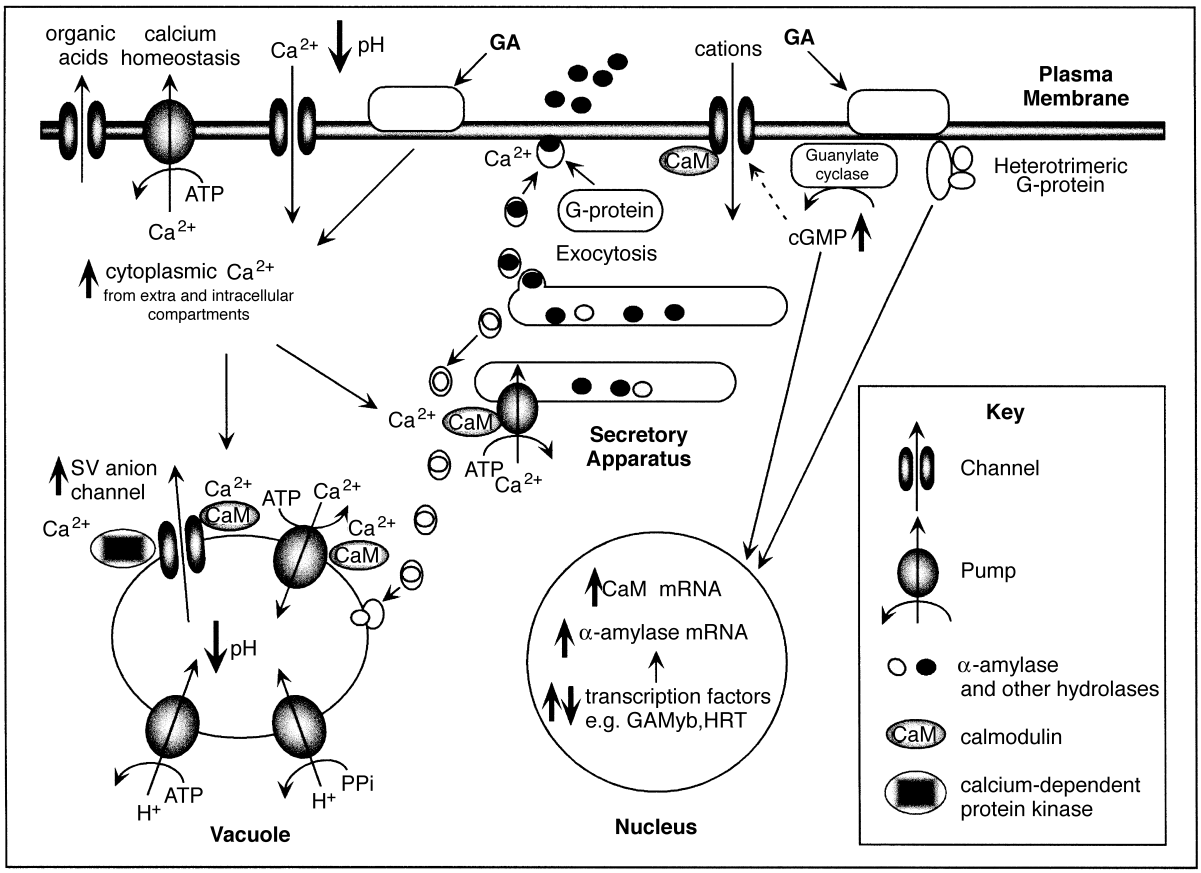

Figure 5. A model of the signalling and cellular events that occur in the cereal aleurone cell in response to GA. Calmodulin, calcium-dependent protein kinase and hydrolytic enzymes which are processed through the endomembrane system are indicated as in the key. Various channels and pumps which have been identified either at the biochemical or molecular level are illustrated. (Ca^{2+}, Bush and Jones, 1987, 1988; Wang *et al.*, 1991; Gilroy and Jones, 1992; Bush, 1996; Gilroy, 1996; Van der Meulen *et al.*, 1996; CaM, Gilroy and Jones, 1993; Schuurink *et al.*, 1996; CDPKs, Huttly and Philips, 1995; Ritchie and Gilroy, 1998b; cGMP, Penson *et al.*, 1996; ER Ca^{2+}/ATPase, Bush *et al.*, 1989, 1993; Gilroy and Jones, 1993; Chen *et al.*, 1997; GAMyb, Gubler *et al.*, 1995; G-protein, Homann and Tester, 1997; Jones *et al.*, 1998; Fujisawa *et al.*, 1999; HRT, Raventos *et al.*, 1998; organic acids, Macnicol and Jacobsen, 1992; Heimovaara-Dijkstra *et al.*, 1994; Drozdowicz and Jones, 1995; plasma membrane ion channel, Schuurink *et al.*, 1998; SV anion channel, Bethke and Jones, 1994, 1997; vacuolar H^+/ATPase and PPIase, Bush and Wang, 1995; Swanson and Jones, 1996; vacuolar pH, Davies *et al.*, 1996; Swanson and Jones, 1996.) See text for specific details of signalling activities.

affect the responsiveness to GA and ABA in oat and barley aleurone (Jones *et al.*, 1996, 1998; Fig. 5), although Kuo *et al.* (1996) reported no effect on wheat aleurone function. Recently it was found that a dwarf mutant of rice, which is impaired in the GA response of the aleurone, has a lesion in a Gα gene (Fujisawa *et al.*, 1999), providing genetic evidence that G-proteins are involved in GA signalling in the aleurone (as well as other plant tissues). Using patch clamping, Homann and Tester (1997) have also shown a G-protein requirement in the aleurone secretory pathway. All these studies imply an important role for G-proteins in aleurone hormone responses, either at the level of signalling elements within the cell or in the machinery of membrane trafficking within the secretory apparatus (Bomsel and Mostov, 1992).

cGMP

3′-5′-cyclic guanosine monophosphate (cGMP) is involved in transducing stimulus-coupled responses in mammalian cells (Bentley and Beavo, 1992). cGMP is now emerging as a central regulator of plant signal transduction networks involved in GA (Penson *et al.*, 1996) and light (Brown *et al.*, 1989; Bowler and Chua, 1994) responses. Thus, in the aleurone, GA, but not ABA, induces a two- to three-

fold increase in cGMP levels (Penson *et al*., 1996). Although adding cGMP was unable to elicit the entire GA response, blocking the normal GA-related rise in cGMP blocked GA action. These results imply that cGMP is involved in some of the early events of the GA signal transduction/regulation system. As the increase in cGMP levels is rapid, the activity producing the cGMP may be close to interactions with the activated GA receptor. The cellular factors that react to the cGMP signal remain to be determined. However, a putative Ca^{2+}-calmodulin- and cGMP-regulated K^+ channel has been identified in the aleurone (Schuurink *et al*., 1998). The channel has homologues in *Arabidopsis* (Leng *et al*., 1999), and characterization of one of these homologues by expression in yeast, *Xenopus* oocytes and human kidney cells showed cyclic nucleotide-dependent inward K^+ channel activity. Therefore, defining the function of this channel in the aleurone may also yield insight into GA responses in other plants and response systems.

Ca^{2+} and calmodulin

Changes in the levels of cytoplasmic Ca^{2+}, often mediated through the regulatory protein calmodulin (CaM), are recognized as ubiquitous signal transduction elements in animal and plant cells (reviewed in Poovaiah and Reddy, 1993; Gilroy *et al*., 1993; Bush, 1995). The cytoplasmic [Ca^{2+}] of barley and wheat aleurone increases more than five-fold (from its basal 100 nM) in response to GA. Conversely, ABA reduces Ca^{2+} levels (Bush and Jones, 1987, 1988; Wang *et al*., 1991; Gilroy and Jones, 1992; Bush, 1996; Gilroy, 1996; Van der Meulen *et al*., 1996). GA also causes increases in CaM levels (Gilroy and Jones, 1993; Schuurink *et al*., 1996), and these changes in Ca^{2+} and CaM are essential components of the aleurone's response to hormones (Gilroy, 1996). In combination with other regulators such as cytoplasmic pH (Van der Veen *et al*., 1992; Heimovaara-Dijkstra *et al*., 1995), this Ca^{2+}-dependent regulatory system appears to act as an integrator of aleurone cell function (Bush, 1996; Gilroy, 1996; Homann and Tester, 1997).

Calcium-regulated events abound in the cytoplasm of the aleurone cell (Fig. 5). The exocytotic process of plant cells seems to be regulated by elevated cytoplasmic Ca^{2+} levels (Battey *et al*., 1999). In the barley aleurone there is a localized increase in cytoplasmic [Ca^{2+}] focused to the plasma membrane of the cell (Gilroy and Jones, 1992; Bush, 1996; Gilroy, 1996) which should promote secretory vesicle fusion and exocytosis (Zorec and Tester, 1992; Homann and Tester, 1997). Candidates for the Ca^{2+}- and Ca^{2+}/CaM-responsive elements include putative Ca^{2+}-dependent, CaM-like domain protein kinases (CDPKs; Huttly and Philips, 1995; Ritchie and Gilroy, 1998b), a tonoplast Ca^{2+} transporter (Bush and Wang, 1995), a slow vacuolar-type ion channel in the protein storage vacuole membrane (which may be CDPK regulated; Bethke and Jones, 1994, 1997), and a Ca^{2+}/CaM-sensitive Ca^{2+}-ATPase in the ER (Bush *et al*., 1989, 1993; Gilroy and Jones, 1993; Chen *et al*., 1997). GA activates this ER Ca^{2+}-ATPase, probably via increased CaM levels (Gilroy and Jones, 1993). This transporter is thought to supply the increased Ca^{2+} levels needed for chaperone activity (Jones and Bush, 1991) and for correct α-amylase folding (α-amylase is a Ca^{2+}-requiring metalloprotein) in the ER lumen of GA-treated cells. One general theme emerging from these studies is that Ca^{2+} appears to co-ordinate diverse aspects of membrane trafficking and transport in the aleurone cell.

However, Ca^{2+} is only one of an array of cellular regulators found in aleurone cells. ABA may act through changes in aleurone cytoplasmic pH (Van der Veen *et al*., 1992; Heimovaara-Dijkstra *et al*., 1994) and phospholipase D activity (Ritchie and Gilroy, 1998a). ABA can also activate a MAP-kinase-like activity in aleurone cells (Knetsch *et al*., 1996). Conversely, GA down-regulates expression of *ASPK9*, an aleurone transcript that has homology to the MAP kinases of mammalian cells (Huttly and Philips, 1995). MAP kinases are rapidly emerging as ubiquitous regulators of eukaryotic cell function (Jonak *et al*., 1999), and MAP kinase activity may represent one further point where GA and ABA interact to co-ordinate aleurone cell function.

In addition, homologues of the PKABA1 class of protein kinases appear important for the aleurone ABA response. PKABA1 was first identified in wheat embryos as an ABA-induced kinase (Anderberg and Walker-Simmons, 1992), and homologues exist in the aleurone of oat (Huttly and Philips, 1995) and barley (Gomez-Cadenas *et al*., 1999). Transiently expressing PKABA1 in barley aleurone mimics ABA action, inhibiting GA-induced amylase gene expression. Similar experiments with an *Arabidopsis* CDPK indicated it had no parallel effect on the ABA-sensitive reporter gene, implying that PKABA1 effects are not specific kinase-related events (Gomez-Cadenas *et al*., 1999).

Aleurone responses to GA and secretion of hydrolases

Along with proliferation and modification of the aleurone Golgi and ER after GA perception, profound changes occur in the protein storage vacuoles to mobilize the cellular resources that support intense secretory activity. Energy for protein synthesis and secretion is derived from mobilization of the stored fats in the oleosomes. The lipases that catalyse

breakdown from triacylglycerols to free fatty acids show GA-induced transfer from the protein storage vacuoles to the oleosomes (Fernandez and Staehelin, 1987). As mobilization of lipid occurs, the number of oleosomes decreases, and the numerous protein storage vacuoles coalesce into one large vacuole (Jones and Price, 1970).

Prior to GA perception, aleurone protein storage vacuoles have an unusually high pH of 6.6–7.0 (Davies *et al.*, 1996; Swanson and Jones, 1996). GA causes an acidification of this organelle to pH 5.8 or lower. Although the mechanism is through activation of the existing vacuolar H^+-ATPase and H^+-translocating pyrophosphatase, the molecular machinery causing this to occur is unknown. The H^+-ATPase and pyrophosphatase may be regulated by factors such as cytoplasmic pH, Mg^{2+}, Ca^{2+}, redox state and phosphorylation (Rea and Poole, 1993; Davies *et al.*, 1994; Muller *et al.*, 1996; Swanson and Jones, 1996), many of which are proposed elements of the GA signalling system in the aleurone. This GA-induced vacuolar acidification may activate vacuolar phytases and proteases with acidic pH optima (Runeberg-Roos *et al.*, 1991; Holwerda and Rogers, 1992; Bethke *et al.*, 1996). These enzymes would then break down vacuolar reserves to fuel the synthesis of the hydrolases to be secreted from the cell.

Facilitating hydrolase transit across the aleurone wall

The aleurone cells also have morphological adaptations to their function. The aleurone cell wall has no secondary thickening and forms a thin inner wall and a thicker outer cell wall matrix. Rather than cellulose, the major carbohydrate components are arabinoxylans and 1–3,1–4 β-glucans (Fincher, 1989). Channels in the outer walls of the aleurone cells form during germination and are thought to permit the diffusion of secreted proteins to the starchy endosperm. The mechanism of channel formation, and the production and activation of the enzymes responsible, have yet to be specifically determined (Fincher, 1989). Likewise, how the secreted hydrolases penetrate the inner wall has not been elucidated. However, 1–3,1–4 β-glucanase is secreted by the aleurone cell in response to GA, and this may then break down the bulk of the inner wall sufficiently, leaving only a framework of molecules through which the hydrolases can pass (Taiz and Jones, 1970).

Transcriptional regulation

In addition to subcellular organelle and morphological changes, increased hydrolase production by the aleurone in response to GA is supported by massive hydrolase gene induction. Figure 6 shows that amylases are central to starch degradation, and so it is not surprising that up to 70% of the newly synthesized and secreted enzyme from the aleurone is α-amylase. This large induction has led to the use of the α-amylase gene family as a tool to understand the molecular basis of GA- and ABA-regulated gene transcription and as markers for GA induction of hydrolase synthesis and secretion in the aleurone cell.

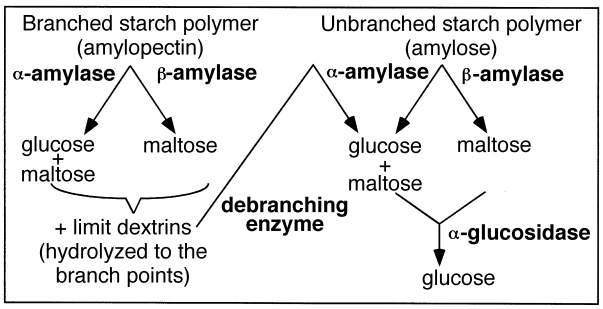

Figure 6. The central role of the amylases in starch mobilization. Hydrolases are shown in bold.

Expression of the α-amylase gene family from the cereal aleurone is tightly controlled by GA, ABA, other germination-related signals, and also by the product of amylase activity, glucose. Hence, studying these genes has provided a model system to unravel not only the elements of GA regulation but also how this system integrates the many factors needed for the co-ordinated regulation in the stimulus-rich germination environment (Bethke *et al.*, 1997; Ritchie and Gilroy, 1998c; Lovegrove and Hooley, 2000). Although the critical link between cytoplasmic signalling events and gene transcriptional regulators remains to be found, these two research areas are slowly converging towards the goal of a complete understanding of the hormone regulatory system (Bethke and Jones, 1998).

Many genes have been identified to date that regulate α-amylase gene expression and other ABA- and GA-regulated genes (Table 1). The most well-characterized, positively regulating protein is GAMyb. GAMyb expression promotes α-amylase gene transcription, suggesting a Myb-based regulation of this GA response. However, expressing a maize Myb (C1) in barley aleurone had the opposite effect, partially inhibiting α-amylase expression (Gubler *et al.*, 1995). In addition, when aleurone proteins were probed with antibodies against the maize Myb, several proteins were evident, two of which decreased in GA-treated tissue (Gubler *et al.*, 1995). These experiments suggest that whilst GAMyb may be activating amylase transcription in response to GA, competition between activator and repressor Mybs binding to the GA-responsive regions of the

Table 1. Regulators of α-amylase gene expression in aleurone cells. GAMyb (Gubler *et al*., 1995, 1997, 1999), UBC (Chen *et al*., 1995), Vp1 (Hoecker *et al*., 1995), HvSPY (Robertson *et al*., 1998), HRT (Raventos *et al*., 1998), PKABA1 (Anderberg and Walker-Simmons, 1992; Gomez-Cadenes *et al*., 1999). GARE, GA response element; GARC, GA response complex; n.d., not determined; ↑, increase in transcriptional activity; ↓, decrease in transcriptional activity

Gene and function of protein (functions in parentheses are inferred from homology only)	How identified	Effect on GA-responsive reporter constructs	Effect on ABA-responsive reporter constructs	Expression levels with		Binds to promoter elements	Other comments
				GA	ABA		
Positive regulators							
GAMyb Transcription factor	Amylase promoter-binding motif has homology to Myb consensus motif. Cloned by homology from barley and rice	↑ (in absence of GA) high and low pI α-amy, β-glucanase and cathespin-B like	n.d.	↑ mRNA and protein	n.d.	GARE in all α-amy and other GA-responsive promoters	The GAMyb protein contains both activation and repression domains
UBC (Ubiquitin-conjugating enzyme)	Differential display of 1 h GA-treated rice endosperm	Inhibits the repression of a truncated α-amy promoter, containing a repressor element	n.d	↑	n.d	n.d.	The promoter of this gene itself contains GAREs required for GA-induced expression
Negative regulators							
Vp1 Transcription factor	Maize viviparous mutant identified as lesion in this transcription factor	↓ in mature aleurone. Unlike wild-type, Vp1 mutant cells can support GA-inducible reporter expression on immature aleurone	↑, but domains in promoters are different for ABA and Vp1 activation	n.d.	n.d.	n.d.	Elevated levels of expression during seed maturation
HvSPY (Acetyl-glucosamine transferase)	GA constitutive response mutant of *Arabidopsis*, barley gene identified by homology	↓ high pI α-amy	↑ dehydrin	n.d	n.d	n.d	
HRT Transcription factor	Southwestern screen with high pI α-amy GAREs	↓ high and low pI α-amy, also ↓ CaMV 35S and ubiquitin promoters	No effect	↓	n.d.	GARC + GARE	Present throughout immature seed, very little in dormant tissue. Nuclear localization signal required for repression activity
PKABA1 Protein kinase	Increased mRNA in wheat seedlings after ABA or dehydration treatment	↓ high and low pI α-amy, and GA-up-regulated cysteine protease	↑ (minor) HVA1	↓ (minor)	↑	n.d	

amylase promoter may be an equally important aspect of this gene's regulation by GA.

Several other proteins have been implicated in repression of α-amylase gene expression (Table 1). Two of these, Vp1 and HRT, are transcription factors while others are likely to act by post-translational modification of other proteins. These include HvSpy (a putative *O*-linked *N*-acetylglucosamine transferase; Robertson *et al*., 1998), UBC (a ubiquitin-conjugating enzyme; Chen *et al*., 1995) and PKABA1 (a protein kinase, Gomez-Cadenas *et al*., 1999). Therefore, the *HvSpy*, *UBC* and *PKABA1* genes are likely to act upstream of *Vp1* and *HRT*. *Vp1* acts in concert with ABA to prevent premature germination of maize grains. Likewise, *HvSpy* is capable of up-regulating an ABA-responsive reporter construct (Robertson *et al*., 1998). Thus, these two regulators seem intimately involved not only in repression of GA-related α-amylase gene expression, but also in ABA-induced responses. On the other hand, whilst PKABA1 and HRT repress α-amylase production, they have little effect on other ABA-regulated genes (Raventos *et al*., 1998; Gomez-Cadenas *et al*., 1999). A likely scenario is that Vp1 (either directly or via a C1-like Myb), HvSpy and HRT are involved in repressing α-amylase expression during maturation; once the grain is germinating and the GA signal is present, the GAMyb competes with these and other repressors at the GA response region of the amylase promoter. Other ABA-responsive elements such as PKABA1 and the UBC are likely to add additional layers of regulation.

Another mechanism whereby GA alters gene expression patterns has been proposed from studies of some GA-repressed genes in the aleurone. GA down-regulates expression of thionins, proteins that are ascribed a defensive role (Heck and Ho, 1996), in addition to peroxiredoxin, a protein that may protect against oxidative damage (Stacy *et al*., 1996; Aalen, 1999). The authors concluded that GA affects the stability of the mRNA of these genes, rather than regulating the expression at the level of transcription.

These studies at the level of gene and message regulation are revealing a complex interaction of many regulators. Many other regulatory *cis* elements and *trans*-acting factors are known to be involved in the expression of the α-amylase genes (reviewed in Ritchie and Gilroy, 1998c), and the likelihood is that even more regulators will emerge as the transcriptional control complex of GA-up-regulated genes such as α-amylase are studied further. This network of interacting regulators presumably builds a sensitive, tightly regulated, yet flexible, control system that can integrate the input of many signals. This idea of a web of interacting and competing control elements is likely an important conceptual starting point for understanding the integrating control systems of many other aspects of the aleurone hormone response systems.

Role of the starchy endosperm during germination and early seedling growth

Although we have focused on hormonal control of aleurone cellular processes, aleurone function cannot be completely understood without placing it in the context of the starchy endosperm undergoing hydrolysis. While the starchy endosperm is a dead tissue at grain maturity, its contents have a major impact on seed and seedling activities, not only as the source of nutrients for growth of the embryo, but also as a likely controlling element of aleurone and scutellar function. For example, pH has been proposed to underlie regulation of enzyme activities in the extracellular milieu of the endosperm. The secreted enzymes that break down endosperm storage polymers have pH optima around pH 4.5 (Wilson, 1975; Koelhler and Ho, 1990; Green, 1994). In addition, proteolytic activation of several hydrolases in the endosperm is mediated by endoproteases with acidic pH optima (Guerin *et al*., 1992; Longstaff and Bryce, 1993). An acidic environment also favours transport across the scutellum to the embryo (e.g. Hardy and Payne, 1991; Bush, 1993), dissociation of endogenous hydrolase inhibitors (Halayko *et al*., 1986), and solubilization of otherwise inaccessible hydrolase substrates (Hamabata *et al*., 1988). The cereal endosperm is acidic at imbibition owing to high levels of malic acid, and this low pH is maintained following germination by GA-induced release of phosphoric and citric acids from the aleurone (Macnicol and Jacobsen, 1992; Heimovaara-Dijkstra *et al*., 1994; Drozdowicz and Jones, 1995). Thus, pH appears to be an important regulator of endosperm activities.

In addition, starch, sugars produced by its hydrolysis, and the resulting changes in osmotic potential all appear to be potentially important regulators. In rice, the expression of two α-amylase genes has been studied with respect to inhibition by sugars. Both the *Ramy3D* and *αAmy8* genes are expressed transiently in the scutellum and aleurone after imbibition (*Ramy3D*, Karrer *et al*., 1991; *αAmy8*, Yu *et al*., 1996), but their mRNA levels differ from other amylase genes whose expression is more sustained during imbibition. In embryos, mRNA levels of both genes are repressed by treatment with specific sugars, e.g. glucose (Karrer and Rodriguez, 1992; Yu *et al*., 1996), and in the case of *αAmy8*, sugar-induced repression was also shown to occur in aleurone tissue (Yu *et al*., 1996). The glucose concentration in the endosperm appears to be high enough to account for the transient expression of *αAmy8* (Yu *et al*., 1996), and in rice-suspension cells its expression is induced by depriving the cells of sugars (Chan *et al*., 1994; Mitsunaga *et al*., 1994). For both genes the regulation of expression may be through

transcriptional control via specific sugar-sensitive elements in the gene promoters (Huang *et al*., 1993; Chan *et al*., 1994), although mRNA stability has also been implicated for induction of *αAmy8* in suspension cells (Sheu *et al*., 1994).

In barley, the α-amylase genes in the embryo are sensitive to sugars while these same genes are GA-regulated in aleurone (Perata *et al*., 1997), suggesting a level of regulation specific to the embryo tissue. A further interesting observation on the interaction between sugars and amylase gene expression comes from the shrunken-2 (*sh-2*) maize mutant. The lesion in this mutant is in a gene involved in the formation of complex carbohydrates and results in the endosperm containing more simple sugars than normal starchy lines. While activation of amylase genes from the wild-type maize aleurone is GA independent, exogenous application of GA is required to restore normal levels of amylase production in the *sh-2* mutant (Sanwo and DeMason, 1994).

Sugars produced from starch degradation in the endosperm are osmotically active, and the resultant decrease in the water potential of the starchy endosperm is also likely to affect aleurone and embryo cell activity. Yu *et al.* (1996) showed that expression of specific α-amylase isoforms was down-regulated in different ways by glucose or mannitol (mannitol is osmotically active, but metabolically inactive). In addition, the secretory activity of the aleurone cell can be affected by high osmoticum (Jones and Armstrong, 1971).

Other components of the starchy endosperm may be further regulators of aleurone function. For example, the Steptoe variety of barley produces very low levels of grain α-amylase. When GA was applied to de-embryonated grains, the levels of amylase gene induction and enzyme were still low. However, aleurone layers isolated from the de-embryonated grain produced levels of amylase comparable to a more "normal" GA-responsive variety (Skadsen, 1993). In many other plants where GA response is impaired in the aleurone, there are GA-related phenotypes, such as stunted shoots. However, the Steptoe phenotype appears unusual in that it is selective for seed responses. Levels of ABA in the seedling were tested to see if this could account for the reduced aleurone activity. Interestingly, ABA levels were not significantly higher than in another barley variety in which the aleurone is GA responsive in the presence of the starchy endosperm. These observations suggest that there is an unidentified factor within the starchy endosperm that represses the GA response in this barley variety.

Conclusions

The more we identify elements of the systems that underlie the mobilization of endosperm reserves during germination and early seedling growth, the more we appreciate the subtle interplay between structure, both molecular and developmental, and function in this system. We can anticipate that modern molecular techniques will continue to unearth the subtleties of the GA- and ABA-regulated signalling systems. The fact that elements such as the GAMyb transcription factors and Cr4 receptor kinase have effects in seed and vegetative tissues also suggests that these studies will have implications for signalling and development in areas far ranging from seed physiology. Similarly, the elements of the hormone signalling system (e.g. cGMP, calcium, calmodulin, phospholipases, and protein kinases) identified in seed responses appear conserved in the response systems of vegetative tissues (e.g. phospholipase D involvement in the ABA response of barley aleurone, Ritchie and Gilroy, 1998a and *Vicia fava* guard cells, Jacob *et al*., 1999). Thus, information from seed signalling systems may well help uncover the complexities of hormone responses in other parts of the plant.

Both structural and developmental studies are keys to understanding how the endosperm functions during germination and seedling growth. We can anticipate that as more molecular regulators of seed development are uncovered, our appreciation of how the germination response is "hard-wired" into the structure of the mature seed will undoubtedly grow. The second theme emerging is that molecular regulators form a network of interacting and competing systems that integrate information at a cellular level from many sources. Aleurone cell activity is governed not only by GA and ABA, but through feedback of information from the mobilizing starchy endosperm. Understanding molecular information processing systems against the backdrop of seed structure is at the heart of our understanding of how cereal grain germination and early seedling growth are controlled.

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