



RESEARCH PAPER

α -Amylase and programmed cell death in aleurone of ripening wheat grains

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Received 14 September 2005; Accepted 16 November 2005

Abstract

Late maturity α -amylase (LMA) in wheat is a genetic defect that may result in the accumulation of unacceptable levels of high pl α -amylase in grain in the absence of germination or weather damage. During germination, gibberellin produced in the embryo triggers expression of α -Amy genes, the synthesis of α -amylase and, subsequently, cell death in the aleurone. LMA also involves the aleurone and whilst LMA appears to be independent of the embryo there is nevertheless some evidence that gibberellin is involved. The aim of this investigation was to determine whether the increase in α -amylase activity in LMA-prone genotypes, like α -amylase synthesis by aleurone cells in germinating or GA-challenged grains, is followed by aleurone cell death. Programmed cell death was seen in aleurone layers from developing, ripe and germinated grains using confocal microscopy and fluorescent probes specific for dead or living cells. Small pockets of dying cells were observed distributed at random throughout the aleurone of ripening LMA-affected grains and by harvest-ripeness these cells were clearly dead. The first appearance of dying cells, 35 d post-anthesis, coincided with the later part of the 'window of sensitivity' in grain development in LMA-prone wheat cultivars. No dead or dying cells were present in ripening or fully ripe grains of control cultivars. In germinating grains, dying cells were observed in the aleurone adjacent to the scutellum and, as germination progressed, the number of dead cells increased and the affected area extended further towards the distal end of the grain. Aside from the obvious differences in spatial distribution, dying cells in 20–24 h germinated grains were similar to dying cells in developing LMA-affected

grains, consistent with previous measurements of α -amylase activity. The increase in high pl α -amylase activity in developing grains of LMA-prone cultivars, like α -amylase synthesis in germinating grains, is associated with cell death, providing further evidence for the involvement of gibberellin in the LMA response.

Key words: Aleurone layer, cell viability, germination, late maturity α -amylase, wheat.

Introduction

Cereal endosperm is composed of two distinct tissues: the starchy endosperm that is primarily a storage organ filled with starch and protein reserves and the surrounding aleurone layer. In the wheat grain, the aleurone tissue is a single layer, which can be distinguished from about 8 d post-anthesis (dpa) (Morrison *et al.*, 1975, 1978). Only the aleurone layer remains alive in the mature grains (Bradbury *et al.*, 1956) and during germination, or in response to exogenous gibberellic acid (GA), synthesizes and secretes a range of hydrolytic enzymes (principally α -amylase) that participate in the breakdown of (endosperm) starch, protein, lipid, and cell walls in the endosperm. Following completion of its secretory function, the aleurone layer is programmed to die (Haberlandt, 1884; Kuo *et al.*, 1996; Wang *et al.*, 1996; Bethke *et al.*, 1999; Fath *et al.*, 2000). Death of the aleurone cells has been described as a form of programmed cell death (PCD) since it is regulated by GA and ABA (Kuo *et al.*, 1996; Bethke *et al.*, 1999). While GA stimulates the onset of PCD in the aleurone layer of barley (Wang *et al.*, 1996; Bethke *et al.*, 1999) and wheat (Kuo *et al.*, 1996), ABA-treated aleurone protoplasts remain viable and PCD is postponed (Fath *et al.*, 2000).

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Late maturity α -amylase (LMA), also termed prematurity α -amylase activity (PMAA) in the UK (Gale *et al.*, 1983), is a genetic defect present in particular wheat genotypes that has been inadvertently disseminated to wheat breeding programmes around the world. Grain of these genotypes may develop high pI α -amylase activity either under normal growing conditions or, more commonly, as a result of cool temperature stress (Mares and Gale, 1990; Mares and Mrva, 1992; Mares *et al.*, 1994) during the middle stages of ripening.

The α -amylase isozyme patterns observed in grains affected by LMA are very similar to the early stages of germination, consisting predominantly of high pI isozymes controlled by α -Amy-1 gene families located on the long arms of the homoeologous group 6 chromosomes of wheat. In LMA-affected grains, high pI α -amylase isozymes appear to be synthesized throughout the entire aleurone layer and released into adjacent endosperm (Mrva and Mares, 1996). This is significantly different from the spatial pattern of enzyme production typical of germination or sprouting where the initial enzyme synthesis is concentrated at the embryo end of the grain (Mares *et al.*, 1994; Mrva and Mares, 1996) adjacent to the site of GA biosynthesis. In germinating grains, the scutellum appears to be the site of initial α -amylase synthesis (Cejudo *et al.*, 1995; Mrva and Mares, 1999) with the aleurone being activated later in germination as GA is transported distally through the grain. This difference in distribution of α -amylase activity within the grain can be used to differentiate LMA from preharvest sprouting or germination.

Comparisons of grain α -amylase phenotype for LMA-prone genotypes grown in several seasons and at different locations around Australia suggested that there was a strong interaction with environment (Mares *et al.*, 1994). In genotypes such as Spica or Lerma 52 (Mares and Gale, 1990), LMA was detected in all of the environments examined, although the amount of enzyme activity appeared to be inversely related to temperature. By contrast, most LMA-prone genotypes maintained a low grain α -amylase phenotype unless they experienced a cool temperature shock during mid-grain development (Mrva and Mares, 2001a). The α -amylase is retained in ripe grain resulting in low falling numbers, downgrading quality at receipt, and potentially adverse effects on end-product quality. Cool temperature stress had no effect on α -amylase activity in non-LMA genotypes that fortunately represent the greatest proportion of wheat cultivars.

Genetic analysis identified two independent QTL associated with variation in LMA phenotype, one highly significant ($P < 0.001$) QTL located on the long arm of chromosome 7B and a second significant QTL ($P < 0.05$) near the centromere on chromosome 3B (Mrva and Mares, 2001b). These QTL are clearly not linked genetically to the *Amy-1* genes on 6A, 6B, and 6D that encode the sequences

for the high pI α -amylase isozymes, however, when present can result in co-ordinated expression of the *Amy-1* genes.

The aim of this study was to determine whether synthesis of late maturity α -amylase, like α -amylase synthesis in germination or GA challenged aleurone, was accompanied by programmed cell death and hence provide further evidence for the role of hormonal action in LMA. Aleurone layers from developing and ripe grains of LMA and non-LMA genotypes were compared with aleurone isolated from germinating grains using confocal microscopy and fluorescent probes specific for dead and live cells.

Materials and methods

Plant material and germination

Wheat (*Triticum aestivum* L. em Thell) LMA genotypes Spica and Seri (produce LMA under normal growing conditions without a cool temperature stress), BD159 and Kennedy (LMA-prone genotypes that only produce LMA if grains are subjected to a cool temperature stress midway through grain development) and control cultivars, Sunco and Janz, were grown at the University of Adelaide, Waite Campus in 2002. Spikes were tagged at anthesis, samples taken at 4 d intervals from 21 d after anthesis (dpa) until harvest-ripeness (12% grain moisture) and stored at -80°C .

Grain samples (30–40 grains) of control cultivar Sunco (non-LMA) were incubated on moist filter paper in Petri dishes at 20°C . Samples of 20 grains were harvested every 24 h until 120 h after imbibition and stored at -80°C .

Induction of LMA and confirmation of the period in grain development that is sensitive to cool temperature

LMA was induced according to the detached tiller method of Mrva and Mares (2001a). For the developmental study, plants of the LMA-prone wheat cultivar Kennedy were grown in pots and from anthesis were maintained in a warm growth chamber (18 – 28°C). At 3–4 d intervals from 7 dpa to ripeness, individual pots were transferred to a cool growth chamber (12 – 18°C) for 7 d, and then transferred back to the warm chamber to complete ripening. Treatments at different times during grain development were compared with controls that remained in the warm chamber for the duration of the experiment.

Estimation of total α -amylase activity and high pI α -amylase enzyme protein in grain samples

α -Amylase activity was determined on single grains using a modification of the method of McCleary and Sheehan (1989) as described by Mares *et al.* (1994) and activities were expressed as α -amylase units per grain, calculated as optical density reading \times appropriate dilution factor. Five spikes per cultivar were analysed with α -amylase activity measured on 40 individual grains in total.

High pI α -amylase protein was assayed in extracts of grains or grain sections with a high pI-specific ELISA (Value Added Wheat CRC, North Ryde, NSW, Australia) (Mrva and Mares, 2001a).

Statistical analysis

In the experiment designed to identify a window of sensitivity to cool temperatures in grain development, simple means, standard deviations (SD), and standard errors (SE) are presented. More detailed statistical analysis was not performed due to the extremely high variability of α -amylase in the individual grains of cool temperature-treated plants.

Preparation of wheat aleurone layers

The proximal (embryo) and distal (brush) portion of grain was removed, followed by the ventral portion including the crease (Fig. 1). For ripe grains, the remaining sections, dorsal and lateral portion of endosperm and seed coat, were imbibed in deionized water for 24 h. In the case of germinating or developing grain, imbibition was not necessary. Aleurone layers were isolated by gently scraping away the starchy endosperm with a metal spatula, by removing the seed coat using tweezers and carefully washing in sterile water (Fig. 1).

Determination of cell viability

Examination and imaging of aleurone layers was done from the endosperm side of the aleurone layer (Fig. 1) using a Bio-Rad MRC 1000 UV confocal laser scanning microscope system in combination with Kr/Ar (krypton/argon) laser and a Nikon Diaphot 300 inverted microscope. Viability of cells in intact aleurone layers was determined by staining aleurone layers with two fluorescent probes; Calcein ($2 \mu\text{l ml}^{-1}$ in 20 mM CaCl_2 ; Molecular Probes) for 15 min followed by *N*-(3-triethylammoniumpropyl)-4-(6-[4-(diethylamino)phenyl]-hexatrienyl)pyridinium dibromide (FM 4-64; $20 \mu\text{M}$ in 20 mM CaCl_2 , Molecular Probes) for 3 min (Bethke *et al.*, 1999).

Calcein was visualized using excitation 488/10 nm and emission at 522/32 nm and FM 4-64 was visualized using excitation 488/10 and 568/10 nm, and emission 680 DF 32. FM 4-64 will only penetrate compromised membranes and results in dead cells fluorescing red whilst Calcein is taken up by viable cells and results in a green fluorescence. Images were collected using $\times 20$ lens, applying 1.5 electronic zoom, with kalman filtering over eight scans. The images of live (green fluorescence) and dead (red fluorescence) cells were collected separately from the same region on the tissue and merged using the Confocal Assistant 4.02 software. The confocal images of aleurone layers from non-LMA and LMA-affected grains were taken from random locations throughout the aleurone segment.

Results

Harvest-ripe grains

Unlike the multi-layered aleurone of barley grains, the aleurone of wheat consists of a single cell layer and it was consequently more difficult to isolate completely intact

layers from ripe grains. Some aleurone cells appeared to have been ruptured during preparation of the layers and did not fluoresce with either probe leaving dark, unstained patches in the micrographs.

Aleurone cells from ripe grains of non-LMA genotypes, Janz and Sunco, fluoresced bright green, or appeared unstained, irrespective of the spatial location within the aleurone segment (Fig. 2A) and there was no evidence of red-stained cells. Images for Sunco and Janz were identical and only an image from Sunco is presented. Similarly, there was no evidence of red fluorescence in the aleurone from control samples of LMA-prone genotypes, Kennedy and BD159, which had not received a cool temperature stress during grain development (images not shown).

By contrast, aleurone layers of the LMA-affected grains of Spica and cool temperature-treated Kennedy contained small groups of cells or individual cells (Fig. 2B, C), that stained red and these appeared to be randomly distributed throughout the aleurone. High pI α -amylase protein was present in extracts of grain sections taken from the dorsal region of these grains. Dead cells (red stained) represented only a small fraction of the total aleurone cells (Table 1), but the results were consistent for all LMA-affected genotypes examined and between different samples from individual genotypes. Other cells were stained green or were unstained. No red-stained cells were observed in aleurone cells adjacent to the crease of the grain.

LMA and cell death in developing grains

Developing grains of three wheat cultivars with contrasting LMA phenotype, Kennedy (cool temperature required for high α -amylase activity), Spica (high α -amylase activity in a wide range of environments), and Sunco (control, non LMA), were examined at intervals after anthesis for evidence of cell death.

Transfer of plants of cultivar Kennedy to the cool temperature environment prior to 25 dpa or after 35 dpa did not result in elevated levels of α -amylase activity at maturity (Fig. 3) and no high pI α -amylase protein was detected with the high pI-specific antibodies (ELISA). The α -amylase phenotype of the ripe grains from these plants was not significantly different from controls that were maintained in the warm chamber from anthesis through to ripeness. By comparison, transfers within the period 25–35 dpa resulted in substantially higher levels of α -amylase activity in ripe grains (Fig. 3) and, in parallel, high pI α -amylase protein was detected with the ELISA. The proportion of grains with high α -amylase activity varied between 5% and 25%. High pI α -amylase protein was not detected in developing grains of any of the cultivars prior to 35 dpa or in the control cultivars at any stage from anthesis to maturity.

Aleurone from grains of Kennedy sampled at 25 dpa (Fig. 4A), or during a cool temperature treatment that lasted from 25–32 dpa, was indistinguishable from controls and

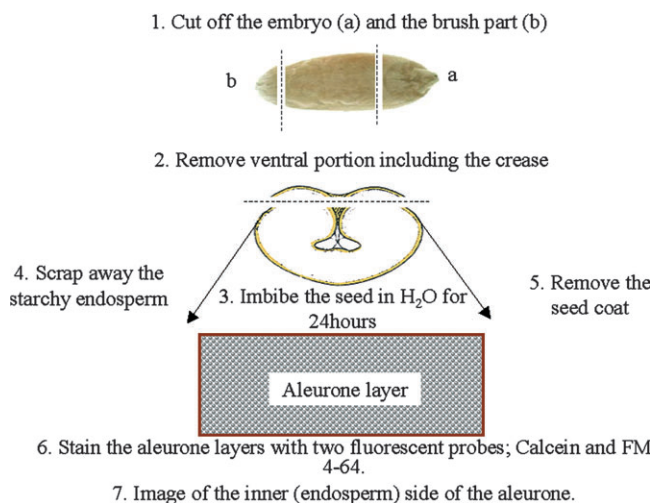


Fig. 1. Preparation of wheat aleurone layers.

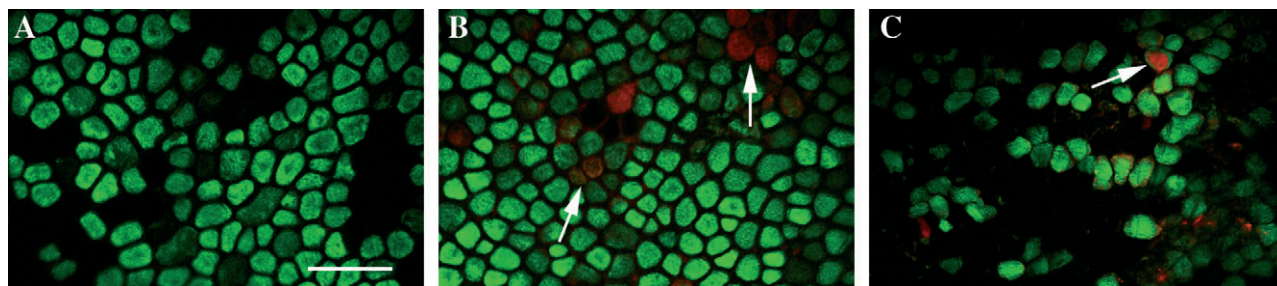


Fig. 2. Merged confocal images of aleurone isolated from ripe grain of a non-LMA wheat cultivar, Sunco (A) and LMA-affected grains of Kennedy (B) Spica (C). Cells fluorescing green represent live cells whereas those fluorescing red are dead. Red-stained (dead) cells are highlighted with arrows. Scale bar=200 μ m, shown in (A) and applies to all frames.

Table 1. The mean percentage of dead cells, \pm SE from the mean, from aleurone layers of LMA genotypes Spica, BD159, and Kennedy from the cool temperature treatment, control cultivar Sunco, and Sunco germinated for 24 h

Genotype	Dead cells (%)
Sunco: control	0
Germinated Sunco 24 h	2.3 \pm 0.08
Spica: LMA	2.1 \pm 0.17
Kennedy: LMA	1.9 \pm 0.14
BD159: LMA	2.0 \pm 0.16

all cells were stained a uniform bright green. At 35 dpa, cells with a trace of red stain or discrete red globules (Fig. 4B) were observed a low frequency in cool-treated samples of Kennedy. By 42 dpa, aleurone cells containing red globules in a green-stained cytoplasmic background appeared to be more common although still at very low frequency compared with cells that stained a uniform bright green (Fig. 4C). Cells with this mixed red–green staining pattern were also present in the aleurone at maturity. Aleurone cells stained completely red were only observed in mature, ripe grain (Fig. 4D).

The cultivar Spica was more convenient for investigation of changes in the aleurone during grain development due to (i) a cool temperature not being required for expression of LMA, and (ii) the high proportion of grains (70–90%) normally affected by LMA and hence the greater certainty of locating early changes associated with enzyme synthesis and cell death. Prior to 29 dpa, the aleurone of Spica appeared very similar to the control, Sunco, with all cells showing a strong green fluorescence (Fig. 5A, F). Sunco retained this appearance through to ripeness (Fig. 5F–J). By contrast, isolated cells or small groups of cells in which part of the cell contents were stained orange–red whilst the rest of the cell showed green fluorescence appeared in the aleurone of Spica at 29–37 dpa (Fig. 5B–D). These changes were presumed to represent dying cells, were similar to those seen in aleurone cells 24 h after imbibition/germination in ripe grains (Fig. 6A) and were only

obvious following merging of the red and green images. It was not practicable to count the numbers of dead and dying cells accurately during grain development and, whilst it appeared that the amount of red stain in affected cells increased with time, there did not appear to be a substantial increase in the number of cells showing evidence of cell death. This trend was similar to that observed in Kennedy subjected to cool temperature treatment. In mature Spica grains, the isolated cells or small groups of cells showed more orange and less green fluorescence, or were completely red (Fig. 5E), and were similar to cells from 48 h-germinated grains (Fig. 6B).

Response to germination: changes in the aleurone layers

In germinating grains, significant changes were first observed in the section of aleurone layer adjacent to the embryo and scutellum. After 24 h the aleurone cells were still stained green, however, for some cells the outer parts of the cells were orange/red (Fig. 6A). With increasing germination time, 48–96 h, the number of aleurone cells with orange and red fluorescence progressively increased (Fig. 6B–D). By 120 h all the aleurone cells in the proximal portion of the aleurone were stained uniformly red (Fig. 6F). In germinating grain there was a time-dependent increase in the proportion of dead cells in the aleurone adjacent to the scutellum (Fig. 7) and progression of cell death towards the distal end of the grains.

Discussion

The confocal images of germinated grains showed that changes in aleurone cells indicative of early, and subsequently final, stages of cell death first appeared in the aleurone from the proximal part of the grain, i.e. adjacent to the embryo and scutellum (Figs 6, 8). The intensity of red fluorescence and the number of dying or dead cells increased with time after imbibition and the area of affected aleurone extended towards the distal end of the grain. After 120 h of germination, the proximal part of the aleurone

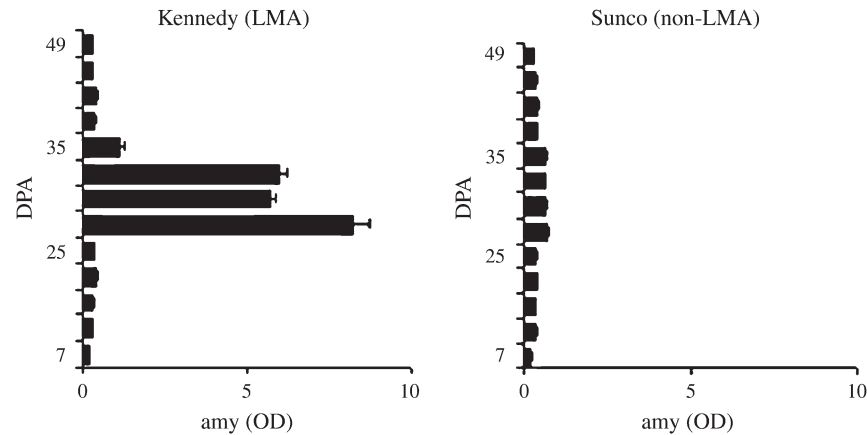


Fig. 3. Effect of cool temperature stress, applied at different stages after anthesis, on final grain α -amylase activity in the wheat cultivars Kennedy and Sunco.

layer was almost uniformly red and devoid of green fluorescent cells. These observations are consistent with previous work reported by Sugimoto *et al.* (1998) and Ritchie *et al.* (1999). During germination, GA is released from the embryo into the starchy endosperm. The aleurone cells nearer the embryo are exposed to GA sooner than those in the distal part of the aleurone. Sugimoto *et al.* (1998) used the accumulation of α -amylase mRNA as a marker for the GA response to demonstrate that there is a wave of activation that travels through the seed following imbibition. Ritchie *et al.* (1999) suggested that the wave of activation is imposed by a combination of the GA concentration and a proximal/distal gradient in aleurone sensitivity to GA.

In aleurone layers from developing and ripe LMA-affected grains, Spica and Seri, and cool temperature-stressed BD159 and Kennedy, small numbers of red fluorescent/dying cells appeared to be scattered randomly throughout the aleurone layer (Fig. 8). Whilst there was no obvious difference between proximal and distal parts of the tissue, these changes were not observed in the aleurone adjacent to the crease region. This is consistent with previous work with LMA-prone germplasm of diverse origin grown in Australia, but inconsistent with observations involving UK-grown wheat. The studies in the UK have consistently shown that the α -amylase activity in PMAA-prone cultivars was concentrated in the crease region (Cornford *et al.*, 1987; Evers *et al.*, 1995; Tjin Wong Joe *et al.*, 2005). The explanation for this difference remains unclear, but possibly relates to the contrasting ripening environments in the two regions. UK cultivars, Huntsman and Rialto, have been tested under Australian growing conditions and the response to a cool temperature shock during the middle stages of grain development appeared very similar to comparable Australian genotypes (data not presented). Aleurone layers from LMA-prone cultivars that required a cool temperature stress, did not show any

evidence of cell death when plants were grown in a warm temperature growth chamber and, in this instance, resembled control non-LMA cultivars such as Sunco and Janz. The initial appearance of cells showing changes associated with cell death coincided with the latter part of the window of sensitivity to cool temperature (25–35 dpa) and with the observed increase in high pI α -amylase activity (Mrva and Mares, 1999, 2001a, b). Mrva and Mares (2001a) used high pI α -amylase-specific ELISA to confirm that the increased activity was due to high pI α -amylase protein. The antibodies did not cross-react with low pI α -amylase protein known to be abundant in early grain development (Mares and Gale, 1990; Mrva and Mares, 1999). Since the ELISA did not detect any high pI α -amylase protein in developing grains (<30 dpa), in developing grains of controls at any stage of development, or in grains of LMA-prone cultivars such as Kennedy that did not receive a cool temperature treatment, the increased α -amylase activity associated with LMA would appear to be the result of new synthesis. New synthesis of high pI α -amylase in aleurone of germinating grains (Cejudo *et al.*, 1995) and isolated aleurone challenged with GA (Gubler *et al.*, 1995) has been convincingly established using gene expression experiments. The similarity of changes observed in aleurone cells in LMA-affected grains with those in germinating grains suggested that, in each case, the underlying mechanism or sequence of events shared many common features. Obvious quantitative differences included the spatial distribution of cell death, the restriction of cell death to individual cells or small groups of cells, and the absence of progressive increases in the number of cells affected with time. The similarity further suggests that GA, variation in sensitivity to GA, or differences in GA/ABA ratios are involved in LMA. A role for GA is supported by previous reports that expression of LMA is reduced in the presence of *Rht* (semi-dwarfing)/*Gai* (GA insensitivity) genes (Mrva and Mares, 1996). Comparison

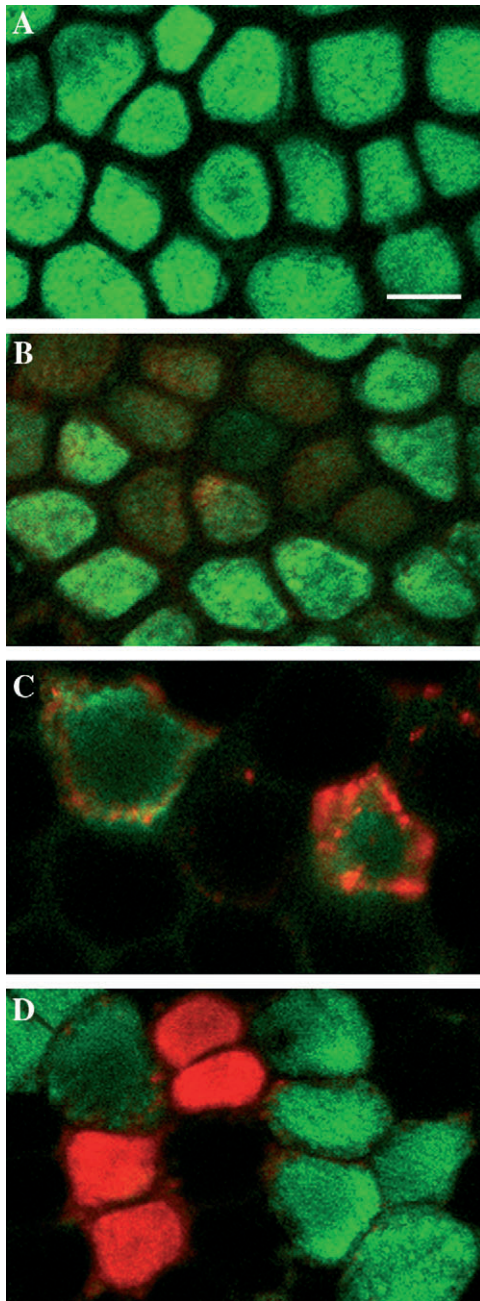


Fig. 4. Merged confocal images from aleurone of developing grains of Kennedy before (A: 22 dpa), at the end of (B: 35 dpa), and following the cool temperature treatment (C: 42 dpa) compared with mature grain (D). Scale bar=100 μ m, shown in (A) and applies to all frames.

of the α -amylase levels between germinated grains and LMA-affected grains are consistent with previous work of Mrva and Mares (1996). The authors indicated that the levels of α -amylase in LMA genotype Spica corresponded approximately to germinated grains at 20 h after imbibition at 20 °C.

These observations raise a number of interesting questions, the first of which relates to whether there is any

evidence for an increase in biologically active GAs, or a change in GA/ABA ratio, in LMA cultivars prior to the observed synthesis of high pI α -amylase. King (1989) reviewed the results from a number of studies of hormones in developing wheat grains and suggested that GA content peaked during the early stages of grain development and declined to low levels by maximum grain dry weight. Conversely, ABA content peaked around the time of maximum dry weight before declining to lower levels by harvest-ripeness. As a consequence, the curves describing the decline in GA and the increase in ABA overlap at about the time grain aleurone becomes sensitive to GA (King and Gale, 1980; Cornford *et al.*, 1987). Gale and Lenton (1987) were unable to demonstrate any peak of new GA synthesis preceding α -amylase synthesis in an LMA-prone UK wheat cultivar, whilst recent efforts by the authors (K Mrva, DJ Mares, P Chandler, unpublished data) have been similarly unsuccessful. Chandler (personal communication) has speculated that LMA might be an indication of perturbation in the normal patterns of GA and ABA synthesis and degradation such that aleurone, having acquired sensitivity to GA, is exposed to a transient period where GA content exceeds that of ABA. The brief phase of response to cool temperature in most LMA-prone genotypes and the apparent absence of increases in cell death with time might be consistent with this proposal. Whilst this conjecture is highly speculative, the technology required to trace the changes in ABA and GA accurately are now available and may enable this question to be resolved in the not too distant future.

A second question relates to the restriction of cell death in LMA genotypes to single cells or small groups of cells. Both Schuurink *et al.* (1997) and Ritchie *et al.* (1999) have reported evidence of heterogeneity in barley aleurone with individual cells varying in sensitivity to GA. Similar heterogeneity in wheat aleurone would be consistent with the observed pattern of cell death, with only cells at the most sensitive end of the spectrum being stimulated to synthesize α -amylase.

The final question relates to the increased expression of LMA in response to cool temperature stress. Bewley and Black (1982) reviewed the literature on the effect of chilling on hormone activity and cited a number of examples in which cool temperature leads to an increase in GA activity in mature seeds. More specifically, several studies have demonstrated that cool temperature can reverse the GA insensitivity associated with the *Rht/Gai* genes in wheat grains (Singh and Paleg, 1984a, b) and wheat coleoptiles (Pinthus and Abraham, 1996; Pereira *et al.*, 2002). In this context, the majority of wheat genotypes that require cool temperature stress for expression of LMA are either *Rht1* or *Rht2* semi-dwarfs. When grain of these LMA-prone cultivars ripens under warm conditions, the presence of the *Rht/Gai* genes may push even the most sensitive cells below the response threshold.

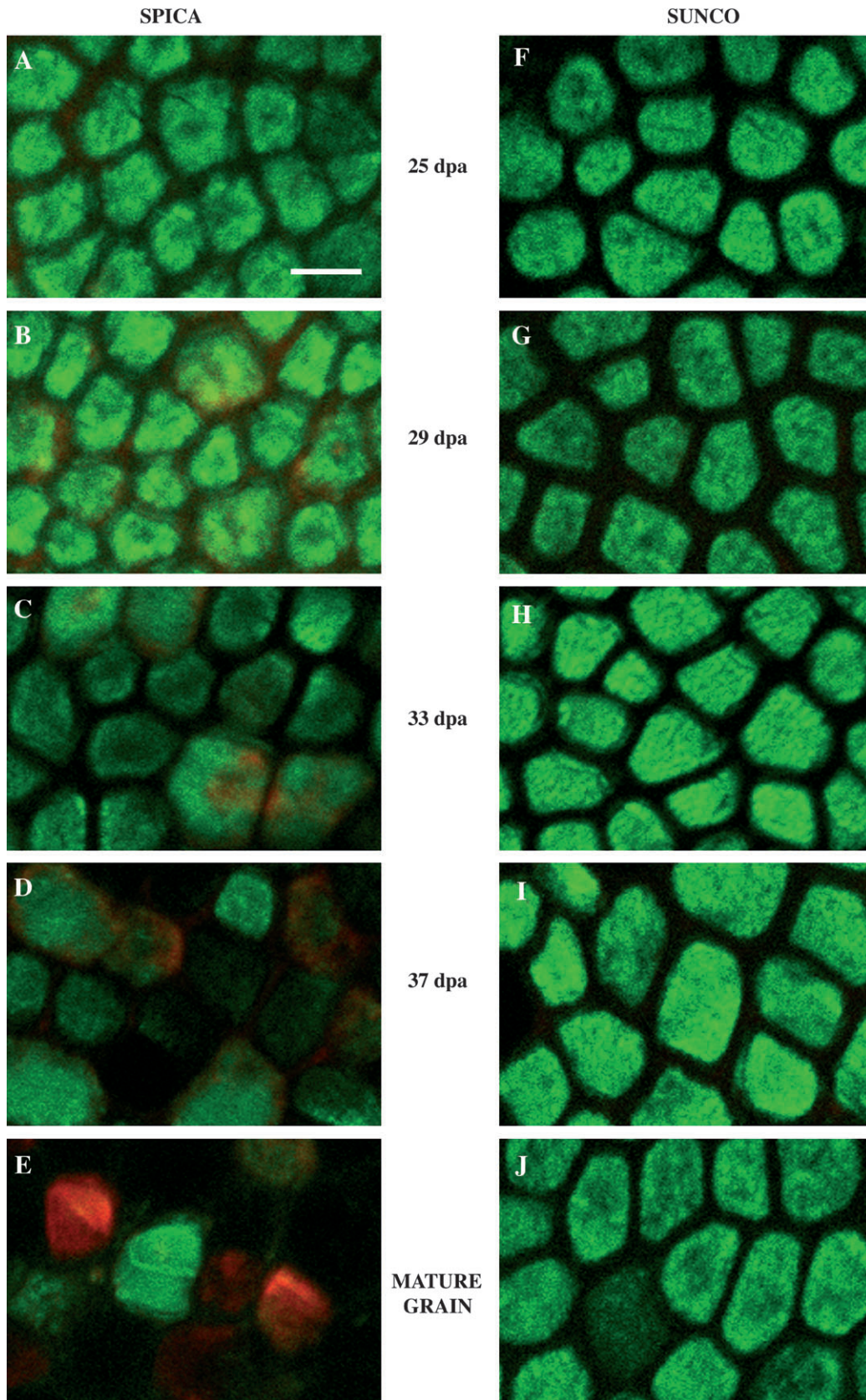


Fig. 5. Merged confocal images of aleurone from developing grains of Spica, LMA (A–E), and Sunco, non-LMA control (F–J), at 25, 29, 33, and 37 dpa compared with mature grain. Scale bar=100 μ m, shown in (A) and applies to all frames.

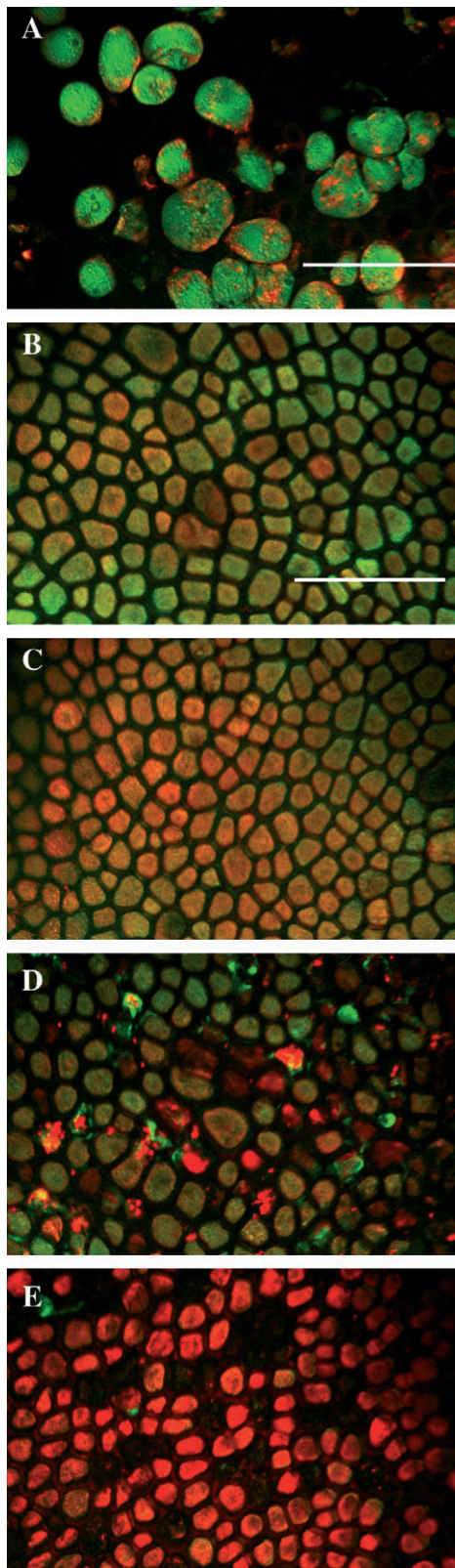


Fig. 6. Merged confocal images from aleurone isolated from germinating grains of wheat cultivar, Sunco, at 24 (A), 48 (B), 72 (C), 96 (D), and 120 (E) h after imbibition. All images were taken from the region of aleurone adjacent to the embryo/scutellum. Scale bar=100 μ m (A), 200 μ m (B, C, D, E).

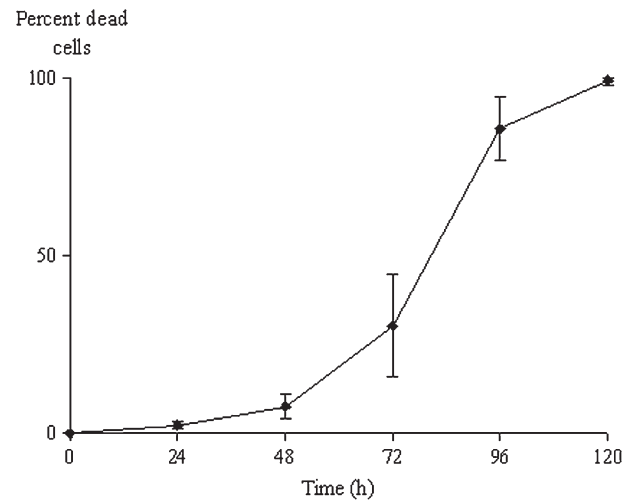


Fig. 7. Time-course of programmed cell death in aleurone layers from germinated grains of wheat cultivar Sunco. Each point represents a mean for five aleurone layers, with three observations per layer \pm SE.

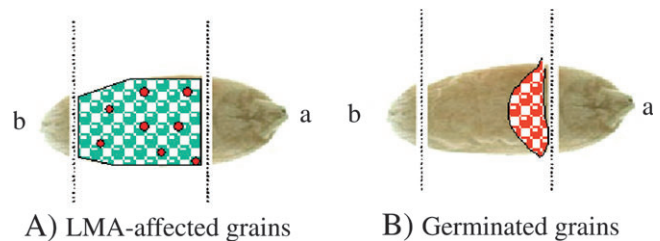


Fig. 8. A model showing the distribution of dead cells in aleurone layers of LMA-affected (A) and germinated grains (B). Note: dotted lines indicate boundaries of aleurone tissue sampled.

Acknowledgements

This work was supported by a research grant from the Grains Research and Development Corporation of Australia. The authors would like to thank Professor Russell L Jones (University of California, Berkeley, California, USA) and Dr Peter Chandler (CSIRO, Plant Industry, Canberra, Australia) for their critical comments during the preparation of this manuscript.

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