The second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA

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Abstract

The role of abscisic acid (ABA) in the weakening of the endosperm cap prior to radicle protrusion in tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) seeds was studied. The endosperm cap weakened substantially in both water and ABA during the first 38 h of imbibition. After 38 h the force required for endosperm cap puncturing was arrested at 0.35 N in ABA, whereas in water a further decrease occurred until the radicle protruded. During the first 2 d of imbibition endo-β-mannanase activity was correlated with the decrease in required puncture force and with the appearance of ice-crystal-induced porosity in the cell walls as observed by scanning electron microscopy. Prolonged incubation in ABA resulted in the loss of endo-β-mannanase activity and the loss of ice-crystal-induced porosity, but not in a reversion of the required puncture force. ABA also had a distinct but minor effect on the growth potential of the embryo. However, endosperm cap resistance played the limiting role in the completion of germination. It was concluded that (a) endosperm cap weakening is a biphasic process and (b) inhibition of germination by ABA is through the second step in the endosperm cap weakening process.

Key words: Abscisic acid, cryo-scanning electron microscopy, endosperm weakening, endo-β-mannanase, tomato seed germination.

Introduction

Endosperm weakening is a prerequisite for the completion of seed germination in a number of species. Several studies have been undertaken to investigate the role of cell wall enzymes in endosperm weakening. In a few species enzyme activity has been identified and associated with cell wall hydrolysis and completion of germination (Bewley, 1997). A class I β-1,3-glucanase was found in the micropylar endosperm of *Nicotiana tabacum* L. seeds and was shown to correlate with endosperm rupture. The enzyme was induced by GA₄ in photodormant seeds and inhibited by ABA (Leubner-Metzger et al., 1996). In *Datura ferox* L. phytochrome-induced β-mannosidase and endo-β-mannanase activities were found to be associated with the weakening of the micropylar endosperm, although activity of these enzymes was not restricted to the micropylar part of the endosperm (Sanchez and de Miguel, 1997). Also phytochrome-induced cellulase activity, mainly located in the radicle half of the endosperm, correlated with endosperm weakening and germination (Sanchez et al., 1986). In *Lactuca sativa* L. seeds cell wall-bound endo-β-mannanase activity was found to correlate with germination (Dutta et al., 1997), although this mannanase was not capable of hydrolysing native lettuce endosperm cell walls. In *Capsicum annuum* L. seeds endosperm weakening was found to play a role in germination (Watkins and Cantliffe, 1983). Germination related galactomannan degrading activity was observed, although this was probably a post-germination event (Watkins et al., 1985).
Endo-β-mannanase activity also has been studied extensively for its role in the germination of tomato (Lycopersicon esculentum Mill. cv. Moneymaker) seeds. Nonogaki et al. have shown that mannanase activity occurred in the endosperm tip opposite the radicle before radicle protrusion (Nonogaki et al., 1992). This mannanase activity was enhanced by red light and fully inhibited by far red light, which correlated with final germination (Nomaguchi et al., 1995). Gibberellin-induced weakening of the endosperm cap leading to the completion of germination (Groot and Karssen, 1987) has been suggested to be mediated by mannanase activity (Groot et al., 1988). ABA clearly inhibits germination of tomato seeds, although there is some controversy as to its site of action. An inhibitory effect of ABA on endosperm cap weakening (Groot and Karssen, 1992) and mannanase activity (Nomaguchi et al., 1995), has been reported. However, others found that ABA was not capable of inhibiting mannanase activity in the endosperm cap, while germination was inhibited considerably (Toorop et al., 1996; Still and Bradford, 1997). Apart from an effect on the endosperm, ABA also influences expansion of the embryo. ABA acts on the water potential of the embryo (Schopfer and Plachy, 1984; Nomaguchi et al., 1995; Ni and Bradford, 1993) by inhibiting the uptake of water by the radicle. Since germination is the final result of two counteracting forces, embryo ‘growth potential’ and endosperm restraint, both should be taken into account when studying tomato seed germination.

A number of studies have focused on the structure of the cell walls of the endosperm cap during germination. Using scanning electron microscopy, the inner surface of the micropylar endosperm of D. ferox seeds was studied (Sanchez et al., 1990) and apparent erosion was found upon red light irradiation which was claimed to be caused by degradation of a mannan-type polysaccharide. Micropylar endosperms of primed tomato seeds appeared to contain eroded surfaces as well (Nonogaki et al., 1992). Lettuce endosperm showed degradation at 12 h imbibition correlating with germination (Pavlista and Valdovinos, 1978). However, these studies focused on the surface of the endosperm; no details of the cell walls throughout the endosperm cap were studied. Jacobsen et al. observed changes in cell walls prior to germination in Apium graveolens L. using conventional light microscopy (Jacobsen et al., 1976). Watkins et al., also using light microscopy, found that the cells in the endosperm opposite the radicle appeared compressed during imbibition (Watkins et al., 1985). Nonogaki et al. have shown cell wall degradation in the endosperm cap of tomato seeds prior to radicle protrusion, but the effect of ABA was not studied (Nonogaki et al., 1998).

In this study there has been an attempt to identify one of the processes in the ABA-inhibition of germination that forms the restricting step for radicle protrusion. The role of ABA in the weakening of the endosperm cap with the use of puncture force measurements and cryo-scanning electron microscopy, and the role of endo-β-mannanase in this process was determined. The influence of ABA on the growth potential of the embryo was also investigated. The role of the endosperm cap weakening in the completion of germination is discussed.

Materials and methods

Plant materials

Seed material was generated as described previously (Toorop et al., 1996). Briefly, tomato plants were grown in a greenhouse in 1992 for seed production of the wild type (Lycopersicon esculentum Mill. cv. Moneymaker) and the GA-deficient genotype gibl. Seeds were stirred in 1% (v/v) HCl for 2 h to remove the locular tissue, rinsed, dried at ambient conditions and stored at 5 °C. Seeds were surface-sterilized in 1% sodium hypochlorite, rinsed in demineralized water and imbibed in demineralized water. ABA solution (racemic mixture; Sigma, St Louis, Mo., USA) or in PEG solution. For some experiments seeds were transferred from water to ABA solution and vacuum infiltrated for 1 min. After vacuum infiltration no visible germination was observed. Vacuum infiltration in water allowed normal germination. Volumes used for imbibition were 2 ml in 50 mm diameter Petri dishes or 6 ml in 100 mm diameter Petri dishes. During imbibition seeds were kept in the dark at 25±1 °C. The denotations lateral endosperm and endosperm cap were used as described previously (Toorop et al., 1996).

Diffusion assay

A gel diffusion assay was used for determining endo-β-mannanase activity (Toorop et al., 1998). Calculation of enzyme activity in the samples was according to Downie et al. (Downie et al., 1994). Single-seed analysis was used to assay two parameters per seed: endo-β-mannanase activity as well as required puncture force.

Required puncture force

Generally, the required puncture force of individual endosperm caps was measured as described previously (Groot and Karssen, 1987). An S100 material tester (Overload Dynamics Inc., Schiedam, The Netherlands) was used with a JP10 load cell (Data Instruments Inc., Lexington, MA, USA) and a range of up to 10 lb. A needle with a hemispherical tip and a diameter of 0.38 mm was placed on the load cell. Upon imbibition endosperm caps were cut from the seeds and the radicle tips removed. The endosperm cap (the testa included) was placed on the needle and was pierced by moving the needle down into a polyvinyl chloride block with a conic hole with a minimum diameter of 0.7 mm. The force required to puncture the endosperm cap was used as a parameter for the mechanical restraint of this tissue. All data points are averages of 24 measurements.

Growth potential

To determine the growth potential of the embryo, 15 seeds were detipped prior to imbibition and placed in different concentrations of PEG, according to Michel and Kaufmann (Michel and Kaufmann, 1973), for 24 h. Seeds were photographed with a digital camera and radicle length was measured. The difference in growth potential between ABA-treated and control seeds
was expressed in Newtons to compare the growth potential of the embryos with values obtained by required puncture force measurements of the endosperm cap. The difference in growth potential was calculated by multiplying the observed difference (expressed in MPa) with the half surface of a sphere (expressed in m²) using the size of the mechanical probe (diameter 0.38 mm), to approach the situation of the radicle tip in the intact seed prior to completion of germination.

Cryo-scanning electron microscopy
Cryo-scanning electron microscopy was performed as described previously (Toorop et al., 1998). At least five seeds were observed per treatment. The occurrence of apparent porosity in the cell walls was caused by ice crystals that were formed during cryo-fixation due to the presence of free water. Freeze-drying after cryo-fixation results in the disappearance of ice crystals, resulting in the apparent porosity (Jeffree and Reid, 1991). This apparent porosity is referred to as ice-crystal-induced porosity.

Statistical analysis
ANOVA and linear regression (SPSS 7.5.2, 1997) were used to analyse data on growth potential and required puncture force. Values were considered significant at probability values lower than 0.05 ($P<0.05$).

Results

Endosperm weakening
The force required to puncture the endosperm was measured of endosperm caps of gibberellin-deficient gib1 seeds imbibed in water and wild-type seeds imbibed in water or 10 μM ABA that had not completed germination (Fig. 1). Seeds of the gib1 mutant lack endo-β-mannanase activity (Groot et al., 1988) and did not show a decrease in the required puncture force. In wild-type seeds there was a decrease from 0.6 N to 0.35 N during the first 38 h of imbibition. No differences were observed between seeds imbibed in water or ABA until this point. After 38 h the required puncture force of ABA-imbibed seeds remained at approximately 0.35 N ($P=0.818$), whereas in water-imbibed seeds there was a significant further decrease down to 0.28 N at 67 h ($P=2.7 \times 10^{-5}$). Prolonged incubation in 10 μM ABA up to 11 d showed the same value of 0.35 N (Fig. 1); from 11 d until 51 d only small variations in the required puncture force were observed (data not shown).

Endo-β-mannanase activity
Upon required puncture force measurements the endosperm caps were incubated in McIlvaine buffer (pH 5.0) for 2 h and the endo-β-mannanase activity in the leachate was assayed. The endo-β-mannanase activity increased for both water and ABA imbibed seeds in a similar way (Fig. 2A). The increase was transient for ABA-imbibed seeds, which is comparable to what was found by other authors (Still and Bradford, 1997; Dahal et al., 1997).

![Fig. 1. The required puncture force of wild-type seeds in water (●) or in 10 μM ABA (○), and of gib1 seeds in water (■). No measured seed had completed germination. Data points are averages of at least 23 measurements of independent batches; error bars indicate standard error of means. The arrow indicates the start of germination of wild-type seeds in water.](image)

![Fig. 2. (A) The endo-β-mannanase activity versus the incubation time. Data points are averages of at least 23 measurements; error bars indicate standard error of mean. (B) The required puncture force of ungerminated wild-type seeds incubated in water (●) or 10 μM ABA (○) for up to 147 h versus the corresponding endo-β-mannanase activity leached from the endosperm caps. The line drawings represent the exponential curve fits ($r^2=0.970$ and 0.945, respectively).](image)
The required puncture forces of the endosperm caps were plotted against the corresponding endo-β-mannanase activities (Fig. 2B). The initial decrease in required puncture force correlated with an initial increase in mannanase activity. In water an exponential relationship was found, which differed from ABA in the region with a low required puncture force, due to a significant difference in required puncture force just prior to radicle protrusion.

Cryo-SEM

Cryo-SEM was performed with the wild-type and gib1 seeds. In the wild-type seeds the lateral endosperm contained four layers of cells at the periphery, of which the outermost cell wall bordering the testa was almost as thick as the remainder of the cells (Fig. 3A). The cells in the lateral endosperm always contained intact cell walls prior to radicle protrusion (Fig. 3B). In the endosperm cap the outermost cell wall was also thicker than the other cell walls, but only marginally compared to the lateral endosperm (Fig. 3C). The cell walls of the endosperm cap appeared intact at 17 h of imbibition (Fig. 3D). In the endosperm required puncture force of the endosperm cap for seeds incubated with or without ABA (Figs 1, 2B).

Discussion

Weakening of the endosperm cap in tomato has been described for wild-type seeds imbibed in water and gib1 seeds imbibed in GA$_4$+$7$ (Groot and Karssen, 1987). It was found that endosperm caps of wild-type seeds that were isolated and subsequently incubated did not weaken in ABA (Groot and Karssen, 1992). However, Fig. 1 shows that during incubation for up to 44 h in ABA, the endosperm cap of wild-type seeds weakened in a similar way as in water. This discrepancy may be explained by the use of isolated endosperm caps by Groot and Karssen (1992), which may have disabled the diffusion of GAs from the embryo to the endosperm cap required for weakening as hypothesized by Groot and Karssen (1987). In this paper intact seeds were incubated, and endosperm caps were isolated only shortly before measuring the required puncture force. After a drop to 0.35 N the required puncture force levelled off in ABA-imbibed seeds, whereas in water-imbibed seeds the required puncture force decreased below this value, eventually resulting in radicle emergence when the germination threshold was crossed. This second step in the weakening process in water was marked by a slower decrease in the required puncture force compared to the first step. A relationship was found, both for water- and ABA-imbibed seeds, between mannanase activity and required puncture force, as was described for wild-type seeds during priming in PEG (Toorop et al., 1998). An exponential relationship was found, with mannanase activity still increasing when required puncture force levelled off. This implies that an initial required puncture force decrease is associated with an increase in endo-β-mannanase activity. Further increase of mannanase activity did not result in a change of required puncture force, and seemed excessive. This

Embryo growth potential

To compare the effect of ABA on the embryo growth potential with the effect of ABA on the endosperm cap weakening, wild-type seeds were dipipped and placed in decreasing concentrations of PEG with or without 10 μM ABA. There was a significant effect of ABA ($P = 1.2 \times 10^{-5}$) and PEG ($P = 9.9 \times 10^{-20}$) on the radicle length. The radicle length measured after 24 h of incubation showed a shift of 0.068 MPa to higher osmotic potentials in the presence of ABA, as calculated with the linear regression equations (Fig. 5). The osmolality of the ABA solution was $10^{-4}$ Osmol, which was insufficient to cause this difference (data not shown). The difference in osmotic potential between the two treatments was converted into Newtons yielding 0.016 N. This value was four times lower than the difference of 0.07 N in the required puncture force of the endosperm cap for seeds incubated with or without ABA (Figs 1, 2B).
Fig. 3. Scanning electron micrographs showing the lateral endosperm (le) of a wild-type seed that was imbibed in water for 2 d (A), of a detail of the cell wall (CW) of the lateral endosperm as in the top left panel (B), the endosperm cap (ec) of a wild-type seed that was imbibed in water for 2 d (C), the cell wall (CW) of the endosperm cap of a wild-type seed that was imbibed in water for 17 h (D). Testa (t) and embryo (em) are indicated. Bar = 10 μm, 1 μm, 10 μm, and 1 μm, respectively. The seeds had not completed germination. Note the intact appearance of the cell wall.

excess could be a mechanism of the seed to make sure that endo-β-mannanase activity can never be limiting for radicle protrusion, which would result in radicle protrusion to fail.

A difference in required puncture force between water and ABA treatment did not become clear until after 44 h of incubation. In ABA the required puncture force stabilized around 0.35 N whereas in water it dropped significantly to 0.28 N at 67 h after which radicle protrusion made measurements impossible. Since endo-β-mannanase activity is not ABA-inhibited this suggests that additional enzymes that are ABA-regulated are involved in the weakening of the endosperm cap (as hypothesized by Toorop et al., 1996), or ABA-regulated modulators of those enzymes. Karssen et al. also hypothesized the existence of a second step in the weakening of the
endosperm cap that precedes radicle emergence, resembling a cell separation process (Karssen et al., 1989). Seeds transferred from ABA to water did not show an increase in mannanase activity, and no further mannanase activity was necessary for radicle protrusion (Dahal et al., 1997). Therefore it was concluded that mannanase action was only required for an initial period during germination, and other factors then became limiting for the completion of germination. These results support this hypothesis.

Using cryo-SEM with germinating lettuce seeds, Nijsse et al. found no changes in the appearance of the endosperm cell walls prior to radicle protrusion (Nijsse et al., 1998). Using the same technique at higher magnifications, it has been shown here that the endosperm cap cell walls

Fig. 4. Scanning electron micrographs showing the cell wall (CW) of the endosperm cap of a *gib1* seed that was imbibed in 10 μM GA₄₊₇ for 24 h (A), the cell wall (CW) of the endosperm cap of a wild-type seed that was imbibed in 10 μM ABA for 2 d (B) or for 7 d (C), and the border between endosperm cap and lateral endosperm of a seed that just completed radicle protrusion (D). Endosperm cap (ec), lateral endosperm (le), embryo (em), and testa (t) are indicated. Bar = 1 μm, 1 μm, 1 μm, and 10 μm, respectively. The seeds in A–C had not completed germination. Note the porosity along the plasma membrane in (A, arrow), of the entire cell wall in (B), and of the patches with both a porous (arrow) and intact (arrowhead) appearance in C.
in tomato seeds do change. Scanning electron micrographs displayed an increase in porosity in the cell walls in the endosperm cap in both water-and ABA-imbibed seeds prior to radicle protrusion. This porosity is caused by ice crystal formation during cryo-fixation and subsequent evaporation of water during the freeze-drying process (Jeffree and Reid, 1991). The ice-crystal-induced porosity corresponded with both a decrease in required puncture force and an increase in leachable endo-β-mannanase activity. Mannose is the major component of tomato seed cell walls (Groot et al., 1988; Dahal et al., 1997) and galacto(gluco)mannans are likely to be present. It is plausible that the hydrolysis products of mannanase activity, possibly in collaboration with other enzymes, are either transported out of the cell wall or rearranged in such a way that pockets of space are created and filled with water. Ice-crystal-induced porosity was visible along the plasma membrane after 1 d of incubation, at which point mannanase activity was low. After 2 d the porosity was found throughout the endosperm cap cell walls and mannanase activity was high, while the seeds had not yet completed germination. Upon radicle protrusion ice-crystal-induced porosity was observed in the lateral endosperm, concurring with endo-β-mannanase activity (Nonogaki et al., 1995). Seeds of the gib1 mutant imbibed in water consistently showed no porous cell walls as well as no decrease of required puncture force, and mannanase activity was absent. Seeds of the gib1 mutant imbibed in GA4+7 displayed ice-crystal-induced porosity, a decrease in required puncture force (Groot and Karssen, 1987), and mannanase activity (Groot et al., 1988). The correlation between an increase in porosity and the increase in mannanase activity and the decrease in required puncture force was strongly supported by observations with wild-type seeds in PEG, which led to the conclusions that ice-crystal-induced porosity was caused by endo-β-mannanase activity (Toorop et al., 1998). A porous appearance was also reported by Williamson and Duncan, who found swollen, eroded and porous cell walls in raspberry fruit cells upon infection by Botrytis cinerea, which could be related to enzymatic activity (Williamson and Duncan, 1989). A change in the appearance of the cell walls in the endosperm cap using TEM has also been shown (Nonogaki et al., 1998), but the effect of ABA was not studied. Several publications reported the degradation of tissues opposing the radicle tip (Leviatov et al., 1995; Welbaum et al., 1995; Watkins et al., 1985). Intact cells only were found in the endosperm cap in this work. Some cells in the inner layer did appear to be compressed (Fig. 3C), but this morphology was also found in shortly imbibed seeds in which the embryo was not fully turgid and in which no endo-β-mannanase activity could be detected.

During prolonged incubation in ABA endo-β-mannanase activity declined (Fig. 2A; Dahal et al., 1997; Still and Bradford, 1997), which corresponded with the lesser degree of ice-crystal-induced porosity in the scanning micrographs. After 6 weeks cell walls appeared intact except for the innermost cell walls of the endosperm cap bordering the embryo. However, this apparent restoration of cell walls was not paralleled by an increase in required puncture force, which remained at 0.35 N. A possible explanation for this discrepancy could be the accumulation in the cell walls of low molecular weight molecules like sugars, which would prevent the formation of water crystals during low temperature fixation of the samples. This in its turn would result in the absence of apparent porosity.

The sharp border between the endosperm cap and the lateral endosperm in germinated seeds (Fig. 4D) suggested that weakening and cell wall hydrolysis prior to radicle protrusion is restricted to the endosperm cap only. It also implies that within the endosperm a spatial regulation is required to create this functional and morphological difference, as the endo-β-mannanase in the endosperm cap obviously did not affect the cell walls in the bordering lateral endosperm cells. Nonogaki and Morohashi found that there is a slight difference in the action pattern of the enzymes from the two parts of the endosperm (Nonogaki and Morohashi, 1996). This difference may be reflected in the different endo-β-mannanase isoforms found on IEF-gels (Toorop et al., 1996) and Western-bolts (Nonogaki and Morohashi, 1996) for the endosperm cap and lateral endosperm. However, a differential pattern of action per se does not explain the inability of the isoforms from the cap region to be active in the lateral endosperm. A higher mannose/galactose ratio was found in endosperm caps than in lateral endosperms, and suggests a difference in the galactomannan composition (Toorop, 1998), indicating an isoform-specific substrate specificity or better accessibility of the enzyme at higher concentrations.

**Fig. 5.** Radicle length of detipped wild-type seeds that were incubated for 24 h in different concentrations of PEG with (○, $r^2=0.971$) or without (*) $10 \mu M$ ABA. The line drawings represent the linear regression curves. Error bars indicate standard error of mean.
mannose/galactose ratios (McCleary and Matheson, 1983). The germination of detipped wild-type tomato seeds has been studied by scoring an arbitrary germination percentage (Liptay and Schopfer, 1983; Nomaguchi et al., 1995). In an attempt to quantify the growth potential in relation to the germination event, the radicle length of detipped seeds was measured in the presence of PEG with or without ABA. After conversion, the effect of ABA on the growth potential of the embryo (0.016 N) is four times lower than the effect of ABA on the required puncture force of the endosperm cap (0.07 N). This indicates that the effect of ABA on the embryo probably plays a minor role in terms of mechanical force while the endosperm cap plus testa is limiting for radicle protrusion. An osmotic effect on the water uptake by the embryo has been shown for Brassica napus (L.) (Schopfer and Plachy, 1984, 1985). However, a difference was found in embryo osmotic potential between water-imbibed and ABA-imbibed wild-type and ABA-deficient tomato seeds which could not explain the observed differences in germination (Ni and Bradford, 1993). These authors concluded that the endosperm cap played a decisive role. The growth potential of the embryo is constant between 12 h and 72 h of imbibition in water until radicle protrusion (Liu, 1996), whereas the endosperm cap restraint decreases strongly (Fig. 1). Altogether, these data suggest that in the germination event endosperm cap weakening is limiting rather than the embryo growth potential.

In summary, these results indicate that endosperm cap weakening in tomato is a biphasic process. The first step in the endosperm cap weakening, which is not inhibited by ABA, is correlated with endo-β-mannanase activity and with the porous appearance of the cell walls in the endosperm cap. The second step in the biphasic endosperm cap weakening is critical since it leads to completion of germination. It is controlled by ABA, which may regulate enzymes associated with the further weakening of the endosperm cap.

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