# The role of endo-ß-mannanase activity in tomato seed germination

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# The role of endo-ß-mannanase activity in tomato seed germination

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## Stellingen

1. Endo-ß-mannanase is verantwoordelijk voor de eerste stap in de verweking van de 'endosperm cap' maar niet voor de tweede stap die door ABA gereguleerd wordt, en is daarom niet betrokken bij het remmend effect van ABA op kieming.
   Dit proefschrift.

2. De invloed van kiemrust op de kwaliteit van commerciële partijen zaaizaad wordt onderschat.

3. De kracht van een 'single-seed' assay ligt in het vermogen om binnen een populatie onderscheid te maken tussen individuen aan de hand van een parameter, die anders zou worden uitgemiddeld in een 'bulk' assay.
   Dit proefschrift.
   Bradford KJ (1996) Population-based models describing seed dormancy behaviour: implications for experimental design and interpretation. *In* GA Lang, ed., Plant dormancy - physiology, biochemistry and molecular biology. CAB International, Wallingford, UK, 313-339.

4. Bij de beschrijving van de relatie tussen metabolische processen en kieming is een accurate omschrijving van de kiemingstoestand van zaden vereist om tot goede conclusies te komen.
   Leviatov S et al. (1995) Involvement of endomannanase in the control of tomato seed germination under low temperature conditions. Annals of Botany 76, 1-6.
   Watkins JT et al. (1985) Gibberellic acid stimulated degradation of endosperm in pepper. Journal of the American Society for Horticultural Science 110, 61-65.

5. Een onjuiste proefopzet bij het zoeken naar processen die verantwoordelijk zijn voor kieming kan leiden tot het onterecht interpreteren van processen die betrokken zijn bij onderhoudsmetabolisme als zodanig.

6. Bij de beoordeling van de smaak van etenswaren vormt een smaakpanel een onmisbaar instrument dat niet vervangen kan worden door andersoortige hulpmiddelen.

7. In hun streven om gelukkig te zijn maken veel mensen van hun zoektocht een doel.

8. Het is mogelijk om met een trage computer een proefschrift snel te schrijven.

9. Het positieve effect van knoflook consumptie op de menselijke gezondheid vindt ondermeer zijn oorzaak in de grotere fysieke afstand tussen consumenten en niet-consumenten van knoflook.

10. Om te voorkomen dat naamsverandering bij getrouwde vrouwen de 'follow-up' van een eerdere wetenschappelijke publicatie onmogelijk maakt, kan het gebruik van een artiestennaam uitkomst brengen.
    stelling 11 uit het proefschrift van M.J.M. Hagendoorn, Vrije Universiteit, Amsterdam.

11. Het is verbazingwekkend hoe men steriel kan werken in een vies laboratorium.

12. Het is legitiem om humor als middel te gebruiken om een stelling van meer overtuigingskracht te voorzien; men dient er echter voor te waken dat de inhoud van de stelling niet ondergeschikt is aan de presentatievorm.
    Plasterk R, De geest van Piet Bambergen. Intermediair, 4 dec 1997.

13. De frequentie van proefschriften die vergezeld gaan van 13 stellingen volgt de normaal verdeling voor de frequentie van proefschriften met n stellingen. Hieruit mag men concluderen dat promovendi in Nederland niet bijgelovig zijn.

Stellingen behorende bij het proefschrift "The role of endo-ß-mannanase activity in tomato seed germination".

Wageningen, 20 mei 1998. Peter Toorop

## Abstract

The role of endo-ß-mannanase activity in tomato seed germination was studied using the osmotic agent PEG 6000 and the plant hormone abscisic acid (ABA). Endo-ß-mannanase is known to degrade galactomannans in cell walls, and its activity was found in the lateral endosperm upon radicle protrusion and in the endosperm cap before radicle protrusion. The former activity appeared to be inhibited by ABA, whereas no regulation by this hormone could be detected for the latter activity. Completion of germination was strongly inhibited by ABA. The isozymes that were found in the endosperm cap before radicle protrusion were different from the ones in the lateral endosperm after radicle protrusion. It was concluded that endo-ß-mannanase in the endosperm cap played no role in the ABA regulated inhibition of germination. Studies with ABA analogs confirmed the observations. Puncture force measurements with endosperm caps revealed that during germination two steps can be distinguished in the endosperm cap weakening, required for radicle protrusion. The first step is not inhibited by ABA and correlates with an increase in endo-ß-mannanase activity. The second step is inhibited by ABA and does not correlate with endo-ß-mannanase activity. It was concluded that endo-ß-mannanase activity mediates the first step of the endosperm weakening, and that a putative cell wall degrading enzyme is involved in the second step. Attempts to identify this enzyme were unsuccessful. Osmotic priming is a treatment of seeds resulting in improved germination. Its beneficial action appeared to correlate partly with endo-ß-mannanase activity and step 1 of the endosperm weakening. Activity of endo-ß-mannanase always correlated with a porous appearance of the cell walls in the endosperm cap, as observed with cryo-scanning electron microscopy. The overall conclusion is that endo-ß-mannanase plays a limited role in the completion of germination and acts through the first step of the endosperm weakening.

# Voorwoord

Eindelijk, het proefschrift is af. En dat nodigt uit om even stil te staan bij de totstandkoming hiervan. Zoals te doen gebruikelijk hebben veel mensen in meerdere of mindere mate hun bijdrage geleverd. Allereerst mijn co-promotor, Henk Hilhorst. Je wist altijd de juiste snaar te raken. In je begeleiding gaf je me veel ruimte, en ook je opportunische manier van denken waardeer ik ten zeerste. Je bent een uitstekende leermeester geweest, en ik hoop de komende jaren nog meer van je te kunnen leren. En dan natuurlijk mijn promotor, Cees Karssen. We hebben elkaar niet vaak gezien, maar die weinige keren dat we om de tafel zaten was je bijzonder stimulerend. Met enige bewondering heb ik gemerkt dat je nog steeds in staat bent om zeer goede kritiek te leveren, ondanks dat je nu jaren een functie buiten het onderzoek bekleedt en welhaast onmogelijk op de hoogte kunt blijven van alle ontwikkelingen op het vakgebied. Zonder dat het gepland was heeft Derek Bewley een grote brok begeleiding voor zijn rekening genomen. Onze samenwerking heb ik ervaren als uitermate inspirerend, en heeft intussen geleid tot drie manuscripten. *Thank you Derek, I learned a lot from you and I am grateful that I had the opportunity to work in your lab.*

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# Contents

Chapter 1

# General introduction

## Tomato seed anatomy and germination

The dicotyledonous tomato (*Lycopersicon esculentum* Mill.) seed consists of an embryo that is embedded in a non-starchy, thick-walled endosperm. The embryo has a spiral form with the cotyledons on the inside and the hypocotyl and radicle curving outwards. The embryo usually lies in a symmetrical plane in the flattened seed (Fig. 1.1). The endosperm consists of thick-walled cells that contain the reserves for the growing embryo upon radicle protrusion. The endosperm cell walls opposite the radicle tip are thinner (Karssen et al., 1989a) and this part of the endosperm is called the micropylar endosperm or endosperm cap. The rest of the endosperm that is characterized by thick-walled cells is called the lateral endosperm. This morphological and structural difference suggests that protrusion through the endosperm cap is predestined.

Germination is defined as the event that 'begins with water uptake by the seed (imbibition) and ends with the start of elongation by the embryonic axis, usually the radicle' (Bewley and Black, 1994). In the case of tomato protrusion of the radicle through the testa is

**Fig. 1.1** Scanning electron micrograph of a fractured germinated tomato seed (bar = 1mm).

considered to be the end of germination, and is referred to as the completion of germination. During seed germination the water uptake is triphasic (Bewley and Black, 1994). Phase I is marked by a fast uptake of water, phase II is marked by a constant water content and ends at radicle protrusion. Water uptake studies with excised embryos indicated that the enclosing tissues prevented the embryo from taking up water, and it was suggested that embryo water content was restricted by the constraint on embryo expansion caused by the enclosing endosperm (Haigh and Barlow, 1987). There was no evidence for the lowering of the embryo water potential or the build up of embryo turgor before radicle emergence. It was concluded that lowering of the mechanical restraint of the endosperm and testa was a prerequisite for the expansion of the embryo, resulting in the completion of germination. In *Cucumis melo* a similar mechanism for radicle expansion was hypothesized (Welbaum and Bradford, 1990). In *Datura ferox* seeds the increase in embryo growth potential was insufficient for completion of seed germination, and endosperm softening was proposed as a requirement (De Miguel and Sanchez, 1992). Therefore, endosperm weakening not only seems to be a prerequisite for radicle protrusion in tomato, but also for radicle protrusion in several other species.

## Composition of the endosperm

Galactomannan is one of the hemicelluloses that is found in the cell walls of many seeds, and occasionally in other cell compartments like the vacuole (Brennan et al., 1996), as a reserve for the growing seedling (Bewley and Black, 1994). The galactomannan structure, its formation during development and its depletion during and after germination, have been studied extensively in the past for physiological reasons. The polysaccharide has also been studied for its various applications. The ancient Egypt used the substance for mummification, more recently it is used in food, pharmaceuticals, cosmetics, paper products, paints and plasters, well-drilling and mining, and explosives and fire-fighting (Dey, 1978). In the light of this economical importance it is not surprising that the structure of the polymer is well-known. The polymer consists of a 1→4, ß-linked D-mannan backbone and is substituted to a higher or lesser degree by single-unit α-D-galactopyranoside side chains linked 1→6 to mannose (Reid, 1985) (Fig. 1.2). The degree of substitution varies from 20% to nearly 100%, depending on the species that the galactomannan originates from. The higher the substitution degree the more water soluble the polymer becomes. Presumably, the galactosyl side chains prevent the self-association of the main chain to give crystalline aggregates. Substitution of

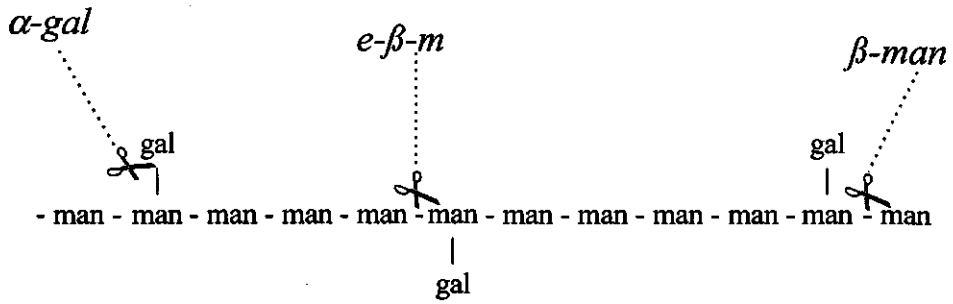


**Fig. 1.2** Schematic structure of galactomannan and the sites of action of endo-ß-mannanase, α-galactosidase, and ß-mannosidase.

the side chains is not necessarily homogeneous throughout the polymer (Courtois and Le Dizet, 1968). The endosperm cell walls of the tomato seed contain large amounts of mannose and lower amounts of glucose and galactose (Groot et al., 1988). It was concluded that the tomato endosperm cell walls contained galactomannans or galactoglucomannans.

Three enzymes are known to be involved in the complete hydrolysis of galactomannans: endo-ß-mannanase (EC 3.2.1.78), α-galactosidase (EC 3.2.1.22) and ß-mannosidase (EC 3.2.1.25). The latter two are exo-enzymes. α-Galactosidase plays the role of debranching enzyme, hydrolyzing the galactosyl side chains. ß-Mannosidase cleaves mannose residues from the mannan backbone. Endo-ß-mannanase is an endo-enzyme, hydrolysing the mannan backbone in between mannose residues. The enzyme requires a minimum chain length to be active: the required number of mannose residues depends on the origin of the enzyme (Nonogaki and Morohashi, 1996). In galactomannans with a high degree of substitution α-galactosidase activity is required before endo-ß-mannanase can become active, cleaving side chains and enabling the endo-ß-mannanase to hydrolyse the backbone. ß-Mannosidase requires the action of endo-ß-mannanase (McCleary, 1983). A ß-galactosidase appeared to be active on galactomannan from locust bean, which was probably caused by non-specificity of this enzyme (Carey et al., 1995).

## Endo-ß-mannanase in the tomato seed

Endo-ß-mannanase has been studied in several species in relation to germination. A distinction should be made between expression of the enzyme in the layers that oppose the radicle and in the rest of the surrounding endosperm tissue. The latter activity is related to depletion of the reserves after radicle emergence, while the former activity could be associated

in at least a number of species with protrusion of the radicle through the surrounding plant tissue. Growth regulators, such as the plant hormones gibberellic acid (GA) and abscisic acid (ABA), are often used to determine their effect on germination as well as on enzyme activity since a similar effect of growth regulators on both processes forms an indication for a causal relationship.

During the depletion of reserves, which is a post-germination event, the galacto(gluco)mannans in tomato seeds are hydrolysed by endo-ß-mannanase (Nonogaki et al., 1992). The mobilized reserves supply the growing embryo with oligosaccharides and, possibly, monosaccharides. Endo-ß-mannanase was found in the endosperm cap opposite the radicle tip prior to radicle protrusion and this activity was associated with the decrease of the endosperm restraint (Nonogaki et al., 1992). The two enzymes, found before and after radicle protrusion, did not only differ in spatial and temporal expression: the action pattern was also different, as was concluded from the reaction products formed *in vitro* (Nonogaki and Morohashi, 1996). Groot and Karssen (1987) hypothesized that a GA is excreted from the embryo to the endosperm cap where it induces a decrease of the mechanical resistance. This weakening was assumed to be mediated by endo-ß-mannanase activity, which was induced by $GA_{4+7}$ in isolated endosperms (Groot et al., 1988). $GA_{4+7}$ also enhanced ß-mannosidase and α-galactosidase activity. From experiments in which wild-type and gibberellin-deficient *gib1* mutant seeds were detipped and placed on different concentrations of osmoticum it was concluded that GAs stimulated the growth potential of the embryo (Karssen et al., 1989b). Dormant seeds did not display a decrease of the puncture force which may imply that endo-ß-mannanase is not induced in dormant seeds. The *gib1* mutant, having a blocked GA synthesis, also does not germinate under favourable conditions. However, it has to be realized that the absence of GA in the endosperm cap may also inhibit other enzymes than endo-ß-mannanase. Plant hormones are known to have pleiotropic effects, and so many processes may be inhibited by the absence of GAs. Endo-ß-mannanase may simply be one of the many enzymes, and cell wall hydrolysis one of the many biochemical activities, that are induced by GAs.

ABA inhibits germination of many species when applied exogenously. Tomato seed germination is also inhibited by ABA, and this effect was accounted for by inhibition of endo-ß-mannanase in the endosperm cap (Nomaguchi et al., 1995) resulting in an unchanged puncture force (Groot and Karssen, 1992). In a few other species a decrease in the restraint of

the tissues surrounding the radicle tip during germination has been described (De Miguel and Sanchez, 1992; Tao and Khan, 1979; Welbaum et al., 1995), yet none of these reports describe the effect of a germination inhibitor like ABA on this mechanical restraint.

## Endo-ß-mannanase in seeds of other species

Lettuce is one of the most widely studied species for galactomannan degrading enzymes (e.g. Halmer et al., 1975, 1976). It appeared that the phytochrome stimulated enzyme activity was expressed under conditions that correlated with germination, but only after the completion of germination (Dulson et al., 1988). Only recently it was shown that a cell wall bound endo-ß-mannanase, that was GA induced and ABA inhibited, was present in excised thermodormant lettuce endosperms prior to the completion of germination (Dutta et al., 1997). Interestingly, this enzyme is hardly capable of hydrolysing purified lettuce endosperm cell walls *in vitro*.

In *Datura ferox* the induction of endo-ß-mannanase in the endosperm cap opposing the radicle tip is under the control of a phytochrome response by the embryo, which can be circumvented by exogenous $GA_3$ (Sanchez and de Miguel, 1997). Paclobutrazol, an inhibitor of gibberellin synthesis, inhibited endo-ß-mannanase more strongly than it inhibited ß-mannosidase, indicating that the latter activity is enhanced and the former induced by gibberellins, as was found for isolated tomato endosperm (Groot et al., 1988). Germination in paclobutrazol treated *D. ferox* seeds was strongly correlated with endo-ß-mannanase activity. In *Capsicum annuum* gibberellins controlled the speed of germination through the lowering of the mechanical resistance of the endosperm opposite the radicle (Watkins and Cantliffe, 1983). Endo-ß-mannanase was postulated as a requirement for this weakening (Watkins et al., 1985). Nevertheless, from the presented data it appeared that post-germinative enzyme activity was observed and not enzyme activity in the endosperm cap prior to radicle protrusion (Bewley, 1997). Therefore, in *C. annuum* seeds the mechanism behind the completion of germination remains to be elucidated. In *Apium graveolens* seeds endo-ß-mannanase was hypothesized to be involved in the hydrolysis of the endosperm to support embryo growth before radicle emergence (van der Toorn, 1989). All experiments lead to the conclusion that endo-ß-mannanase activity as well as endosperm weakening is induced by gibberellins, resulting in the completion of germination. However, for *D. ferox* and *C. annuum* no experiments were performed using inhibitors of germination like ABA or PEG, to see if these

affect endo-ß-mannanase activity.

## Other enzymes and their role in the control of germination

Enzymes other than endo-ß-mannanase have been investigated for their presumed role in the completion of germination. In the photodormant seeds of *Nicotiana tabacum* $GA_4$ substituted for light as a requirement for germination (Leubner-Metzger et al., 1996). ABA delayed seed coat rupture marginally and endosperm rupture considerably. ß-1,3-Glucanase activity followed seed coat rupture and preceded endosperm rupture, and correlated with the completion of germination. Prior to radicle emergence the enzyme was expressed in the micropylar region of the endosperm only. 0.04 M PEG-6000 delayed the onset of endosperm rupture more strongly than ß-1,3-glucanase activity, and could be antagonized by $GA_4$. Interestingly, the enlargement of the radicle was not inhibited by ABA, indicating that the growth potential of the embryo was not decreased and that the inhibition of germination was through the micropylar endosperm only (Leubner-Metzger et al., 1995). In lettuce, a GA that is produced in the axis upon red light irradiation and transported to the cotyledons, induces α-galactosidase activity in the cotyledons from where it presumably diffuses into the endosperm (Leung and Bewley, 1981). However, germination and enzyme activity are not essentially linked.

## The role of GA sensitivity in germination

Not only GA levels, also GA sensitivity plays a role in the control of germination or dormancy (Hilhorst et al., 1986; Hilhorst and Karssen, 1992). In dormant *Arabidopsis thaliana* seeds increased GA sensitivity is induced by prolonged dry storage or chilling, resulting in the breaking of dormancy (Karssen et al., 1989b). In excised sunflower embryos ABA levels did not correlate with release from dormancy during dry storage. On the other hand dry storage for 3 days drastically increased the germination response to $GA_3$ compared to not drying (Bianco et al., 1994). This indicates that during dry storage the sensitivity to GAs plays a decisive role in the relief of dormancy, as was also concluded previously (reviewed in Hilhorst and Karssen, 1992). A similar change in GA sensitivity is conceivable for tomato seeds, but has never been investigated.

## The role of ABA in germination

ABA plays a role in primary dormancy in tomato, as can be concluded from a high correlation between ABA content of seeds and germinability (Hilhorst, 1995). The ABA deficient *sit*$^{W}$ mutant showed low ABA levels during development in the seeds, whereas the wild-type showed high ABA levels (Groot et al., 1991). The maternal ABA levels in the seed reached a maximum around 30 days after pollination (DAP) while the zygotic ABA levels were lower and reached a maximum around 40 DAP. During maturation ABA is present in endosperm, embryo and testa, and drops to low levels in the mature seed (Hocher et al., 1991). The high ABA levels during development, probably in concert with the subsequent dehydration, control premature germination of the maturing seeds (Finkelstein et al., 1985). Maternal ABA appeared to play a role in the induction of primary dormancy next to zygotic ABA (Groot and Karssen, 1992). Due to the absence of ABA the seeds of the *sit*$^{W}$ mutant showed no dormancy and even vivipary in overripe fruits. A subset of harvested *sit*$^{W}$ seeds contained higher amounts of endo-ß-mannanase in the endosperm cap of which the mechanical restraint was lower (Downie et al., 1997). This subset commenced radicle protrusion almost immediately upon imbibition and the seeds were less sensitive to ABA and far-red light irradiation, probably due to the irreversible onset of radicle protrusion. A difference was found in the testa structure of *sit*$^{W}$ seeds. In dry seeds of the mutant the testa was only 1 cell layer thick, while in the wild-type the testa consisted of 4-5 cell layers. This difference in structure might add to the difference in germinability. Sensitivity to ABA throughout development also plays a role in the germinability of several species, regardless of ABA levels (reviewed in Hilhorst, 1995). Changes in ABA sensitivity were not reported for tomato seeds, but may play a role in the germinability.

## Priming

Osmotic pre-treatment, also called osmotic priming, is a good technique to improve the germination of seeds (Heydecker et al., 1973). A good correlation between endo-ß-mannanase activity of tomato seeds during and the increase of germination rate after priming has been reported (Karssen et al., 1989a). Tomato seeds showed endo-ß-mannanase activity during incubation in PEG, which persisted upon redrying (Nonogaki et al., 1992). The level of enzyme activity immediately increases after transfer of primed seeds to water, whereas unprimed seeds do not show activity until after 1 day. Therefore, faster and more uniform

germination after priming may be caused by metabolic activities during priming, reducing the time required until radicle protrusion. Priming also increases the germination rate of seeds of which the micropylar endosperm and testa have been removed (Dahal and Bradford, 1990). This indicates that priming may also have an effect on the embryo. Finch-Savage and McQuistan (1991) found that priming of seeds in ABA was as effective as priming in an osmoticum. ABA priming also resulted in a faster and more uniform germination and a higher germination percentage after redrying and imbibition in water. Seeds had a very different water status during treatment by the two methods, indicating that it is unnecessary for seeds to encounter an osmotic stress to achieve a priming effect.

## Germination models

Ni and Bradford (1992) have proposed a model that provides quantitative values for the germination of a population. Predictions can be made based on the effects of ABA and water potential. Both the interactive and independent action of ABA and water potential, influencing physiological processes required for radicle growth such as the accumulation of osmotic solutes, can be determined. The model also explains the rate and level of germination of the wild-type, the GA-deficient mutant, and the ABA-deficient mutant with or without exogenous hormones (Ni and Bradford, 1993). Hormonal effects on endosperm weakening opposite the radicle tip determine the threshold water potential for germination, which determines the rate and extent of germination. The threshold temperature and water potential for metabolic advancement are considerably lower than the corresponding thresholds for radicle emergence of the same seed lot. This allows the accumulation of hydrothermal priming time, whereas radicle protrusion is blocked. This is expressed as more rapid germination with increasing temperature or water potential (Bradford and Haigh, 1994). Several aspects of this germination model, like metabolic advancement and threshold water potential, will be discussed in this thesis.

## Scope of the thesis

Endo-ß-mannanase is widely accepted as a potential key enzyme in the regulation of the completion of germination in a few species. However, for tomato only preliminary attempts have been made to see if this hypothesis is true. Two reports presented evidence that ABA inhibits both the decrease in mechanical restraint (Groot and Karssen, 1992) and endo-ß-

mannanase activity (Nomaguchi et al., 1995) in the endosperm cap of tomato. Yet no elaborate experiments were performed to validate this hypothesis. This thesis is an attempt to describe the role of endo-ß-mannanase in the germination event in relation to the influence of ABA, GA and osmoticum. The hypothesis that endo-ß-mannanase acts as the limiting factor in the completion of germination was tested. $GA_{4+7}$ and ABA were used to study both the endo-ß-mannanase activity in the endosperm cap and the post-germinative enzyme activity, quantitatively and qualitatively (chapter 2). The role of endo-ß-mannanase in the weakening of the endosperm cap and the influence of ABA on this process were investigated (chapter 3). An attempt was made to visualize the weakening of the endosperm cap with a low-temperature scanning electron microscope. ABA analogs were used to see if certain structures of the ABA molecule could influence the level of endo-ß-mannanase activity similarly as germination (chapter 4). Also the influence of ABA analogs on post-germinative endo-ß-mannanase activity was investigated. A correlation of germination with endo-ß-mannanase activity before and after radicle protrusion was presented. Several other hydrolytic enzymes were investigated to see if they played a role in the inhibition of germination by ABA (chapter 5). The role of endo-ß-mannanase in the mechanical weakening of the endosperm cap during osmotic priming was investigated (chapter 6). The general discussion describes a model for the role of endo-ß-mannanase in the completion of germination.

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Chapter 2

# Endo-ß-mannanase isoforms are present in the endosperm cap and embryo of tomato seeds, but are not essentially linked to the completion of germination

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## Abstract

A current hypothesis is that endo-ß-mannanase activity in the endosperm cap of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) seeds is induced by gibberellin (GA) and weakens the endosperm cap thus permitting radicle protrusion. We have tested this hypothesis. Incubation of isolated endosperm parts shows that the expression of endo-ß-mannanase in the endosperm after germination is induced by gibberellins (GAs), but the expression of endo-ß-mannanase in the endosperm cap prior to radicle protrusion is not induced by GAs. Also, abscisic acid (ABA) is incapable of inhibiting endo-ß-mannanase activity in the endosperm cap, even though it strongly inhibits germination. However, ABA does inhibit enzyme activity in the endosperm and embryo after germination. There are several isoforms in the endosperm cap and embryo prior to radicle protrusion that are tissue-specific. Tissue prints showed that enzyme activity in the embryo spreads from the radicle tip to the cotyledons with time after the start of imbibition. The isoform and developmental patterns of enzyme activity on tissue prints are unaffected when seeds are incubated in ABA, even though germination is inhibited. We conclude that the presence of endo-ß-mannanase activity in the endosperm cap is not in itself sufficient to permit tomato seeds to complete germination.

## Introduction

The tomato seed (*Lycopersicon esculentum* Mill. cv. Moneymaker) consists of an embryo which is embedded in a thick-walled endosperm surrounded by a seed coat. The endosperm walls contain relatively large amounts of galactomannans (Groot et al., 1988) which are a source of stored reserve (Reid, 1985). Three enzymes are involved in the hydrolysis of galactomannans; α-galactosidase (EC 3.2.1.22), a mannohydrolase, and endo-ß-mannanase (EC 3.2.1.78). The latter endo-enzyme hydrolyses 1→4-mannan chains with the required minimum degree of polymerisation (McCleary et al., 1976). Endo-ß-mannanase activity has been reported in seeds of several species following germination (McCleary and Matheson, 1975; Halmer et al., 1976; McCleary, 1978; DeMason at al., 1985) and in storage tissues of bulbs (Wozniewski et al., 1992) and is involved in the degradation of gluco- or galactomannans, which are present in the cell walls of numerous higher plants (Bewley and Reid, 1985).

In the tomato seed, endo-ß-mannanase not only plays a role in reserve food mobilization but has also been suggested to play an important role in germination. The thin-walled endosperm cap opposite the radicle tip exhibits endo-ß-mannanase activity prior to completion of germination (Nonogaki et al., 1992) and has been associated with endosperm weakening, thus allowing radicle protrusion (Groot and Karssen, 1987). Only after radicle protrusion can enzyme activity be detected in the rest of the endosperm.

GAs stimulate and ABA inhibits germination of tomato seeds. Also GAs induce and ABA inhibits endosperm cap weakening (Groot and Karssen, 1992). GAs induce endo-ß-mannanase activity in the GA-deficient *gib1* mutant (Groot et al., 1988). Hence expression of endo-ß-mannanase activity is expected to be controlled by ABA, as predicted by Ni and Bradford (1993) in their model for GA-induced cell wall hydrolases.

Studies of endo-ß-mannanase have been facilitated by the recent development of a gel diffusion assay by which it is possible to quantify endo-ß-mannanase activity; this assay is based on the staining of galactomannans by the Congo Red dye (Downie et al., 1994). The same staining procedure has been used to detect endo-ß-mannanase isoforms in an activity overlay from isoelectric focusing (IEF) gels (Dirk et al., 1995). Tissue printing is an easy and effective tool in locating enzyme activity *in situ* (Varner, 1992), but so far no suitable technique has been available to detect endo-ß-mannanase. In this paper, we have used the diffusion assay, activity overlays from IEF gels, and a tissue printing technique to further characterize the occurrence of endo-ß-mannanase activity in the different seed tissues during and after germination. These

techniques have allowed us to question the hypothesis that endo-ß-mannanase activity is an integral part of tomato seed germination.

## Materials and methods

*Seed material.* Tomato plants were grown in a greenhouse in 1993 or 1994 for seed production of wild-type (*Lycopersicon esculentum* Mill. cv. Moneymaker) and the GA-deficient genotype *gib1*. Seeds were stirred in 1% (v/v) HCl for 2 h to remove the locular tissue, rinsed, dried 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 $GA_{4+7}$ solution (Sigma). Volumes used for imbibition were 2mL in 50mm diameter Petri dishes or 6ml in 100 mm diameter Petri dishes. During imbibition seeds were kept at 25 (± 1) °C in the dark. Seeds were dissected laterally in halves with a razor blade, and embryo halves were separated from the endosperm halves. Endosperm caps adjacent to the radicle tip were cut from the remaining lateral endosperm (Fig. 2.1). These seed parts still contained part of the testa. After a short rinse in demineralized water, leachates were collected by incubating isolated seed parts from 10 seeds in 250 µL Hepes (Sigma) buffer (100 mM pH 8.0) for up to 24 h at 4 °C to minimize interfering activity by infection. Extracts were made by grinding parts from 10 fresh seeds in a mortar in 250 µL Hepes buffer (100 mM pH 8.0). The samples were centrifuged in a microfuge for 10 min at 14000 rpm. The supernatant was used for protein and enzyme assays.

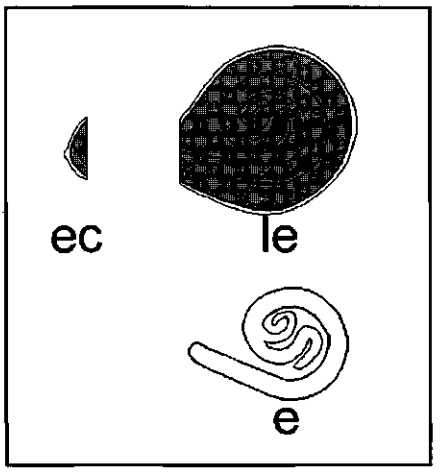


**Fig. 2.1** Schematic representation of tissue parts of a tomato seed; endosperm cap (ec), lateral endosperm (le), embryo (e). The orientation corresponds with the tissue prints in Fig. 2.4.

*Diffusion assay and protein assay.* A modified diffusion assay (Downie et al., 1994) was used for determining endo-ß-mannanase activity. Gels (0.5 mm thick) were used containing 7.5% (w/v) polyacrylamide and 0.07% (w/v) locust bean gum (Sigma) in 0.1 M citric acid - 0.2 M disodium phosphate buffer (pH 5.0). Holes were punched in the gel with a 2 mm paper punch, and samples of 2 µL were applied. Gels were incubated for 16 to 18 h at 25°C, and then

washed with deionized water for 20 min, stained with 0.4% (w/v) Congo Red (Sigma) for 30 min, washed with 96% (w/v) ethanol for 10 min, and destained in 1 M NaCl. Commercial endo-ß-mannanase from *Aspergillus niger* (Megazyme; North Rocks, Sydney, Australia) in various concentrations from 0.03 to 14.00 pkat was used to create a standard curve. Calculation of activity in extracts was according to Downie et al. (1994). α-Galactosidase was purchased from Megazyme.

*Isoelectric focusing and overlays.* Isoelectric focusing gels were cast between 125 x 260 mm glass plates with a 0.5 mm spacer. Ampholytes with a pH range of 5 to 7 or a combination of pH range 5 to 7 and 6 to 8 were used (Bio-Rad). Gels were run for 2 h at 2000V while cooled at 5 °C. After focusing, the IEF gel was laid on top of an activity gel, containing 7.5% (w/v) polyacrylamide and 0.07% (w/v) locust bean gum (Sigma) in 0.1 M citric acid - 0.2 M disodium phosphate buffer (pH 5.0) (Torrie et al., 1990; Dirk et al., 1995). The gel-sandwich was incubated at 25 °C for 16 to 18 h in a closed dish lined with moist paper towels to prevent drying out. Activity gels were stained with Congo Red as described above. Isoforms were visible as clear bands on a red background. A pH gradient was measured by cutting 1-cm strips from each IEF gel before incubation with the overlay gel, and incubating the strips overnight in 2 mL deionized water at 25 °C. Isoelectric points were determined by measuring the distance from each isoform to the cathode, using the pH gradient as a standard.

*Tissue printing.* Imbibed seeds were cut in half with a razor blade and the embryo was separated from the endosperm; sometimes the endosperm was divided into endosperm cap and lateral endosperm. At least 10 seed parts per sample were washed briefly with deionized water, blotted dry on a filter paper, and laid on top of an activity gel, cut side down. Activity gels were incubated for 2 h at room temperature. The seed parts were then washed from the gel with deionized water. After incubation the gel was stained with Congo Red as described above.

## Results

*Activity pattern.* In the lateral endosperm no endo-ß-mannanase activity was detected in the diffusion assay using extracts from seeds sampled prior to completion of germination, while in the endosperm cap there was an increasing amount of activity prior to radicle protrusion (Fig. 2.2a). After radicle protrusion, activity increased sharply in both the lateral endosperm and endosperm cap (not shown). In the axis there was an increase in activity starting after 16 h which reached a constant level after about 30 h from the start of imbibition. The cotyledons showed an

increase in activity only after 24 h from the start of imbibition, followed by a slower increase after 40 h. Germination was not completed until 96 h by seeds incubated in 1 µM ABA (not shown). Until 72 h the same pattern of endo-ß-mannanase activity occurred in the lateral endosperm (no activity), endosperm cap, axis and cotyledons (increasing activity) as in seeds imbibed in water (Fig. 2.2b). The activity of endo-ß-mannanase in the lateral endosperm, endosperm cap, axis, or cotyledons of ungerminated seeds was the same at all concentrations of ABA (0.1 µM, 1 µM, and 10 µM) used, regardless of germination capacity (data not shown).

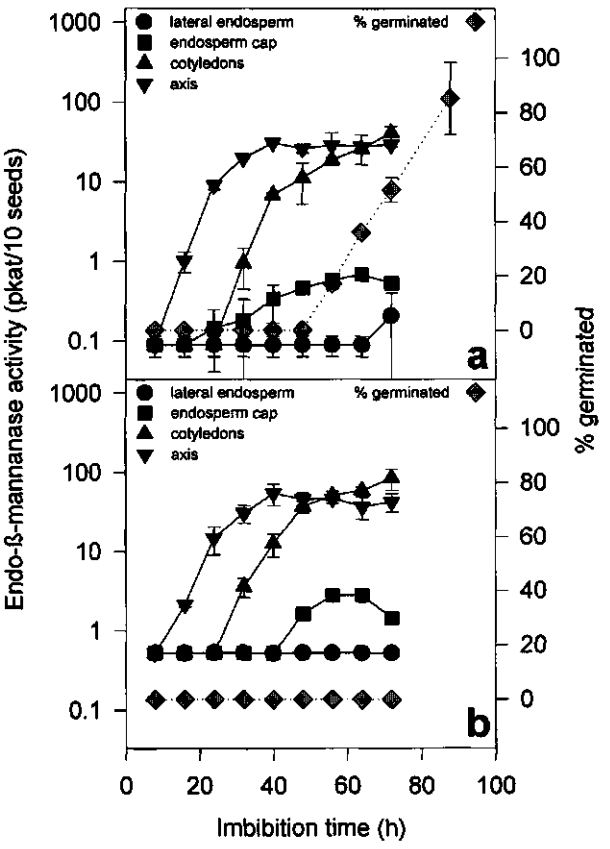


**Fig. 2.2** Endo-ß-mannanase activity of ungerminated seeds imbibed in water (a) or in 1 µM ABA (b).

*Enzyme activity in isolated parts of the seed.* Wild-type seeds were dissected into endosperm halves and whole embryos, and incubated separately in water under sterile conditions. No enzyme activity could be detected in the endosperm when incubated in water (Fig. 2.3a). Activity was induced when endosperm halves were incubated in 10 µM $GA_{4+7}$. Further dissection into lateral endosperm and endosperm cap, and incubation separately in 10 µM $GA_{4+7}$, yielded no activity in the endosperm cap, but increased activity in the lateral

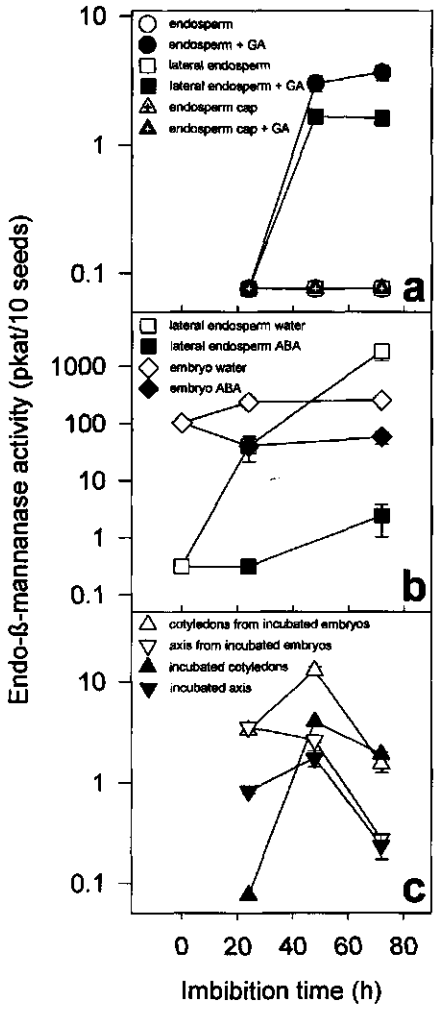


**Fig. 2.3** Endo-ß-mannanase activity after incubation of isolated parts of the wild-type tomato seed. Activity in endosperm halves, lateral endosperm, and endosperm cap, with or without 10 µM $GA_{4+7}$ (a); activity in the lateral endosperm and embryo after detipping and subsequent incubation in water or 10 µM ABA (b); activity in axis and cotyledons after isolation and subsequent incubation of the intact embryo or incubation of the separated embryo parts (c).

endosperm after 48 h. Incubation in water in the presence of isolated embryos did not induce detectable activity in either the lateral endosperm or endosperm cap (not shown). When 2-day-imbibed ungerminated seeds were detipped and incubated in water or in 10 µM ABA, endo-ß-mannanase activity in the lateral endosperm was strongly inhibited by ABA, while activity in the embryo was slightly inhibited (Fig. 2.3b). Incubation of isolated embryos in water resulted in activity within 24 h. Incubation of cotyledons and axis separately resulted in activity in both parts of the embryo, although the cotyledons did not show activity until 48 h (Fig. 2.3c).

*Tissue printing.* Tissue printing was used to locate endo-ß-mannanase activity in the several tissues of a single seed (Fig. 2.4a). The lateral endosperm caused no clearing on the tissue prints before completion of germination, but after germination the clearing became detectable. Activity in the

endosperm cap was initiated at 12 h of imbibition. This activity increased and reached a maximum at 48 h prior to radicle protrusion. Enzyme activity in embryos was also initiated at 20 h from the start of imbibition. The radicle tip was the first site within the embryo to display enzyme activity, and activity then spread throughout the embryo with time, the cotyledons being the last parts to show enzyme activity at 40 to 48 h after the start of imbibition. When the outside of the embryo was used for tissue printing by putting the embryo on the activity gel cut side up, a weaker clearing was found, and no leakage from the cotyledons was observed (Fig. 2.4b).

Endosperm caps and embryos from seeds imbibed in 1 µM ABA, 10 µM ABA, or 100 µM ABA showed the same pattern of enzyme activity on tissue prints at 24 h, 48 h, and 72 h of imbibition when compared to seeds imbibed in water (not shown). Again this was regardless of germination capacity due to the differences in ABA concentration. Lateral endosperms showed activity only after seeds had completed germination.

In *gib1* seeds, deficient for gibberellins, enzyme activity was not present and the seeds did not germinate. When the seeds were imbibed in 10 µM $GA_{4+7}$ both enzyme activity in the seeds and germination capacity were restored (not shown). Imbibition in 10 µM $GA_{4+7}$ plus 10 µM ABA restored enzyme activity in the endosperm cap and embryo in tissue prints, even though germination was inhibited completely (Fig. 2.4c).

*IEF.* Activity overlays of IEF gels run with leachates from embryos and endosperms showed differences in their endo-ß-mannanase isoforms. The embryo contained several isoforms before the completion of germination, of which the ones with pI 4.3 and 4.1 were more abundant than the one with pI 4.7 (Fig. 2.5b). The axis showed more activity at an earlier stage of germination than cotyledons (Fig. 2.5c), supporting the tissue print data showing that the initial activity in the radicle tip spread throughout the embryo with time. The pattern of isoforms did not change until after germination, when additional bands became visible. The endosperm cap showed the presence of several isoforms, of which three were more apparent (pI 5.2, 5.5, and 5.8; Fig. 2.5a). Activity started to become detectable at 40 h after the start of imbibition. After radicle emergence more isoforms were visible on the gels. The lateral endosperm as well as the endosperm cap, showed traces of isoforms which correspond well with the two abundant isoforms in the embryo (pI 4.3 and 4.1). This activity was not detectable in the diffusion assay. Only after germination were high amounts of endo-ß-mannanase isoforms detected in the lateral endosperm, and the isoform pattern was identical to that in the endosperm cap after germination

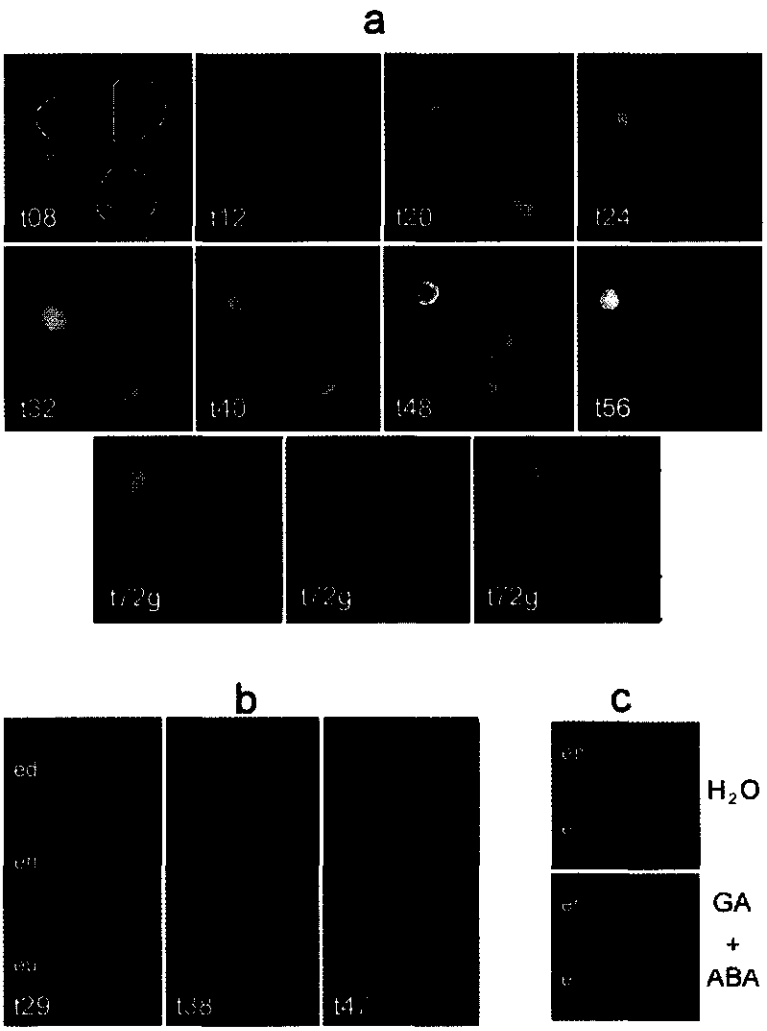


**Fig. 2.4** **a**: Tissue prints using parts of ungerminated (t8-t64) or germinated (t72 G) seeds after imbibition in water for various times. Endosperm cap (ec), lateral endosperm (le) and embryo (e) were separated before printing. The line drawing in the first panel illustrates the position of the tissues. **b**: Tissue prints of ungerminated seeds showing embryo cut side down (ed), endosperm cut side down (en), and embryo cut side up (eu), after 29, 38, and 47 h imbibition in water. **c**: Tissue prints of ungerminated *gib1* seeds imbibed for 42 h in water, or 10 µM $GA_{4+7}$ and 10 µM ABA; endosperm (en) and embryo (e). Tissue prints shown are representative examples of 10 seed parts.

(not shown).

In all tissues a dark red band with an pI of about 4.1 was found. This was presumed to be α-galactosidase. Commercially prepared α-galactosidase showed isoforms with the same red colour, but never a clearing zone of the type caused by endo-ß-mannanase. Galactose side chains cleaved from the galactomannan leaves a mannan backbone that probably binds more strongly to Congo Red, causing the dark red coloration in a red background.

## Discussion

Endosperm caps weaken prior to radicle protrusion (Groot and Karssen, 1987). Endosperm cells opposite the radicle tip appear eroded after prolonged incubation in -0.8 MPa PEG, which is correlated with increased activity of endo-ß-mannanase (Nonogaki et al., 1992). From these observations it was concluded that endo-ß-mannanase facilitates radicle protrusion through the surrounding endosperm by weakening it in the area close to the radicle tip. Tissue printing not only confirmed the presence of endo-ß-mannanase in this area, but also allowed the detection of low amounts of activity in the endosperm cap and embryo of early imbibed seeds. The increase in activity in the endosperm cap coincided with the decrease in endosperm cap restraint (Groot and Karssen, 1987). Leachates used for IEF overlays, presumably containing proteins present in the apoplast (including mature endo-ß-mannanase enzyme), showed only after 40 h that the activity in the endosperm cap was due to isoforms that were specific for the endosperm cap. Both the diffusion assay and tissue prints showed very low, if any, endo-ß-mannanase activity in the lateral endosperm prior to germination. Enzyme activity in the lateral endosperm probably did not contribute to radicle protrusion but more likely was involved in cell wall reserve mobilisation after germination.

In the diffusion assay, the embryo showed a typical pattern of endo-ß-mannanase activity, increasing first in the axis and, with a delay of approximately 16 h, in the cotyledons. Tissue prints consistently showed the same pattern of endo-ß-mannanase activity in the embryo before radicle protrusion. Activity started simultaneously in the radicle tip and the endosperm cap, and then spread throughout the embryo with time. Detipping did not influence this pattern, and neither did cutting the endosperm at the opposite end from the endosperm cap (not shown). Thus a diffusion gradient within the embryo during imbibition as a result of facilitated water uptake through the endosperm cap in the intact seed was not responsible for this specific pattern. Isolation of cotyledons and axis and separate incubation resulted in activity in both parts of the

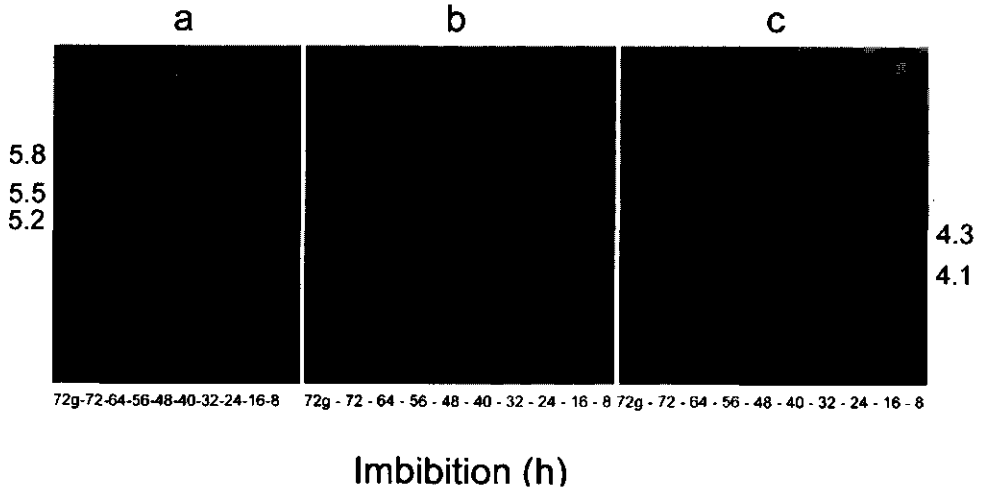


**Fig. 2.5** Endo-ß-mannanase activity overlays from IEF gels with leachates from endosperm caps at a pH range 5-7/6-8, measured pH gradient 4.1-7.0 (a), axes at a pH range 5-7, measured pH gradient 3.5-5.6 (b), cotyledons at a pH range 5-7, measured pH gradient 3.5-5.1 (c). Activity spots in the top of each panel are the sites of the application paper containing the sample and the electrode wick.

embryo. Development of endo-ß-mannanase activity in cotyledons, therefore, was independent of the axis and vice versa. Tissue prints of the outside of embryos showed slight activity compared to the inside. The reason for the developmental pattern of endo-ß-mannanase activity in the embryo remains unknown.

Detipping of seeds also showed that endo-ß-mannanase activity in the embryo developed independently of the endosperm cap. On the other hand the decrease in puncture force of the endosperm cap was dependent on a diffusable factor from the embryo (Groot and Karssen, 1987) which is probably a GA. Decrease in puncture force was ascribed to endo-ß-mannanase activity in the isolated endosperms (Groot et al., 1988). However, separation of isolated endosperms into endosperm caps and lateral endosperms yielded a different result. Incubation of isolated endosperm caps with 10 μM $GA_{4+7}$ did not result in endo-ß-mannanase activity within the endosperm caps, while incubation of isolated lateral endosperms with 10 μM $GA_{4+7}$ did. This result suggests that the expression of endo-ß-mannanase involved in the hydrolysis of galactomannan after germination is under the direct control of a GA, while the expression of the enzyme prior to radicle protrusion is not. Therefore the postulation that endosperm cap

weakening is mediated by an endo-ß-mannanase that is induced by a GA cannot be supported by our data (Groot et al., 1988). When seeds were detipped before germination and transferred to 10 µM ABA, endo-ß-mannanase activity in the embryo was only slightly decreased while activity in the lateral endosperm was strongly inhibited. Expression of endo-ß-mannanase in the lateral endosperm, involved in the hydrolysis of the endosperm after completion of germination, therefore seems to be regulated by ABA as well as GA.

The several isoforms present within germinating and germinated tomato seeds might be correlated with site-specific functions of the enzyme. The presence of endo-ß-mannanase in the endosperm cap can be correlated with a physiological function, namely weakening the endosperm cap, thus facilitating radicle protrusion. In the lateral endosperm the enzyme plays a role in the catabolic processes, allowing the embryo to resorb all galactomannan resources from the endosperm. The reason for there being endo-ß-mannanase in the embryo, however, is not so clear. The enzyme may be involved in cell wall extensibility, like other cell wall proteins (Schopfer and Plachy, 1985; Karssen et al., 1989; Fry, 1993; Hilhorst, 1995; McQueen-Mason and Cosgrove, 1995), thus promoting cell growth.

Groot and Karssen (1992) showed that the puncture force of the endosperm cap does not decrease in the presence of ABA. Nomaguchi et al. (1995) found that both endo-ß-mannanase activity in endosperm caps and germination were completely inhibited by ABA, and $GA_3$ could restore both. We have found that ABA hardly influences endo-ß-mannanase activity in the embryo or endosperm cap from wild-type seeds before radicle protrusion, although germination is strongly inhibited. Also, in the *gib1* mutant, ABA could prevent germination but not enzyme activity, both induced by $GA_{4+7}$. Apparently, ABA can antagonize the action of GA in the germination process, but ABA cannot inhibit the expression of endo-ß-mannanase prior to radicle emergence. Removing the endosperm cap of imbibed seeds always results in growth of the embryo of at least 1 mm when incubated in ABA (data not presented). This indicates that a lack of germination cannot be due to the inability of the radicle to develop enough thrust to extend. Consequently the mechanical restraint of the endosperm cap needs to be the limiting factor in radicle protrusion. A possible explanation for the inability to positively correlate inhibition by ABA of germination and of endo-ß-mannanase activity is that this enzyme is not (exclusively) responsible for the weakening of the endosperm cap. ABA might inhibit the activity of other (GA-inducible) enzymes that are responsible for breakdown of cell wall components, thus influencing the mechanical restraint of the endosperm cap.

## Acknowledgment

We thank Dr. Bruce Downie for the seemingly endless but inspiring discussions.

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Chapter 3

# ABA controls the second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination

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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 investigated. The endosperm cap weakened substantially in both water and ABA up to 45 h of imbibition. In the second phase of imbibition after 45 h the puncture force levelled off in ABA, whereas in water a further decrease occurred until the radicle protruded. During the first 2 days of imbibition endo-ß-mannanase activity correlated with the decrease in puncture force and with the appearance of porous cell walls as observed by scanning electron microscopy. Prolonged incubation in ABA resulted in the loss of mannanase activity and the disappearance of porosity, but not in a reversion of the puncture force. ABA did not inhibit transcription of the mannanase gene, gibberellins appeared to be necessary for its transcription in the gibberellin deficient *gib1* mutant. 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) inhibition of germination by ABA is through the endosperm cap b) endosperm cap weakening is a biphasic process.

## Introduction

Endosperm weakening is a prerequisite for the completion of germination of tomato (*Lycopersicon esculentum* Mill. cv Moneymaker) seeds. 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, 1998). A class I ß-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 $GA_4$ in photodormant seeds, and inhibited by ABA (Leubner-Metzger et al., 1996). In *Datura ferox* L. phytochrome-induced ß-mannosidase and endo-ß-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-ß-mannanase activity was found to correlate with germination conditions (Dutta et al., 1997), although this mannanase was not capable of hydrolysing native lettuce endosperm cell walls. Weakening of the endosperm was found prior to radicle protrusion, yet no clear correlation with germination conditions was found (Tao and Khan, 1979). 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-ß-mannanase activity also has been investigated extensively for its role in the germination of tomato seeds. Nonogaki et al. (1992) showed that the mannanase activity, found before radicle protrusion, was present in the endosperm tip opposite the radicle. This mannanase activity was enhanced by red light compared to dark treatment, 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 in tomato seeds, although there is some controversy as to its site of action. Groot and Karssen (1992) and Nomaguchi et al. (1995) reported an inhibitory effect of ABA on endosperm cap weakening and mannanase activity, respectively. However, others found that ABA was not capable of inhibiting mannanase activity, 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. It was shown that 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, Sanchez et al. (1990) studied the inner surface of the micropylar endosperm of *D. ferox* seeds and found apparent erosion upon red light irradiation which was claimed to be caused by degradation of a mannan type polysaccharide. Micropylar endosperm 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 conditions (Pavlista and Valdovinos, 1978). However these studies focused on the surface of the endosperm, instead of the cell walls throughout the endosperm cap. Jacobsen et al. (1976) observed changes in cell walls prior to germination in *Apium graveolens* L., using conventional light microscopy. In lettuce achenes it was demonstrated that the micropylar endosperm cells became highly vacuolated, cytoplasmic reserve materials were mobilized, the cells became swollen whereas the cell walls remained PAS positive, indicating that despite morphological changes the actual mobilisation of the endosperm did not occur prior to tissue rupture (Psaras et al., 1981). Watkins et al. (1985), also using light microscopy, found that the cells in the endosperm opposite the radicle appeared compressed during imbibition. Unfortunately, this technique lacks the resolution that scanning electron microscopy offers.

In the present study we investigated 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-ß-mannanase in this process. Also the influence of ABA on the growth potential of the embryo was investigated. The role of the endosperm weakening and the embryo growth potential in the completion of germination was 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 *gib1*. Seeds were stirred in 1% (v/v) HCl for 2 h to remove the locular tissue, rinsed, dried 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. 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. Mean time until germination (MTG) was used to express the germination rate, and was calculated as the time to 50% final germination of the batch. The denotations lateral endosperm and endosperm cap were used as described previously (Toorop et al., 1996).

*Diffusion assay.* A modified diffusion assay (Downie et al., 1994) was used for determining endo-ß-mannanase activity. Gels (0.5 mm thick) were used containing 0.1 % (w/v) locust bean gum (Sigma) in McIlvaine buffer (pH 5.0) and 0.8% type III-A agarose (Sigma) and made on Gel bond film (Pharmacia). Leachates of endosperm caps were made by incubating single endosperm caps in 20 µL McIlvaine buffer (pH 5.0) for 2 h at room temperature according to Still and Bradford (1997). Samples of 2 µL were applied to holes that were punched in the gel with a 2-mm paper punch. Gels were incubated for 20 h at 25°C, and then washed in McIlvaine buffer (pH 5.0) for 30 min, stained with 0.5% (w/v) Congo Red (Sigma) for 30 min, washed with 96% ethanol for 10 min, and destained in 1 M NaCl for at least 24 h. All staining steps were performed on a rotating platform while rotating gently. Commercial endo-ß-mannanase from *Aspergillus niger* (Megazyme, North Rocks, Sydney, Australia) was used to generate a standard curve. Calculation of enzyme activity in the samples was according to Downie et al. (1994).

*Puncture force.* Generally, the puncture force of individual endosperm caps was measured as described by 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 lbs. A needle with a hemispherical tip and a diameter of 0.38 mm was placed on the load cell. 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. Significance was tested using a two-sample t-test according to Meilgaard et al. (1987).

*Growth potential.* In order to determine the growth potential of the embryo, seeds were detipped and placed in different concentrations of polyethylene glycol 6000, according to Michel and Kaufmann (1973). The difference in growth potential between ABA treated and control seeds was expressed in Newton in order to compare the growth potential of the embryo's with values obtained by puncture force 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 $m^2$) 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.

*RNA isolation.* RNA was isolated as described by Wan and Wilkins (1994). Seeds were dissected into lateral endosperm, endosperm cap and embryo and stored at -80 °C until further use. Each sample containing approximately 0.2 g fresh weight, was prepared by homogenising for 3 min at 1600 rpm in a micro-dismembrator U (B. Braun Biotech International, Melsungen, Germany) using 2 mL Eppendorf tubes. All grinding materials were kept in liquid nitrogen to prevent RNase activity. Per sample 700 µL buffer of 80 °C was added (0.2 M sodium borate decahydrate, 30 mM EGTA, 1% (w/v) SDS, 1% (w/v) sodium deoxycholate pH 9.0) containing 14 mg PVP and 1.078 mg DTT. After adding 0.35 mg proteinase K (Boehringer Mannheim) and vortexing for 1 min, samples were incubated at 42 °C for 1.5 h while shaking. After adding 56 µL 2 M KCl the samples were incubated on ice for 1 h and centrifuged in a microfuge for 20 min. All centrifuging was done at 4 °C. The supernatant was added to 270 µL 8 M LiCl and incubated on ice overnight. After centrifuging for 20 min the supernatant was discarded and the pellet was resuspended and washed three times in 1 mL cold 2 M LiCl. The pellet was resuspended in 400 µL 10 mM Tris (pH 7.5), centrifuged for 10 min and the supernatant was added to 40 µL 2 M potassium acetate (pH 5.5). After 15 min incubation on ice and centrifuging for 10 min the supernatant was added to 1320 µL ethanol and incubated at -20 °C overnight. The RNA was pelletted by centrifuging for 30 min. The pellet was washed with 400 µL 70% ethanol and dried in a speedvac for 5 min. The pellet was

resuspended in 40 µL water and 4 µL 3 M sodium acetate (pH 6.0) and 132 µL cold ethanol was added. After 2 h incubation at -20 °C the RNA was pelletted by centrifuging for 20 min, the RNA was washed with 70% ethanol and dried in a speedvac for 5 min. The RNA was resuspended in 60 µL water and stored at -80 °C until further use. Electrophoresis of formaldehyde gels for separation of RNA and Northern blotting was performed according to Sambrook et al. (1989). For each sample 6 µg of total RNA was loaded onto the formaldehyde gel. The gel was blotted overnight on Genescreen Plus (NEN Life Science Products, Boston, MA), the blot was prehybridized in hybridization mix for 1 h at 55 °C and hybridized overnight at 55 °C. The hybridisation mix contained 7% (w/v) SDS, 1% (w/v) BSA, 1 mM EDTA (pH 8.0) and 0.5 M phosphate buffer (pH 7.2). The blot was washed twice at 55 °C in 2x SSC containing 0.1% (w/v) SDS for 15 min, and exposed to Kodak X-ray film for 7 d at -70 °C with an intensifying screen. The X-ray films were scanned, loaded onto a computer and printed on photographic paper.

*Cryo-scanning electron microscopy.* Imbibed seeds were fixed on a stub with colloidal carbon adhesive (Leit-C, Neubauer, Germany), plunge-frozen in liquid nitrogen and affixed to a stub carrier. This was transferred under vacuum at near liquid nitrogen temperature to the cold stage of a cryo-preparation chamber (CT 1500-HF, Oxford Instruments, UK). The seeds were freeze-fractured with a cold scalpel knife, heated up to -80°C, partially freeze-dried and sputter-coated with 3 nM of Pt. The seeds were placed in the cryostage of the scanning electron microscope equipped with a cold field emission electron gun (JEOL 6300F). Observations were made at -180°C using a 2.5-5 kV accelerating voltage. Digital images were taken, loaded onto a computer and printed on photographic paper.

## Results

The puncture force was measured of endosperm caps of gibberellin deficient *gib1* seeds that were imbibed in water and wild-type seeds that were imbibed in water or 10 µM ABA and that had not completed germination (Fig. 3.1). Seeds of the *gib1* mutant lack endo-ß-mannanase activity (Groot et al., 1988) and did not show a decrease in the puncture force. After a plateau of 0.6 Newton in wild-type seeds the puncture force decreased to 0.35 Newton during the first 40 h of imbibition. No differences were observed between seeds imbibed in water or ABA until this point. After 45 h the puncture force of ABA imbibed seeds remained at approximately 0.35 Newton, while in water imbibed seeds there was a significant further

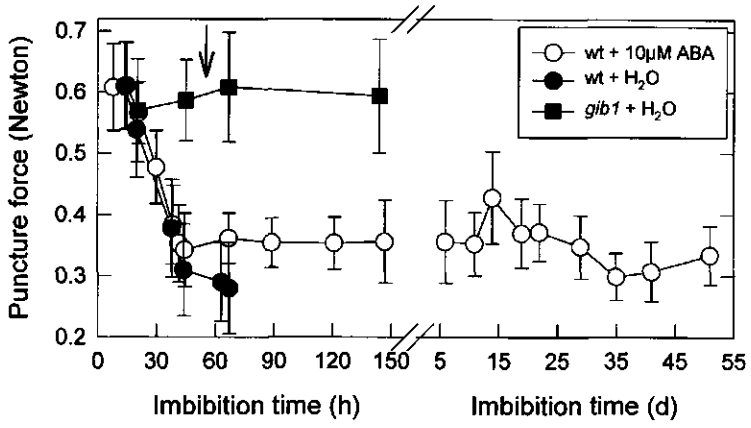



**Fig. 3.1** The puncture force of wild-type seeds in water(●) or in 10 µM ABA (○) and of *gib1* seeds in water (■) during incubation. No measured seed had completed germination. Data points are averages of 24 measurements of independent batches, error bars are standard deviations. The arrow indicates the start of germination in water.

**Table I** *Puncture force (Newton) of seeds at 88 h, 144 h, and 212 h that were transferred to 10 µM ABA after 67 h incubation in water*

Seeds were transferred after 67 h from water to 10 µM ABA under vacuum, and the puncture force was measured after 88 h, 144 h and 212 h of total imbibition time. Puncture force was compared in a two-tailed t-test with the puncture force of seeds imbibed in water for 67 h or in 10 µM ABA for 45 h. Values are average and standard deviation of 24 seeds.

| | | | compared with: | |
|---|---|---|---|---|
| | | | 67 h $H_2O$ | 45 h ABA |
| time (h) | mean | sd | | |
| 88 | 0.306 | 0.054 | NS[a] | *[b] |
| 144 | 0.283 | 0.058 | NS | * |
| 212 | 0.263 | 0.071 | NS | * |

[a] NS, not significant. [b] *, significant at the level of 0.025.

decrease down to 0.28 Newton at 67 h. When ungerminated seeds were transferred after 67 h from water to 10 µM ABA through vacuum infiltration germination was inhibited. The puncture force of those seeds did not alter during further incubation and was significantly lower than the puncture force at 45 h of the seeds that were incubated in 10 µM ABA from the start (Tab. 1). Prolonged incubation in 10 µM ABA up to 51 days showed hardly any change in the puncture force compared to 45 h incubation in the same medium (Fig. 3.1).

After puncture force measurements the endosperm caps were incubated in McIlvaine buffer for 2 h and the endo-ß-mannanase activity in the leachate was assayed. The endo-ß-mannanase activity increased for both water and ABA imbibed seeds and showed a plateau between 67 and 121 h in ABA (Fig. 3.2 inset), which is slightly different from the results of Still and Bradford (1997). The average puncture force of the individual endosperm caps was plotted against the corresponding endo-ß-mannanase activity (Fig. 3.2). The initial decrease in 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 low puncture force, due to a significant difference in puncture force just prior to radicle protrusion.

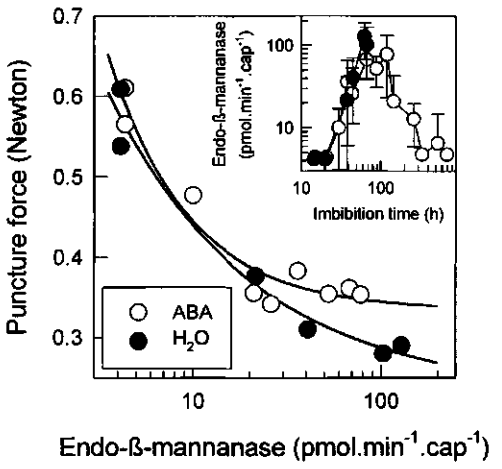



**Fig. 3.2** The puncture force of ungerminated wild-type seeds incubated in water (●) or 10 µM ABA (○) up to 147 h versus the corresponding endo-ß-mannanase activity leached from the corresponding endosperm caps. The line drawings represent the exponential curve fits ($r^2$ = 0.970 and 0.945 respectively). Inset: the endo-ß-mannanase activity versus the incubation time. Data points are averages of at least 24 measurements, error bars are standard deviations.

Total RNA was isolated from parts of ungerminated wild-type seeds incubated in water or ABA, and *gib1* seeds incubated in water (Fig. 3.3). The lateral endosperm and the endosperm cap showed constant levels of RNA during imbibition

of all treatments and genotypes. The embryo on the other hand showed a peak in the RNA content after 24 h in the water imbibed wild-type seeds. The RNA content in the embryo of ABA imbibed wild-type seeds was consistent with this. A Northern blot was made using the endo-ß-mannanase clone (Bewley et al., 1997) as a probe (Fig. 3.4). The lateral endosperm consistently lacked expression of endo-ß-mannanase. The endosperm cap showed expression of endo-ß-mannanase in wild-type seeds that were imbibed in water or 10 µM ABA for 24 h or 70 h. In water the signal was stronger after 70 h than after 24 h. After 27 days incubation in 10 µM ABA only faint expression was detectable in the endosperm cap and embryo. No expression was seen in the endosperm cap in 5 h imbibed wild-type seeds or in *gib1* seeds that were imbibed in water for 58 h. Except for 5 h imbibition in water and 24 h imbibition in 10 µM ABA the embryo consistently showed a signal in all samples.

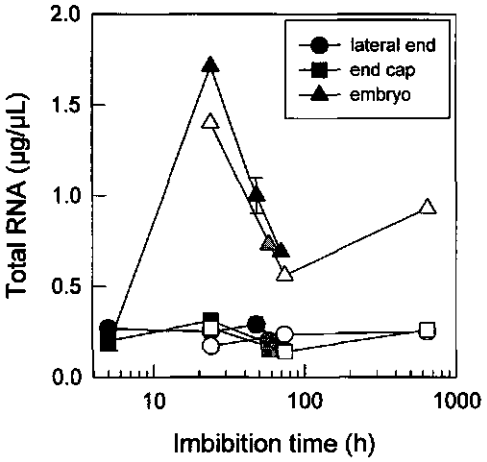



**Fig. 3.3** Total RNA content of lateral endosperm (●), endosperm cap (■) or embryo (▲) of ungerminated wild-type seeds imbibed in water (black symbols), ungerminated wild-type seeds imbibed in 10 µM ABA (open symbols), or ungerminated *gib1* seeds imbibed in water (grey symbols). Standard deviations were only available for 48 h water imbibed seed parts.

Cryo-SEM was performed with the wild-type and *gib1* seeds. In the wild-type seeds the lateral endosperm contained 4 layers of cells at the periphery, of which the outer most cell wall bordering the testa was almost as thick as the cells that they belonged to (Fig. 3.5). The cells in the lateral endosperm always contained intact cell walls in the ungerminated state (Fig. 3.6). In the endosperm cap the outer most cell wall was also thicker than the other cell walls, but only marginally compared to the lateral endosperm (Fig. 3.7). The cell walls of the endosperm cap appeared fully intact at 17 h of imbibition (Fig. 3.8), at which point the seeds

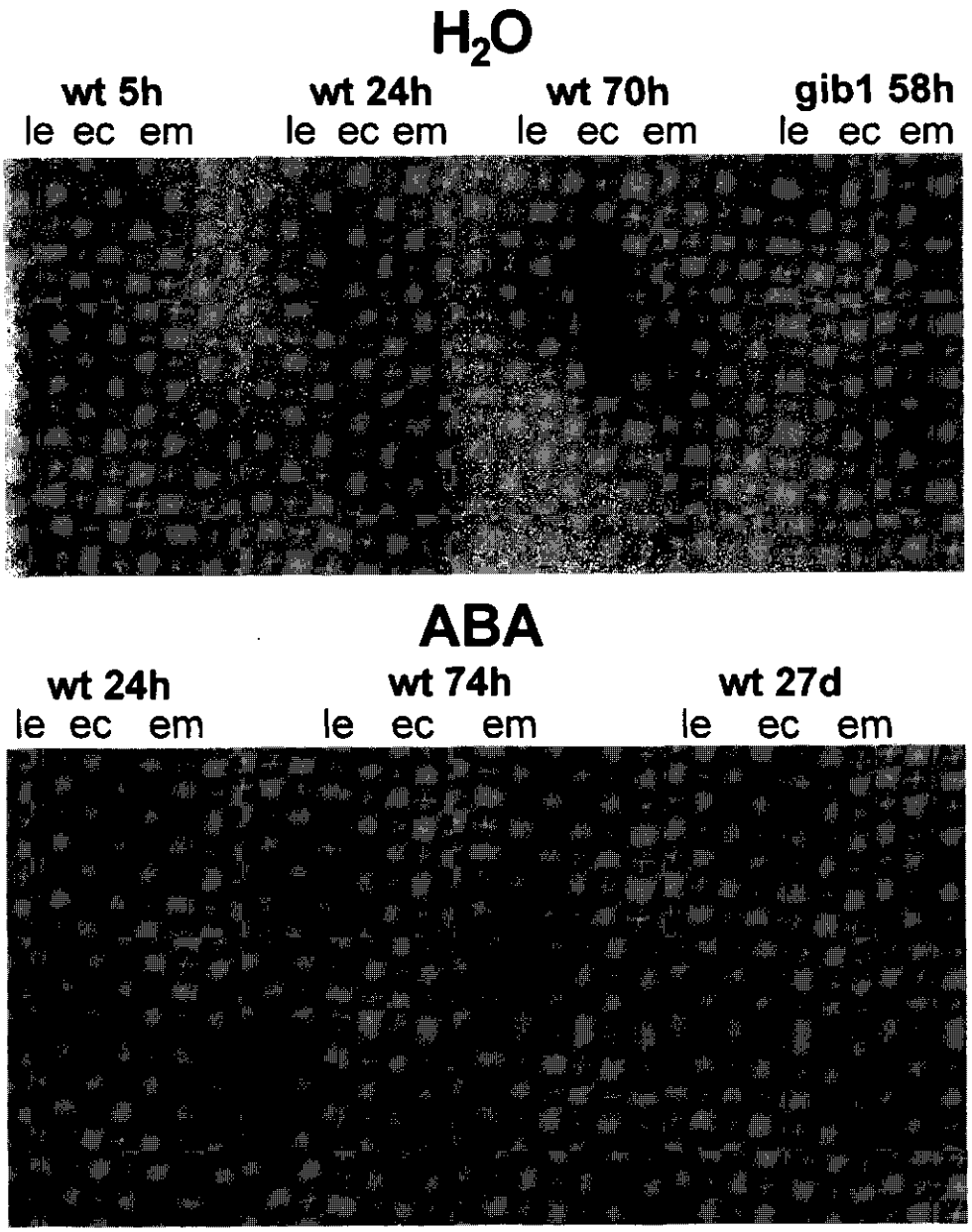
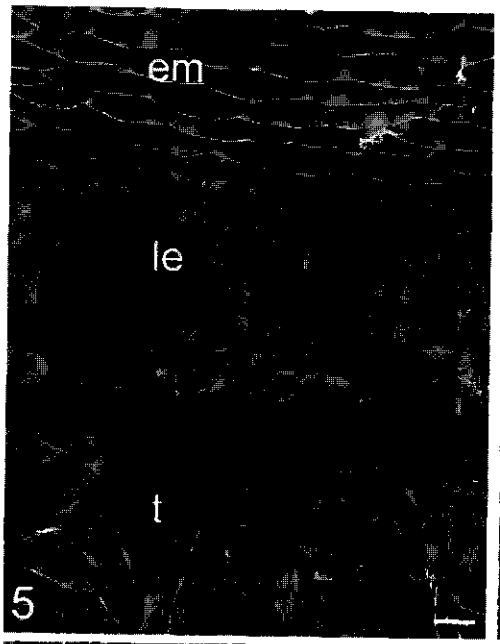



**Fig. 3.4** Northern blot of wild-type (wt) and *gib1* RNA isolated from lateral endosperm (le), endosperm cap (ec) and embryo (em) from ungerminated seeds incubated in water (top panel) or in 10 µM ABA (bottom panel) for various time as indicated. The blot was hybridized with $^{32}P$ labelled probe for endo-ß-mannanase.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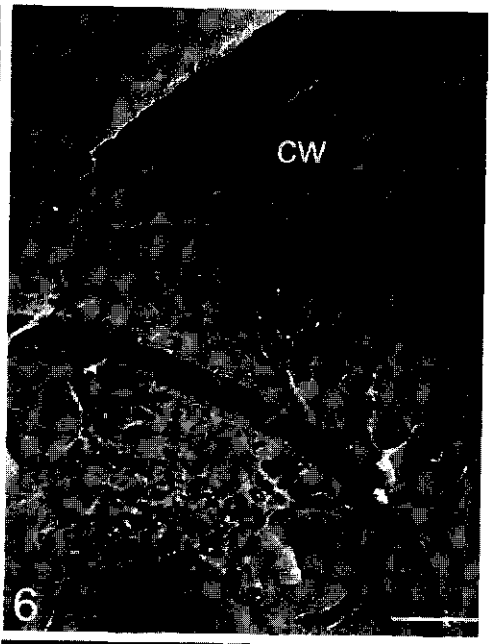
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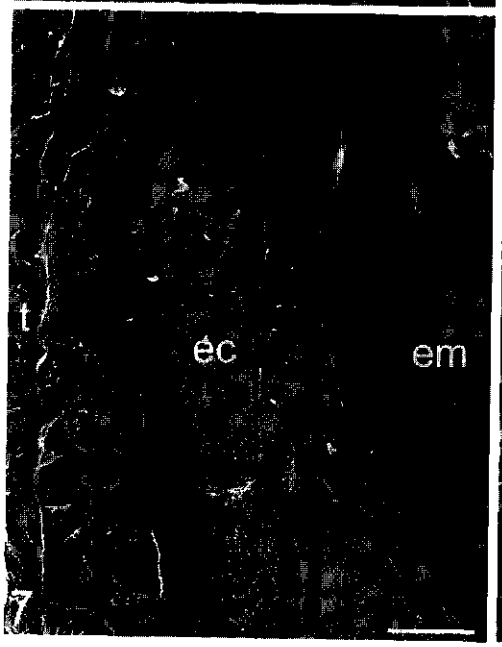
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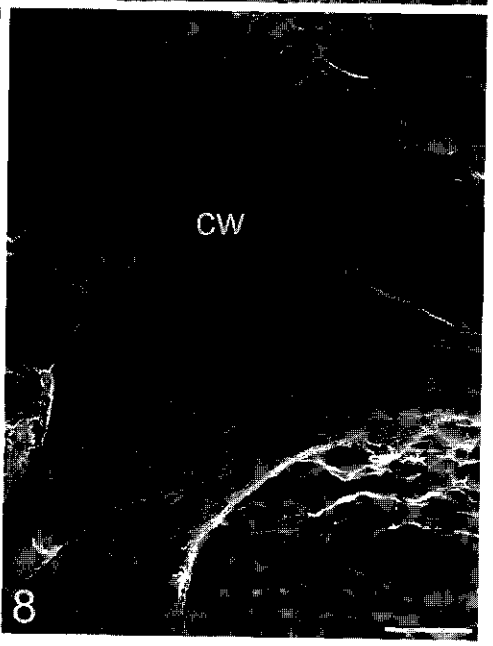
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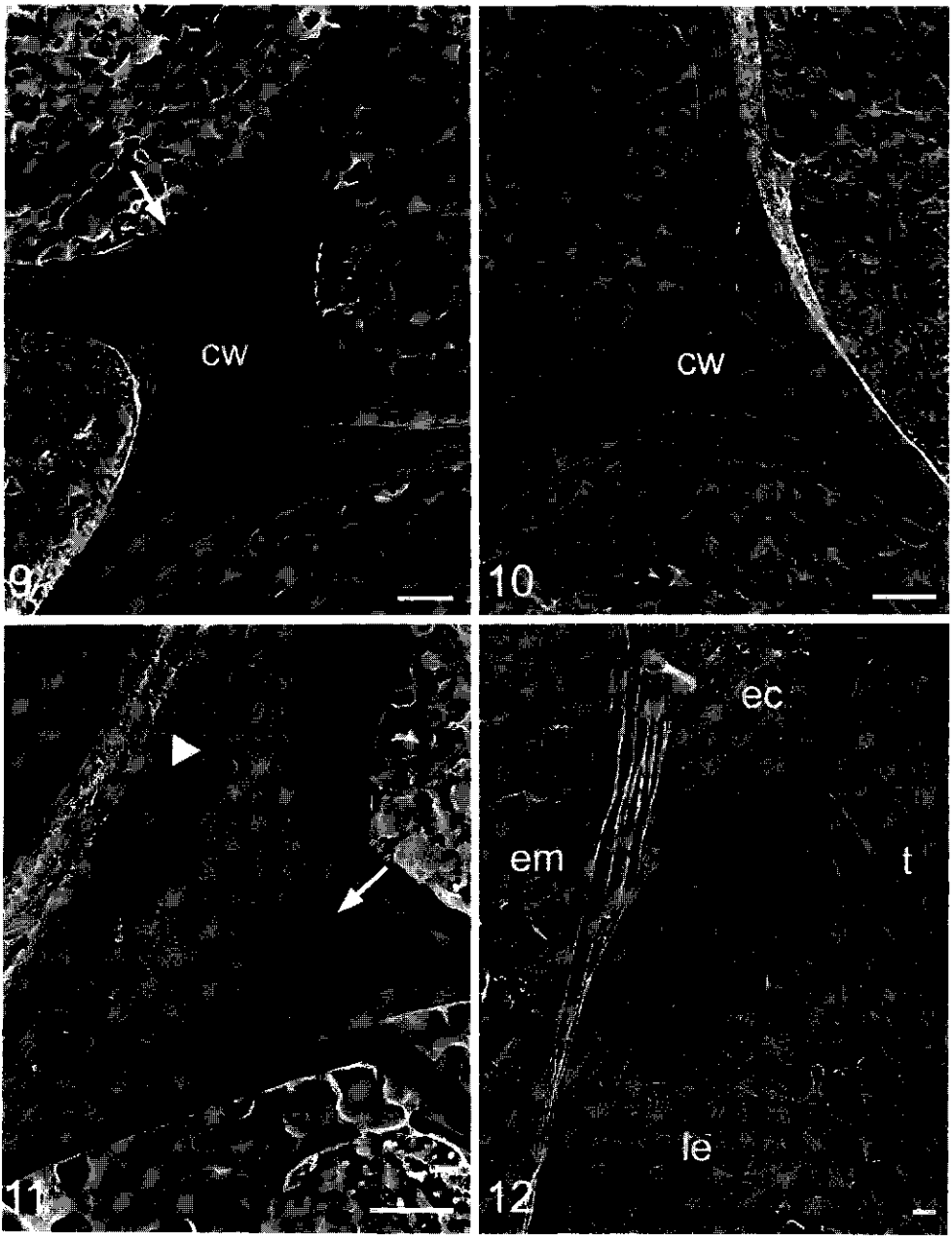
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were fully imbibed and endo-ß-mannanase activity was not detectable (Fig. 3.2). Seeds of the *gib1* mutant that were incubated for 24 h in 10 µM $GA_{4+7}$ showed porosity of the cell wall along the plasma membrane in the endosperm cap while the cell wall further from the plasma membrane appeared more or less intact (Fig. 3.9). The cell walls in the endosperm cap displayed a fullyporous appearance after 2 d of imbibition in wild-type seeds imbibed in water or 10 µM ABA (Fig. 3.10) and in *gib1* seeds imbibed in 10 µM $GA_{4+7}$ (not shown). Seeds of the *gib1* mutant that were incubated in water showed no traces of porosity after 4 days (results not shown). Long incubations in ABA showed a gradual restoration of the cell walls in the endosperm cap. After 7 days cell walls in the endosperm cap were observed that were porous as well as cell walls that showed intermediate porosity (Fig. 3.11), displaying patches of intact appearance next to patches of porous appearance. There appeared to be a gradient of almost intact cell walls next to the testa to intermediately porous cell walls next to the radicle tip (results not shown). After 6 weeks all cell walls appeared to be intact except for the cell walls bordering the radicle tip which always showed traces of porosity. Scanning micrographs of seeds that had just completed germination showed that the endosperm cap was ruptured due to the protrusion of the embryo (Fig. 3.12). The ruptured endosperm cap and the intact lateral endosperm were separated by only a small zone with intermediate morphology.

Wild-type seeds were detipped and placed in several concentrations of PEG with or without 10 µM ABA. The radicle length measured after 6 d showed a shift of 0.11 MPa to

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**Left: Figs. 3.5 - 3.9** Scanning electron micrographs of the lateral endosperm (le) of a wild-type seed that was imbibed in water for 2 days (Fig. 3.5; bar = 10 µm), of a detail of the cell wall (CW) of the lateral endosperm as in Fig. 3.5 (Fig. 3.6; bar = 1 µm), of the endosperm cap (ec) of a wild-type seed that was imbibed in water for 2 days (Fig. 3.7; bar = 10 µm), and of the cell wall (CW) of the endosperm cap of a wild-type seed that was imbibed in water for 17 h (Fig. 3.8; bar = 1 µm). Testa (t), embryo (em). The seeds had not completed germination. Note the intact appearance of the cell wall.

**Right: Figs. 3.9 - 3.12** Scanning electron micrographs of the cell wall (CW) of the endosperm cap of a *gib1* seed that was imbibed in 10 µM $GA_{4+7}$ for 24 h (Fig. 3.9; bar = 1 µm), of the cell wall (CW) of the endosperm cap of a wild-type seed that was imbibed in 10 µM ABA for 2 days (Fig. 3.10; bar = 1 µm) or for 7 days (Fig. 3.11; bar = 1 µm), and of a seed that just completed germination showing the endosperm cap (ec), lateral endosperm (le), embryo (em) and testa (t) (Fig. 3.12; bar = 10 µm). The seeds in Figs. 3.9 - 3.11 had not completed germination. Note the porosity along the plasma membrane in Fig. 3.9 (arrow), of the entire cell wall in Fig. 3.10, and of the patches with both a porous (arrow) and intact (arrowhead) appearance in Fig. 3.11.

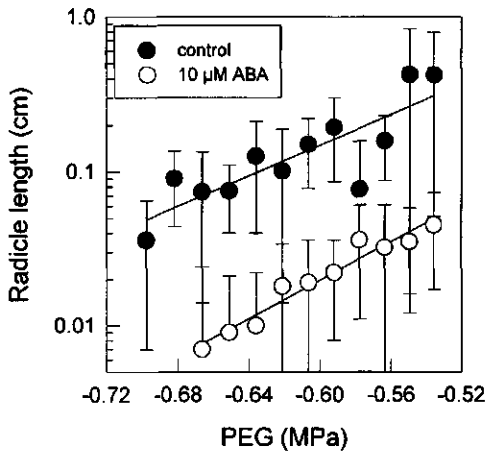


**Fig. 3.13** Radicle length of detipped wild-type seeds that were incubated for 144 h in different concentrations of PEG with (○, $r^2 = 0.948$) or without (●, $r^2 = 0.705$) 10 µM ABA. The line drawings represent the linear regression curves.

higher osmotic potentials in the presence of ABA, as calculated with the linear regression equations (Fig. 3.13). The osmolality of the ABA solution was $10^{-4}$ osmol, which was insufficient to cause this difference. The difference in osmotic potential between the two treatments was converted into Newton yielding 0.025 Newton. This value was in the same order of magnitude as the difference of 0.07 Newton in the puncture force of the endosperm cap for seeds incubated with or without ABA (Fig. 3.2).

Wild-type seeds that were incubated for various times in ABA, washed, dried and germinated in water displayed a negative log-linear relationship between the time incubated in ABA and the germination speed, expressed as mean time until germination (Fig. 3.14). Improved germination was found with incubation times between 136 and 336 h, as calculated with the regression equation, after which germination speed appeared to level off. Germination after ABA incubation did not correlate with endo-ß-mannanase activity, porosity of the endosperm cap cell walls, or puncture force at the time of drying.

## Discussion

Weakening of the endosperm cap in tomato has been described for wild-type seeds in water and *gib1* seeds in $GA_{4+7}$ (Groot and Karssen, 1987). It was found that endosperm caps of wild-type seeds did not weaken in ABA (Groot and Karssen, 1992). However, Fig. 3.1 shows that during incubation up to 45 h in ABA the endosperm cap of wild-type seeds weakened in a similar way as in water. After a drop to 0.35 Newton the puncture force levelled off in ABA imbibed seeds, whereas in water imbibed seeds the puncture force

decreased beyond 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 puncture force compared to the first step. It should be noted that the experiments of Groot and Karssen (1992) were different in that in those experiments endosperm caps were used that were isolated from the start of imbibition. A correlation was found between mannanase activity and puncture force, as earlier described for ABA-deficient *sit*$^{W}$ seeds imbibed in water (Downie et al., 1997). We found an exponential relationship, with mannanase activity still increasing when puncture force levelled off. This implies that an initial puncture force decrease is associated with an increase in endo-ß-mannanase activity. Further increase of mannanase activity did not result in a change of puncture force. An alternative function for the seemingly excessive amounts of mannanase in the endosperm cap between 45 h and radicle protrusion might be a role in the early mobilisation of reserves in the endosperm cap by hydrolysing the mannans into oligosaccharides.

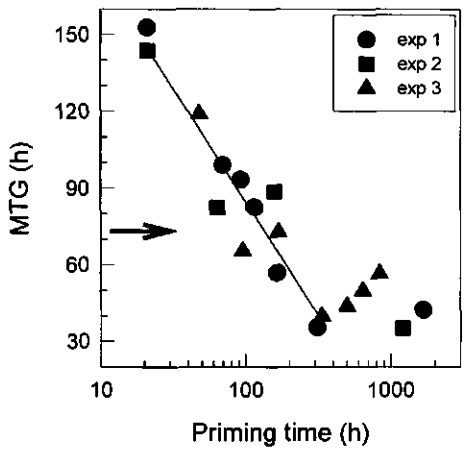


**Fig. 3.14** Germination speed (mean time until 50% germination) versus incubation time in 10 µM ABA. After incubation seeds were rinsed in water, dried back and germinated in water. Data points are from three independent experiments (●, ■ and ▲). Unprimed seeds showed 50% germination after 73 h, as indicated by an arrow. The line drawing represents the linear regression curve of the data points up to 336 h ($r^2$ = 0.895).

Not until after 45 h of incubation a difference in puncture force between water and ABA treatment became clear. This strongly suggests that additional enzymes that are ABA-regulated are involved in the weakening of the endosperm cap, as was hypothesized by Toorop et al. (1996), or that an ABA-regulated enzyme is induced that actively inhibits the weakening process. Karssen et al. (1989) also hypothesized the existence of a second step in

the weakening of the endosperm cap that precedes radicle emergence, resembling a cell separation process. After 67 h the endosperm caps of seeds transferred from water to ABA stopped lowering their puncture force, implying that the ABA-regulated step in endosperm weakening can be arrested at any time as long as radicle protrusion has not occurred. 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. The testa has been described as an important component of the mechanical resistance that is imposed on the embryo before radicle protrusion takes place (Hilhorst and Downie, 1996). Leviatov et al. (1994) however concluded that at low temperature the main barrier to germination of tomato seeds is imposed by the endosperm, and not by the testa. Similarly, Groot and Karssen (1987) showed that the testa only gave a restraint of 0.1 Newton at any point during germination, and that the endosperm was the main component of the mechanical resistance. These findings were supported by the results of Fooland and Jones (1991), who concluded that the embryo and the testa played a minor role in germination behaviour, while the endosperm is the principal site of genetic determinants controlling germination performance. Our data showed that the minimum puncture force in ABA was approximately 0.35 Newton, which was considerably higher than the puncture force for the testa alone. Therefore, it is likely that the endosperm is the tissue that plays a major role in the ABA inhibited decrease in puncture force.

Scanning electron micrographs showed increased porosity of the cell walls in the endosperm cap in both water and ABA imbibed ungerminated seeds. These pores, caused by evaporation of water during the freeze-drying process, corresponded with both a decrease in puncture force and an increase in leachable endo-ß-mannanase activity. Porosity was visible along the plasma membrane after 1 day of incubation, at which point mannanase activity was low. After 2 days the porosity was found throughout the endosperm cap cell walls and mannanase activity was high, while the seeds had not yet completed germination. Seeds of the *gib1* mutant in water consistently showed no porous cell walls as well as no decrease of puncture force, and mannanase activity was absent. $GA_{4+7}$ induced porous cell walls, decrease of puncture force (Groot and Karssen, 1987), and mannanase activity (Groot et al., 1988). The correlation of an increase in porosity with the increase of mannanase activity and the decrease

of puncture force strongly suggested that the porosity was caused by mannanase activity. Mannose is the major component of tomato seed cell walls (Groot et al., 1988) and galacto(gluco)mannans are likely to be present. It is plausible that the hydrolysis products of mannanase, 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. A similar result was found by Williamson and Duncan (1989) who found swollen, eroded and porous cell walls in raspberry fruit cells upon infection by *Botrytis cinerea*, which could be related to enzymatic activity. Leviatov et al. (1995) previously mentioned destruction of endosperm cells opposite the radicle tip of tomato seeds in an anatomical study. Welbaum et al. (1995) showed cellular degradation and weakening of the perisperm envelope preceding radicle emergence in muskmelon seeds. Watkins et al. (1985) found that the endosperm cells opposite the radicle tip in pepper seeds appeared compressed and had lost some of their integrity. We never found any trace of deterioration of these cells; only cell walls were affected, as was the case in *D. ferox* (Sanchez et al., 1990). Some cells in the inner layer did appear to be compressed, but this morphology was also found in shortly imbibed seeds of which the embryo was not fully turgescent. Therefore this phenomenon could not be attributed to deterioration due to the weakening of the endosperm.

During long incubation in ABA endo-ß-mannanase activity faded (Still and Bradford, 1997), which corresponded with the lesser degree of porosity found in the scanning micrographs. After 6 weeks all walls appeared intact except for the inner most cell wall of the endosperm cap bordering the embryo. However, this apparent restoration of cell walls was not paralleled by an increase in puncture force; the puncture force remained at 0.35 Newton. The apparent restoration might be explained either by a rearrangement of hydrolysis products in such a way that there is no more space for water to accumulate, or similarly by a replacement of water by other molecules. The synthesis of cell wall polymers is unlikely since no increase in puncture force is observed. However, it cannot be excluded.

While the total RNA remained approximately constant in both the lateral endosperm and the endosperm cap, it showed a transient rise in the embryo. Remarkably, no differences were observed between wild-type seeds imbibed in water and ABA, and *gib1* seeds imbibed in water. Obviously RNA accumulation is not influenced by these hormones. Most of the total RNA was likely to be ribosomal RNA since no other bands could be seen on both agarose and formaldehyde gels (data not shown). Therefore the embryo seemed to transiently increase its

translation apparatus anticipating the completion of germination, regardless of the presence of GA or ABA. Using the mannanase clone as a DNA-probe Northern blotting showed that the endo-ß-mannanase coding sequence was expressed in the embryo and endosperm cap and not in the lateral endosperm of ungerminated seeds (Fig. 3.4). Seeds that were imbibed for 5 h did not show transcription, indicating that the mRNA is newly synthesized. ABA partially suppressed the transcription in the endosperm cap although a clear signal was still detectable. Given the unreduced levels of mannanase activity, any effect of ABA on the transcription can be questioned. GAs seemed to be necessary for the induction of the mRNA in the endosperm cap of the *gib1* mutant, supporting previous results showing that mannanase activity is GA-regulated (Groot and Karssen, 1987). In wild-type seeds upon imbibition mannanase transcription is likely to be induced by endogenous gibberellins. Prolonged incubation of wild-type seeds in ABA resulted in the total loss of mRNA coding for endo-ß-mannanase, corresponding with the loss of enzyme activity. Surprisingly, the embryo of water incubated *gib1* seeds displayed a signal, indicating that the transcription of endo-ß-mannanase in the embryo is not GA-regulated, which differs from the endosperm cap.

The sharp border between the endosperm cap and the lateral endosperm in germinated seeds (Fig. 3.12) 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 strict regulation is required at the cellular level to create this functional and morphological difference, as the endo-ß-mannanase in the endosperm cap obviously did not affect the cell walls in the lateral endosperm. No traces of hydrolysis in these cell walls were observed, and so the enzyme obviously is not capable of being active in the cell walls of the lateral endosperm. Nonogaki and Morohashi (1996) found on TLC that there is a slight difference in the action pattern of the enzymes from the two parts of the endosperm. A different action pattern in itself however does not explain the inability of the mannanase from the cap region to be active in the lateral endosperm. Additional structural differences in the mannans are required.

ABA had an inhibiting effect on the growth potential of the radicle of detipped seeds when taking longitudinal growth of the radicle of detipped seeds into account. An osmotic effect on the water uptake by the embryo has been shown for *Brassica napus* (L.) (Schopfer and Plachy, 1984, 1985). Liptay and Schopfer (1983) and Nomaguchi et al. (1995) only studied arbitrary germination of detipped wild-type tomato seeds, while length of the radicle

more accurately describes the growth potential in relation to the germination event. After conversion, the effect of ABA on the growth potential of the embryo (0.025 Newton) is of the same order of magnitude as the effect of ABA on the puncture force of the endosperm cap (0.07 Newton). Ni and Bradford (1993) found a difference 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. They concluded that the endosperm cap played a decisive role. Our results indicated 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, even after considerable weakening in ABA.

Priming in ABA has previously been described by Finch-Savage and McQuistan (1991), who also found an improved germination with longer priming time in tomato seeds. ABA priming also positively affected mean germination time in *A. graveolens* L. seeds (Biddington et al., 1982) *Daucus carota* L. seeds (Finch-Savage and McQuistan, 1989) and *Pisum sativum* L. seeds (Sivritepe and Dourado, 1995). Prolonged incubation in ABA, as described in this paper, may be considered as ABA priming. An optimum in priming time was found. Priming times up to 136 h resulted in slower germination than seeds that were not primed; priming longer than 336 h had no effect on germination rate. Interestingly, the optimum priming time window showed normal green cotyledons upon germination and light treatment, while longer priming resulted in yellowish cotyledons, and shorter priming resulted in purple hypocotyls (data not shown), probably due to anthocyanin. This indicated that only at the optimum priming time the seedlings developed normally. The germination speed after ABA priming did not correspond with the mannanase activity in the endosperm cap, the puncture force or the apparent cell wall restoration during priming. Other factors must be involved in the regulation of the germination speed as affected by ABA-priming, possibly factors related to the embryo. Repair of age-induced genetic damage as was found for pea seeds (Sivritepe and Dourado, 1995), or prevention of dehydration injury as in celery seeds (Biddington et al., 1982) are processes that also cannot be excluded to play a role.

In summary, our results indicate that ABA acts in two ways in the inhibition of germination; (i) by interference with the water uptake by the embryo, and (ii) by interference with the weakening process of the endosperm cap, thus preventing the seed from completion of germination. The endosperm weakening plays the limiting role in the completion of germination. The first step in the endosperm weakening, which is not inhibited by ABA, is

correlated with endo-ß-mannanase activity and with the porous appearance of the cell walls in the endosperm cap. ABA can neither fully inhibit mannanase activity nor transcription of the mannanase gene. The second step in the biphasic endosperm weakening is inhibited by ABA. It is hypothesized that ABA-regulated enzymes are involved in the second step of endosperm weakening in tomato seeds.

## Acknowledgement

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Chapter 4

# Structure-activity studies with ABA analogs on germination and endo-ß-mannanase activity in tomato and lettuce seeds

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## Abstract

The effects of several analogs of ABA were tested on tomato (*Lycopersicon esculentum* Mill.) and lettuce (*Lactuca sativa* L.) seed germination and on the ability of these seeds to produce endo-ß-mannanase during and following germination. The (+)-enantiomers of ABA were more effective than the (-)-enantiomers in inhibiting the germination of seeds of both species. Endo-ß-mannanase activity in the tomato endosperm cap during germination was not influenced by ABA or its analogs, regardless of whether the seeds completed germination or not. Thus while there was a structure-activity relationship of the ABA analogs with respect to tomato seed germination, there was no such relationship with respect to endo-ß-mannanase activity in the endosperm cap during this event. Post-germination enzyme activity in the tomato lateral endosperm region, on the other hand, was inhibited by the (+)-enantiomers of ABA. PBI-51, a purported competitive inhibitor of ABA, did not reverse the inhibitory effects of (S)- or (R)-ABA on tomato seed germination; instead, it enhanced the inhibition. Lettuce seed endo-ß-mannanase activity was suppressed by most analogs of ABA, and in the presence of analogs PBI-59 and 89 very low amounts of enzyme were produced in the endosperms of seeds that were able to complete germination. This observation supports the suggestion that there is no cause-and-effect relationship between lettuce seed germination and endo-ß-mannanase production.

## Introduction

Abscisic acid exhibits optical activity because of the asymmetric carbon at position C-1', resulting in two enantiomers, (S)-(+)-ABA and (R)-(-)-ABA. ABA analogs with different structures and substitutions have proven to be useful tools for studying which components and arrangements of the ABA molecule are necessary for its action. Generation of a new chiral centre at C-2', by reduction of the double bond, has provided new molecules for studying ABA action (Walker-Simmons et al., 1992), as has removal of the methyl group at C-7' (Walker-Simmons et al., 1994). Differences in the structure of ABA have resulted in different physiological responses to the analogs. For example, ABA analogs which were effective in altering the germination capacity of wheat embryos showed differential effects on ABA-responsive genes (Walker-Simmons et al., 1992). Another analog displayed antagonistic properties to natural (S)-(+)-ABA through direct competition at a hormone-recognition site (Wilen et al., 1993).

In tomato seeds, endo-ß-mannanase is present in the endosperm cap prior to the completion of germination. It is thought to play an important role in germination by weakening the endosperm at the micropylar (radicle) end to permit protrusion of the radicle (Nonogaki et al., 1992; Groot and Karssen, 1987; Groot et al., 1988). ABA inhibits the completion of germination, i.e. radicle protrusion, and also prevents an increase in endo-ß-mannanase activity (Nomaguchi et al., 1995). Additionally, the puncture force of the endosperm cap does not decrease in the presence of ABA (Groot and Karssen, 1992). Recent studies have cast some doubt on the causal relationship between an increase in endo-ß-mannanase activity and the completion of tomato seed germination, for the two events are not always essentially linked (Still and Bradford, 1997; Toorop et al., 1996). To investigate this relationship, ABA analogs were applied to tomato seeds to determine if there were any that would affect germination, but not endo-ß-mannanase activity, and vice versa, and thus clearly separate the two events. In addition similar experiments were conducted on lettuce seeds. In these seeds ABA (racemic mixture) inhibits mannanase production and prevents germination (Halmer and Bewley, 1979). Because the increase in endo-ß-mannanase activity in the endosperm is a post-germination phenomenon, it has been suggested that, unlike in some seeds (e.g. tomato), weakening of the cell wall by this enzyme is not a pre-requisite for radicle emergence. The possibility that there is a low, but effective amount of enzyme present in the endosperm prior to radicle emergence that causes weakening of its cell walls cannot be ruled

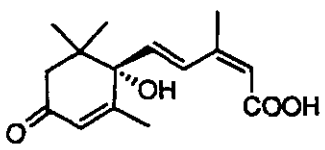
out, however. As a different approach to the cause-and-effect of endo-ß-mannanase activity and germination, the impact of the various ABA analogs on production of this enzyme was tested, to see if the sensitivity of this was different from that of germination.

## Materials and methods

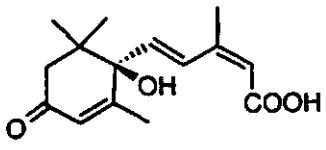
*Seed material.* Wild-type tomato seeds (*Lycopersicon esculentum* cv. Moneymaker) and *sit*$^{w}$ mutant seeds (ABA-deficient) harvested in 1992 were stirred in 2% HCl for 2 h to remove the surrounding locular tissue, rinsed, dried and stored at 5°C. Seeds were surface sterilized in 1% sodium hypochlorite, rinsed in demineralized water and imbibed in 1.5 ml ABA or ABA analog solution on two layers of Whatman No. 1 filter paper in 50-mm-diam Petri dishes, or in 6 ml ABA or ABA analog in 100-mm-diam Petri dishes. Seeds were kept at 25°C in the dark. Mean time to germination (MTG) expressed the time in which 20% of the batch had completed germination. Seeds were dissected into lateral endosperm, endosperm cap and embryo as described by Toorop et al. (1996). Extracts were made by homogenizing the different tissues from 10 fresh seeds in 200 µL McIlvaine buffer (0.1 M citric acid - 0.2 M disodium phosphate buffer, pH 5.0) in a micro-dismembrator U (Braun Biotech International, Melsungen, Germany) for 3 min at 1600 rpm. Part of the testa remained attached to the endosperm after dissection. The samples were centrifuged in a microfuge for 10 min at 14000 rpm. The supernatant was used for enzyme assays.

Non-photosensitive lettuce seeds (*Lactuca sativa*, cv. Grand Rapids) (Ferry Morse Seed Co) were germinated at 25°C on two layers of Whatman No. 1 filter paper wetted with 5 ml liquid in 100-mm-diam Petri dishes. For experiments involving isolated endosperms, these were dissected from seeds after 4 h imbibition and placed in 1 ml water in a 5 cm-diam-Petri dish for 1 h. Extraction and assay of endo-ß-mannanase was as for tomato seeds and seed parts, except that 300 µl 0.1 M Hepes buffer pH 8 was used for the extraction.

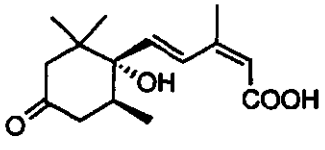
*ABA and analogs.* Seeds were incubated in ABA and 6 of its analogs. PBI-58 is the natural form of the hormone (+)-S-ABA, and PBI-145 the unnatural (-)-R-ABA. PBI-89 is the dihydroABA of (+)-S-ABA, and PBI-59 the dihydroABA of (-)-R-ABA. The dihydroacetylenic acids are designated PBI-82 and PBI-185, being the (+) and (-) enantiomer respectively. PBI-51 differs from PBI-185 by an alcohol group at C1 instead of an acid. The structure of PBI-51 is described by Wilen et al. (1993), and that of the other molecules by Walker-Simmons et al. (1992), and shown in Fig. 4.1. The analogs were initially dissolved in



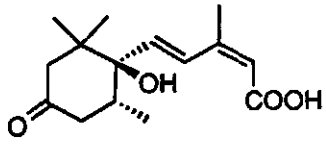

(S)-ABA PBI-58



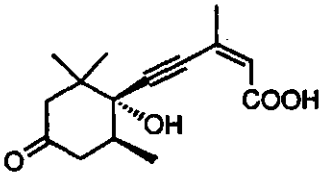

(R)-ABA PBI-145



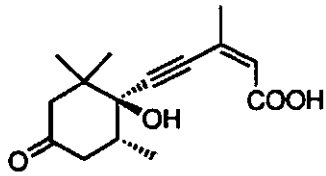

PBI-89



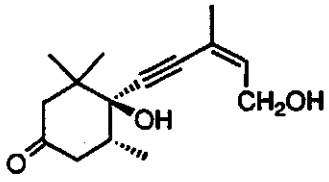

PBI-59




PBI-82




PBI-185




PBI-51

**Fig. 4.1** Chemical structures of the PBI analogs of ABA.

ethanol and then diluted to the required concentrations with distilled water; the final ethanol concentration was <0.025%.

*Endo-ß-D-mannanase assay.* For the lettuce seed experiments the gel plate method of Downie et al. (1994) was used for determining endo-ß-mannanase activity in 10 µl samples.

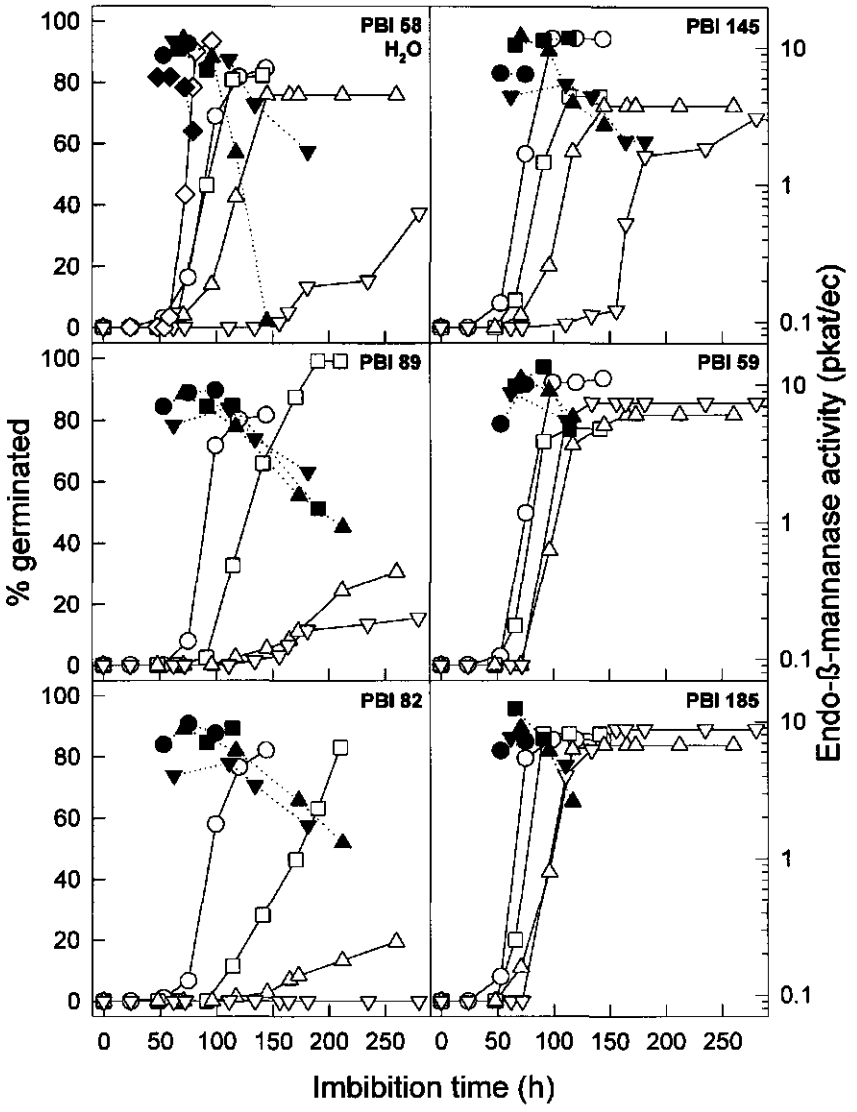



**Fig. 4.2** Germination of tomato seeds (open symbols) and endo-ß-mannanase activities (closed symbols) in endosperm caps of ungerminated seeds incubated in water (◆), 1 μM (●), 2 μM (■), 5 μM(▲) or 10 μM (▼) of PBI-145, PBI-89, PBI-59, PBI-82 or PBI-185. For PBI 58 the concentrations were 0.1 μM (●), 0.2 μM (■), 0.5 μM (▲) and 1 μM (▼) respectively.

For tomato seeds a modified assay involved using gels (0.5 mm thick) containing 0.1 % (w/v) locust bean gum (Sigma) in McIlvaine buffer (pH 5.0) and 0.8% type III-A agarose (Sigma) made on Gel Bond film (Pharmacia) instead of Petri dishes. Holes were punched in the gel with a 2-mm paper punch, and samples of 2 µL were applied. Gels were incubated for 20 h at 25°C, and then washed in McIlvaine buffer (pH 5.0) for 30 min, stained in a 0.5% (w/v) Congo Red (Sigma) solution for 30 min, washed with 96% ethanol for 10 min, and destained in 1 M NaCl for at least 24 h. All staining steps were performed while rotating the gel gently. Commercial endo-ß-mannanase from *Aspergillus niger* (Megazyme, North Rocks, Sydney, Australia) was used to obtain a standard curve. Calculation of enzyme activity in the samples was according to Downie et al. (1994).

## Results

*Tomato.* Wild-type tomato seeds were incubated in several concentrations of ABA or ABA analogs. The (+)-enantiomers PBI-58, PBI-89 and PBI-82 inhibited germination at lower concentrations than the (-)-enantiomers (Fig. 4.2). PBI-58 showed strong inhibition, at only 0.1 µM. Seeds of the *sit*$^{w}$ mutant generally showed the same pattern of inhibition of germination (results not shown). To assay endo-ß-mannanase activity, extracts were made from embryos and endosperm caps of imbibed wild-type seeds prior to the completion of germination. At low concentrations of the ABA analogs, allowing a germination pattern close to the germination pattern in water, endo-ß-mannanase activity developed normally in the embryo and endosperm cap prior to germination (Figs. 4.2 and 4.3). Incubation in higher concentrations of most of the analogs, which inhibited or slowed germination, resulted in an eventual decline in enzyme activity in the endosperm cap of the ungerminated seeds. However, endo-ß-mannanase activity was initially the same in this region of the seed at both inhibitory and non-inhibitory concentrations. Embryo endo-ß-mannanase activities were generally slightly lower than activities in the endosperm but showed an overall consistency with the response of the endosperms to the analogs (Fig. 4.3), with no inhibition of enzyme activity regardless of the capacity of the analog to inhibit germination.

PBI-51 has been previously described as an ABA antagonist (Wilen et al., 1993), and so a germination test was performed to see if this was also the case in tomato seeds. Seeds were imbibed in combinations of a high concentration of PBI-51 and several concentrations of PBI-58 and PBI-145 (the natural ABA and its mirror image) (Fig. 4.4). Incubation of seeds in 10

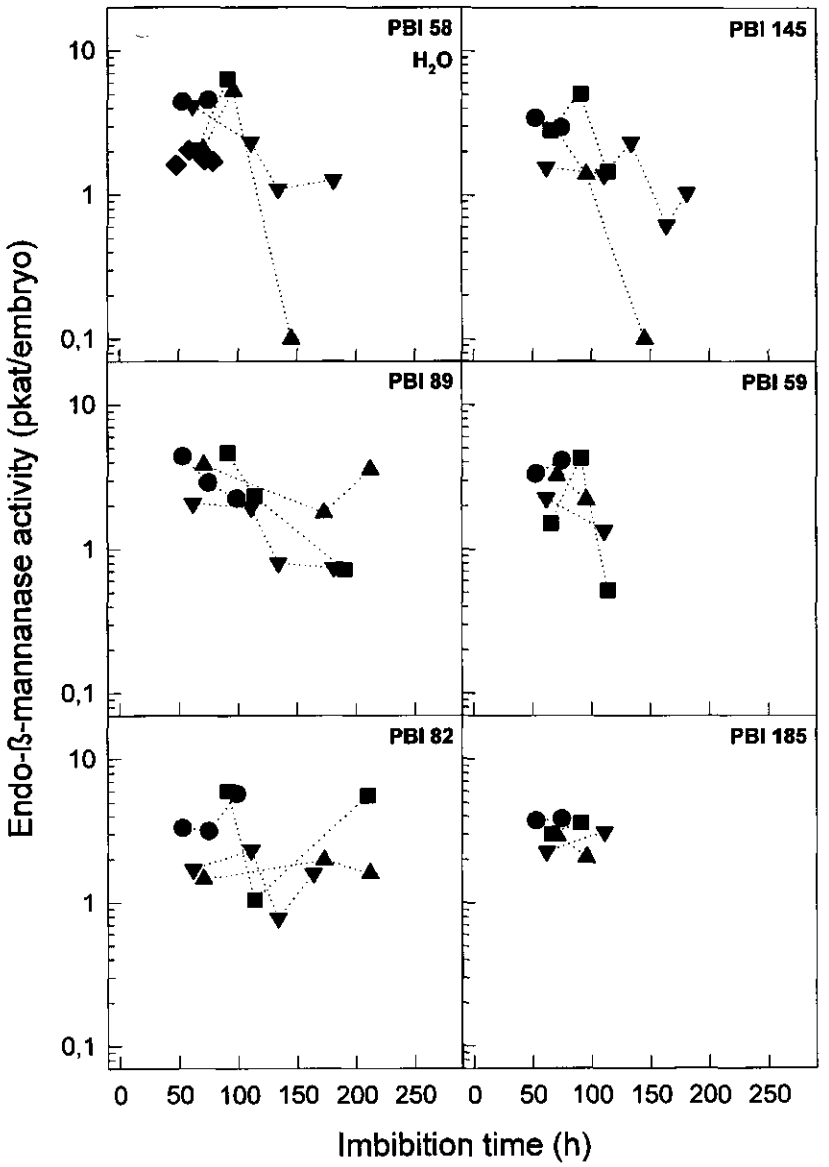



**Fig. 4.3** Endo-ß-mannanase activities of embryos of ungerminated seeds incubated in water (◆), 1 μM (●), 2 μM (■), 5 μM(▲) or 10 μM (▼) of PBI-145, PBI-89, PBI-59, PBI-82 or PBI-185. For PBI-58 the concentrations were 0.1 μM, 0.2 μM, 0.5 μM and 1 μM respectively.

μM PBI-51 delayed germination by approximately 50 h, whereas of 0.1 μM PBI-58 resulted in a lesser delay of 25 h. Both 0.5 μM and 1.0 μM PBI-58 caused a much stronger inhibition of approximately 150 and 350 h, respectively. PBI-145 applied in the same concentrations showed a weaker inhibition of germination (Fig. 4.4). A combination of 10 μM PBI-51 and PBI-58 or PBI-145 in concentrations of 0.1 μM, 1 μM, or 10 μM resulted in greater effect on the delay of germination.

To analyse the influence of the ABA analogs on post-germinative enzyme activity, seeds were incubated in several concentrations of them, detipped after 3 days, incubated for another 3 days and endo-ß-mannanase activity extracted and assayed in the remaining endosperm and embryo. The (+)-enantiomers appeared to be considerably more effective in inhibiting endo-ß-mannanase activity than the (-)-enantiomers. With the exception of PBI-58, the natural form of ABA, only small differences were observed amongst the individual (+)-enantiomers and (-) -enantiomers (Fig. 4.5). Endo-ß-mannanase activity of detipped seeds correlated negatively with MTG, indicating that ABA and its analogs had a similar effect on germination and post-germinative endo-ß-mannanase activity in the embryo and lateral endosperm (Fig. 4.6).

*Lettuce.* Imbibing lettuce seeds in the various analogs of ABA resulted in a general slowing of germination, and sometimes a reduction in total germination achieved after 7 days (Fig.4.7). The concentrations required to inhibit germination were different for each analog, and only representative data of the several concentrations of each that were used are shown. PBI-89, for example, was much more effective in preventing germination than PBI-59 at the same concentration ($5 \times 10^{-5}$ M) (Fig. 4.7), although whether this was due to less uptake or a more effective chemical structure for inhibition of the germination process is not known. PBI-82 slowed germination considerably for 72-96 h. The ABA analogs were also capable of inhibiting germination of the light-sensitive cv. Ferrari of lettuce, with the effectiveness of the analogs being similar to those for the Grand Rapids cultivar (results not shown).

To see if the sensitivity of endo-ß-mannanase production to ABA analogs was different from that of germination, and following emergence of the radicle, endosperms were dissected from the seeds, and enzyme activity determined (Table 1). In many endosperms from germinated seeds, some endo-ß-mannanase activity was detectable, although it was always

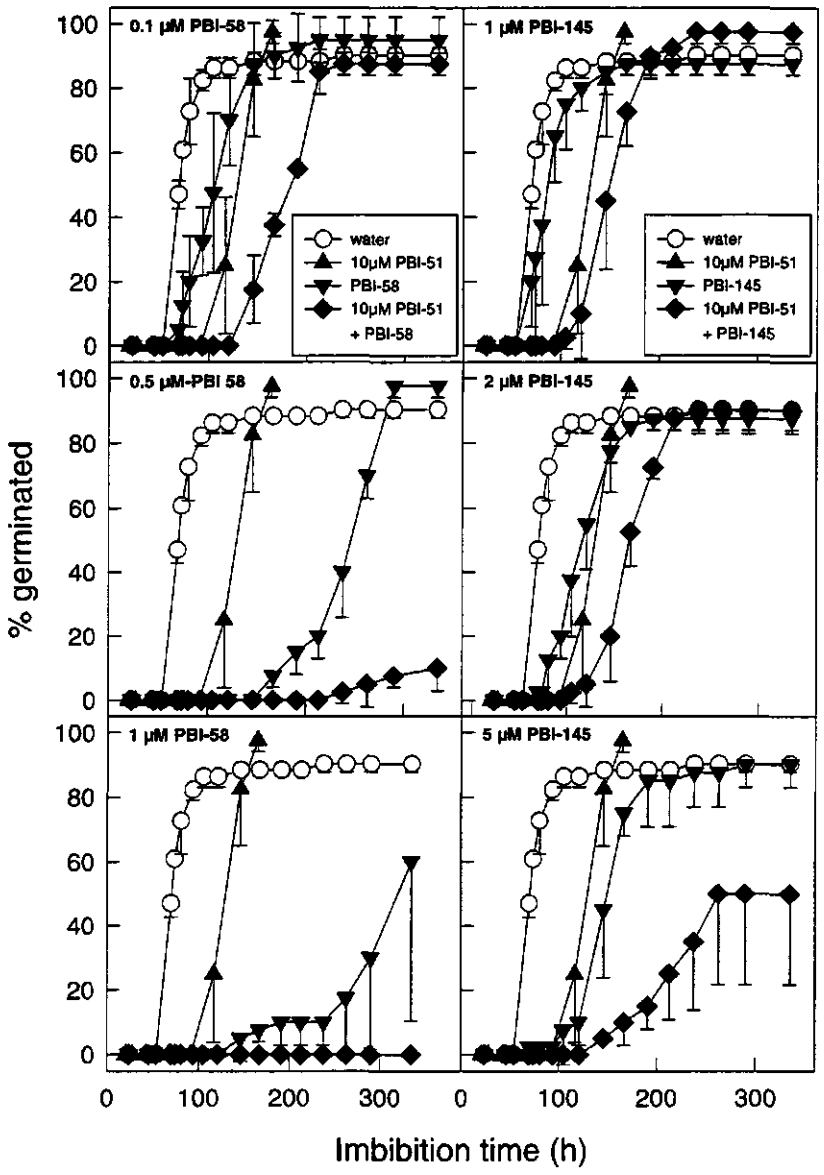



**Fig. 4.4** Left panels: germination of tomato seeds in water, 10 µm PBI-51, PBI-58 or 10 µM PBI-51 + PBI-58. The concentration of PBI-58 is indicated in each panel. Right panels: germination of tomato seeds in water, 10 µM PBI- 51, PBI-145, or 10 µM PBI-51 + PBI-145. The concentration of PBI-145 is indicated in each panel.

suppressed in relation to endosperms from water-imbibed seeds. Non-germinated seeds imbibed on the ABA analogs showed very low activity for all analogs (max. 0.02 - 0.03 pkat). In germinated seeds the least activity was detected in the endosperms of seeds incubated in 5 x $10^{-5}$ M PBI-89 after 48 h. Radicles had protruded from the seeds by approx. 1 cm, although there was no root hair development. The endosperm was still capable of being dissected intact. In water-control seeds by this time the radicle would have extended about 1.5 - 2 cm, there would be copious root hair development, and the endosperm would have been completely digested (usually by 30 - 36 h after the start of imbibition). Endo-ß-mannanase activity in the endosperms was about 1 - 2% of that in germinated water control seeds, and given the subjective nature of the assay at such low activities, might not have been present at all.

Isolated endosperms of lettuce, when placed in a sufficiently high volume of water, produce endo-ß-mannanase (Halmer and Bewley, 1979). When 3-h-imbibed seeds were dissected and their endosperms placed in water for 24 h, copious enzyme activity could be detected, in the endosperms itself and even more was secreted into the surrounding solution

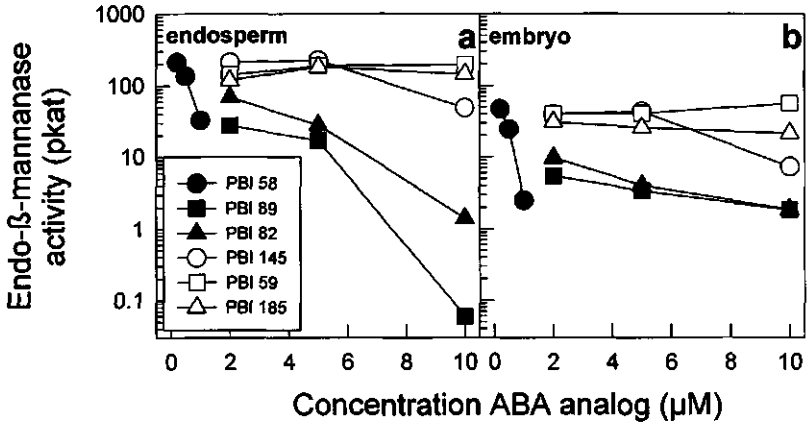



**Fig. 4.5** Post-germinative endo-ß-mannanase activity in (a) the endosperm and (b) the embryo of detipped tomato seeds at different concentrations of ABA analogs. Seeds were detipped after 3 days of incubation and incubated for another 3 days in the same incubation medium.

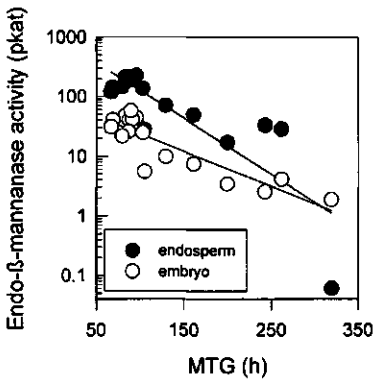



**Fig. 4.6** Post-germinative endo-ß-mannanase activity of endosperm and embryo and mean time to germination of intact tomato seeds. Correlation equations are for endosperm Y = 3.04 - 0.0094 X ($r^2$ = 0.695) and for embryo Y = 1.95 - 0.0058 X ($r^2$ = 0.783).

(Table 2). The enzyme was strongly suppressed by the two analogs that were tested; not only was there negligible activity secreted into the surrounding medium, but no increase in activity in the endosperm either.

As an addendum to the experiment in which PBI-59 ($10^{-4}$ M) inhibited endo-ß-mannanase production in whole seeds, after 48 h the endosperms were dissected from non-germinated seeds and washed in 2 ml distilled water for 2 h, with shaking. They were then transferred to 1 ml water overnight, and enzyme activity measured. Some endo-ß-mannanase production was achieved, it being present both in the endosperms and the surrounding incubation solution, while total enzyme activity was only 25% of water controls in the isolated and washed endosperms, this exceeded the 12% of water

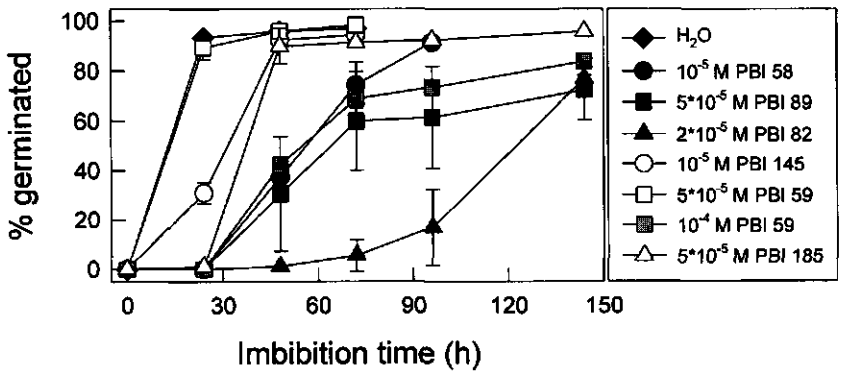



**Fig. 4.7** Time course of germination of lettuce seeds (cv. Grand Rapids) in ABA and its analogs.

**Table 1.** *Reduction in endo-β-mannanase production by ABA analogs in germinated intact lettuce seeds.*

The assay was conducted on seeds which had germinated to a radicle length of 2-5 mm. These were collected at the appropriate times, frozen in liquid nitrogen, and pooled for the enzyme assay at the appropriate times. All incubations and assays were duplicated, and maximum variability in activity was 10%.

| Analog | Concentration (M) | Enzyme activity (pkat/seed) |
|---|---|---|
| PBI-58 | $10^{-5}$ | 0.17 |
| PBI-59 | $10^{-4}$ | 0.03 |
| PBI-82 | $10^{-5}$ | 0.18 |
| PBI-89 | $5 \times 10^{-5}$ | 0.03 |
| PBI-145 | $10^{-5}$ | 0.15 |
| PBI-185 | $2.5 \times 10^{-5}$ | 0.26 |
| Water | | 0.35 |

**Table 2.** *Reduction in endo-β-mannanase production by ABA analogs in isolated lettuce endosperms.*

Endo-ß-mannanase activity in endosperms, and in the surrounding incubation solution, from lettuce seeds isolated after 3 h and placed in water with or without ABA analogs PBI-58 and 59 ($10^{-5}$ M) for 48 h. For PBI-59 → water, endosperms from 48-h-analog-imbibed ($10^{-4}$ M) seeds were washed and placed in water for 48 h and enzyme activity measured. All incubations and assays were duplicated and maximum variability in activity was 8%.

| Analog | Enzyme activity in endosperm (pkat/endosperm) | Enzyme activity in incubation liquid (pkat/endosperm) |
|---|---|---|
| PBI-58 | 0.001 | 0.001 |
| PBI-59 | 0.001 | 0.001 |
| water | 0.094 | 0.038 |
| PBI-59 → water | 0.015 | 0.018 |

controls achieved by endosperms in the intact seed.

## Discussion

The three-dimensional shape of the molecule played an important role in the influence of ABA on the rate of seed germination of tomato and lettuce. Changes to the configuration of the molecule at C-1', altering the (S)- into an (R)-configuration, and to the C-2' methyl position, by reduction of the double bond, caused a significant change in the germination response. In both species the (+)-enantiomers appeared to be much stronger inhibitors of germination than the (-)-enantiomers (Figs. 2 and 7). This is similar to the effects of (+)- and (-)-ABA on cress seed germination (Gusta et al., 1992). Although the effect on germination of tomato and lettuce seeds was stronger, it is similar to the effect on growth of maize cells (Balsevich et al., 1994). The configuration of the molecule at C-1' probably plays an important role in the binding of the molecule to the putative ABA receptor. The dihydroABA (PBI-89) and the dihydroacetylenic acid (PBI-82) of both PBI-58 and PBI-145 only had moderate effects on the germination rate of both species, indicating that the C-2', C-3' double bond in the ring structure played a less significant role in the recognition by the ABA receptor. These results differ from those of Walker-Simmons et al. (1992) who described an equal biological activity for PBI-58 and PBI-145, and similar activities for the dihydroABA and the dihydroacetylenic acid of PBI-58 in dormant wheat embryos. The strongest changes in inhibition of wheat embryo germination were caused by an alteration to the ring structure, which in our study only played a minor role. Churchill et al. (1992) also found an important role of the C-2', C-3' double bond in the freezing tolerance of cultured bromegrass cells. However, in tomato and lettuce seeds the C4-C5 triple bond did not result in a change of inhibition pattern. This was similar to what was found for wheat embryos.

The germination response of seeds of the ABA-deficient *sit*$^{w}$ mutant to ABA or its analogs was similar to that of wild type seeds, indicating that the sensitivity to ABA or its analogs was not altered by the mutation.

The pattern of endo-ß-mannanase activity in the tomato endosperm cap during germination was not influenced by ABA and its analogs. In all cases the highest values of endo-ß-mannanase activity were obtained between 50 and 100 h of imbibition. There was some variation in the activity pattern between the treatments with the different ABA analogs, but this was not consistent with the differences in germination (Fig. 4.2). Previous studies

have shown that ABA did not influence endo-ß-mannanase activity (Still and Bradford, 1997; Toorop et al. 1996). The results of the present study are consistent with these studies. Seeds that germinated slower at higher concentrations of ABA analog developed high amounts of activity that decreased during incubation up to 300 h, as was described for (±)-ABA previously (Still and Bradford, 1997). Even seeds germinated in weak germination inhibitors like PBI-185 showed this decrease in activity, suggesting this process is independent of ABA. Since the similar decrease in enzyme activity was found in treatments with a wide range of germination responses, the transient expression of the enzyme appears not to be related to the moment of radicle protrusion. More likely, the decrease in activity indicates a change in the turnover of the enzyme. The occurrence of endo-ß-mannanase activity in the endosperm cap at any concentration of ABA or ABA analog, as well as the consistent increase and decrease of enzyme activity strongly suggests that one or more additional processes are required for radicle emergence, as was hypothesized by Still and Bradford (1997). Endo-ß-mannanase activity in the embryo displayed more variation in the ungerminated seed during imbibition but again no correlation with germination patterns was found.

Endo-ß-mannanase activity in the lateral endosperm of detipped seeds was hardly influenced by the (-)-enantiomers, but strongly reduced by the (+)-enantiomers (Fig. 4.5). There was a negative linear correlation between endo-ß-mannanase activity and mean time to germination, both for the lateral endosperm ($r^2 = 0.64$) and embryo ($r^2 = 0.86$). ABA strongly inhibits the enzyme activity in the lateral endosperm and embryo of detipped seeds (Toorop et al., 1996). The present study shows that the (+)-enantiomer is the active compound causing this inhibition. It is difficult to see how post-germinative endo-ß-mannanase activity can play a role in the completion of germination, and it is therefore not likely that germination and enzyme production are causally related. However, ABA may have a similar effect on the two different processes. Inhibition of endo-ß-mannanase activity is correlated with a slower growth rate of the emerging radicle (data not shown), supporting the postulation that enzyme activity in the radicle is associated with growth (Toorop et al., 1996).

Lettuce seed endo-ß-mannanase activity could be inhibited by analogs that were not effective in tomato seed. For example, PBI-59 was ineffective in tomato at 10 µM (Fig. 4.5) but inhibited mannanase activity in lettuce endosperms to trace values, which was comparable to the action of natural ABA (Table 2). As in tomato, there was no consistency between the effect of ABA and its analogs on germination and endo-ß-mannanase activity in lettuce (Fig.

4.7; Table 1). For example, PBI-82 was the strongest inhibitor of germination but had only a moderate effect on enzyme activity.

The use of the ABA analogs also allows the separation of germination from an increase in endo-ß-mannanase activity. There exists a controversy in the literature as to whether weakening of the endosperm cell walls by endo-ß-mannanase, which is produced almost exclusively in the endosperm (Halmer et al., 1976), is essential to allow the embryonic radicle to emerge, thus completing germination (reviewed in Bewley, 1997). The results presented here show those intact seeds which were able to complete germination on analogs PBI- 58, 82, 145 and 185 exhibited reduced endo-ß-mannanase activity compared to those which germinated on water; on two analogs the seeds produced negligible amounts of enzyme activity (PBI-59 and 89) in the germinated seeds. When incubated in the latter analogs, therefore, it appears that germination was completed in the absence of appreciable enzyme activity, the amount produced being less than 10% of the water controls (Table 1). Isolated endosperms were considerably more sensitive to the applied analogs, presumably because their uptake is greater. Inhibition of enzyme production in the intact seeds was reversible when the endosperms are isolated and the analogs washed out by copious rinsing in water (Table 2).

PBI-51 has been described as a competitive inhibitor of ABA (racemic) in *Brassica napus* (Wilen et al., 1993). However, when used in combination with either the natural ABA (PBI-58) or its mirror image (PBI-145) no competitive effect could be detected on tomato seed germination (Fig. 4.4). On the contrary, addition of PBI-51 showed synergistic effects with PBI-58 and PBI-145. Obviously the structure of ABA makes a difference, depending on the system in which ABA is active, as is the case for the inhibitory action of other ABA analogs. The existence of several (putative) ABA receptors and/or multiple transduction pathways has already been hypothesized (Hill et al., 1995). The inconsistency of synergism in one species and antagonism in the other species could be explained by this hypothesis. In tomato seeds the post-germinative endo-ß-mannanase activity and the inhibition of germination showed a similar response, suggesting that only one ABA receptor is involved in both processes. However, in lettuce these responses differed, which is suggestive of the involvement of more than one ABA-receptor. It should be noted, however, that uptake characteristics and stability of the different analogs may vary between species.

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Chapter 5

# Galactomannan-, cellulose-, and pectin-degrading enzymes are not involved in the inhibition of tomato (*Lycopersicon esculentum*) seed germination by abscisic acid

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## Abstract

The endosperm cell walls of tomato seeds are probably weakened prior to radicle protrusion by (an) enzyme(s) that hydrolyses cellulose and/or hemicellulose. The hemicellulosic fraction of the tomato endosperm wall is predominantly comprised of galactomannans or galactoglucomannans, but also consists of fucose, rhamnose, arabinose and xylose. The enzyme(s) responsible for endosperm cell wall weakening is presumably inhibited by ABA. Therefore, seven cell wall degrading enzymes, chosen based on endosperm cell wall composition, were assayed in extracts from tomato seeds imbibed in either water or ABA. No differences in any of the enzyme activities were found between seeds incubated in water or ABA in the lateral endosperm, endosperm cap or embryo. Since the enzyme activities we chose to study in the endosperm cap were not affected by ABA, it can be concluded that these enzymes do not control the second step in endosperm weakening resulting in the completion of germination.

## Introduction

Abscisic acid (ABA) is a known inhibitor of tomato seed germination. It was postulated that an ABA inhibited enzyme was responsible for inhibition of germination (Ni and Bradford, 1993). ABA was found to inhibit endo-ß-mannanase (EC 3.2.1.78) activity (Nomaguchi et al., 1995), which was held responsible for endosperm cap weakening (Groot and Karssen, 1992). However, others found that endo-ß-mannanase was not inhibited by ABA (Still and Bradford, 1997; Toorop et al., 1996). Recent work has shown that only the second phase of endosperm cap weakening, which was not related to water extractable endo-ß-mannanase activity, was inhibited by ABA (Toorop et al., unpublished results). Obviously, at least one other enzyme must be responsible for the second step of the endosperm cap weakening, playing a key role in the completion of germination. Evidently, an important characteristic of this enzyme would be its inhibition by ABA. A number of enzymes can be taken into consideration for this key role.

Osmoticum or ABA did not influence endo-ß-mannanase in endosperm caps of tomato seeds, while germination and mannose release were inhibited (Dahal et al., 1997). In *Datura ferox* seeds ß-mannosidase (EC 3.2.1.25) activity in the micropylar endosperm correlated with germination, albeit to a lesser extent than endo-ß-mannanase (Sanchez and de Miguel, 1997). This makes ß-mannosidase a potential enzyme that is regulated by ABA. Galactomannan (Groot et al., 1988) has been hypothesized as the main reserve food component of the endosperm cell walls in tomato seeds. α-Galactosidase (EC 3.2.1.22) is an enzyme that aids in the degradation of galactomannans by means of its debranching activity, cleaving off galactose side-chains from the mannose backbone. In guar this debranching activity is a prerequisite for the action of endo-ß-mannanase (McCleary and Matheson, 1975; McCleary, 1983).

Enzymes affecting cell wall components other than galactomannan can also play a role in endosperm weakening, e.g. cellulose or pectin degrading enzymes. Like endo-ß-mannanase and ß-mannosidase activity, cellulase (EC 3.2.1.4) activity is phytochrome-regulated in *Datura ferox* seeds and is only expressed in the endosperm part containing the radicle (Sanchez et al., 1986). Cellulase activity was also found in ungerminated tomato seeds (Leviatov et al., 1995), but no effort was made to determine the spatial distribution of the enzyme. It has been hypothesized that a cell separation process in the endosperm cap of tomato seeds immediately precedes radicle emergence and would be involved in the lowering

of the mechanical resistance (Karssen et al., 1989). Recently, an exo-polygalacturonase (EC 3.2.1.67) was found predominantly in radicle tips of tomato seeds (Sitrit et al., 1996) and it is conceivable that its presence could also occur in the endosperm cap. Therefore, cellulose and polygalacturonic acid degrading enzymes may also be involved in the completion of germination.

In lettuce seeds a high salt extractable endo-ß-mannanase was found in the endosperm, which correlated with germination conditions (Dutta et al., 1997). High salt extractability of endo-ß-mannanase activity has never been studied in tomato seeds, although it is conceivable that a cell wall bound form of this enzyme is present as in lettuce seeds. For this reason not only endo-ß-mannanase, but all hydrolytic enzymes that were tested for ABA inhibition were extracted with high salt concentrations. The hemicellulosic composition of the different tissues in the tomato seed was analysed and compared.

## 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). Seeds were stirred in 1% (v/v) HCl for 2 h to remove the locular tissue, rinsed, dried and stored at 5°C. Seeds were surface-sterilized in 1% sodium hypochlorite, rinsed in demineralized water and imbibed in demineralized water. 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). For determination of enzyme activity parts from ten fresh seeds that showed no radicle protrusion were homogenized for 3 min at 1600 rpm in a Micro-dismembrator U (B. Braun Biotech International, Melsungen, Germany) using 2 mL Eppendorf tubes. All grinding materials were kept in liquid nitrogen. Crude extracts were made by adding 900, 300 or 600 µL of demineralized water to lateral endosperms, endosperm caps or embryos, respectively. The extracts were sonicated for 20 min and centrifuged in a microfuge at 4°C for 10 minutes at 14000 rpm. The supernatant was removed and used for enzyme assays. The pellet was re-extracted and sonicated in water or NaCl solution of different molarity.

*Cell wall neutral sugar analysis.* For hemicellulosic composition of cell walls seeds were incubated at 4°C for 2 h, seed parts were collected on ice, plunged into liquid nitrogen and stored at -80°C until cell wall isolation. All cell wall composition determinations were repeated using three (embryo, testa, lateral endosperm) or two (micropylar endosperm) independent samples, depending on the yield of cell wall material from the various seed components. Cell walls from approximately 300 seed parts were isolated by grinding seed parts in ice-cold 70% ethanol and washing the homogenate 7 times with 10 mL ice-cold 70% (v/v) ethanol while on a fine nylon mesh in a glass funnel (Fry, 1988). The alcohol-insoluble residue (AIR) was suspended in 35 mL 70% ethanol and agitated constantly for 16 h at 3°C. The AIR was recovered by centrifugation at 3000 g for 15 min. The cell wall preparations were agitated (2 x 24 h) in 90% (v/v) dimethyl sulfoxide (DMSO) at 23°C (Fry, 1988). The DMSO-insoluble cell wall preparations were lyophilized to dryness, the DMSO-soluble material was dialysed and lyophilized. Dry weights of the fractions were determined prior to hydrolysis. The samples were resuspended in 1 mL 2M trifluoroacetic acid (TFA) in gas chromatography vials and the lids crimped in place. The vials were heated for 1 h at 120°C in a heating block (Fry, 1988). After hydrolysis the supernatant was collected upon centrifugation at 2000 g for 5 min and lyophilized to dryness. Dried hydrolysates were reconstituted in 1 mL demineralized water on ice for 4 h with intermittent vortexing, centrifuged at 14000 g for 10 min and diluted 10-fold with demineralized water. Monosaccharides were identified and quantified by Pulsed Amperometric Detection (PAD) (Townsend et al., 1988) using a Dionex Series 4500 HPLC and a CarboPAC-PA1 tm pellicular anion exchange column with column guard (Dionex, Sunnyvale, CA). Throughout the study, the PAD electrode potentials were set to cycle at E1 0.05 V, E2 0.6 V, and E3 -0.06 V with 500, 10, and 5 ms durations. Column and eluent temperature was maintained at 23°C. Samples were eluted isocratically in 22.5 mM sodium hydroxide (NaOH) over a 30-min period and the column was washed in 1 M sodium acetate (NaOAc), 150 mM NaOH for 5 min. The column was recharged by passing 150 mM NaOH through it for 10 min, and re-equilibrated in 22.5 mM NaOH for 10 min prior to injection of the next sample. This protocol resulted in optimal (near baseline) resolution of glucose, mannose and xylose.

*Enzyme assays.* Endo-ß-mannanase. A modified diffusion assay (Downie et al., 1994) was used for determining endo-ß-mannanase activity. Gels (0.5 mm thick) were used containing 0.1 % (w/v) locust bean gum (LBG; Sigma) in McIlvaine buffer (pH 5.0) and 0.8%

type III-A agarose (Sigma) and made on Gel bond film (Pharmacia). Samples of 2 µL were applied to holes that were punched in the gel with a 2-mm paper punch. Gels were incubated for 20 h at 25°C, and then washed in McIlvaine buffer (pH 5.0) for 30 min, stained with 0.5% (w/v) Congo Red (Sigma) for 30 min, washed with 96% ethanol for 10 min, and destained in 1 M NaCl for at least 24 h. All staining steps were performed on a gently rotating platform. Commercial endo-ß-mannanase from *Aspergillus niger* (Megazyme, North Rocks, Sydney, Australia) was used to generate a standard curve. Calculation of enzyme activity in the samples was according to Downie et al. (1994).

α-Galactosidase. A modified assay was used as described by Groot et al. (1988). A reaction volume of 300 µL containing 150 µL McIlvaine buffer (pH 5.0), 30 µL p-nitrophenyl-α-D-galactopyranoside (10mM) (Sigma) and 60 µL enzyme extract was incubated at 35°C for 10 min. The reaction was stopped by addition of 150 µL $Na_2CO_3$ (1 M). Extinction was determined with a microtiter plate reader at 405 nm in a microtiter plate. Activities were calculated on the basis of *p*-nitrophenol released.

ß-Mannosidase A modified assay was used as was described by Groot et al. (1988). The reaction was similar to the α-galactosidase assay, except for the use of p-nitrophenyl-ß-D-mannopyranoside (Boehringer Mannheim) as the substrate and an incubation time of 2 h.

LBG-degrading activity. Reducing sugars were determined according to the method used by Knegt et al. (1988) using locust bean gum instead of polygalacturonic acid as substrate. A volume of 0.5 mL extract was added to 2.0 mL buffer (0.2 M sodium acetate pH 4.0 - 0.4 M NaCl) and 2.5 mL substrate (0.2% locust bean gum). The reaction temperature was 35°C. The typical incubation time was 1 h; longer incubation times were applied when activities were very low.

Polygalacturonase (EC 3.2.1.15) Reducing sugars were determined according to the method used by Knegt et al. (1988). The activity of polygalacturonase from tomato seeds showed an optimum at pH 5.0 (data not shown). A volume of 0.5 mL extract was added to 2.0 mL buffer (0.2 M sodium acetate pH 5.0 - 0.4 M NaCl) and 2.5 mL substrate (0.1% polygalacturonic acid). The reaction temperature was 35°C. The typical incubation time was 1 h; longer incubation times were applied when activities were very low.

Exo-polygalacturonase The reaction was similar to the α-galactosidase assay, except for the use of p-nitrophenyl-ß-D-galacturonide (Sigma) as substrate, 120 µL enzyme extract and an incubation time of 24 h.

Cellulase Reducing sugars were determined according to the method used by Knegt et al. (1988) using carboxymethyl-cellulose instead of polygalacturonic acid as substrate. A volume of 0.25 mL extract was added to 1.0 mL buffer (0.2 M sodium acetate pH 5.0 - 0.4 M NaCl) and 1.0 mL substrate (0.2% carboxymethyl-cellulose). The reaction temperature was 35°C. The typical incubation time was 2 h; longer incubation times were applied when activities were very low.

ß-glucosidase (EC 3.2.1.21) The assay was similar to the α-galactosidase assay, except for the use of p-nitrophenyl-ß-D-glucopyranoside (Sigma) as a substrate and an incubation time of 5 h.

*Separation of enzymes.* Gel filtration was performed according to Knegt et al. (1988) using Sephacryl HR S-200 (Pharmacia) as the matrix.

## Results

The identity and quantities of cell wall neutral sugars in the hemicellulosic fraction of tomato seeds were determined for lateral endosperm, endosperm cap, testa and embryo. The results for the endosperm are generally consistent with previous reports (Fig. 5.1a) (Groot et al., 1988; Dahal et al., 1997). The sugar contents of the lateral endosperm and endosperm cap

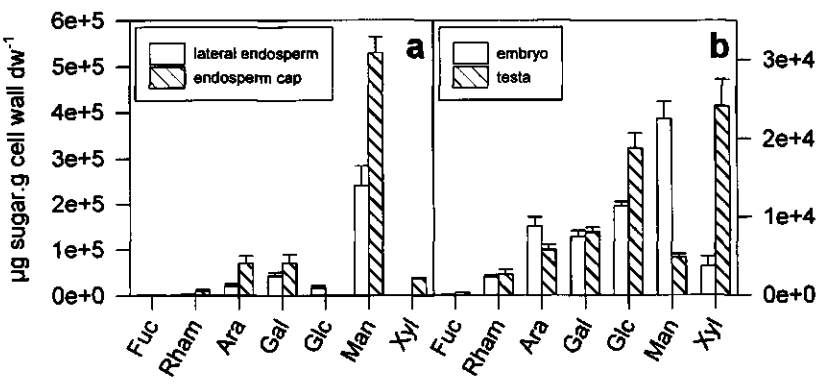


**Fig. 5.1** Neutral sugar composition of the hemicellulose fraction of cell walls from tomato seed parts: lateral endosperm and endosperm cap (a) and embryo and testa (b). Data represent the average of 3 (embryo, lateral endosperm, testa) or 2 (endosperm cap) independent samples. Error bars represent the standard deviation.

were similar except for the absence of glucose in the latter and of xylose in the former tissue. Mannose was the major sugar component in both the lateral endosperm and endosperm cap (74% in both tissues). The mannose/galactose ratio was 5.7 in the lateral endosperm and 8.0 in the endosperm cap, however, this difference was not significant due to the low sampling number. The hemicellulosic cell wall component of the embryo contained less sugar than the endosperm (Fig. 5.1b). Again mannose was the major sugar component (40%). In the testa xylose and glucose were the main components (Fig. 5.1b), implying the predominance of xylans or xyloglucans.

Wild-type seeds were incubated for 2 days in water or 10 µM ABA and ungerminated seeds were dissected into lateral endosperm, endosperm cap and embryo. Sequential extracts were made using a step-wise increase in the concentration of NaCl from 0 to 5 M in the re-extraction medium. No endo-ß-mannanase activity could be observed in any fraction in the lateral endosperm (Fig. 5.2a). The endosperm cap contained 50% of the endo-ß-mannanase activity in the water extracts and 50% in the 0.1 and 0.5 M salt extracts (Fig. 5.2b). In the embryo most of the endo-ß-mannanase activity (92%) was found in the water extracts, while only 8% was found in a wide range of the salt extracts (Fig. 5.2c). Small amounts of ß-mannosidase activity were found in the water extract and in the 0.5 M extract of both the lateral endosperm and endosperm cap, whereas the amounts of this enzyme were more substantial in the embryo (Fig. 5.2d-f). Some of the ß-mannosidase activity in the embryo was in the water extracts, while most of the activity (54%) was found in the 0.5 M extract. α-Galactosidase activity was found in the water extracts in all parts of the seed (Fig. 5.2g-j). No substantial amounts were detected in any of the salt extracts. No major differences in the activity of any of these enzymes were found between water and ABA imbibed seeds. In seeds of which the radicle had protruded most of the ß-mannosidase activity in the embryo was located in the cotyledons (74%) while only small amounts of activity were in the radicle and hypocotyl (26%); for α-galactosidase 58% of the total activity in the embryo was located in the cotyledons (data not shown).

When reducing sugars were determined with LBG as a substrate, a combination of the results of endo-ß-mannanase, ß-mannosidase and α-galactosidase was found for all of the seed parts (Fig. 5.2k-m). The lateral endosperm showed activity in the water extracts and the higher salt extracts, reflecting α-galactosidase and ß-mannosidase activity. The endosperm cap contained activity in the water extracts and in a wide range of salt extracts, representing

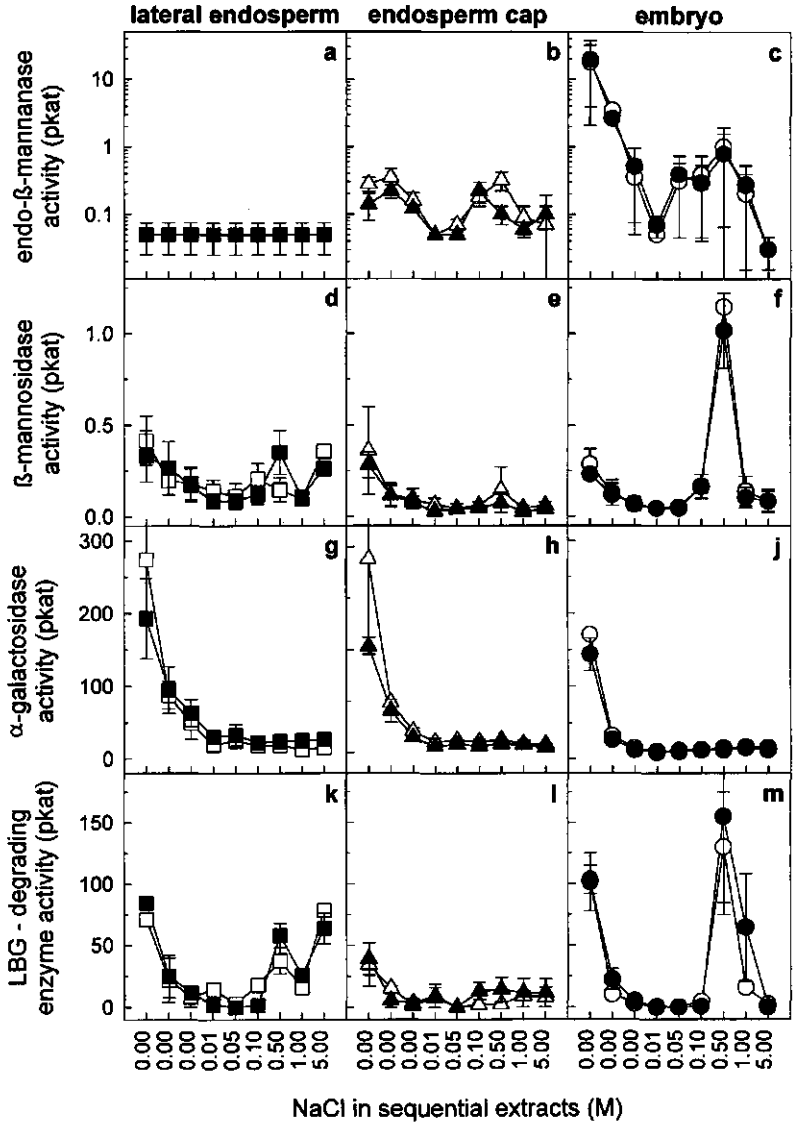


**Fig. 5.2** Endo-ß-mannanase activity (a-c), ß-mannosidase activity (d-f), α-galactosidase activity (g-j) and LBG degrading activity (k-m) in lateral endosperm (a, d, g, k), endosperm cap (b, e, h, l) and embryo (c, f, j, m) of 2 days imbibed ungerminated tomato seeds. Seeds were imbibed in water (open symbols) or 10 µM ABA (closed symbols) and sequentially extracted in water (3x), 0.01 M, 0.05 M, 0.1 M, 0.5 M, 1.0 M and 5.0 M NaCl. Data represent the average and standard deviation of two independent experiments.

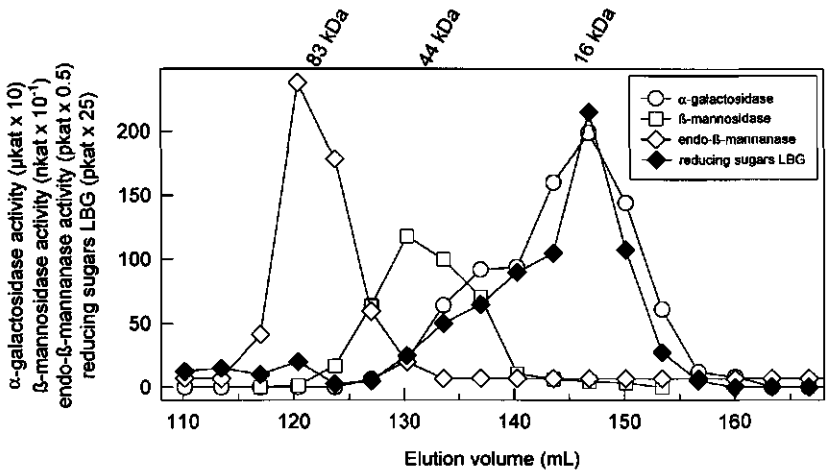


**Fig. 5.3** α-Galactosidase, ß-mannosidase, endo-ß-mannanase, and LBG degrading activity upon elution over a gelfiltration column of a 1.0 M NaCl extract of ungerminated seeds that were incubated in water for 52 h.

activity of all three enzymes. The embryo contained high activity in the first water extract and in the higher salt extracts, again reflecting activity of α-galactosidase, ß-mannosidase and endo-ß-mannanase. A 1.0 M NaCl extract of 52 h imbibed ungerminated seeds, eluted over a gel filtration column, contained an endo-ß-mannanase of approximately 83 kDa, a ß-mannosidase of approximately 44 kDa, and an α-galactosidase of approximately 16 kDa (Fig. 5.3). Measuring reducing sugars with LBG as a substrate yielded activity that overlapped with endo-ß-mannanase, ß-mannosidase and α-galactosidase activity. Most of the activity detected by the reducing sugars coeluted with α-galactosidase activity, only a very small fraction coeluted with endo-ß-mannanase activity.

Polygalacturonase activity was also present in the ungerminated seed, both in the endosperm and in the embryo (Fig.5.4 a-c). The testa contained negligible amounts of polygalacturonase activity (data not shown), making it likely that the enzyme did not originate from any remnants of the locular tissue of the fruit. In the endosperm the fraction extracted by salt was as large as the fraction extracted by water; in the embryo the fraction extracted by salt was smaller. The same result was observed when exo-polygalacturonase activity was determined (Fig. 5.4 d-f). No differences could be seen between ABA and water imbibed seeds. The overall similarity and amounts of polygalacturonase and exo-polygalacturonase

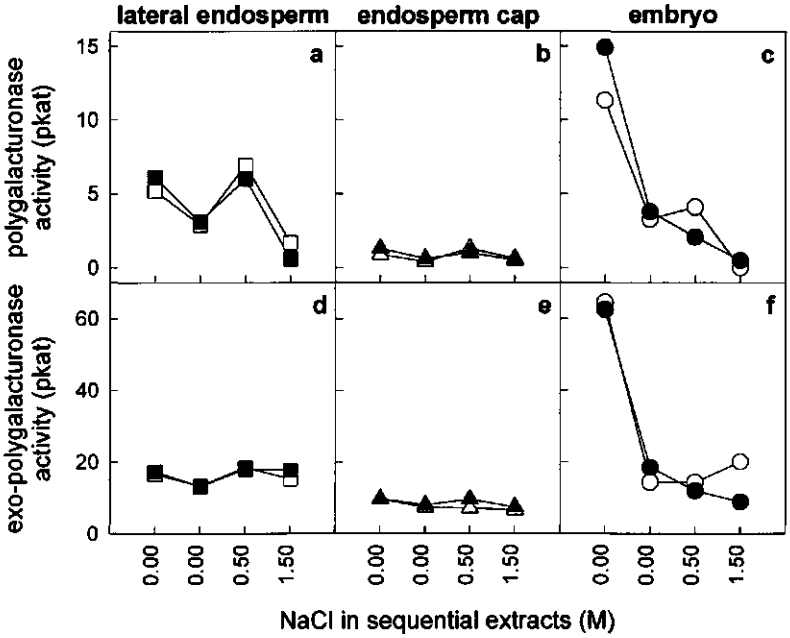


**Fig. 5.4** Polygalacturonase (a-c) and exo-polygalacturonase (d-f) activity in lateral endosperm (a, d), endosperm cap (b, e) and embryo (c, f) of 2 days imbibed ungerminated tomato seeds. Seeds were imbibed in water (open symbols) or 10 µM ABA (closed symbols) and extracted sequentially in water (2x), 0.5 M and 1.5 M NaCl. Data represent the result of a single experiment.

activity in the extracts suggested that the former activity was completely accounted for by the latter. This hypothesis could not be affirmed by gel filtration. After elution over a gel filtration column no distinct peak for either enzyme was detected in a water or a 1.0 M NaCl extract of seeds incubated for 52 h. Instead, activity was evenly spread over a range of fractions (data not shown).

Cellulase as well as ß-glucosidase activities were also found in the ungerminated seed (Fig. 5.5). ß-Glucosidase activity was found in the first water extract and in the 0.5 M NaCl extract. For cellulase activity a similar pattern was observed, with relatively more activity in the 1.5 M NaCl extract. Both enzyme activities were not inhibited by ABA; although enzyme activity in the endosperm cap was reduced in the water and 0.5 M NaCl extract, it was higher in the 1.5 M NaCl extract. Gel filtration of the water extract of 52 h imbibed ungerminated seeds gave a clear peak for ß-glucosidase at 50 kDa, while cellulase activity was not detected (data not shown). Gel filtration of the NaCl extract displayed cellulase and ß-glucosidase

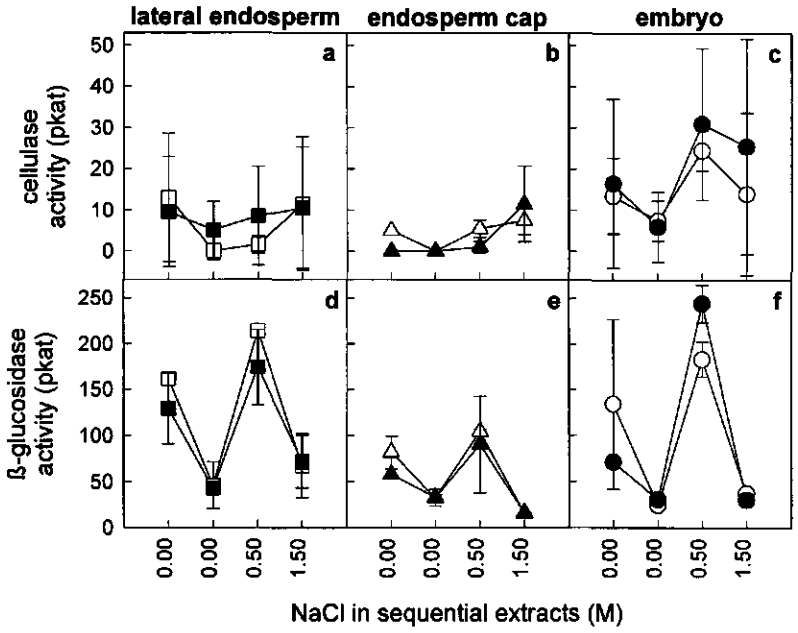


**Fig. 5.5** Cellulase (a-c) and ß-glucosidase (d-f) activity in lateral endosperm (a, d), endosperm cap (b, e) and embryo (c, f) of 2 days imbibed ungerminated tomato seeds. Seeds were imbibed in water (open symbols) or 10 µM ABA (closed symbols) and extracted sequentially in water (2x), 0.5 M and 1.5 M NaCl. Data represent the average and standard deviation of two independent experiments.

activity at 43 and 56 kDa respectively (Fig. 5.6); coelution as in the case of LBG degrading activity was not observed.

## Discussion

The cell wall analysis of the hemicellulosic fraction of tomato seeds showed that mannose was the major component in the embryo as was shown by Dahal et al. (1997). This supports the hypothesis by Toorop et al. (1996) that the occurrence of endo-ß-mannanase in the embryo is involved in growth. The analysis also confirmed the result of Groot et al. (1988) and Dahal et al. (1997) that mannose is the major component in the endosperm. No difference was found in the mannose content between lateral endosperm and endosperm cap. The lateral endosperm contained only 5% glucose, while the endosperm cap did not contain any

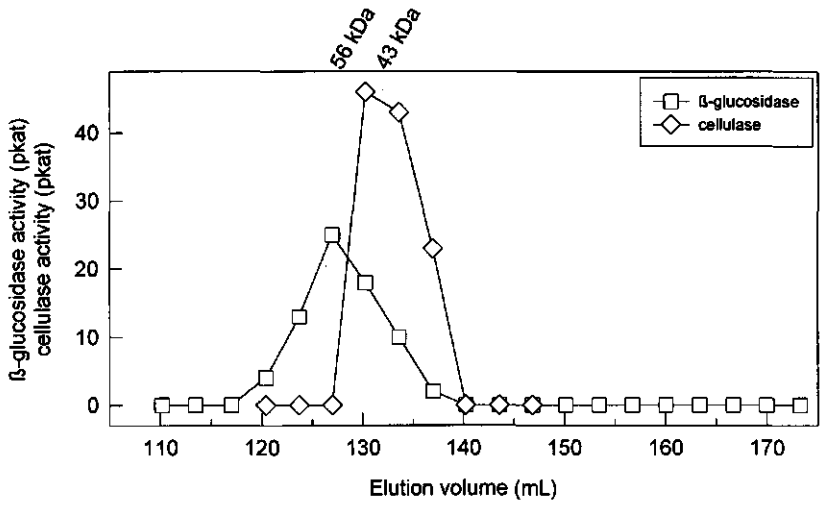


**Fig. 5.6** ß-Glucosidase and cellulase activity upon elution over a gel filtration column of a 1.0 M NaCl extract of seeds that were incubated in water for 52 h.

detectable glucose at all. This is considerably less than the 21% as recalculated from Groot et al. (1988), which was probably due to cellulose. Based on this result, the occurrence of galactoglucomannans can at least be excluded in the endosperm cap. Galactomannans remained a likely component of the endosperm cell wall and its degrading enzymes were likely candidates for the ABA-regulated completion of germination.

Sequential salt extraction with increasing concentrations of NaCl showed that endo-ß-mannanase activity in the endosperm cap was present in both the water soluble and the intermediate (0.1 and 0.5 M) NaCl soluble fractions. In the embryo most of the enzyme activity was found in the water soluble fraction. It was hypothesized that an ABA inhibited enzyme in the endosperm cap would be responsible for tomato seed germination (Ni and Bradford, 1993). No major differences between water and ABA imbibed seeds were found for endo-ß-mannanase in any salt soluble fraction in endosperm cap or embryo. Therefore it can be concluded that no high-salt soluble endo-ß-mannanase is responsible for ABA inhibited germination. This is in accordance with the results of Still and Bradford (1997) and Toorop et al. (1996). A discrepancy was found between endo-ß-mannanase activity and LBG-degrading activity (Figs. 2 and 3) since LBG-degrading activity has been mentioned to represent endo-ß-

mannanase activity (Dutta et al., 1997). Here it hardly represents endo-ß-mannanase activity, but α-galactosidase activity instead (Fig. 5.3).

For guar it was hypothesized that a high degree of substitution of the mannan backbone with galactose would require α-galactosidase as a debranching enzyme thus creating free (unsubstituted) mannose residues and enabling the endo-ß-mannanase to hydrolyse the backbone (McCleary and Matheson, 1975; McCleary, 1983). In tomato the mannose/galactose ratio was 8.0 for the endosperm cap. The α-galactosidase activity appeared not to be inhibited by ABA (Fig. 5.2g-j). Hence this enzyme is not a candidate for causing the inhibition of germination by ABA.

Mannose release from endosperm caps, but not endo-ß-mannanase activity, was suppressed during imbibition in osmoticum or ABA which correlated with germination (Dahal et al., 1997), suggesting that ß-mannosidase is responsible for the inhibition of germination and mannose release. However, our results showed that ß-mannosidase activity is not controlled by ABA, and therefore not responsible for the inhibition of germination. Moreover, the enzyme is mostly present in the cotyledons, implying that during degradation of the endosperm oligomannans are imported into the cotyledons and subsequently hydrolysed by ß-mannosidase prior to further metabolization, similar to the process in guar (McCleary, 1983).

Cellulase was previously not detected in the endosperm of ungerminated tomato seeds (Groot et al., 1988). Enzyme activity was found in ungerminated seeds prior to germination (Leviatov et al., 1995), however, no distinction was made between endosperm and embryo. Our data showed that cellulase activity is present in the lateral endosperm, endosperm cap and embryo. Also ß-glucosidase, the exo-enzyme that hydrolyses 1→4 linked glucans, was detected in all seed parts. Neither enzyme activity was inhibited by ABA, and therefore neither cellulase nor ß-glucosidase account for the inhibition of germination by ABA.

An enzyme causing cell separation has previously been mentioned as a candidate for the ABA-regulation of germination (Karssen et al., 1989). We found polygalacturonase activity which was probably entirely accounted for by exo-polygalacturonase activity. No differences were found between water and ABA imbibed ungerminated seeds at 52 h after the start of imbibition, at which point 31% of the seeds had completed germination. Therefore exo-polygalacturonase activity is not responsible for the inhibition of germination by ABA. Sitrit et al. (1996) found an exo-polygalacturonase which was associated with the radicle tip of

tomato seeds, and proposed the involvement of this enzyme in cell wall loosening necessary for radicle protrusion. No polygalacturonase activity was detected by these authors, confirming our conclusion that most of the polygalacturonase activity was accounted for by exo-polygalacturonase activity.

Interestingly, the molecular weight of endo-ß-mannanase after gelfiltration was 83 kDa, approximately twice the weight of 39 kDa after SDS-PAGE (Nonogaki and Morohashi, 1996). Perhaps a different extraction method caused dissociation of the dimeric enzyme. However, under the circumstances a 15-fold enzyme activity is found in the embryo. Since the enzyme detected after gel filtration originated from an extract made of intact ungerminated seeds, the 83 kDa protein most likely represents the enzyme of embryonic origin while the enzyme from the endosperm cap is negligable.

Summarising, we may conclude that none of the investigated enzymes (endo-ß-mannanase, α-galactosidase, ß-mannosidase, cellulase, ß-glucosidase or exo-polygalacturonase) are inhibited by ABA. According to our hypothesis these enzyme activities in the endosperm cap contribute to the state of endosperm weakening. Since no enzyme activity declined in the presence of ABA, the difference in the second step of the puncture force decrease between water and ABA imbibed seeds (Toorop et al., unpublished results) cannot be attributed to one of these enzymes. Another enzyme, yet unknown, must be responsible for the further weakening of the endosperm cap.

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Chapter 6

# Endosperm cap weakening and endo-ß-mannanase activity during priming of tomato (*Lycopersicon esculentum* cv. Moneymaker) seeds are initiated upon crossing a threshold water potential

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## Abstract

The relationship between the endosperm cap weakening and endo-ß-mannanase activity during priming and the germination speed after priming was studied in tomato (*Lycopersicon esculentum* Mill.) seeds. During priming of seeds in -0.4 MPa PEG the mechanical restraint of the endosperm cap decreased while the endo-ß-mannanase activity in the endosperm cap increased. There was no decrease of puncture force and no increase in endo-ß-mannanase activity in seeds in -1.0 MPa PEG. A decrease of puncture force correlated with a porous appearance of the endosperm cap cell walls on scanning micrographs. During priming in -0.7 MPa two classes of seeds could be distinguished; one with decreased puncture force and one without. A strong correlation was found between the lowering of the mechanical restraint and endo-ß-mannanase activity. It was concluded that individual seeds have to cross a threshold water potential in order to develop enzyme activity and lower their puncture force. Germination speed after priming correlated positively with puncture force during priming, depending on the osmotic potential. Seeds in -1.0 MPa PEG improved their germination speed, however they did not show a decrease in puncture force. Therefore it was concluded that lowering of the endosperm restraint during priming positively affects the germination speed of primed seeds but is not a prerequisite.

## Introduction

Osmotic priming is a widely used method to improve seed quality. It causes an arrest in seed germination after phase II of the triphasic pattern of water uptake, when no changes in water content occur and which comprises major metabolic events preparing the seed for radicle emergence (Bewley and Black, 1994). The seed is refrained from entering phase III, which includes radicle elongation and completion of germination. In tomato seeds osmotic priming generally causes a faster germination, higher final germination percentage at reduced osmotic potential (Mauromicale and Cavallaro, 1995) and faster field emergence (Haigh et al., 1986) which may result in greater mean plant dry weights, leaf areas, and ground cover percentages (Alvarado et al., 1987). A linear relationship was found for germination rate and hydrothermal priming time, indicating that both the external osmotic potential during priming and the duration of the priming period add up to the improved germination rate (Bradford and Haigh, 1994). The threshold temperature and threshold water potential for metabolic advancement were considerably lower than the corresponding thresholds for radicle emergence, thus allowing for metabolic events during phase II and explaining the improved performance after priming.

Several metabolic events occur during priming that may play a beneficial role in radicle emergence upon rehydration. DNA synthesis was detectable in leek embryos during priming in -1.0 MPa PEG, which was suggested to reflect DNA repair, thus contributing to rapid germination and embryo growth upon priming. Protein synthesis was enhanced during priming and correlated with the rate of germination (Bray et al., 1989). Nuclear replication in embryo root tips of tomato seeds progressed in -1.0 MPa PEG in a similar way as in water, yet slower and with a lag period of 3 days instead of 1 day (Bino et al., 1992). A strong correlation was found between increase in replicated nuclei 12 h before radicle emergence and germination speed, indicating the relevance of nuclear replication. A higher concentration of the osmotic agent used for the priming solution (-1.5 MPa) caused a lower percentage 4C nuclei, possibly due to water limitation in the embryo, and resulted in nullification of improved germination (Lanteri et al., 1994). ß-Tubulin accumulation, like nuclear replication a cell-cycle-related process, occurred in the radicle tip prior to radicle protrusion and was hypothesized to be a prerequisite for completion of germination (De Castro et al., 1995). It accumulated in the radicle tip during osmopriming and coincided with nuclear replication. During priming in -0.6 MPa PEG rRNA levels increased and the 25S:18S ratio was restored

to a near theoretical value (Coolbear and Grierson, 1979). In low-vigour leek embryos damaged rRNA was replaced by intact rRNA during osmopriming (Davison et al., 1991).

Weakening of the endosperm cap has been proposed as the mechanism that controls tomato seed germination (Groot and Karssen, 1987; Haigh and Barlow, 1987). Endo-ß-mannanase appeared to play a key role in the weakening of the endosperm cap (Groot et al., 1988). It was shown that seeds develop endo-ß-mannanase activity in the endosperm cap during priming (Karssen et al., 1989; Nonogaki et al., 1992; Still and Bradford, 1997). The puncture force of seeds decreased during priming in -1.2 MPa PEG, which correlated with a faster germination upon drying (Karssen et al., 1989). However, none of these studies showed a clear relationship between endo-ß-mannanase activity, puncture force and improved seed performance, nor studied seeds in different concentrations of the priming agent. Still et al. (1997) showed that a single seed assay of endo-ß-mannanase is more accurate in the biochemical description of a population of seeds. In the present study we attempted to correlate endo-ß-mannanase activity, puncture force and seed performance using single seed assays for enzyme activity and puncture force upon priming in different concentrations of PEG.

## Materials and methods

*Plant materials.* Seed material was generated as described previously (Toorop et al., 1996). Briefly, tomato plants (*Lycopersicon esculentum* Mill. cv Moneymaker) were grown in a greenhouse in 1992 for seed production. Seeds were stirred in 1% (v/v) HCl for 2 h to remove the locular tissue, dried and stored at 5°C. Seeds were surface-sterilized in 1% sodium hypochlorite, rinsed in demineralized water and imbibed in demineralized water or in PEG solutions of different osmotic potential, according to Michel and Kaufmann (1973). 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. Mean time until germination (MTG) was used to express the germination rate, and was calculated as the time to 20% final germination of the batch. The denotations lateral endosperm and endosperm cap were used as described previously (Toorop et al., 1996).

*Puncture force.* Generally, the puncture force of individual endosperm caps was measured as described by 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 lbs. A needle with a hemispherical tip and a diameter of 0.38 mm was placed on the load cell. 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 at least 24 measurements. Significance was tested using a two-sample t-test according to Meilgaard et al. (1987).

*Diffusion assay.* A modified diffusion assay (Downie et al., 1994) was used for determining endo-ß-mannanase activity. Gels (0.5 mm thick) were used containing 0.1 % (w/v) locust bean gum (Sigma) in McIlvaine buffer (pH 5.0) and 0.8% type III-A agarose (Sigma) and made on Gel bond film (Pharmacia). Leachates of single endosperm caps were made upon measuring the puncture force by incubating in 20 µL McIlvaine buffer (pH 5.0) for 2 h at room temperature according to Still et al. (1997). Samples of 2 µL were applied to holes that were punched in the gel with a 2-mm paper punch. Gels were incubated for 20 h at 25°C, and then washed in McIlvaine buffer (pH 5.0) for 30 min, stained with 0.5% (w/v) Congo Red (Sigma) for 30 min, washed with 96% ethanol for 10 min, and destained in 1 M NaCl for at least 24 h. All staining steps were performed on a rotating platform while rotating gently. Commercial endo-ß-mannanase from *Aspergillus niger* (Megazyme, North Rocks, Sydney, Australia) was used to generate a standard curve. Calculation of enzyme activity in the samples was according to Downie et al. (1994).

*Cryo-scanning electron microscopy.* Imbibed seeds were fixed on a stub with colloidal carbon adhesive (Leit-C, Neubauer, Germany), plunge-frozen in liquid nitrogen and affixed to a stub carrier. This was transferred under vacuum at near liquid nitrogen temperature to the cold stage of a cryo-preparation chamber (CT 1500-HF, Oxford Instruments, UK). The seeds were freeze-fractured with a cold scalpel knife, heated up to -80°C, partially freeze-dried and sputter-coated with 3 nM of Pt. The seeds were placed at the cryostage of the scanning electron microscope equipped with a cold field emission electron gun (JEOL 6300F). Observations were made at -180°C using a 2.5-5 kV accelerating voltage. Digital images were taken, loaded onto a computer and printed on photographic paper.

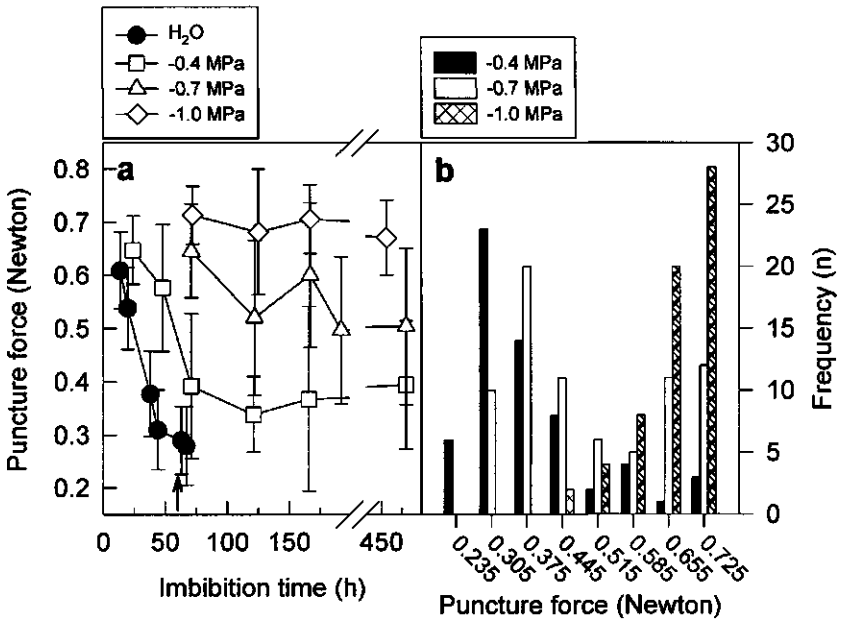



**Fig. 6.1** The puncture force of seeds in water, -0.4 MPa, -0.7 MPa and -1.0 MPa PEG versus imbibition time (a), and a frequency distribution of the puncture force of seeds after 456 h in -1.0 MPa PEG and 480 h in -0.4 and -0.7 MPa PEG (b). Data points in panel A are averages of at least 24 measurements, error bars are standard deviations. The arrow in panel A indicates the start of radicle emergence in water. The histogram in panel b represents at least 61 measurements per treatment.

## Results

Wild-type seeds were imbibed in water or PEG solutions of -0.4, -0.7 or -1.0 MPa. Incubation in -0.7 and -1.0 MPa did not show germination, incubation in -0.4 MPa showed 3% germination after 20 days. The puncture force was measured and plotted against the imbibition time (Fig. 6.1a). The seeds incubated in -0.4 MPa PEG solution showed a decrease in puncture force which was slower compared to the decrease in water. After 121 h the minimum puncture force was reached, which was significantly higher than the puncture force of water imbibed seeds after 63 or 67 h, followed by a small increase at 480 h. When seeds were incubated in -1.0 MPa PEG a small decrease in puncture force was only seen after 456 h. Seeds that were incubated in -0.7 MPa PEG showed a decrease that was intermediate compared to -0.4 and -1.0 MPa. A frequency distribution of the puncture force after 20 days

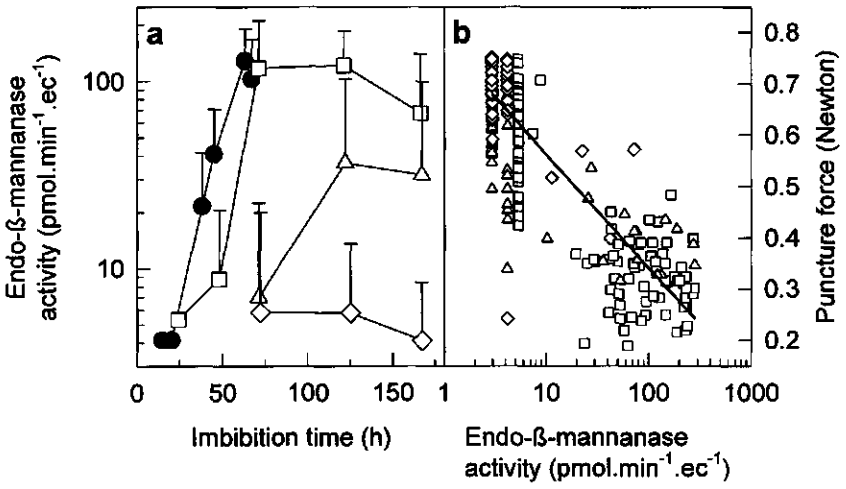



**Fig. 6.2** The endo-ß-mannanase activity of endosperm caps of seeds in water, -0.4 MPa, -0.7 MPa and -1.0 MPa PEG versus imbibition time (a), and the puncture force of individual seeds in -0.4 MPa, -0.7 MPa and -1.0 MPa PEG after incubation for 3, 5 and 7 days versus the corresponding endo-ß-mannanase activity (b). The line drawing represents a linear fit (Y = 0.783 - 0.221 X, $r^2 = 0.734$). Data points in panel A are averages of at least 24 measurements, error bars are standard deviations, symbols as in Fig. 6.1a.

showed a low median for the seeds in -0.4 MPa PEG, a high median for the seeds in -1.0 MPa PEG and a bimodal distribution for the seeds incubated in -0.7 MPa PEG (Fig. 6.1b). Seeds incubated in -0.7 MPa PEG for 5 or 7 days showed the same bimodal distribution (not shown). The median of the seeds with decreased puncture force incubated in -0.7 MPa PEG was still higher than the median of the seeds incubated in -0.4 MPa PEG. Endo-ß-mannanase activity developed in seeds incubated in water after 38 h, in -0.4 MPa PEG after 48 h, in -0.7 MPa after 122 h while in -1.0 MPa no enzyme activity was detected within 7 days (Fig. 6.2a). The puncture force of all seeds imbibed in PEG at all sampling times was plotted against the corresponding endo-ß-mannanase activity (Fig. 6.2b). Two classes could be distinguished; one with a decreased puncture force and an increased endo-ß-mannanase activity, and one without.

After incubation in PEG seeds were washed, dried back and imbibed in water. The MTG was plotted against the priming time (Fig. 6.3a). Seeds incubated in -0.4 MPa PEG

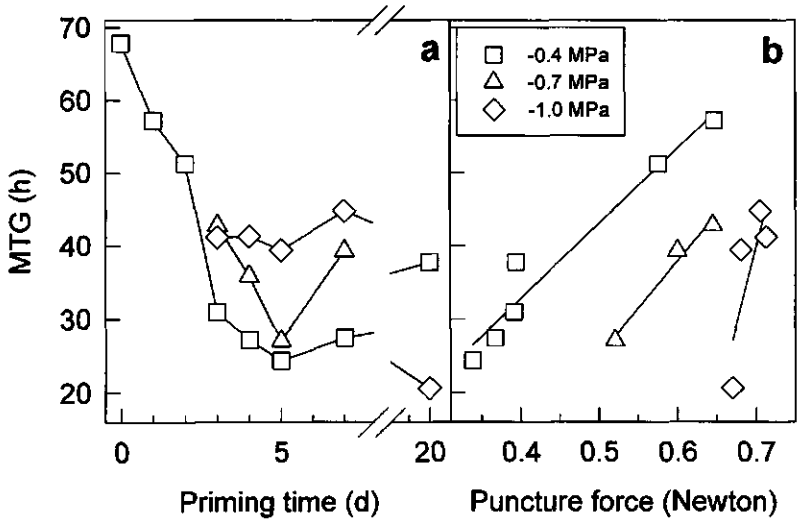
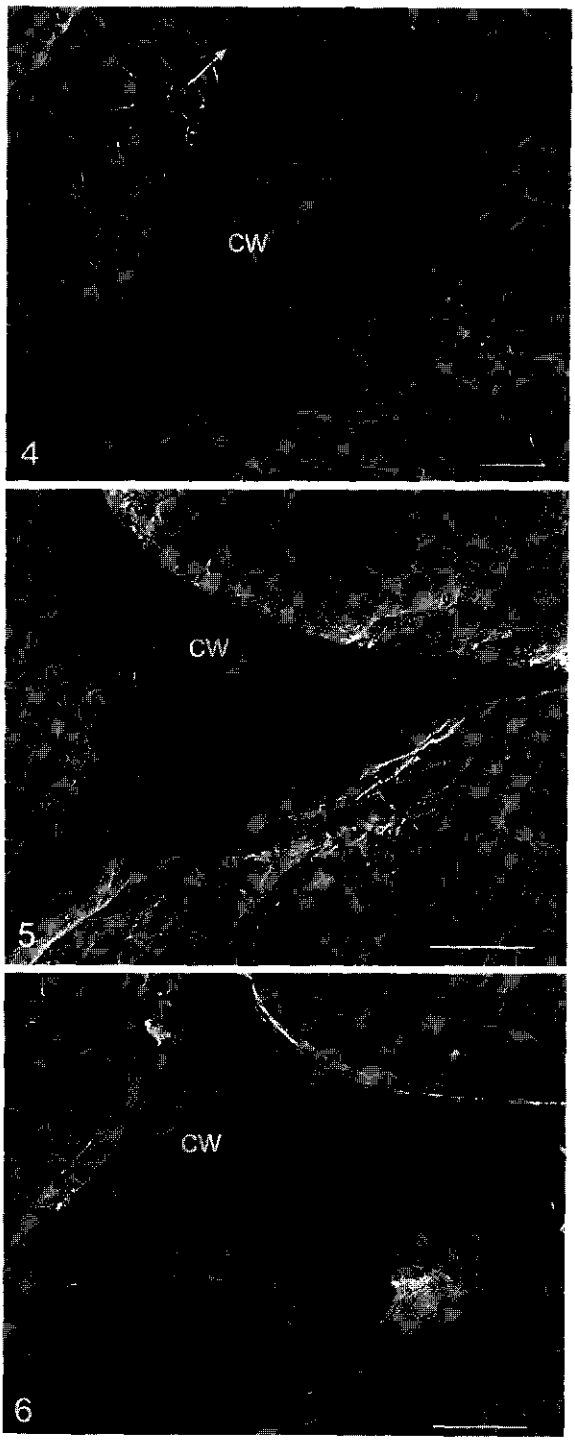



**Fig. 6.3** Germination speed after priming (mean time until 20% germination) versus priming time in -0.4 MPa, -0.7 MPa and -1.0 MPa PEG (a) and germination speed after priming versus puncture force during priming (b). After priming seeds were rinsed in water, dried back and germinated in water. Unprimed seeds showed 20% germination after 68 h. Symbols as in Fig. 6.1a.

showed a decrease in MTG, reaching a minimum of 24 h after 5 days of priming. This was followed by an increase up to 38 h after 20 d of pre-incubation. Seeds incubated in -0.7 MPa followed the same pattern. Seeds incubated in -1.0 MPa did not show this transient decrease, although the low value after 20 d suggested that the same process acts on a different time scale. Uniformity of germination was similar for all priming treatments and the unprimed control (data not shown), which shows that the MTG reflects the lag time until the start of radicle protrusion. A positive correlation was found for MTG after drying back and germination in water, and puncture force of the batch just prior to drying back (Fig. 6.3b). Although seeds in -0.7 and -1.0 MPa PEG did not reach a puncture force as low as in -0.4 MPa PEG, a low MTG was still acquired in a few batches.

Cryo-SEM was performed with seeds incubated in water and in -0.4 and -1.0 MPa PEG. Seeds incubated for 7 days in -1.0 MPa PEG showed an embryo that was not fully turgescent and a lateral endosperm of which the cytoplasm appeared not fully hydrated compared to the endosperm cap (data not shown). The endosperm cap showed intact cell walls, although slight




**Figs. 6.4 - 6.6** Scanning electron micrographs of the endosperm cap of a seed that was imbibed in -1.0 MPa PEG for 7 days (Fig. 6.4), of the endosperm cap of a seed that was imbibed in -0.4 MPa for 5 days (Fig. 6.5) and of the endosperm cap of a seed that was imbibed in water for 60 h (Fig. 6.6). Note the narrow zone of porosity in the cell wall (CW) along the plasma membrane (arrow) in Fig. 6.4 while the rest of the cell wall appears intact, and the porous appearance throughout the cell wall in Figs. 6.5 and 6.6. Bar = 1 μm.

porosity was observed in the cell wall along the plasma membrane (Fig. 6.4). Seeds incubated for 5 days in -0.4 MPa PEG showed porosity throughout all the cell walls in the endosperm cap (Fig. 6.5). The embryo was almost fully turgescent, and again the lateral endosperm appeared to be incompletely hydrated as in -1.0 MPa PEG (data not shown). Seeds incubated in water for 60 h also showed porous cell walls in the endosperm cap (Fig. 6.6), while the embryo appeared fully turgescent and the lateral endosperm fully hydrated (not shown). The cell walls in the lateral endosperm always appeared intact, regardless of the treatment. The porosity of the cell walls in the endosperm cap correlated with the occurrence of mechanical weakening (Fig. 6.1a).

## Discussion

The osmotic potential of the imbibition medium appeared limiting for the decrease in puncture force and the initiation of endo-ß-mannanase activity in the endosperm cap of tomato seeds (Figs. 1a and 2a). There was a good correlation between puncture force and endo-ß-mannanase activity in the endosperm cap for all osmotic potentials of the PEG solution (Fig. 6.2b). This supports the hypothesis that endo-ß-mannanase activity is involved in the weakening of the endosperm cap, but which does not necessarily result in the completion of germination. The limitation for endosperm weakening and development of endo-ß-mannanase activity was different for the individual seeds in a population and showed a wide variation, as was described by Still et al. (1997). This was particularly true for seeds incubated in -0.7 MPa, allowing two classes to be distinguished: one class with low endo-ß-mannanase activity and high puncture force and one class with high enzyme activity and low puncture force. The two classes of seeds in the different PEG concentrations (Fig. 6.2b) and the bimodal distribution of the puncture force in -0.7 MPa after 20 days (Fig. 6.1b), indicated that the individual seeds had to cross an osmotic threshold in order to produce endo-ß-mannanase and lower their puncture force. This result shows the value of single-seed analysis, as was stressed by Still and Bradford (1997), since pooled samples would not have given a clear distinction of the two classes in -0.7 MPa. The weakening of the endosperm cap in -0.7 MPa PEG was not to the full extent as could be observed in -0.4 MPa PEG, as can be concluded from the difference in the median (Fig. 6.1b). PEG was found to influence the ABA levels in both embryo and endosperm cap (Ni and Bradford, 1992), but these differences were small and unlikely to have significant physiological impact. Apparently an additional PEG effect must

be limiting for further decrease of puncture force, possibly incomplete hydration. In analogy, an additional PEG effect was found by Leubner-Metzger et al. (1996), who noticed that the onset of endosperm rupture in *Nicotiana tabacum* L. seeds was delayed more than ß-1,3-glucanase activity by PEG. In *Datura ferox* seeds PEG was found to affect the red light induced decrease in puncture force, however, no threshold behaviour was reported (De Miguel and Sanchez, 1992).

The finding of reduced endo-ß-mannanase activity in the endosperm cap with decreased osmotic potential is different from Dahal et al. (1997), who found that -1.5 MPa PEG could not inhibit the endo-ß-mannanase activity although germination was completely blocked. In another study endo-ß-mannanase activity after 44 h in -0.3 MPa PEG and germination after 8 days were reduced to less than 50% (Hilhorst and Downie, 1996), which concurs with our results. Variation in physiological and biochemical parameters between cultivars and even seed batches may play a role in general. Seeds germinated in -0.6 MPa PEG according to Dahal et al. (1997) and to Still and Bradford (1997), although slower and with a decreased maximum percentage. Both reports described enhanced levels of endo-ß-mannanase activity compared to water incubation. Our results indicate a strong inhibition of germination and no change in the maximum level of endo-ß-mannanase activity in -0.4 MPa PEG. Karssen et al. (1989) showed that the puncture force decreased in -1.2 MPa within 15 days while in our study no major decrease in puncture force was detected after 20 days in -1.0 MPa PEG.

Seeds with a low puncture force during priming germinated faster upon drying back than seeds with a high puncture force (Fig. 6.3b). The conclusion can be drawn that seeds with an already advanced weakened endosperm cap germinate faster due to this lower restraint, similar to what was found by Karssen et al. (1989) and de Miguel and Sanchez (1992). However, although all different osmotic treatments displayed a positive correlation between puncture force and germination speed, they did not share the same regression line. Moreover, regardless of the lowering of the puncture force, all treatments resulted in increased germination rates, albeit after different times of priming. Obviously, the endosperm cap restraint during priming does not play an essential role in the germination speed upon hydration. This is in contrast with a study by Fooland and Jones (1991) who found that the endosperm is the principal site of genetic determinants controlling germination performance. A shift in base water potential occurred during imbibition of tomato seeds in a low (<-0.5 MPa) osmotic potential, which was attributed to an accumulation of solutes in the embryo (Ni

and Bradford, 1992). This may explain the faster germination rates of seeds primed at lower osmotic potential (-0.7 and -1.0 MPa) without substantial weakening of the endosperm cap during priming (Fig. 6.3b). Cell-cycle-related activities during priming in the radicle tip of tomato seeds have been shown to correlate positively with germination (Bino et al., 1992; De Castro et al., 1995). Cell elongation in the embryo was initiated during priming of lettuce seeds (Cantliffe et al., 1984). Repair of DNA, rRNA and protein synthesis played a role in the embryo of leek during priming (Bray et al., 1989; Davison et al., 1991). These processes may all play a role in the priming of tomato seeds, which would explain why endosperm cap weakening during priming at low osmotic potential is not a prerequisite for improved germination rate.

Micrographs obtained with cryo-SEM showed porosity of the cell walls in the endosperm cap under conditions that allowed weakening. This result is consistent with the results that were found for seeds imbibed in water or ABA, and supports the hypothesis that the porous appearance of the cell walls is caused by endo-ß-mannanase (Toorop et al., unpublished results). Nonogaki et al. (1992) observed eroded cell walls in primed and rehydrated seeds. However, we never noticed any change in the cell wall surface of the endosperm during priming. Surprisingly, the lateral endosperm showed restricted imbibition at both low and high osmotic potential, while the endosperm cap showed apparently normal imbibition and a normal development of endo-ß-mannanase activity in -0.4 MPa PEG. Based on these observations, it cannot be excluded that the hydration of the lateral endosperm plays a role in the onset of phase III in germination.

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Chapter 7

# General discussion

Abscisic acid is an inhibitor of germination, and is involved in the regulation of dormancy in seeds (Hilhorst, 1995). So far, actions have been proved through inhibition of the expansion of the embryo (Schopfer and Plachy, 1985), and through inhibition of the weakening of the tissues surrounding the radicle tip (Groot, 1987; Haigh and Barlow, 1987). Completion of germination in tomato is regulated by both ABA and GA and it was hypothesized that endo-ß-mannanase is regulated by these hormones, thus mediating weakening of the endosperm cap (Groot, 1987). However, our results indicate that endo-ß-mannanase activity is not inhibited by ABA while germination is, which forms a discrepancy with the previously mentioned results. The role of endo-ß-mannanase in the completion of germination was investigated in this thesis, and is discussed further in this chapter.

## Induction of endo-ß-mannanase by GA

In the GA-deficient *gib1* seeds the concentration of exogenously applied $GA_{4+7}$ was related to the final germination percentage. A more than 400-fold greater GA concentration was required to induce germination of the least sensitive 10% of the seed population compared with the most sensitive 10%, indicating the wide variation in the response to applied GAs for the individual seeds in a population (Bradford and Trewavas, 1994). This requirement for GAs could be circumvented by detipping the seeds, which resulted in growth of the embryo and a seedling with a dwarfy phenotype (Groot and Karssen, 1987). Detipping of *gib1* seeds resulted in increased levels of 4C DNA in the radicle tip, indicating that the endosperm cap forms the inhibiting factor for the onset of this embryonic process (Liu et al., 1997). On the other hand, DNA replication is not a prerequisite for radicle protrusion in cabbage and tomato seeds (Górnik et al., 1997). These results suggest that lowering the endosperm restraint is essential, whereas DNA replication in the radicle tip is not essential for the completion of germination.

The *gib1* seeds did not germinate and the endosperm cap did not display endo-ß-mannanase activity when imbibed in water (Groot and Karssen, 1987; chapter 2). Also no weakening of the endosperm cap occurred (Groot and Karssen, 1987). In isolated endosperms of the *gib1* mutant, consisting of both lateral endosperm and endosperm cap, endo-ß-mannanase activity could be induced by $GA_{4+7}$ (Groot et al., 1988). The enzyme activity could also be induced by $GA_{4+7}$ in the endosperm cap of intact *gib1* seeds (chapter 2). It was postulated that in the intact seed a GA, emanating from the embryo, induced endosperm

weakening through the action of endo-ß-mannanase[1] (Groot et al., 1988). However, actual transport of a GA from the embryo to the endosperm cap was never shown. Another weak point in this work is that no distinction between lateral endosperm and endosperm cap was made in the induction of endo-ß-mannanase. Also, the possibility that other GA induced enzymes may play a role in the completion of germination can not be ruled out.

When shortly imbibed wild-type endosperms were isolated and separated into lateral endosperm and endosperm cap and subsequently incubated in $GA_{4+7}$, endo-ß-mannanase activity was induced in the isolated lateral endosperm of tomato seeds[2], while no detectable activity was found in the endosperm cap (chapter 2). From these data it can be concluded that GAs do not induce the enzyme activity in the endosperm cap. Still, incubation of intact *gib1* seeds in $GA_{4+7}$ induced endo-ß-mannanase activity in the endosperm cap[1] (chapter 2), endosperm weakening and radicle protrusion (Groot and Karssen, 1987). Obviously, some controversy exists about the action of exogenous GAs. A possible explanation for this discrepancy is that GA induces a factor from the embryo, that is required for the induction of endo-ß-mannanase activity in the endosperm cap. This would explain the lack of endo-ß-mannanase activity in isolated endosperm caps in the presence of $GA_{4+7}$ but in the absence of the embryo, whereas in the intact *gib1* seed, in the presence of the embryo, the enzyme is induced by $GA_{4+7}$. The presumption then would be that in the intact seed GAs are indirectly involved in the induction of endo-ß-mannanase in the endosperm cap.

The induction of endo-ß-mannanase activity in isolated lateral endosperms in the presence of $GA_{4+7}$ strongly implies that in the intact seed the post-germinative enzyme activity is induced by GAs[2]. The hypothesized factor from the embryo, required for induction of endo-ß-mannanase in the endosperm cap, apparently is not required for induction of the enzyme in the lateral endosperm. Clearly, GAs are not sufficient for the induction of post-germinative endo-ß-mannanase in the intact seed: radicle protrusion is another prerequisite since the enzyme is never observed in the lateral endosperm of the ungerminated seed despite the presumed presence of GAs.

## Inhibition of endo-ß-mannanase by ABA

Endo-ß-mannanase activity in the endosperm cap was not inhibited by exogenously applied ABA up to 100 µM in either wild-type or *gib1* seeds (chapter 2). Radicle emergence

[1, 2] See corresponding number in Fig. 1

on the other hand was strongly inhibited by ABA. This lead to the hypothesis that endo-ß-mannanase did not play a key role in the completion of germination, and that at least one other ABA-inhibited enzyme would be responsible for endosperm weakening, resulting in radicle protrusion. Puncture force measurements of water imbibed wild-type seeds showed that endosperm weakening occurs in two phases (chapter 3). In the first phase a decrease in puncture force concurred with an increase in endo-ß-mannanase activity. In the second phase the puncture force decreased further but slower, while the enzyme activity increased at the same rate. The first step in endosperm weakening, marked by a relatively fast decrease in puncture force, was not inhibited by ABA. However, the second step, marked by a slower decrease, appeared to be inhibited by ABA. This was in contradiction with previous results, showing complete inhibition of endosperm weakening by ABA (Groot and Karssen, 1992). It was concluded that endo-ß-mannanase mediated the first step in the puncture force decrease, which was not inhibited by ABA[3] . It is hypothesized that the second step in the puncture force decrease, which is controlled by ABA, is mediated by a, yet unknown, ABA-regulated cell wall degrading enzyme[4]. Since no endosperm weakening occurred in *gib1* seeds when incubated in water it can be hypothesized that this second step in the endosperm weakening is either controlled by GA[5] or by the first step.

Two reports mentioned that exogenous ABA inhibits endo-ß-mannanase (Nonogaki et al., 1995; Voigt and Bewley, 1996). Voigt and Bewley (1996) found that both the enzyme activity in the endosperm cap and the number of its isoforms decreased when seeds were imbibed in 20 µM ABA, whereas in 2 µM ABA no decrease was observed. For this study developing seeds were used, harvested 45 days after pollination (DAP). It is believed that during development high levels of endogenous ABA cause primary dormancy in tomato (Hilhorst, 1995). Dormant wild-type seeds showed no decrease in puncture force (Groot and Karssen, 1992) and no endo-ß-mannanase activity (Still and Bradford, 1997). It was hypothesized that during imbibition the lack of endosperm weakening in dormant wild-type seeds is caused by high endogenous ABA levels[6] that impair the action of GAs from the embryo (Bewley, 1997). Alternatively, the lack of endosperm weakening may be explained by the state of dormancy, which is reflected in the sensitivity to ABA, and not by the endogenous ABA levels. The 45 DAP seeds that were used by Voigt and Bewley (1996) might have been deeper dormant than the mature dried seeds that were used in our study, which is reflected in

[3, 4, 5, 6] See corresponding number in Fig. 1

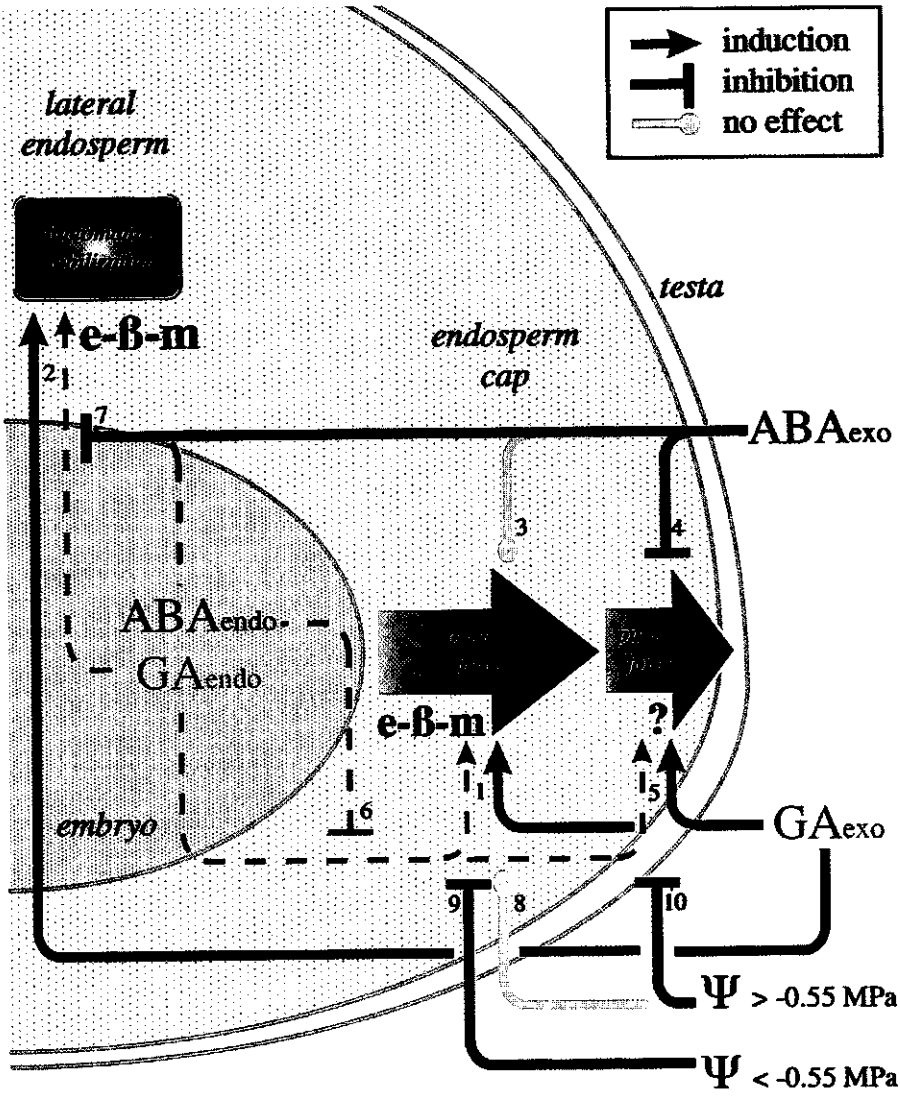


Fig. 7.1 A germination model for tomato describing the influence of GA, ABA and PEG (Ψ) on the endosperm cap weakening preceding radicle protrusion, and of GA and ABA on the mobilization of galactomannans in the lateral endosperm: modified from Groot (1987) and Ni and Bradford (1993). The numbers in the diagram are referred to in the text. The solid lines represent the investigated steps in this thesis, whereas the dotted lines represent the steps that were postulated, either in this thesis or in previous publications (see text). The question mark indicates the postulated cell wall hydrolyzing enzyme, additionally required for endosperm weakening.

the difference in sensitivity to exogenous ABA in the induction of endo-ß-mannanase. Demir and Ellis (1992) found a change in germination time and percentage normal germination during developing tomato seeds, showing that developmental stage can make a difference in the physiological response. Apart from a change in the sensitivity to ABA, a change in the sensitivity to GAs during the release from dormancy may also explain the discrepant behaviour of the different seed batches (Karssen et al., 1989b). Exogenous ABA would then inhibit germination by interference with the release of and/or sensitivity to GAs, causing either low levels of or low sensitivity to GAs.

The finding that exogenous ABA could not inhibit endo-ß-mannanase activity was confirmed by Still and Bradford (1997), who also found that the enzyme activity declined when no radicle emergence occurred. The authors concluded that a previous report, showing inhibition of endo-ß-mannanase activity by ABA, might have described the enzyme activity sampled after the decline, and thus lead to the wrong conclusion (Nomaguchi et al., 1995). An alternative explanation for the discrepancy with the results of Nomaguchi et al. (1995) could be a difference in the ABA-sensitivity due to the state of after-ripening, as described previously.

Groot and Karssen (1992) showed inhibition of endosperm weakening by ABA. However, for their experiments they used isolated endosperm caps instead of intact seeds. The degree of penetration of the seed by exogenous ABA may play an important role in the response. The ABA molecule is known to enter *Chenopodium album* seeds with difficulty, and a discriminating mechanism was suggested (Karssen, 1976). Although tomato seeds are different from *C. album*, it is conceivable that the concentration of ABA in the seed is not as high as in the incubation medium. Using isolated endosperm caps, the ABA concentration is likely to be higher than in intact seeds. This may explain why Groot and Karssen (1992) found complete inhibition of endosperm weakening using isolated endosperm caps, whereas we found no effect using intact seeds (chapter 3).

ABA analogs also had no inhibitory effect on the endo-ß-mannanase activity in the endosperm cap, while germination was affected to a higher or lesser degree depending on the three-dimensional structure of the analog (chapter 4). The configuration at the C-1' position had the biggest impact on changes in germination, the C-2', C-3' double bond was less significant while the C4-C5 double bond played no role at all. These changes in germination response, induced by mutations in the ABA molecule, probably reflect a lesser recognition by

the putative ABA receptor. The results confirmed that ABA does not inhibit endo-ß-mannanase activity, and that this enzyme does not play a key role in the weakening of the endosperm.

(±)-ABA (chapter 2) and the (+)-enantiomers and (-)-ABA that were used (chapter 4) appeared to inhibit post-germinative endo-ß-mannanase activity in both endosperm[7] and embryo. The post-germinative endo-ß-mannanase activity of detipped seeds correlated with the germination speed of intact seeds imbibed on the same analogs. It was concluded that ABA has an identical effect on the completion of germination and on the mobilisation of the mannan reserves. The two processes are not likely to be causally related. It is more likely that they are both the result of an induction pathway that they have in common.

Recently Garciarrubio et al. (1997) found that in *Arabidopsis thaliana* (L.) seeds ABA inhibits germination by restricting the availability of energy and metabolites. Addition of sugars and amino acids alleviated the inhibitory action of ABA. The finding that availability of energy and metabolites are restricted by ABA during germination is a new aspect of the action of ABA, which has not been studied in tomato yet.

## Inhibition of endo-ß-mannanase by osmoticum

Osmotic priming improves tomato seed germination under dark conditions (Coolbear and Grierson, 1979) and even under unfavourable far-red light conditions (Georghiou et al., 1982). Karssen et al. (1989a) showed that during PEG priming endo-ß-mannanase activity developed in the endosperm cap, and that this enzyme activity was higher in primed seeds upon reimbibition in water. Generally, endosperm cap weakening was found in a high[8] (>-0.55 MPa), but not in a low[9] (<-0.55 MPa) osmotic potential during priming (chapter 6). Lowered endosperm cap restraint correlated strongly with the presence of endo-ß-mannanase activity, supporting the hypothesis that the two are causally related. It was previously described that in a partly dormant population of wild-type seeds two sub-populations can be distinguished, using a single-seed analysis for endo-ß-mannanase activity (Still and Bradford, 1997). In chapter 6 single-seed analysis revealed that the puncture force decreased and the endo-ß-mannanase activity increased upon crossing a threshold water potential, which was estimated at -0.55 MPa, indicating that the metabolic response to water limitation is a discontinuous event in seeds.

[7, 8, 9] See corresponding number in Fig. 1

The level of endo-ß-mannanase activity in seeds incubated in -0.4 MPa PEG was similar to that of seeds incubated in ABA (chapter 3). From the latter data it was concluded that an ABA-regulated enzyme was responsible for the rate-limiting step in the germination of tomato seeds. This putative enzyme could also be regulated by osmotic stress[10], which would explain the absence of radicle protrusion in low concentrations of PEG, allowing lowering of the puncture force and development of endo-ß-mannanase activity.

Germination speed improved upon priming, and this improvement correlated with decreased puncture force (Karssen et al., 1989a; chapter 6). However, decrease of puncture force was dependent on the osmotic potential of the priming solution. In the lower osmotic potential (-1.0 MPa) this resulted in improved germination speed without decreased puncture force (chapter 6). From this it was concluded that endosperm weakening is not a prerequisite for acquiring faster germination upon priming. Embryo parameters might be affected by priming, independently of the endosperm cap, resulting in the observed change in germination speed. DNA replication seems to be a good embryo parameter that is possibly involved in the improved germination upon priming. Upon three days priming in PEG-6000 the 4C DNA signal increased in root tips (Bino et al., 1992). $GA_{4+7}$ enhanced the replication of DNA during priming in -1.0 MPa PEG (Liu et al., 1996). The 4C:2C signal ratio was constant after redrying and probably attributed to a more rapid and equal germination of the primed seeds. An inverse correlation between the frequency of root tip cells expressing 4C DNA signals and the mean time to germination for individual seed lots stressed the importance of nuclear replication in the root tip for the germination event (Lanteri et al., 1994). However, recently it was postulated that DNA replication is an independent cell cycle event that is not required for radicle protrusion (Górnik et al., 1997). Therefore it remains unclear what embryo parameter is involved in improvement of germination upon osmotic priming.

## Endo-ß-mannanase in the embryo

Endo-ß-mannanase activity was also detected in the embryo of tomato seeds (Dirk et al., 1995; Voigt and Bewley, 1996; chapter 2). The development of the enzyme activity started in the radicle tip, and progressed towards the cotyledons before completion of germination (chapter 2). The presence of the enzyme in the embryo was somewhat puzzling, since one would not expect the cell walls of the embryo to degrade, like in the endosperm. Other cell

[10] See corresponding number in Fig. 1

wall proteins have been suggested to be involved in cell wall extensibility (Schopfer and Plachy, 1985; Karssen et al., 1989b; Fry, 1993; Hilhorst, 1995; McQueen-Mason and Cosgrove, 1995; Sitrit et al., 1996). It was hypothesized that endo-ß-mannanase was involved in partial degradation of the cell wall, allowing elongation of the embryo (Dirk et al., 1995). A necessary assumption would be the presence of substrate in the cell walls of the embryo. It was shown that the occurrence of galactomannans in the embryo is likely (chapter 5). Organs of some species, e.g. the megagametophyte of conifers, also contain endo-ß-mannanase although they are not known to contain mannans (Dirk et al., 1995). Interestingly, all studied vegetative tissues of alfalfa (*Medicago sativa*) contained endo-ß-mannanase, supporting the hypothesis that this enzyme is involved in cell elongation and growth. Post-germinative endo-ß-mannanase activity in the embryo was inhibited by ABA (chapter 2 and 4), supporting the hypothesis that this enzyme is involved in growth of the seedling. Like endo-ß-mannanase activity in the embryo prior to radicle protrusion, DNA replication in the root tip was not inhibited (Liu et al., 1994). This indicates that the metabolic advancement of the embryo towards the start of radicle growth prior to radicle protrusion is not blocked by ABA, making it likely that the endosperm cap is the conclusive site of action involved in the inhibition of germination by ABA.

### Expression of endo-ß-mannanase

Recently, three endo-ß-mannanase isoforms were isolated from endosperms of germinated seeds (Nonogaki et al., 1995). The isoforms M1, M2 and M3 differed slightly in molecular mass (37.5 and 38 kDa) and isoelectric point (5.15, 5.40 and 5.45), but were much different from endo-ß-mannanase from tomato fruit (43 kDa, pI 9.3) (Pressey, 1989). Prior to radicle protrusion, an endo-ß-mannanase Mα (39 kDa) was detected in the endosperm opposing the radicle tip using an antibody raised against one of the isoforms in the lateral endosperm (Nonogaki and Morohashi, 1996). A second isoform Mß (38 kDa) was found during assaying as the result of a proteolytic hydrolysis, and therefore was considered an artefact. This indicates that the different isoforms (IEP 5.2, 5.5, 5.8) found after isoelectric focusing (chapter 2) might not be all present *in vivo*. Mα appeared to have a different action pattern than the isoforms from the lateral endosperm, since a presumed galactosyl mannotriose but not mannobiose was found as a hydrolysis product. Recently, the gene coding for endo-ß-mannanase was cloned (Bewley et al., 1997) and the mRNA coding for

endo-ß-mannanase was used as a probe in Northern blotting. This technique showed that the mRNA coding for endo-ß-mannanase was detected in the endosperm cap after 24 and 70 h imbibition of the wild-type seed in water or 10 µM ABA, and only faintly after 27 days in 10 µM ABA (chapter 4). No positive signal was found in the lateral endosperm of seeds that had not completed germination. This expression pattern concurred with enzyme activity. The mRNA was not present in dry seeds or seeds of the *gib1* mutant, indicating that *de novo* transcription occurs and that GA regulates at the transcription level. ABA could not inhibit expression of the gene, which confirmed the results obtained with enzyme activity. The probe also hybridized with an embryo mRNA, which might encode for the endo-ß-mannanase in the embryo. This result is in contradiction with previous results showing no detection of transcripts in embryo tissues (Bewley et al., 1997). Surprisingly, expression in the embryo was observed in the *gib1* mutant. This indicated that endo-ß-mannanase in the embryo is not under the control of GA. Nevertheless, no enzyme activity was found, possibly caused by an impaired translation of the mRNA.

## Other hydrolytic enzymes

Since endo-ß-mannanase activity appeared not to be inhibited by ABA, other enzymes were studied to see if these were rate-limiting for germination (chapter 5). Guar seeds contain galactomannan that is highly substituted with galactose. In these seeds α-galactosidase activity was a prerequisite for the action of endo-ß-mannanase (McCleary and Matheson, 1975; McCleary, 1983). In tomato the structure of galactomannan is unknown, but a similar way of inhibition is conceivable. Release of mannose was suppressed in the presence of osmoticum or ABA (Dahal et al., 1997), therefore ß-mannosidase activity was a candidate for the ABA inhibition of germination. Exo-polygalacturonase mRNA was found in the radicle tip of embryos, and was associated with radicle protrusion (Sitrit et al., 1996). Cellulase activity was associated with phytochrome-regulated germination in *Datura ferox* (Sanchez et al., 1986), and was previously detected in ungerminated tomato seeds (Leviatov et al., 1995). Therefore cellulase, and perhaps ß-glucosidase, activity formed a potential key enzyme in the ABA inhibition of germination. Nevertheless none of the enzymes mentioned were inhibited by ABA in any part of the tomato seed. Therefore none of these enzymes can be considered as a key enzyme in the completion of tomato seed germination.

## Visualisation of hydrolytic activity

Two methods appeared useful in the determination of hydrolytic enzyme activity in the cell walls of the endosperm. The tissue printing technique has proven to be a quick method in screening several seeds simultaneously and was used to detect endo-ß-mannanase activity at the sub-organ level. Although the method suffers from low resolution it appeared to be as sensitive as the diffusion assay, and combined the benefits of localization of the enzyme with those of a single seed assay. Low temperature scanning electron microscopy was used to study cell walls at the sub-cellular level. Porosity of the cell wall correlated with endo-ß-mannanase activity, and therefore it was concluded that the porosity was the direct result of the hydrolysis of the mannan-rich cell walls. The lateral endosperm of the ungerminated seed did not show endo-ß-mannanase activity on tissue prints and also did not show porosity of the cell walls. Previously, hydrolysis of cell walls was visualized with light microscopy (Jones, 1974; van der Toorn, 1989), transmission electron microscopy (Benhamou and Chet, 1996; Dawidowicz-Grzegorzewska, 1997), and confocal laser scanning microscopy (Travis et al., 1997). Eroded surfaces of endosperm cell walls were visualized with scanning electron microscopy (Sanchez et al., 1990; Nonogaki et al., 1992) and cracks in seed tissues enveloping the radicle tip were interpreted as signs of degradation (Pavlista and Valdovinos, 1978; Welbaum et al., 1995). A recent study showed that the cracks in the endosperm of lettuce are artefacts due to the fixation method (Nijsse et al., 1998), confirming light microscopic observations that could not detect structural modifications that would be responsible for surface alterations (Georghiou et al., 1983). Porosity of cell walls associated with hydrolysis has only been reported by Williamson and Duncan (1989) in red raspberry infected by *Botrytis cinerea* using scanning electron microscopy.

## Germination model

Groot (1987) and Ni and Bradford (1993) proposed a germination model, displaying the role of the embryo and the endosperm cap. Here a modified model is presented describing the role of ABA, GA and osmoticum in germination and the involvement of endo-ß-mannanase and other hydrolytic enzymes, putatively involved in the endosperm weakening, based on the results that were discussed in this chapter (Fig. 7.1). Several questions still need to be answered, e.g. whether the loss of ABA sensitivity during after-ripening that was hypothesized in this chapter actually occurs, and whether this change is responsible for the

differences in response found for the expression of endo-ß-mannanase. More insight into this process may lead to a better design of experiments. The presence of endo-ß-mannanase in the embryo is also yet unexplained, and one can only speculate about its function. The factors from the embryo that are involved in the regulation of endosperm weakening should be investigated. The most urgent question that remains to be answered is what enzyme is limiting for the second step of the endosperm weakening. A few enzymes were eliminated, although this did not clarify the situation. Recently a GA-upregulated $H^+$-ATPase was found in the endosperm cap of tomato (Yang et al., 1996), which was associated with vacuolisation during germination. This observation reflects the concept that processes other than endo-ß-mannanase induced endosperm weakening may play a role in the completion of germination. General methods, such as cDNA differential display analysis, may indicate whether a cell wall degrading enzyme or possibly other enzymes are involved in the ABA controlled completion of germination (Li and Foley, 1997).

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# Summary

ABA is an inhibitor of tomato seed germination. Earlier studies have shown that the inhibitory action of ABA lies in the inhibition of both the endo-ß-mannanase activity in the endosperm cap (Nomaguchi et al., 1995) and the weakening of the endosperm cap (Groot and Karssen, 1987). It was hypothesized that endo-ß-mannanase mediates the weakening of the endosperm cap (Groot et al. 1988). However, initial experiments revealed that ABA had no effect on the amounts of endo-ß-mannanase in the endosperm cap (chapter 2). Also, no differences could be made in the isoform pattern in extracts from water and ABA treated seeds. On the other hand, ABA appeared to be capable of inhibiting the post-germinative endo-ß-mannanase activity, located in the lateral endosperm, in detipped seeds. Using isolated parts of the endosperm GA was shown to be a prerequisite for expression of endo-ß-mannanase activity. However, GA could induce endo-ß-mannanase activity in the lateral endosperm, but not in the endosperm cap. Therefore, we could not confirm the hypothesis that GAs emanating from the embryo induced expression of the enzyme in the tissue opposing the radicle tip, resulting in endosperm weakening (Groot and Karssen, 1987). Activity of the enzyme was also found in the embryo, both prior to radicle protrusion and in the growing seedling (chapter 2). Its isoforms were distinct from the ones in the endosperm cap. Tissue printing showed that its induction started in the radicle tip in the early stage of imbibition, and spread towards the cotyledons before radicle protrusion. In this stage ABA could not inhibit the enzyme activity. The post-germinative enzyme activity was inhibited by ABA, although not as strongly as in the lateral endosperm. The reason for the presence of endo-ß-mannanase in the embryo was unknown. It was postulated that its activity is associated with growth.

The puncture force appeared to be only partly inhibited by ABA (chapter 3). After an initial decrease from 0.6 to 0.35 Newton the puncture force arrested at this level in seeds incubated in ABA, whereas in water imbibed seeds the puncture force decreased further until the radicle protruded. An exponential relationship was found between puncture force and endo-ß-mannanase activity, both in water and ABA imbibed seeds. It was concluded that endo-ß-mannanase mediated the first step in the decrease of puncture force down to 0.35 Newton. It was hypothesized that, yet unknown, ABA-regulated enzymes are involved in the second step of the weakening leading to radicle protrusion. An apparent correlation was found

between endo-ß-mannanase activity and porosity of the cell walls in the endosperm cap. The pores possibly represent the hydrolytic activity of this enzyme in the cell wall.

ABA analogs confirmed the finding that endo-ß-mannanase activity is not inhibited by ABA (chapter 4). Analogs with different mutations to the structure displayed a varying inhibition capacity using radicle protrusion as a parameter. Endo-ß-mannanase activity in the endosperm cap was not inhibited by any of the analogs, nor was its activity in the embryo prior to radicle protrusion. Endo-ß-mannanase activity in the lateral endosperm and embryo of detipped seeds incubated in the various ABA analogs was strongly inhibited. This was consistent with the results from chapter 2. A correlation was found between enzyme activity in detipped seeds and germination speed of intact seeds in the different ABA analogs. It was concluded that ABA has a similar effect on radicle protrusion, growth of the embryo and depletion of the endosperm.

Attempts to identify the ABA-regulated enzyme that is involved in radicle protrusion failed. α-Galactosidase, ß-mannosidase, cellulase, ß-glucosidase and exo-polygalacturonase activities appeared not to be inhibited by ABA.

Endo-ß-mannanase played a role in the degradation of the endosperm cap cell walls during osmotic priming. A clear relationship was found between the decrease of puncture force, the occurrence of endo-ß-mannanase activity and the porous appearance of the cell walls in the endosperm cap. These events occurred in a high (-0.4 MPa) but not in a low (-1.0 MPa) osmotic potential of the imbibition medium. An intermediate osmotic potential (-0.7 MPa) showed two classes of seeds: one with and one without puncture force decrease and endo-ß-mannanase activity. It was concluded that individual seeds have to cross a threshold water potential to lower their puncture force. Puncture force decrease only partly explained the beneficial effects on the germination speed of osmotic priming upon subsequent germination.

A germination model is presented, in which the hormonal and osmotic regulation of the endosperm cap weakening is described, as well as the hormonal regulation of the post-germinative depletion of the galactomannans in the endosperm. The model describes the limited role of endo-ß-mannanase in this process. The overall conclusion is that endo-ß-mannanase activity is a prerequisite in the completion of germination, but is not the sole factor. Other putative cell wall hydrolases, probably GA- and ABA-regulated, are involved as well.

# Samenvatting

ABA is een remmer van de kieming van tomatenzaden. Eerdere studies wezen uit dat de remmende werking van ABA ligt in de remming van zowel de endo-ß-mannanase activiteit in de 'endosperm cap' (Nomaguchi et al., 1995) als de verweking hiervan (Groot en Karssen, 1987). De hypothese werd geponeerd dat endo-ß-mannanase een rol speelt in de verweking van de endosperm cap. Inleidende experimenten lieten echter zien dat ABA geen effect had op het niveau van endo-ß-mannanase activiteit in de endosperm cap (hoofdstuk 2). Ook konden geen verschillen worden waargenomen in het patroon van isovormen in extracten van zaden die met water of ABA zijn behandeld. Daarentegen bleek ABA in staat te zijn om de endo-ß-mannanase activiteit die gelocaliseerd is in het lateral endosperm te remmen in zaden waarvan de endosperm cap was verwijderd. Gebruik makende van geisoleerde delen van het endosperm kon worden aangetoond dat GA een voorwaarde was voor expressie van endo-ß-mannanase activiteit. GA kon echter endo-ß-mannanase activiteit induceren in het lateral endosperm, maar niet in de endosperm cap. Derhalve kon de hypothese dat GAs vanuit het embryo expressie van het enzyme in de endosperm cap induceren (Groot and Karssen, 1987) niet worden bevestigd. Enzymactiviteit was ook aanwezig in het embryo, zowel voor als na worteldoorbraak. De isovormen in het embryo verschilden van die in de endosperm cap. Tissue prints lieten zien dat endo-ß-mannanase activiteit begon in de worteltop, en verspreidde naar de cotylen gedurende imbibitie. ABA kon de enzymactiviteit in het embryo voor worteldoorbraak niet remmen, maar wel na worteldoorbraak. Deze remming was niet zo sterk als in het laterale endosperm. De aanwezigheid van endo-ß-mannanase in het embryo kon niet worden verklaard. Een mogelijke functie van het enzym is betrokkenheid bij groei.

De weerstand van de endosperm cap kon alleen gedeeltelijk worden geremd met ABA (hoofdstuk 3). Na een afname van 0,6 naar 0,35 Newton bleef de weerstand op dit niveau bij ABA geïmbibeerde zaden, terwijl bij zaden in water de weerstand verder omlaag ging totdat worteldoorbraak plaatsvond. Er werd een exponentiele relatie gevonden tussen endosperm weerstand en endo-ß-mannanase activiteit, zowel bij zaden in water als in ABA. De conclusie was dat endo-ß-mannanase verantwoordelijk was voor de eerste stap in de verlaging van de endosperm weerstand. De hypothese werd gesteld dat onbekende ABA gereguleerde enzymen betrokken zijn in de tweede stap van de verlaging van de endosperm weerstand die tot worteldoorbraak leidt. Een correlatie werd gevonden tussen endo-ß-mannanase activiteit en

porositeit van de celwanden in de endosperm cap. De poriën houden mogelijk verband met de hydrolyse van de celwand door dit enzym.

Het gebruik van ABA analogen bevestigde de conclusie dat endo-ß-mannanase niet geremd wordt door ABA (hoofdstuk 4). Analogen met verschillende veranderingen aan de structuur hadden een variabele remmende werking op worteldoorbraak. Zowel de endo-ß-mannanase activiteit in de endosperm cap als in het embryo voor worteldoorbraak werden niet geremd door deze analogen. Zaden waarvan de endosperm cap was verwijderd lieten een sterke remming zien van de endo-ß-mannanase activiteit in het laterale endosperm en het embryo, overeenkomstig de resulaten uit hoofdstuk 2. Er was een sterke correlatie tussen enzym activiteit in zaden waarvan de endosperm cap was verwijderd, en kiemingspercentage van intacte zaden in de verschillende ABA analogen. De conclusie luidde dat ABA een vergelijkbaar effect had op zowel worteldoorbraak als groei van het embryo en mobilisatie van het reservevoedsel. Pogingen om een ABA gereguleerde enzym dat betrokken is bij de remming van de worteldoorbraak te identificeren, lukten niet. Wel is duidelijk dat α-galactosidase, ß-mannosidase, cellulase, ß-glucosidase en exopolygalacturonase activiteiten niet door ABA worden geremd.

Endo-ß-mannanase speelt een rol in de afbraak van de celwanden in de endosperm cap tijdens osmotisch 'priming'. Er was een duidelijke relatie tussen de mechanische verweking, endo-ß-mannanase activiteit, en porositeit van de celwanden in de endosperm cap. Deze processen kwamen voor bij incubatie in hoge (-0,4 MPa) maar niet in lage (-1,0 MPa) osmotische potentiaal. Bij intermediaire osmotische potentiaal (-0,7 MPa) waren twee klassen te zien: een klasse met mechanische verweking en endo-ß-mannanase activiteit, en een zonder. De conclusie werd getrokken dat individuele zaden een drempelwaarde voor de osmotische potentiaal moeten overschrijden om voordat zij mechanische verweking laten zien. Verweking is slechts ten dele de oorzaak van het positieve effect van priming op kiemsnelheid, wanneer de zaden na terugdrogen in water worden geïmbibeerd.

In hoofdstuk 7 wordt een model voor kieming beschreven met de hormonale en osmotische regulatie van de verweking van de endosperm cap. Tevens wordt de hormonale regulatie beschreven van de mobilisatie van galactomannanen in het endosperm na kieming. Het model schetst de beperkte rol van endo-ß-mannanase in het verwekingsproces. De algehele conclusie is dat endo-ß-mannanase activiteit een voorwaarde is voor worteldoorbraak. Activiteit van het enzym is echter niet de enige voorwaarde. De hypothese

wordt gesteld dat andere celwand hydrolyserende enzymen, die vermoedelijk gereguleerd worden door GA en ABA, ook een rol spelen.

# List of publications

### papers

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Toorop PE, van Aelst AC, Hilhorst HWM. ABA controls the second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination. Submitted for publication.

Toorop PE, Bewley JD, Abrams SR, Hilhorst HWM. Structure-activity studies with ABA analogs on germination and endo-ß-mannanase activity in tomato and lettuce seeds. Submitted for publication.

### abstracts

van der Schoot C, Toorop P, van Bel AJE (1987) Xylem-to-phloem transfer in the petiole of the third leaf of tomato. *Acta Botanica Neerlandica* **36**, 220. Poster presentation.

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Toorop PE (1995) Multiple isoforms of endo-ß-mannanase during germination. Fifth International Workshop on Seeds, Reading. Oral presentation.

Toorop PE, van Aelst AC, Hilhorst HWM (1997) ABA controls the second step of the biphasic endosperm cap weakening that mediates tomato seed germination. Autumn Meeting of the Dutch Society for Microscopy, Arnhem. Oral presentation.

Hilhorst HWM, Toorop PE (1997) Is endo-ß-mannanase a good marker for seed performance? Combined meeting of 'E.C. Concerted Action AIR-CT94-1863' and 'COST Action 828, WG4', Wageningen.

# Curriculum vitae

Peter Toorop werd geboren op 26 april 1964 te 's-Gravenhage. Na het atheneum doorlopen te hebben werd in 1982 begonnen aan de studie Biologie aan de Rijksuniversiteit Utrecht. Als hoofdvak koos hij Plantenfysiologie en als bijvak Moleculaire Genetica. In 1988 werd het doctoraal examen succesvol afgerond. Na een gastmedewerkschap van enkele maanden bij Dr. Lanfermeijer en Prof. Kollöffel aan de vakgroep Algemene Plantkunde te Utrecht was hij van 1990 tot 1992 werkzaam als analist bij dr. Woldringh aan de vakgroep Electronenmicroscopie en Moleculaire Cytologie van de Universiteit van Amsterdam. Na opnieuw een gastmedewerkerschap van enkele maanden te hebben bekleed bij Prof. Kollöffel aan de vakgroep Botanische Ecologie en Evolutiebiologie te Utrecht begon hij in 1993 als assistent in opleiding bij de vakgroep Plantenfysiologie te Wageningen, wat resulteerde in dit proefschrift. Sinds 1998 is hij werkzaam als post-doc bij het laboratorium voor Plantenfysiologie van het departement Biomoleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen.