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Class I ß-1,3-Glucanases in the Endosperm of Tobacco during Germination

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Rupture of the seed coat and rupture of the endosperm are separate events in the germination of *Nicotiana tabacum* L. cv. "Havana 425" seeds. Treatment with 10^{-5} M abscisic acid (ABA) did not appreciably affect seed-coat rupture but greatly delayed subsequent endosperm rupture by more than 100 h and resulted in the formation of a novel structure consisting of the enlarging radicle with a sheath of greatly elongated endosperm tissue. Therefore, ABA appears to act primarily by delaying endosperm rupture and radicle emergence. Measurements of ß-1,3-glucanase activity, antigen content, and mRNA accumulation together with reporter gene experiments showed that induction of class I ß-1,3-glucanase genes begins just prior to the onset of endosperm rupture but after the completion of seed-coat rupture. This induction was localized exclusively in the micropylar region of the endosperm where the radicle will penetrate. ABA treatment markedly inhibited the rate of ß-1,3-glucanase accumulation but did not delay the onset of induction. Independent of the ABA concentration used, onset of endosperm rupture was correlated with the same ß-1,3-glucanase content/seed. These results suggest that ABA-sensitive class I ß-1,3-glucanases promote radicle penetration of the endosperm, which is a key limiting step in tobacco seed germination.

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Abbreviations: *GLB*, gene encoding tobacco class I ß-1,3-glucanase B; PR, pathogenesis-related.

Germination of seeds is a complex physiological process triggered by imbibition of water. Under favorable conditions this results in a burst of respiration and rapid growth of the embryo culminating in rupture of the covering layers of the seed and emergence of the radicle. Molecular events associated with imbibition and post-germination mobilization of storage materials in the endosperm have been studied in detail (reviewed by Bewley and Black, 1994). Far less is known about the molecular basis for the rupture and physical penetration of the covering layers.

Depending on the species, either the seed coat or the endosperm can act as a major physical barrier to embryo enlargement and radicle emergence (Bewley and Black, 1994). In the case of endosperm-limited germination, penetration is often preceded by breakdown or weakening of the endosperm proximal to the radicle tip, which has been shown to be a prerequisite for radicle emergence in tomato (Groot and Karssen, 1987; Groot and Karssen, 1992; Liptay and Schopfer, 1983). Ikuma and Thimann (1963) first suggested that endosperm weakening is the consequence of enzymatic action. Later studies of the time course of enzyme accumulation and the incidence of germination support the view that cellulases in *Datura ferox* and endo-ß-mannanases in tomato have this function (Sanchez et al., 1986; Groot et al., 1988; Karssen et al., 1989).

The present report deals with the function of ß-1,3-glucanases (glucan endo 1,3-ß-glucosidase, E.C. 3.2.1.39) in tobacco seed germination. ß-1,3-Glucanases are abundant proteins found in all higher plants examined (reviewed by Stone and Clarke, 1992). There is evidence that they help defend plants against infection by pathogenic and potentially pathogenic fungi (reviewed by Bowles, 1990). Based on the localization of the enzymes or their putative substrate, callose, it has been proposed that ß-1,3-glucanases are also important for diverse physiological processes in the uninfected plant including pollen development, fertilization, mobilization of storage reserves in the endosperm, and cell division (reviewed by Stone and Clarke, 1992).

The ß-1,3-glucanases have been assigned to several classes based on primary structure. In tobacco, the vacuolar class I enzymes are localized primarily in the epidermis of lower leaves and in roots of healthy plants (Keefe et al., 1990). They are induced by treatment with the stress hormone ethylene and by microbial infection and are down-regulated in cultured cells by combinations of the growth hormones auxin and cytokinin (Felix and Meins, 1985, 1986, 1987). The class II and class III enzymes are secreted proteins similar in amino-acid sequence to the class I enzymes. Members of these classes include the pathogenesis-related (PR) proteins PR 2, PR N, PR O and PR Q' (Kauffmann et al., 1987; Payne et al., 1990; Ward et al., 1991a), as well as two glycoproteins localized in the style of the flower (Ori et al., 1990). Finally, novel ß-1,3-glucanases have been recently identified in anthers (Hird et al., 1993) and in virus-infected class I ß-1,3-glucanase-deficient mutants (Beffa et al., 1993).

In the mature tobacco seed, the embryo is surrounded by 3-5 layers of rather thick-walled endosperm cells. The periphery of the endosperm is pressed against the seed coat. The integument, except for a thin outer layer consisting of cutinized and lignified dead cells, is completely obliterated (Avery, 1933). Following imbibition in the light, tobacco seeds begin to accumulate class I ß-1,3-glucanases at roughly the time of radicle emergence (Vögeli-Lange et al., 1994a). This finding and the observation that expression of a class I ß-1,3-glucanase reporter gene is localized in the endosperm where the radicle will penetrate led us to suggest that ß-1,3-glucanase has a role in tobacco seed germination. Treatment with abscisic acid (ABA), which is thought to be important for the onset and maintenance of seed dormancy (reviewed by Bewley and Black, 1994), delays germination of seeds of many species including tobacco (Khalil, 1992). In the work described here we used ABA to explore the relationship between ß-1,3-glucanase induction and events in tobacco seed germination. We provide evidence that endosperm rupture is the limiting factor in radicle emergence and suggest as a working hypothesis that rupture of the endosperm depends on the ABA-sensitive induction of ß-1,3-glucanases in a specific region of the endosperm, which helps promote radicle penetration.

MATERIALS AND METHODS

Plant Materials

Seed of *Nicotiana tabacum* L. cv. "Havana 425" 6-12 months after harvest were used. The *GLB-GUS* transformant, which is homozygous for the transgene, was transformed with a construct containing 1.6 kb of the 5' flanking sequence of the tobacco class I ß-1,3-glucanase B gene fused to the GUS reporter gene and has been described (Vögeli-Lange et al., 1994a).

Germination Analysis

Seeds (100-200) were surface-sterilized for 10 min with 2% (w/v) NaOCl, rinsed with sterile water and sown in 9-cm-diameter plastic Petri dishes containing a double layer of filter paper (MN 713, Machery-Nagel). The paper was wetted with 6 ml autoclaved rooting medium consisting of dilute inorganic nutrients and thiamine (Meins and Binns, 1977). Where indicated, *cis*-S(+)-abscisic acid (ABA, Sigma) was added to the medium as a neutralized filter-

sterilized 10 mM stock solution. Petri dishes were sealed with Parafilm and incubated at 24 ˚C under continuous light. To score different stages of germination, seeds from 1-3 Petri dishes were counted for each time point. Plant material was harvested and stored at -80˚C for subsequent analysis.

Protein Assays

Tissue extracts were prepared (Felix and Meins, 1985) and clarified by centrifugation for 15 min at 12,000 x g. ß-1,3-Glucanase activity was measured radiometrically using [^{3}H]-laminarin as substrate (Keefe et al., 1990). Western-blot analysis of ß-1,3-glucanase and measurement of GUS activity with 4-methylumbelliferyl-ß-D-glucuronide as substrate were as described (Vögeli-Lange et al., 1994a). Protein was measured by the method of Bradford (1976) using bovine γ-globulin as standard.

Northern Analysis

Total RNA was extracted from batches of 50-150 seeds by the method of Chomcynski (1993) using TRIzol^{TM} reagent (Life Technologies). Electrophoresis of glyoxal-denatured total RNA in 1.1% (w/v) agarose gels, blotting onto nylon membranes, and hybridization (6 x SSC, 5 x Denhardt's reagent, 0.1% (w/v) SDS, 0.1 mg/ml fragmented, denatured salmon sperm DNA at 65˚C), and autoradiography were carried out according to standard procedures (Sambrook et al., 1989). A constant amount of total RNA (30 µg) as judged by absorbance at 260 nm was loaded in each lane. Hybridized membranes were washed at high stringency (30 min at 65˚C, 0.2 x SSC, 0.1% (w/v) SDS) and analyzed by autoradiography. The intensity of labelling was measured with a PhosphoImager (Molecular Dynamics) and is expressed in arbitrary units. The hybridization probes used were: the 1 kb *Pst*I fragment of tobacco class I ß-1,3-glucanase cDNA clone pGL43 (Shinshi et al., 1988), the 1.2 kb *Xho*I/*Xba*I fragment of a tobacco PR 2 cDNA clone (Ward et al., 1991b), the 1.4 kb *Eco*RI fragment of a tobacco PR Q' cDNA (Ward et al., 1991b), the 0.65 kb *Xho* I-*Bam* HI fragment of the tobacco stylar ß-1,3-glucanase cDNA clone sp41a (Ori et al., 1990), and a 1.8 kb *Eco*RI fragment of genomic DNA encoding tomato 18S ribosomal RNA (Schmidt-Puchta et al., 1989). The probes were labelled with [α-^{32}P]-dATP by random priming (Boehringer).

Histochemistry

Imbibed seeds were stained for GUS activity by a modification of the method of Jefferson *et al.*(1987). In brief, seeds were fixed for 20 min in cold 90% (v/v) acetone (Rodrigues-Pousada et al., 1993), rinsed thoroughly with water, and stained in 1 mM 5-bromo-4-chloro-3-indoxyl glucuronide (Research Organics), 0.2% (v/v) Tween 20, 50 mM Na phosphate buffer, pH 7.0 for 24 h at 37˚C in a moist chamber.

RESULTS

Rupture of the Seed Coat and the Endosperm are Separate Events in Tobacco Seed Germination

In our initial experiments we used morphological landmarks to stage events in tobacco seed germination. Surface-sterilized Havana 425 tobacco seeds were germinated in continuous light on filter paper moistened with a dilute solution of nutrient salts. Examination of the time course for germination in Figure 1a clearly shows that rupture of the seed coat and rupture of the endosperm are two distinct, roughly synchronous events well separated in time. Representative micrographs illustrating the different stages of germination are shown in Figure 2. We define stage I (0-30 h) as the period from the start of imbibition to the first signs of seed-coat rupture (Fig. 2a). Stage II (30-60 h) begins with the splitting of the seed coat at the micropylar end of the seed. During this stage the length of the seed increases ca. 1.3-fold and the growing radicle, still encased in the endosperm, gradually protrudes through the gap in the seed coat (Fig. 2b). The last stage in germination, stage III (≥60 h) is the rupture of the protruding endosperm and emergence of the radicle (Fig. 2c), which then continues to grow rapidly (Fig. 2d).

Induction of ß-1,3-Glucanases Begins Just Prior to the Onset of Endosperm Rupture

The timing of ß-1,3-glucanase induction was established on the basis of enzyme activity, protein and mRNA content, and expression of a reporter gene. ß-1,3-Glucanase activity was measured using as substrate the algal ß-1,3-glucan laminarin, which can be digested by all known tobacco ß-1,3-glucanases (Kauffmann et al., 1987; Beffa et al., 1993). Accumulation of class I ß-1,3-glucanase antigen was measured semiquantitatively by western blot analysis. Abundance of the mRNA for class I ß-1,3-glucanase was estimated by quantitating the intensity of labelled bands on northern blots of total seed RNA. Finally, activity of the tobacco class I ß-1,3-glucanase B (*GLB*) promoter was estimated from ß-glucuronidase (GUS) activity using seed of tobacco plants transformed with a *GLB-GUS* reporter-gene construct (Vögeli-Lange et al., 1994a; Vögeli-Lange et al., 1994b). We also measured chitinase activity as an independent marker. This enzyme was chosen because it is coordinately regulated with class I ß-1,3-glucanase at the mRNA level in response to hormone treatment and pathogen infection (Shinshi

et al., 1987; Vögeli-Lange et al., 1988; Meins and Ahl, 1989).

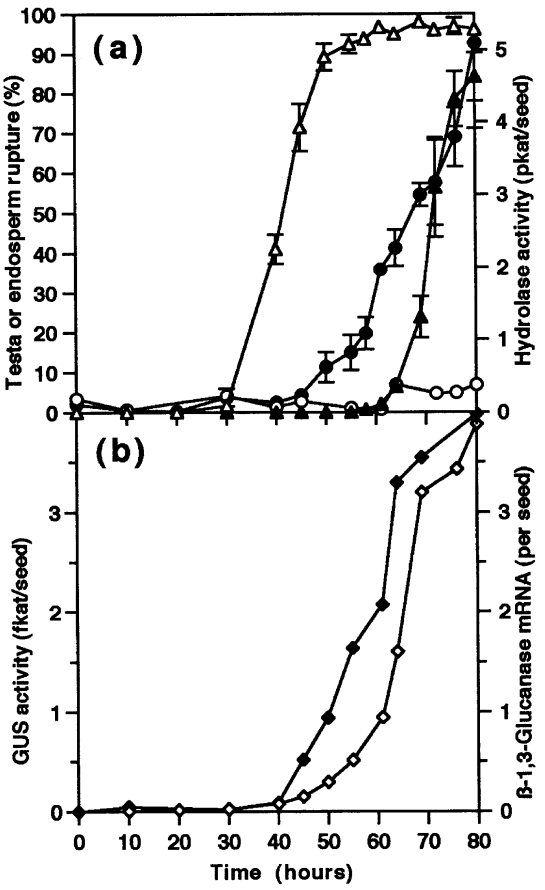

Figure 1. The time course of ß-1,3-glucanase induction during tobacco seed germination. (a) The incidence of seed-coat (testa) rupture (Δ) and endosperm rupture (▲) expressed as % of 100-200 seeds scored with time after the start of imbibition. The activities of ß-1,3-glucanase (●) and chitinase (❍), are expressed in pkat/seed. The values presented are the means ± SEM obtained with at least two batches of seed in two independent experiments. (b) The time course for accumulation of class I ß-1,3-glucanase mRNA (♦) and GUS activity (◊) resulting from expression of the class I *GLB* promoter. The content of mRNA is expressed in arbitrary PhosphoImager units per seed. A similar pattern of induction was found when mRNA was normalized for the signal obtained with 18S rRNA. GUS activity, expressed as fkat/seed, was measured in seeds from plants homozygous for the *GLB-GUS* transgene. The time course for covering-layer rupture and ß-1,3- glucanase activity was the same for GLB-GUS and wild-type seed lots.

Very low, roughly constant ß-1,3-glucanase activity was detected in stage I seeds. Activity, expressed on a per seed basis, first increased in stage II, ca. 50 h after the start of imbibition, at the time when seed-coat rupture was complete, but ca. 10 h before the onset of endosperm rupture (Fig. 1a). Induction was greater than 50-fold by 80 h when endosperm rupture was complete. A similar pattern of induction was observed when the data were expressed on a protein basis. Over the same period of time, there was essentially no induction of chitinase activity.

Figure 3a shows a western blot stained with an antibody directed against class I tobacco ß-1,3-glucanase, which cross-reacts with all known tobacco ß-1,3-glucanases (Neuhaus et al., 1992; Beffa et al., 1993). The major labelled band was at the 33 kDa position in the gel corresponding to class I ß-1,3-glucanase. The signal at this position increased with increasing ß-1,3-glucanase activity. No antigen was detected at the 34-36 kDa position of the gel indicating that there was no appreciable induction of the class II and III pathogenesis-related ß-1,3-glucanases PR 2, PR N, PR O, and PR Q' during germination (Fig. 3a). Additional faint bands were sometimes detected. In the experiment shown, a faint band at 45 kDa decreased with time during germination. On the other hand, the doublet at 40 kDa and 41 kDa, which corresponds in size to the class II stylar ß-1,3-glucanases (Ori et al., 1990), was detectable 30 h after the start of imbibition and remained constant in intensity or increased slightly in intensity during stage III. In other experiments a faint band at ca. 64 kDa was also detected, which appears to be enriched in extracts prepared from isolated endosperms (Vögeli-Lange et al., 1994a). The nature of these antigens is not known. Their appearance was not correlated with ß-1,3-glucanase activity.

The pattern of induction obtained for the 1.6 kb class I ß-1,3-glucanase mRNA was very similar to that of the class I antigen and enzyme activity except, as expected, the onset of mRNA accumulation was ca. 5-10 h earlier (Fig. 1b and Fig. 3b). No transcripts were detected by northern analysis using probes for stylar sp41 (class II), PR 2 (class II) or PR Q' (class III) ß-1,3-glucanases confirming the results of the western analyses (data not shown). Finally, activity of the *GLB* promoter, as judged by GUS activity, showed essentially the same time course as ß-1,3-glucanase activity (Fig. 1b). Taken together, the results indicate that induction of ß-1,3-glucanase activity starts in stage II before the endosperm ruptures and continues throughout stage III of germination. The close agreement of the kinetics obtained for promoter activity, mRNA accumulation, antigen accumulation, and enzyme activity show that the activity induced is due to class I ß-1,3-glucanase and that induction is regulated predominately at the transcriptional level.

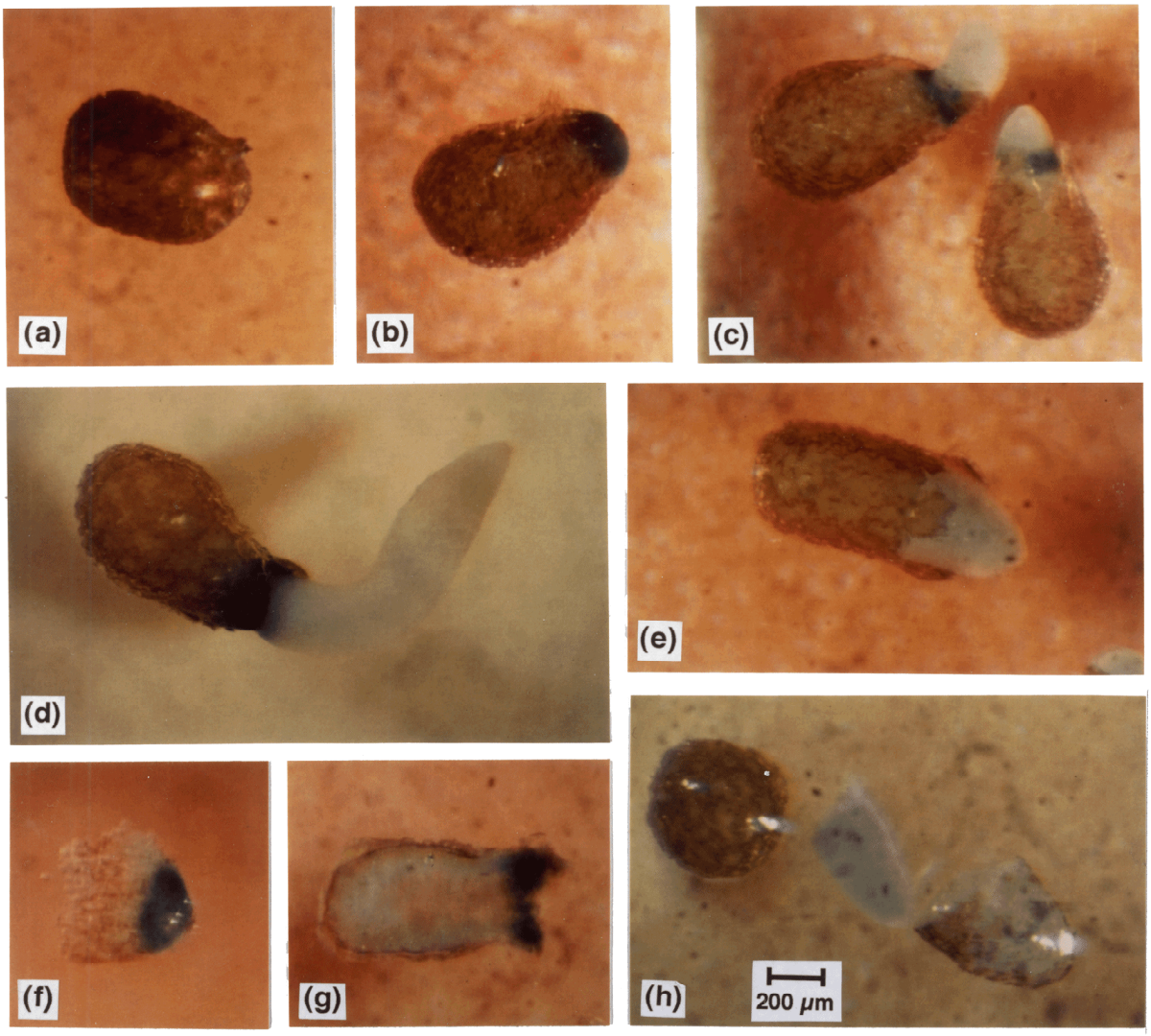

Figure 2. Stages in the germination of tobacco seed homozygous for the *GLB-GUS* transgene. Seeds were germinated in continuous light at 24˚C with 10^{-5} M ABA (ABA) and without ABA (control) added to the medium. At the times indicated after the start of imbibition, seeds were stained for GUS activity. The blue staining is indicative of transcriptional activity of the class I ß-1,3-glucanase B promoter. **(a)** Stage I (control, 3 h): intact seed prior to seed-coat rupture. **(b)** Stage II (control, 60 h): seed with ruptured seed coat and protruding endosperm. **(c)** Stage III (control, 72 h): seeds with ruptured endosperm showing GUS staining at the rupture site and emerging radicles, which do not stain for GUS. **(d)** Stage III (control, 96 h): seed with ruptured endosperm and elongating radicle. GUS staining is localized in a collar of endosperm tissue at the site of radicle penetration. **(e)** Stage II (ABA, 144 h): ABA treatment markedly delays endosperm rupture and results in a novel structure consisting of the enlarging radicle completely enclosed in a sheath of intact endosperm, which does not stain for GUS. **(f)** Stage II (control, 60 h): endosperm dissected prior to rupture showing GUS stain localized in the micropylar region. **(g)** Stage III (control, 96 h): endosperm dissected after rupture showing that the radicle penetrates the region which stains for GUS. **(h)** Stage II (ABA, 144 h): a seed arrested in stage II by ABA treatment dissected to show that the elongated radicle is enclosed in a sheath of endosperm. Magnification: 40X.

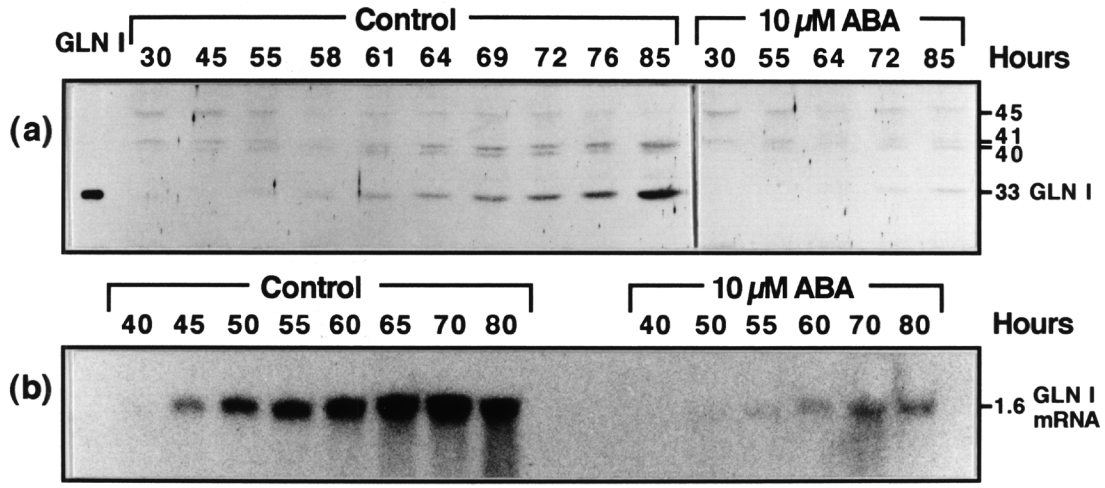

Figure 3. The time course of class I ß-1,3-glucanase antigen and mRNA accumulation during and following the germination of tobacco seed with 10^{-5} M ABA (ABA) and without ABA (control) added to the medium. **(a)** Western-blot analysis of antigens reacting with antibody directed against tobacco class I ß-1,3-glucanase. Equal amounts of protein (150 µg) were applied to each lane. GLN I, 10 ng of tobacco class I ß-1,3-glucanase. The apparent size in kDa of immunoreactive bands is indicated. **(b)** Northern-blot analysis of total RNA (30 µg/lane) using a hybridization probe for tobacco class I ß-1,3-glucanase mRNA. The size of the transcript in kb is indicated. Note the marked reduction in ß-1,3-glucanase mRNA accumulation in the ABA-treated seeds. Very similar patterns of accumulation were obtained when hybridization was quantitated with a PhosphoImager and normalized for the signal obtained with 18S rRNA used as an internal standard (data not shown).

ABA Inhibits the Rate of ß-1,3-Glucanase Accumulation and Delays Endosperm Rupture

Addition of ABA to the medium is known to delay germination of tobacco seeds in the light (Khalil, 1992). Figure 4 shows the effect of different ABA concentrations on the time course of ß-1,3-glucanase induction, seed-coat rupture, and endosperm rupture. At the physiological concentrations tested, ABA had little effect on seed-coat rupture (Fig. 4a) but markedly delayed the onset of endosperm rupture in a dose-dependent fashion (Fig. 4b). For example, treatment with 10^{-5} M ABA delayed endosperm rupture by more than 100 h, as judged from the time required for 50% of seeds to exhibit a ruptured endosperm. There was no effect on the final incidence of rupture; and, the time from the onset to completion of rupture in seed populations was only extended at the highest ABA concentration tested.

ABA had a marked effect on the morphology of germinating seeds. The treated seeds were elongated relative to untreated seeds and showed a conspicuous protuberance through the broken seed coat (Fig. 2e). Dissection of this novel structure showed that it consists of a greatly elongated sheath of endosperm covering the enlarged radicle (Fig. 2h). Although we cannot rule out the possibility that ABA also inhibits embryo growth, the results suggest that ABA delays germination primarily by preventing penetration of the endosperm by the radicle.

Examination of the time courses in Figures 3 and 4c shows that ABA also inhibited the induction of ß-1,3-glucanase activity, class I ß-1,3-glucanase antigen, and class I ß-1,3-glucanase mRNA. The pattern of inhibition was, however, very different from that observed with endosperm rupture. Based on activity measurements, the rate of ß-1,3-glucanase accumulation was inhibited with increasing ABA concentration; but, the onset of induction was not delayed. Independent of the ABA concentration used and the timing of germination events, the onset of endosperm rupture in the seed population was associated with roughly the same ß-1,3-glucanase activity per seed. This is more apparent when the data from Figure 3c are plotted as the incidence of endosperm rupture versus ß-1,3-glucanase content shown in Figure 5. There was a sharp transition in incidence of rupture over a narrow range of ß-1,3-glucanase activities (2-3 pkat/seed).

ABA-Sensitive ß-1,3-Glucanase is Localized in Micropylar Endosperm

Experiments were performed to establish the location of ß-1,3-glucanase expression during germination. Control and 10^{-5} M ABA- treated stage II seeds were harvested 3 d after the start of imbibition, which is just prior to the onset of endosperm rupture in control seeds. The embryos and endosperm with adhering seed coat were separated and

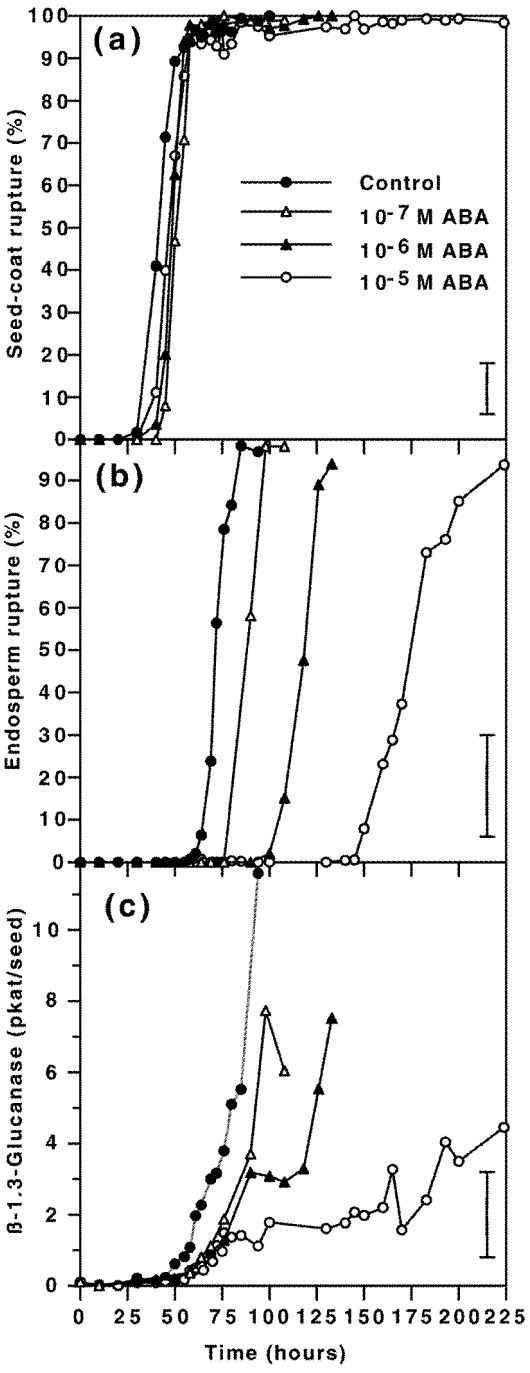

Figure 4. The effect of different ABA concentrations on tobacco seed germination. The incidence of ruptured seed coats **(a)**, ruptured endosperm **(b)**, and ß-1,3-glucanase activity expressed as pkat/seed **(c)**. Seeds were germinated in continuous light at 24˚C in the presence of 0 (Control), 10^{-7} M, 10^{-6} M, and 10^{-5} M ABA added to the medium at the start of the experiment. The data are presented as mean values, usually for at least two samples in two independent experiments. The error bars represent the maximum ±SEM.

the endosperm was dissected into the micropylar region surrounding the radicle and the remaining, non-micropylar region. Intact wild-type seeds and their components were assayed for ß-1,3-glucanase and chitinase activity. Similar preparations from seed homozygous for the *GLB-GUS* construct were also assayed for GUS activity. The results in Table I show that essentially all of the ß-1,3-glucanase activity is localized in the micropylar region of the endosperm and that this activity is inhibited by ABA treatment. Western- and northern-blot analyses confirmed that class I ß-1,3-glucanase expression is confined to the micropylar region of the endosperm and is down-regulated by ABA (data not shown). GUS activity indicative of class I ß-1,3-glucanase promoter activity was localized exclusively in the micropylar region of the endosperm and was completely blocked by ABA treatment (Table 1). Taken together, these results and those obtained from time course experiments show that essentially all the ß-1,3-glucanase activity present in seeds immediately before endosperm rupture is localized in the micropylar region of the endosperm and that this ABA-sensitive activity can be accounted for by expression of class I ß-1,3-glucanase genes.

A higher resolution localization of ABA-sensitive ß-1,3-glucanase expression was obtained by histological examination of homozygous GLB-GUS seeds stained for GUS activity (Fig. 2). Staining was localized exclusively in the micropylar endosperm.

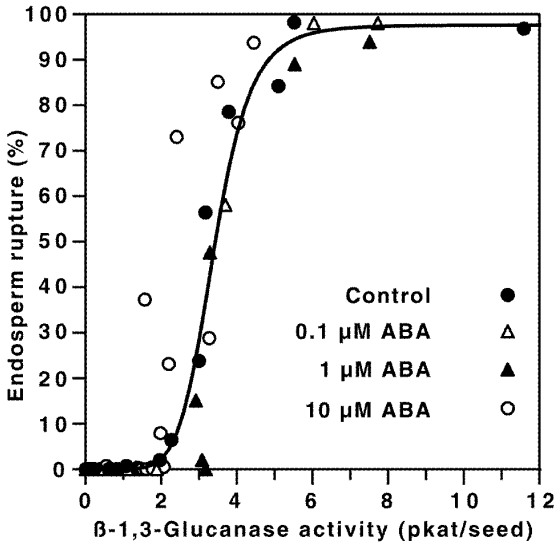

Figure 5. Relationship of the incidence of endosperm rupture to ß-1,3-glucanase content. The data from Figure 4b and 4c are plotted as incidence of endosperm rupture versus ß-1,3-glucanase content expressed in pkat/seed for seeds treated with 0 (Control), 10^{-7}, 10^{-6} and 10^{-5} M ABA. The solid line is a Hill Function fit of the data. The ß-1,3-glucanase content calculated for 50% endosperm rupture is ca. 3.4 pkat/seed.

Table I *Localization of ß-1,3-glucanase and chitinase expression in stage II germinating seeds just prior to endosperm rupture*

Seed component [a]	ß-1,3-Glucanase		*GLB* promoter activity		Chitinase	
	Control	ABA	Control	ABA	Control	ABA
	pkat/seed		*fkat/seed*[b]		*pkat/seed*	
Entire seed	2.37 ± 0.26 [c]	0.56 ± 0.11	1.63 ± 0.43	0.12 ± 0.05	0.30 ± .05	0.29 ± 0.07
Embryo	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	ca. 0 [d]	0.14 ± 0.04	0.12 ± 0.01
Entire endosperm	2.57 ± 0.27	0.56 ± 0.18	1.94 ± 0.46	0.09 ± 0.06	0.19 ± 0.05	0.23 ± 0.01
Micropylar endosperm	2.67 ± 0.06	0.69 ± 0.31	1.30	ca. 0	0.08 ± 0.01	0.11 ± 0.08
Non-micropylar endosperm	0.11 ± 0.03	ca. 0	ca. 0	ca. 0	0.07 ± 0.05	0.05 ± 0.04

[a] Batches of 100 seeds were dissected after 3 d incubation in continuous light with 10^{-5} M ABA (ABA) and without ABA (Control). The endosperm fractions contain a thin layer of adhering seed coat. [b] Expressed as GUS activity measured using seeds of a homozygous GLB-GUS transformant

[c] Mean values ± SEM for at least two independent experiments [d] below the limit of detection, 0.004

Late in stage II, the tip of micropylar endosperm protruding from the seed is stained (Fig. 2b and f). After rupture of the endosperm, a thin collar of endosperm surrounding the emerging radicle is stained (Fig. 2c, d and g). No stain was detected in the conspicuous endosperm sheath of seeds treated with ABA (Fig. 2e and h).

DISCUSSION

We found that rupture of the seed coat and the endosperm are distinct events in tobacco-seed germination. Rupture of the seed coat following imbibition leaves the embryo enclosed in the endosperm. The first signs of endosperm rupture and emergence of the radicle are only apparent after rupture of the seed coat is complete. When seeds are treated with ABA, seed-coat rupture and initial growth of the embryo are unaffected; but, the elongating radicle remains surrounded by an endosperm sheath and further growth of the embryo is restricted until the endosperm eventually ruptures. Thus, rupture of the endosperm appears to be the limiting factor in radicle emergence. The only similar examples for this two-step mechanism are germination of pepper and *Chenopodium album* seeds (Watkins et al., 1985; Karssen, 1976a; Karssen, 1976b; Cumming, 1963). In the case of *C. album*, light triggers the splitting of the outer layer of the seed coat and initial growth of the radicle. Depending on environmental conditions, the embryo can remain encased in the inner layer of the seed coat and endosperm for up to one year until conditions favor emergence of the radicle (Bouwmeester and Karssen, 1993).

Delay of germination due to inhibitory effects of ABA acting primarily on the covering layers has also been reported for *C. album* (Karssen, 1976a; Karssen, 1968) and tomato seeds (Groot and Karssen, 1992; Ni and Bradford, 1992; Liptay and Schopfer, 1983). On the other hand, in *Brassica napus* seeds the embryo is surrounded by a thin, fragile seed coat and ABA was found to delay germination by reducing the expansive force of the embryo (Schopfer and Plachy, 1985). In lettuce, ABA treatment appears to delay germination by reducing both the expansive force of the embryo (Carpita et al., 1979) and weakening of the endosperm (Dutta et al., 1994).

The present study shows that class I ß-1,3-glucanase is spatially and temporally regulated during seed germination. The important question that arises is, what is the physiological function of ß-1,3-glucanase induction? One possibility is suggested by the fact that class I ß-1,3-glucanases are induced as part of the defense reaction of plants to infection with microbial pathogens (reviewed by Fincher, 1989). Thus, accumulation of ß-1,3-glucanases at the site of rupture might protect against infection once the radicle begins to emerge. The main argument against this hypothesis is that class I ß-1,3-glucanases and chitinases are coordinately induced as part of the defense reaction; and, ß-1,3-glucanases are only effective in inhibiting fungal growth in combination with chitinases (reviewed by Ward et al., 1991a; Meins et al., 1992). Neither class I chitinase nor class II and class III ß-1,3-glucanases associated with the defense reaction are induced during seed germination.

We favor the working hypothesis that class I ß-1,3-glucanases contribute to the weakening of the endosperm and thereby facilitate penetration by the radicle. This hypothesis is consistent with several observations. First, the endosperm rather than the seed coat is the physical barrier to radicle emergence.

Second, the onset of ß-1,3-glucanase induction, which is almost exclusively due to expression of class I ß-1,3-glucanase genes, is just prior to endosperm rupture but after the completion of seed coat rupture. Third, ß-1,3-glucanase is only expressed in the micropylar region of the endosperm where the radicle will penetrate. Finally, ABA delays the onset of endosperm rupture and inhibits ß-1,3-glucanase accumulation at the site where rupture will occur.

We suggest that ß-1,3-glucanases weaken the endosperm by helping to hydrolyze cell-wall polysaccharides. Several cell-wall hydrolases have been implicated in endosperm weakening. Cellulase activity is localized in the micropylar region of the endosperm of *Datura ferox* seeds, and is induced before the earliest signs of either endosperm weakening or radicle emergence (Sanchez et al., 1986). It has been proposed that the timing of tomato seed germination, which is regulated by ABA and gibberellin, is mediated by cell-wall hydrolase activity leading to endosperm weakening (Groot et al., 1988; Ni and Bradford, 1993). Weakening of the endosperm proximal to the radicle has been shown to be required for radicle emergence and is delayed by ABA (Groot and Karssen, 1987; Groot and Karssen, 1992; Ni and Bradford, 1992). Moreover, surgical rupture of the endosperm abolished this inhibitory effect of ABA (Liptay and Schopfer, 1983). The walls of tomato and lettuce endosperm cells are composed primarily of mannans (Halmer et al., 1975; Groot et al., 1988). In tomato, endo-ß-mannanase, but not cellulase activity is induced prior to radicle emergence and is promoted by gibberellin, which also promotes seed germination (Groot et al., 1988; Karssen et al., 1989). In contrast, endo-ß-mannanase activity in lettuce seeds increased after the emergence of the radicle (Halmer et al., 1976) and recent studies have shown that breakdown of micropylar-endosperm cells prior to radicle emergence is caused by an as yet unidentified endohydrolase (Dutta et al., 1994). Thus, from the few cases studied in detail it is still unclear if combinations of hydrolases are involved in wall weakening, as seems likely, and to what extent the enzymes and substrates vary from species to species.

Direct evidence for a function of ß-1,3-glucanase in endosperm rupture is still lacking. It remains to be demonstrated that the cell wall of tobacco endosperm contains substrates for ß-1,3-glucanases and that these molecules decrease in degree of polymerization at the onset of endosperm rupture. Another problem is compartmentation. In tobacco leaves, and presumably in the endosperm as well, class I ß-1,3-glucanases are localized in the vacuole (Keefe et al., 1990). If the ß-1,3-glucanases act on the cell wall as we suggest, then mechanisms should exist for transporting the enzymes to the cell surface.

Class I ß-1,3-glucanases show a novel pattern of expression during seed germination. Several cell-wall associated hydrolases, including ß-1,3-glucanases in cereals, have been implicated in the mobilization of reserve polysaccharides associated with germination. In these cases, the enzymes are induced in the endosperm, but expression eventually spreads throughout the tissue and is not confined to the micropylar end (e.g. Halmer et al., 1976; Fincher, 1989; Malek and Bewley, 1991; Dutta et al., 1994). Class I ß-1,3-glucanases are down-regulated in cultured pith cells of tobacco by combinations of the growth hormones auxin and cytokinin and are induced in plants by ethylene treatment, wounding, and microbial infection. These forms of regulation are tightly coordinated with the regulation of class I chitinases and, in the case of microbial infection, with class II and class III ß-1,3-glucanase (reviewed by Ward et al., 1991a; Meins et al., 1992). The present studies show that during seed germination class I ß-1,3-glucanases can also be down-regulated by ABA and that induction of this enzyme is not coordinated with induction of the other PR proteins tested.

The close correlation between promoter activity, mRNA accumulation, and antigen accumulation provide strong evidence that ß-1,3-glucanase is regulated primarily at the level of transcription during seed germination. The *GLB* promoter has several regions that could play a role in transcriptional regulation. The highly conserved TGTAAAG core of the -300 element, which is required for endosperm-specific expression of storage proteins in cereals and is active in tobacco, is present at a comparable position in the *GLB* promoter (Vögeli-Lange et al., 1994a). The promoter of genes encoding basic a-amylase in barley contain a negative-acting TAACAAA box at -130 important for down-regulation by ABA, but do not contain the ABA response elements found in genes induced by ABA (Gubler and Jacobsen, 1992). It is of particular interest that the *GLB* promoter, which shows ABA regulation similar to basic a-amylase, also has the negative element but not the positive element. There are two copies of this element in the *GLB* promoter: a distal copy at -1545 in a region important for high-level induction; and, a proximal copy at -93 in a region important for root-specific expression (Vögeli-Lange et al., 1994b).

The physiological mechanism for highly localized expression in the micropylar endosperm is not known. Even assuming that the -300 element is responsible for endosperm-specific expression, other factors must be important in specifying expression in the micropylar region just prior to radicle penetration. It has been proposed that during tomato seed germination, endosperm weakening mediated by cell-wall hydrolases is regulated by inductive effects of gibberellins and inhibitory effects of ABA (Ni and Bradford, 1993). Thus, these hormones, or other signals from the elongating radicle might specify when and where in the endosperm ß-1,3-glucanase is induced.

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