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Effects of gibberellins, darkness and osmotica on endosperm rupture and class I ß-1,3-glucanase induction in tobacco seed germination

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Abstract. The "Havana 425" cultivar of *Nicotiana tabacum* L. is photodormant. Gibberellins (e.g. 10^{-5} M GA_4 or GA_7) can substitute for light in releasing dormancy. Measurements of ß-1,3-glucanase activity, mRNA accumulation and the activity of the class I ß-1,3-glucanase B promoter indicate that class I ß-1,3-glucanases are induced by GA_4 in the dark in association with germination. As in the light, this induction occurred prior to endosperm rupture and was localized exclusively in the micropylar region of the endosperm where the radicle will penetrate. Abscisic acid (ABA, 10^{-5} M) did not appreciably affect GA-induced release of photodormancy or seed coat rupture, but it delayed endosperm rupture and inhibited the rate of class I ß-1,3-glucanase accumulation. Seeds imbibed in the light in the presence of osmotica, e.g. 0.04 M polyethylene glycol 6000, showed delayed seed-coat and endosperm rupture, delayed onset of ß-1,3-glucanase induction, and decreased rates of ß-1,3-glucanase accumulation. These delays were shortened by GA_4 treatment. Our results suggest that GAs and ABA act at two distinct sites during germination and that expansive growth of the embryo acts in two ways by triggering ß-1,3-glucanase induction and by providing force for endosperm penetration. This provides further support for our working hypothesis that class I ß-1,3-glucanases promote endosperm weakening and facilitate radicle penetration.

Key words: Endosperm – Germination – Gibberellins - ß-1,3-Glucanase – Photodormancy -Tobacco

Abbreviations: ABA= *cis*-S(+)-abscisic acid; GA= gibberellin; GUS= ß-glucuronidase; PEG= polyethylene glycol

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Introduction

In seeds of several species, the endosperm functions as a mechanical restraint to germination which must be overcome by the growth potential of the embryo (review, Bewley and Black, 1994). In these seeds, emergence is often preceded by breakdown or weakening of the endosperm proximal to the radicle tip (Juntilla, 1973; Watkins et al. 1985; Sanchez et al. 1990; Dutta et al. 1994). Studies with tomato seeds have shown that weakening of the micropylar endosperm is a prerequisite for germination and suggest that hydrolytic enzymes, in particular endo-ß-mannanases, contribute to this process (Liptay and Schopfer, 1983; Groot and Karssen, 1987; Groot et al. 1988; Groot and Karssen, 1992). Similar functions for cellulases in *Datura ferox* (Sanchez et al. 1986) and ß-1,3-glucanases in tobacco (Vögeli-Lange et al. 1994; Leubner-Metzger et al. 1995) have been proposed.

Plant growth regulators play important roles in seed germination. Abscisic acid (ABA) is involved in induction and maintenance of seed dormancy in many plants; whereas, the gibberellins (GA) have actions that appear to be antagonistic to ABA (review, Bewley and Black, 1994). They can break dormancy, promote germination, and substitute for light in the release of photodormancy. There is evidence that these growth regulators applied to germinating seeds of some species have antagonistic effects on the endosperm, e.g. in tomato seeds GA promotes weakening and ABA reduces weakening (Groot and Karssen, 1987; Karssen et al. 1989; Groot and Karssen, 1992; Ni and Bradford, 1993).

Earlier we provided evidence that endosperm rupture is a limiting factor in the germination of Havana 425-variety tobacco seeds imbibed in the light (Leubner-Metzger et al. 1995). Treatment of seeds with 10^{-5} M ABA greatly delayed endosperm rupture and resulted in the formation of a novel structure consisting of the enlarging radicle with a sheath of greatly elongated endosperm tissue. Of particular interest was the finding that ß-1,3-glucanase activity is induced just prior to the onset of endosperm rupture and that ABA treatment markedly inhibited the rate of ß-1,3-glucanase accumulation. The tobacco ß-1,3-glucanases consist of a group isoforms that differ in cellular localization, primary structure, and regulation (review, Meins et al. 1992). Measurements of ß-1,3-glucanase activity, the concentration of ß-1,3-glucanase isoforms and mRNA in combination with reporter gene experiments using ß-glucuronidase regulated by the ß-1,3-glucanase B promoter established that most if not all of the enzyme activity detected is due to the induction of the class I vacuolar isoform at the level of transcription. Moreover, induction of the class I isoform was localized exclusively in the micropylar region of the endosperm where the radicle will penetrate. Taken together, these results suggested as a working hypothesis that ABA-sensitive ß-1,3-glucanases contribute to the weakening and subsequent penetration of the endosperm.

In the present report, we have investigated the interaction of light, GA, ABA, and osmotica in their effects on seed germination and ß-1,3-glucanase induction in Havana 425 tobacco. We show that light is required to trigger germination of this variety; that GA can substitute for light in breaking dormancy; that GA and ABA are likely to act at different sites; and, that under the different conditions tested there is close correlation between class I ß-1,3-glucanase induction and the onset of endosperm rupture.

Materials and methods

Plant Materials. Seeds of *Nicotiana tabacum* L. cv. "Havana 425" (Agricultural Experiment Station, University of Wisconsin, Madison, WI, USA) 6-12 months after harvest were used. The *GLB-GUS* transformant, which is homozygous for the transgene, contained a construct consisting of 1.6 kb 5' flanking sequence of the tobacco class I ß-1,3-glucanase B gene fused to the GUS reporter gene as described by Vögeli-Lange et al. (1994).

Germination analysis. Germination experiments have been described (Leubner-Metzger et al. 1995). Where indicated, *cis*-S(+)-abscisic acid (ABA, Sigma, St. Louis, MO, USA) and the gibberellins GA_3, GA_4, or GA_7 (Sigma) were added to the medium as neutralized filter-sterilized 10 mM stock solutions. Osmotica were added from autoclaved stock solutions of polyethylene glycol 6000 (PEG 6000, Merck, Darmstadt, Germany) or D-mannitol (Fluka, Buchs,

Switzerland).

Analysis of proteins and RNA. Procedures for extracting of proteins and RNA, assays of enzyme activity, immunoblot analysis, and RNA blot analyses have been described (Leubner-Metzger et al. 1995). In brief, ß-1,3-glucanase activity was assayed radiometrically using ^{3}H-laminarin as the substrate. ß-Glucuronidase (GUS) activity was measured fluorometrically with 4-methylumbelliferyl-ß-D-glucuronide as the substrate. The rabbit anti-tobacco ß-1,3-glucanase antibody used for immunoblot analyses detects the class I, class II and class III isoforms of the enzyme (Neuhaus et al. 1992; Beffa et al. 1993). The radiolabelled probe used in RNA blot analyses was the 1 kb *Pst* I fragment tobacco class I ß-1,3-glucanase cDNA pGL43 (Shinshi et al. 1988). Hybridized membranes were washed at high stringency (30 min at 65 ˚C in 0.1 % (w/v) sodium dodecyl sulfate, 30 mM NaCl, 3 mM sodium citrate, pH 7.0). Protein was measured by the method of Bradford (1976) using bovine γ-globulin as standard.

Results

Effects of GA and ABA on light- and dark-incubated seeds. In initial experiments we examined the effects of light and GA_4 on the germination of Havana 425 tobacco. Under our standard conditions, i.e., imbibition in continuous light at 25 ˚C on filter paper moistened with a dilute solution of nutrient salts, seed coat rupture and subsequent endosperm rupture are separate events well separated in time: seed coat rupture begins 30 h after the start of imbibition and endosperm rupture begins 30 h later (Leubner-Metzger et al. 1995). Seeds were incubated in continuous light and in darkness with and without 10^{-5} M GA_4 added to the nutrient solution at the time of imbibition. The incidence of seeds with ruptured seed coat and ruptured endosperm was measured daily for 4 days. After scoring, groups of ≈ 100 seeds were assayed for ß-1,3-glucanase activity 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).

When incubated in continuous light, nearly 100% of the seeds showed seed-coat and endosperm rupture 4 days after imbibition (Fig. 1A,B). In contrast, when incubated in darkness 80-90% of the seeds did not germinate (Fig. 1D,E) and the incidence

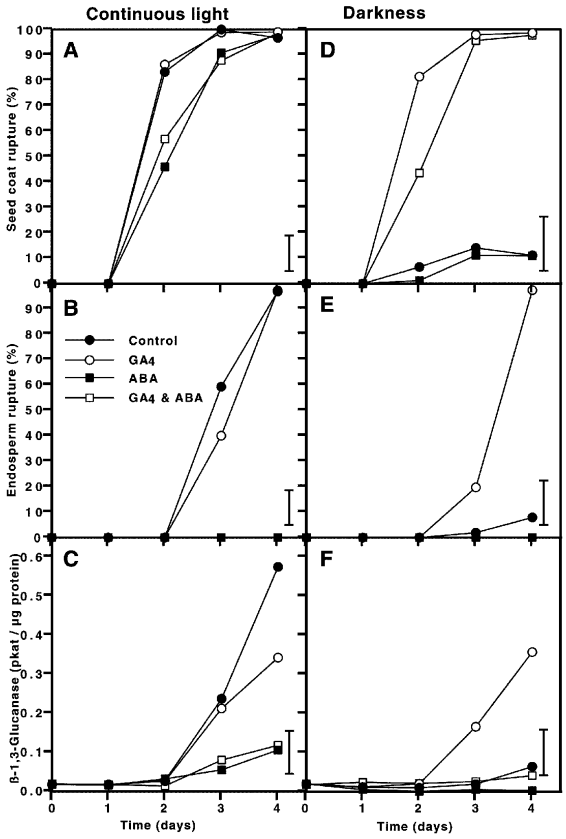

Fig. 1A-E. The effect of GA_4 and ABA treatment on the time course of tobacco seed germination and ß-1,3-glucanase activity in seeds incubated in continuous light (**A-C**) or in the dark (**D-F**). The incidence of seed coat rupture (**A** and **D**) and endosperm rupture (**B** and **E**) expressed in per cent of 100-200 seeds scored. The activity of ß-1,3-glucanase (**C** and **F**) expressed in pkat·(µg protein)$^{-1}$. Seeds were incubated in the absence (control) or presence of 10^{-5} M GA_4, 10^{-5} M ABA, or GA_4 and 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. Error bars= maximum ±SE

of seed-coat and endosperm rupture did not increase further for up to 5 weeks. These results indicate that Havana 425 is photodormant. As found for other photodormant varieties of tobacco (Avery, 1933), there was also a small subpopulation of 10-20% able to germinate even in the dark. GAs have been reported to substitute for light in releasing seed

dormancy in other photodormant varieties of tobacco (Hashimoto, 1958; Hashimoto and Yamaki, 1959; Khalil, 1992). GA_4 at a concentration of 10^{-5} M released seed dormancy of Havana 425 tobacco in the dark (Fig. 1D,E). We also confirmed for this variety the finding of Hashimoto and Yamaki (1959) that GA_3 is ≈20% as effective as GA_4 and found that GA_7 is also as effective as GA_4 at the same concentration (data not shown). GA_4 at a concentration of 10^{-5} M had no appreciable effect on the incidence of either seed-coat or endosperm rupture in populations of seeds imbibed in continuous light (Fig. 1A,B).

Treatment of Havana 425 tobacco seeds in light with 10^{-5} M ABA has been shown to delay seed-coat rupture by 5-10 h and endosperm rupture by more than 100 h (Leubner-Metzger et al. 1995). Although no endosperm rupture was detected during the 4 day period investigated (Fig. 1B,E), eventually endosperm rupture occurs in non-photodormant seeds indicating that ABA delays but does not block this process. ABA treatment also slightly delayed seed coat rupture of light-imbibed seeds, of non-photodormant dark-imbibed seeds, and of dark-imbibed seeds induced to germinate by GA_4 treatment (Fig. 1A,D). The inhibitory effects of ABA on seed-coat and endosperm rupture in the light were not prevented by GA_4 added at the same time to the cultures (Fig. 1A,B). Taken together, the results show that GA_4 can substitute for light in its effects on both seed-coat and endosperm rupture and that treatment with equal concentrations of GA_4 cannot reverse the inhibitory effects of ABA on endosperm rupture.

Earlier we showed that the induction ß-1,3-glucanase activity in light-imbibed Havana 425 tobacco seeds begins after completion of seed-coat rupture but ≈10 h before the onset of endosperm rupture (Leubner-Metzger et al. 1995). The present experiments confirmed that ß-1,3-glucanase induction is associated with endosperm rupture, the lag between enzyme induction and the onset of endosperm rupture is not evident because measurements were made on a daily basis. In dark-imbibed seeds, there was a slight increase in ß-1,3-glucanase activity which was correlated with the onset of endosperm rupture in the non-photodormant subpopulation of seeds (Fig. 1F). Separate measurements of photodormant and non-photodormant seeds showed that this activity was associated exclusively with the non-photodormant seeds undergoing endosperm rupture (data not shown). The induction of ß-1,3-glucanase activity was also correlated with the onset of endosperm rupture when dormancy was broken by GA_4 treatment (Fig. 1F). In light-imbibed seeds, GA_4-treatment appeared to slightly decrease the rate of ß-1,3-glucanase accumulation after endosperm rupture, but did not affect the onset of induction (Fig. 1C). In summary, under the conditions tested ß-1,3-glucanase activity was only induced in populations of seeds showing endosperm rupture and induction occurred just preceeding or at the onset of rupture.

Class I ß-1,3-glucanase is induced during the germination of dark-imbibed seeds. Most if not all of the ß-1,3-glucanase activity induced in light-imbibed seeds is due to the class I isoforms of the enzyme (Vögeli-Lange et al. 1994; Leubner-Metzger et al. 1995). Immunoblot- and RNA blot analyses were used to establish whether or not the same ß-1,3-glucanase isoforms are induced during the germination of dark-imbibed seeds in response to GA_4 treatment. Immunoblot analysis was performed using 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). Figure 2A shows the time course for the accumulation of ß-1,3-glucanase antigens in response to 10^{-5} M GA_4 treatment in the dark. A sample of seeds germinated in light for 4 days is included as a positive control. Accumulation of an antigen corresponding to the ≈33 kDa class I isoform began 3 days after the start of imbibition. Weak signals were also obtained at the position corresponding to the ≈40 and ≈41 kDA class II stylar ß-1,3-glucanases (Ori et al. 1990). There was no appreciable induction of the 34-36 kDa class II and class III pathogenesis-related ß-1,3-glucanases (Kauffmann et al. 1987; Payne et al. 1990; Ward et al. 1991).

Figure 2B shows that the ≈1.6 kb tobacco class I ß-1,3-glucanase transcript was detected in GA_4-treated and in non-photodormant seeds but not in photodormant seeds imbibed for 4 days in the dark. The timing of mRNA induction following GA_4 treatment was similar to that observed at the protein level. No signals were obtained using probes for the stylar sp41 transcript, the class II PR 2 transcript, or the class III PR Q' transcript (data not shown).

Seeds from Havana 425 tobacco homozygous for a tobacco class I ß-1,3-glucanase B promoter-GUS

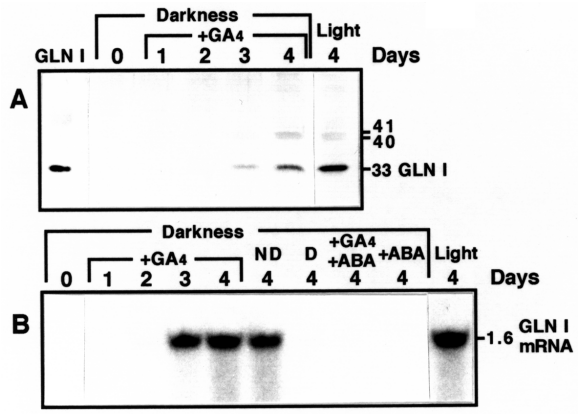

Fig. 2A,B. The effect of 10^{-5} M GA_4 and 10^{-5} M ABA on the accumulation of class I ß-1,3-glucanase antigen and mRNA in dark-incubated seeds. (**A**) Immunoblot analysis of antigens reacting with antibody directed against tobacco class I ß-1,3-glucanase. Equal amounts of protein (80 µg) were applied to each lane. GLN I, 10 ng of purified tobacco ß-1,3-glucanase. The apparent size in kDa of immunoreactive bands is indicated. (**B**) RNA-blot analysis of total RNA (30 µg per lane) prepared from photodormant (D) and non-photodormant (ND) subpopulations of seeds incubated in the dark. The hybridization probe used is for tobacco class I ß-1,3-glucanase mRNA. The size of the transcript in kb is indicated

reporter gene fusion (Vögeli-Lange et al. 1994) were used to localize ß-1,3-glucanase-gene expression induced by GA_4 treatment. The seeds were treated for 3 days in the dark with 10^{-5} M GA_4. Samples of entire seeds and seed parts from the subpopulation of seeds showing seed-coat rupture but no endosperm rupture were assayed for GUS and ß-1,3-glucanase activity. Essentially all of the ß-1,3-glucanase and GUS activities in these seeds prior to endosperm rupture could be accounted for by the micropylar region of the endosperm (Table 1). In contrast, no increase in activity was detected in control seeds showing neither seed coat rupture nor endosperm rupture. These results were confirmed by histological staining of GUS activity (data not shown).

In summary, the results show that most if not all of the ß-1,3-glucanase activity induced by GA_4 treatment is due to class I isoforms. Based on the activity of the ß-1,3-glucanase B promoter, this induction is localized in the micropylar region of the endosperm prior to rupture and is regulated, at least in part, at the level of transcription.

Effects of osmotica on seed germination and ß-1,3-glucanase induction. Treatment with osmotica is known to delay seed germination and is believed to act primarily by reducing the expansive growth of the embryo (review, Bewley and Black, 1994). The time

Table 1 Effect of GA_4 on the accumulation and localization of ß-1,3-glucanase and GUS activity in dark-incubated seeds prior to endosperm rupture

	ß-1,3-Glucanase[b]		GUS[c]	
Seed component[a]	Control	+GA_4	Control	+GA_4
Entire seed	0.03 ± 0.01[d]	2.63 ± 0.28	0.07	0.48
Embryo	n.d.[e]	0.02	n.d.	<0.001
Entire endosperm	n.d.	3.00	n.d.	0.48
Micropylar endosperm	n.d.	2.98	n.d.	0.48
Non-micropylar endosperm	n.d.	0.02	n.d.	<0.001

[a] GA_4 treated seeds were dissected after 3 d incubation just before the onset of endosperm rupture; as controls, entire photodormant seeds were measured after 3 d incubation.
[b] data obtained with 100 wild-type seeds expressed as pkat per seed
[c] data obtained with 100 homozygous GLB-GUS seeds expressed as fkat per seed
[d] values obtained for two or more experiments are expressed as the mean ±SE
[e] not determined

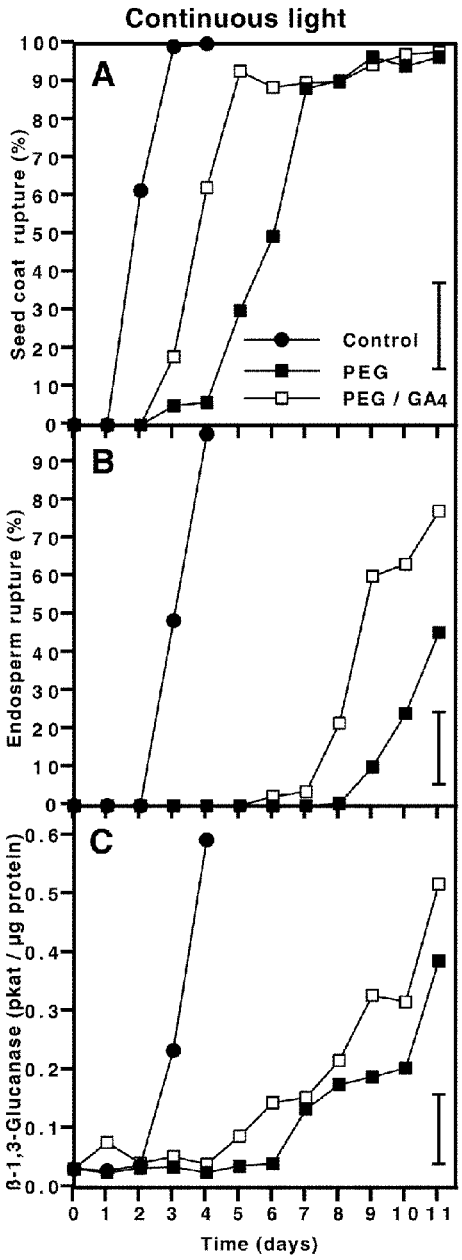

Fig. 3A-C. The effect the osmoticum and GA_4 treatment on the time course of tobacco seed germination and ß-1,3-glucanase activity in seeds incubated in continuous light. The incidence of seed coat rupture (**A**) and endosperm rupture (**B**) expressed in per cent of 100-200 seeds scored. The activity of ß-1,3-glucanase (**C**) expressed in pkat/µg protein. Seeds were incubated without PEG 6000 or GA_4, with 0.04 M PEG 6000, and with 0.04 M PEG 6000 + 10^{-5} M GA_4 added at 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. Error bars= maximum ±SE

courses for seed-coat rupture, endosperm rupture, and ß-1,3-glucanase activity of Havana 425 tobacco seeds imbibed in light with and without 0.04 M of the osmoticum PEG 6000 are shown in Figure 3. As judged from the time required for 50% of the seeds to rupture, PEG 6000 treatment delayed seed coat rupture by ≈4 days and delayed endosperm rupture by ≈9 days. PEG 6000 treatment also delayed the onset of ß-1,3-glucanase induction and decreased the rate of enzyme accumulation (Fig. 3C). These effects depended on the concentration of PEG 6000 and were similar when another osmoticum, mannitol, was used (data not shown).

As reported for seeds of other species (Juntilla, 1973; Karssen et al. 1989; Ni and Bradford, 1993), treatment with 10^{-5} M GA_4 decreased the effects of the osmoticum on germination. GA_4 reduced the delay in seed-coat rupture, endosperm rupture, and ß-1,3-glucanase induction due to PEG 6000 (Fig. 3). Of particular interest was the finding that treatment with PEG 6000 alone or in combination with GA_4 delayed the onset endosperm rupture longer than it delayed the onset of ß-1,3-glucanase induction. Thus, induction of ß-1,3-glucanase was earlier relative to endosperm rupture than in the untreated controls.

Discussion

The present results confirm and extend our previous finding that class I ß-1,3-glucanase induction is closely linked to endosperm rupture during tobacco seed germination (Leubner-Metzger et al. 1995). Treatments resulting in endosperm rupture, e.g. imbibition in continuous light and GA_4 treatment in the dark resulted in class I ß-1,3-glucanase accumulation which started before or at the time of endosperm rupture. On the other hand, treatments that prevent or delay endosperm rupture, e.g. incubation in the dark or the addition of ABA or osmotica to the culture medium, also prevented or delayed ß-1,3-glucanase induction.

Some, but not all varieties of tobacco are photodormant: brief treatment with red light activates the phytochrome signal transduction pathway resulting in the release of dormancy (Avery, 1933; Kincaid, 1935; Ogawara, 1954; Toole et al. 1955). This effect of light can be replaced by treating the dormant seeds with GA in the dark (Hashimoto, 1958; Hashimoto and Yamaki, 1959; Khalil, 1992).

We showed that Havana 425 is a photodormant variety of tobacco and that 10^{-5} M GA_4 effectively released dormancy in the dark.

Germination of tobacco seeds is restricted by the covering layers of the embryo. Removal of the seed coats and endosperm from the micropylar end of photodormant seeds results in radicle growth in the absence of light (Bihlmeier, 1927; Böhmer, 1928; Kincaid, 1935). Reciprocal crosses of photodormant and non-photodormant tobacco varieties indicate that both maternal and patternal genes contribute to covering-layer imposed dormancy (Honing, 1930; Kasperbauer, 1968). Seed coat rupture and endosperm rupture of Havana 425 tobacco seeds are separate events well separated in time. Although ABA treatment of light-imbibed seeds delayed seed coat rupture slightly and may slow down embryo growth, the primary effect was on endosperm rupture (Leubner-Metzger et al. 1995). Taken together, these findings indicate that the endosperm rupture is the major step in limiting tobacco seed germination.

We found that ABA can delay GA_4-induced endosperm rupture in the dark and that the inhibitory effects of ABA in the light were not prevented by GA_4. This suggests that ABA and GA act at different regulatory sites in tobacco seed germination. These results are in agreement with the revised hormone-balance theory for seed dormancy proposed by Karssen and Laçka (1985). According to this theory, which is supported by experiments with deficiency mutants of *Arabidopsis* and tomato, ABA and GA act at different times and sites during seed life. ABA induces primary dormancy during seed development and GA play a key role in promotion of germination. Light stimulates both GA-biosynthesis and sensitivity towards GAs and the GA requirement for germination depends on the degree of dormancy established earlier by ABA (reviews, Karssen and Lacka, 1985; Bewley and Black, 1994).

GA can promote the initiation of the germination process and the subsequent proliferation of cells in the embryo (review, Bewley and Black, 1994; Liu et al. 1994). The major site of action of GA, however, appears to be the endosperm. GA treatment can induce endosperm weakening and rupture in pepper (Watkins and Cantliffe, 1983; Watkins et al. 1985), celery (Jacobsen and Pressman, 1979), *Syringa* (Juntilla, 1973), lettuce (Dutta et al. 1994), *Chenopodium album* (Karssen, 1976), and tomato (Groot and Karssen, 1987; Groot et al. 1988; Karssen et al. 1989; Ni and Bradford, 1993). In many seeds, including tobacco, in which dormancy is imposed by the covering layers germination is preceded by weakening or breakdown of the endosperm. There is indirect evidence that hydrolytic enzymes acting on cell-wall polysaccharides contribute to endosperm weakening and facilitate radicle protrusion (Sanchez et al. 1986; Groot and Karssen, 1987; Groot et al. 1988; Ni and Bradford, 1993; Dutta et al. 1994; Leubner-Metzger et al. 1995). In the case of tomato, which has been studied in detail, weakening of the endosperm cap surrounding the radicle was found to be a prerequisite for germination (Groot and Karssen, 1987; Groot and Karssen, 1992). Seeds of the GA-deficient mutant ga-1 do not germinate unless treated with GAs or the endosperm cap is mechanically removed. In the latter case, radicle growth was slower than in wild-type seeds, but could be restored by GA treatment indicating that radicle growth does not require, but is promoted by GA. GA-treatment of isolated endosperms of this mutant resulted in the induction of endo-ß-mannanase activity and hydrolysis of cell walls at times prior to radicle protrusion (Groot et al. 1988; Karssen et al. 1989). These findings and other studies with GA- and ABA- deficient mutants lead to the hypothesis that in tomato control of germination is regulated primarily by ABA and GA, which alter endosperm weakening, at least in part, by modulation of cell wall hydrolase activity (Ni and Bradford, 1993).

Our working hypothesis is that class I ß-1,3-glucanase contributes to the weakening of the endosperm of tobacco seeds and promotes radicle penetration (Leubner-Metzger et al. 1995). This hypothesis was based on the observation that induction ß-1,3-glucanase in light treated seeds begins just prior to the onset of endosperm rupture in a highly localized region at the site where radicle penetration will occur. Induction of this enzyme was inhibited by ABA, which at the same concentrations, also delayed endosperm rupture. ABA decreased the rate of ß-1,3-glucanase accumulation but not the time of onset of accumulation. Independent of the delay in endosperm rupture in response to ABA, the onset of rupture was correlated with the same ß-1,3-glucanase content per seed suggesting that a critical ß-1,3-glucanase concentration was required for endosperm

rupture. The present studies show that ß-1,3-glucanase induction and endosperm rupture are also correlated in response to GA_4 in the dark and to treatment with osmotica in the light.

According to our working hypothesis, penetration of the endosperm depends on both wall weakening facilitated in part by ß-1,3-glucanase and sufficient growth of the embryo to overcome the mechanical resistance of the endosperm. We speculate that early in the germination process the "program" timing the various events including ß-1,3-glucanase induction is established and growth of the embryo required for radicle penetration is initiated. Osmotica are thought to delay germination primarily by lowering the expansive growth of the embryo. Unlike ABA, treatment with osmotica alone or in combination with GA_4 delayed both the onset and rate of ß-1,3-glucanase induction. The onset of endosperm rupture under these conditions occurred at the time when the ß-1,3-glucanase content of the seeds reached the "threshold" concentrations found earlier for ABA treatment. This suggests that expansive growth might act in two ways by triggering ß-1,3-glucanases induction and by providing force for endosperm penetration.

In conclusion, little is known about enzymes associated with endosperm weakening in dicot seeds, the genes encoding these enzymes, or their regulation. ß-1,3-Glucanases provide a useful molecular marker for investigating the regulation of tobacco seed germination. Although there is as yet no direct evidence that class I ß-1,3-glucanase contributes to wall weakening, our studies provide the molecular tools needed to test this hypothesis.

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References

Avery GSJr (1933) Structure and germination of tobacco seed and the developmental anatomy of the seedling plant. Am J Bot 20: 309-327

Beffa RS, Neuhaus J-M, Meins FJr (1993) Physiological compensation in antisense transformants: Specific induction of an ersatz glucan endo-1,3-ß-glucosidase in plants infected with necrotizing viruses. Proc Natl Acad Sci USA 90: 8792-8796

Bewley JD, Black M (1994) Seeds- Physiology of development and germination. Plenum Press, New York

Bihlmeier M (1927) Der Einfluss der Vorquellung und der Samenschale auf die Keimung lichtgeförderter Samen. Jahrb wiss Bot 67: 702-732

Böhmer K (1928) Die Bedeutung der Samenteile für die Lichtwirkung und die Wechselbeziehung von Licht und Sauerstoff bei der Keimung lichtempfindlicher Samen. Jahrb wiss Bot 68: 549-601

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72: 248-254

Dutta S, Bradford KJ, Nevins DJ (1994) Cell-wall autohydrolysis in isolated endosperms of lettuce (*Lactuca sativa* L.). Plant Physiol 104: 623-628

Groot SPC, Kieliszewska-Rokicka B, Vermeer E, Karssen CM (1988) Gibberellin-induced hydrolysis of endosperm cell walls in gibberellin-deficient tomato seeds prior to radicle protrusion. Planta 174: 500-504

Groot SPC, Karssen CM (1987) Gibberellins regulate seed germination in tomato by endosperm weakening: A study with gibberellin-deficient mutants. Planta 171: 525-531

Groot SPC, Karssen CM (1992) Dormancy and germination of abscisic acid-deficient tomato seeds. Plant Physiol 99: 952-958

Hashimoto T (1958) Increase in percentage of gibberellin-induced dark germination in tobacco seeds by N-compounds. Bot Mag (Tokyo) 71: 430-431

Hashimoto T, Yamaki T (1959) On the physiological effects of gibberellins A_1, A_2, A_3, and A_4. Bot Mag (Tokyo) 72: 178-181

Honing JA (1930) Nucleus and plasma in the heredity of the need of light for germination in *Nicotiana* seeds. Genetica 12: 441-476

Jacobsen JV, Pressman E (1979) A structural study of germination in celery (*Apium graveolens* L.) seed with emphasis on endosperm breakdown. Planta 144: 241-248

Juntilla O (1973) The mechanism of low temperature dormancy in mature seeds of *Syringa* species. Physiol Plant 29: 256-263

Karssen CM (1976) Two sites of hormonal action during germination of *Chenopodium album* seeds.

Physiol Plant 36: 264-270

Karssen CM, Zagorsky S, Kepczynski J, Groot SPC (1989) Key role for endogenous gibberellins in the control of seed germination. Ann Bot 63: 71-80

Karssen CM, Lacka E (1985) A revision of the hormone balance theory of seed dormancy: studies on gibberellin and/or abscisic acid-deficient mutants of *Arabidopsis thaliana*. In: Bopp M (ed) Plant Growth Substances. Springer-Verlag, Berlin, Heidelberg, pp. 315-323

Kasperbauer MJ (1968) Dark-germination of reciprocal hybrid seed from light-requiring and -indifferent *Nicotiana tabacum*. Physiol Plant 21: 1308-1311

Kauffmann S, Legrand M, Geoffroy P, Fritig B (1987) Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-ß-glucanase activity. EMBO J 6: 3209-3212.

Khalil MK (1992) Nature of growth regulators effects on *Nicotiana tabacum* seed germination. Angew Bot 66: 106-108

Kincaid RR (1935) The effects of certain environmental factors on the germination of Florida cigar-wrapper tobacco seeds. Tech Bull Univ Florida, Agr Expt St 277: 5-47

Leubner-Metzger G, Fründt C, Vögeli-Lange R, Meins FJr (1995) Class I ß-1,3-glucanases in the endosperm of tobacco during germination. Plant Physiol

Liptay A, Schopfer P (1983) Effect of water stress, seed coat restraint, and abscisic acid upon different germination capabilities of two tomato lines at low temperature. Plant Physiol 73: 935-938

Liu Y, Bergervoet JHW, De Vos CHR, Hilhorst JWM, Kraak HL, Karssen CM, Bino RJ (1994) Nuclear replication activities during imbibition of abscisic acid- and gibberellin-deficient tomato (*Lycopersicon esculentum* Mill.) seeds. Planta 194: 368-373

Meins FJr, Neuhaus J-M, Sperisen C, Ryals J (1992) The primary structure of plant pathogenesis-related glucanohydrolases and their genes. In: Boller T, Meins FJr (eds) Genes Involved in Plant Defense. Springer Verlag, Vienna/New York, pp. 245-282

Neuhaus J-M, Flores S, Keefe D, Ahl-Goy P, Meins FJr (1992) The function of vacuolar ß-1,3-glucanase investigated by antisense transformation. Susceptibility of transgenic *Nicotiana sylvestris* plants to *Cercospora nicotianae* infection. Plant Molec Biol 19: 803-813

Ni BR, Bradford KJ (1993) Germination and dormancy of abscisic acid- and gibberellin-deficient mutant tomato (*Lycopersicon esculentum*) seeds. Sensitivity of germination to abscisic acid, gibberellin, and water potential. Plant Physiol 101: 607-617

Ogawara K (1954) Interaction of light and darkness in the germination of the light-favored tobacco seeds. Proc Jpn Acad 30: 504-509

Ori N, Sessa G, Lotan T, Himmelhoch S, Fluhr R (1990) A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related protein superclass. EMBO J 9: 3429-3436

Payne G, Ward E, Gaffney T, Ahl-Goy P, Moyer M, Harper A, Meins FJr, Ryals J (1990) Evidence for a third structural class of ß-1,3-glucanase in tobacco. Plant Molec Biol 15: 797-808

Sanchez RA, De Miguel L, Mercuri O (1986) Phytochrome control of cellulase activity in *Datura ferox* L. seeds and its relationship with germination. J Exp Bot 37: 1574-1580

Sanchez RA, Sunell L, Labavitch JM, Bonner BA (1990) Changes in the endosperm cell walls of two *Datura* species before radicle protrusion. Plant Physiol 93: 89-97

Shinshi H, Wenzler H, Neuhaus JM, Felix G, Hofsteenge J, Meins FJr (1988) Evidence for N- and C-terminal processing of a plant defense-related enzyme. Primary structure of tobacco prepro-ß-1,3-glucanase. Proc Natl Acad Sci USA 85: 5541-5545.

Toole EH, Toole VK, Borthwick HA, Hendricks SB (1955) Interaction of temperature and light in germination of seeds. Plant Physiol 30: 473-478

Vögeli-Lange R, Fründt C, Hart CM, Beffa R, Nagy F, Meins FJr (1994) Evidence for a role of ß-1,3-glucanase in dicot seed germination. Plant J 5: 273-278

Ward ER, Payne GB, Moyer MB, Williams SC, Dincher SS, Sharkey KC, Beck JJ, Taylor HT, Ahl-Goy P, Meins FJr, Ryals J (1991) Differential regulation of ß-1,3-glucanase messenger RNAs in response to pathogen infection. Plant Physiol 96: 390-397

Watkins JT, Cantliffe DJ, Huber DJ, Nell TA (1985) Gibberellic acid stimulated degradation of endosperm in pepper. J Am Soc Hortic Sci 110: 61-65

Watkins JT, Cantliffe DJ (1983) Mechanical resistance of the seed coat and endosperm during germination of *Capsicum annuum* at low temperatures. Plant Physiol 72: 146-150