The Plant Journal (2000) **23**(2), 215–221

Sense transformation reveals a novel role for class I β-1,3-glucanase in tobacco seed germination

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Summary

'Coat-enhanced' seed dormancy of many dicotyledonous species, including tobacco, is released during after-ripening. Rupture of the endosperm, which is the limiting step in tobacco seed germination, is preceded by induction of class I β-1,3-glucanase (βGLU I) in the micropylar endosperm where the radicle will penetrate. Treating after-ripened tobacco seeds with abscisic acid (ABA) delays endosperm rupture and inhibits βGLU I induction. Sense transformation with a chimeric ABA-inducible βGLU I transgene resulted in over-expression of βGLU I in seeds and promoted endosperm rupture of mature seeds and of ABA-treated after-ripened seeds. Taken together, these results provide direct evidence that βGLU I contributes to endosperm rupture. Over-expression of βGLU I during germination also replaced the effects of after-ripening on endosperm rupture. This suggests that regulation of βGLU I by ABA signalling pathways might have a key role in after-ripening.

Keywords: abscisic acid, after-ripening, endosperm-limited seed germination, gene function, β-1,3-glucanase, sense transformation.

Introduction

Little is known about the molecular basis for the after-ripening and germination of dicotyledonous seeds (Bewley, 1997a). During seed maturation, water content decreases, abscisic acid (ABA) accumulates, and primary dormancy is established (Hilhorst, 1995; Li and Foley, 1997; Rock and Quatrano, 1995). Primary dormancy of many species can be altered by after-ripening, i.e. the storage of mature seeds for several months under dry, warm conditions (Hilhorst, 1995; Kasperbauer, 1968; Koornneef and Karssen, 1994; Li and Foley, 1997). Under favourable conditions, imbibition of water by non-dormant seeds results in a burst of respiration, rapid growth of the embryo, rupture of the covering layers, and finally emergence of the radicle. Many of these seeds exhibit 'coat-enhanced' dormancy in which the emergence of the radicle is physically restrained by the covering layers (Baskin and Baskin, 1998; Bewley, 1997a; Hilhorst, 1995). In the case of endosperm-limited germination, it is believed that hydrolytic enzymes facilitate weakening of the endosperm surrounding the radicle tip by hydrolysing cell-wall materials (Bewley, 1997b; Black, 1996; Ni and Bradford, 1993; Sitrit *et al.*, 1999); however, direct evidence for this hypothesis from studies with transgenic seeds is lacking.

The first morphological event following imbibition of tobacco seeds is rupture of the seed coat (testa). This is followed by rupture of the endosperm, which is the limiting step of tobacco seed germination (Arcila and Mohapatra, 1983; Leubner-Metzger *et al.*, 1995). β-1,3-glucanase (βGLU) activity is induced after testa rupture and just prior to the onset of endosperm rupture. This activity is localized in the micropylar endosperm at the site where the radicle will emerge, and results from transcriptional induction of class I β-1,3-glucanase (βGLU I) (Leubner-Metzger *et al.*, 1995). The induction of βGLU I and endosperm rupture are tightly linked in response to physiological factors known to affect the incidence and timing of germination (Leubner-Metzger *et al.*, 1996; Leubner-Metzger *et al.*, 1998). For example, ABA treatment specifically delays endosperm rupture and inhibits the induction of βGLU I. Kinetic analysis of this effect strongly suggests that the onset of endosperm rupture depends on a critical threshold concentration of βGLU I.

In the present study, we established by sense transformation that βGLU I has a causal role in endosperm rupture. We also provide less direct evidence that after-ripening

could affect endosperm rupture, at least in part, by a βGLU I-mediated process.

Results

High-level expression of βGLU I can promote endosperm rupture

Tobacco plants were transformed with the expression vector pKSG7 carrying a chimeric tobacco *βGLU I* gene regulated by the castor bean *Cat1* gene promoter. The *Cat1* promoter was chosen because it directs high-level expression of a reporter gene in the endosperm of germinating seeds of transgenic tobacco (Suzuki *et al.*, 1995). Unless indicated otherwise, after-ripened seeds obtained by self-fertilization of primary sense (TKSG7) and empty-vector (TCIB1) transformants were used. Seeds were imbibed in the presence of ABA to delay the onset of germination and inhibit the induction of host *βGLU I* genes (Leubner-Metzger *et al.*, 1995).

In our initial screen, we scored the incidence of germination, i.e. percentage of endosperm rupture, at 73 h after the start of imbibition in the presence of 1 μM ABA in continuous light. Under these conditions, the germination rate of the 27 empty-vector TCIB1 lines used as controls was 6.8 ± 1.2% (mean ± SE) and the population of seeds contained a mean βGLU activity of 0.9 ± 0.1 pkat/seed. In contrast, 41.9% of the 43 TKSG7 lines germinated at rates at least twofold higher than the TCIB1 controls and exhibited a 7.5-fold higher mean βGLU activity. Thus, germination was correlated with elevated βGLU activity in numerous, independent sense transformants.

Four representative TKSG7 lines with high germination rates in the presence of 1 μM ABA were chosen that differed in transgene dose and βGLU content. The time course for testa rupture, endosperm rupture and βGLU activity accumulation was determined for lines carrying one (TKSG7-43), two (TKSG7-34 and TKSG7-42) and three or more (TKSG7-10) transgene loci and for four control TCIB1 lines imbibed with and without 10 μM ABA in the incubation medium. The time course for testa rupture was not affected by ABA treatment and was very similar for TKSG7 and TCIB1 seeds (data not shown). Figure 1(a) shows that after-ripened seed of TKSG7 and TCIB1 lines

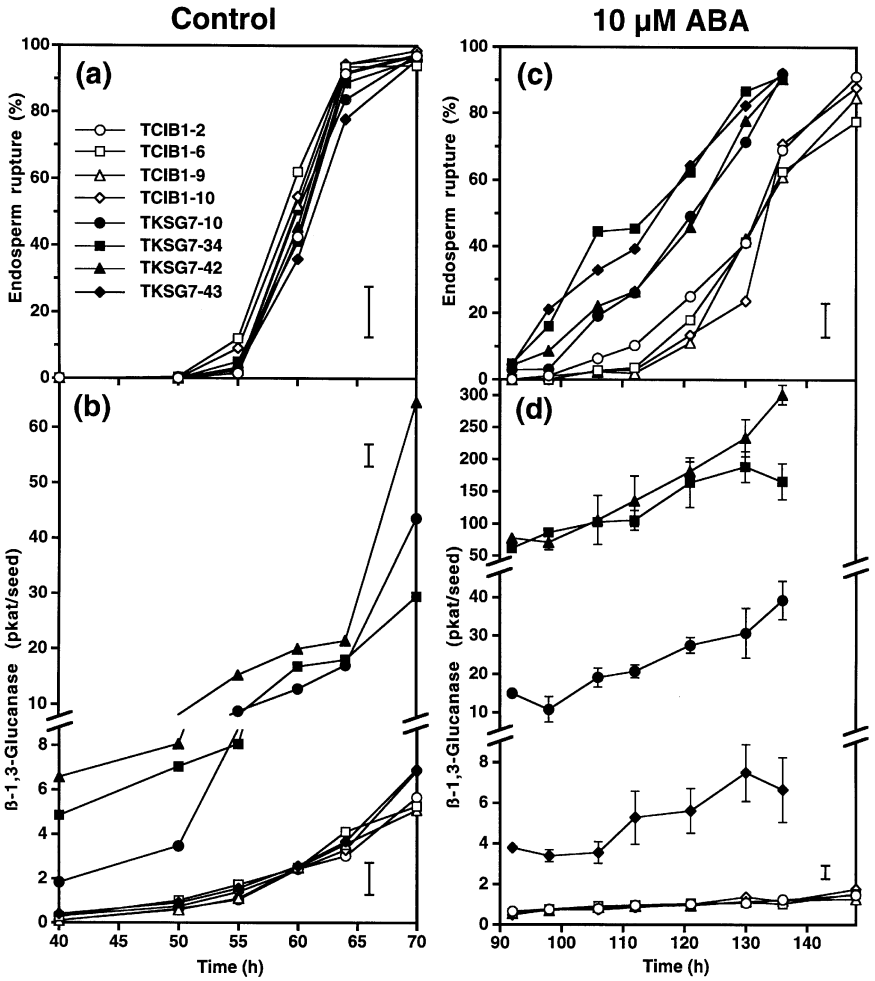

Figure 1. The effect of 10 μM ABA on the time course of endosperm rupture and βGLU accumulation in after-ripened S_1 seeds of independent sense βGLU I (TKSG7) and empty-vector (TCIB1) transformants.
(a) and (c) The incidence of endosperm rupture expressed as the percentage of 100–150 seeds scored over time since the start of imbibition in continuous light without (a) and with (c) 10 μM ABA added. Segregating seed populations of lines carrying one (TKSG7-43), two (TKSG7-34 and TKSG7-42) and three or more (TKSG7-10) transgene loci were used. The means of two replicates from one experiment are presented. Similar results were obtained in a second experiment. Error bars indicate the largest standard error value. (b) and (d) The βGLU enzyme activities of imbibed TKSG7 and TCIB1 seed populations without (b) and with (d) 10 μM ABA. Error bars (b) indicate the largest standard error values of lines TKSG7-10, TKSG7-34 and TKSG7-42 (upper corner) and of lines TCIB1 and TKSG7-43 (lower corner). Error bars (d) indicate individual standard error values (TKSG7 lines) and the largest standard error value (TCIB1 lines). Note the difference in scale of (b) and (d).

did not differ in timing of endosperm rupture when imbibed in medium without added ABA. As reported earlier for wild-type seeds (Leubner-Metzger *et al.*, 1995), treatment with 10 μM ABA dramatically delayed by approximately 70 h the time for 50% endosperm rupture of TCIB1 seeds (Figure 1c). This delay in endosperm rupture was substantially reduced by about 15 h in TKSG7 seeds.

Three of the TKSG7 lines accumulated considerably more βGLU activity than did the empty-vector control lines when imbibed without added ABA. The activity of line TKSG7-43 was comparable to the TCIB1 lines (Figure 1b). The finding that over-expression of βGLU I in sense lines did not promote endosperm rupture under these conditions is consistent with studies showing that βGLU activities above approximately 5 pkat/seed at the onset of rupture do not increase the incidence of endosperm rupture (Leubner-Metzger *et al.*, 1995). ABA treatment inhibited βGLU accumulation of TCIB1 seeds (Figure 1d) as reported earlier for wild-type seed (Leubner-Metzger *et al.*, 1995). In contrast, ABA treatment markedly increased the βGLU activities of the TKSG7 seeds. Although the level of βGLU activity and the induction in response to 10 μM ABA differed considerably for the different TKSG7 lines, even the least responsive line, TKSG7-43, showed at least fivefold higher βGLU activities relative to the TCIB1 controls throughout the experiment. Quantitative immunoblot analysis using antibodies that detect all known βGLU classes and RNA-blot hybridization using a probe specific for βGLU I mRNA confirmed that the increased βGLU activity of the TKSG7 lines is due exclusively to increased amounts of βGLU I (data not shown). Taken together, these findings indicate that expression of βGLU I under the regulation of the *Cat1* promoter increases βGLU I levels during endosperm rupture and results in a partial reversal of the delay in rupture due to ABA.

After-ripening and photodormancy effects

We compared germination in the light of 'fresh' seeds, i.e. mature seeds 40 days after pollination (DAP), and of after-ripened seeds stored at room temperature for at least 6 months after harvest. When imbibed without added ABA, essentially all tobacco seeds eventually germinate. Thus, changes in the onset of germination can be detected by measuring the germination rate at a fixed time after the start of imbibition. Table 1 shows that after-ripening increased the germination rate of wild-type and TCIB1-2 seed. This indicates that after-ripening can release dormancy of Havana 425 tobacco seeds. Fresh sense seeds (TKSG7) germinated at approximately 1.5-fold higher rates than did the controls, whereas after-ripened sense and control seeds germinated at comparable rates. The βGLU activity at the time of endosperm rupture was higher in after-ripened than in fresh control seeds. Independent of after-ripening, the βGLU content of sense-transformed seeds was higher than that of the control seeds. Taken together, these results suggest that over-expression of βGLU can replace the effect of after-ripening on endosperm rupture in the light.

Havana 425 tobacco seeds exhibit photodormancy, i.e. fresh seeds do not germinate in the dark, even after prolonged periods of time (Leubner-Metzger *et al.*, 1996). Table 1 shows that after-ripening contributes to the release of photodormancy and that this effect varies greatly for different seed batches as reported for other tobacco cultivars (Kasperbauer, 1968). Sense transformation did not have detectable effects on either photodormancy of

Table 1. Effect of after-ripening on endosperm rupture, βGLU activity and photodormancy of sense βGLU I seeds

	Continuous light				Darkness	
	Endosperm rupture (%)[b]		βGLU (pkat/seed)[c]		Non-photodormancy (%)[d]	
Lines[a]	'Fresh' seed	After-ripened seed	'Fresh' seed	After-ripened seed	'Fresh' seed	After-ripened seed
Wild-type	49.8 ± 5.2	79.2 ± 6.8	2.4 ± 0.1	3.2 ± 0.2	0.4 ± 0.2	50.3 ± 16.5 (0.0–80.6)
TCIB1-2	49.7 ± 2.1	81.4 ± 5.2	2.2 ± 0.1	2.9 ± 0.2	0.3 ± 0.2	40.0 ± 12.6 (9.1–80.1)
TKSG7-32	74.4 ± 2.1	79.0 ± 2.4	9.1 ± 0.2	5.3 ± 0.2	0.3 ± 0.3	29.7 ± 13.5 (3.0–63.6)
TKSG7-38	77.1 ± 2.1	73.5 ± 0.1	8.9 ± 0.2	4.9 ± 0.3	0.9 ± 0.2	39.0 ± 18.9 (0.0–82.4)
TKSG7-43	75.3 ± 3.6	75.8 ± 7.4	8.2 ± 0.5	4.7 ± 0.4	0.4 ± 0.2	43.6 ± 19.6 (9.4–90.2)

[a]Wild type and independent, homozygous, monogenic vector-control (TCIB1) and sense βGLU I (TKSG7) tobacco lines selected from the S_2 seed generation. [b]Mean ± SE of 'fresh' (directly after harvest) and after-ripened (≥6 months of dry storage) seed from 3–6 capsules scored 72 h and 67 h, respectively, after the start of imbibition in continuous light in control medium without antibiotics. [c]Mean ± SE βGLU activities of protein extracts prepared from seed samples described under [b]. [d]Mean ± SE of 'fresh' and after-ripened seed from 3–6 capsules scored after 10 days incubation in the dark in control medium without antibiotics. The range obtained with different capsules is shown in parentheses.

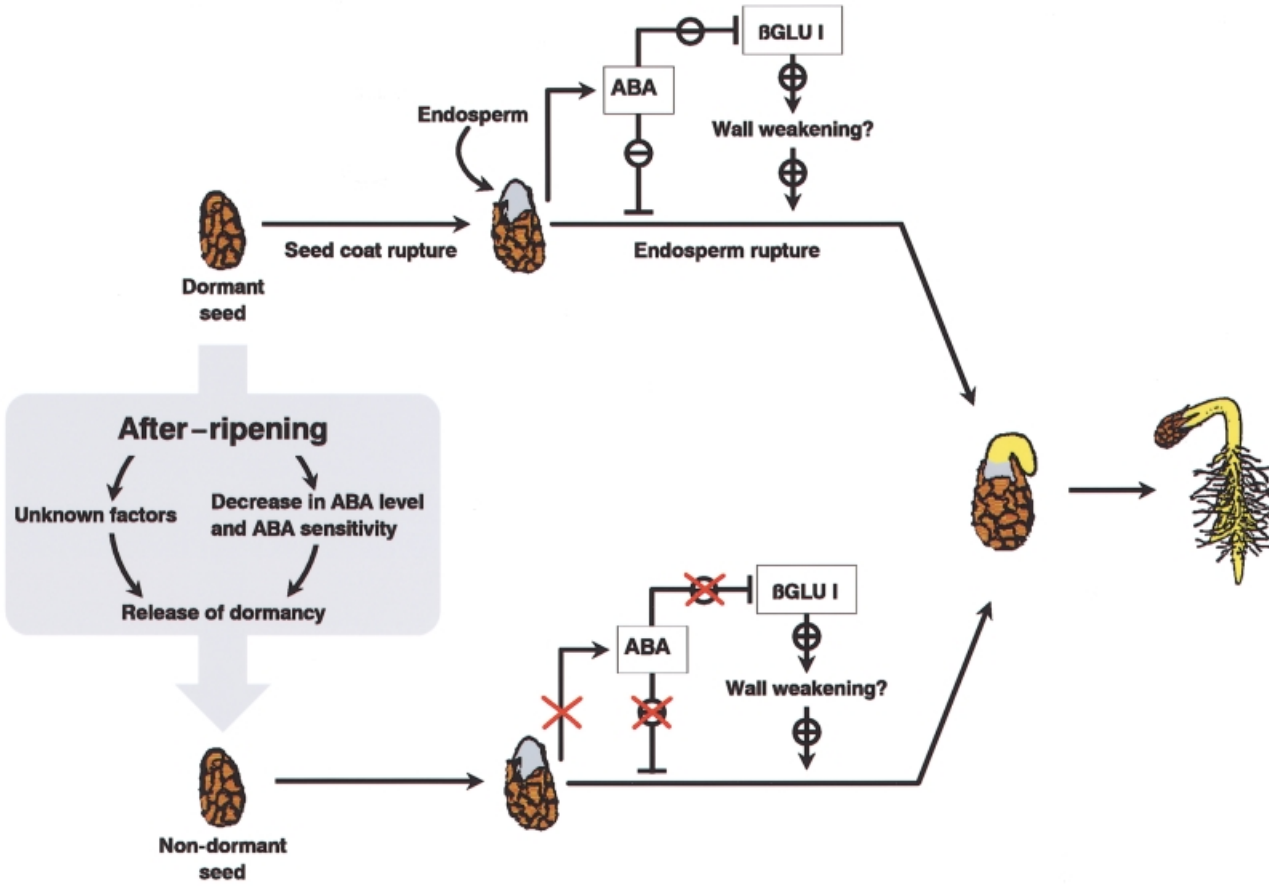

Figure 2. A speculative model relating after-ripening, βGLU I and endosperm rupture.
According to the model, βGLU I, which is transcriptionally down-regulated by ABA, contributes to endosperm wall weakening and breaking of coat-enhanced dormancy. ABA accumulated during seed maturation is sufficient to delay βGLU I induction and endosperm rupture. After-ripening decreases ABA levels and possibly sensitivity to ABA, allowing the induction of βGLU I during imbibition, which helps modulate coat-enhanced dormancy. The model does not exclude the possibility that other factors contribute to after-ripening and modulation of coat-enhanced dormancy, or that ABA has additional functions, e.g. in photodormancy.

fresh seed or on the release of photodormancy due to after-ripening.

Discussion

βGLU I contributes to endosperm rupture

βGLU I induction in the micropylar endosperm is tightly correlated with endosperm rupture under a variety of physiological conditions that delay or promote tobacco-seed germination (reviewed in Leubner-Metzger and Meins, 1999). In the present study, we provide direct evidence that βGLU I has a role in endosperm rupture. The castor bean *Cat1* promoter shown earlier to confer micropylar-endosperm expression in tobacco seeds (Suzuki *et al.*, 1995) was used to regulate expression of a *βGLU I* sense transgene. Expression of maize and *N. plumbaginifolia* orthologues of the castor bean *Cat1* gene is strongly induced by ABA during seed maturation and germination (Bueno *et al.*, 1998; Guan and Scandalios, 1993; Willekens *et al.*, 1994; Williamson and Scandalios, 1992). We found that ABA treatment induces the castor bean *Cat1* promoter in tobacco seeds. This effect was exploited to achieve high-level βGLU I expression in seeds treated with ABA to delay endosperm rupture and block host βGLU I expression. The results indicate that expression of βGLU I reduced the delay in endosperm rupture of after-ripened TKSG7 seeds due to ABA treatment, but did not affect the onset of testa rupture. Increased βGLU I expression also promoted germination of fresh TKSG7 seeds that were not treated with ABA. Taken together, these results show that βGLU I substantially contributes to endosperm rupture, which is the limiting step in tobacco seed germination.

We also attempted to block βGLU I induction during endosperm rupture by antisense transformation using the *Cat1* promoter. As is often the case for antisense experiments, this approach was not successful. Although numerous independent transformants were screened, none were found with reduced βGLU activity, βGLU I

protein or βGLU I mRNA levels, and none showed effects on endosperm rupture.

In cases of endosperm-limited germination, radicle emergence depends on both wall weakening and sufficient growth of the embryo to overcome the mechanical resistance of the endosperm (Bewley, 1997b; Ni and Bradford, 1993). Ultrastructural studies suggest that the endospermic hole formed at the micropylar end of tobacco seeds results from endosperm 'dissolution' rather than from the 'pushing' action of radicle growth (Arcila and Mohapatra, 1983). Earlier studies showed that ABA treatment of wild-type tobacco seeds delayed the onset of endosperm rupture and inhibited βGLU I induction in a dose-dependent fashion (Leubner-Metzger *et al.*, 1995). Independent of the ABA concentration, the onset of endosperm rupture was correlated with a βGLU activity of roughly 2 pkat/seed, and the incidence of endosperm rupture did not increase with βGLU activities greater than roughly 5 pkat/seed. In the present experiment, the onset of endosperm rupture of control TCIB1 seeds treated with 10 μM ABA was somewhat lower, roughly 1 pkat/seed. The important point is that all independent sense lines exhibited earlier germination and βGLU activities above this threshold value. This further supports the hypothesis that a threshold βGLU content is required for endosperm rupture, and suggests that accumulation of βGLU is necessary but not sufficient for endosperm weakening.

The mechanism underlying the effect of βGLU I on endosperm weakening is not known. One possibility is that βGLU I helps to hydrolyse extracellular polysaccharides important for strengthening the endosperm wall. This would imply that βGLU I, which is a vacuolar protein (Keefe *et al.*, 1990), can be alternatively targeted for secretion. Recent studies suggesting that tobacco βGLU I (Kunze *et al.*, 1998) and other vacuolar proteins (Kjemtrup *et al.*, 1995) can be secreted support this view. A possible target for βGLU I is callose, which is a substrate for the enzyme (Stone and Clarke, 1992). Callose has been found in the impermeable covering layers of seeds of some dicotyledonous species, e.g. *Cucumis melo* (Welbaum *et al.*, 1995; Yim and Bradford, 1998), but not in others, e.g. tomato and pepper (Beresniewicz *et al.*, 1995). The presence of callose or other potential substrates of βGLU in the endosperm of tobacco has not been demonstrated and is an interesting area for further investigation.

The effect of after-ripening on endosperm could involve βGLU I

We found that after-ripening of Havana 425 tobacco seed had two effects on germination: it breaks photodormancy, which blocks germination prior to testa rupture (Leubner-Metzger *et al.*, 1996; Mohapatra and Johnson, 1978), and it modulates coat-enhanced dormancy, which involves endosperm rupture. Little is known about the molecular basis for dormancy or the modulation of dormancy during after-ripening (Bewley, 1997a; Li and Foley, 1997). Although several genes that are differentially expressed in imbibed dormant and non-dormant seeds have been identified, none have been shown to be directly involved in the maintenance or breaking of dormancy (Bewley, 1997a; Li and Foley, 1997). Over-expression of βGLU I during germination replaced the after-ripening effect on endosperm rupture, but did not influence photodormancy. Endogenous production of ABA is needed to establish and maintain dormancy in many species including tobacco (Artsaenko *et al.*, 1995; Bewley, 1997a; Fry *et al.*, 1999; Grappin *et al.* 2000; Koornneef and Karssen, 1994; Li and Foley, 1997; Rock and Quatrano, 1995). The onset of dormancy in tobacco is correlated with a peak in ABA content, which declines rapidly during further seed maturation (approximately 15–25 DAP) (Jiang *et al.*, 1996; Phillips *et al.*, 1997; Yamaguchi-Shinozaki *et al.*, 1990). The fresh tobacco seeds we used were sampled at approximately 40 DAP, which is after maturation and establishment of primary dormancy is complete. After-ripening is often correlated with a further decline in ABA content and decreased sensitivity to ABA (Benech-Arnold *et al.*, 1999; Bewley, 1997a; Grappin *et al.* 2000; Hilhorst, 1995; Li and Foley, 1997; Ren and Kermode, 1999).

Recent work with *Nicotiana plumbaginifolia* suggests that *de novo* synthesis of ABA in imbibed fresh seed also contributes to dormancy (Grappin *et al.* 2000). ABA treatment at concentrations in the range found in mature wild-type tobacco seeds transcriptionally down-regulates βGLU I expression in the micropylar endosperm and markedly delays endosperm rupture (Leubner-Metzger *et al.*, 1995). Moreover, expression of host βGLU I in control seeds, which is down-regulated by ABA, was increased by after-ripening; whereas expression of the *Cat1*-regulated *βGLU I* gene in sense seeds, which is up-regulated by ABA, was decreased by after-ripening (Table 1). This leads us to speculate that the expression of βGLU I needed for endosperm weakening is inhibited by ABA present in the fresh control seed. During after-ripening, decreasing ABA levels and decreasing sensitivity to ABA eventually permit βGLU I expression, which results in the release of dormancy (Figure 2).

Experimental procedures

Plasmid construction and plant transformation

The chimeric sense-βGLU I construct KSG7 was obtained by transcriptional fusion between the 2.7 kb *Eco*RI–*Bam*HI genomic DNA fragment of pCBC1 that contains the promoter of the castor

bean *Cat1* gene (Suzuki *et al.*, 1994; Suzuki *et al.*, 1995) and the 2.9 kb *Bam*HI–*Sph*I genomic DNA fragment of the *βGLU I B* (*Glb*) gene which contains an introduced *Bam*HI site located at the transcription start site, the entire coding sequence, the intron and 0.9 kb of 3′ flanking region (Hart *et al.*, 1993). The resulting 5.6 kb *Eco*RI–*Sph*I DNA fragment replaced the cauliflower mosaic virus (CaMV) 35S promoter in the pDH51 vector (Pietrzak *et al.*, 1986) and is terminated by the CaMV 35S RNA polyadenylation signal. The binary vector pCIB200 (Neuhaus *et al.*, 1992), which carries the chimeric neomycin phosphotransferase gene (*Npt II*) under the control of the nopaline synthase (*Nos*) promoter and terminator, was linearized in the polylinker with *Kpn*I and *Eco*RI and ligated to the *Kpn*I–*Eco*RI fragment that contained the chimeric sense-βGLU I gene. The resulting expression vector pKSG7 carried the chimeric sense-βGLU I and *Nos–NptII* genes transcribed in the same direction.

The methods for introducing the pCIB200 expression vectors into *Agrobacterium tumefaciens*, Ti-plasmid transformation of *Nicotiana tabacum* L. cv. Havana 425 leaf discs, regeneration of plants, and segregation tests using the kanamycin resistance marker have been described previously (Beffa *et al.*, 1993). TCIB1 transformants obtained with the empty-vector plasmid pCIB200 were used as controls. Segregation tests were performed with S_1 seeds obtained by self-fertilization of independent primary transformants. Homozygous, monogenic S_2 seeds were obtained by self-fertilization of S_1 plants using kanamycin resistance as the marker.

Germination experiments

Seed from mature capsules of wild-type or transformed Havana 425 tobacco were used either at approximately 40 DAP (fresh seed) or after at least 6 months of dry storage at room temperature (after-ripened seed), as indicated. Germination analyses was performed as described by Leubner-Metzger *et al.* (1998). In brief, 100–150 seeds were sown on plastic Petri dishes containing filter paper wetted with a nutrient solution supplemented as indicated with 50 μg ml^{-1} kanamycin, 100 μg ml^{-1} Claforan (Hoechst-Pharma AG, Zürich, Switzerland), and 1 or 10 μM *cis*-(±)-abscisic acid (ABA). Petri dishes were incubated at 25°C in continuous white light (3000 lux, Philips 'TL'D 35 W/33 lamps) or in darkness. After scoring for germination, seeds were stored at –80°C for subsequent analysis.

Analysis of proteins and RNA

Procedures for extracting proteins, assays for enzyme activity, immunoblot analysis, protein determination, preparation of total RNA and RNA-blot hybridization have been described previously (Leubner-Metzger *et al.*, 1995). In brief, βGLU activity was assayed radiometrically using [^{3}H]-laminarin as the substrate. The rabbit anti-tobacco *βGLU I* antibody used for immunoblot analysis detects the class I, class II and class III isoforms of the enzyme (Beffa *et al.*, 1993; Neuhaus *et al.*, 1992). The 'ECF Western blotting system' (Amersham Pharmacia Biotech, Amersham, UK) was used for quantitative immunoblot analysis. The DNA probes used for RNA-blot hybridization were the 1 kb *Pst*I fragment of the tobacco βGLU I cDNA pGL43 (Shinshi *et al.*, 1988) and the 1.8 kb *Eco*RI fragment of genomic DNA encoding tomato 18S ribosomal RNA (Schmidt-Puchta *et al.*, 1989). Hybridized membranes were washed at high stringency. Signals were detected and quantified with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) and corrected for RNA loading based on the 18S ribosomal RNA signal.

Acknowledgements

We thank Masaharu Suzuki and Waltraud Schmidt-Puchta for kindly providing plasmids, Rosa Waldvogel and Monique Thomas for expert technical assistance, Sjoerd van Eeden and Markus Briker for care of plants, and Thomas Boller, Ramamurthy Baskar and Alejandro Iglesias for their critical comments.

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