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β-1,3-Glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening

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Summary

An air-dry developmental state with low-hydrated tissues is a characteristic of most plant seeds. Seed dormancy is an intrinsic block of germination and can be released during after-ripening, that is air-dry storage of mature seeds. Both seed-covering layers, testa and endosperm, cause the coat-imposed dormancy of tobacco (*Nicotiana tabacum*). After-ripening and over-expression of class I β-1,3-glucanase (βGlu I) confer maternal effects on testa rupture and dormancy release. Very little is known about the molecular mechanisms of after-ripening and whether gene expression is possible in low-hydrated seeds. Transient, low-level βGlu I transcription and translation was detected during tobacco seed after-ripening. ^{1}H NMR 2D micro-imaging showed uneven distribution of proton mobility in seeds. βGlu I gene expression is associated spatially with the inner testa and temporally with the promotion of testa rupture. Local elevation in moisture content seems to permit local, low-level βGlu I gene transcription and translation in the maternal tissues of air-dry, low-hydrated seeds. *De novo* gene expression is therefore proposed to be a novel molecular mechanism for the release of coat-imposed dormancy during oilseed after-ripening.

Keywords: after-ripening, coat-imposed dormancy, β-1,3-glucanase, gene expression, low-hydrated state, seed germination, testa rupture.

Introduction

In many organisms the passage through arrested life stages like plant seeds, crustacean cysts, or microbial spores is associated with an air-dry state and low-hydrated tissues (Bewley, 1997b; Potts, 1994). The uptake of water is sufficient for the germination of non-dormant plant seeds under favorable environmental conditions. In contrast to non-dormant seeds, dormant seeds have endogenous mechanisms that block germination under favorable conditions. Defined environmental cues can release dormancy in the imbibed state, but in many species dormancy release is also evident in the air-dry state during after-ripening (Bewley, 1997b; Hilhorst, 1995; Koornneef *et al.*, 2002). Very little is known at the molecular level about the mechanisms involved in the after-ripening-mediated dormancy release. Using 'run-on' transcription analyses with isolated nuclei from air-dry seeds Comai and Harada (1990) showed that a subset of genes is transcriptionally competent in the air-dry, low-hydrated state. Whether gene expression is actually possible at the low seed moisture during after-ripening is not known. Parameters that determine seed after-ripening are moisture and oil contents, seed-covering structures, the plant hormone abscisic acid (ABA), and temperature (Bewley, 1997b; Grappin *et al.*, 2000; Koornneef *et al.*, 2002; Murdoch and Ellis, 2000). After-ripening is prevented in very dry seeds, it requires seed moisture contents above a threshold value. This threshold moisture content is species-specific and lower in oilseeds compared with starchy seeds. After-ripening is also prevented during storage at very high air humidity and for several species the conditions that generate optimal low-hydration values for after-ripening have been determined (e.g. Chapman and Robertson, 1987; Ellis *et al.*, 1995; Hay *et al.*, 2003; Mazza and Jayas, 1991; Mohamed *et al.*, 1998; Murdoch and Ellis, 2000; Pixton and Warburton, 1971; Shatadal and Jayas, 1990; Watanabe and

Tsuda, 1983). The overall seed moisture contents that allow optimal after-ripening are similar for different oilseeds including tobacco and correspond to the 'low-hydrated' seed state, that is the transition between region 2 and region 3 of the moisture sorption isotherm. There is evidence for low-level *de novo* transcription and translation in cyanobacteria in the air-dry state (Potts, 1985, 1994; Xie *et al.*, 1995). We therefore explored whether low-level gene expression is possible in low-hydrated seeds and whether this is a novel developmental mechanism for dormancy release during after-ripening.

Tobacco, *Nicotiana tabacum*, was chosen for the studies because it is an established model system for seed dormancy release, after-ripening, and germination (Koornneef *et al.*, 2002; Leubner-Metzger, 2003). The embryo in mature tobacco seed is surrounded by three to five layers of endosperm cells and a thin testa, which consists of an outer layer of cutinized and lignified dead cells and a living inner testa layer. Seed dormancy of tobacco and other Solanaceae is coat-imposed and both covering layers, the testa and the endosperm, are a hindrance to seed germination. Testa rupture and endosperm rupture, which always occur at the micropylar seed end, are distinct and temporally separate events during the germination of tobacco. This important experimental advantage was used to study the hormonal regulation and to pinpoint the sites for β-1,3-glucanase (βGlu) action during seed dormancy release and germination (Krock *et al.*, 2002; Leubner-Metzger, 2003; Petruzzelli *et al.*, 2003; Schwachtje and Baldwin, 2004). βGlu are encoded by multigene families, are widespread and abundant in microbes and plants, and also found in snails and sea urchins. In sea urchins βGlu is involved in egg fertilization by influencing the hydration state at the fertilization envelope and in embryo hatching by serving as an envelope-modifying or -hatching enzyme (Bachman and McClay, 1996; Sova *et al.*, 2003). The various plants βGlu are classified into at least four structural classes that differ by a minimum of 40–50% in amino acid sequence identity of the mature proteins (Leubner-Metzger, 2003). Plant βGlu are involved in pathogen defense and in diverse physiological and developmental processes in the uninfected plant, including the release of bud dormancy (Rinne *et al.*, 2001) and the promotion of seed germination (Leubner-Metzger, 2003).

^{1}H nuclear magnetic resonance (NMR) micro-imaging is a non-invasive *in vivo* technique that allows the acquisition of sequential cross-sectional 2D micro-images of the spatial distribution of proton mobility (Fountain *et al.*, 1998; Jenner *et al.*, 1988; Schneider *et al.*, 2003). It relies on the principle that ^{1}H nuclei carry a spin and therefore a magnetic moment. The interaction of this magnetic moment with externally applied magnetic fields and radio frequencies can be used to obtain two-dimensional micro-images. ^{1}H NMR 2D micro-imaging of relatively large seeds and of crustacean cysts shows redistribution of water and tissue-specific differences in moisture content (Fountain *et al.*, 1998; Jenner *et al.*, 1988; Seitz *et al.*, 1981). Different seed tissues and organs hydrate at different extents and the seed-covering layers seem to act as water reservoirs for the embryo in several species (ASAE, 2001; Hegarty, 1978; Karon, 1947; Mazza and Jayas, 1991). Neither such dissecting experiments nor ^{1}H NMR 2D micro-imaging studies have been published yet for seeds as small as tobacco, that is <1 mm.

Transcription and translation are both required for the completion of tobacco seed germination and ABA inhibits endosperm rupture and gene expression of class I βGlu (βGlu I) in the micropylar endosperm covering the radicle tip (Arcila and Mohapatra, 1992; Leubner-Metzger, 2003). Sense transformation of tobacco with a chimeric ABA-inducible βGlu I transgene provided direct evidence that βGlu I contributes to endosperm rupture (Leubner-Metzger, 2002; Leubner-Metzger and Meins, 2000). The release of coat-imposed dormancy during tobacco seed after-ripening has been studied by comparing freshly harvested mature seeds ('fresh' seeds, dormant state, approximately DAP40, i.e. 40 days after pollination) with seeds after several months of air-dry storage at room temperature (after-ripened seeds, non-dormant state). Germination in darkness of fresh tobacco seeds is blocked and this photodormancy can be released during after-ripening. Germination in the light of fresh tobacco seeds is slow and the after-ripening-mediated release of coat-imposed dormancy causes a promotion of testa rupture and a similar promotion of subsequent endosperm rupture. After-ripened tobacco seeds have lower ABA contents, decreased ABA sensitivity, and enhanced βGlu I expression during imbibition (Grappin *et al.*, 2000; Leubner-Metzger, 2002; Leubner-Metzger and Meins, 2000). Reciprocal crosses between wild-type tobacco and sense-βGlu I transformant lines (TKSG7) showed that βGlu I over-expression in the seed-covering layers can replace the promoting effect of after-ripening on testa rupture, but only if the mother plant is a TKSG7 line. This maternal effect supports a model for two sites of βGlu I action (Leubner-Metzger, 2003): (i) ABA-sensitive expression of βGlu I in the micropylar endosperm, which contributes to endosperm rupture. (ii) βGlu I action in the after-ripening-mediated release of coat-imposed dormancy in the air-dry state, which is manifested in the promotion of testa rupture during imbibition.

The work presented in this research paper demonstrates that *de novo* βGlu I gene expression occurs in air-dry, low-hydrated tobacco seeds. ^{1}H NMR 2D micro-imaging showed uneven proton-mobility distribution in low-hydrated seeds. Enzyme activity assays, immunoblot analyses, RT-PCR, and reporter gene assays detected transient, low-level βGlu I transcript and protein expression in the inner testa during after-ripening. Together with the use of sense- and anti-sense-βGlu I seeds, these results provide strong evidence that transcription and translation are involved in the

promotion of testa rupture during tobacco after-ripening. *De novo* gene expression is therefore proposed to be a novel molecular mechanism for the after-ripening-mediated release of coat-imposed seed dormancy.

Results

Promotion of testa rupture occurs during tobacco seed after-ripening and depends on βGlu I

Initial after-ripening experiments were performed by air-dry storage of a number of independent tobacco seed batches from different transgenic lines. TCIB1 (transformed with the 'empty-vector'), and *GlbGus* [transformed with a β-glucuronidase (Gus)-reporter gene regulated by the βGlu I promoter] tobacco lines have wild-type (WT) expression of the βGlu genes and WT seed germination physiology (Leubner-Metzger and Meins, 2000; Leubner-Metzger *et al.*, 1998). WT and the transgenic lines TCIB1 and *GlbGus* do not differ with regard to the βGlu I genes and the seed phenotype and are therefore referred to as 'control seeds.' Figure 1(a) shows the time courses of βGlu activity expression during after-ripening storage of mature seeds harvested at approximately DAP40 (i.e. fresh seeds). βGlu activity was at elevated levels during the entire storage period in seeds of TKSG7 transformants (Figure 1a). This βGlu I over-expression in TKSG7 seeds was due to the transformation with a chimeric sense-tobacco βGlu I-transgene regulated by the castor bean *Cat1* promoter (Leubner-Metzger and Meins, 2000). Interestingly, transiently elevated levels of βGlu activity and βGlu I protein were detected around DAH60 (i.e. 60 days after harvest) in after-ripening TCIB1 (Figure 1a), WT (data not shown) and *GlbGus* (Figure 2) seeds. This suggests that βGlu I gene expression occurs during control seed after-ripening.

Comparison of fresh and after-ripened seeds demonstrates that the time to reach 50% testa rupture (TR_{50}) was similarly promoted by 15–18 h during the after-ripening of

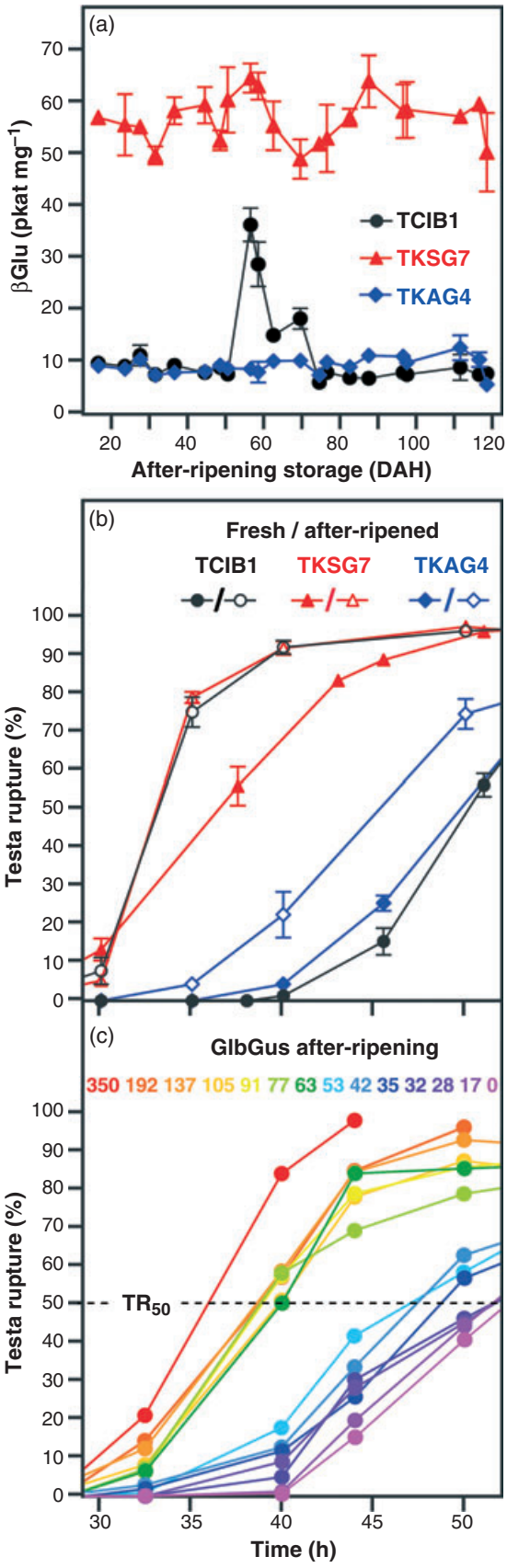
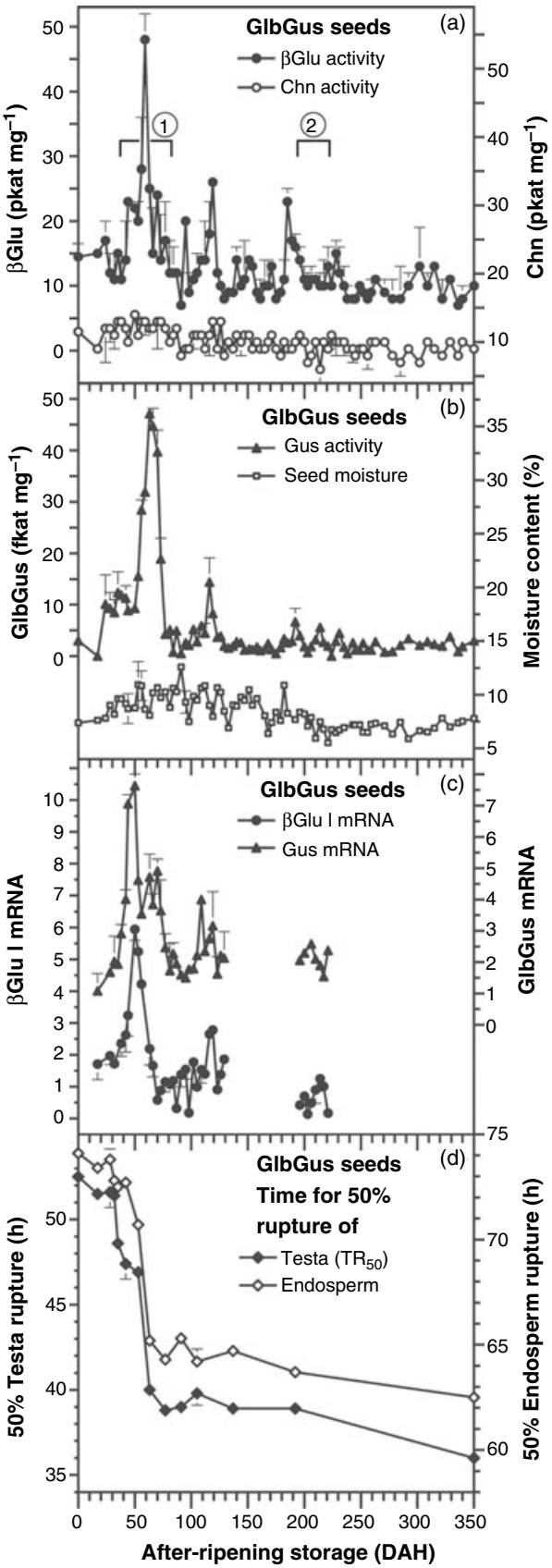


Figure 1. Effect of sense- and antisense-βGlu I transformation on the time courses of βGlu I expression and testa rupture during tobacco seed after-ripening.
(a) βGlu enzyme activities in sense-βGlu I (TKSG7), antisense-βGlu I (TKAG4), and 'empty vector' control seeds (TCIB1) during after-ripening; DAH, days after harvest; mature seeds were harvested at approximately DAP40 (i.e. fresh seeds at DAH0).
(b) Kinetics of testa rupture of fresh and after-ripened (>6 months of air-dry storage) TCIB1, TKSG7, and TKAG4 seeds. Note that over-expression of βGlu I in the covering layers of TKSG7 seeds caused promoted testa rupture already in the fresh state and that antisense-βGlu I transformation prevented the after-ripening-mediated promotion of testa rupture of TKAG4 seeds.
(c) Promotion of *GlbGus* seed testa rupture during after-ripening. Time courses of testa rupture of *GlbGus* seeds at various DAH. The curves and corresponding DAH numbers are indicated by rainbow colors. TR_{50} is the time needed to reach 50% testa rupture. Note that these TR_{50} values are presented in Figure 2(d) and that endosperm rupture is equally promoted by after-ripening. (a–c) Mean ± SE of usually three samples for each line are presented. When error bars are not shown, the ±SE values are <2.0 (a, b). For presentation reasons error bars are not shown in (c), the ±SE values are comparable to (b).



control seeds. TR_{50} (fresh/after-ripened) were 50.5/34.9 h for WT, 51.0/33 h for TCIB1 (Figure 1b), and 51.5/36.0 h for *GlbGus* (Figure 1c). This further supports our earlier findings that the release of coat-imposed dormancy and the promotion of testa rupture are hallmarks of tobacco seed after-ripening (Leubner-Metzger, 2002, 2003; Leubner-Metzger and Meins, 2001). In TKSG7 seeds βGlu I over-expression is confined to the seed-covering layers, it replaced the after-ripening effect and promoted testa rupture already in the fresh stage (Figure 1b, Leubner-Metzger, 2002).

The TKAG4 tobacco antisense-βGlu I transformants harbor an antisense-tobacco βGlu I-transgene regulated by the castor bean *Cat1* promoter (Leubner-Metzger and Meins, 2001). In contrast to control seeds, during after-ripening storage βGlu activity remained low in TKAG4-type antisense-βGlu I seeds (Figure 1a) and in several other antisense-βGlu I lines tested. A comparison of seed samples harvested from single capsules between DAH10 and DAH60 showed at least twofold elevated βGlu I contents (enzyme activity and 33 kDa protein) in 16 of 66 control seed extracts and no elevation in 48 antisense-βGlu I seed extracts. In contrast to control seeds, the promoting effect of after-ripening on testa rupture was prevented in antisense-βGlu I (TKAG4) seeds (Figure 1b). This is in agreement with the earlier finding that the after-ripening-mediated promotion of subsequent endosperm rupture is prevented in antisense-βGlu I seeds and that photodormancy release is blocked in antisense-βGlu I seeds (Leubner-Metzger and Meins, 2001).

Comparison with the sense- and antisense-βGlu I lines suggested that promotion of testa rupture during control seed after-ripening is mediated, at least in part, by βGlu expression during prolonged air-dry storage. The *GlbGus* reporter gene line was utilized to further test this hypothesis with detailed time course experiments. Fresh *GlbGus* seeds were stored air-dry at room temperature for 1 year, and samples were taken every 3–4 days during this after-ripening period. Figure 1(c) shows for *GlbGus* seeds that the

Figure 2. Time course analyses of tobacco after-ripening investigated by air-dry storage of *GlbGus* seeds.
(a) βGlu and Chn enzyme activities measured in the same protein extracts by equally sensitive and specific radiometric assays. Further analyses of regions 1 and 2 are presented in Figure 3.
(b) Gus enzyme activities measured in the same protein extracts. Seed moisture content expressed as percentage (w/w) H_2O per FW.
(c) Relative levels of βGlu and Gus mRNAs determined by semi-quantitative RT-PCR with 18S rRNA as internal standard. The same RNA samples at several DAH were analyzed by TaqMan real-time RT-PCR presented in Figure 3(e).
(d) Times in hours for 50% testa rupture (TR_{50}; Figure 1c) or endosperm rupture as determined from kinetic analyses of imbibed seed populations. Note that a decrease in these values represents a promotion of testa or endosperm rupture. Mean ± SE of at least three protein and two RNA samples are presented for each time point. For each RNA sample different amounts, 25–50 ng were used in the RT-PCR assays as described in Experimental procedures. When error bars are not shown, the ±SE values are <2.0 for enzyme activities (a, b) and rupture (d), <1.0 for moisture (b), and <0.25 for mRNA content (c).

promotion of testa rupture is not equally distributed over the entire after-ripening period. The major promotion of testa rupture occurs at a defined time window around DAH60 (Figure 1c). Figure 2(d) shows that the promotion of testa rupture is not only evident by a decrease of the TR_{50} times (i.e. an increase in testa rupture speed) during after-ripening, but also by a similar promotion of subsequent endosperm rupture, that is radicle protrusion.

Evidence for transient βGlu I gene expression in low-hydrated tobacco seeds during after-ripening

The *GlbGus* seed after-ripening process presented in Figure 2 shows that the βGlu enzyme activities of fresh seeds and of seeds during the very early storage period until approximately DAH40 are at low levels of approximately 10 pkat mg^{-1} protein (Figure 2a). A major peak of βGlu activity was detected between DAH40 and DAH70 with βGlu activities above 20 pkat mg^{-1} protein at several time points and a maximum of approximately 50 pkat mg^{-1} protein around DAH60 (region 1 in Figure 2a). In addition, several minor peaks were observed at later times, but after approximately DAH196 (starting with region 2 in Figure 2a) βGlu activities remained at low levels. The highly sensitive and specific radiometric assay that was used for the measurement of these low βGlu activities utilized reduced [^{3}H]-laminarin, an algal β-1,3-glucan, known to be specifically digested by all endo-type βGlu isoforms (Beffa *et al.*, 1993; Leubner-Metzger, 2003). βGlu and chitinase (Chn) are often co-induced, for example in seedling roots (Petruzzelli *et al.*, 2003) and therefore a corresponding radiometric assay of equal sensitivity and specificity with [^{3}H]-chitin as the substrate was used to measure the Chn activities as internal controls in the same seed protein extracts (Figure 2a). In contrast to βGlu, no Chn peaks were detected and very low, roughly constant levels of chitinase enzyme activity (approximately 10 pkat mg^{-1} protein) were found during the entire after-ripening period (Figure 2a). This was further supported by comparative measurements at DAH60 and DAH210 of single-seed βGlu and Chn activities (data not shown). The single-seed βGlu activities were considerably higher in DAH60 seeds compared with DAH210 seeds, whereas the single-seed Chn activities did not differ between DAH60 and DAH210.

The anti-tobacco βGlu I antibody is known to detect the class I, class II and class III βGlu isoforms (Beffa *et al.*, 1993; Leubner-Metzger, 2003). Figure 3(a) shows that this antibody detected the transient accumulation of the 33 kDa βGlu I antigen in the seed extracts of the major βGlu activity peak (region 1 in Figure 2a). The signal intensities for the 33 kDa were already increased at DAH50, were maximal around DAH60, and decreased thereafter. Only faint, roughly constant immunoreactive bands were detected in region-2 seed extracts (Figures 2a and 3a). The temporal pattern of the 33 kDa βGlu I protein abundance and the βGlu activities coincide.

Fluorimetric measurements of the Gus activities in the *GlbGus* seed protein extracts detected a major peak in region 1 of the early after-ripening period and, in addition, several minor peaks (Figure 2b). As the βGlu activity peaks, the Gus activity peaks were transient and their increase coincided in time with the transient βGlu activity peaks. The major Gus activity peak remained somewhat longer at a high level compared with the major βGlu activity peak. In agreement with the Gus activity profile an anti-Gus antibody detected the transient accumulation of the 72-kDa Gus antigen in the seed extracts of region 1, while this antigen was not detected in region 2 (Figure 3c). It is known that the Gus protein, which has a predicted molecular weight of 68.2 kDa, runs in denaturing gels at a higher apparent molecular weight of approximately 72 kDa (Jefferson *et al.*, 1987; To *et al.*, 1996; Yang *et al.*, 1996). A commercial Gus protein preparation and a protein extract from a Gus-over-expressing tobacco plant were used as additional controls and further verified that the 72-kDa immunoreactive band is the Gus antigen (Figure 3c). These findings demonstrate that the βGlu I promoter is transiently active during seed after-ripening and suggest that transcription of the *GlbGus* transgene and the βGlu I gene is coordinately induced.

In agreement with this, TaqMan real-time RT-PCR and semi-quantitative RT-PCR analyses performed with RNA preparations of the after-ripening *GlbGus* seeds detected transient accumulation of βGlu I and Gus transcripts in region 1, but only faint signals in region 2 (Figures 2c and 3b,d,e). The relative mRNA quantities obtained were based on 18S rRNA as the internal standard by using a specific 18S rRNA TaqMan probe in the real-time PCR and specific primers in combination with fluorimetric measurement of the product yield in the semi-quantitative RT-PCR. Real-time PCR with specific TaqMan probes showed 6.9- and 3.3-fold increases of βGlu I and Gus mRNA levels from DAH38 to DAH50, respectively (Figure 3e). Approximately threefold increases of βGlu I and Gus mRNA levels were detected by semi-quantitative RT-PCR (Figure 2c). By using TaqMan real-time RT-PCR and semi-quantitative RT-PCR the same temporal pattern for the βGlu I and Gus mRNA levels during seed after-ripening was obtained (Figures 2c and 3e). The maxima of the transient βGlu I and Gus mRNA peaks at approximately DAH50 preceded the corresponding maxima of the major protein and enzyme activity peaks by approximately 10 days. That transcription and mRNA accumulation precede translation and protein accumulation is expected, the relatively long time difference between the peak maxima is evidence for a slow process of gene expression. Consistent with the finding that Gus activity and protein remain longer at elevated levels compared with βGlu I, the Gus mRNA contents also remained longer at elevated levels compared with the βGlu I mRNA contents (Figures 2 and 3).

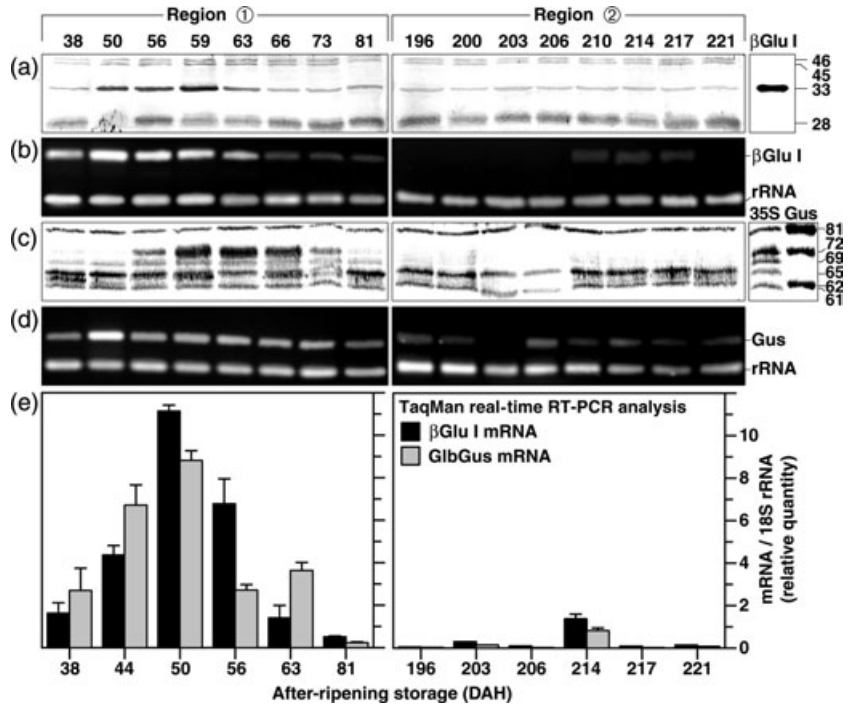


Figure 3. Time course immunoblot (a, c) and RT-PCR (b, d, e) analyses of *GlbGus* tobacco seed after-ripening. Regions 1 and 2 represent the early (major βGlu peak and promotion of testa rupture) and the late after-ripening period, respectively (see Figure 2).
(a) Immuno-blot analyses with the anti-tobacco βGlu I antibody of protein extracts (100 μg per lane) at different DAH during after-ripening. 'βGlu I', 10 ng of pure tobacco 33 kDa βGlu I protein. The apparent size in kDa of immunoreactive bands is indicated.
(b) Semi-quantitative RT-PCR of βGlu I transcripts. RT-PCR and subsequent nested PCR with βGlu I-mRNA-specific primers (one of which spans the intron-splice site) generated a 708-bp βGlu I cDNA amplification product. The 443-bp rRNA PCR product was co-amplified with primers specific for 18S rRNA and served as internal standard.
(c) Immuno-blot analyses with the anti-*Escherichia coli* Gus antibody (details as in a). '35S', protein extract from Gus-over-producing transgenic 35SGus tobacco (Beffa *et al.*, 1993); 'Gus', commercial *E. coli* Gus protein extract used as standard. Note that the intact Gus protein corresponds to the 72-kDa antigen and that this antibody is known to detect additional bands (Jefferson *et al.*, 1987; To *et al.*, 1996; Yang *et al.*, 1996).
(d) Semi-quantitative RT-PCR of Gus mRNA generated a 620-bp Gus PCR amplification product (rRNA and details as in b).
(e) TaqMan real-time RT-PCR analysis of βGlu I and Gus mRNAs. Transcript quantification with specific TaqMan MGB probes was performed in multiplex assays with 18S rRNA as internal standard. The RNA samples analyzed at the given DAH were identical to the RNA samples used to obtain the results in Figure 2(c).

Taken together, these results strongly suggest that the βGlu I genes are transiently expressed during tobacco seed after-ripening and that low-level *de novo* transcription and translation are possible in air-dry, low-hydrated oilseeds.

Transient βGlu I gene expression during after-ripening is localized in the seed-covering layer and is associated with the promotion of testa rupture

Figure 2(d) shows by the decrease of the time to reach 50% testa rupture (TR_{50}) that the major promotion of testa rupture during tobacco seed after-ripening occurs between DAH40 and DAH70 and therefore coincides in time with the major transient peak of βGlu I gene expression. The TR_{50} value of 51.5 h for fresh *GlbGus* seeds decreased to approximately 39 h between DAH40 and DAH70, and this was followed by a further slower decrease to approximately 36 h in fully after-ripened seeds (Figure 2d). The promotion of testa rupture was associated with a similar promotion pattern for the time to reach 50% endosperm rupture (Figure 2d). In agreement with a role of βGlu I in the after-ripening-mediated release of coat-imposed dormancy, over-expression of βGlu I in the covering layers of TKSG7 seeds replaced the after-ripening effect on testa rupture and 50% testa rupture was already approximately 39 h in fresh TKSG7 seeds (Figure 1b); and βGlu I expression and the promotion of testa rupture are prevented in TKAG4 antisense-βGlu I seeds (Figure 1a,b).

The spatial pattern of βGlu I expression in after-ripening seeds was investigated by histological Gus staining and by *in situ* immunohistological analyses using the anti-tobacco βGlu I antibody. Figure 4 shows that βGlu I-promoter activity and βGlu I antigen are localized in the outer living cell layer(s) of the DAH60-*GlbGus* seed coverings. From assays of dissected seeds βGlu I expression localized to the living inner testa layer and was not detected in the inner endosperm cell layers or the embryo (Figure 4c). Neither Gus staining nor βGlu I-antigen was detected in *GlbGus* seeds at DAH210 (Figure 4d). Interestingly, Gus and βGlu I expression did not cover the entire DAH60 seeds, only about 30% of

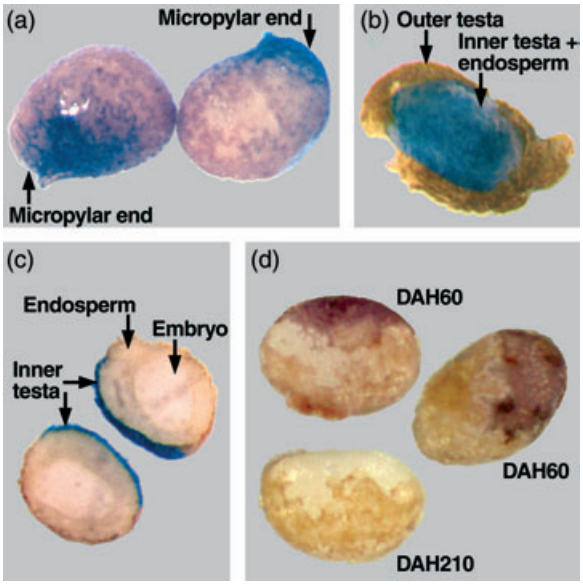


Figure 4. Spatial analyses of βGlu I expression in air-dry *GlbGus* tobacco seeds during after-ripening.
(a–c) Histological Gus staining during the release of coat-imposed dormancy (region 1). Blue staining due to βGlu I-promoter activity is localized in the inner testa. The staining was achieved with intact DAH60-seeds (a) or with seeds after removal of the outer testa (b, c), and either without (a, b) or with (c) seed dissection. No Gus staining was detected in DAP210 seeds and in wild-type seeds.
(d) *In situ* immunohistological analyses of DAP60 and DAP210 seeds with the outer testa removed. Staining is indicative of the localization of βGlu-antigens detected by the βGlu I-specific antibody. (b–d) To make the seeds permeable, to permit uptake of the reagents and to remove the outer testa seeds frozen in liquid nitrogen were gently rubbed with a cover slide.

the seed surface was stained and βGlu I expression appeared to be either localized at the micropylar end (Figure 4a), the seed flank regions (Figure 4b–d), or at the non-micropylar end (Figure 4d). These results suggest: (i) The βGlu I promoter has the potential to generate βGlu I expression in all areas of the inner testa. (ii) Other probably general factors may limit gene expression to some areas of the inner testa. (iii) βGlu I expression in only a part of the inner testa seems to be sufficient for the after-ripening-mediated promotion of testa rupture. Together with the earlier results that after-ripening and βGlu I over-expression confer maternal effects on testa rupture (Leubner-Metzger, 2002), these new results about the temporal and spatial expression pattern support a role for βGlu I during the after-ripening-mediated promotion of testa rupture and the release of coat-imposed dormancy.

Uneven distribution of moisture in low-hydrated tobacco seeds during after-ripening

The moisture content of the fresh seeds used for the after-ripening experiment was approximately 8% H_2O (w/w) on a fresh weight (FW) basis, that is 0.08 g H_2O g^{-1} FW. Figure 2(b) shows that similar and roughly constant moisture contents were also measured during the late after-ripening period including region 2. In contrast to this, the early after-ripening period including region 1 with the major βGlu I peak was characterized by higher values and by fluctuations of the seed moisture content. Increases up to approximately 13% H_2O per FW were detected during this first half of the after-ripening period. Tobacco seeds contain high amounts of triacylglycerols stored in oil (lipid) bodies. A triacylglycerol content of approximately 43% (w/w) per FW was measured for the Havana-425 cultivar, a value which is in agreement with other tobacco cultivars (Frega *et al.*, 1991). As water is excluded from the oil bodies and the overall moisture content of the after-ripening tobacco seeds is 8–13%, one can calculate that the overall seed moisture content of the non-oil regions is approximately 14–23%.

^{1}H NMR 2D micro-imaging was used as a non-invasive *in vivo* technique to determine how the proton mobility is spatially distributed in tobacco seeds (Figure 5). Proton

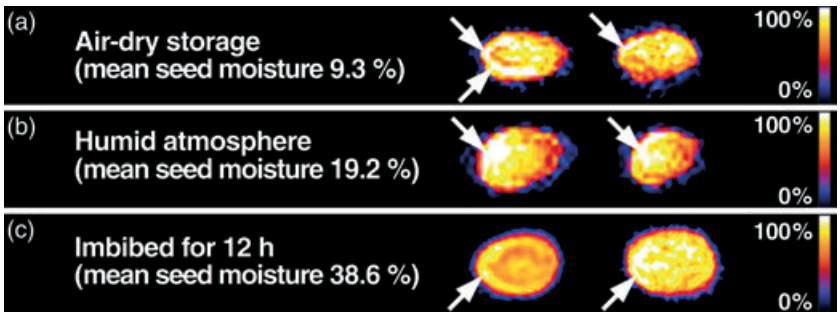


Figure 5. Spatial analyses of the proton mobility distribution in tobacco seeds by non-invasive *in vivo* ^{1}H NMR 2D micro-imaging. Representative cross-sectional *GlbGus* seed micro-images with 31 μm resolution. The spatial distribution of proton mobility within the tissues is visualized by the false colors (relative scale from 0 to 100%).
(a) Seed during air-dry storage (moisture content 9.3%).
(b) Seed during storage in humid atmosphere (moisture content 19.2%).
(c) Seed imbibed for 12 h (moisture content 38.6%).
(a–c) Arrows indicate areas with high proton mobility in the covering layers. Note that high proton mobility is specific for water and/or lipids in (a) and becomes specific for water at increasing moisture contents in (b, c).

mobility distribution is uneven and there are areas of high proton-mobility content in the outer seed-covering layer. Seeds analyzed were stored air-dry, stored in humid atmosphere, and imbibed for 12 h; they contained increasing moisture contents of 9.3, 19.2, and 38.6%, respectively. While in air-dry seeds the high proton mobility could be due either to high water or to high lipid content, in the other two conditions with higher seed moisture content, the high proton mobility becomes indicative of high water content. These high-resolution ^{1}H NMR 2D micro-images are consistent with the presence of localized areas of higher water content within the seed-covering layers. It is proposed that local βGlu expression in the seed-covering layer requires a local increase in the moisture content, but depends on the overall low-hydrated seed state. It occurs during a window of competence successively in most or all seeds, and finally results in the after-ripening-mediated promotion of testa rupture.

Discussion

Transient, low-level transcription and translation in air-dry, low-hydrated seeds

The most interesting finding presented in this publication is that gene expression is possible in air-dry, low-hydrated (i.e. 8–13% overall moisture content) tobacco seeds during after-ripening and seems to constitute a novel developmental mechanism for seed dormancy release. The transient βGlu I gene transcription and translation detected in the air-dry seeds is approximately 10- or 200-fold lower compared with germinating seeds or seedling roots, respectively (Leubner-Metzger, 2002; Petruzzelli *et al.*, 2003). The finding that low-level *de novo* gene expression can occur in air-dry, after-ripening seeds is novel. It has been shown earlier that in nuclei isolated from air-dry *Brassica napus* seeds a subset of genes is transcriptionally competent (Comai and Harada, 1990). Among the transcripts that elongated *in organello* by 'run-on' transcription analyses were genes that are active during late embryogenesis (e.g. cruciferin) and very early during imbibition (e.g. isocitrate lyase), but not genes specifically expressed in seedlings. The nuclear RNA profiles showed that transcription complexes remain intact in air-dry seeds. The transcriptional activities in the dry seed nuclei indicate the timing of the transition from an embryonic to a post-germinative program after seed rehydration of *B. napus* (Comai and Harada, 1990). As 'run-on' transcription with the dry-seed nuclei requires aqueous *in vitro* assays, they cannot be used to demonstrate that competent genes are transcribed actively *in vivo* in the air-dry seed state. In the present work the βGlu I promoter activities and mRNA contents showed that the βGlu I genes not only belong to the transcriptionally competent gene subset in air-dry tobacco seeds, but also that they are actively transcribed in the low-hydrated seed state. In contrast, the Chn genes are not expressed in air-dry tobacco seeds, but the Chn genes are specifically expressed in seedlings (Petruzzelli *et al.*, 2003). Preformed, long-lived mRNAs are stored in air-dry seeds either in association with proteins as mRNP complexes or sequestered within the nucleus (Bewley, 1997b; Payne, 1976). The nature of the stored transcripts in seeds has not been studied extensively, but *in vitro* translation of these long-lived mRNAs is possible (Bewley, 1997b; Lalonde and Bewley, 1986). In contrast to mature tobacco seeds which have an abundant endosperm layer, mature seeds of Arabidopsis have only a single-cell layer remnant of the endosperm. Recent work on the Arabidopsis seed proteome demonstrates the importance of translation for radicle protrusion (Rajjou *et al.*, 2004). Inhibitor experiments strongly suggest that transcription is dispensable for the initial embryo elongation needed for germination *sensu stricto*. The proteins and mRNAs stored in air-dry mature Arabidopsis seeds are sufficient for radicle protrusion. This seems to be different in seeds like tobacco with an abundant endosperm layer. Experiments with inhibitors of transcription and translation (Arcila and Mohapatra, 1992) and work with transgenic seeds (Leubner-Metzger and Meins, 2000) show that tobacco endosperm rupture and radicle protrusion require transcription and translation. However, as in Arabidopsis, testa rupture of tobacco appears to depend exclusively on stored protein and translation from stored mRNAs. Storage of functional mRNAs has also been reported from other air-dry propagation units like cysts of crustaceans (Bruni *et al.*, 1989) and ciliates (Gutiérrez *et al.*, 2001), and spores of fungi (Linz and Orlowski, 1982) and other microbes (Potts, 1994). Transcripts stored in air-dry seeds are also transiently translated very early during imbibition (Bewley, 1997b; Comai and Harada, 1990; Leubner-Metzger *et al.*, 1998).

The transient pattern of βGlu I gene expression during tobacco after-ripening is of different nature. First, it is found in air-dry, low-hydrated seeds without imbibition. Secondly, the elevation of βGlu I transcription and translation product levels starts from background levels and not from residual mRNA. Thirdly, TaqMan real-time RT-PCR, semi-quantitative RT-PCR and Gus measurements (βGlu I promoter activity) strongly suggest that transcriptional gene induction is needed for this rise in βGlu I expression. Finally, the subsequent decline of βGlu I and Gus transcript and protein contents occurs simultaneously, but an unknown post-transcriptional mechanism seems to cause a slightly faster degradation of βGlu I transcripts. The finding that translation is possible in air-dry tobacco seeds is in agreement with evidence for low-rate incorporation of amino acids into protein of air-dry seeds of charlock (Edwards, 1976). The presence of active proteasome complexes in dry pea seeds and a proteasome-mediated seed dormancy release of after-ripening sunflower seeds has

been reported (Borghetti *et al.*, 2002; Skoda and Malek, 1992). Furthermore, activation of respiration and of enzymes involved in amino acid metabolism has been demonstrated in air-dry seeds (Hilhorst, 1998; Linko and Milner, 1959; Obroucheva and Antipova, 1997). This is further supported by results from *in vitro* systems for low water content showing that enzymes can work, and in some cases even exhibit 'superactivity', under low-hydration conditions that are far from those that exist in living organisms (De Gómez-Puyou and Gómez-Puyou, 1998). Evidence also suggests that *de novo* transcription and translation occurs in cyanobacteria in the air-dry state (Potts, 1985, 1994; Xie *et al.*, 1995). Air-dry seeds are, in contrast to air-dry cyanobacteria, composed of different tissues and this raises the question about the spatial localization of βGlu I gene expression and about the spatial distribution of moisture in air-dry seeds.

Spatial localization of βGlu I gene expression and uneven moisture distribution in after-ripening seeds

The βGlu I gene expression was localized to the living inner testa. The existence of this living cell layer interposed between the endosperm and the dead outer testa and the identification of its maternal origin as parenchyma cells of the integument is based on gene promoter studies and genetic ablation experiments (Czakó *et al.*, 1992; Fobert *et al.*, 1994; Matzke *et al.*, 1993). As for the seed coat-specific cryptic promoter of tobacco (Fobert *et al.*, 1994), the βGlu I expression is absent from the embryo and is localized to the inner testa. Thus, the βGlu I expression pattern in air-dry, low-hydrated tobacco seeds is spatially and temporally associated with a function in the after-ripening-mediated promotion of testa rupture.

The after-ripening time window with the major promotion of testa rupture and the major transient βGlu I peak was characterized by fluctuations of the overall moisture contents between 8% and maximal values of 13% H_2O per FW. Tobacco seeds are oilseeds and contain 43% triacylglycerol per FW, which is stored in the oil bodies of the embryo and the endosperm (this work, Frega *et al.*, 1991). In the sorption isotherm for oilseeds 13% overall moisture content corresponds already to the beginning of the steep part of the curve and small fluctuations in humidity can cause large changes in water activity (Chapman and Robertson, 1987; Mazza and Jayas, 1991; Pixton and Warburton, 1971; Shatadal and Jayas, 1990). As water is excluded from the oil bodies the corresponding overall seed moisture content is 14–23% H_2O per FW in the non-oil body cell portions. Activation of respiration, phytochrome, and amino acid metabolism, and even incorporation of amino acids into proteins has been reported from non-oil seeds at such low moisture contents (Edwards, 1976; Linko and Milner, 1959; Obroucheva and Antipova, 1997). Redistribution of water and tissue-specific differences in moisture content has been detected by ^1H NMR micro-imaging of cereal and legume seeds (Fountain *et al.*, 1998; Jenner *et al.*, 1988). These seeds are relatively large and do not store oil, the spatial analyses of the moisture content could therefore be performed without significant interference by lipids. In contrast to this, tobacco seeds are small and store oil. The ^1H NMR 2D micro-imaging of low-hydrated (9.3% moisture content) tobacco seeds shows that there are localized tissue areas in the covering layers with higher proton mobility. Indirect evidence supports the view that these are localized areas with higher water content. First, localized areas with higher water content are evident in the covering layers when the overall moisture content of the seeds is doubled by incubation in a humid atmosphere or further increased by imbibition. In these cases of higher moisture content the proton mobility increase becomes indicative of an increase in water content. Secondly, it is known from dissection studies of cereal and legume seeds that the hydration properties of seed tissues differ and that the covering layers, the endosperm, and the cotyledons can act as water reservoirs (Allen *et al.*, 2000; Hegarty, 1978). This is also true for oilseeds and has been determined by comparing the sorption isotherms for different tissues. Based on these measurements the seed-covering layers of cotton, sunflower, peanut, and other species with high oil content have a significantly higher equilibrium moisture content curve compared with the embryos (ASAE, 2001; Karon, 1947; Mazza and Jayas, 1991). Thirdly, the occurrence of localized areas with higher water content and the importance of the seed-covering layers in moisture uptake is further supported by ^1H NMR 2D analysis of the tobacco seed imbibition and germination process (Manz *et al.*, 2004).

It is therefore likely that the βGlu I gene expression in the inner testa layer is associated with such areas of higher moisture content. This may also explain the spatial differences in size and localization of the area in the inner testa exhibiting βGlu I gene expression. Similar to βGlu I gene expression, the areas of higher moisture content also exhibited spatial differences in size and localization within the covering layer. βGlu is proposed to influence the tissue hydration state (Bachman and McClay, 1996; Sova *et al.*, 2003) and is known to decrease the viscosity of malting solutions. One can therefore speculate that the spatial βGlu I expression pattern in air-dry tobacco seeds could be generated from punctual initial expression which causes local elevation of the osmotic potential followed by local elevation of the moisture content and spread of the area of βGlu I expression and higher moisture content. Taken together, these findings suggest that the covering layers of air-dry, low-hydrated tobacco seeds contain regions with moisture contents high enough above a threshold level to permit transient, low-level βGlu I gene expression.

Gene expression as a novel mechanism for the release of coat-imposed dormancy during oilseed after-ripening

The developmental process of seed after-ripening depends on moisture and oil contents, temperature, seed coverings, and ABA level and sensitivity (Bewley, 1997b; Ellis *et al.*, 1995; Grappin *et al.*, 2000; Koornneef *et al.*, 2002; Mohamed *et al.*, 1998; Murdoch and Ellis, 2000). In the starchy grains of cereals and wild grasses, at very low moisture contents (<7%) the rate of after-ripening is minimal, it is greatest between 11 and 15% moisture content (low-hydrated seeds) and is again minimal at higher moisture content (>22%). While these are typical values for starchy seeds, the overall moisture contents for optimal after-ripening and dry storage of oilseeds are lower. Seed moisture is absorbed almost exclusively by carbohydrates and proteins, but not triacylglycerols (oil). Thus, if the moisture content values above are present in the 'oil-free' seed portion of tobacco seeds (with a 43% 'oil' content), the corresponding (lower) overall moisture contents can be calculated. Based on this, very low moisture contents would be <5% for tobacco seeds and should not allow after-ripening-mediated dormancy release, for example photodormancy release measured as germination percentage in darkness. Dormancy release of tobacco did not occur during several months of dry storage under conditions where the seed moisture content was only approximately 5% (Watanabe and Tsuda, 1983). Tobacco after-ripening should be greatest in low-hydrated seeds between 7 and 10% moisture content and again minimal at higher moisture content >14%. Photodormancy release (Kasperbauer, 1968; Leubner-Metzger, 2002; Leubner-Metzger and Meins, 2001), after-ripening-mediated promotion of germination speed in the light (Leubner-Metzger and Meins, 2000, 2001), and after-ripening-mediated promotion of testa rupture (this work, Leubner-Metzger, 2002) was optimal at the seed moisture contents of 8–13%. In the sorption isotherms for oilseeds 8% corresponds to the end of the flat part and 13% overall moisture content corresponds to the already steep part of the curve (Chapman and Robertson, 1987; Hay *et al.*, 2003; Mazza and Jayas, 1991; Pixton and Warburton, 1971). The seed-covering layers of many oilseeds are known to have higher water uptake capacities compared with the embryos, for example the equilibrium moisture content of the cotton or sunflower seed-covering layers is approximately 4% higher compared with the embryos (ASAE, 2001; Karon, 1947; Mazza and Jayas, 1991; Shatadal and Jayas, 1990). In lettuce, tomato, tobacco, legumes, and other species the seed-covering layers have high contents of galactomannan-type hemicelluloses (Bewley, 1997a; Reid *et al.*, 2003). In legumes they are known to function as water reservoirs during seed germination and are commercially used as gelling agents (Cunningham and Walsh, 2002). Thus, in tobacco seeds a combination between a high hemicellulose-caused water reservoir function of the covering layers (endosperm and inner testa) and a high triacylglycerol content could result in locally elevated water contents in the covering layers. This locally elevated water content is high enough to permit *de novo* βGlu I gene expression in the covering layers during seed after-ripening, that is in (overall) low-hydrated seeds. *De novo* gene expression is therefore proposed to be a novel molecular mechanism for the release of coat-imposed dormancy during oilseed after-ripening.

The new findings support a model integrating tobacco seed dormancy, after-ripening, βGlu I and germination (Figure 2 in Leubner-Metzger, 2003). According to the model, βGlu I, which is transcriptionally downregulated by ABA, contributes to the release of coat-imposed dormancy and the promotion of germination at two sites. The first site of βGlu I action is the after-ripening-mediated promotion of testa rupture. Decreasing ABA level and sensitivity during tobacco after-ripening (Grappin *et al.*, 2000; Leubner-Metzger and Meins, 2000) is likely to be a major determinant for setting the time window for βGlu I gene induction. Within this competence window locally elevated water content eventually permits βGlu I expression. βGlu action via β-1,3-glucan degradation at plasmodesmata has been demonstrated for the release of bud dormancy (Rinne *et al.*, 2001). An endogenous β-1,3-glucan has not yet been identified in tobacco seeds. As an alternative mechanism indirect βGlu action by releasing elicitor-active oligo-β-1,3-glucans that act as signaling molecules has been proposed (Leubner-Metzger, 2003). Evidence for both modes of action, direct and indirect, has also been suggested from work in the animal field. βGlu seems to be involved in fertilization and hatching of sea urchins, but here also a demonstration of a substrate is still lacking (Bachman and McClay, 1996). Strong support for the role of βGlu I in the after-ripening-mediated release of photodormancy and the promotion of testa rupture comes from antisense- and sense-βGlu I seeds. Photodormancy is not released and testa rupture is not promoted during after-ripening storage of antisense-βGlu I seeds (this work, Leubner-Metzger and Meins, 2001). βGlu I over-expression in the covering layers of TKSG7 seeds replaced the after-ripening effect on testa rupture (this work, Leubner-Metzger, 2002). The second site of βGlu I action in tobacco seeds is the final ABA-controlled step of endosperm rupture. Sense-βGlu I transformation provided direct evidence for a causal contribution of βGlu I to endosperm rupture (Leubner-Metzger and Meins, 2000).

In conclusion, the presented results strongly suggest that local elevation of moisture content above a threshold level can permit local, transient, and low-level βGlu I gene transcription and translation in the maternal tissues of air-dry, low-hydrated tobacco seeds. This mediates the maternal effect of after-ripening on the promotion of testa rupture. *De novo* gene expression is therefore proposed to be a novel molecular mechanism for the release of coat-imposed dormancy during oilseed after-ripening.

Experimental procedures

Plant materials, after-ripening, and germination conditions

Mature seeds of wild-type or transgenic *N. tabacum* L. cv. Havana 425 were harvested at approximately DAP40 (fresh seeds). Air-dry after-ripening storage was performed in an incubator at approximately 22°C (constant setting) and approximately 30–40% relative humidity (measured at the beginning of the experiment). For the main experiment (e.g. Figures 1c and 2a–d) several seed samples were taken every 3–4 days during this storage period of more than 1 year. For additional experiments sampling was either less frequent (e.g. Figure 1a) or fresh seeds were compared with fully after-ripened seeds stored for approximately 12 months (e.g. Figure 1b). Seeds were stored at −70°C directly after sampling and the same seed batches were therefore compared in parallel in the same experiments. Germination analyses was performed as described earlier (Leubner-Metzger *et al.*, 1998). In brief, 100–150 seeds were sown in 9-cm-diameter plastic petri dishes containing filter paper wetted with a nutrient solution and incubated at 24°C in continuous white light. Triplicate plates for each time point were used for scoring testa and endosperm rupture. The homozygous, monogenic *GlbGus* tobacco line carries a chimeric *Gus* reporter gene regulated by the β*GluIB*(*Glb*) promoter (Leubner-Metzger *et al.*, 1998). The chimeric sense-βGlu I and antisense-βGlu I-transgenes of the TKSG7 and TKAG4 lines, respectively, are regulated by the ABA-inducible *Cat1* promoter and TCIB1 (empty vector) lines are the proper controls (Leubner-Metzger, 2002, 2003; Leubner-Metzger and Meins, 2000, 2001). The homozygous, monogenic lines TCIB1-2, TKAG4-31, TKSG7-32, TKSG7-38, and TKSG7-43 were used for this study; TKSG7 results presented are mean values of the three TKSG7 lines.

Analyses of proteins

Procedures for extracting proteins, assays for βGlu, Chn, and Gus enzyme activities, immunoblot analyses, and protein determination were with at least three independent protein extracts for each time point and have been described (Leubner-Metzger, 2002; Leubner-Metzger *et al.*, 1998). In brief, βGlu and Chn activities were assayed radiometrically using [^{3}H]-laminarin and [^{3}H]-chitin as substrates, respectively. The polyclonal rabbit anti-tobacco βGlu I antibody used for immunoblot analyses detects the class I, class II, and class III isoforms of the enzyme (Beffa *et al.*, 1993; Leubner-Metzger, 2003). This antibody was also used for the *in situ* immunohistological localization of βGlu I antigens in air-dry tobacco seeds, which was performed using the same procedure as for the immunoblots. Histochemical Gus analyses with 5-bromo-4-chloro-3-indolyl glucuronide and fluorometric Gus enzyme activity assays with 4-methylumbelliferyl-β-D-glucuronide as substrates were as described (Jefferson *et al.*, 1987; Leubner-Metzger *et al.*, 1998). A polyclonal rabbit anti-*Escherichia coli*-Gus antibody (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) and purified Gus protein type IX-A from *E. coli* (Sigma, Munich, Germany) were used for the immunoblot analyses of Gus.

Analyses of mRNAs by semi-quantitative and TaqMan real-time RT-PCR

Total seed-RNA was extracted from approximately 200 air-dry seeds by using the 'RNeasy plant extraction kit' with the inclusion of a DNAse digestion step (Qiagen, Hilden, Germany). The RNA concentration was determined spectrometrically and by semi-quantitative RT-PCR amplification of rRNA. A PTC-100 thermocycler (MJ Research, Inc., Biozym Diagnostik GmbH, Oldendorf, Germany) was used for the 20-μl RT-PCR ('Qiagen OneStep RT-PCR Kit') and nested PCR ('Platinum Taq DNA Polymerase'; Invitrogen, Karlsruhe, Germany) reactions. One-tube RT-PCR reactions with 100, 50, 25, or 10 ng of total seed-RNA as template for the reverse transcription (30 min, 55°C) was followed by inactivation of reverse transcriptase, activation of *Taq* polymerase and template denaturation (15 min, 95°C), by 25 cycles of denaturation (0.5 min, 95°C), annealing (0.5 min, 68°C for βGlu I; 66°C for Gus), and extension (1 min, 72°C), by a final extension cycle (7 min, 72°C) and subsequent cooling. These one-tube RT-PCR reactions usually did not result in visible PCR fragment amplification from control seeds (except for weak rRNA signals from 100 ng RNA). Aliquots (0.2 μl) of these RT-PCR reactions were used as templates for nested PCR reactions: 2 min 95°C followed by 25 cycles of denaturation (0.5 min, 95°C), annealing (0.5 min, 68°C for βGlu I; 62°C for Gus), and extension (1 min, 72°C); final extension as above. Specific primers were designed for the co-amplification of partial cDNAs of the near-identical tobacco β*GluI-A* (*Gla*; M60403) and *-B* (*Glb*; M60402) transcripts (Leubner-Metzger, 2003) or the Gus transcripts (Jefferson *et al.*, 1987) and the tobacco 18S rRNA (AJ236016). Primer sequences (5′–3′) for βGlu I: GLF2 (AGCCACTCTCGGACACAACAATCC; RT primer), GLF4 (CTAGGATTACTACTTGTTGCCAGC), GLF6 (CATTGASATAGCAGGGGCTCA), GLF13 (GATCGCTCGAGGGCAGCATA); for Gus: GUSF1 (GATCGCGAAAACTGTGGAATTGATCA), GUSF2 (GCGAAGCGGGTAGATATCACACT; RT primer), GUSF3 (TCAGTTCGCCGATGCAGATAT), GUSF4 (GTGACGCACAGTTCATAGAGA); and for rRNA: RRNA2 (CGAGCTGATGACTCGCGCTTA; RT primer), RRNA5 (GAGTGGAGCCTGCGGCTTA). βGlu I-RT-PCR reactions contained GLF2, GLF4, RRNA2 and RRNA5; Gus-RT-PCR reactions contained GUSF2, GUSF1, RRNA2, and RRNA5. In the nested PCR reactions the primers GLF6 (spans the intron splice site sequences of *Gla* and *Glb*) and GLF13 yielded a 708-bp βGlu I, GUSF3 and GUSF4 a 620-bp Gus, and RRNA2 and RRNA5 a 443-bp rRNA PCR fragment. Co-amplification of βGlu I or Gus PCR fragments with rRNA PCR fragments from different amounts of RNA template was used to semi-quantitatively evaluate and visualize the βGlu I and Gus contents by gel electrophoresis (Figure 2). Twenty-five to 50 ng RNA templates were optimal and therefore used for single-transcript nested PCR reactions from the RT-PCR reactions with subsequent fluorometric quantification of the single-PCR fragment amounts ('PicoGreen dsDNA Quantitation Kit;' Molecular Probes, MoBiTec GmbH, Göttingen, Germany). Relative contents of βGlu I or Gus transcripts were calculated based on the roughly constant 18S rRNA amounts of the same RNA samples (Figures 2c and 3).

TaqMan real-time PCR assays were performed with first-strand cDNAs as templates that were obtained from total seed RNAs by using the TaqMan RT-kit with random hexamers (Applied Biosystems, Darmstadt, Germany). Primers and TaqMan MGB probes were designed for βGlu I (to detect *Gla* and *Glb* cDNAs) and Gus sequences using the Primer Express software (Applied Biosystems). The ABI PRISM 7000 sequence detection system was used for the real-time PCR assays and the eukaryotic 18S rRNA control kit (PDAR system with VIC as fluorescent dye; Applied Biosystems) served as internal standard. The primer βGlu1forward (TGCAATGCTGGATTCTGTGTATG) and βGlu1reverse (CGGACACAACAATCCCTACAGAT) generated a 71-bp βGlu I amplicon which was detected by the TaqMan βGlu1-MGB probe (6FAM-CCCTCGAGCGATCA-MGB). The primer Gus1forward (TGGCCTGGCAGGAGAAACT) and Gus1reverse (CGTATCCACGCCGTATTCG) generated a 59-bp Gus amplicon which was detected by the TaqMan Gus1-MGB probe (6FAM-TCAGCCGATTATCATCA-MGB). The concentrations of the TaqMan MGB probes and primers and the thermal cycler program

(initial 95°C for 15 min, 45 cycles with 95°C for 15 sec and 60°C for 1 min) were according to the manufacturer's instructions. cDNA obtained from 50 ng mRNA was utilized in the 23 μl assays with ABsolute QPCR mix (ABgene, Hamburg, Germany). Comparison of single-probe assays with βGlu I/18S rRNA- or Gus/18S rRNA-double probe assays showed that multiplex analyses are possible. Multiplex real-time PCR with triplicates of unknown cDNAs and with standard curves on every 96-well plate was performed and analyzed by the ABI PRISM software (Applied Biosystems). Relative contents of βGlu I or Gus transcripts were calculated based on the contents of 18S rRNA of the same RNA samples from the linear reaction phase. Mean values from two to three independent RNA samples are presented in Figure 3(e).

Seed moisture analyses and ^{1}H NMR 2D micro-imaging

Tobacco seed moisture contents were determined by weighing triplicate samples before and after heating for 3 h at 100°C (initial experiments showed that longer heating times give equal results). ^{1}H NMR 2D micro-imaging (Fountain *et al.*, 1998; Jenner *et al.*, 1988; Schneider *et al.*, 2003) experiments were carried out at the Fraunhofer-Institute of Biomedical Engineering (IBMT), St Ingbert, Germany (http://www.nmr.fraunhofer.de/). The seed sample inside a glass capillary was placed inside a superconducting magnet with a static field strength of 9.4 T. The images of the proton density were acquired on a Bruker Avance 400 NMR spectrometer (Bruker, Rheinstetten, Germany) equipped with standard micro-imaging set and using a standard three-dimensional spin-echo pulse sequence ($T_e = 1$ msec, $T_r = 0.8$ sec) with an isotropical spatial resolution of 0.03 mm. The total acquisition time was 110 min per image.

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