Planta (2002) 215: 959–968
DOI 10.1007/s00425-002-0837-y

ORIGINAL ARTICLE

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Seed after-ripening and over-expression of class I β-1,3-glucanase confer maternal effects on tobacco testa rupture and dormancy release

Received: 19 January 2002 / Accepted: 4 May 2002 / Published online: 25 July 2002

Abstract 'Coat-imposed' seed dormancy of many non-endospermic and endospermic species is released during after-ripening. After-ripening-mediated promotion of tobacco (*Nicotiana tabacum* L.) seed germination is mainly due to a promotion of testa rupture and a similar promotion of subsequent endosperm rupture. Treatment of after-ripened or freshly harvested mature seeds with abscisic acid (ABA) delays endosperm rupture and inhibits the induction of class I β-1,3-glucanase (βGlu I) in the micropylar endosperm, but does not affect the kinetics of testa rupture. After-ripening-mediated release of photodormancy is correlated with a decreased gibberellin (GA) requirement for testa rupture during dark-imbibition. Reciprocal crosses between wild-type tobacco and sense-βGlu I transformant lines showed that βGlu I over-expression in the seed covering layers can replace the promoting effect of after-ripening on testa rupture in light, but only if the mother plant is a sense-βGlu I line. This maternal effect supports the model of two sites for βGlu I action: (i) βGlu I contribution to the after-ripening-mediated release of dormancy in the dry seed state, which is manifested in the promotion and ABA-insensitivity of testa rupture during imbibition. (ii) ABA-sensitive expression of βGlu I in the micropylar endosperm, which contributes to endosperm rupture. The importance of GA-signaling and testa characteristics appear to be a common feature during the after-ripening-mediated release of coat-imposed dormancy in endospermic and non-endospermic seeds.

Keywords After-ripening · Gibberellin requirement · β-1,3-Glucanase · Maternal effect · *Nicotiana* (seed dormancy) · Testa rupture

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Abbreviations ABA: abscisic acid · DAP: days after pollination · GA: gibberellin · βGlu I: class I β-1,3-glucanase · WT: wild type

Introduction

In many plant species with coat-imposed dormancy the seed envelope tissues impose a physical constraint to radicle protrusion (Kelly et al. 1992; Bewley 1997b; Li and Foley 1997; Welbaum et al. 1998). Dormancy can be released during after-ripening, i.e. a period of dry storage of mature seeds. Little is known about the molecular basis for the transition from the dormant to the non-dormant state during after-ripening. Gibberellin (GA) requirement for seed germination and dormancy release is determined by the seed's covering structures and abscisic acid (ABA) content (e.g. Hilhorst 1995; Debeaujon and Koornneef 2000; Grappin et al. 2000). In *Arabidopsis thaliana*, seed dormancy appears to be a quantitative trait, its release during after-ripening results in the promotion of seed germination, and altered testa (seed coat) characteristics of testa mutants affects seed after-ripening, coat-imposed dormancy and storability (e.g. Debeaujon and Koornneef 2000; Debeaujon et al. 2000). Thus, although the seeds of *A. thaliana* also contain a single cell layer of endosperm, the testa appears to be more important for the coat-imposed dormancy of this species. While in non-endospermic seeds the testa characteristics are responsible for the degree of coat-imposed dormancy, in endospermic seeds, such as from *Nicotiana tabacum*, the contributions of both the testa and the endosperm layers have to be considered (e.g. Arcila and Mohapatra 1983; Watkins and Cantliffe 1983; Hilhorst 1995). In the mature seed of tobacco, the embryo is surrounded by three to five layers of rather thick-walled endosperm cells. The periphery of the endosperm is pressed against the thin testa, which consists of an outer layer of cutinized and lignified dead cells and a living inner parenchyma layer (Avery 1933; Matzke et al. 1993). The existence of a 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 suggested by gene-promoter studies and by genetic ablation (Czakó et al. 1992; Matzke et al. 1993; Fobert et al. 1994). Rupture of the testa precedes rupture of the micropylar endosperm and microscopic studies show that the endospermic hole, formed at the micropylar end of germinating tobacco seeds, results from endosperm 'dissolution' rather than from the 'pushing' force of the extending radicle (Arcila and Mohapatra 1983). In members of the Solanaceae (e.g. tomato, tobacco, *Datura*) weakening of the micropylar endosperm cell walls surrounding the radicle tip is likely to be achieved by the action of several phytochrome- and GA-regulated cell-wall hydrolases (e.g. Black 1996; Bewley 1997a; Welbaum et al. 1998).

Two sites of class I β-1,3-glucanase (βGlu I) action on tobacco seed germination, dormancy and after-ripening became evident from sense and antisense experiments (Leubner-Metzger and Meins 1999, 2000, 2001). Tobacco seed germination commences with the uptake of water by imbibition, followed by embryo expansion growth, testa rupture, and finally endosperm rupture, which is visible by the emergence of the radicle, i.e. is the completion of germination. βGlu I is transcriptionally induced in the micropylar endosperm after testa rupture, but just prior to endosperm rupture. ABA delays endosperm rupture of imbibed seeds and inhibits βGlu I gene expression. Sense transformation with a chimeric βGlu I transgene regulated by the ABA-inducible *Cat1* promoter causes over-expression of βGlu I in imbibed seeds and promotes endosperm rupture of ABA-treated after-ripened seeds (Leubner-Metzger and Meins 2000). These results provide strong evidence that βGlu I contributes to ABA-controlled endosperm rupture, i.e. the completion of germination.

Moreover, sense and antisense transformation also provide indirect evidence for a second, novel site of βGlu I action on the release of dormancy during seed after-ripening (Leubner-Metzger and Meins 2000, 2001). Freshly harvested, mature tobacco seeds are photodormant, i.e. they do not germinate during imbibition in darkness. Photodormancy can be released during after-ripening and this release is inhibited in antisense-βGlu I seeds, but is not affected in sense-βGlu I seeds. Treatment with GA can replace after-ripening in releasing photodormancy, but the requirements of GA for the release of photodormancy during dark-imbibition are not known. After-ripened seeds from antisense-βGlu I transformant lines unexpectedly show a delayed onset of testa rupture, which subsequently causes a similar delay in endosperm rupture. Neither sense-βGlu I transformation nor ABA-treatment affect the timing of testa rupture during imbibition of after-ripened seeds. In contrast to after-ripened seeds, germination in the light is not affected in freshly harvested, mature antisense-βGlu I seeds, but is promoted in sense-βGlu I seeds. Sense-βGlu I transformation therefore replaces the promoting effects of after-ripening on the timing of germination in the light. The relative contributions of the testa and the endosperm layers during the promotion of seed germination in the light by after-ripening and βGlu I over-expression have not been investigated.

In the present study, reciprocal crosses between wild-type (WT) tobacco and sense-βGlu I transformant lines show that the after-ripening-mediated promotion of seed germination is mainly manifested by a promoted onset of testa rupture, which subsequently causes a similar promotion of endosperm rupture. βGlu I over-expression in the maternal seed tissues can replace the promoting effect of after-ripening on testa and endosperm rupture. Decreased GA-requirement seems to be important for the release of photodormancy during after-ripening. Endospermic and non-endospermic seeds appear to share GA-requirements and testa characteristics as common factors in the after-ripening-mediated release of coat-imposed dormancy.

Materials and methods

Plant materials and germination conditions

Mature seeds of WT or transformed *Nicotiana tabacum* L. cv. Havana 425 (Agricultural Experimental Station, University of Wisconsin, Madison, Wis., USA) were used either at approximately 40 days after pollination (DAP; 'fresh' seed) or after about 1 year of dry storage at room temperature (after-ripened seed), as indicated. Germination kinetics of fresh and after-ripened seeds were determined in parallel in the same experiments. The fresh seeds were from the same batches as the after-ripened seeds and the fresh seeds had been stored at –70 °C for about 1 year. Fresh seeds from the next-year's harvest from plants grown in the greenhouse in the same season yielded the same results as seeds stored at –70 °C (data not shown). Sense-βGlu I transformed lines (TKSG7) carry a chimeric tobacco βGlu I gene regulated by the castor bean *Cat1* gene promoter, which confers over-expression and ABA-inducibility in transgenic tobacco seeds (Suzuki et al. 1995; Leubner-Metzger and Meins 2000). TCIB1 transformants obtained with the empty-vector plasmid pCIB200 were used as a control. Reciprocal genetic crosses were performed between WT tobacco and homozygous, monogenic TKSG7 or TCIB1 plants. Self-crosses on the same plants were used as a control. Germination analyses were 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 (control medium) supplemented as indicated with 10 µM *cis*-(±)-ABA (Sigma), and 0.01, 0.1, 1, or 4 µM gibberellin A_4 (GA_4; Sigma). Petri dishes were incubated at 25 °C in continuous white light (3,000 lux; Philips 'TL'D 35 W/33 lamps) or in darkness. After scoring for testa and endosperm rupture, seeds were stored at –70 °C for subsequent analyses.

Analysis of proteins and in situ immunohistological βGlu I detection

Procedures for extracting proteins, assays for enzyme activity, immunoblot analysis, and protein determination have been described previously (Leubner-Metzger et al. 1995). In brief, βGlu activity was assayed radiometrically using [^{3}H]laminarin as the substrate. The polyclonal rabbit anti-tobacco βGlu I antibody used for immunoblot analysis detects the class-I, class-II and class-III isoforms of the enzyme (Leubner-Metzger and Meins 2000). For the in situ immunohistological detection of βGlu I by fluorescence microscopy the outer testa tissue of dry seeds was removed to

decrease the amount of background autofluorescence. The seed material was then fixed for 1 h at room temperature and subsequently overnight at 4 °C in 3.7% (w/v) paraformaldehyde dissolved in post-fixation buffer (50 mM potassium phosphate, 5 mM EGTA, pH 6.8). After several washings with post-fixation buffer, gradual infiltration was performed [30 min 30% (v/v), 30 min 60%, 1 h 100%] with a mixture of 2 vol. 20% (w/v) sucrose and 1 vol. absolute Tissue Tek optimal cutting temperature compound (Ted Pella, Redding, Calif., USA; Itaya et al. 1998). Seeds were embedded in absolute Tissue Tek, frozen in a cryostat, 14-µm cryosections were obtained, attached to microscope slides coated with 1% (w/v) gelatin and 0.1% (w/v) chrome alum, and incubated on a warming plate at 40 °C for at least 2 h before further processing. The slides were incubated in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 3% (w/v) bovine serum albumin (BSA) and 1% (v/v) Nonidet P-40 (Sigma) for 30 min at room temperature. Afterwards, the sections were incubated for 2 h at 37 °C with 20 µg/ml rabbit anti-tobacco βGlu I antibody in binding solution [PBS, 1% (w/v) BSA, 0.05% (v/v) Triton X-100]. Following PBS washes for 10–15 min, the sections were incubated for 1.5 h at 37 °C with an ALEXA-Fluor488-conjugated goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) at 1:50 dilution in binding solution. The sections were washed with PBS, embedded in mounting medium (Vector Laboratories, Burlingame, Calif., USA), covered with a coverslip and placed in the dark for at least 1 h prior to examination with either a Leitz DMRBE fluorescence microscope (Leica, Wetzlar, Germany) or a Fluoview confocal microscope (Olympus Optical Co., Hamburg, Germany). Images were recorded using the SPOT RT Software (Diagnostic Instruments) with user-defined constant-exposure-time profiles for the fluorescence in the green (ALEXA-Fluor488) or the red (autofluorescence) channel.

Results

Sense-βGlu I transformation and seed after-ripening promote testa rupture

Time course analyses presented in Fig. 1 show that the after-ripening-mediated promotion of germination of WT or vector-control (TCIB1-2) seeds (Leubner-Metzger and Meins 2000) is due to a promotion of testa and endosperm rupture. Fresh seeds, i.e. mature seeds harvested 40 DAP, and after-ripened seeds, i.e. seeds stored

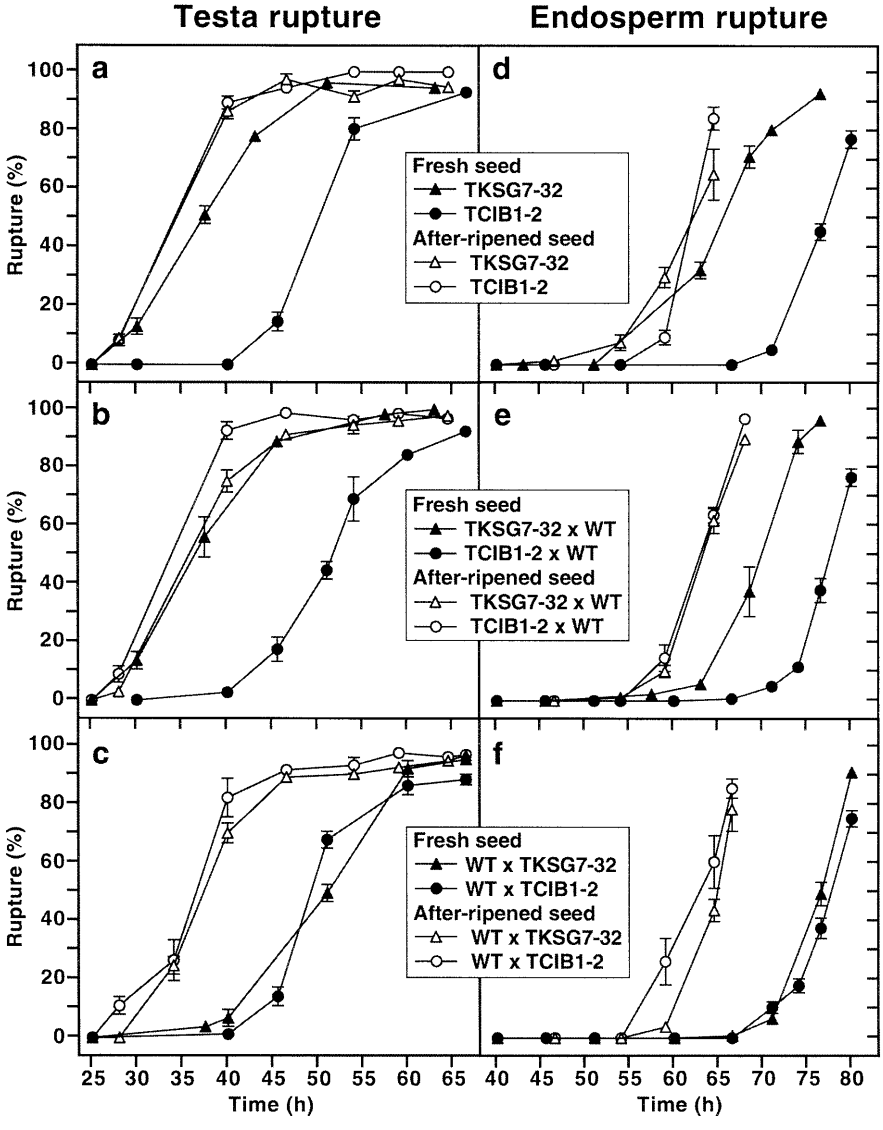

Fig. 1. The effect of after-ripening and sense-βGlu I transformation on testa (**a–c**) and endosperm (**d–f**) rupture of germinating tobacco (*Nicotiana tabacum*) seeds. Fresh (*closed symbols*) and after-ripened (*open symbols*) progeny seeds from reciprocal crosses of a homozygous monogenic sense-βGlu I (TKSG7-32) or an empty-vector (TCIB1-2) line with WT were compared. The incidence of testa and endosperm rupture expressed as a percentage was scored over time from the start of imbibition in continuous light in control medium. Mean values ± SE of four populations each with 100-150 seeds from independent capsules

dry at room temperature for 1 year, were imbibed in the light on filter paper moistened with control medium. Testa rupture and subsequent endosperm rupture of after-ripened control seeds (WT, data not shown; and TCIB1-2, Fig. 1a, d) was about 15 h earlier than for fresh control seeds. Thus, after-ripening promoted testa rupture and subsequent endosperm rupture of control seeds. In contrast to control seeds, the time required for 50% testa rupture and subsequent endosperm rupture of fresh sense-βGlu I transformant seeds (TKSG7-32) was almost reduced to the time that control seeds required in the after-ripened state, and there was no appreciable further promotion during after-ripening (Fig. 1a, d). Thus, sense-βGlu I transformation can substitute for the after-ripening effect on seed germination by promoting testa rupture. This was also obvious in TKSG7 seeds of other independent lines (Table 1): the time periods for 50% testa rupture did not differ between after-ripened control seeds and TKSG7 seeds from independent transformant lines, but they were advanced by about 10 h in fresh TKSG7 seeds compared to fresh control seeds. Thus, not only can the promoting effect of after-ripening on seed germination be replaced by sense-βGlu I transformation, the effect of both factors can already be detected at the stage of testa rupture.

Maternal effect of sense-βGlu I transformation on after-ripening-mediated promotion of testa rupture

Seeds obtained from reciprocal crosses between WT and homozygous, single-locus TKSG7 lines were used to investigate maternal effects of sense-βGlu I transformation. The testa is entirely of maternal origin and therefore carries the βGlu I-transgene (two copies per diploid genome) only if a TKSG7 plant is used as the female parent. The triploid endosperm tissue carries either one (male parent TKSG7) or two (female parent TKSG7) sense-βGlu I transgene copies. Testa and endosperm rupture of fresh progeny seeds was only promoted if the female parent in the reciprocal cross was a TKSG7 line (TKSG7×WT) (Fig. 1b; Table 1). When the male parent was the sense-βGlu I transformant line (WT×TKSG7), testa rupture of fresh progeny seed was not promoted (Fig. 1c; Table 1), as was the case for fresh seed from control crosses with TCIB1-2 either as female (TCIB1-2×WT) or male (WT×TCIB1-2) parent. As for TKSG7× (self-crosses), testa rupture of fresh seeds from TKSG7×WT crosses was promoted by about 10 h compared to fresh seeds from WT×TKSG7 and from control crosses. While treatment of imbibed tobacco seeds with ABA delayed endosperm rupture, it did not affect the kinetics of testa rupture of after-ripened seeds (Leubner-Metzger and Meins 2000) or of fresh-progeny seeds from any of the crosses (Table 1).

βGlu I is not expressed during the early phase of imbibition of WT seeds prior to testa rupture (Leubner-Metzger et al. 1995). In agreement with this, only background βGlu activities were measured during the early phase of imbibition of seeds from crosses between TCIB1-2 and WT (Table 1). βGlu activities had already increased in dry seeds from crosses involving TKSG7 lines, and remained roughly constant during the early phase of imbibition as measured at the time of 50% testa rupture (Table 1). βGlu activities differed among the various types of crosses involving TKSG7 in a transgene-dose-dependent manner with respect to the triploid endosperm and compared to the background levels measured in TCIB1-2, i.e. TKSG7× (10- to 20- fold) > TKSG7×WT (5- to 15-fold) > WT×TKSG7 (2- to 5-fold). Immunoblot analyses showed that the different βGlu activities are due to equally different contents of the 33-kDa βGlu I isoforms (data not shown). The onset of further βGlu I accumulation of imbibed seeds was after testa rupture, but prior to endosperm rupture and transgene-dose-dependent βGlu I activities with respect to the triploid endosperm were detected at the time of 50% endosperm rupture (Table 1). ABA-treatment of fresh or after-ripened sense-βGlu I seeds did not cause higher βGlu I activities prior to testa rupture, but ABA further enhanced the accumulation of βGlu I prior to endosperm rupture, as had been demonstrated earlier for after-ripened seeds (Leubner-Metzger and Meins 2000). In situ immunohistological analyses of dry TKSG7 seed sections with fluorescence microscopy shown in Fig. 2 demonstrated strong accumulation of βGlu I antigen within the entire endosperm, the endosperm–embryo border, but not within the embryo tissue (green fluorescence in Fig. 2a, d). Green fluorescence was also detected within the thin inner layer of the testa (Fig. 2a, d, f, h, j), but a high degree of autofluorescence of this layer was also obvious in the red channel (Fig. 2b, e), making it difficult to distinguish between background and βGlu I-specific fluorescence within this tissue. Autofluorescence of the inner layer of the testa was evident in sections of dry seeds without antibody treatment and βGlu I-specific fluorescence was not detected in TCIB1 seed sections (Fig. 2f–j). Although testa rupture was not promoted in fresh seeds from WT×TKSG7 crosses, their βGlu I activities at 50% testa rupture were slightly increased compared with fresh control seeds (Table 1). Taken together, these results demonstrate that high expression of βGlu I in the endosperm or expression of βGlu I in the entirely maternal inner testa layer can replace the effect of after-ripening and causes an anticipated promotion of testa rupture.

Effects of after-ripening on photodormancy release and GA-sensitivity

Germination in darkness of after-ripened, photodormant tobacco seeds is blocked at a step before testa rupture, but addition of GA releases photodormancy and induces testa rupture and endosperm rupture, i.e. non-photodormancy and dark-germination (Leubner-Metzger et al. 1996). Our earlier results (Leubner-Metzger and Meins 2000) demonstrated that after-ripening contributes to

Table 1. Effect of tobacco (*Nicotiana tabacum*) seed after-ripening and sense βGlu I transformation on testa rupture, endosperm rupture and βGlu enzyme activity of reciprocal crosses

Crosses (lines)[a]	50% Testa rupture (continuous light)					50% Endosperm rupture (continuous light)				
	Time (h)[b]			βGlu (pkat/seed)[d]		Time (h)[b]			βGlu (pkat/seed)[d]	
	'Fresh'	After-ripened	Difference[f]	'Fresh'	After-ripened	'Fresh'	After-ripened	Difference[f]	'Fresh'	After-ripened
TKSG7-32×	37.3 (37.5)[c]	34.5		3.3	1.5	65.6	62.0		7.4	5.2
TKSG7-38×	42.0	34.3		2.7	1.7	68.9	62.7		7.6	4.8
TKSG7-43×	–[e]	34.7		–	2.0	–	61.4		–	5.3
TKSG7× (mean)	**39.6**	**34.5**	**5.1**	**3.0**	**1.7**	**67.2**	**62.0**	**5.2**	**7.5**	**5.1**
TKSG7-32×WT	36.5 (36.0)	36.0		1.3	0.8	69.9	63.3		4.2	2.4
TKSG7-38×WT	43.4 (40.0)	34.3		2.2	1.6	68.3	62.4		5.6	2.8
TKSG7-43×WT	–	35.2		–	1.2	–	63.8		–	5.1
TKSG7×WT (mean)	**39.9**	**35.2**	**4.8**	**1.7**	**1.2**	**69.1**	**63.2**	**5.9**	**4.9**	**3.5**
WT×TKSG7-32	51.4 (50.0)	37.0		0.3	0.2	76.6	64.8		2.9	2.2
WT×TKSG7-38	50.2 (49.5)	34.8		0.8	0.2	77.9	63.0		4.1	2.5
WT×TKSG7-43	–	35.4		–	0.8	–	63.5		–	3.2
WT×TKSG7 (mean)	**50.8**	**35.7**	**15.0**	**0.6**	**0.4**	**77.3**	**63.8**	**13.5**	**3.5**	**2.6**
TCIB1-2×	50.2 (47.0)	34.3		0.2	0.2	76.9	62.0		2.6	2.5
TCIB1-2×WT	51.8	34.2		–	0.1	77.6	62.9		2.6	2.3
WT×TCIB1-2	49.5 (49.0)	36.3		0.2	0.2	77.7	62.8		2.4	2.3
TCIB1-2/WT (mean)	**50.5**	**34.9**	**15.6**	**0.2**	**0.2**	**77.4**	**62.6**	**14.8**	**2.5**	**2.4**

[a]Reciprocal crosses of independent, monogenic vector-control (TCIB1) or sense-βGlu I (TKSG7) tobacco lines with WT; female parent in cross is on the left and the male parent is not shown in self-crosses; ''(mean)'' designates mean values of presented data obtained for the independent lines

[b]Time to reach 50% testa or endosperm rupture, respectively, determined from the time-course of seeds imbibed in continuous light in control medium; 100–150 'fresh' (directly after harvest) or after-ripened (about 1 year of dry storage) seeds from four independent capsules were scored at each time-point; time-course data for TKSG7-32 and relevant statistics are presented in Fig. 1

[c]Time to reach 50% testa rupture in the presence of 10 μM ABA is shown in parenthesis

[d]βGlu enzyme activities at the time of 50% testa or endosperm rupture, respectively, determined in protein extracts from seed samples described under [b]

[e]Not determined

[f]Time differences of 'fresh' and after ripened seeds to reach 50% testa or endosperm rupture, respectively

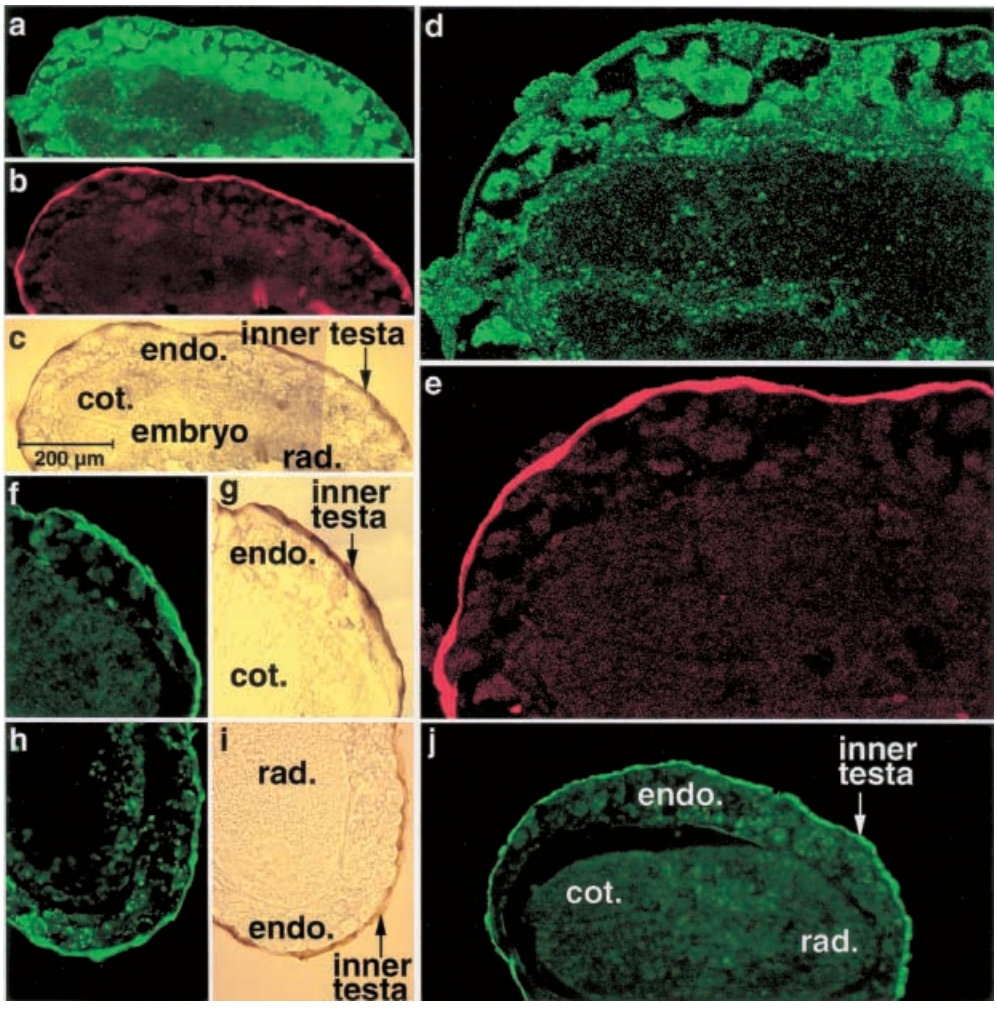

Fig. 2a–j. In situ immunohistological localization of βGlu I in mature, dry sense-βGlu I tobacco seeds. Cryosections (14 µm) of TKSG7 seeds (**a–e**) were used to detect βGlu I as green fluorescence (**a, d**) with the rabbit anti-tobacco βGlu I antibody and fluorescence-labeled secondary antibody against rabbit IgG (ALEXA-Fluor488); **d** and **e** were obtained by confocal microscopy. Red autofluorescence of TKSG7 seeds (**b, e**), green fluorescence of TCIB1 seeds (**f–i**) and green autofluorescence of WT seeds (**j**) served as controls. Note that the outer testa layer of the seed was removed before sectioning to decrease the amount of autofluorescence, and that the thin inner testa exhibits autofluorescence. *endo*. Endosperm, *cot*. cotyledons, *rad*. radicle

photodormancy release of Havana 425 tobacco. This effect varies greatly for different batches, but was not affected by sense-βGlu I transformation. In agreement with this, no detectable effects on either photodormancy of fresh seed or after-ripening-mediated release of photodormancy were obvious in seed batches of reciprocal crosses of TKSG7 or TCIB1 lines with WT (data not shown). Results presented in Fig. 3 show that GA_4 treatment released photodormancy of fresh and after-ripened seeds, which resulted in the ability to germinate during dark-imbibition. Approximately 0.73 µM GA_4 was required to achieve 50% dark-germination of fresh seed batches, which are almost 100% photodormant in medium without hormones. The after-ripened seed batches were grouped into five equally sized classes depending on their degree of photodormancy in control medium. As with the fresh seeds, the photodormant after-ripened seed batches (class A; Fig. 3) showed a dose-dependence with about 0.73 µM GA_4 required for 50% dark-germination. The other four classes were characterized by a higher percentage of dark-germination in medium without hormones (class B 5–10%, C 10–25%, D 25–50%, E 50–100% non-photodormancy; Fig. 3) and correlated with lower GA_4 concentrations sufficient to obtain 50% dark-germination (class B 0.2, C 0.1, D 0.02 µM GA_4; Fig. 3). However, if only the photodormant seed population of classes B to E is considered, an approximately equal concentration of 0.2–0.4 µM GA_4 is sufficient to release photodormancy and induce 50% dark-germination of the photodormant seeds of classes B to E. Thus, photodormancy release during after-ripening is associated with a decrease in GA dependence of dark-germination.

Discussion

In non-endospermic dicot species coat-imposed dormancy is determined by the testa characteristics (e.g. Duran and Retamal 1989; Debeaujon and Koornneef 2000; Debeaujon et al. 2000), whereas in endospermic species the contribution of both covering layers, the testa and the endosperm, has to be considered. Testa and endosperm rupture are distinct and temporally separate events during the germination of tobacco (Arcila and Mohapatra 1983). Sense and antisense transformation of tobacco revealed at least two sites of βGlu I action on

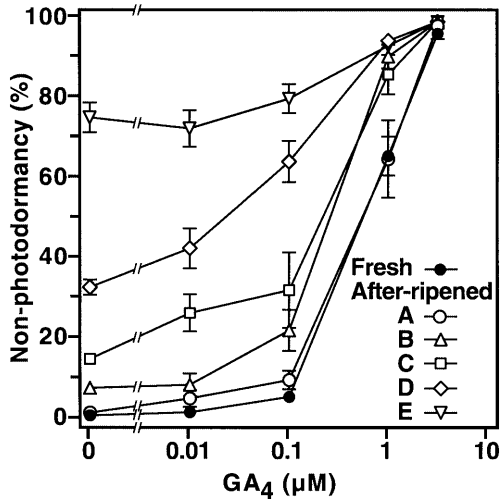

Fig. 3. Gibberellin-requirement of photodormancy release of fresh and after-ripened Havana-425 tobacco seeds imbibed in darkness. Fresh seed batches (*closed symbols*) are completely photodormant in control medium, i.e. no germination occurs during dark-imbibition. After-ripened seed batches (*open symbols*) were grouped into five equal-sized classes depending on their degree of non-photodormancy in control medium: *class A* 0–5 %, *B* 5–10%, *C* 10–25%, *D* 25–50%, *E* 50–100% germination during dark-imbibition, respectively. The percentage germination of approximately 100 seeds was scored after 2 weeks of imbibition in darkness in medium without (control) or with 0.01, 0.1, 1, or 4 μM GA_4 added. The GA_4 concentrations for 50% photodormancy release were 0.73 μM for fresh seeds, and 0.73, 0.2, 0.1, 0.02 μM GA_4 for after-ripened seed batches of classes A–D, respectively. Mean values ± SE of 11 independent WT or TCIB1 seed batches per class

seed germination, after-ripening and coat-imposed dormancy (Leubner-Metzger and Meins 2000, 2001). In agreement with this, the results of the present study demonstrate that over-expression of βGlu I in the covering layers of dry seeds of sense-βGlu I transformant lines (TKSG7) promotes testa rupture in fresh seeds and therefore replaces the effect of after-ripening on germination in the light. In our earlier study (Leubner-Metzger and Meins 2000) we showed that sense transformation with a chimeric ABA-inducible βGlu I transgene causes over-expression of βGlu I in TKSG7 seeds and promotes endosperm rupture of ABA-treated after-ripened seeds. This study provided direct evidence that βGlu I contributes to endosperm rupture. This study also showed that light-imbibed after-ripened TKSG7 and after-ripened control seeds do not differ in their kinetics of testa rupture in control medium (without ABA added) or in medium with ABA added. The effects of sense-βGlu I transformation and ABA on testa rupture of fresh seeds were not studied. The fresh tobacco seeds used in the earlier studies and in the present study were sampled approximately 40 DAP, which is after maturation and establishment of primary dormancy are completed (Yamaguchi-Shinozaki et al. 1990; Jiang et al. 1996; Phillips et al. 1997). Reciprocal genetic crosses and the advantage that testa rupture and endosperm rupture are temporally separated events were utilized in the present study to assign βGlu I effects to either of the two covering layers.

The most important finding is that maternal βGlu I appears to contribute to the after-ripening-mediated promotion of tobacco seed germination manifested by a promotion of testa rupture. The after-ripening-mediated gain in germination speed of light-imbibed WT and TCIB1 seeds was mainly due to an earlier onset of testa rupture, which subsequently caused a similarly earlier onset of endosperm rupture. In mature progeny seed from reciprocal crosses between homozygous, monogenic sense-βGlu I TKSG7 lines with WT, the tissues are distinct in transgene dosage. The equal contributions of the maternal and paternal parent genomes result in an embryo genome that is always hemizygous for the transgene. In contrast, two-thirds of the triploid endosperm genome is derived from the mother plant, and the triploid endosperm genomes of the crosses TKSG7× (self-cross), TKSG7×WT, and WT×TKSG7 therefore have distinct transgene dosages of 3, 2, and 1, respectively. The testa, which develops from the integuments of the ovule, is entirely of maternal origin. In mature tobacco seeds it consists of an outer layer of dead cells and a living inner parenchyma layer (Avery 1933; Matzke et al. 1993). The promotion of testa rupture was obvious in fresh sense-βGlu I seeds, but only if the mother plant was TKSG7. Only seeds of TKSG7× and TKSG7×WT crosses exhibited an earlier onset of testa rupture in the fresh state, whereas the testa-rupture kinetics of fresh seeds of the reciprocal cross WT×TKSG7 was as for fresh control seeds. The fresh seeds with promoted testa rupture (TKSG7× and TKSG7×WT) are characterized by TKSG7-derived testas (two βGlu I-transgene copies) and a substantially increased βGlu I content in the dry state and during early imbibition (5- to 20-fold). In contrast, the fresh WT×TKSG7 seeds exhibit WT-like kinetics of testa rupture, have WT-derived testas (no βGlu I transgene), and have only 2- to 5-fold βGlu I contents. The molecular mechanism underlying the promotion of testa rupture by after-ripening and its anticipated promotion by sense-βGlu I transformation is not known. In agreement with reporter-gene studies (Suzuki et al. 1995), in situ immunohistological studies presented here showed that over-expression caused high βGlu I contents in dry TKSG7 seeds, which were spatially associated with the thick endosperm layer and were absent from the embryo. βGlu enzyme activity (Table 1) and βGlu I immunoblot analyses (data not shown) of seeds from reciprocal crosses showed transgene-dosage-dependent βGlu I contents that are consistent with endosperm-specific expression during seed maturation and/or after-ripening. Due to the high autofluorescence of the testa tissue, no conclusion can be drawn regarding the inner parenchyma layer of the testa as an additional tissue for βGlu I expression in TKSG7 seeds. Since WT×TKSG7 seeds have WT-derived testas, the 2- to 5-fold increase in βGlu I contents present during testa rupture must originate from expression in the endosperm. This

suggests that either this amount of βGlu I in the endosperm is not sufficient to cause promoted testa rupture or that βGlu I expression in the inner testa layer is needed. The βGlu I contents of TKSG7×WT seeds were sufficient for promoting testa rupture in fresh seed; further βGlu I accumulation in TKSG7× seeds did not result in a stronger effect. Although an effect on testa rupture of high-level βGlu I expression in the endosperm is also possible, it seems more likely that βGlu I expression in the maternal TKSG7-derived testa tissue is necessary and sufficient for replacing the promoting effects of after-ripening on the onset of testa rupture. This interpretation is also in agreement with the finding that antisense-βGlu I transformation prevents the promotion of testa rupture during after-ripening (Leubner-Metzger and Meins 2001). Thus, the maternal effect on the after-ripening-mediated release of tobacco coat-imposed dormancy seems to involve βGlu I action with testa rupture as the target. Possible relations between testa characteristics, coat-imposed dormancy and its release during after-ripening are also evident from other species (e.g. Duran and Retamal 1989; Kelly et al. 1992; Welbaum et al. 1998; Debeaujon and Koornneef 2000; Debeaujon et al. 2000).

That the effects of βGlu I on testa rupture during after-ripening and on endosperm rupture during imbibition are distinct events is further supported by the distinct effects of ABA. Treatment of fresh (this study) and after-ripened (Leubner-Metzger et al. 1995) control seeds with ABA specifically delays endosperm rupture and inhibits the induction of βGlu I, but it does not affect the kinetics of testa rupture. The kinetics of testa rupture of fresh (this study) and after-ripened (Leubner-Metzger and Meins 2000) TKSG7 seeds also are not affected by ABA, but ABA causes βGlu I over-expression and promotes endosperm rupture. Similarly, distinct ABA effects seem to be associated with two phases of tomato seed germination (e.g. Bewley 1997a; Nonogaki et al. 2000; Toorop et al. 2000; Wu et al. 2000). The first phase is characterized by major endosperm weakening, is not inhibited by ABA, and is associated with endosperm cap-specific and ABA-independent expression of endo-β-mannanase, but not with βGlu I expression. ABA clearly inhibits the completion of tomato seed germination and the second phase just prior to the completion of germination is ABA-dependent. It has been proposed that tomato endosperm cap weakening is a biphasic process and that the second ABA-dependent phase also includes a second weakening step (Toorop et al. 2000). The ABA-controlled final step is critical since it leads to radicle emergence and is associated with the ABA-sensitive expression of tomato βGlu I in the micropylar endosperm, which could contribute to this key step (Wu et al. 2000). The testa accounts for approximately 20% of the mechanical resistance during the early phase of tomato seed imbibition (see Fig. 4 of Groot and Karssen 1987), and the mechanical resistance of the testa appears to decrease during the late phase just prior to radicle protrusion. In agreement with this, a much thinner testa (one cell layer) is correlated with faster seed germination of the ABA-deficient *sit*^{w} mutant compared to WT (four to five cell layers) tomato (Hilhorst and Downie 1995). Germination of intact *sit*^{w} seeds occurred at lower external osmotic potentials, and removal of the micropylar testa did not affect seed germination of the *sit*^{w} mutant, but significantly promoted WT seed germination. These authors conclude that, although the testa resistance is smaller than the endosperm resistance, it is the micropylar testa that finally controls the completion of tomato seed germination, i.e. radicle emergence (Hilhorst and Downie 1995). Interestingly, faster seed germination of the ABA-deficient *sit*^{w} mutant is also associated with substantially increased βGlu I expression than in WT tomato (data not shown). Although tomato and tobacco might differ in the processes of testa and endosperm rupture, in both species both covering layers contribute to coat-imposed dormancy. This is further support for the view that βGlu I action on tobacco seed germination and coat-imposed dormancy has at least two target sites: (i) testa rupture, which is not affected by ABA during imbibition, but seems to be affected by βGlu I during after-ripening, and (ii) endosperm rupture, which is inhibited by ABA during imbibition, and depends on the contribution of ABA-sensitive expression of βGlu I during germination.

According to the revised hormone-balance hypothesis for seed dormancy proposed by Karssen and Laçka (1986), ABA and GA act at different times and sites during ''seed life''. ABA induces primary dormancy during seed maturation and GA plays a key role in the promotion of germination. A decline in ABA content, decreased sensitivity to ABA and increased sensitivity to GA are involved in the after-ripening-mediated transition from the dormant to the non-dormant state of many species (Hilhorst 1995; Li and Foley 1997; Debeaujon and Koornneef 2000; Grappin et al. 2000). Work with *Nicotiana plumbaginifolia* seeds showed such an after-ripening-mediated decline in ABA content and decrease in ABA sensitivity, and in addition de novo synthesis of ABA only in fresh (dormant) seed during imbibition (Grappin et al. 2000). Freshly harvested tobacco seeds are photodormant, i.e. neither testa nor endosperm rupture occurs during dark-imbibition, and after-ripening contributes to the release of photodormancy (Kasperbauer 1968; Leubner-Metzger and Meins 2000). Results obtained in the present study demonstrate that the GA requirements for photodormancy release of fresh seed batches and completely photodormant after-ripened seed batches are equal. In contrast, after-ripened seed batches with higher degrees of non-photodormancy exhibited decreased GA requirements for inducing dark-germination, and appeared to contain two seed populations: (i) non-photodormant seeds which do not require GA treatment for dark-germination; and (ii) photodormant seeds which require a similar concentration of exogenous GA for dark-germination. Thus, after-ripening-mediated release of photodormancy and the

ability to germinate in darkness is correlated with decreased GA dependence, which could be due to increased GA-sensitivity and/or increased endogenous GA. Germination of photodormant tobacco seeds is blocked at a step prior to testa rupture and genetic and physiological experiments suggest it to be mainly under maternal control (e.g. Honing 1930; Kincaid 1935; Kasperbauer 1968). Testa rupture due to after-ripening- or GA-mediated photodormancy release, as well as subsequent stimulation of ABA degradation, inhibition of ABA biosynthesis, and βGlu I accumulation in the micropylar endosperm of *Nicotiana* seeds seem to be GA-promoted processes (Leubner-Metzger et al. 1996; Grappin et al. 2000; Leubner-Metzger 2001). Results with *Arabidopsis* testa mutants showed that reduced coat-imposed dormancy is correlated with increased sensitivity to GA and suggest that GA requirement for seed germination is determined by the testa constraints and by ABA (Debeaujon and Koornneef 2000).

In conclusion, the importance of GA-signaling and testa characteristics appears to be a common feature in the after-ripening-mediated release of coat-imposed dormancy of endospermic and non-endospermic seeds.

Acknowledgements I thank Katrin Hermann and Maren Sachtlebe for expert technical assistance; Peter Schopfer, Tim Kunkel and Birgit Eiter from the Institut für Biologie II (Albert-Ludwigs-Universität, Freiburg i. Br., Germany) for critical reading of the manuscript; Sjoerd van Eeden, Monique Thomas, Andreas Gisel and Frederick Meins from the Friedrich Miescher Institute (Basel, Switzerland) for their kind support with plant raising, crosses, and confocal microscopy. This research was supported by a grant from the Deutsche Forschungsgemeinschaft (LE 720/3), which is gratefully acknowledged.

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