Seed Science Research (2003) **13**, 139–153 DOI: 10.1079/SSR2003132

Distinct expression patterns of β-1,3-glucanases and chitinases during the germination of Solanaceous seeds

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Abstract

The expression patterns of β-1,3-glucanases (βGlu) and chitinases (Chn) were investigated during the seed germination of members of the *Cestroideae* (three *Nicotiana* species, *Petunia hybrida*) and the *Solanoideae* (*Capsicum annuum*, *Physalis peruviana*) subgroups of Solanaceous species. Rupture of the micropylar testa (seed coat) and rupture of the micropylar endosperm, i.e. radicle emergence, were distinct and temporally separate events during the germination of *Cestroideae*-type seeds. βGlu accumulation in imbibed *Cestroideae*-type seeds, occurring after testa rupture but prior to endosperm rupture, was inhibited by abscisic acid (ABA) and promoted by gibberellins (GA) and light, in strict association with germination, and appeared to be caused by transcriptional regulation of the class I βGlu genes. The micropylar cap of *Solanoideae*-type seeds does not allow a distinction between testa and endosperm rupture, but βGlu accumulation occurred prior to radicle emergence of pepper and *P. peruviana* seeds. ABA inhibited endosperm rupture and βGlu accumulation in the micropylar cap of pepper seeds. In contrast to tomato, βGlu accumulation in pepper seeds was not only confined to the micropylar cap, was due to distinct, tissue-specific βGlu isoforms, and was not accompanied by Chn accumulation. In conclusion, ABA inhibition of germination and βGlu accumulation in the micropylar endosperm appears to be a widespread event during the seed germination of Solanaceous species. In contrast, accumulation of Chn and distinct βGlu isoforms in the embryo, prior to germination, appears to be a species-specific phenomenon within the *Solanaceae*. In addition, a post-germination co-induction of βGlu and Chn in the root of the emerged seedling was found in endospermic and non-endospermic species and could represent an evolutionarily conserved event during dicot seedling development.

Keywords: abscisic acid, *Capsicum*, chitinase, gibberellin, β-1,3-glucanase, *Nicotiana*, seed germination, *Solanaceae* family

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Abbreviations: ABA = abscisic acid; Chn = chitinase; Chn I = class I chitinase; GA = gibberellin; βGlu = β-1,3-glucanase; βGlu I = class I β-1,3-glucanase.

Introduction

The intrafamilial relationships of Solanaceous species have been investigated using morphological and molecular criteria. On the morphological level, the *Solanaceae* family can be divided into two large subgroups (Judd *et al.*, 1999): (1) The *Cestroideae* subgroup, e.g. *Nicotiana* and *Petunia*, is characterized by straight to slightly bent embryos and prismatic to subglobose seeds, and typically by capsules; and (2) the *Solanoideae* subgroup, e.g. *Capsicum*, *Lycopersicon* and *Physalis*, is characterized by curved embryos and flattened, discoid seeds and often by berries. Seed germination of tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*), type members for each of the two subgroups, is regulated by the balance of forces between the growth potential of the embryo and the micropylar layers that cover the radicle tip and function as a constraint to radicle protrusion (reviewed by Hilhorst, 1995; Bewley, 1997b; Koornneef *et al.*, 2002; Leubner-Metzger, 2003). These covering layers are the testa (seed coat), an entirely maternal tissue, and the triploid endosperm, a predominantly maternal tissue. Both micropylar layers are involved in controlling tobacco and tomato coat-imposed dormancy (e.g. Hilhorst and Downie, 1996; Nonogaki *et al.*, 2000; Wu *et al.*, 2000; Leubner-Metzger, 2002).

Surgical removal of the micropylar testa and the endosperm tissues permits radicle growth under conditions that inhibit germination of intact seeds of tobacco (Bihlmeier, 1927; Kincaid, 1935) and tomato (Liptay and Schopfer, 1983; Hilhorst, 1995). Microscopic studies in tobacco showed that storage

reserves are degraded in the micropylar endosperm cells prior to protrusion by the radicle and that the endospermic hole, which has a smooth outline and is always formed at the micropylar end of germinating tobacco seeds, results from tissue dissolution rather than from the pushing action of the protruding radicle (Arcila and Mohapatra, 1983; Leubner-Metzger, 2002). Similar observations are obvious for other Solanaceous species and are correlated with weakening of the micropylar endosperm, e.g. in tomato (Hilhorst, 1995; Bewley, 1997a; Toorop *et al.*, 2000) and in pepper (Watkins and Cantliffe, 1983; Watkins *et al.*, 1985).

Rupture of the micropylar testa and the micropylar endosperm, resulting in radicle emergence, are distinct and temporally separate events during the germination of tobacco seeds (Arcila and Mohapatra, 1983; Leubner-Metzger *et al.*, 1995; Web site: http://www.leubner.ch/). Treatment of imbibed tobacco seeds with abscisic acid (ABA) specifically delays endosperm rupture, but not testa rupture, and results in the formation of a novel structure, consisting of the enlarging radicle covered by a sheath of greatly elongated micropylar endosperm.

As in *Cestroideae*-type seeds, βGlu also accumulates prior to endosperm rupture in *Solanoideae*-type tomato seeds (Wu *et al.*, 2000). The micropylar-covering layers of these types of seeds are organized in cap-like structures, but a distinction between testa and endosperm rupture is not possible (Watkins and Cantliffe, 1983; Watkins *et al.*, 1985; Hilhorst, 1995; Judd *et al.*, 1999; Nonogaki *et al.*, 2000). ABA also inhibits tomato endosperm rupture, i.e. radicle emergence of tomato seeds (Hilhorst, 1995; Toorop *et al.*, 2000; Wu *et al.*, 2000). Gibberellin (GA)-induced weakening of the micropylar endosperm cap is required for tomato and pepper seed germination, and the GA-deficient *gib1* mutant of tomato does not germinate without GA treatment (e.g. Watkins and Cantliffe, 1983; Watkins *et al.*, 1985; Hilhorst, 1995; Bewley, 1997a; Nonogaki *et al.*, 2000; Wu *et al.*, 2000). Weakening of the micropylar endosperm is likely to be achieved by cell-wall hydrolysis by the action of GA-induced cell-wall hydrolases.

Prior to radicle protrusion, two phases can be distinguished in tomato: (1) the early phase is not inhibited by ABA and includes ABA-insensitive endosperm weakening associated with micropylar-endosperm-specific, ABA-independent expression of endo-β-mannanase and other proteins; ABA inhibits βGlu I expression (e.g. Bewley, 1997a; Toorop *et al.*, 1998, 2000; Nonogaki *et al.*, 2000; Wu *et al.*, 2000). Endo-β-mannanase, which can hydrolyse isolated micropylar endosperm cell walls *in vitro*, appears to be necessary for endosperm weakening, but is not sufficient for the completion of tomato germination, and its accumulation is not inhibited by ABA. (2) The late phase is critical, since it includes the final ABA-controlled step of radicle emergence. It is associated with ABA-sensitive βGlu I expression in the micropylar endosperm, and βGlu I, therefore, could contribute to radicle emergence of tomato (Wu *et al.*, 2000; Leubner-Metzger, 2003). It has been proposed that the late phase includes a second, ABA-controlled step of endosperm weakening, which thereby is a biphasic process in tomato (Toorop *et al.*, 2000).

On the molecular level, phylogenetic analyses of the multigene families of β-1,3-glucanases (βGlu) and chitinases (Chn) showed that their organization is highly conserved within the *Solanaceae* (e.g. Sperisen *et al.*, 1991; Meins *et al.*, 1992; Van Buuren *et al.*, 1992; Simmons, 1994). Based on the amino acid sequences of the mature proteins, the various βGlu and Chn have been grouped into structural classes that differ in sequence identity by at least 40–50%. At least three structural classes are identified for the highly homologous βGlu isoforms of tobacco, tomato, potato and pepper. These conserved evolutionary relationships are also manifested on the level of the hormonal regulation of the orthologous genes. For example, ethylene treatment results in the transcriptional co-induction of the class I isoforms of βGlu (βGlu I) and Chn (Chn I) in the leaves of tobacco and tomato. ABA transcriptionally down-regulates βGlu I, but not Chn I, of tobacco and tomato (Rezzonico *et al.*, 1998; Leubner-Metzger and Meins, 1999; Wu *et al.*, 2000).

Seed germination of tobacco and tomato is associated with the transcriptional induction of the βGlu I genes just prior to radicle emergence (Leubner-Metzger *et al.*, 1995; Wu *et al.*, 2000; Leubner-Metzger, 2001). This induction is highly confined to the micropylar endosperm, and βGlu I accumulation and endosperm rupture of both species are promoted by GA and inhibited by ABA. Sense transformation of tobacco with a chimeric ABA-inducible βGlu I transgene provided direct evidence that βGlu I contributes to endosperm rupture (Leubner-Metzger and Meins, 2000). In tomato, but not in tobacco, Chn I also accumulates in the micropylar endosperm prior to radicle emergence. In contrast to βGlu I, and in agreement with the situation in vegetative tissues, Chn I accumulation in imbibed tomato seeds is not inhibited by ABA. Thus, the hormonal regulation of βGlu I induction and endosperm rupture of *Cestroideae*-type (tobacco) and *Solanoideae*-type (tomato) seeds is similar, but the two seed types differ with respect to Chn expression. However, since only one type-member of each subgroup has been investigated so far, no general conclusions with respect to similarities and differences in the tissue-specific and hormonal regulation of βGlu and Chn among Solanaceous seeds can be drawn.

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Materials and methods

Plant materials and germination conditions

Mature seeds of *Capsicum annuum* L. cv. Toro (ESASEM, Milano, Italy), *Physalis peruviana* L. (Wyss Samen und Pflanzen AG, Zuchwil-Solothurn, Switzerland), *Petunia hybrida* Hort. (Vilm.) (Plantania seeds, OBI and Royal Lemkes, Bleiswijk, The Netherlands), *Nicotiana sylvestris* Speg & Comes, *Nicotiana plumbaginifolia* Viv., and *Nicotiana tabacum* L. cv. Havana 425 (Agricultural Experimental Station, University of Wisconsin, Madison, Wisconsin, USA) were used for germination analyses, performed as described earlier (Leubner-Metzger *et al.*, 1998). In brief, 100–150 seeds (*c.* 20 seeds for *C. annuum* and *P. peruviana*) 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*-(±)-abscisic acid (ABA, Sigma, St. Louis, Missouri, USA) and 10 µM gibberellin A_4 (GA_4, Sigma). Petri dishes were incubated at 24°C in continuous white light (3000 lux, Philips 'TL'D 35W/33 lamps) or in darkness. After scoring for germination, seeds were stored at –70°C for subsequent analyses.

Analysis of proteins and RNA

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 and Chn activities were assayed radiometrically using reduced [^{3}H]laminarin and [^{3}H]chitin as the substrates, which are specifically digested by endo-type βGlu and Chn, respectively. The polyclonal rabbit anti-tobacco βGlu I antibody used for immunoblot analysis is known to detect the class I, class II and class III isoforms of *N. tabacum* and *N. sylvestris* (Neuhaus *et al.*, 1992; Beffa *et al.*, 1993; Kunz *et al.*, 2001) and is known to cross-react with the βGlu I of tomato (Wu *et al.*, 2000) and pea (Petruzzelli *et al.*, 1999). The polyclonal rabbit anti-tobacco Chn I antibody used for immunoblot analysis is known to detect the Chn I isoforms of *N. tabacum*, *N. sylvestris* (Kunz *et al.*, 2001) and tomato (Wu *et al.*, 2000). Preparation of total RNA and RNA-blot hybridization were as described by Leubner-Metzger *et al.* (1995). The probes were radiolabelled with [α-^{32}P]dCTP by random priming (rediprime kit; Amersham, Buckinghamshire, UK), using as templates the *c.* 1 kb *Pst*I fragments of the tobacco cDNAs for βGlu I (Shinshi *et al.*, 1988) and Chn I (Shinshi *et al.*, 1987), and the 1.8 kb *Eco*RI fragment of genomic DNA encoding tomato 18S ribosomal RNA (Schmidt-Puchta *et al.*, 1989). Hybridized membranes were washed at high stringency [20 min at 62°C in 0.1% (w/v) SDS, 30 mM NaCl, 3 mM sodium citrate, pH 7.0].

Results

ABA-sensitive βGlu is induced after testa rupture, but prior to endosperm rupture in Cestroideae-type seeds

Testa rupture and endosperm rupture are distinct and temporally separate events during the germination of tobacco seeds (Arcila and Mohapatra, 1983; Leubner-Metzger *et al.*, 1995). In initial experiments we investigated whether this is also the case for other *Nicotiana* species and for *Petunia hybrida*, which all belong to the *Cestroideae* subgroup of *Solanaceae* (Judd *et al.*, 1999). Testa rupture also precedes endosperm rupture of *N. plumbaginifolia*, *N. sylvestris* and *P. hybrida* (Table 1). Seed germination of many *Nicotiana* species requires light, and, in agreement with this, neither testa rupture nor endosperm rupture was observed in *N. tabacum*, *N. plumbaginifolia* and *N. sylvestris* seeds imbibed in darkness (Table 1; Leubner-Metzger, 2003). In contrast to *N. tabacum*, *N. sylvestris*, and *P. hybrida*, light alone was not sufficient to induce testa rupture and germination of imbibed *N. plumbaginifolia* seeds, but treatment with 10 µM GA_4 induced testa rupture and subsequent endosperm rupture (Table 1). Furthermore, as in tobacco, ABA treatment of seeds inhibited endosperm rupture, but did not affect testa rupture of the three *Nicotiana* species or of petunia.

Reduced [^{3}H]laminarin, an algal β-1,3-glucan known to be specifically digested by all endo-type βGlu isoforms (Leubner-Metzger *et al.*, 1995), was utilized for the βGlu assays. As in tobacco, βGlu activity accumulated after testa rupture, but prior to endosperm rupture in germinating seeds of *N. plumbaginifolia*, *N. sylvestris* and *P. hybrida* (Table 1). No accumulation of βGlu activity was found under the conditions where these species did not germinate, i.e. in dark-imbibed *N. sylvestris* seeds and in light-imbibed *N. plumbaginifolia* seeds without GA treatment. ABA treatment not only inhibited endosperm rupture, but also inhibited the accumulation of βGlu activities in the different *Nicotiana* species and in petunia (Table 1; Leubner-Metzger *et al.*, 1995). βGlu activity accumulation in the micropylar endosperm during tobacco seed germination appears to be due to the transcriptional induction of the βGlu I genes. RNA-blot and immunoblot analyses presented in Fig. 1 support the view that this is also the case in the different *Nicotiana* species and in petunia. A tobacco βGlu I cDNA probe detected a *c.* 1.6 kb transcript in germinating *N.*

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Table 1. Effect of gibberellin (GA) and abscisic acid (ABA) on germination and endo-type β-1,3-glucanase (βGlu) enzyme activity accumulation in imbibed Solanaceous seeds

				Control			10 μM GA_4			10 μM ABA		
				Rupture (%)[b]		βGlu	Rupture (%)		βGlu	Rupture (%)		βGlu
Species	Incubation conditions	Time (hours)	Seed state[a]	Testa	Endosperm	pkat[c]	Testa	Endosperm	pkat	Testa	Endosperm	pkat
Cestroideae subgroup of *Solanaceae*												
Nicotiana plumbaginifolia	Cont. light	25		0.0	0.0	–	71.2	0.0	0.01 ± 0.00	0.0	0.0	–
		45		0.0	0.0	0.01 ± 0.00	95.8	23.8	0.22 ± 0.01	0.0	0.0	0.01 ± 0.00
		45	NG				100	0.0	0.19 ± 0.03			
		45	G				100	100	0.26 ± 0.03			
Nicotiana sylvestris	Cont. light	0		0.0	0.0	0.04 ± 0.01	–	–	–	0.0	0.0	0.04 ± 0.01
		60	NG	100	0.0	0.20 ± 0.02	–	–	–	100	0.0	0.06 ± 0.01
		90	G	100	100	0.79 ± 0.05	–	–	–	100	0.0	0.10 ± 0.01
	Darkness	90	NG	0.0	0.0	0.05 ± 0.01	–	–	–	0.0	0.0	0.04 ± 0.01
Petunia hybrida	Cont. light	20		3.5	0.0	0.22 ± 0.01	9.7	0	0.19 ± 0.02	4.8	0.0	0.22 ± 0.01
		40		46.5	0.0	0.21 ± 0.02	57.1	1.2	0.18 ± 0.01	47.8	0.0	0.19 ± 0.00
		50		65.3	0.7	0.29 ± 0.02	68.5	3.9	0.24 ± 0.02	57.6	0.0	0.16 ± 0.01
		60		75.3	29.2	0.39 ± 0.03	77.8	24.2	0.31 ± 0.02	79.9	3.1	0.19 ± 0.03
		70		77.7	45.0	0.39 ± 0.02	79.7	48.1	0.35 ± 0.03	80.8	3.4	0.21 ± 0.02
		70	NG			0.31 ± 0.03			–			–
		70	Endosperm[d]			0.45 ± 0.01			–			0.20 ± 0.01
		70	Embryo[d]			0.37 ± 0.04			–			0.10 ± 0.00
Solanoideae subgroup of *Solanaceae*												
Physalis peruvianum	Cont. light	0			0.0	0.05 ± 0.01		0.0	0.05 ± 0.01		0.0	0.05 ± 0.01
		20			0.0	0.28 ± 0.03		0.0	–		0.0	–
		70			0.0	0.48 ± 0.04		0.0	0.88 ± 0.12		0.0	0.76 ± 0.09
		140			65.2	2.08 ± 0.18		100	1.77 ± 0.21		0.0	1.30 ± 0.15
		140	NG		0.0	1.23 ± 0.11						
		140	G		100	2.53 ± 0.27						

Cont., continuous.

[a] NG, Non-germinated; G, germinated.

[b] Germination of 100–150 seeds (20 seeds for *P. peruviana*) incubated at 24°C; mean values of usually 3 samples.

[c] Mean values ± SE of enzyme activities in pkat/seed (*P. peruviana*) or pkat/μg protein (*P. hybrida*, *N. plumbaginifolia*, *N. sylvestris*).

[d] βGlu activities of endosperms and embryos from control seeds (germinated) or ABA-treated seeds (non-germinated) were analysed separately.

plumbaginifolia seeds, but only upon GA treatment and not in non-germinating seeds (Fig. 1A). The rabbit anti-tobacco βGlu I antibody used for immunoblot analysis is known to detect the class I, class II and class III βGlu isoforms *of N. tabacum* and *N. sylvestris* (Neuhaus *et al.*, 1992; Beffa *et al.*, 1993). This antibody detected the accumulation of immunoreactive bands in germinating seeds of *N. sylvestris* imbibed in the light (*c.* 33 kDa; data not shown) and of *N. plumbaginifolia* imbibed in the light and treated with GA (*c.* 34 kDa; Fig. 1B). The sizes of these bands are in agreement with the known sizes of the βGlu I of these species (Castresana *et al.*, 1990; Gheysen *et al.*, 1990; Neuhaus *et al.*, 1992), and the bands were not detected in seeds imbibed under conditions that prevent or inhibit germination. The initial βGlu activities in seeds of the three *Nicotiana* species are essentially background level (Table 1), and the βGlu I accumulation during imbibition is localized exclusively in the endosperm (data not shown; Leubner-Metzger *et al.*, 1995). In contrast to *Nicotiana* seeds, the initial βGlu activities of petunia seeds were considerably higher, and βGlu activity accumulation during imbibition was localized in the endosperm and in the embryo (Table 1). Figure 1C shows that the anti-tobacco βGlu I antibody detects several immunoreactive bands (27, 30, 35, 43, 47 and 50 kDa in size) in the protein extracts of germinating petunia seeds. The 27, 30, 47 and 50 kDa bands did not show appreciable regulation with respect to the germination process and to the hormone treatments. In contrast, the 35 kDa and the 43 Da bands appeared to be down-regulated by ABA and up-regulated by imbibition and/or GA, in agreement with the βGlu activities. These two putative βGlu isoforms are located in the endosperm and in the embryo, with a predominance of the 43 kDa bands in the embryo. Taken together, these findings in four species suggested that the accumulation of ABA-sensitive βGlu I in the endosperm after testa rupture, but prior to endosperm rupture, appears to be a common phenomenon during the germination of *Cestroideae*-type seeds.

*β*Glu and Chn are co-induced in the roots of Solanaceous seedlings as a post-germination event

βGlu I induction in germinating tobacco seeds prior to endosperm rupture is confined to the micropylar endosperm and is absent from the lateral endosperm and the embryo (Fig. 2A; Leubner-Metzger *et al.*, 1995). When we extended our studies to germinated tobacco seedlings at 90 h and 150 h, we found an additional, post-germination induction phase of βGlu I that is localized in the root, but not appreciably in the shoot of the seedlings (Fig. 2A; Table 2). Chn is not induced prior to the completion of endosperm rupture of *N. tabacum* (Fig. 2B, C; Leubner-Metzger *et al.*, 1995) and *N. sylvestris* (data not shown). However, in germinated tobacco seedlings at 90 h and 150 h, the mRNA and the 32 kDa and 34 kDa antigens of Chn I are detected (Fig. 2B, C). As for the βGlu I, Chn I accumulation is also mainly localized to the root of the tobacco seedlings (Table 2). In contrast to βGlu I, which is transcriptionally down-regulated by ABA in seeds and in vegetative tissues, Chn I expression in vegetative tissues is not down-regulated by ABA (Leubner-Metzger *et al.*, 1995; Rezzonico *et al.*, 1998). This was also the case in ABA-treated tobacco seeds and, since ABA delays endosperm rupture, this leads to the finding that Chn I accumulates prior to endosperm rupture in ABA-treated seeds (Fig. 2B, C). This is in contrast to seeds imbibed in medium without ABA added, and the Chn I levels found in 150 h seedlings were similar to those found in 150 h seeds treated with ABA (Fig. 2B). In contrast to tobacco, Chn I is also induced prior to germination in tomato seeds in medium lacking ABA (Wu *et al.*, 2000). As in tobacco, βGlu and Chn activity accumulations are associated with the root, but appreciably less with the shoot, of tomato and pepper seedlings (Table 2). Thus, a post-germination co-induction of βGlu and Chn in the root of seedlings seems to be a general phenomenon in both subgroups of the *Solanaceae*.

Temporal and spatial pattern of βGlu and Chn accumulation are distinct among Solanoideae*-type seeds*

Tomato, *Physalis peruviana* and *Capsicum annuum* belong to the *Solanoideae* subgroup of *Solanaceae* and are characterized by flattened, discoid seeds with curved embryos, and the micropylar endosperm and testa layers of pepper and tomato form a cap-like structure (Watkins and Cantliffe, 1983; Watkins *et al.*, 1985; Judd *et al.*, 1999). βGlu I and Chn I are both co-expressed in the micropylar endosperm of tomato prior to radicle emergence (Wu *et al.*, 2000). Using highly sensitive radiometric assays with [^{3}H]laminarin and [^{3}H]chitin as substrates, respectively, similar contents and kinetics of specific βGlu and Chn activities were obtained in tomato seeds. Figure 3 shows that by using the same highly sensitive radiometric assays, only the accumulation of βGlu activity, but not of Chn activity, was detected in germinating pepper seeds. The onset of radicle protrusion of imbibed pepper seed populations was not detected in the 72 h samples, but started slightly later. As in tomato, βGlu activity accumulates in the micropylar cap of pepper seeds prior to its rupture by the protruding radicle and germination is inhibited by ABA (Fig. 3B). In contrast to tomato, where βGlu accumulation is confined to the micropylar

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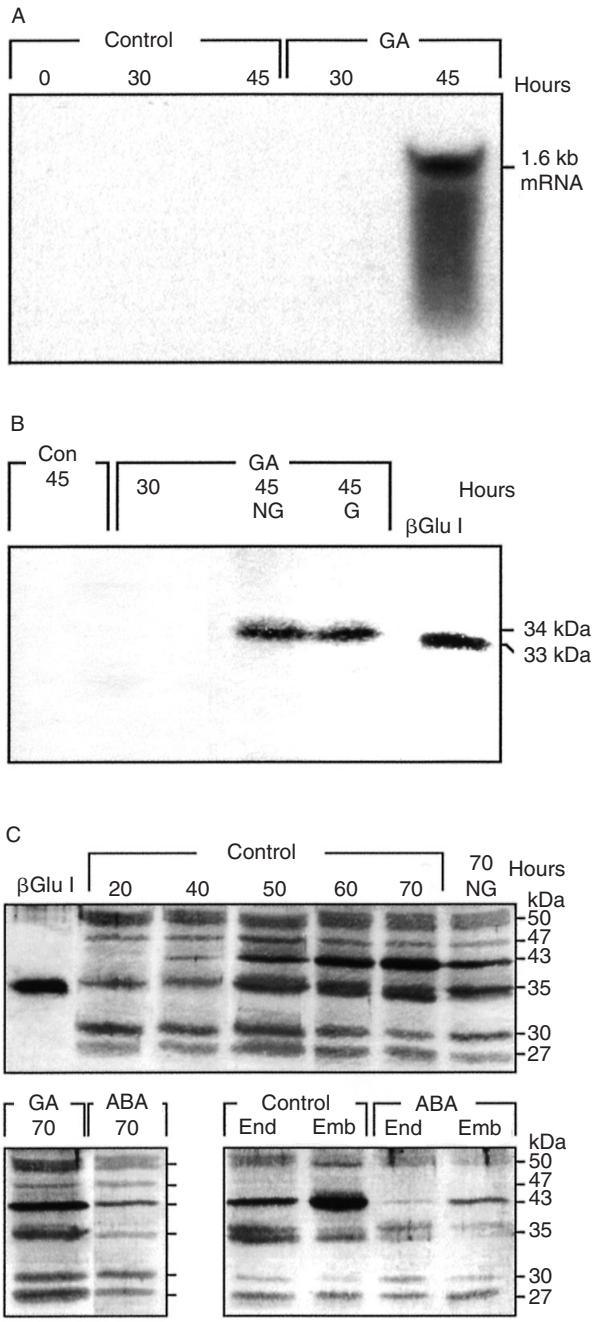

Figure 1. Hormone-regulated accumulation of tobacco βGlu I-related mRNAs and antigens during the germination of *Cestroideae*-type *Solanaceae* seeds. (A) RNA-blot hybridization of total RNA (25 µg/lane) prepared from *Nicotiana plumbaginifolia* seeds imbibed in continuous light either in the absence (Control) or presence of 10 µM GA_4 (GA); only GA-treated seeds germinate. The RNA-blot was hybridized with a cDNA probe for tobacco βGlu I. (B) Immunoblot analysis of *N. plumbaginifolia* seed extracts (80 µg protein/lane) probed with the rabbit anti-tobacco βGlu I antibody. G = germinated seeds only; NG = ungerminated seeds only. βGlu I = 10 ng of the authentic 33 kDa tobacco enzyme. No signals were obtained in control blots with rabbit anti-tobacco Chn I antibody or with rabbit pre-immune serum. (C) Immunoblot analysis of *Petunia hybrida* seed extracts using the anti-tobacco βGlu I antibody; GA = 10 µM GA_4; ABA = 10 µM ABA, End = endosperm, Emb = embryo; analysis as described in (B) with the difference that 50 µg protein/lane (entire seeds) and 30 µg protein/lane (End, Emb) were applied.

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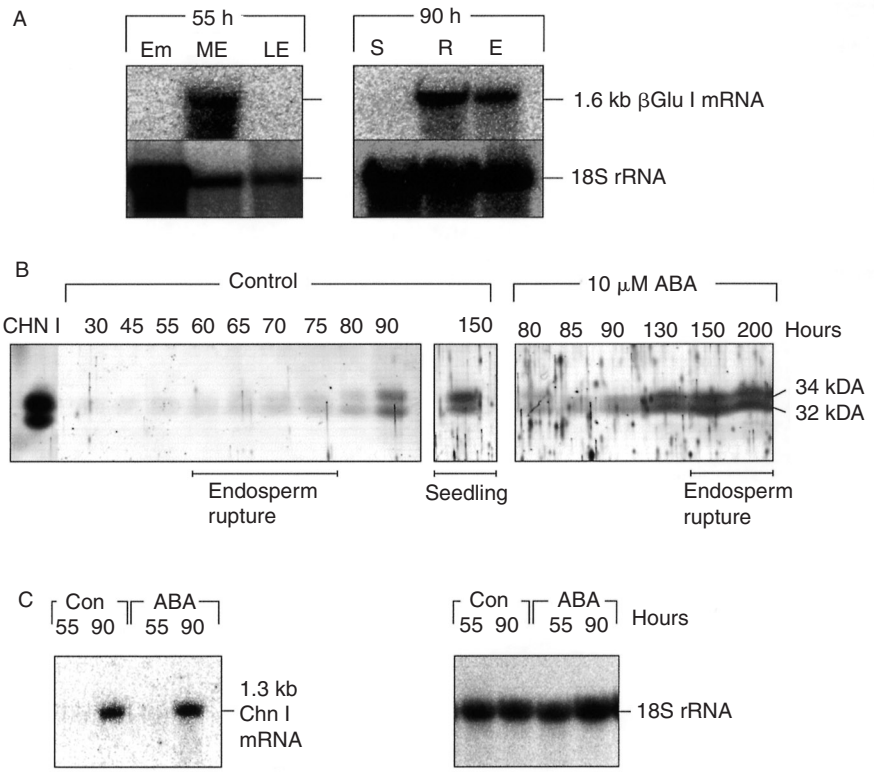

Figure 2. Tissue specificity and regulation by abscisic acid (ABA) of βGlu I and Chn I in imbibed seeds and in seedlings of *Nicotiana tabacum*. (A) RNA-blot hybridization of total RNA (25 μg/lane) prepared from seeds prior to endosperm rupture (55 h) during imbibition in continuous light and from 90 h seedlings. The RNA-blot was hybridized with a cDNA probe for tobacco βGlu I, and a probe for 18S rRNA was used as a loading standard. Em = embryo; ME = micropylar endosperm; LE = lateral endosperm; S = shoot; R = root; E = 90 h endosperm remnant. (B) Immunoblot analysis of seed and seedling extracts (80 μg protein/lane) probed with the rabbit anti-tobacco Chn I antibody. Seeds and seedlings were imbibed in continuous light either in the absence (Control) or presence of 10 μM ABA. CHN I = 10 ng of the authentic 32 kDa and 34 kDa tobacco enzymes. (C) RNA-blot hybridization of total RNA prepared from seeds and seedlings incubated in medium without (Con) or with 10 μM ABA added (ABA). The RNA-blot was hybridized with probes for tobacco Chn I and 18S rRNA as described in (A).

Table 2. Tissue-specificity of β-1,3-glucanase (βGlu) and chitinase (Chn) enzyme activity in Solanaceous seedlings

	βGlu[a]			Chn[a]		
Species [b]	Root (R)	Shoot (S)	R/S ratio[c]	Root (R)	Shoot (S)	R/S ratio
Pepper	3.3 ± 0.6	0.1 ± 0.0	33	8.2 ± 0.9	0.1 ± 0.0	82
Tomato	1.0 ± 0.5	0.1 ± 0.0	10	–	–	–
Tobacco	11.7 ± 1.1	2.0 ± 0.6	6	31.1 ± 5.5	6.8 ± 2.6	5

[a] Mean values ± SE of enzyme activities in pkat/μg protein of four protein samples, each from 10–15 seedling tissues.
[b] Seedlings from germinated seeds incubated for 150 h (tobacco) or 160 h (pepper, tomato) in continuous light at 24°C.
[c] Ratio between the specific enzyme activities of the seedling root and shoot tissues.

endosperm, βGlu activity also accumulated in the embryo (Fig. 3A) and the rest of the pepper seed (Fig. 3C). The βGlu activity accumulation in all three compartments was inhibited by ABA (Fig. 3). βGlu activity also accumulated in imbibed seeds of *P. peruviana* and the enzyme activity accumulation was only partially inhibited by ABA (Table 1).

To further investigate the tissue- and species-specific accumulation of βGlu isoforms, we performed immunoblot analyses with the different pepper seed tissues. The rabbit anti-tobacco βGlu I antibody, which is known to also detect the 35 kDa tomato βGlu I in the micropylar endosperm (Wu *et al.*, 2000), detected a distinct temporal and spatial pattern

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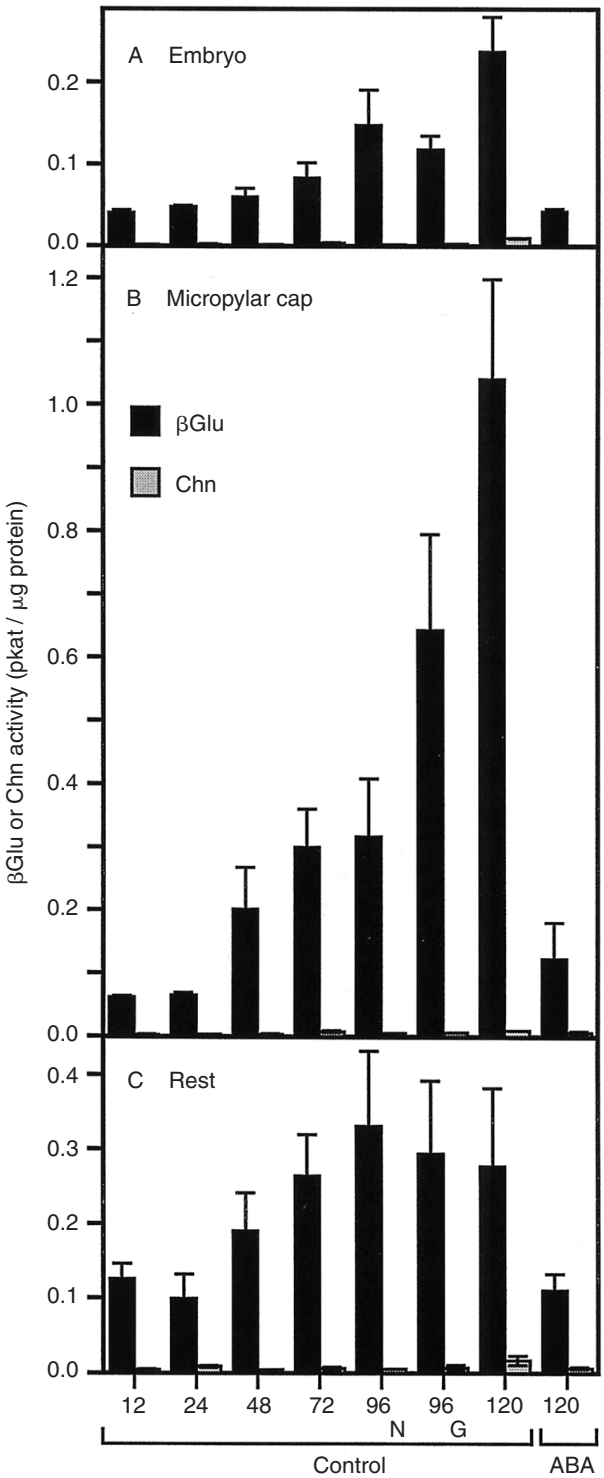

Figure 3. Tissue specificity and regulation by ABA of βGlu and Chn activities during the seed germination of *Capsicum annuum*, measured in (A) embryo, (B) micropylar cap and (C) the rest of the seed. Populations of 20 seeds were imbibed in continuous light, either in the absence (Control) or presence of 100 μM ABA (ABA), and seeds were dissected at the times indicated. Specific βGlu and Chn activities are expressed in pkat/μg protein. Mean values ± SE are presented from four to ten replicate samples at each time point. Radicle protrusion of imbibed pepper seeds: 0% until 72 h, 11.6% at 96 h, 100% at 120 h (Control) and 0% for ABA-treated seeds at 120 h. At 96 h non-germinated (N) and germinated (G) seeds were measured separately.

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of immunoreactive bands in the different seed compartments and in seedlings (Fig. 4). A major, *c*. 39.5 kDa band increased in intensity in the embryo prior to and during endosperm rupture, but was not detected in the micropylar cap prior to endosperm rupture or in the rest of the seed. The 39.5 kDa band was also present in the micropylar cap of 120 h germinated seeds, but not in ABA-treated ungerminated seeds. The appearance of the 39.5 kDa band in the embryo was inhibited by ABA (Fig. 4A), which also inhibited βGlu activity accumulation (Fig. 3A). The 39.5 kDa band was the only band detected in seedling shoots and was absent from seedling roots (Fig. 4A). Additional bands, *c*. 27 kDa and 31 kDa in size, were detected at roughly constitutive levels in all seed tissues (Fig. 4). The 27 kDa band was especially strong in the embryo, but was absent from seedlings. Roughly constitutive levels of a *c*. 24 kDa band were found in the endosperm, but not in embryos or seedlings. Treatment with ABA did not affect the levels of the 24, 27 and 31 kDa bands, but the transition from the embryo state to the seedling state was associated with the disappearance of these bands. A *c*. 33 kDa band was detected in seedling roots. Thus, a complex tissue-specific pattern of putative βGlu was detected in imbibed seeds and in seedlings of pepper. In contrast to pepper, the rabbit anti-tobacco βGlu I antibody did not detect any antigen in protein extracts from seed extracts of *P. peruviana*. Finally, in agreement with the finding that Chn activity did not accumulate during pepper seed germination, the rabbit anti-tobacco Chn I antibody did not detect immunoreactive bands in imbibed pepper seeds (data not shown); but it did detect Chn I in tomato seeds (Wu *et al*., 2000).

Discussion

Two developmentally regulated sites of βGlu induction seem to be common among Solanaceous species: (1) βGlu accumulation in imbibed seeds prior to endosperm rupture, and (2) βGlu accumulation in roots of young seedlings as a post-germination event. Whereas co-induction of Glu and Chn in seedling roots appears to be a common phenomenon, significant differences exist among Solanaceous species during seed germination. The various βGlu isoforms of tobacco, tomato, potato, pepper and other species have been classified into at least three structural classes that differ by a minimum of 40–50% in amino acid sequence identity of the mature proteins (Meins *et al*., 1992; Simmons, 1994; Leubner-Metzger and Meins, 1999). The endo-type class I enzymes (βGlu I) include the highly homologous 33 kDa isoforms of *N. tabacum* and *N. sylvestris* (Sperisen *et al*., 1991; Neuhaus *et al*., 1992), the 34 kDa isoforms of *N. plumbaginifolia* (*Gn2* and *Gn1* share 98% and 76% amino acid sequence identity with tobacco βGlu I, respectively; Castresana *et al*., 1990; Gheysen *et al*., 1990), the 35 kDa tomato isoform Glu B (*c*. 90% amino acid sequence identity with tobacco βGlu I; Van Kan *et al*., 1992; Wu *et al*., 2000), and the βGlu I of pepper exhibits a similarly high degree of sequence identity (Kim and Hwang, 1997; Jung and Hwang, 2000b; Pflieger *et al*., 2001). These βGlu I isoforms are usually basic, intracellular proteins, but there is strong evidence that they can also be secreted (Simmons, 1994; Leubner-Metzger and Meins, 1999). In contrast to βGlu I, the class II and III βGlu are secreted into the apoplast. Characteristic differences between class I and II βGlu genes are also evident at the level of gene regulation by ethylene, ABA, salicylic acid, pathogens and other factors. βGlu I gene expression is induced transcriptionally in vegetative tissues and in seeds by ethylene, and down-regulated by ABA (e.g. Leubner-Metzger *et al*., 1995; Rezzonico *et al*., 1998; Jung and Hwang, 2000b; Wu *et al*., 2000). As for βGlu, similar structural classes exist for the Chn isoforms, and βGlu I and Chn I are often co-induced, e.g. in leaves by ethylene or pathogens. In contrast to βGlu I, Chn I is not transcriptionally down-regulated by ABA and does not accumulate in the micropylar endosperm of germinating tobacco seeds (Leubner-Metzger *et al*., 1995), but does accumulate in the micropylar endosperm of germinating tomato seeds (Wu *et al*., 2000). The present study shows that *Cestroideae*- and *Solanoideae*-type seeds not only differ in their morphology (Judd *et al*., 1999), but that they also exhibit common and distinct aspects with regard to the hormonal and developmental regulation of βGlu and Chn.

*β*Glu induction in the endosperm, and endosperm rupture but not testa rupture, are inhibited by ABA in Cestroideae-type seeds

Our most intriguing findings with *Cestroideae*-type seeds (three *Nicotiana* species and petunia) are the common distinction between testa rupture and endosperm rupture, and that ABA-sensitive βGlu accumulation occurs prior to endosperm rupture, but after testa rupture. As in tobacco (Arcila and Mohapatra, 1983; Leubner-Metzger *et al*., 1995), testa rupture and endosperm rupture of *N. sylvestris*, *N. plumbaginifolia* and petunia are distinct and sequential events. As in tobacco, ABA does not appreciably inhibit testa rupture, but inhibits βGlu accumulation in the endosperm and endosperm rupture (Table 3). GA treatment is required to release dormancy and induce βGlu accumulation and germination of *N. plumbaginifolia* seeds imbibed in the light and *N. sylvestris* seeds imbibed in darkness. These results are

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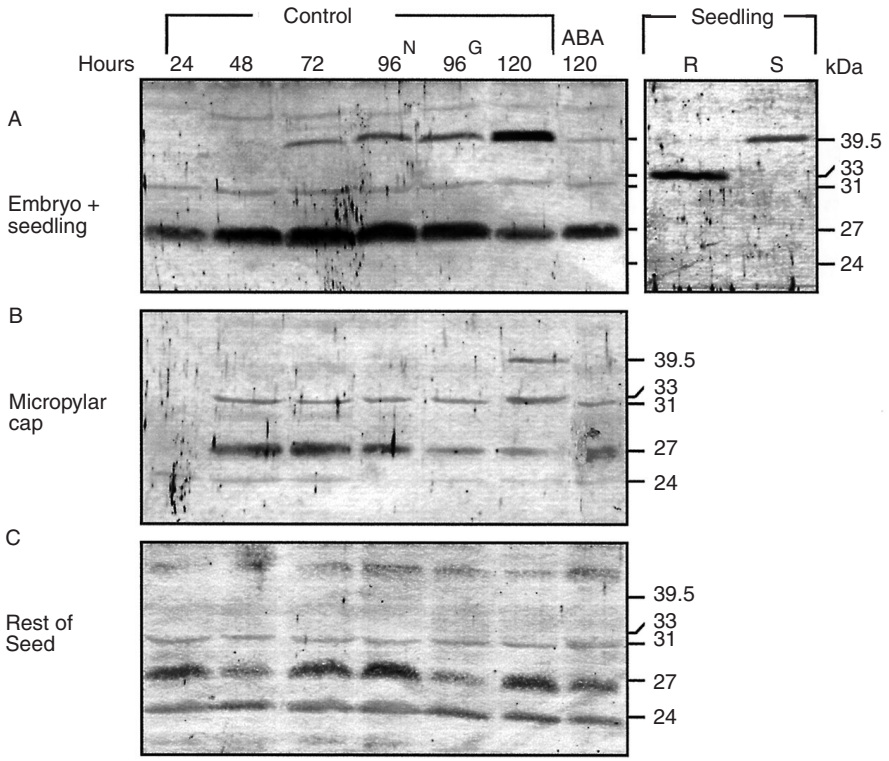

Figure 4. Tissue specificity and regulation by ABA of βGlu antigens during germination of *Capsicum annuum* seeds, in (A) embryo and 160 h-seedling, (B) micropylar cap and (C) the rest of the seed. Immunoblot analysis of seed and seedling extracts (40 µg protein/lane) probed with the rabbit anti-tobacco βGlu I antibody, which recognizes all known tobacco β-1,3-glucanases and is also known to cross-react with tomato βGlu I (Neuhaus *et al.*, 1992; Beffa *et al.*, 1993; Petruzzelli *et al.*, 1999; Wu *et al.*, 2000; Kunz *et al.*, 2001). No signals were obtained in control blots with rabbit anti-tobacco Chn I antibody or with rabbit pre-immune serum. Extracts and germination are as described in Fig. 3. R = root, S = shoot.

in agreement with the roles of ABA and GA in regulating dormancy and germination of *Nicotiana* species (e.g. Leubner-Metzger *et al.*, 1996; Frey *et al.*, 1999; Grappin *et al.*, 2000; Leubner-Metzger, 2001) and of petunia (Sink, 1984; Girard, 1990). The highly homologous 34 kDa and 33 kDa βGlu I isoforms of *N. plumbaginifolia* (Castresana *et al.*, 1990; Gheysen *et al.*, 1990) and *N. sylvestris* (Sperisen *et al.*, 1991; Neuhaus *et al.*, 1992), respectively, accumulate after testa rupture, but prior to endosperm rupture. As in tobacco, germination and βGlu I accumulation seem to depend on the light/GA pathway and are inhibited by ABA (Leubner-Metzger *et al.*, 1996; Leubner-Metzger, 2001). No sequence information is available for petunia βGlu isoforms, but the anti-tobacco βGlu I antibody detected several constitutively expressed immunoreactive bands, as well as two immunoreactive bands, 35 kDa and 43 kDa in size, that exhibit ABA-inhibited regulation. In contrast to *Nicotiana* seeds, enzyme activity and antigen accumulation in petunia are present in both endosperm and embryo. It seems likely, but not proven, that the 35 kDa band that accumulates in association with endosperm rupture is a petunia βGlu I. Taken together, these findings suggest that the accumulation of βGlu I prior to endosperm rupture and the regulation of βGlu I expression by light/GA and ABA could be an evolutionarily conserved developmental event in *Cestroideae*-type seeds (Leubner-Metzger, 2003).

Distinct expression pattern of βGlu and Chn isoforms in germinating Solanaceous seeds

ABA also inhibits endosperm rupture of *Solanoideae*-type seeds such as tomato (Hilhorst, 1995; Toorop *et al.*, 2000; Wu *et al.*, 2000) and pepper, but not *P. peruviana* (this study). As in *Cestroideae*-type seeds, βGlu also accumulates prior to endosperm rupture of tomato, pepper and *Physalis* seeds (Table 3). As in tobacco, βGlu accumulation in the micropylar endosperm of tomato and pepper is inhibited by ABA. In contrast to tobacco and tomato, βGlu accumulation in pepper and petunia seeds is not

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Table 3. β-1,3-Glucanase (βGlu) and chitinase (Chn) activity induction in imbibed Solanaceous seeds and in young seedlings

	Seeds prior to endosperm rupture						Seedling roots
	βGlu induction		Chn induction		Inhibition by ABA of endosperm		
Species[a]	Endosperm[b]	Embryo	Endosperm	Embryo	βGlu induction	rupture	βGlu and Chn co-induction
Cestroideae subgroup of *Solanaceae*							
Nicotiana tabacum	+[c]	–	–	–	+	+	+
Nicotiana sylvestris	+	–	–	–	+	+	+
Nicotiana plumbaginifolia	+	–	n.d.	n.d.	+	+	n.d.
Petunia hybrida	+	+	n.d.	n.d.	+	+	n.d.
Solanoideae subgroup of *Solanaceae*							
Lycopersicon esculentum	+	–	+	+	+	+	+
Capsicum annuum	+	+	–	–	+	+	+
Physalis peruviana	+	n.d.[d]	n.d.	n.d.	(–)[e]	+	n.d.

[a] Based on this study and on additional results for *Nicotiana* species (Vögeli-Lange *et al.*, 1994; Leubner-Metzger *et al.*, 1995; Kunz *et al.*, 2001) and tomato (Wu *et al.*, 2000).

[b] Localization of βGlu induction in the micropylar part of the endosperm has been demonstrated for *N. tabacum*, *N. sylvestris*, *L. esculentum*, and *C. annuum*; for the other species only accumulation in the overall endosperm has been demonstrated.

[c] A '+' sign means presence and a '–' sign absence of a feature or process.

[d] n.d. = not determined.

[e] No inhibition by ABA of overall seed βGlu activity accumulation, which does not *per se* exclude the existence of an ABA-responsive βGlu isoform in the endosperm.

confined exclusively to the micropylar tissue, but also accumulates in other seed parts, especially in the embryo. At least ten basic and acidic βGlu isoforms have been described in healthy and infected vegetative pepper tissues, corresponding to sizes of 25, 27, 29, 34, and 36 kDa in denaturing gels (e.g. Kim and Hwang, 1994, 1997; Egea *et al*., 1999). The limited sequence information on pepper available in databases includes a 39.3 kDa ethylene-inducible βGlu I (Jung and Hwang, 2000a, b; Pflieger *et al*., 2001). Using the anti-tobacco βGlu I antibody, we detected a major *c*. 39.5 kDa band in the embryo prior to and during germination, and in seedling shoots. This band is not present during early imbibition, is not induced in the micropylar cap prior to endosperm rupture, nor in the rest of the seed or in seedling roots. Its appearance is inhibited by ABA and it corresponds in size to the ethylene-inducible 39.3 kDa βGlu I described by Jung and Hwang (2000b). The anti-tobacco βGlu I antibody also detected several other immunoreactive bands that were 24, 27 and 31 kDa in size, roughly constitutive and not affected by ABA. The nature of these bands is not known, and the limited sequence information, together with expected cultivar differences, does not allow an unambiguous identification of these isoforms. ABA-sensitive βGlu activity accumulation was especially strong in the micropylar endosperm of pepper seeds, but none of the detected bands correlated with this activity pattern. Thus, a serologically distinct ABA-sensitive βGlu isoform must account for the βGlu accumulation in the micropylar cap of imbibed pepper seeds. The anti-tobacco βGlu I antibody also detected a *c*. 33 kDa band, but only in seedling roots. Considering the known serological relatedness of the tobacco and tomato βGlu I with the 34 kDa pepper βGlu I (e.g. Kim and Hwang, 1997; Wu *et al*., 2000), we propose that this is a pepper βGlu I, probably the basic 34 kDa isoform described by Kim and Hwang (1997). The tissue-specific and ABA-regulated differences in band patterns suggest that several distinct βGlu isoforms account for the βGlu activities of pepper and petunia. ABA did not inhibit overall βGlu activity accumulation in seeds of *P. peruviana*; however, it is still a reasonable, but unproven, hypothesis that several distinct βGlus accumulate and that, among them, are ABA-sensitive and -insensitive isoforms. The finding that the anti-tobacco βGlu I antibody did not detect any signals in seed extracts of *P. peruviana* is a complication for testing this hypothesis. Prior to endosperm rupture, βGlu I expression in *Nicotiana* and tomato seeds is confined to the micropylar endosperm, whereas in pepper and petunia seeds, distinct isoforms confer βGlu activity accumulation in several tissues (this study; Leubner-Metzger *et al*., 1995; Wu *et al*., 2000).

Chn is not expressed in *Nicotiana* and pepper seeds prior to endosperm rupture, but Chn I is expressed in the micropylar endosperm of tomato (Table 3). Interestingly, jasmonates or wounding induce Chn I expression in the micropylar endosperm of tomato (Wu and Bradford, 2002), but do not induce βGlu I in tomato (Wu and Bradford, 2002) or tobacco seeds (Leubner-Metzger, personal communication). Thus, βGlu I and Chn I induction in the micropylar endosperm may be regulated by distinct signalling pathways. Therefore, we propose that signalling of ABA-sensitive βGlu induction in the micropylar endosperm is an evolutionarily conserved pathway that is widespread in Solanaceous seeds and serves a developmental function during endosperm rupture. This is in agreement with direct evidence obtained by sense transformation of tobacco, which showed that βGlu I contributes to endosperm rupture (Leubner-Metzger and Meins, 2000). In contrast, βGlu expression in other seed tissues and Chn expression in seeds occur only in some Solanaceous species, is regulated by distinct and diverse pathways, and therefore might serve different developmental or pathogenesis-related functions (Wu and Bradford, 2002; Leubner-Metzger, 2003).

Evolutionarily conserved induction of βGlu in seedling roots of endospermic and non-endospermic species

We found that a post-germination induction of βGlu and Chn occurred mainly in the roots of young seedlings and appears to be an additional evolutionarily conserved site of βGlu I induction (Table 3). In contrast to the micropylar endosperm, βGlu I and Chn I are co-induced in the roots of young tobacco seedlings. βGlu and Chn are also co-induced in the roots of tomato and pepper seedlings, and appear to be the class I isoforms. βGlu I is also induced in seedlings of *N. sylvestris*, and increased βGlu I and Chn I levels are present in older seedlings of this species (Vögeli-Lange *et al*., 1994; Kunz *et al*., 1996, 2001). βGlu I and Chn I accumulate at high concentrations in the roots and in lower leaves of mature, healthy Solanaceous plants (Van de Rhee *et al*., 1993; Beerhues and Kombrink, 1994; Vögeli-Lange *et al*., 1994; Leubner-Metzger and Meins, 1999). The establishment of this gradient of βGlu I and Chn I expression is regulated by ethylene. In agreement with these findings, endogenous ethylene also induces the accumulation of a βGlu I in the root, but not the shoot, of pea seedlings directly after germination (Petruzzelli *et al*., 1999, 2000, 2003). Within the pea root, ethylene responsiveness and βGlu I accumulation are localized to the elongation and differentiation zones where the root hairs form. In contrast to Solanaceous seeds, pea seeds are non-

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dormant and non-endospermic. This supports the view that the post-germination induction of βGlu I (and possibly also Chn I) is widespread among dicot seedlings and represents an evolutionarily conserved event (Leubner-Metzger, 2003).

Acknowledgements

We thank Waltraud Schmidt-Puchta for kindly providing plasmids and Sjoerd van Eeden (Friedrich Miescher Institute, Basel, Switzerland) for care of plants. This research is supported by a grant from the Deutsche Forschungsgemeinschaft (LE 720/3) to G.L.-M., which is gratefully acknowledged.

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Received 10 July 2002
accepted after revision 8 January 2003

https://doi.org/10.1079/SSR2003132 Published online by Cambridge University Press