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Distinct ethylene- and tissue-specific regulation of β-1,3-glucanases and chitinases during pea seed germination

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Abstract. The expression of β-1,3-glucanase (βGlu) and chitinase (Chn) was investigated in the testa, cotyledons, and embryonic axis of germinating *Pisum sativum* L. cv. 'Espresso generoso' seeds. High concentrations of βGlu and Chn activity were found in the embryonic axis. Treatment with ethylene alone or in combination with the inhibitor of ethylene action 2,5-norbornadiene showed that an early, 4-fold induction of βGlu activity in the embryonic axis during the first 20 h after the start of imbibition is ethylene-independent. This initial increase was followed by a later 4-fold ethylene-dependent induction in the embryonic axis starting at 50 h, which is after the onset of ethylene evolution and after completion of radicle emergence. The βGlu activity in cotyledons increased gradually throughout germination and was ethylene-independent. In contrast, the ethylene-independent Chn activity increased slightly after the onset of radical emergence in the embryonic axis and remained at a constant low level in cotyledons. Immunoinactivation assays and immunoblot analyses suggest that early βGlu activity in the embryonic axis is due to a 54-kDa antigen, whereas late induction is due to a 34.5-kDa antigen, which is likely to be the ethylene-inducible class I βGlu G2 described for immature pea pods. Increases in Chn in the embryonic axis were correlated with a 26-kDa antigen, whereas amounts of the additional 32- and 20-kDa antigens remained roughly constant. Thus, ethylene-dependent and ethylene-independent pathways regulate βGlu and Chn during pea seed germination. The pattern of regulation differs from that of leaves and immature pods, and from that described for germinating tobacco seeds. The functional significance of this regulation and its underlying mechanisms are discussed.

Key words: Chitinase – Ethylene – β-1,3-Glucanase – *Pisum* – Seed germination – Signalling

Introduction

The glucanohydrolases β-1,3-glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14) are abundant, highly regulated enzymes widely distributed in seed plants (review: Meins et al. 1992). The β-1,3-glucanases (βGlu) and chitinases (Chn) exist as multiple structural isoforms that differ in their size, isoelectric point, primary structure, cellular localization, and pattern of regulation. There is compelling evidence that βGlu and Chn are part of the defense of plants against pathogenic fungi (reviews: Boller 1988; Bowles 1990). There is also growing evidence that βGlu and Chn serve important functions in diverse physiological and developmental processes including embryogenesis (Van Hengel et al. 1998), seed germination (Cordero et al. 1994; Leubner-Metzger et al. 1995, 1996), mobilization of storage reserves in the endosperm (Fincher 1989), stem growth (Wong and Maclachlan 1980; Inouhe and Nevins 1998), and regulation of transport through vascular tissues and plasmodesmata (Wong and Maclachlan 1980).

This report deals with the ethylene- and tissue-specific regulation of βGlu and Chn isoforms during the germination of pea seeds. Ethylene is involved in seed germination of many plant species (reviews: Esashi 1991; Kepczynski and Kepczynska 1997). Radicle protrusion through the testa (seed coat) of non-endospermic pea seeds is accompanied by an early increase in ethylene evolution (Gorecki et al. 1991; Petruzzelli et al. 1995). Germination of endospermic tobacco seeds, which involves the successive rupture by the radicle of the testa and the endosperm layer (Arcila and Mohapatra 1983; Leubner-Metzger et al. 1995), is also accompanied by a burst of ethylene production (Khalil 1992). Earlier we showed that class I βGlu, but not class I Chn, are transcriptionally induced during tobacco seed

Abbreviations: Chn = chitinase(s); βGlu = β-1,3-glucanase(s); NBD = 2,5-norbornadiene

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germination (Leubner-Metzger et al. 1995, 1996). The induction started after completion of testa rupture just prior to the onset of endosperm rupture, and was localized exclusively in the micropylar region of the endosperm where the radicle will penetrate. Further studies with tobacco seeds (Sisler and Wood 1986; Leubner-Metzger et al. 1998) provided strong evidence that endogenous ethylene is required both for high levels of class I βGlu expression in the micropylar endosperm and for endosperm rupture. In contrast to tobacco, the mature pea seeds are non-endospermic, i.e. the endosperm tissue is resorbed during seed development, the fleshy storage cotyledons make up most of the seed mass, and the testa is the only covering layer (review: Bewley and Black 1994). Several isoforms of βGlu and Chn have been characterized from pea and other non-endospermic species (e.g. Mauch et al. 1984, 1988a; Vögeli et al. 1988; Broglie et al. 1989; Chang et al. 1992; Vad et al. 1993; Dassi et al. 1996), but almost nothing is known about their regulation and function during seed germination.

The present report provides evidence that the temporal and spatial accumulation of βGlu and Chn during pea seed germination is regulated by ethylene-dependent and ethylene-independent pathways, and that the pattern of regulation differs from that reported for tobacco seeds. This suggests that Chn, βGlu, and the ethylene-signalling pathway might serve different functions in endospermic and non-endospermic seeds.

Materials and methods

Plant material and germination experiments. Seeds of *Pisum sativum* L. cv. 'Espresso generoso' (Sais, Cesena, Italy) were surface-sterilized with 1% (w/v) NaOCl, washed with distilled water for 15 min, and then incubated in the dark at 20 °C in 9-cm-diameter plastic Petri dishes containing a double layer of filter paper wetted with 10 ml distilled, sterile water as described by Petruzzelli et al. (1995). Seeds were harvested at different times after the start of imbibition and dissected into testa, cotyledons, and embryonic axis. Tissues were frozen immediately and stored at −80 °C for subsequent analyses. For experiments with a controlled ethylene atmosphere, Petri dishes with seeds were incubated for the 24-h period prior to harvesting in gas-tight 10-l desiccators. Where indicated, ethylene (40 µl/l; Siad, Milano, Italy) or 2,5-norbornadiene (100 µl/l; NBD; Aldrich Chemical Co, Buchs, Switzerland) were introduced into the air phase as described by Petruzzelli et al. (1995). The final concentration of ethylene was verified by gas chromatography. Air used in the control desiccators was first passed through a Dreschel bottle packed with silica gel coated with $KMnO_4$ to remove ethylene.

Protein assays. Procedures for extracting proteins, measuring enzyme activity, immunoblot analysis, and protein determination have been described previously (Leubner-Metzger et al. 1995). In brief, Chn and βGlu activities were assayed radiometrically, with [^{3}H]chitin and the algal β-1,3-glucan [^{3}H]laminarin reduced with $NaBH_3$ as substrates, respectively (Boller et al. 1983). Protein was measured by the method of Bradford (1976) using bovine γ-globulin as standard. Immunoblot analyses were performed as described previously (Beffa et al. 1993; Leubner-Metzger et al. 1995) using rabbit antibodies directed against the class I βGlu and Chn purified from bean leaves (Vögeli et al. 1988). Immunoinactivation was assayed as described previously (Beffa et al. 1993), except that protein extracts were preincubated with antibody for 10 min at 37 °C followed by 2 h on ice.

Results

Distinct ethylene- and tissue-specific accumulation of β-1,3-glucanase and chitinase activities during pea seed germination. In an initial experiment we examined the effect of ethylene treatment on the accumulation of βGlu and Chn during germination of *Pisum sativum* L. cv. 'Espresso generoso' seeds. Under our standard conditions, i.e. imbibition in the dark at 20 °C, there was an early increase in ethylene evolution after 15–20 h, followed by radicle protrusion through the testa, which started at ca. 30 h and was complete by 50 h (Petruzzelli et al. 1995). Imbibed seeds were incubated for increasing intervals of time and were treated with 40 µl/l of ethylene for the last 24 h prior to harvesting. Ethylene treatment did not affect either the incidence or time of germination (data not shown). After harvesting, the seeds were dissected into the embryonic axis, the fleshy storage cotyledons, and the testa, which were then assayed for βGlu and Chn activities.

Figure 1A shows the time courses for βGlu and Chn activities in the embryonic axis expressed on a protein basis. The activity of βGlu in embryonic axes increased ca. 4-fold during the first 20 h after the start of imbibition, remained approximately constant from the onset of ethylene evolution at 20 h until the completion of radical emergence at 50 h, and then showed a second increase of ca. 4-fold at the last, 65-h time point (Fig. 1A). Ethylene treatment markedly increased βGlu accumulation after 50 h by up to 12-fold. The activity of Chn in embryonic axes was approximately constant until the onset of radicle emergence at ca. 30 h and then increased by ca. 1.5-fold (Fig. 1A). Accumulation of Chn activity was not affected by ethylene treatment.

The activity of βGlu and Chn detected in cotyledons was roughly 10-fold less than that detected in the embryonic axis (Fig. 1B). The βGlu activity increased ca. 3-fold until the completion of radicle emergence at 50 h, and then appeared to decrease slightly and was not affected by ethylene treatment. The Chn activity in cotyledons remained approximately constant during imbibition and was not affected by ethylene treatment. Enzyme activities in the testa (data not shown) were low and showed a pattern of accumulation similar to that of cotyledons.

To determine if early and late enzyme accumulation depended on ethylene, seeds were treated from 0 to 24 h or from 41 to 65 h with combinations of ethylene and 2,5-norbornadiene (NBD), which inhibits ethylene action (Sisler and Pian 1973). Table 1 shows that NBD markedly inhibited late induction of βGlu in the embryonic axis and that this effect was partially reversed by simultaneous treatment with ethylene. This strongly suggests that late induction of βGlu in the embryonic axis is an ethylene-dependent process. In contrast, NBD treatment (Table 1) did not have an appreciable effect on the early accumulation of βGlu in the embryonic

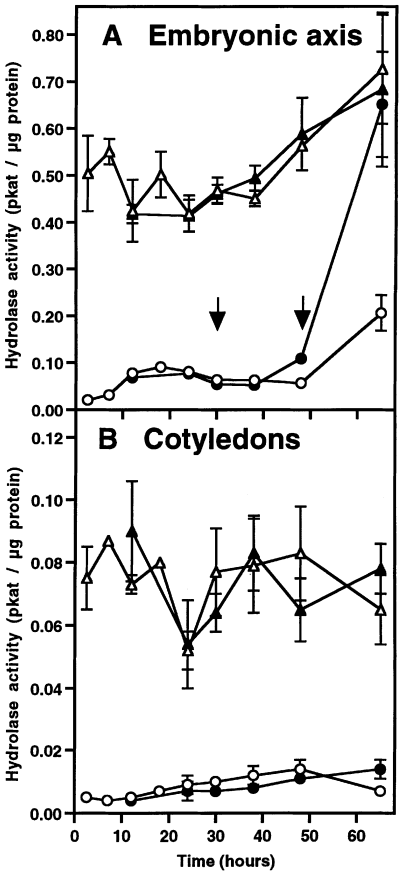

Fig. 1A,B. The effect of ethylene on the time courses of βGlu and Chn activities in embryonic axis (**A**) and cotyledons (**B**) during pea seed germination. The enzyme activities of βGlu (○, ●) and Chn (△, ▲) are expressed in pkat/µg protein. Seeds were incubated at 20 °C in the dark in the absence (control, *open symbols*) or presence of 40 µl/l ethylene (*closed symbols*) for the 24-h period before harvest (24- to 65-h time points) or for the entire incubation time (2- to 24-h time points). *Arrows* indicate the onset and the completion of radicle emergence. Mean values ±SE of three samples from one of two independent experiments are presented. When error bars are not shown, the largest SE values are ±0.006 (**A**) and ±0.003 (**B**)

axes, on the accumulation of βGlu in cotyledons, or on the accumulation of Chn in either tissue.

Taken together the results show that βGlu and Chn exhibit distinct patterns of regulation during imbibition. The Chn content remains approximately constant in cotyledons and in the embryonic axis before the onset of radicle emergence and does not appear to depend on ethylene. In contrast, βGlu in embryonic axes, but not in cotyledons, appears to show a late period of accumulation after radicle emergence is complete, which depends on ethylene, and an early period of accumulation in both tissues, which does not depend on ethylene.

An ethylene-regulated, embryonic axis-specific 34.5-kDa βGlu is induced after radicle emergence. Immunoblot analysis was used to partially characterize βGlu isoforms induced during pea seed germination. The blots were stained with antibody specific for the ethylene-inducible, 36-kDa class I βGlu of bean leaves (Vögeli et al. 1988; Mauch et al. 1992). To determine if the anti-bean class I βGlu antibody cross-reacts with βGlu responsible for the enzyme activity found in pea seeds, βGlu activity was compared in protein extracts incubated with and without the antibody (Table 1). The bean antibody inhibited 88% and 95% of the enzyme activity in extracts of embryonic axes harvested after 24 h and 65 h, respectively. This indicates that in the embryonic axis, essentially all the late activity and most of the early activity is due to βGlu isoforms detected by the antibody. In contrast, only 56–57% of the low levels of βGlu found in cotyledons was inhibited, indicating that this activity is due to both cross-reacting and serologically different isoforms of βGlu.

Figure 2A,B shows immunoblots of protein extracts from germinating pea seeds probed with anti-bean class I βGlu antibody. The major immunoreactive band was a 34.5-kDa antigen localized exclusively in the embryonic axis after germination (Fig. 2A). The appearance of this band was correlated with the late, ethylene-responsive βGlu activity (Fig. 1A). The 34.5-kDa immunoreactive band was also correlated with βGlu activity after treatments with combinations of NBD and ethylene (Fig. 2A, Table 1). These results suggest that the ethylene-dependent βGlu activity found in the embryonic axis late in imbibition can be accounted for by the 34.5-kDa antigen.

Table 1. Effect of ethylene and NBD treatment on the accumulation of βGlu and Chn enzyme activities in germinating pea seeds

Treatment[a]	Embryonic axis				Cotyledons			
	βGlu		Chn		βGlu		Chn	
	24 h	65 h	24 h	65 h	24 h	65 h	24 h	65 h
Control	81 ± 6[b]	206 ± 38	419 ± 38	726 ± 116	9 ± 3	7 ± 0	52 ± 6	65 ± 11
	(88 ± 1)[c]	(95 ± 1)	(99 ± 1)	(99 ± 1)	(56 ± 3)	(57 ± 2)	(95 ± 2)	(98 ± 2)
Ethylene	77 ± 6	651 ± 112	414 ± 34	683 ± 164	7 ± 3	14 ± 3	54 ± 14	78 ± 8
NBD	63 ± 3	70 ± 7	397 ± 14	534 ± 31	6 ± 1	10 ± 1	47 ± 4	60 ± 19
Ethylene + NBD	72 ± 7	139 ± 13	347 ± 16	687 ± 19	6 ± 1	6 ± 2	40 ± 3	65 ± 8

[a]Treated, as indicated, with 40 µl/l ethylene and 100 µl/l NBD for 24 h prior to harvest
[b]Mean activity (fkat/µg protein) ± SE in 3 replicate extracts prepared from 10 embryonic axes or 1 cotyledon 24 and 65 h after the start of imbibition
[c]Mean % inhibition of enzyme activity ± SE in replicate extracts preincubated with 10 µg anti-bean class I antibody for βGlu or Chn, as indicated

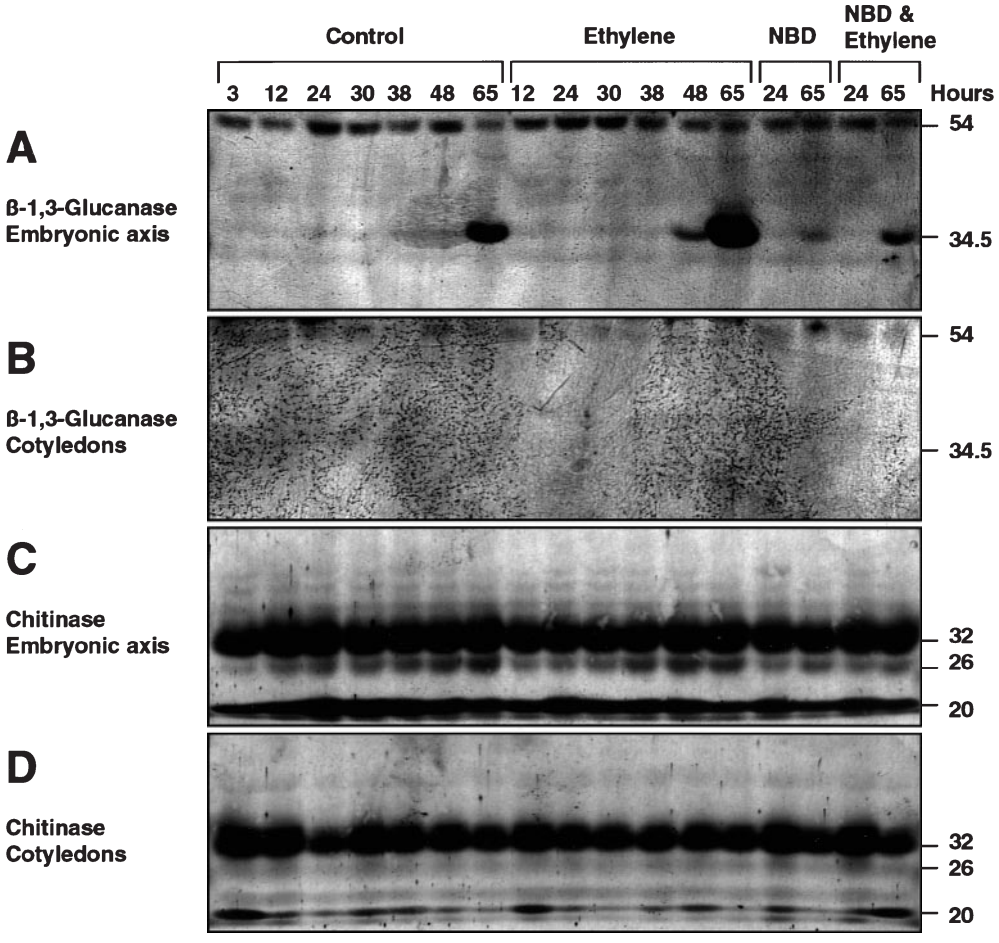

Fig. 2A–D. Immunoblot analyses of βGlu and Chn antigens during the germination of pea seeds in the absence (control) or presence of ethylene or NBD. Seeds were incubated at 20 °C in the dark and treated as indicated with 40 μl/l ethylene and 100 μl/l NBD for the 24-h period before harvest. The antigens of the pea seed extracts (100 μg protein per lane) were detected with antibodies directed against bean class I βGlu (**A,B**) and Chn (**C,D**). The apparent size in kDa of immunoreactive bands is indicated at the right

A 54-kDa immunoreactive band was detected in both embryonic axis and cotyledons (Fig. 2A,B). This antigen did not change appreciably with time, did not show ethylene-dependent regulation, and was present at lower abundance in the cotyledon tissue. Immunoinactivation studies with the same antibody suggest that the 54-kDa antigen could account for almost all of the early βGlu activity in the embryonic axis and ca. 50% of the low-level βGlu activity in the cotyledons.

Tissue-specific and ethylene-independent expression pattern of putative chitinase isoforms during pea seed germination. We used an anti-bean class I Chn antibody, which detects an ethylene-inducible 32.5-kDa class I Chn, and two additional 20-kDa and 40-kDa antigens in bean leaves (Vögeli et al. 1988; Mauch et al. 1992), to partially characterize Chn isoforms present in germinating pea seeds. This antibody completely inhibited the Chn activity in extracts of embryonic axes and cotyledons harvested 24 h and 65 h after the start of imbibition (Table 1). Thus, all Chn activity present in both tissues is due to Chn isoforms detected by the antibody.

Figure 2C,D shows that three major immunoreactive bands at the 20-, 26- and 32-kDa positions are present in extracts of germinating embryonic axes and cotyledons. The 32-kDa antigen in the embryonic axis did not change with time. In contrast, the weaker 26-kDa antigen started to increase late in germination and this increase was correlated with the increase in Chn activity (Fig. 1A). The 32-, 26-, and 20-kDa antigens were also present, but at lower abundance, in cotyledons. Figure 2D shows that the strong 32-kDa signal and the weak 26-kDa signal did not change with time. In contrast, the 20-kDa antigen declined rapidly between 12 and 24 h after the start of imbibition and thereafter remained at a low level. Comparison of the immunoblot analyses and measurements of Chn activity obtained after treatment with ethylene or ethylene inhibitor (Table 1) and the results of immunoinactivation assays suggest that none of the Chn antigens present during seed germination are ethylene-responsive.

Discussion

Regulated expression of βGlu and Chn isoforms during pea seed germination. Our most important finding was that βGlu and Chn show novel patterns of regulation during the germination of pea seeds. Low levels of βGlu activity were detected in the embryonic axis early in germination. The strong increase late in germination after completion of radicle emergence depends on ethylene. Several βGlu isoforms have been characterized from pea leaves, roots, stems, and immature pea pods (siliques) (Wong and Maclachlan 1979, 1980; Mauch

et al. 1984, 1988a,b; Chang et al. 1992, 1993; Dassi et al. 1996). We propose that the increased βGlu activity late in germination is due to the class I βGlu G2 reported for immature pea pods (Mauch et al. 1984, 1988a,b). Several lines of evidence support this conclusion: First, the late induction of βGlu activity is tightly correlated with the timing and response of the 34.5-kDa antigen to ethylene. Second, this activity was almost completely inhibited by an anti-bean βGlu antibody shown to be highly specific for the class I isoforms (Vögeli et al. 1988; Mauch et al. 1992). Finally, both the 34.5-kDa βGlu in the embryonic axis and βGlu G2 are induced by ethylene and have similar apparent molecular masses (Mauch et al. 1984, 1988a).

Compared with the testa and cotyledons, the embryonic axis also contained high concentrations of a 54-kDa βGlu antigen. The content of this antigen did not change during germination and did not depend on ethylene. The finding that βGlu activity of embryonic axes early in germination is inhibited by anti-βGlu antibody when the 34.5-kDa antigen is not detectable, indicates that the early activity might be due to the 54-kDa antigen. Although measurements were made near the limit of detection, ca. 54% of the βGlu activity found in cotyledons was not inhibited by anti-bean βGlu antibody. Thus, at least part of the low-level βGlu activity in cotyledons is due to serologically distinct isoforms.

The highest Chn activity, like that of βGlu activity, was detected in the embryonic axis, but the pattern of regulation of the two enzymes differed. The Chn activity did not change markedly during germination in either the embryonic axis or the cotyledons, and was not dependent on ethylene. Immunoinactivation studies and immunoblot analyses suggest that Chn activity is due to one or more of the three major 32-, 26- and 20-kDa Chn antigens present in both tissues. Based on the correlation of antigen content and Chn activity, it seems likely that the late, ethylene-insensitive increase in Chn activity in the embryonic axis is due to the less-abundant (or less immunoreactive) 26-kDa antigen, whereas most of the activity, which remains constant during germination may be due to the more-abundant 32- and 20-kDa antigens. Assignment of these antigens to known pea Chn is complicated by differences in the apparent molecular masses of homologs in different pea cultivars (Dumas-Gaudot et al. 1994). The 26-kDa antigen might correspond to the 25-kDa basic Chn isozyme B from leaves (Vad et al. 1991) and the 27-kDa Chn found in roots (Dumas-Gaudot et al. 1994). The identity of the 32-kDa antigen is unclear, since it differs in molecular mass and ethylene-inducibility from Chn found in immature pea pods (Mauch et al. 1984, 1988a). One possibility is that the 32-kDa antigen may be a homolog of the acidic 34-kDa class IV P4-Chn expressed in germinating bean seeds (Margis-Pinheiro et al. 1994).

Ethylene-responsiveness of βGlu and Chn induction changes during seed germination. Ethylene coordinately induces class I βGlu and Chn in immature pea pods (Mauch et al. 1984, 1988a) and in leaves of several plant species (Felix and Meins 1987; Boller 1988; Vögeli et al. 1988; Samac et al. 1990; Mauch et al. 1992; Hart et al. 1993). In pea seeds, detectable ethylene production begins 15–20 h after the start of imbibition (Gorecki et al. 1991; Petruzzelli et al. 1995). Nevertheless, ethylene-dependent induction of 34.5-kDa βGlu starts considerably later, after 50 h, and is limited to the embryonic axis. This finding, as well as results showing that other βGlu antigens and Chn are not induced by ethylene treatment, suggests that responsiveness to ethylene depends on the stage of germination and is tissue-specific. In the case of tobacco seed germination, class I βGlu, but not other βGlu isoforms or Chn isoforms are transcriptionally induced during germination; and, class I βGlu induction is highly localized in the micropylar endosperm (Leubner-Metzger et al. 1995, 1998).

A putative ethylene receptor and several components of the ethylene signalling pathway have been identified (review: Fluhr 1998). Far less is known about the developmental regulation of ethylene-responsiveness with regard to glucanohydrolase expression. Ethylene-sensitivity of class I Chn induction is developmentally regulated in an organ-specific and age-dependent manner in soybean and *Arabidopsis* (Samac et al. 1990; Xie et al. 1996). Of particular interest is the finding that high-level expression of class I βGlu depends on the interaction of ethylene-responsive elements (ERE) with ethylene-responsive-element binding proteins (EREBP), which are transcription factors believed to be targets of ethylene-dependent signal transduction (Ohme-Takagi and Shinshi 1995; Leubner-Metzger et al. 1998). Class I Chn genes, which are transcriptionally induced by ethylene and have EREs in their promoters, are likely to be regulated in the same way (Hart et al. 1993; Shinshi et al. 1995). There is evidence that induction of high levels of βGlu during germination of tobacco seeds depends on ethylene-induced transcription mediated by EREBP-3, whereas ethylene-independent signalling pathways acting on proximal promoter elements determine spatial and temporal patterns of expression (Leubner-Metzger et al. 1998). We speculate that similar pathways could account for tissue-specific and temporal regulation of βGlu and Chn expression in pea seeds.

Possible functions of βGlu and Chn in seed germination. The function of the multiple isoforms of βGlu and Chn induced during seed germination is not known. The limiting step in germination of many seeds is the testa, the endosperm, or both covering layers (reviews: Black 1996; Bewley 1997). In the case of photodormant tobacco seeds, the limiting step is endosperm rupture, which occurs after testa rupture is complete (Arcila and Mohapatra 1983; Leubner-Metzger et al. 1995, 1996). There is strong, but indirect, evidence that class I βGlu promotes endosperm rupture and radicle protrusion in tobacco, leading to the hypothesis that βGlu activity contributes to the hydrolysis of cell wall components resulting in endosperm weakening at the site of radicle penetration (Leubner-Metzger et al. 1995).

The situation is quite different in the case of pea seed germination. Unlike tobacco, pea is a non-endospermic

species, the seeds are non-dormant, and covering layers are not a mechanical constraint to radical protrusion (review: Bewley and Black 1994; Gorecki et al. 1991; Petruzzelli et al. 1995). High constitutive levels of Chn are present in cotyledons and the embryonic axis. Thus, after the completion of radicle emergence, when βGlu is induced in the embryonic axis, antifungal combinations of the enzymes might be present. Both βGlu and Chn could be part of a preemptive strategy to protect seeds against microbial attack, as has been proposed for cereal grains (Fincher 1989). Testing this hypothesis and the possibility that these enzymes have developmental functions, as has been proposed for a specific βGlu isoform in germinating maize kernels (Cordero et al. 1994), are interesting areas for future study.

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