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Ethylene responsive element binding protein (EREBP) expression and the transcriptional regulation of class I ß-1,3-glucanase during tobacco seed germination

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Abstract

Class I ß-1,3-glucanase (ßGLU I) is transcriptionally induced in the micropylar endosperm just before its rupture prior to the germination (i.e., radicle emergence) of *Nicotiana tabacum* L. cv "Havana 425" seeds. Ethylene is involved in endosperm rupture and high-level ßGLU I expression; but, it does not affect the spatial and temporal pattern of ßGLU I expression. A promoter deletion analysis of the tobacco ßGLU I B gene suggests that (1) the distal -1452 to -1193 region, which contains the positively-acting ethylene-responsive element (ERE), is required for high-level, ethylene-sensitive expression, (2) the regions -1452 to -1193 and -402 to 0 contribute to down-regulation by abscisic acid (ABA), and (3) the region -402 to -211 is necessary and sufficient for low-level micropylar-endosperm specific expression. Transcripts of the ERE binding proteins (EREBPs) showed a novel pattern of expression during seed germination: light or gibberellin was required for EREBP-3 and EREBP-4 expression; EREBP-4 expression was constitutive and unaffected by ABA or ethylene; EREBP-3 showed transient induction just before endosperm rupture, which was earlier in ethylene-treated seeds and inhibited by ABA. No expression of EREBP-1 and EREBP-2 was detected. In contrast to ßGLU I, EREBP-3 and EREBP-4 were not expressed specifically in the micropylar endosperm. The results suggest that transcriptional regulation of ßGLU I could depend on: activation of ethylene signalling pathways acting via EREBP-3 with the ERE as the target, and ethylene-independent signalling pathways with targets in the proximal promoter region that are likely to determine spatial and temporal patterns of expression.

Introduction

ß-1,3-Glucanases (glucan endo-1,3-ß-glucosidase, EC 3.2.1.39) are abundant, highly regulated hydrolytic enzymes widely distributed in seed plants (review [37]). There is considerable evidence to suggest that ß-1,3-glucanases are part of the defense of plants against pathogenic fungi (reviews [2, 6]). They are also thought to have important functions in diverse physiological and developmental processes including pollen development [7, 46], mobilization of storage reserves in the endosperm [13], and seed germination [8, 21, 22, 43].

This report deals with tobacco class I ß-1,3-glucanases (ßGLU I) (reviews [2, 25]). These ß-1,3-glucanases show complex hormonal, developmental, and pathogenesis-related regulation. They accumulate in high concentrations in the roots and in lower leaves [42, 44]. They are up-regulated in leaves by treatment with ethylene [12, 16] and ozone [10, 41] and in response to pathogen infection [23, 42, 44]. They are down-regulated in cultured cells and in leaf disks by combinations of cytokinin and auxin [27, 44] and in germinating seeds by ABA treatment [22]. Measurements of steady-state mRNA levels and reporter gene experiments provide strong evidence that many of these responses, including induction by ethylene and tissue specific localization are regulated, at least in part, at the transcriptional level (e.g. [22, 44]).

Several tobacco ßGLU I genes have been partially characterized, including the closely related homeologues *Gla* and *Glb* cloned from Havana 425 tobacco [40] and *gln2* cloned from Bright Yellow tobacco, which is identical to *Gla* [29]. Promoter deletion analyses of the promoters indicate that a distal region, corresponding to positions -1193 to -1452 of *Glb*, is required for high levels of expression in leaves. This region contains a 61-bp enhancer of transcription that binds ethylene-inducible proteins present in nuclear extracts of leaves [16, 44]. A slightly modified 49-bp sequence from the comparable enhancer region of the *gln2* promoter is an ethylene-responsive element (ERE) essential for ethylene responsiveness when combined with a minimal cauliflower mosaic virus 35S RNA promoter [30]. Enhancer activity, ethylene responsiveness, and binding of nuclear proteins depend on the integrity of two copies of the AGC box, AGCCGCC, present in the promoters of several ethylene-responsive genes. cDNA clones have been identified representing 4 novel DNA-binding proteins, called ethylene-responsive element binding proteins (EREBPs), that specifically bind the ERE AGC box [30]. Accumulation of mRNAs for these EREBPs is induced by ethylene treatment. Thus, it is likely that the EREBPs are transcription factors important for ethylene-dependent, high-level transcription of ßGLU I genes.

Earlier we showed that ßGLU I is transcriptionally induced prior to germination (i.e., radicle emergence) of *Nicotiana tabacum* L. cv. "Havana 425" seeds [22]. Induction started just prior to endosperm rupture (but after testa rupture) and was localized exclusively in the micropylar region of the endosperm where the radicle will penetrate. In the present report, we have examined the possible role of ethylene and EREBPs in the transcriptional regulation of ßGLU I during seed germination. Ethylene and regions of the *Glb* promoter containing the ERE were required for high level induction; but, they did not affect temporal and spatial expression. EREBP mRNAs showed a novel pattern of expression in germinating seeds. In contrast to leaves and roots, neither EREBP-1 nor EREBP-2 were expressed during seed germination, and ethylene did not stimulate expression levels of EREBP-3 and EREBP-4.

Materials and methods

Plant materials

Seed of *Nicotiana tabacum* L. cv. Havana 425 were used 1-2 years after harvest. The line GLB-GUS, which is homozygous for the transgene, was transformed with a construct containing the "full-length" ca. 1.6 kb of the 5' flanking sequence of *Glb* from -1630 to +6 fused to the GUS reporter gene and has been described [43]. Deletion analyses were performed with monogenic transformants carrying a series of 5'-deleted *Glb*-promoter fragments fused to *GUS*. Seed was obtained by selfing the two high-, two medium- and one low-expressing primary transformants for each construct described by Vögeli-Lange *et al.* [44].

Germination experiments

Transgenic seeds (100-200) were sown in 9-cm-diameter plastic Petri dishes containing a double layer of filter paper (MN713, Machery-Nagel, Düren, Germany). The paper was wetted with 6 ml of autoclaved rooting medium consisting of dilute inorganic nutrients and thiamine [24], supplemented with 50 µg/ml kanamycin sulfate (Serva, Heidelberg, Germany), 100 µg/ml Claforan (Hoechst-Pharma AG, Zürich, Switzerland), and, where indicated, 10 µM *cis*-(±)-abscisic acid (ABA; Sigma, St. Louis, Mo., USA) and 10 µM gibberellin A_4 (GA_4; Sigma). Supplements were added as neutralized, filter-sterilized stock solutions. No microbial contamination was visible after prolonged incubation of seeds on the antibiotic-containing medium used,

and surface-sterilized and antibiotic-treated seeds gave comparable patterns of ßGLU and GUS activity. Petri dishes were sealed with Parafilm and incubated at 25 °C, as indicated in continuous white light (3000 lux, Philips 'TL'D 35W/33 lamps) or in the dark. Petri dishes incubated in the dark were set up under dim light. To score different stages of germination, seeds from two to three Petri dishes were counted for each time point. Plant material was harvested and stored at -80 °C for subsequent analysis.

In ethylene experiments, open 2-cm-diameter plastic Petri dishes were incubated in gas-tight 150-ml Erlenmeyer flasks fitted with rubber caps. Ethylene (Siad, Italy) was injected through the septum of the rubber caps at the start of imbibition to give a final concentration of 100 ppm. To reduce the ethylene concentrations in the control flasks, the air was first passed through a Dreschel bottle containing silica gel coated with $KMnO_4$ as an ethylene absorbent, and a small tube with this absorbent was placed inside the flasks. The ethylene concentration in the flasks were confirmed by gas-chromatography. Where indicated, 1 mM (final concentration) aminoethoxyvinylglycine (AVG; Sigma) was added to the medium and liquid 2,5-norbornadiene (NBD; Aldrich Chemical Co, Buchs, Switzerland) was injected into a small glass tube inside the flasks to give a final concentration in the gas phase of 50 ppm.

Analysis of proteins

Procedures for extracting proteins, assays for enzyme activity, immunoblot analysis, and protein determination have been described previously [22]. In brief, ß-1,3-glucanase activity was assayed radiometrically using [^{3}H]-laminarin as the substrate. ß-Glucuronidase activity was measured fluorometrically with 4-methylumbelliferyl-ß-D-glucuronide and histochemically with 5-bromo-4-chloro-3-indolyl glucuronide as the substrates. The rabbit anti-tobacco ß-1,3-glucanase antibody used for immunoblot analysis detects the class I, class II and class III isoforms of the enzyme [3, 28].

Analysis of RNA

Preparation of total RNA and RNA-blot hybridization were as described by Leubner-Metzger *et al.* [22]. The probes were radiolabelled with [α-^{32}P]dCTP by random priming (rediprime kit; Amersham, Buckingshamshire, UK) using as template DNAs the 1-kb PstI fragment of the tobacco class I ß-1,3-glucanase cDNA pGL43 [36], the ca. 1-kb XbaI-EcoRI fragments of the cDNAs of EREBP-1, EREBP-2, EREBP-3, and EREBP-4 [30], and the 1.8 kb EcoRI fragment of genomic DNA encoding tomato 18S ribosomal RNA [34]. 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

Ethylene is involved in seed germination and endosperm-specific induction of ßGLU I

Earlier studies suggest that ethylene is required for the germination of tobacco seeds [20, 39]. In initial experiments we confirmed this effect for the tobacco variety used in our studies (Table 1) and examined the possibility that ethylene treatment induces ßGLU I during seed germination. Figure 1a shows that endosperm rupture started 6-10 h earlier in GLB-GUS seeds treated with 100 ppm ethylene than in control seeds without ethylene. The timing of ßGLU I induction was established by measuring enzyme activity, ßGLU I antigen and mRNA, as well as the GUS resulting from activity of the *Glb* promoter. Accumulation of ß-1,3-glucanase and GUS activity started 6-10 h earlier, and higher levels were induced with ethylene treatment (Fig. 1b). The earlier onset of endosperm rupture and ß-1,3-glucanase induction was confirmed in two independent experiments each with triplicate samples.

Figure 2a shows immunoblot analyses of ß-1,3-glucanase induction obtained with antibody that reacts with all known tobacco ß-1,3-glucanases [3, 28]. The only major antigen detected corresponded to the ca. 33 kDa class I isoform (Fig. 2a). Accumulation of this antigen started >6 h earlier and reached higher induction levels in ethylene-treated populations of seeds. These results confirm that only the class I isoform is induced just prior to endosperm rupture [22, 43] and show that ethylene treatment did not induce any additional isoforms of the enzyme.

Seeds of Havana 425 tobacco exhibit photodormancy, i.e. most seeds in the population do not germinate when imbibed in darkness [21]. Table 1 shows that roughly 95% of seeds imbibed for 80 h in darkness were photodormant. Under these conditions, ßGLU I is localized exclusively in the micropylar endosperm of the small portion of germinated, non-dormant seeds [22]. Ethylene treatment increased the ß-1,3-glucanase and GUS contents of the small proportion of non-dormant seeds, but not of the dormant seeds. These results show that although ethylene treatment induces ß-1,3-glucanase in the non-dormant seeds, it does not break photodormancy.

To detect effects of ethylene on the spatial pattern of ß-1,3-glucanase induction, GLB-GUS seeds with ruptured testas but intact endosperms were harvested 54 h after the start of imbibition in the light with and without ethylene. The embryos and covering

Table 1. Effect of ethylene (100 ppm) on ß-1,3-glucanase and *Glb* promoter activity in *Glb-GUS* tobacco seeds

		ß-1,3-Glucanase (pkat / seed)		GUS (fkat / seed)		Endosperm rupture (%)	
Incubation conditions	Seed component [1]	Control	Ethylene	Control	Ethylene	Control	Ethylene
54 h light						0.2 ± 0.2	8.2 ± 0.8
	Embryo	0.12 ±0.00[2]	0.16 ± 0.00	0.02 ± 0.01	0.02 ± 0.00		
	Endosperm	1.22 ± 0.02	2.07 ± 0.11	0.61 ± 0.03	1.59 ± 0.16		
65 h light [3]							
No addition	Entire seed	1.65 ± 0.09	3.00 ± 0.31	1.00 ± 0.14	3.27 ± 0.36	79.3 ± 3.5	87.5 ± 1.6
1 mM AVG	Entire seed	0.62 ± 0.07	1.32 ± 0.02	0.25 ± 0.09	0.67 ± 0.00	12.6 ± 4.0	59.1 ± 0.6
50 ppm NBD	Entire seed	1.04 ± 0.10	2.43 ± 0.00	0.59 ± 0.05	1.91 ± 0.06	17.2 ± 2.6	62.8 ± 0.4
80h darkness						3.5 ± 0.9	4.7 ± 0.7
	Entire dormant seed	0.10 ± 0.01	0.17 ± 0.05	0.02 ± 0.01	0.03 ± 0.00		
	Entire non-dormant seed	1.45 ± 0.31	3.40 ± 0.92	0.30 ± 0.02	0.93 ± 0.19		

1 Batches of 100 dissected or entire seeds were assayed. Samples of embryos and endosperms were obtained from seeds with intact endosperms. Note that dormant seeds have intact testas and intact endosperms.

2 Mean values ± SE for at least two independent experiments.

3 Testa rupture ≥ 96 % in all 65 h light samples.

layers consisting of the endosperm with adhering remnants of the seed coats were then assayed separately. Table 1 shows that accumulation of ß-1,3-glucanase and GUS activity as well as induction of these activities by ethylene treatment was associated with the covering layers of the seed. Histochemical assays of GUS activity, not shown, established that induction of *Glb*-promoter activity was localized in the micropylar region of the endosperm both in control and ethylene-treated seeds. Thus, ethylene treatment enhances induction but does not change the spatial pattern of *Glb*-promoter activity.

We examined the effect of the inhibitor of ethylene synthesis, aminoethoxyvinylglycine (AVG; [31]), and the inhibitor of ethylene action, 2,5-norbornadiene (NBD; [38]), on germination and ßGLU I induction in Glb-GUS seeds. Measurements were made after 65 h when ca. 80 % of the seeds had ruptured endosperms in control populations, and ßGLU I is localized exclusively in the micropylar endosperm [22]. Additional unpublished results show that ßGlu I first appears in roots considerably later, ca. 90 hours after imbibition at the late root-hook stage. At the times tested in the present experiments, ß-1,3-glucanase and GUS activities of entire seeds therefore reflect ßGLU I localized exclusively in the micropylar endosperm. Table 1 shows that 1 mM AVG inhibited accumulation of ß-1,3-glucanase (62%) and GUS activity (75%) as well as the per cent of seeds with ruptured endosperms (84%). Relative to ethylene-treated controls, ethylene treatment partially reversed the effect of AVG by 44% for ßGLU, 21% for GUS activity, and 68% for the incidence of endosperm rupture. NBD has a narrow effective concentration range [12]. At a concentration of 50 ppm, NBD inhibited ßGLU by 40%, GUS by 41%, and endosperm rupture by 78%. Ethylene partially reversed these effects by 81% for ßGLU, 58% for GUS, and 72% for endosperm rupture. These results show that although AVG and NBD have some nonspecific inhibitory effects; inhibition was reversed, but only partially, by simultaneous treatment with 100 ppm ethylene. More than 96 % of the seeds treated with 1 mM AVG germinated when incubated longer than 80 h; and, more than 96 % of the seeds treated for 65 h with 50 ppm NBD germinated when incubated longer than 100 h

(data not shown). These results indicate that the concentrations of the inhibitors used did not kill the seeds. Taken together this provides evidence that ethylene is required both for high levels of ßGLU I expression in the micropylar endosperm and for endosperm rupture.

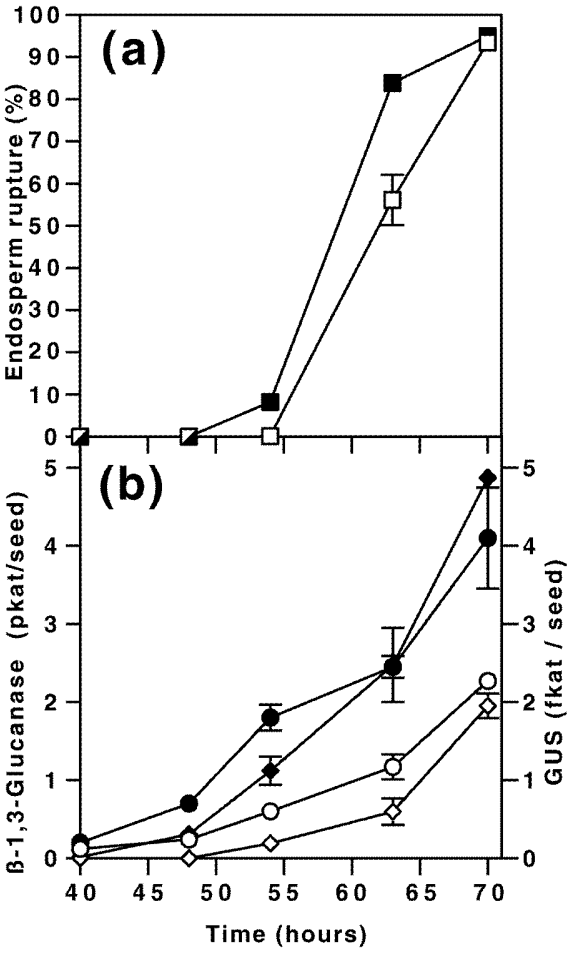

Figure 1. The effect of ethylene treatment on ß-1,3-glucanase accumulation and germination of GLB-GUS tobacco seeds in the light. Populations of 100-200 seeds were imbibed in continuous light either in the absence (open symbols) or presence of 100 ppm ethylene (filled symbols). Means values ± SE are presented for two independent experiments each with at least two samples (triplicate samples for 48, 54, and 63 h). When error bars are not shown, the SE values were ± 2 in (a) and ± 0.1 in (b). (a) The incidence of endosperm rupture (□ and ■). The onset of endosperm rupture was consistently earlier in populations of ethylene-treated seeds in the two independent experiments. (b) The accumulation of ß-1,3-glucanase, expressed as pkat enzyme activity / seed (○ and ●) and *Glb*-promoter activity expressed as fkat GUS / seed (◇ and ◆).

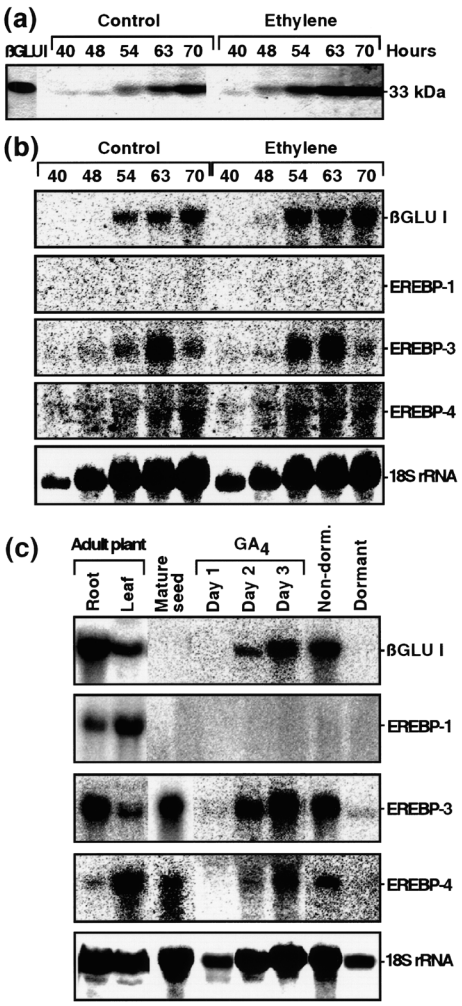

Figure 2. EREBP- and ßGLU I-mRNA expression during seed germination and in mature seeds, roots, and leaves of Glb-GUS tobacco. (a) Immunoblots of seed extracts (80 µg protein/lane) probed with an antibody that recognizes all known tobacco ß-1,3-glucanases. Seeds were imbibed in continuous light either in the absence (Control) or presence of 100 ppm ethylene. The onset of endosperm rupture was ca. 58 h in control and ca. 50 h in ethylene-treated seed populations. ßGLU I, 10 ng of the authentic 33 kDa tobacco enzyme. (b) RNA-blot hybridization of total RNA (25 µg/lane) prepared from seeds germinated under the conditions indicated in (a). The blots were hybridized with probes for ßGLU I, EREBP-1, EREBP-3, EREBP-4, and 18S rRNA used as a loading standard. The recovery of 18S rRNA was consistently reduced early in germination. (c) RNA-blots of total RNA (20 µg/lane) were hybridized with probes for ßGLU I, EREBP-1, EREBP-3, EREBP-4, and 18S rRNA. Seeds were imbibed in the dark with 10 µM GA_4 added; endosperm rupture was 19.7 % (day 3) and 0 % (days 1 and 2). Photodormant (Dormant) and non-photodormant (Non-dorm.) seeds without added GA_4 were sampled 4 days after the start of imbibition in the dark. Root and leaf RNA were extracted from an adult plant (4 months old, ca. 1 meter in height). With the exception of mature seed RNA, the samples are on the same blot.

Functional analysis of Glb-promoter activity during germination

Transformants representing a series of promoter deletions fused to the *GUS* reporter gene were used to identify regions of the *Glb* promoter important for micropylar-endosperm specific expression of ßGLU I. Seeds obtained by selfing the five monogenic transformants for each construct, described by Vögeli-Lange *et al.* [44], were imbibed in light for 68 h. Extracts were prepared and fluorometrically assayed for GUS activity. The results are expressed as GUS activity per seed. Similar results were obtained when the data were expressed on a protein basis.

Figure 3a shows that deletion of the region from -1452 to -1193 resulted in a marked drop in GUS activity indicating that this region is important for *Glb*-promoter activity during seed germination. GUS

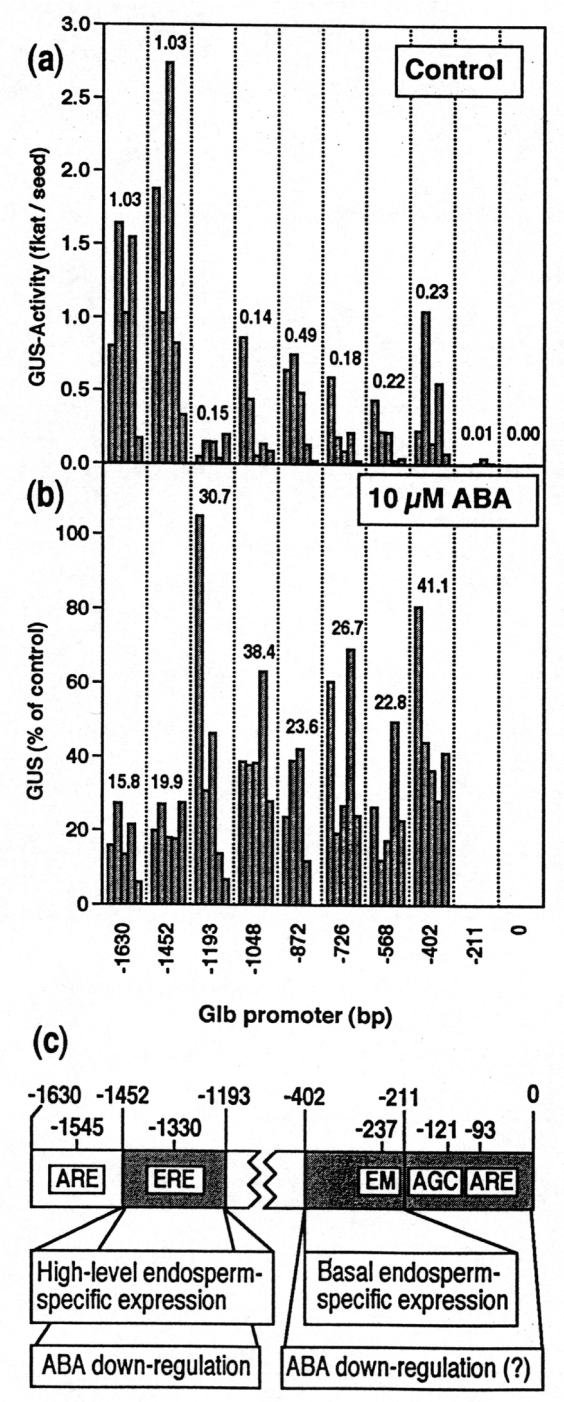

Figure 3 Deletion analysis of the the *Glb* promoter in germinating Havana 425 tobacco seeds. (a) GUS activity (fkat/seed) of control seeds. (b) Down-regulation by 10 µM ABA expressed as % GUS activity relative to the corresponding control populations of seeds shown in (a). Seeds of a *Glb-GUS* promoter deletion series with 5 independent transformants for each deletion were imbibed in continuous light with and without 10 µM ABA. Extracts were prepared from seeds after 3 days when ca. 70 % of controls and none of ABA treated seeds had ruptured endosperms. The extracts were assayed for GUS activity. For each construct, bars in the same relative position represent seed of the same independent transformant shown in the order: 2 high-, 2 medium-, and 1 low-expressing line as judged from tests of the transformants described by Vögeli-Lange *et al.* [44]. Each bar represents the mean value, usually of replicate assays, in two independent experiments. The numbers above the bars are the median value obtained with each deletion. (c) Diagram illustrating regions of the *Glb* promoter relevant to regulation in germinating seeds. Filled boxes: The distal region (-1452 to -1193) important for high-level expression and ABA down-regulation; the proximal region -402 to -211 necessary and sufficient for low-level expression in the micropylar endosperm; and, the proximal region downstream of -402 that may also contribute to ABA down-regulation. Symbols: ARE, an ABA-responsive element (TAACAAA) important for down-regulation by ABA of α-amylase genes in barley endosperm [15]; AGC, one copy in inverse orientation of the AGC box (AGCCGCC) present as two copies (-1346 and -1314) in the ERE [16]; EM, an EM box (TGTAAAG) important for endosperm-specific expression of cereal storage-protein genes in tobacco [4]; and, ERE, the *Glb* ethylene-responsive element [30]. Numbers refer to position relative to the start of transcription.

values obtained with deletions in the region from -1048 to -211 were roughly 3 to 4-fold lower than the values obtained with the full length 1630 bp promoter. Essentially background GUS activity was detected for the -211 promoter. These results indicate that the region from -402 to -211 is required for promoter activity. Activity of the full length 1630 bp promoter is exclusively localized in the micropylar endosperm [22, 43]. Fluorometric and histochemical measurements of GUS showed that activity of the minimal -402 promoter had the same localization (data not shown). Thus, the region -402 to -211 is necessary and sufficient for proper spatial

expression of the *Glb* promoter during seed germination.

Endosperm-specific *Glb* expression is transcriptionally down-regulated by ABA [22]. To identify regions of the promoter important for this down-regulation, we compared expression obtained with the promoter deletion series in the presence and absence of 10 µM ABA. Figure 3b shows the GUS activity of seeds treated with ABA expressed as per cent of the GUS activity of control seed populations in the same experiment. Deletion of the distal -1452 to -1193 region reduced down regulation relative to shorter promoters suggesting that this region is concerned with ABA regulation. The fact that GUS expression was lower in ABA-treated seeds than in control seeds for all the deletions giving expression suggests that the region closer to the start of transcription than -402 might also be concerned with ABA regulation.

Regulation of EREBP expression during seed germination

Four EREBPs, EREBP-1 and EREBP-2 in the same class and EREBP-3 and EREBP-4, each in different classes, specifically bind the ßGLU I ERE [30]. The distal -1452 to -1193 region of the *Glb* promoter important for localized, high-level expression in the micropylar endosperm contains the ERE. This prompted us examine the pattern of EREBP mRNA expression during seed germination. RNA blots were hybridized with probes for the four EREBP mRNAs and ßGLU I mRNA and the signals quantitated with a PhosphorImager. The values presented are normalized for the signals obtained with 18S ribosomal RNA used as an internal standard.

The RNA blots in Figure 2b and the PhosphorImager values in Table 2 show that in control seeds ßGLU I mRNA content began to increase between 48 and 54 h. In ethylene-treated seeds the onset of induction was earlier, 40 to 48 h, and accumulation was ca. 2-fold higher at 54 h and ca. 10-fold higher at 48 h than in control seeds. The different EREBPs showed distinctive patterns of expression. Accumulation of EREBP-3 mRNA was temporally regulated. In control seeds, EREBP-3 mRNA began to increase between 48 and 54 h, reached a peak at 63 h, and then declined at 70 h to the level observed at 40 h. The timing and the level of EREBP-3 mRNA accumulation in control and ethylene-treated seeds was very similar, although ethylene increased the content at 54 h suggesting that the onset of induction might be slightly earlier. EREBP-4 mRNA was barely detectable and showed a roughly constitutive pattern of expression, i.e. mRNA content did not change with time and was not increased by ethylene treatment. Finally, EREBP-1 (Fig. 2b) and EREBP-2 (data not shown) mRNAs were not detected either in the control or the ethylene treated seeds.

We also investigated the effect on EREBP expression of ABA treatment, and of GA treatment of dark-incubated seeds, shown earlier to inhibit or promote ß-1,3-glucanase induction and endosperm rupture, respectively [21, 22]. ABA treatment in the light down-regulates ßGLU I and greatly delays endosperm rupture. Table 2 confirms this ABA effect for ßGLU I mRNA and shows that ABA inhibits and greatly delays EREBP-3 mRNA accumulation. ABA did not appreciably affect EREBP-4 mRNA expression. No EREBP-1 or EREBP-2 mRNAs were detected after ABA treatment.

Treatment with 10 µM GA_4 breaks the photodormancy of Havana 425 tobacco seeds imbibed in the dark and induces ßGLU I accumulation [21]. The RNA blots in Figure 2c show that ßGLU I, EREBP-3, and EREBP-4 mRNAs accumulate in the small population of non-dormant seeds but not in photodormant seeds 80 h after imbibition in the dark. Treatment with 10 µM GA_4 resulted in a parallel induction of ßGLU I, EREBP-3, and EREBP-4 mRNAs starting 2 days after imbibition. EREBP-1 (Fig. 2c) and EREBP-2 (data not shown) mRNAs were not detected in either the control or GA_4 treated seeds.

As a positive control, RNA blot hybridization was performed with total RNA prepared from lower leaves and roots of Havana 425 tobacco plants. Although their relative amounts differed, both organs accumulated ß-1,3-glucanase, EREBP-1, EREBP-3 and EREBP-4 mRNAs in essential agreement with earlier reports [27]; [30] (Fig. 2c). Signals for EREBP-3 and EREBP-4 mRNAs, but not for ßGLU I or EREBP-1 mRNAs were detected in dry, mature seeds. The levels of EREBP-3 and EREBP-4 transcripts in dry, mature seeds were comparable to the highest levels found in germinating seeds at the onset of endosperm rupture. Hybridization with sense and antisense RNA probes confirmed that the EREBP signals were due to mRNAs (data not shown). The EREBP-3 values are intriguing because they suggest that EREBP-3 transcripts stored in mature seeds are rapidly degraded early during imbibition, remain at low levels in dormant seeds incubated in the dark, and are resynthesized during germination in the light and in the dark after GA_4 treatment.

The spatial pattern of EREBP mRNA expression was investigated by RNA blot hybridization of RNA extracted from embryos, micropylar endosperm, and non-micropylar endosperm dissected from seeds. Seeds were sampled at 54 h, which is after the onset of ß-1,3-glucanase induction but before endosperm rupture, and at 63 h when ca. 56 % of the seeds had ruptured endosperms. Seeds with intact and with ruptured endosperms were sampled separately. Because of weak signal strength, only a rough

Table 2. Effect of ethylene (100 ppm) and ABA (10 μM) on the levels of EREBP-3, EREBP-4, and βGLU I mRNA (in arbitrary units)[1] during the germination of GLB-GUS tobacco seeds in continuous light.

	EREBP-3			EREBP-4			βGLU I		
Time (h)	control	ethylene	ABA	control	ethylene	ABA	control	ethylene	ABA
40	0.19	0.16	——	0.54	0.46	——	0.00	0.45	0.00
48	0.19	0.15	0.16	0.55	0.58	——	0.17	1.37	0.20
54	0.25	0.60	0.16	0.46	0.60	——	2.16	5.67	0.24
63	0.73	0.84	0.09	0.48	0.58	0.42	2.53	5.15	0.41
70	0.20	0.21	0.15	0.60	0.52	——	3.70	5.92	0.37
94	——[2]	——	0.81	——	——	0.68	——	——	0.38
147	——	——	0.25	——	——	0.32	——	——	0.24

[1] Batches of 100 entire seeds were used for the extraction of total RNA. mRNA contents are expressed as Phosphor-Imager units of the RNA-blot hybridization signals normalized for the signals obtained with 18S rRNA used as a loading standard. Equal amounts of RNA (20 μg) were loaded in each lane.

[2] Not determined.

Table 3. Relative contents of βGLU I, EREBP-3, and EREBP-4 mRNAs in dissected parts of GLB-GUS tobacco seeds imbibed in continuous light.

Seeds sampled	Tissue [1]	mRNA (arbitrary units)[2]		
		βGLU I	EREBP-3	EREBP-4
Seeds after βGLU I induction, but before endosperm rupture (54 h)	Embryo	4	73	43
	Micropylar endosperm	42	91	22
	Non-micropylar endosperm	9	105	50
Seeds with intact endosperm (63 h)	Embryo	1	216	104
	Micropylar endosperm	32	64	7
	Non-micropylar endosperm	5	60	40
Seeds with ruptured endosperm (63 h)	Embryo	11	135	87
	Micropylar endosperm	84	74	22
	Non-micropylar endosperm endosperm	11	124	65

[1] Batches of 100 seed parts were used for the extraction of total RNA.

[2] mRNA contents are expressed as PhosphorImager units of the RNA-blot hybridization signals normalized for the signals obtained with 18S rRNA used as a loading standard. Equal amounts of RNA (20 μg) were loaded in each lane.

estimate of mRNA content was obtained. Nevertheless, the semi-quantitative data shown in Table 3 confirm that in seeds with intact as well as ruptured endosperms, ßGLU I mRNA is localized almost exclusively in the micropylar region of the endosperm. In contrast, signals for EREBP-3 and EREBP-4 mRNAs were detected in all the seed parts assayed.

Discussion

Role of ethylene in tobacco seed germination

Little is known about the signal transduction pathways that regulate germination of dicotyledonous seeds. Ethylene is involved in seed germination, breaking of seed dormancy, or both processes in many plant species (reviews [5, 11, 19]. Earlier studies provided evidence that ethylene is required for germination of tobacco seeds but is unable to break photodormancy [20, 39]. Our experiments confirm this, show that ethylene is involved in endosperm rupture, and establish ßGLU I as a molecular target of ethylene action in the germination of a dicotyledonous seed. These conclusions are based on several lines of evidence: First, for seeds imbibed in the light, endosperm rupture occurred slightly earlier in ethylene-treated seeds than in control seeds; whereas, release of photodormancy in the light was not affected. Second, ethylene treatment resulted in an earlier and increased induction of ß-1,3-glucanase activity, of ßGLU I protein and mRNA, and of *Glb*-promoter activity, but did not result in ectopic expression of ßGLU I. Therefore, ethylene promotes micropylar-endosperm specific transcription of ßGLU I genes. Third, ethylene has been shown to be produced by germinating tobacco seeds [20]. This indicates that endogenous ethylene could act as a regulator of germination and provides a plausible explanation for the relatively small effects of exogenous ethylene we observed. Fourth, the ethylene inhibitors AVG and NBD inhibited ßGLU I expression and endosperm rupture, but did not affect the breaking of photodormancy in the light. These inhibitory effects were partially reversed by simultaneous treatment with ethylene. Finally, for seeds imbibed in the dark, ethylene did not increase the incidence of non-dormant seeds although it did increase the ß-1,3-glucanase content of the small population of non-dormant seeds. Thus, ethylene is unable to break photodormancy. Taken together our results suggest that ßGLU I induction in the micropylar endosperm and endosperm rupture depend on ethylene; whereas, breaking of photodormancy is an ethylene-independent process.

Transcriptional regulation of ßGLU I

Regions of the *Glb* promoter and elements thought to be relevant to transcriptional regulation are summarized in Figure 3c. Ethylene-inhibitor studies and deletion analyses suggest that the distal -1193 to -1452 region of the *Glb* gene, which contains the ERE, is important for high-level ßGLU I expression in response to ethylene. Ethylene treatment did not result in ectopic ßGLU I expression, and GUS expression obtained with the promoter lacking the -1193 to -1452 region was still localized exclusively in the micropylar endosperm. This suggests that ethylene and the ERE are not involved in determining the spatial pattern of expression.

Although the -402 promoter is not the minimum promoter -- the shorter -211 promoter gave expression in roots [44] -- no reporter-gene expression was observed during germination when the -402 to -211 region was deleted. This suggests that the region -402 to -211 is necessary and sufficient for low-level expression in the micropylar endosperm. This region contains a highly conserved endosperm motif (EM) present in the endosperm box (EB) important for endosperm-specific expression of cereal storage-protein genes, which also functions in tobacco [1, 4, 18]. If EB has a role in ßGLU I expression, then additional signals or elements in the proximal region of the *Glb* promoter are probably required since EB-mediated expression in the endosperm is not confined to the micropylar region and occurs during seed development [4].

Deletion analysis of ABA down-regulation was difficult to interpret owing to the low and variable expression level of deletions shorter than -1193 in different transformants. Based on the decreased down-regulation in deletions shorter than -1193, it is likely that the region -1452 to -1193, which contains the ERE, is important for down-regulation by ABA. Deletions in the region -1193 to -402 had a weaker effect on ABA regulation. This might be due to the presence of a single copy of the AGC box at -121, which, in combination with other *cis* elements, may also be concerned with ethylene regulation. The *Glb* promoter contains two copies of a TAACAAA box at -1545 and at -93 important for ABA down-regulation of α-amylase genes in barley endosperm [15]. The role of this box in ßGLU I regulation is unclear since deletion of the region containing a copy at -1545 did not appreciably effect ABA down regulation.

The expression of EREBPs during seed germination

A putative ethylene receptor and several upstream components of the ethylene signalling pathway have been identified [32, 33, 45]; reviews [9, 14]. Far less is known about downstream components closer to the activation of transcription. Three classes of EREBPs

in tobacco can interact with an ERE in the enhancer region of ßGLU I genes and impart ethylene-sensitive transcription [30]. Accumulation of ßGLU I, ßGLU I mRNA and EREBP mRNA is correlated in leaves, roots, and cultured cells. Moreover, EREBP transcripts are upregulated by ethylene suggesting that ethylene might increase ethylene responsiveness.

EREBP mRNAs showed a novel pattern of expression during seed germination: light or gibberellin (in darkness) were required for EREBP-3 and EREBP-4 expression; EREBP-4 expression was constitutive and unaffected by ABA or ethylene; EREBP-3 showed transient induction just before endosperm rupture, which was earlier in ethylene-treated seeds and inhibited by ABA, but was later than the onset of ßGLU I induction. No expression of EREBP-1 and EREBP-2 was detected. Based on the assumption that EREBP transcripts are translated, we speculate that EREBP-3 and EREBP-4 with ERE as a target are important for high-level ßGLU I expression in response to ethylene during germination. According to this view, additional up-stream regulation of ßGLU I by ABA and ethylene would also be possible at the level of EREBP-3 expression. Nevertheless, it is unlikely that regulation EREBP-3 and EREBP-4 can account for the temporal and spatial pattern of ßGLU I expression: EREBP-3 and EREBP-4 expression is not localized in the micropylar endosperm and short promoters with the ERE-containing region deleted are sufficient for correctly timed and localized low-level ßGLU I expression. Thus, it is likely that ethylene-independent signalling pathways determine spatial and temporal patterns of expression. It is of interest in this regard that although the promoters of CHN I genes have the ßGLU I ERE [35], these genes are not expressed in germinating seeds [22] even after ethylene treatment (data not shown).

In conclusion, there is hierarchical regulation of ßGLU I by light, GA, ABA, and ethylene during tobacco seed germination. At the top of the hierarchy are light and GA, which are believed to act by a common pathway to break photodormancy of seeds [17, 21, 26]. Further downstream, ABA acts negatively and ethylene acts positivity possibly via EREBP-3 as well as more directly on elements in the *Glb* promoter. A causal role for EREBPs in regulating ßGLU I during germination has not as yet been established. Our studies provide a starting point for more rigorous tests of this hypothesis employing informative sense, antisense, and reporter-gene constructs of *Glb* and genes encoding EREBPs.

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