3. Functions and regulation of plant ß-1,3-glucanases (PR-2)

Gerhard Leubner-Metzger and Frederick Meins Jr. (1999)

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3.1. Introduction

Several classes of proteins, called pathogenesis-related (PR) proteins, are induced in response to the infection of plants with microbial pathogens. [1-3] This chapter deals with the family of PR-2 proteins, which are ß-1,3-glucanases (glucan endo-1,3-ß-glucosidases, E.C. 3.2.1.39) able to catalyze endo-type hydrolytic cleavage of the 1,3-ß-D-glucosidic linkages in ß-1,3-glucans. The ß-1,3-glucanases are abundant, highly regulated enzymes widely distributed in seed-plant species (reviews 4-7). Although the major interest in ß-1,3-glucanases stems from their possible role in the response of plants to microbial pathogens, there is strong evidence that these enzymes are also implicated in diverse physiological and developmental processes in the uninfected plant including cell division, [8,9] microsporogenesis, [10,11] pollen germination and tube growth, [12,13] fertilization, [14,15] embryogenesis, [16,16a] fruit ripening, [17] seed germination, [18,19] mobilization of storage reserves in the endosperm of cereal grains, [20] bud dormancy, [21] and responses to wounding, cold, ozone and UV B. [22-26]

In this chapter we focus on progress being made in understanding the function and regulation of ß-1,3-glucanases in reproductive development and pathogenesis. For more general information on plant ß-1,3-glucanases, the reader is referred to the extensive reviews by Stone and Clarke, [7] Meins et al., [4] Simmons, [6] and Høj and Fincher. [5]

3.2. Structural classes of ß-1,3-glucanases and PR-2 nomenclature

ß-1,3-Glucanases (ßGlu) exist as multiple structural isoforms that differ in size, isoelectric point, primary structure, cellular localization, and pattern of regulation. [4] The most detailed sequence information for these isoforms is available from cDNA and genomic clones of tobacco ßGlu, which form a multigene family. Based on amino acid sequence identity, the various ßGlu of the genus *Nicotiana* have been be classified into three structural classes. [4,27,28] The classification, nomenclature and salient features of these ßGlu are summarized in Table 3.1. Similar structural isoforms have been reported for tomato, potato and other plant species [29-32] (reviews 4,6).

The ca. 33 kDa class I enzymes (ßGlu I) of *Nicotiana tabacum*, which constitute the PR-2e subgroup of tobacco PR-proteins, are basic proteins localized in the cell vacuole. [33-35] ßGlu I is produced as a preproprotein with a N-terminal hydrophobic signal peptide, which is co-translationally removed, and a C-terminal extension N-glycosylated at a single site. The proprotein is transported from the endoplasmic reticulum via the Golgi compartment to the vacuole where the C-terminal extension is removed to give the mature, ca. 33 kDa enzyme, which is not glycosylated. [35,36] There is considerable indirect evidence that, in analogy to tobacco class I chitinases [37] and barley lectin, [38] the C-terminal extension contains a signal for targeting to the vacuole. [10,39] Recent results obtained with cultured

Table 3.1
PR-2 family members (ß-1,3-glucanases) of tobacco and other *Nicotiana* species

Class [a]	Member name	Trivial name	Origin [b]	MW (kDa) [c]	pI	Localization	References
I	PR-2e	Glb	Nt (T)	33	basic	Vacuole	42,36,18,19,71, 24
I	PR-2e	Gla	Nt (S)	33	Basic	Vacuole	36, 42
I	PR-2e	Gglb50	Nt (S)		Basic	Vacuole	44, 125
I	PR-2e	Gln2	Nt (S)		Basic	Vacuole	132, 126
I		Gn2 [d]	Np		Basic	Vacuole	40
I		Gn1 [d]	Np	34	Basic	Vacuole	41, 127
I I	PR-2a	PR-2 (I, b_4)	Nt	35	Acidic	Secreted	27
I I	PR-2b	PR-N (b_5), G19	Nt	35	Acidic	Secreted	27, 44, 125
I I	PR-2c	PR-O ($b_{6(b)}$, O')	Nt	35	Acidic	Secreted	27, 142
I I		PR-2d	Nt		Acidic	Secreted	128, 129
I I	stylar ßGLU [e]	Sp41a	Nt	41	Acidic	Secreted	14, 15, 60
I I	stylar ßGLU [e]	Sp41b	Nt	41	Acidic	Secreted	14, 15
I I I	PR-2d	PR-Q'	Nt	35	Acidic	Secreted	28
IV [f]	anther ßGLU [e]	Tag1	Nt	35	Acidic	Secreted	11

a Classification according to amino acid sequence identity of the mature proteins (ref. 4)
b *Nicotiana tabacum* (Nt); *N. plumbaginifolia* (Np); T and S refer to the *N. tometosiformis* and *N. sylvestris* progenitors of tobacco, respectively.
c Approximate molecular weight of mature protein; Selected values from the literature, which might differ between publications.
d Amino acid sequence identity to tobacco ßGLU I enzymes of the *N. plumbaginifolia* enzymes is ca. 98 % for Gn2, but only ca. 76 % for Gn1
e Not induced by pathogens, i.e. a "PR-like protein" (ref. 1).
f Tag1 is assigned to a new class, since it shares only 37-38 % amino sequence identity to Gla, PR-2, and PR-Q'.

tobacco cells provide strong evidence that vacuolar class I ßGlu and chitinases can be secreted into the medium via a novel pathway. [39a]

The known mature ßGlu I of tobacco and Gn2 of *Nicotiana plumbaginifolia* share ca. 98 % amino acid identity. [40] In contrast, with only ca. 76 % similarity, the Gn1 of *N. plumbaginifolia* is structurally more distinct. [41] The tobacco ßGlu I multigene family consists of very similar homeologues derived from the *N. sylvestris* and *N. tomentosiformis* progenitors of tobacco as well as recombinants of the two progenitor types. [42]

In contrast to ßGlu I, the class II and III members of the PR-2 family are secreted into the extracellular space (reviews 2,4,6). The tobacco class II ßGlu PR-2a, PR-2b, PR-2c and the class III ßGlu PR-2d, also known as PR-2, PR-N, PR-O, PR-Q', respectively, [1] are acidic proteins without the C-terminal extension present in the class I enzymes ranging in apparent size from ca. 34 to 36 kDa in denaturing gels. [43] The class II tobacco isoforms are at least 82 % identical in amino acid

sequence and differ from the class I enzymes at a minimum of 48.8 % of the positions. [4,6,27,44] Class II also includes the two acidic 41 kDa stylar ßGlu isoforms, Sp41a and Sp41b, which are exclusively expressed in the style of tobacco flowers. [15] They do not appear to be induced by pathogen infection and, hence, are referred to as "PR-like proteins". [1]

The acidic ca. 35 kDa PR-2d (PR-Q') is the sole representative of tobacco class III ßGlu and differs in sequence by at least 43 % from the class I and class II enzymes. [28] Two highly homologous cDNA clones for class III ßGlu have been isolated from tomato plants infected with a viroid. [31] Based on deduced amino-acid sequences, TomPR-Q'a is an acidic isoform 86.7 % identical to tobacco PR-Q' and TomPR-Q'b is a basic isoform 78.7 % identical to tobacco PR-Q'.

Tag1 appears to represent a novel class of tobacco ßGLU. It is a "PR-like" protein which is expressed specifically in tobacco anthers. [11] Like the tobacco class I ßGLU, Tag1 is encoded by a small gene family with at least two members derived from the *N. sylvestris* and *N. tomentosiformis* progenitors of tobacco. Based on deduced amino acid sequence, *Tag1* encodes a polypeptide with an N-terminal signal peptide, but no C-terminal extension, suggesting that the protein may be secreted. The mature form of Tag1 is an acidic, 35 kDa protein, which shares absolutely conserved sequences found in all classes of tobacco ßGLU. It is 37-38 % identical in sequence to the mature forms of tobacco class I Gla, class II PR-2, and class III PR-Q'. Based on the criteria used earlier to for tobacco ßGlu, [4] we have assigned Tag1 to a new class, IV (Table 3.1).

The specific enzymatic activities and substrate specificities of different ßGlu vary considerably. The ßGlu I and class II PR-2c appear to be 50 to 250 times more active in degrading the ß-1,3-glucan substrate laminarin than the class II PR-2a and PR-2b and the class III PR-2d enzymes. [24,45] The mechanism of catalysis has been recently reviewed by Davies and Henrissat [46] and by Høj and Fincher. [5] The complete structure three-dimensional structure of a barley ßGlu has been determined. [47]

3.3. Biological functions of ß-1,3-glucanases

3.3.1. Plant reproductive biology

3.3.1.1. Anther ß-1,3-glucanases

Pollen development begins with the division of diploid sporophytic cells within the anther, which gives rise to tapetal cells and pollen mother cells (reviews 48,49). The tapetum forms a single layer of cells around the anther locule in which the pollen develops. The pollen mother cells undergo meiosis to form tetrads of haploid microspores. In almost all higher plants each individual microspore of the tetrad is surrounded by a thick callose wall composed of a ß-1,3-glucan, which is laid down

between the cellulose cell wall and the plasma membrane. At a critical stage of microspore development, the callose wall of the tetrads is degraded by callase activity, which is secreted by the tapetal cells. The microspores are then released into the anther locule where they develop into mature pollen grains. Although the callose wall is essential for production of fertile pollen, its function is unknown. Proposed functions include physical and chemical isolation of the developing gametes from sporophytic tissues, mechanical isolation of the meiocytes and tetrads, protection from environmental and osmotic stress, and formation of the pollen cell wall. [11]

In the anthers of petunia and lily, expression and secretion of callase activity is under strict developmental control. [50,51] The callase complex of lily consists of a 32 kDa endo-ßGlu and a 62 kDa exo-type ß-1,3-glucanase. [52] The endo-type enzyme seems to be most important for the degradation of the callosic walls, while the exo-type ß-1,3-glucanase is involved in the further hydrolysis of released oligosaccharides. Alterations in the timing of ßGlu expression, or failure to express ßGLU, leads to abnormal dissolution of the tetrad callose walls, which has been shown to be a primary cause of male sterility in cytoplasmic male-sterile lines of petunia, [53] sorghum, [54] and soybean. [55]

Two ßGlu genes have been identified that are expressed during microsporogenesis. The *A6* gene was originally identified as an anther-specific *Brassica napus* cDNA, which was then used to isolate genomic clones of the *Arabidopsis thaliana* homologues. [56] Based on deduced amino acid sequences and immunoblotting experiments, *A6* encodes a polypeptide with a domain similar in sequence to ßGlu and a 114 amino-acid long C-terminal domain, which is not present in other known ßGLU. Reporter gene studies established that *A6*-gene expression is tapetum specific and temporally correlated with the expression of callase activity. Transcripts of the class IV tobacco anther ßGlu Tag1 are also expressed exclusively in the tapetum and show a callase-like pattern of expression. [11] *Tag1* mRNA is not detectable in flower buds, pistil, sepals, petals, roots, healthy and TMV-infected leaves.

Further evidence for a role of ßGlu in callose-wall dissolution and microsporogenesis has come from sense-transformation experiments. Worrall et al. [10] transformed tobacco with a gene encoding a tobacco ßGlu I with the C-terminal extension deleted, which is secreted into the extracellular space. Tobacco plants that express the recombinant extracellular ßGlu I under the control of the tapetum-specific promoters of the Arabidopsis *A3* and *A9* genes exhibited premature degradation of callose in microspore cell walls, production of abnormal microspores, and partial to complete male sterility. No male sterility was observed in transformants obtained with the extracellular ßGlu I regulated by the cauliflower mosaic virus (CaMV) 35S RNA promoter. Similar results were obtained in lettuce, [56a] and by expressing in transgenic tobacco the cDNA of a pathogenesis-related extracellular endo-ßGlu from soybean using the rice, tapetum-specific *Osg6B* promoter. [57] Taken together the sense transformation experiments indicate that

premature callose degradation is sufficient to cause male sterility and suggest that formation of the callose cell wall and its proper developmental degradation by endo-ßGlu are critical for microsporogenesis.

3.3.1.2. Stylar ß-1,3-glucanases

As part of the fertilization process, pollen tubes grow through the transmitting tissue of the style toward the ovary (reviews 58,59). The transmitting tissue consists of elongated, secretory cells, connected end-to-end through plasmodesmata. It is believed that interactions between the transmitting tissue and the growing pollen tube are important for guiding pollen tube growth toward the ovules and successful fertilization.

The class II ßGlu Sp41 of tobacco is a "PR-like" protein encoded by the two closely related *Sp41a* and *Sp41b* genes. [14,15] It is a major component -- up to 20% -- of the soluble protein in the stylar transmitting tissue of the tobacco flower. The mature form of Sp41 is a ca. 41 kDa, acidic, glycoprotein, which is secreted into the extracellular matrix. Measurements of Sp41 protein, Sp41 steady-state mRNA, and activity of the *Sp41a*-gene promoter indicate that Sp41 is expressed exclusively in the style in a developmentally regulated fashion. [14,15,60] Accumulation of the protein begins 4 days before anthesis and reaches a broad peak from 2 days before anthesis until style senescence. No expression is found in leaf, root, sepal, petal, anther, pollen sac, and ovary. Moreover, the protein is not induced by TMV in leaves or by treatment with abiotic elicitors of PR proteins in leaves and styles. Reporter-gene experiments have shown that 2.5 kb 5'-noncoding region of the *Sp41a* gene is sufficient to confer transmitting-tissue specific expression.

Sessa et al. [60] used antisense transformation to find out if expression of Sp41 has an essential role in reproductive development. Tobacco plants were transformed with a construct containing a partial Sp41a cDNA in reverse orientation fused to a CaMV 35S promoter, which is active in the style, and a TMV Ω enhancer of translation. The results are difficult to interpret: Although neither Sp41 protein nor ßGlu activity was detected and fertility was reduced in 3 of 18 primary transformants, progeny obtained by outcrossing the Sp41-deficient transformants were fully fertile even though many of them had undetectable or greatly reduced levels of Sp41. Moreover, no direct effects on stylar development or pollen tube growth related to Sp41 deficiency were observed. Sessa et al. [60] suggest as possible explanations that either the Sp41 deficiency is significant only under specific environmental conditions not met in their experiments; or, that Sp41 has a role in defense against pathogen infection rather than in the fertilization process. Another possibility is that the plants can compensate for the deficiency by producing other proteins with Sp41-like functions as has been reported for ßGlu I induced in tobacco by TMV infection. [43]

3.3.1.3. Endosperm ß-1,3-glucanases

Germination of seeds is a complex physiological process triggered by imbibition of water. Under favorable conditions rapid growth of the embryo culminates in rupture of the covering layers and emergence of the radicle. In many species the enclosing tissues act as a physical barrier which must be overcome by the growth potential of the embryo if the seed is to complete its germination (reviews 61,62). Little is known about the molecular basis for the rupture and physical penetration of these covering layers.

In the case of tobacco, the embryo is surrounded by three to five layers of rather thick-walled endosperm cells in the mature seed. The periphery of the endosperm is pressed against the thin seed coat (testa), consisting of cutinized and lignified dead cells. [63] Rupture of the testa and rupture of the endosperm are separate events in the germination of tobacco seeds (Fig. 3.1) [19,64] and there is strong evidence that endosperm rupture is the limiting factor in the germination of these seeds. Electron microscopic studies support the view that the

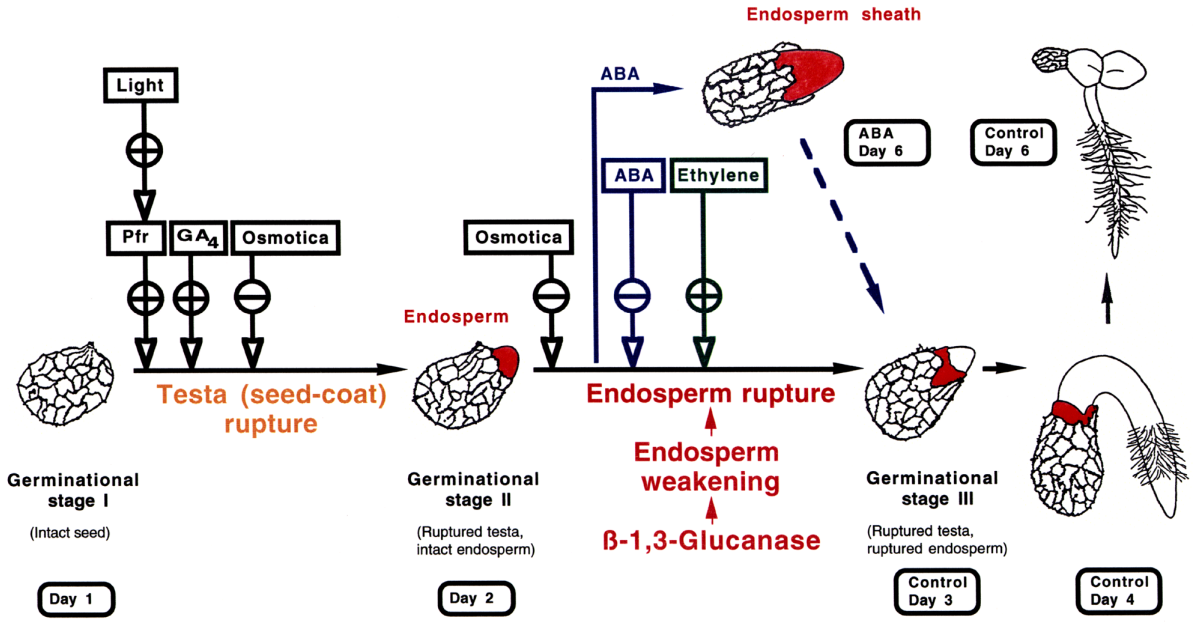

Figure 3.1: Working model for tobacco seed germination. Rupture of the testa and rupture of the endosperm are separate events in *Nicotiana tabacum*. Class I ß-1,3-glucanase accumulates just prior to endosperm rupture and is proposed to promote radicle protrusion by weakening of the endosperm. Plant hormones and environmental factors alter the germination process and in strict correlation with this either promote (+) or inhibit (-) class I ß-1,3-glucanase induction. GA = gibberellin(s); ABA = abscisic acid; Pfr = Phytochrome. The model summarizes results from Leubner-Metzger *et al.* [19,65,71]

endospermic hole of the germinated seed, which is always at the micropylar end, is formed by "dissolution" rather than by "pushing" action. [64] In photodormant varieties of tobacco, both the seed-coat and endosperm remain intact in the 80-90 % of seeds that do not germinate in darkness. [63,65-67] However, when the seed coat and endosperm are mechanically removed, there is radicle growth even in the absence of light. [68-70] Finally, treatment of tobacco seeds with 10 µM abscisic acid (ABA) greatly delays endosperm rupture and results in the formation of a novel structure consisting of the enlarging radicle with a sheath of greatly elongated endosperm tissue [19] (Fig. 3.1).

The first hint that ßGlu may play a role in tobacco seed germination was our observation that ßGlu I is induced during germination. [18,19] Measurements of ßGlu activity, ßGlu I protein and mRNA in combination with reporter-gene experiments with the *E. coli uidA* gene (*Gus* reporter-gene) fused to the promoter of the tobacco class I ß-1,3-glucanase B gene (*Glb*) established that most if not all of the ßGlu activity is due to transcriptional induction of the ßGlu I isoforms. ßGlu I is induced exclusively in the micropylar region of the endosperm where the radicle will penetrate, but prior to endosperm rupture. Finally, ßGlu I induction during germination is not a classical defense-type response, since chitinases (Chn) and the known acidic class II and III ßGlu are not induced. Based on these findings, we proposed as a working hypothesis that ßGlu I weaken the endosperm by digestion of cell-wall material and that this promotes radical protrusion to facilitate germination (Fig. 3.1).

In support of this hypothesis, treatment of tobacco seeds incubated in the light with ABA does not affect seed-coat rupture but greatly delays subsequent endosperm rupture and inhibits the rate of endosperm-specific ßGlu I accumulation in a concentration dependent manner. [19] Gibberellins (GA), which can substitute for light in releasing dormancy, induced ßGlu I in the dark in association with germination. [65] Recent results [71] provide evidence that ethylene is required both for high levels of ßGlu I expression in the micropylar endosperm and for endosperm rupture. Although the close correlation between ßGlu I induction and the onset of endosperm rupture under a variety of physiological conditions (Fig. 3.1) is consistent with our working hypothesis, direct evidence is still lacking. One approach we are currently exploring is the use of sense- and antisense-transformation to alter the level of expression of ßGlu I during the germination process.

It is well established that 1,3;1,4-ß-glucanases, which are structurally related to ßGlu but differ in substrate specificity, [5,47,72] hydrolyze the 1,3;1,4-ß-glucan cell walls of the starchy endosperm during the germination of cereals (reviews 5,6,73. Less is known about the function of cereal ßGlu, which are present in ungerminated grain and rise markedly in concentration during germination. However, the putative substrate, ß-1,3-glucan, is not abundant in cereal grains and is restricted to small callosic deposits scattered through the starchy endosperm. Fincher [73] has proposed that the high levels of ßGlu are part of a preemptive strategy to protect the

grain against microbial attack. Cordero et al. [74] found that one ßGlu isoform and three Chn isoforms are induced in germinating maize kernels infected by *Fusarium moniliforme*. In contrast, a second ßGlu isoform is expressed in embryo and radicle tissues, but is not induced by fungal infection. These findings support the view that cereal ßGlu, in addition to their possible role in pathogen defense, might also be involved in embryogenesis and seed germination.

3.3.2. Pathogenesis-related functions

In 1971, Abeles et al. [75] suggested that the glucanohydrolases ß-1,3-glucanase (ßGlu) and chitinase (Chn) might function as a defense against fungal pathogens. At about the same time, the PR-proteins were first described as a novel set of abundant proteins accumulating in leaves of resistant tobacco cultivars reacting with hypersensitive response (HR) to infection with tobacco mosaic virus (TMV). [3,76,77] Later, it was shown that the PR-proteins include ßGlu (the PR-2 family) [45] and Chn (the PR-3 family) (reviews 2,24,78).

There is now compelling evidence that ßGlu and Chn, acting alone and particularly in combination, can help defend plants against fungus infection. It has been proposed that these glucanohydrolases act in at least two different ways: directly, be degrading the cell walls of the pathogen; and, indirectly by promoting the release of cell-wall derived materials that can act as elicitors of defense reactions (reviews 4,79-83).

3.3.2.1. In vitro antifungal activity

ßGlu and Chn can hydrolyze ß-1,3-glucans and chitin, respectively, which are major components of the cell walls of many pathogenic and potentially pathogenic fungi (review 84). Although, in some cases, treatment with ßGlu or Chn can inhibit fungal growth in vitro, more often combinations of the two enzymes are required for antifungal activity (reviews 79,81,85).

Several studies have been made in which different ßGlu and Chn isoforms were tested for in vitro antifungal activity. [86-93] Only class I vacuolar isoforms of tobacco ßGlu and Chn were effective in promoting the lysis of hyphal tips and inhibiting the growth of *Fusarium solani* . [89] These effects were greatly enhanced by using combinations of ßGlu I and class I Chn. In contrast, the class II ßGlu PR-2a, PR-2b, and PR-2c did not exhibit antifungal activity either alone or in any combinations tested. Similar studies with tomato ßGlu and Chn have shown that the vacuolar class I isoforms, but not the secreted class II isoforms inhibit growth of *Alternaria solani* , *Trichoderma viride* and *Phytophthora infestans*; [91,93] and that none of the combinations of ßGlu and Chn tested inhibited growth of the tomato pathogen *Cladosporium fulvum*. [92]

3.3.2.2. Release of fungal elicitors

Plant ßGlu can release oligosaccharides from cell walls of the pathogens, which can then act as elicitors of defense reactions (reviews 81-83). This is well-documented for interactions between soybean and the ß-glucan elicitor from the pathogenic oomycete *Phytophthora megasperma* f. sp. *glycinea.* [94-107] Following fungal attack, soybean ßGlu releases ß-glucans from the fungal cell wall, which then induce accumulation of the phytoalexin glyceollin. The smallest ß-glucan released with elicitor-activity was a ß-1,3-ß-1,6-heptaglucoside [95] and the structural requirements for elicitor activity of these oligosaccharides have been investigated. [96]

Proteins which bind this oligosaccharide elicitor have been purified from soybean membranes. [104,105] Recently Umemoto et al. [97] isolated a cDNA for a ß-glucan elicitor binding protein (GEBP), which is localized in the plasma membrane of soybean root cells. Expression of the soybean GEBP gene has been shown to confer ß-glucan binding activity to *Escherichia coli* and to tobacco cells cultured in suspension, suggesting that GEBP might be an elicitor receptor.

Soybean ßGlu have been purified that are able to release active ß-glucan elicitors from fungal cell walls. [98,99,106,107] The enzyme described by Takeuchi et al. [98] is 63% identical in amino acid sequence to the tobacco class III ßGlu PR-Q', but only 55% and 51% identical to the class I and class II enzymes of tobacco, suggesting that the soybean enzyme is a class III ßGLU. Albersheim and Valent [94] have reported that the fungus *Colletotrichum lindemuthianum* secretes a protein that inhibits an endo-ßGlu of its host, French bean. Recently, Ham et al. [99] presented evidence that fungal pathogens secrete proteins that can selectively inhibit plant ßGlu. They purified two basic pathogenesis-related ßGlu, $EnGL_{soy}$-A and $EnGL_{soy}$-B, from soybean seedlings as well as a ß-1,3-glucanase inhibitor protein (GIP-1) from the culture fluid of *Phytophthora sojae* f. sp. *glycines* (formerly *Phytophthora megasperma* f. sp. *glycinea*). GIP-1 specifically inhibited the soybean $EnGL_{soy}$-A, but not $EnGL_{soy}$-B or several other ßGlu including tobacco class II PR-2c and enzymes secreted by the fungus. GIP-1 does not exhibit proteolytic activity but does appear to physically bind to $EnGL_{soy}$-A. The fungal pathogen can also secrete GIPs with other host ßGlu as targets. Thus, fungal pathogenesis appears to involve a complex interplay between host ßGlu and pathogen ßGlu and ßGlu inhibitors.

3.3.2.3. Enhanced resistance to fungal pathogens resulting from transgene expression

There is strong evidence that expression of ßGlu transgenes alone or in combination with *Chn* transgenes regulated by the strong CaMV 35S RNA promoter can reduce the susceptibility of plants to infection by certain fungi. Transgenic tobacco plants expressing a soybean ß-1,3-glucan-elicitor releasing

ßGlu or the tobacco class II ßGlu PR-2b show reduced symptoms when infected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica* var. *nicotianae* and *Peronospora tabacina*. [102,102a] ß-1,3-Glucans are the major components of the cell walls of oomycetes, a group of fungi that do not contain chitin. [84]

In many cases, a pronounced synergic effect is obtained when ßGlu and Chn transgenes are expressed in combination. Tomato plants expressing tobacco class I ßGlu and Chn transgenes show reduced susceptibility to infection by *Fusarium oxysporum* f.sp. *lycopersici*, whereas expression of either gene alone is not effective. [108] Sela-Buurlage et al. [85,89] transformed tobacco plants with transgenes encoding modified class I tobacco ßGlu and Chn that are secreted. They found that the extracellular wash fluid from the leaves of plants expressing both ßGlu and Chn showed strong antifungal activity against *Fusarium solani*, whereas this effect was less for plants expressing either transgene alone. Tobacco plants expressing a bean class I *Chn* gene show decreased susceptibility to the root pathogen *Rhizoctonia solani.* [109] Resistance to infection was further enhanced by co-expression of this *Chn* gene with barley class II ßGlu and Chn genes. [110] Alfalfa plants expressing alfalfa *Aglu1* acidic ßGlu and rice *Rch10* basic Chn transgenes showed reduced disease symptoms when infected with the oomycete pathogen *Phytophthora megasperma* f. sp *medicaginis*, which does not contain chitin in its cell walls, whereas no reduction in symptoms was observed with several chitin-containing fungal pathogens. [111] Expression the *Rch10* and *Aglu1* transgenes also substantially increased protection of tobacco against the chitin-containing fungus *Cercospora nicotianae*, the causal agent of the frogeye disease, relative to plants expressing either of the transgenes alone. [112] In contrast, susceptibility of *N. sylvestris* to *C. nicotianae* was not affected by high level expression of tobacco class I Chn [113] or deficiencies in host ßGlu I generated by antisense transformation. [114] The latter results suggest that host ßGlu I may not be required for defense against this pathogen.

3.3.2.4. Decreased susceptibility to viral disease of ß-1,3-glucanase-deficient plants

The induction of ßGlu as part of the hypersensitive reaction is a stereotypic response, i.e., the pattern of induction is similar for viral, bacterial, and fungal pathogens (review 115). Although antifungal ßGlu I appears to be tailored for defense against fungi, recent studies of ßGlu I-deficient mutants generated by antisense transformation suggest that these enzymes also play an important role in viral pathogenesis.

TMV infection of leaves of tobacco cultivars showing a local-lesion response induces the expression of all three ßGlu classes. [27] Antisense transformants of Havana 425 tobacco and *Nicotiana sylvestris* transformed with sequences of *Gla*, the *N. sylvestris* homeologue of tobacco ßGlu I, in reverse orientation regulated by the CaMV 35S RNA promoter show greatly reduced levels of ßGlu I. [43,114,116,117]

Expression of class I but not class II or class III ßGlu is effectively and specifically blocked when these antisense lines are infected with TMV. [117] The antigen content for the ßGlu I in lower leaves of healthy transgenic plants is reduced ca. 20-fold. They are fertile, develop normally under greenhouse conditions, and like in the wild-type plants, they accumulate the known pathogen-inducible class II and class III isoforms of ßGlu when infected with necrotizing viruses. [43] A novel intracellular form of ßGlu serologically distinct from any of the known tobacco ßGlu is induced in ßGlu I-deficient plants, but not in wild-type plants by virus infection. Thus, plants can compensate for a deficiency in enzyme activity by producing a functionally equivalent replacement - i.e. "ersatz" protein or proteins. The fact that compensation occurred specifically in response to virus infection suggests an important role of ßGlu in pathogenesis.

Unexpectedly, the ßGlu I-deficient mutants showed markedly reduced lesion size, lesion number, and virus yield in the local-lesion response of Havana 425 tobacco to TMV and *N. sylvestris* to tobacco necrosis virus. [117] In contrast to ßGlu I, no change in resistance to TMV was reported for antisense and sense transformation with constructs for the class II ßGlu PR-2b. [102a] The ßGlu I-deficient *N. sylvestris* mutants [117] also showed decreased severity and spread of mosaic disease symptoms and reduced virus yield in the susceptible response to TMV. Moreover, the symptoms of disease in both plant species were positively correlated with ßGlu I content in a series of independent transformants providing direct evidence for a function of these enzymes in viral pathogenesis.

Callose deposition is known to act as a physical barrier to the spread of virus. [118] Callose deposition in and around TMV-induced lesions is increased in ßGlu I-deficient tobacco suggesting that decreased susceptibility to virus resulted from increased callose deposition in response to infection. These findings are of particular interest because they suggest a novel means, based on antisense transformation with host genes, for protecting plants against viral infection. They also raise the intriguing possibility that viruses can use a defense response of the host against fungal infection -- production of ßGlu I -- to promote their own replication and spread.

3.4. Regulation of ß-1,3-glucanase expression

ßGlu show developmental regulation and regulation in response to treatment with hormones or infection with pathogens. Early studies were made based on the basis of measurements of enzyme activity before it was recognized that there are different classes of ßGlu (e.g. refs. 119-123). More recently, specific ßGlu proteins have been measured immunologically and their mRNAs have been measured semi-quantitatively by RNA-blot hybridization. In a limited number of cases, regulation of transcription has been studied using plants transformed with *Gus* reporter genes under the control of the promoter region of ßGlu genes, namely: 1) The tobacco

class I *N. tomentosiformis* homeologue *Glb,* [18,124] and the tobacco class I *N. sylvestris* homologues *Gglb50* [125,153a] and *Gln2* [126]. 2) The less-related ßGlu I gene *Gn1* of *N. plumbaginifolia.* [41,127] 3) The tobacco class II ßGlu genes *PR-2b* (*G19, PR-N*) [125] and *PR-2d.* [128,129]

In the following sections, we summarize the patterns of ßGlu regulation, and review progress being made in identifying *cis*-acting promoter elements and *trans*-acting factors important in transcriptional regulation and signal transduction.

3.4.1. Developmental and hormonal regulation

3.4.1.1. Class I ß-1,3-glucanases

ßGlu I accumulate at high concentrations in the roots and in lower leaves of mature, healthy tobacco plants. [120,130,131] The ßGlu I content of leaves decreases toward the top of the plant. Within leaves, they are localized in the vacuole of epidermal cells. [34] ßGlu I transcripts accumulate at low levels in developing floral tissues of tobacco [36,132] and potato [133] and accumulate at high levels during *de novo* flower formation of tobacco. [131] The pattern of expression in leaves and roots of ßGlu I proteins and steady state mRNA are very similar and are correlated with promoter activity of the ca. 1.5 to 1.7 kb 5' flanking region. [120,124,125,130,132] Therefore, regulation of ßGlu I in these organs appears to be primarily at the level of transcription. Similar conclusions may be drawn for the less related ßGlu I gene *Gn1* of *N. plumbaginifolia*, which shows low promoter activity in upper leaves and high promoter activity in lower leaves and roots. [41]

The accumulation of ßGlu I protein and mRNA is strongly down-regulated when discs of leaf tissue or callus cultures are incubated for less than 7 days on medium containing combinations of auxin and cytokinin at physiological concentrations. [120,130,134] This is a particularly rapid form of down regulation detectable at the mRNA level one hour after suspension-cultured cells are treated with auxin and cytokinin. [135] Down-regulation by auxin and cytokinin also appears to be at least in part transcriptional since the decrease in steady state RNA is correlated with decreased activity of the *Glb* promoter. [124,136]

Many plant species react to treatment with the stress hormone ethylene with induction of ßGlu I activity, protein, and mRNA in leaves. [30,79,121,137-139] Although ethylene increases the ßGlu I content of epidermal cells slightly, its inductive effect is most pronounced on mesophyll cells of the tobacco leaf. [34] Studies with inhibitors of ethylene production and ethylene action have shown that ethylene is required for the induction of ßGlu I in cultured tobacco cells. [121] Ethylene treatment also dramatically increases the promoter activity of the tobacco ßGlu I gene *Glb.* [124] Similarly, ethephon (2-chloroethylphosphonic acid), which releases ethylene, increases the promoter activity of the tobacco ßGlu I genes *Gglb50* [125] and *Gln2* [126] in leaves of transgenic tobacco. When applied to plants, ethephon also gives rise to HCl and H_3PO_3.[140] Since acids can induce accumulation of PR-protein

transcripts, [141] control experiments have been performed suggesting the induction of *Gglb50* [125] and *Gln2* [126] by ethephon is, in fact, due ethylene. As judged from the ca. 20-fold increase in *Gus* reporter-gene activity following treatment with ethylene or ethephon, ßGlu *I* promoters are highly induced by ethylene. Although activity of the homologous *N. plumbaginifolia Gn2* promoter is also highly induced by ethylene in transgenic tobacco leaves, activity of the less homologous *N.plumbaginifolia Gn1* promoter shows only a weak, ca. 2.5-fold induction. [41]

Regulation of ßGlu I and class I Chn is often tightly coordinated. [4,26,30] In tobacco, their expression gradients in leaves and roots of the mature plant, their kinetics of down-regulation in cultured by auxin and cytokinin, and their responses in leaves to ethylene treatment and infection by pathogens are very similar. In contrast, during the germination of tobacco seeds, ßGlu I, but not class I Chn, are transcriptionally induced in the micropylar endosperm. [19] ABA inhibits this seed-specific induction, [19] and also down-regulates ßGlu I, but not class I Chn at the transcriptional level in tobacco pith-cell suspensions and cultured leaf discs. [136]

3.4.1.2. Class II and class III ß-1,3-glucanases

The class II ßGlu PR-2a, PR-2b, and PR-2c are present in sepals, but not in other floral organs. [128,142] In general, these acidic ßGlu do not appear to accumulate in vegetative tissues of mature, healthy tobacco plants (e.g. refs. 27,28). Reporter gene experiments suggest that the 1.7 kb promoter of the tobacco class II ßGlu *PR-2d* gene is active in sepals, in the base of flowers, and in young seedlings, but not in leaves, roots or the stem of mature tobacco plants. [128] In contrast, the 1.75 kb class II ßGlu *PR-2b* promoter is active in leaves, stem and root of mature tobacco plants, but at levels much lower than that of the tobacco ßGlu I *Gglb50* promoter. [125]

Treatment of plants with ethephon results in no detectable induction, or very weak induction of class II and class III ßGlu in leaves of tobacco and tomato. [26,137,139,143]

3.4.2. Pathogenesis-related regulation

In general, ßGlu and Chn are induced in plants infected with viral, bacterial, and fungal pathogens. Similarly, elicitors including fungal glucans, [144,145] chitosan, [122] *N*-acetylchitooligosaccharides, [146] and glycoprotein [147] can induce the accumulation of the two enzymes (reviews 79,81,82).

Tobacco cultivars carrying the dominant *N* gene from *N. glutinosa* show a local lesion, hypersensitive reaction (HR) response to TMV rather than systemic symptoms of mosaic disease. [2,148,149] These plants also show decreased disease symptoms in response to secondary infection with certain other viral, bacterial and fungal pathogens. This is an example of a type of induced long-lasting, broad spectrum resistance called systemic acquired resistance (SAR) (reviews 150,151;

refs. 152,153).

Class I ßGlu proteins and their mRNAs are induced in TMV-infected leaves of tobacco as part of the local lesion, HR response. [2,4,28,44,45,116] *Gus* reporter-gene experiments with the tobacco ßGlu I *Gglb50* and *Glb* promoters have shown that ßGlu I is transcriptionally induced by up to ca.10-fold in TMV infected leaves showing HR. [124,125,153a] Histological studies indicate that the *Glb* promoter is active in a ring of cells around necrotic lesions induced by TMV infection, but not in cells immediately adjacent to the lesions or in the lesions themselves. Gus activity is also higher in areas with lesions compared to lesion-free areas, [124] which is in agreement with the accumulation of basic PR-2 proteins in and around lesions described by Heitz et al. [154]

Activity of the weakly ethylene-inducible *Gn1* promoter of *N. plumbaginifolia* is strongly induced (ca. 21-fold) as part of the HR of tobacco leaves infected with the incompatible bacterium *Pseudomonas syringae* pv *syringae* and is localized around the necrotic lesions. [41,127] Induction of this promoter is much weaker in leaves infected with the compatible bacterium *Erwinia carotovora* subsp *carotovora* or a saprophytic strain of *Pseudomonas fluorescens.* [41] The *Gn1* promoter is also induced in a *Cf*-gene dependent manner in the interaction of transgenic tomato with incompatible and compatible races of the leaf mold pathogen *Cladosporium fulvum.* [155]

Either no or weak and erratic RNA-blot hybridization signals for ßGlu I mRNAs have been detected in uninfected leaves of TMV-infected tobacco plants indicating that ßGlu I induction is a local response associated with HR. [26-28,142,153,156]

The PR-related class II and III ßGlu are induced both locally in TMV-infected leaves and systemically in noninfected leaves of the same plant. [26-28,44,142,153,156-158] *Gus* reporter gene studies have shown that the promoters of the tobacco class II *PR-2b* and *PR-2d* are induced both locally around necrotic lesions on TMV-infected tobacco and systemically in noninfected leaves. [125,128] The close correlation between systemic induction of class II and class III ßGlu has led to the use of these genes as markers for SAR (reviews 150,151; refs. 152,153). For example, reporter gene constructs based on the *Arabidopsis* ßGlu II *Bgl2* promoter have been used to isolate *Arabidopsis* SAR mutants such as *cpr1* (constitutive expresser of PR genes) and *npr1* (nonexpresser of PR genes). [150,151]

Systemic accumulation of salicylic acid (SA) is associated with the HR of tobacco, *Arabidopsis thaliana*, and certain other plants (reviews 150,151). Treatment of mature, wild-type tobacco plants with SA strongly induces accumulation of mRNAs of PR-related class II and III ßGlu and certain other PR proteins; [153,158a] and, promoter activity of the class II *PR-2b* [125] and *PR-2d* [128] genes is induced in response to SA. While SA is probably not the long-distance systemic signal for SAR activation, it is required for transduction of the this signal in leaves distal from the primary infection site (reviews 150,151). Thus, transgenic tobacco plants expressing a bacterial salicylate hydroxylase gene (*NahG*) that fail to accumulate significant amounts of SA are unable to develop SAR and do not

show increased expression of SAR markers including class II and III ßGlu.

Transcripts of tobacco class I ßGlu and Chn are either not induced or only weakly induced in response to SA. [44,132,153,158a,159] In contrast to the tobacco class I *Gglb50* and *Glb* promoters, the promoter of the less related *N. plumbaginifolia* class I *Gn1* promoter is strongly induced (ca. 14-fold) in transgenic tobacco plants treated with SA. [41]

3.4.3. *Cis*-acting elements

3.4.3.1 Class I ß-1,3-glucanase genes

Expression studies of the *Gus* coding region fused to deletion series of 5'-flanking sequences have been used to identify regions of class I ßGlu promoters important for transcriptional regulation. Elements for responsiveness to SA, ethylene, and TMV are present in the -0.45 to -1.5 kb region of the 1.5 kb tobacco *Gglb50* promoter. [125,153a] The homeologous 1.6 kb tobacco *Glb* promoter confers proper regulation to reporter gene expression in leaves, roots, seeds and cultured cells. [18,19,65,71,124,136] A more detailed analysis of the *Glb* promoter indicates that the distal -1193 to -1452 region is required for high levels of expression in leaves and for responsiveness to ethylene and TMV infection. [124] The distal region contains a 61-bp enhancer of transcription in *N. plumbaginifolia* protoplasts. [138] A slightly modified 49-bp sequence from the highly homologous enhancer region of the Bright Yellow tobacco *Gln2* gene [132] is an ethylene-responsive element (ERE) essential for ethylene responsiveness when combined with a minimal CaMV 35S promoter. [126] Enhancer activity and ethylene responsiveness depend on the integrity of two copies of the AGC-box, AGCCGCC, present in the promoters of several ethylene-responsive genes. [126,138,160,160a]

The same *Glb* deletion series has been used to analyze transcriptional regulation of ßGlu I in the micropylar endosperm of germinating tobacco seeds. [71] The distal -1452 to -1193 region, which contains the ERE, is required for high-level, ethylene-sensitive expression; the regions -1452 to -1193 and -402 to 0 are important for down-regulation by ABA; and the region -402 to -211 is necessary and sufficient for micropylar-endosperm specific expression. The -402 *Glb* promoter is the shortest fragment giving developmental regulated expression in seeds [71] and leaves. [124] It is not, however, the minimal promoter per se since the shorter -211 fragment confers root-specific expression. [124]

Analysis of the *N. plumbaginifolia* class I *Gn1* gene has shown that a short -138 bp promoter is sufficient to confer full activity in transgenic tobacco leaves and is more active than the -736 and -2000 promoters. [127] The region -138 to -98 of the *Gn1* promoter is sufficient for high-level response to *Pseudomonas syringae* pv *syringae* infection.

3.4.3.2. Class II ß-1,3-glucanase genes

Multiple regions of the ca. 1.7 kb tobacco class II *PR-2b* and *PR-2d* promoters contain elements for inducibility by SA and TMV. [125,128] For the *PR-2d* gene this includes a major *cis*-acting element in the region -364 to -288 which confers to a core CaMV 35S promoter high-level expression in response to SA. [129]

3.4.4. Signal transduction and *trans*-acting factors

A putative ethylene receptor and several upstream components of the ethylene signaling pathway have been identified (reviews, 161-164). Far less is known about downstream components closer to the activation of transcription. Nuclear factors from tobacco leaves have been described that bind defined regions of the promoters of the class I *Gn1* of *N. plumbaginifolia,* [127] the class II *PR-2d* of tobacco, [129] and the class I *Glb* of tobacco. [138,153a]

Tobacco cDNA clones have been identified representing four novel DNA-binding proteins, called ethylene-responsive element binding proteins (EREBPs), that specifically bind the ERE AGC box. [126] The mRNAs of EREBP-1 and EREBP-2 in the same class and EREBP-3 and EREBP-4, each in different classes, show distinctive expression patterns in leaves, roots, and cultured cells which are correlated with the pattern of ßGlu I expression. Accumulation of mRNAs for these EREBPs in tobacco leaves 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.

The deduced amino acid sequences of EREBPs are not similar to those of classical DNA binding proteins or transcription factors, i.e. they do not contain a basic leucine zipper or zinc finger motif. [126] The EREBP DNA binding domain is highly homologous in sequence to a domain present in the APETALA2 (AP2) protein, a regulator of meristem identity, floral organ specification, and seed coat development. [164a] Recently, homologues of the tobacco EREBPs have been isolated from *Arabidopsis thaliana* and tomato. The *Arabidopsis* EREBP, AtEBP, binds specifically to TAAGAGCCGCC, a AGC-box containing sequence and confers ethylene-responsiveness to promoters of genes encoding PR-proteins. [160] AtEBP interacts with *ocs* element binding factors (OBFs), belonging to a specific class of basic-region leucine zipper (bZIP) transcription factors. This suggests that cross-coupling between EREBP and bZIP transcription factors occurs important in regulating plant-defense related gene expression.

Further evidence linking EREBPs with the defense response has come from an analysis of the tomato *Pto* resistance gene against *Pseudomonas syringae* pv *tomato*. Three tomato genes, *Pti4*, *Pti5* and *Pti6* have been identified that physically interact with the Pto kinase. [165] Each of these genes encode a protein with characteristics that are typical of transcription factors and are similar to the tobacco EREBPs. These proteins specifically bind a DNA sequence present in the promoter

region of a large number of PR genes. These findings are of particular interest because they establish a direct relationship between EREBPs, a disease-resistance gene, and the specific activation of plant defense genes.

In animals, plants and fungi, cholera toxin (CTX) can activate signaling pathways dependent on heterotrimeric GTP binding proteins (G-proteins). [139] Tissues of transgenic tobacco plants expressing CTX show greatly reduced susceptibility to the bacterial pathogen *Pseudomonas tabaci*, accumulate high levels of SA, and constitutively express PR protein genes including PR-1 and the class II ßGlu. In contrast, the class I ßGlu are not induced and display normal developmental and ethylene-responsive regulation. In good agreement with these results, microinjection experiments demonstrate that CTX or GTP-γ-S induce the expression of a *Gus* reporter gene regulated by the PR-1 promoter, but not by the class I *Glb* promoter. Microinjection and grafting experiments strongly suggest that CTX-sensitive G-proteins are important in inducing the expression of a subset of PR genes and that these G-proteins act locally rather than systemically upstream of SA induction.

Multiple signal transduction pathways in tobacco and *Arabidopsis* and "cross-talk" between these pathways, seem to be utilized for the signaling of the induction of different subsets of PR genes in response to different pathogens. One type of pathway depends on ethylene and also leads to induction of the highly ethylene-responsive tobacco class I ßGlu and Chn. [4,80,139,161,166,167] The SA-dependent pathways associated with SAR induction also activate expression of class II and III ßGlu genes. These pathways appear to be ethylene-independent in tobacco and *Arabidopsis* [139,141,168] (reviews 150,151).

Vidal et al. [159] have recently identified a SA-independent pathway in the interaction between the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* and tobacco. Treatment of tobacco with this bacterium or a sterile culture filtrate (CF) containing elicitor(s) very rapidly induces local and systemic accumulation of transcripts for PR-proteins including ßGlu I, but not of acidic PR-1a, which, in contrast to ßGlu I, is induced by SA but not by ethylene. [2,169] SA is not the signal molecule leading to the early response of plants to *Erwinia,* [159] since induction of ßGlu I transcripts in SA-deficient transgenic *NahG* tobacco plants and wild-type plants in response to CF is comparable. Therefore, the induction of ßGlu- and other PR-protein genes by *Erwinia* and SA appear to involve two distinct pathways.

3.5. Concluding remarks

Over the last 10 years considerable progress has been in understanding the structure and regulation of plant ßGlu. It is now recognized that species of higher plants produce a broad range of ßGlu differing in primary structure, cellular localization, and catalytic activity. The most striking structural variation is the C-terminal extension that distinguishes the vacuolar class I ßGlu from the extracellular class II and III isoforms. This peptide is posttranslationally removed during

intracellular transport and is likely to contain a vacuolar targeting signal.

A major problem is establishing the biological functions of these ßGlu and understanding how intracellular localization and structure are related to these functions. The available evidence suggests that different classes of ßGlu have different functions in plant-microbe interactions. For example, the class I ßGlu, particularly in combination with class I Chn, inhibit the growth of certain fungi both in vitro and when over-expressed in transgenic plants. On the other hand, the extracellular ßGlu have weak antifungal activity, but may be involved in releasing elicitors that activate host defense reactions. These ßGlu are also induced systemically after infection. Their role, if any, in SAR is still an open question.

Several PR-like ßGlu are localized in specific floral organs. Dissolution of the callosic wall of microspores, which can be broken down by class I ßGlu, is required for pollen formation. Correlative evidence suggests that precisely regulated expression of specific anther ßGlu is required for this process. Direct evidence for a causal role of these ßGlu has yet to be established. The results of antisense experiments with the stylar ßGlu are inconclusive and the function of these proteins in the conducting tissues is not known. Class I ßGlu show a novel pattern of expression and regulation during seed germination and may have a role in weakening the endosperm to allow the penetration of the radicle. Although there is a close correlation between ßGlu I induction and endosperm rupture under a variety of physiological conditions, direct evidence for a causal role of these enzymes in germination is lacking.

Comparisons of the patterns of enzyme and steady-state mRNA accumulation and reporter-gene experiments indicate that transcription is an important site of developmental, hormonal, and pathogenesis-related regulation of ßGlu genes. Nevertheless, these studies do not rule out additional regulation, e.g., at the level of translation or protein degradation. Transcriptional regulation involves multiple signaling pathways linking different signal molecules to the same and different target ßGlu genes. Promoters regions have been identified that are important for responses to ethylene, SA, elicitors, and infection with viral and bacterial pathogens. The most interesting of these is the ERE present in class I ßGlu and Chn genes which is responsible for high-level expression in response to ethylene. Several components of the ethylene-signaling pathway have now been identified and studies aimed at relating these components to the EREBP transcription factors will undoubtedly provide insight into the regulation and function of the ßGlu.

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