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Induced expression of pathogenesis-related protein genes in soybean by wounding and the *Phytophthora sojae* cell wall glucan elicitor

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Abstract

The effects of the glucan elicitor (WGE) from the cell wall of *Phytophthora sojae* on the transcriptional upregulation of genes representing five families of pathogenesis-related (PR) proteins have been investigated in soybean under wound and minimal-wound conditions. Although each has its own expression pattern, these PR protein genes can be broadly categorized into two groups. Induced expression of genes for PR-1a and PR-10 is predominantly limited to cells proximal to the point of treatment, while that of genes for the elicitor-releasing endoglucanase (GLU, a PR-2), a WIN-like protein (WIN, a PR-4) and a Kunitz trypsin inhibitor (KTI, a PR-6) is seen in proximal, near-proximal and distal cells. GLU is the only family that shows weak though detectable constitutive (basal) gene expression. Wounding induces only barely detectable to weak expression of genes for PR-1a and KTI and somewhat stronger expression of those for GLU, WIN and PR-10. PR-1a gene expression is essentially induced only by WGE; similarly, that for KTI is greatly enhanced by WGE. While gene expression for PR-10 is induced by wounding, it is also strongly upregulated by WGE in a minimal wound background, suggesting a possibly independent effect of WGE. Finally, WGE also enhances the speed and magnitude of expression of GLU and WIN genes in all cell zones. Methyl jasmonate induces strong gene expression for KTI and the ethylene precursor, ACC, and jasmonic acid (JA) enhance that of GLU. The results are discussed in the context of the recently observed WGE- and JA-induced distal defense potentiation in soybean.

Keywords: Glucanase; WIN-like protein; Kunitz trypsin inhibitor; PR proteins; Elicitor; Wounding; Ethylene; Jasmonic acid; Soybean defense

1. Introduction

The interaction of *Phytophthora sojae* and soybean has been a useful association to elucidate various biochemical aspects of host-pathogen interactions. The secondary product pathways in soybean leading to the accumulation of the pterocarpan phytoalexins, the glyceollins, were among the first to be thoroughly characterized [11] and it was also in soybean that the first comprehensive plant metabolic profiling protocols were developed [19]. In addition, the β-1,3/1,6 glucan elicitor from the cell wall of *P. sojae* (WGE) is one of the most thoroughly characterized defense elicitors [2,32,38,50], and putative receptors for the elicitor have been reported [6,15,43]. The extensive use of metabolic profiling have led to a very clear delineation of the multiplicity of secondary product responses in soybean and their spatial and temporal regulation [17,18,20,21,23]. Responses induced by WGE closely mimic many of those seen in incompatible infections and include the accumulations of the glyceollins and phenolic polymers in cells immediately proximal to treatment and the massive accumulation of conjugates of defense-related isoflavones in cells distal to treatment. Whether cells display the proximal or distal cell response to WGE is regulated in part by the phenomenon of elicitation competency [18,21], a process by which proximal cells are activated to specifically enable the glyceollin and phenolic polymer responses by factors associated with wounded or hypersensitively dying cells.

Recently we demonstrated that treatment with WGE is also highly effective in inducing disease resistance in distal

Abbreviations: ACC, 1-amino-cyclopropane-carboxylic acid; GLU/Glu, elicitor-releasing glucanase protein/gene; EST, expressed sequence tag; HR, hypersensitive response; ISR, induced systemic resistance; JA, jasmonic acid; JAME, jasmonic acid methyl ester; KTI/Kti, Kunitz trypsin inhibitor protein/gene; SA, salicylic acid; SAR, systemic acquired resistance; PR, pathogenesis-related; WGE, wall glucan elicitor; WIN/Win, Win-like protein/gene.

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cells (several hundred cells away from the point of treatment) against subsequent infection by *P. sojae* [34]. Mycolaminaran (a cytoplasmic β-1,3/1,6 glucan from *P. sojae* closely related to WGE), jasmonic acid (JA), jasmonic acid methyl ester (JAME) and the ethylene precursor, 1-amino-cyclopropane carboxylic acid (ACC), were also effective, while salicylic acid (SA) was not [34]. The activities of ACC, JA and JAME and the lack of activity of SA suggest that this distal defense potentiation response in soybeans may share some attributes in common with non-SA mediated induced systemic resistance (ISR or ISR-like) responses [10,45]. Careful analysis of WGE-induced distal protection suggested that two forms of induced resistance could be differentiated, a lesion-limiting resistance and a second form of resistance characterized by nearly complete containment of the pathogen. The well-characterized phenylpropanoid responses appear to contribute to lesion-limiting resistance, but not to containment resistance [34]. It was hypothesized that pathogenesis-related (PR) proteins might play a central role in this latter, more effective form of distal defense potentiation. PR proteins were originally observed in several laboratories in tobacco following viral infections. Defined as proteins that are induced in pathological and related situations, they were later grouped into five major families [46]. As analogous proteins were identified in other species and additional PR proteins were found, they were grouped into ten [28] and then fourteen families [47], with additional families being added with time. The PR proteins are thought to play an important role in induced and in some cases constitutive resistance. The systemic activation of certain PR protein genes has led to their association with various forms of systemic induced resistance [44] in a number of plants.

Although there are some studies of individual PR proteins or their genes in soybean, their role in defense has not been as comprehensively studied as in certain other plants. Accumulations of several acidic peroxidase isoforms occur in proximal and distal cells in response to WGE treatment [17] and expression of mRNA for a basic peroxidase is enhanced in infected hypocotyls [49]. PR-1 clones have been shown to be common in a cDNA library of *P. sojae* infected tissues [37] and several papers describe the induced expression of glucanases (PR-2) or their genes in response to ethylene [35,42] or infection [5,48]. Chitinase enzyme induction has also been observed in infected tissues [48]. Consistent with these results, accumulations of glucanase and chitinases enzymatic activities have been demonstrated in soybean suspension cultures in response to pathogen elicitors [24]. A member of the PR-10 gene family is reported to be activated by incompatible infection in soybean suspension cultures [29]. Finally, recent work in our lab has shown the importance of the soybean Kunitz trypsin inhibitor (KTI) as a regulatory protein in the establishment of elicitation competency [33]. KTI can be considered a member of the PR-6 protease inhibitor family.

Most of the above studies on PR protein responses in soybean were carried out with infected tissues. To our knowledge, no comprehensive study of the effects of elicitor on PR protein gene expression has been published in soybean. Employing the same temporally and spatially defined thin sections used to delineate the multiplicity and coordination of secondary product responses to elicitor described above, we have initiated a series of studies of elicitor-related events regulating PR gene activation in this system. For this work, the ready accessibility of the large public soybean EST database greatly facilitated our efforts. We hope that these studies will contribute to further elucidation of the relative roles of secondary product and PR protein responses in local and distal defense reactions and distal defense potentiation. Results on the effects of WGE on gene expression for representatives of five families of PR proteins are reported here.

2. Materials and methods

2.1. Plant materials

Soybean (cultivar Williams) seedlings were grown and cotyledons harvested from 7 to 8 day old seedlings as described earlier [23]. The cut and snapped (minimal wound) cotyledon assays were also performed as described previously [20,21], respectively, with slight modifications as described below. An advantage of the soybean cotyledon tissue is that it is made up of tightly aligned and uniform columns of mesophyll parenchyma cells, thus allowing precise spatial characterization of responses. For cut cotyledons, three sections of tissue were harvested for analysis at various times after treatment. The first two sections were cut from a column of cells harvested at and below the cut surface as described before [20], except that a number 2 (rather than a number 1) cork borer was used to obtain more tissue. The first section, constituting the *proximal cell section*, was a cross section of the column of cells approximately 0.6 mm thick containing the treated surface and the immediately underlying cells. The second section, constituting the *near-proximal cell section*, consisted of a cross section including the remaining 1.2 mm of tissue. Finally, in most cases a third section, constituting the *distal cell section*, was a 0.6 mm transverse section through the original cotyledon harvested 3–4 mm from the edge of the wound at the point of treatment (see Ref. [34] for a diagram). In the snapped cotyledon assay, three consecutive transverse cross sections from the point of treatment (0.6, 1.2 and 0.6 mm, respectively) were harvested to represent the proximal, near-proximal and distal sections. In all cases, a minimum of 200 mg of tissue was harvested for mRNA isolation. Because of the relatively small sections taken, this normally required the pooling of samples from approximately 20 cotyledons.

2.2. Chemicals

The cell wall glucan elicitor (WGE) from race 1 of *P. sojae* was purified and characterized as reported previously [2,23]. Methyl jasmonate was obtained from Bedoukian Research Inc. (Danbury, CT, USA). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.3. Derivation of probes

The extensive EST database for soybean has allowed us to identify soybean homologs for most classes of the PR proteins [47]. ESTs of interest for initial grouping into PR protein families were identified through annotations in the database and also by BLAST searches of the soybean EST database using the most relevant prototype gene(s) for each class. Contigs for specific family members of genes of interest were assembled from individual ESTs using the contig assembly program, CAP3 [25]. Contigs were then scanned for primer pairs (20 nucleotides in length) that would generate an approximately 300–400 bp product using Primer Designer software (v. 3.0, Scientific and Educational Software, Durham, NC) or Primer-3 software (1996, 1997, Steve Rozen, Helen J. Skaletsky, http://www-genome.wi.mit.edu/genome_software/other/primer3.html). The theoretically primed products were then re-BLASTed against the soybean EST database to help in grouping them, organizing family members and in choosing the probe with the most desired specificity. Oligo primer sets were custom synthesized at Integrated DNA Technologies (Coralville, IA).

PCR was used to obtain probes by priming appropriate products from genomic DNA or reverse transcribed cDNA from total RNA (e.g. from 3 to 4 h elicitor treated tissue) as template. The cultivar Williams was used since it is one of the most commonly used cultivars, especially in library constructions for ESTs. Products were first gel purified and only the band for the correct size was excised. Further purification was achieved by electroelution followed by clean-up with DEAE Sephacel (Sigma Chemical Co., St. Louis, MO) mini-columns or by direct extractions from the agarose block with Qiaquick kits (Qiagen Inc., Valencia, CA). The final PCR products were each subjected to automated sequencing at the Ohio State University Plant-Microbe Genomics Facility to verify their correspondence to the correct genes. Probes were labeled with α-^{32}P-dCTP using Random Priming Kits (Life Technologies/Invitrogen Corp., Carlsbad, CA). Some aspects of the choice and specificity of the probes are further described individually for each gene in Section 3. Table 1 shows the actual primer sets used to prime the probes used.

2.4. RNA isolation and northern blots

An adaptation of a LiCl precipitation RNA miniprep protocol [9] has been routinely used in our lab to accommodate the small samples resulting from the thin sectioning required to enrich specific spatial zones. Northern blotting and hybridization was carried out using standard procedures [3]. Except where noted, 10 ug of total RNA was used per lane for formaldehyde denaturing gels, and Nytran SPC membranes (Schleicher and Schuell Biosciences Inc., Keene, NH) were used for blotting. Hybridizations were carried out with 50% formamide at 42 C and final washings were usually done at 0.2XSSC/0.1% SDS at 38–40 C. Total RNA was quantified by A_{260} to guide in loading of gels. Ethidium bromide staining of ribosomal RNA in the gels was photographed using a ultraviolet trans-illuminator and is presented with the Northern blots to provide loading controls. Filters were usually stripped according to the manufacturer's instructions (in 6XSSPE-50% formamide two times at 65 C for 30 min) for hybridizations to more than one probe. The experiments have been performed over a period of several years. Some experiments were repeated several times in minor variations and only representative data are shown. Each figure represents results with a single probe within the same experiment. Since the data for a given figure is the result of hybridization and washing under identical conditions (in a single reaction) comparisons within a figure are thus appropriate.

Table 1
Primer sets used to obtain products used as probes

Gene family	Template[a]	Specific class	5′ primer	3′ primer	Product size (bp)
PR-1	A	PR-1a	TGATGTTGCCTACGCTCAAG	ATCCAAGACGCACCGAGTTA	365
PR-2	A	Class III β-Glucanase	CCTAGCATCTAGCCAAGACA	GTGAACCATCTTGCACTACC	434
PR-4	B	WIN-like	CTCGTGGCCGTGATTCTTGT	GAGCATCGAGGATGGAGAGT	246
PR-6	A	KTI (general)	GCCTTCACCACCTCATACCT	CAGCTTGCTGTGGACAGAAC	457
PR-6	A	KTI-S	CGGCACGAGGACAAGATAGA	GAATAACGGTCCACCGAGAA	416
PR-10	B	SAM22	AGTTACAGATGCCGACAACG	CCTCAATGGCCTTGAAGAGA	377

[a] A, genomic DNA; B, cDNA from 3 h WGE-elicited cotyledon total RNA.

3. Results

3.1. PR protein gene expression in proximal cells

3.1.1. PR-1a gene expression is transiently induced in proximal cells by WGE

The PR-1 family is one of the most commonly examined PR protein families. Their function remains unclear despite many studies and enigmatic homologies to proteins of various activity from many non-plant species [47]. In tobacco, of three PR-1 proteins, PR-1a is one for which genes are strongly upregulated during infection. Its activation by salicylic acid in tobacco and Arabidopsis has been associated with the establishment of SAR, and its expression is commonly used as a marker for SAR [47]. Although other PR-1-like sequences exist, the clearest homolog we found by analysis of the soybean EST database is a PR-1a homolog. Our derived PCR product is 98% identical to an unpublished, but registered sequence (GenBank Accession AF136636). Gene expression for PR-1a in WGE-treated or control cut cotyledon tissues is shown in Fig. 1. Although there is no detectable constitutive gene expression, PR-1a mRNA is elevated to barely detectable levels at late time points (24–48 h) by wounding alone, but only in cells immediately proximal to the wound. While the same weak wound-induced response is seen as an underlying response in WGE-treated tissues, WGE also leads to an additional earlier and greatly enhanced expression. WGE-activated expression is sharp and transient at 16 h, and like the wound-induced expression, is essentially limited to proximal cells, though some near-proximal expression is detectable. No expression in distal cells was observed (data not shown). This result was repeated in several experiments.

3.1.2. Induced PR-10 gene expression is rapid, prolonged and upregulated by both wounding and WGE

Parsley ‘PR1’ was an early and well-studied prototypic PR-10 gene [39,40]. Homologs of this class of genes were found in many plants in which they have been associated with pathogenesis, general stress, development or as pollen allergens [28,47]. It deviates from the original, classically defined PR protein genes in that it encodes an intracellular product. Although some limited homology to ribonucleases

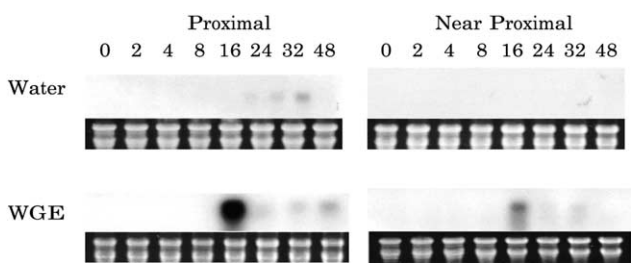

Fig. 1. WGE-induced gene expression of PR-1a. Water or 30 μg/ml WGE (20 μl/cotyledon) were applied to the wounded surface in the cut cotyledon protocol. Proximal and near-proximal sections were harvested at 0, 2, 4, 8, 16, 24, 32 and 48 h and Northern blots were performed as described in Section 2.

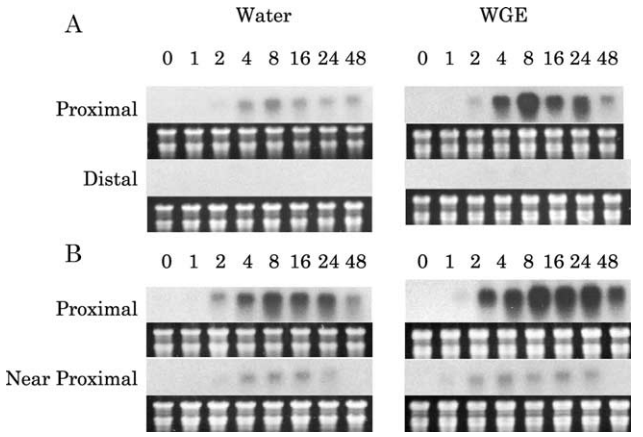

Fig. 2. Wound and WGE-induced gene expression of PR-10. A, water or 30 μg/ml WGE (15 ul/cotyledon) were applied to surface cells in the minimal-wound snapped cotyledon protocol. B, water or 30 ug/ml WGE (30 ul/cotyledon) were applied to the surface cells on freshly cut cotyledons. Proximal, near proximal or distal sections were harvested as indicated at 0, 1, 2, 4, 8, 16, 24 and 48 h and Northern blots were performed as described in Section 2, except that in both of these experiments a total RNA of 5 ug was used per lane.

has been detected, its true function remains to be established [28,47]. In soybean, an earlier cDNA clone encoding a general stress protein (*S*tarvation *A*ssociated *M*essages 22, SAM22) [8] turns out to be homologous to the PR-10 family. An electronic analysis of expression in the soybean EST database done by us suggests that cDNAs for SAM22 homologs are also among the most abundant transcripts associated with pathogenesis libraries. As shown in Fig. 2, using a derived probe 98% identical to Sam22 (from the cultivar Mandarin) in the region of the primed PCR product, we compared its expression in minimally wounded snapped cotyledons [21] and the classical cut cotyledons assay [20]. There is no detectable constitutive expression, but some expression is seen in proximal cells even in minimally wounded, water-treated controls in snapped cotyledons beginning at 2–4 h and continuing out to 48 h (Fig. 2A). Similar, but much stronger upregulation is seen in WGE-treated snapped cotyledon samples, particularly at 8 h. In this minimal wound protocol, there is no detectable expression in distal cells. More severe wounding (Fig. 2B, cut panels) leads to much more pronounced expression in proximal cells. WGE again greatly enhances message accumulation in this section over the same time frame. While there is some expression in near-proximal cells, expression is predominantly limited to proximal cells.

3.2. Some PR protein genes are also upregulated in near proximal and distal cells

3.2.1. Gene expression of a wound-inducible PR-4 is seen in all tissue sections and is more rapidly and strongly induced in WGE-treated tissues

The PR-4 genes are somewhat divergent, but may contain one or more elements with homology to chitin-binding

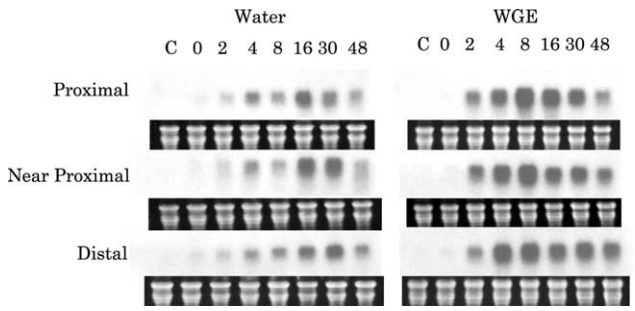

Fig. 3. Wound and WGE-induced gene expression of WIN-like Protein (PR-4). Water (5 μl) was applied to the wounded surface in the cut cotyledon protocol. After 4 h, water or 100 μg/ml WGE (20 μl) were applied to the same cotyledons. Proximal, near-proximal and distal sections were harvested at 0, 2, 4, 8, 16, 30 and 48 h after the second treatment and Northern blots were performed as described in Section 2. The control (C) tissues were treated identically except that the various sections were harvested immediately after wounding.

domains, wound-induced (WIN) proteins of potato, and several plant lectins [28]. Because expression of the WIN-like protein genes is notably activated by wounding [41], a process we have demonstrated to enhance elicitation competency [18], we identified a Win-like homolog in the soybean EST database. We derived a probe that is 100% identical in the PCR product region to a registered Win sequence from soybean (GenBank Accession Z11977) that was cloned through artifactual ligation to a Cox II gene [7]. Its expression in control and WGE-treated cut cotyledons is shown in Fig. 3. Though constitutive expression was not detected, message for this WIN sequence begins to accumulate rapidly in both wounded and WGE-treated tissues. In wounded tissues, its expression is gradual, with maximal induction at about 16–30 h in all sections. Note that in this experiment, WGE treatment was delayed for 4 h after wounding for reasons that are further discussed in Section 3.2.3. Even with this delayed application, WGE enhances both the speed and magnitude of mRNA accumulation in all sections, shifting maximal expression to 4–8 h. In some other experiments (data not shown) expression in near proximal and distal sections was actually stronger than in proximal cells.

3.2.2. Wound-induced gene expression for KTI (a PR-6) is weak, but enhanced by WGE or JAME

Protease inhibitors have recently been categorized as PR-6 proteins [28,47]. The soybean Kunitz trypsin inhibitor (KTI) was originally isolated as a seed storage protein with potential anti-feeding and anti-nutritional effects on herbivores and humans. More recently, in our lab it was identified as a potential regulatory factor contributing to the elicitation competency that enhances phenolic polymer deposition in response to the WGE [33]. Thus we were interested to determine if, in addition to its developmental regulation [27], it is induced as a potential PR protein. There are at least 10 KTI genes in the soybean genome [27] which share strong sequence homology. Initially we derived a probe from the KTI3 gene [27] that is general and non-discriminatory for this family of genes and used it in some preliminary studies. We were able to show that expression of some member(s) of this PR-6 family are induced by wounding and further enhanced by either methyl jasmonate (JAME) or WGE. Fig. 4A shows an example of such a Northern comparing wound and JAME responses in the cut cotyledon protocol. While wounding leads to some detectable induced expression, JAME treatment greatly enhances expression over the period 2–16 h.

The rapidly growing soybean EST database allowed us to undertake electronic expression analysis of specific KTI gene family members. This analysis suggested that a particular family member, corresponding to a previous clone for a variant called KTI-S [16], is specifically enhanced in defense response libraries. The KTI-S gene has some significant sequence differences from the other KTI gene family members. We designed primers based on this specific member and the PCR product used as a probe shows 98% identity to the KTI-S gene. With this probe, induced expression is generally stronger and more discrete than we observed with the general probe. As shown in Fig. 4B, even minimal wounding (snapped cotyledon assay) leads to weakly induced expression of the KTI-S gene in proximal cells. In this situation proximal expression begins before and peaks at 8 h, falling off rapidly after 16 h. In distal cells, expression in the controls is somewhat later (beginning at 8 h, peaking at 16 h), but expression is stronger over the 24–48 h time frame. Expression in WGE-treated tissues follows very similar time courses to the controls in both proximal and distal cells, but expression is

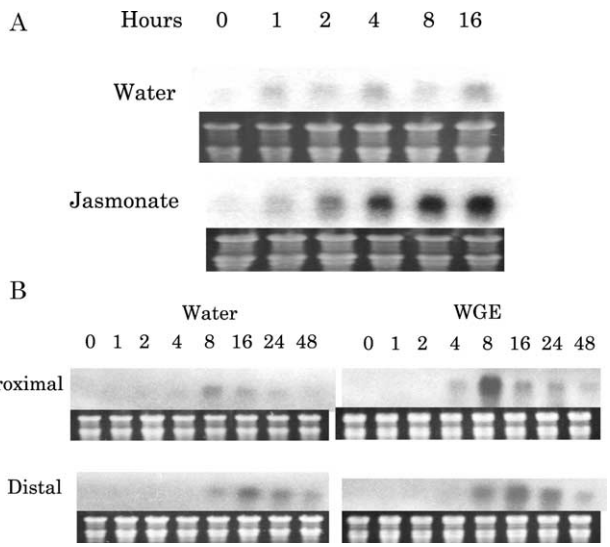

Fig. 4. Induced gene expression of KTI and KTI-S (PR-6). A, water or 25 uM methyl jasmonate (20 μl/cotyledon) were applied to the wounded surface in the cut cotyledon protocol. Proximal sections were harvested at 0, 1, 2, 4, 8, and 16 h and Northern blots were performed as described in Section 2. B, the same filters used for the snapped cotyledon assay in Fig. 2A were used in this experiment with the PCR generated probe for KTI-S as described in Section 2. Loading controls were the same as Fig. 2A.

greatly enhanced at 8 h in proximal cells and somewhat enhanced in distal cells.

3.2.3. Wound-induced expression of the elicitor-releasing endo-β-1,3-glucanase (PR-2) gene is rapid and prolonged and WGE, JA and ACC all have some enhancing effect

Endo-glucanases were among the most early PR proteins to be studied for their involvement in defense and have been designated as the PR-2 class [28,47]. There are multiple distinct β-1,3-glucanases in soybean that fit into the five classes established in tobacco [26]. Our electronic expression analysis of the soybean EST database, which includes several recently added pathogen infection related libraries, suggests that potentially only two of these β-1,3-glucanase genes are likely highly expressed in these soybean defense responses. For the present studies we chose to focus on the specific endo-β-1,3-glucanase that has been implicated in releasing active elicitor fragments from WGE itself [42]. It is also the only β-1,3-glucanase present in the *P. sojae* infection EST library. Our PCR-derived probe was 91% identical to the cDNA clone of this elicitor-releasing glucanase from soybean cultivar Harosoy 63 [42] and 93% identical to another clone, Sgn1, from cultivar Williams [5]. Both of these sequences encode basic proteins belonging to the Class III glucanases (as defined in tobacco) and corresponding to the second of 12 groups of glucanases as defined in soybean by Shoemaker and coworkers [26]. The results of Northern blots using this probe for response to wounding and WGE treatment are shown in Fig. 5A. Note that this experiment again involved a 4 h delay after wounding, but before treatment with elicitor. Wound-induced elicitation competency is maximal at 3–4 h [18]. This maximal state of elicitation competency, also leads to maximal glucan-induced distal defense potentiation [34]. Thus in Fig. 5A, the control (C) represents freshly harvested tissues and the 0 h time point is 4 h after wounding, but 0 h after WGE treatment. As shown in Fig. 5A there is some level of constitutive gene expression of PR-2. This is consistent with several studies showing the presence of constitutive elicitor releasing glucanase activity in cotyledon tissues [1,50]. Although there is some variation, PR-2 gene expression is generally relatively low both in the controls and 4 h after wounding ($t = 0$ h). Depending on the tissue section, it is strongly upregulated by wounding over the 2–16 h time frame (6–20 h after wounding) after which it falls off. It is interesting that distal gene expression of PR-2 is as rapid, even at early time points, as it is in proximal cells, suggesting a very rapid spatial signaling process. WGE treatment leads to some further enhancement of expression in all sections, but particularly over the 2–8 h time points in near-proximal and distal cells, suggesting that WGE may enhance the processes involved in distal signaling.

Figure 5B shows an experiment in which minimally wounded (snapped) cotyledon tissues were treated with jasmonic acid or the ethylene precursor, 1-amino-cyclopropane-caboxylic acid (ACC). Interestingly, even the very minimal wounding associated with this protocol is sufficient

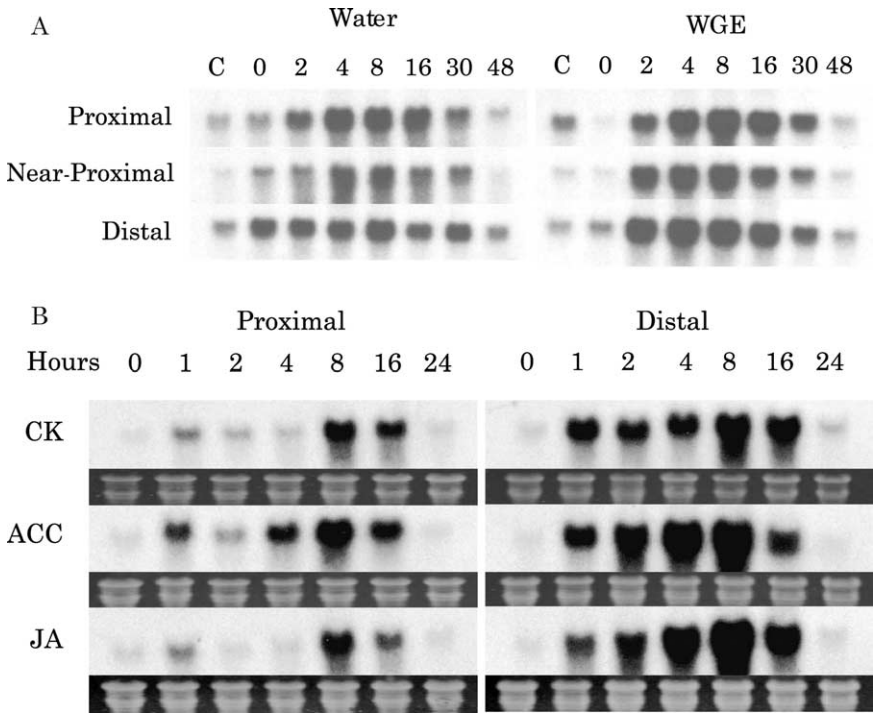

Fig. 5. Induced gene expression of the elicitor-releasing glucanase (PR-2). A, the same filters used for Fig. 3 were used in this experiment with the PCR generated probe for PR-2 as described in Section 2. Loading controls were the same as Fig. 3. B, CK (water), 100 μM ACC or 33 μM JA (15 μl/cotyledon) were applied to the freshly exposed surface in the snapped cotyledon protocol. Proximal and distal sections were harvested at 0, 1, 2, 4, 8, 16, and 24 h and Northern blots were performed as described in Section 2.

for upregulation of this family of genes and this enhanced expression appears to actually be more rapid and of higher magnitude in distal tissues than in the treated proximal sections. ACC enhances PR-2 gene expression somewhat in proximal tissues and both ACC and JA strongly enhance expression in distal tissues.

4. Discussion

Previous analyses of defense responses in soybean have demonstrated that both wounding and treatment with *P. sojae* wall glucan elicitor (WGE) lead to differential spatial and temporal inductions and/or modulations of secondary product defense responses [18,21,22]. In this paper we showed that wounding and WGE treatment also have profound and differential effects on the expression of representatives of five families of PR protein genes. Although each has its own activation kinetics and pattern, we can broadly categorize them into two groups. Induced gene expression for PR-1a and PR-10 is predominantly limited to cells proximal to the treated surface, whereas that for GLU (PR-2), WIN (PR-4) and KTI (PR-6) is seen in proximal, near-proximal and distal cells. GLU is the only family that shows weak though detectable constitutive (basal) gene expression. In terms of response to wounding or elicitor, PR-1a gene expression is essentially induced only by WGE. The extensive upregulation of PR-10 genes by WGE compared to the minimal-wound water control in snapped cotyledons argues that one or more of this family of genes may also respond to this glucan elicitor.

Wounding induces only weak gene expression for KTI and somewhat stronger expression for GLU, WIN and PR-10. This is not surprising in that genes for members of both the PR-2 and PR-4 families are known to be wound or ethylene activated [35,41,42,51]. Consistent with the rapid spatial spread of ethylene-mediated responses, GLU and WIN genes are expressed over a similar time frame in all cell populations. WGE enhances the speed and magnitude of distal gene expression for both GLU and WIN, suggesting that it may enhance or synergize with the underlying wound-mediated signaling process. Expression of KTI genes show a different pattern from all of the other genes in that their activation in distal cells is relatively late and WGE enhances, but does not affect the timing of response. This may suggest a somewhat different wound-mediated mechanism of response and WGE enhancement. Preliminary results reported here suggest that the wound hormone methyl jasmonate (JAME) is capable of greatly enhancing gene expression for KTI. This is also consistent with the known activation of other defense-related serine protease inhibitor genes by jasmonic acid [13]. Further work will be needed to determine the relative contributions of ethylene and jasmonate signaling in these various responses.

In conclusion, then, except for PR1a, and possibly some members of the PR-10 family, the other PR gene families studied are all wound-inducible and enhanced by WGE consistent with ethylene/jasmonate-mediated signaling pathways playing a primary role. Recently, a very detailed analysis of the effects of natural and synthetic analogs of jasmonic acid on phytoalexin and phenylpropanoid enzyme gene activation has been reported [14]. It is thus apparent that jasmonic acid has effects on both secondary product and PR gene defense responses. Therefore, wound-jasmonate/ethylene pathways appear to play central roles in soybean defense.

In studies using the same protocols as used here, we have also shown that WGE treatment leads to distal defense potentiation, characterized by nearly complete protection against subsequent inoculation by *P. sojae* [34]. As noted in the introduction, a lesion-containment resistance, which is part of this response, has been shown to involve the potentiation of unknown non-phenylpropanoid defense responses [34]. It was hypothesized that PR proteins might play a role in this form of induced resistance. While more detailed analyses are needed, the patterns of activation of the PR protein genes described here suggest that GLU, WIN and KTI are potential candidates for involvement. Since wounding alone leads only to relatively weak distal defense potentiation, the strong enhancement of gene expression for GLU and WIN in distal cells by the glucan may be particularly relevant.

PR proteins have been studied in many systems in the context of infection or elicitation. Among them, another system in which elicitor induction of both secondary product and PR protein responses has been comprehensively studied is in the well characterized interaction of parsley with *P. sojae*. Interestingly, in this non-host interaction the cell wall glucan (WGE) from *P. sojae* is inactive, but a glycoprotein elicitor found associated with the pathogen cell wall induces both parsley phenylpropanoid phytoalexins and PR proteins [30,36]. Proteolytic cleavage of the glycoprotein elicitor led to the identification of a thirteen amino acid motif, Pep-13, which is necessary and sufficient for elicitor activity [30]. Likewise, acid [38] and enzymatic [50] cleavage of WGE has confirmed that smaller β-1,3/β1,6 linked oligoglucans are sufficient for activity. Thus, although WGE and the glycoprotein elicitor are chemically very different, both are associated with the cell wall of the same oomycetic species, both contain active motifs that are specifically found in the pathogen but not in the host, and both are capable of inducing what would seem to be very divergent phenylpropanoid and PR protein responses. As pointed out by Brunner et al. [4], such elicitors share many attributes with pathogen-derived or associated molecular patterns or motifs that function in innate immunity in animals [31].

Some members of the WRKY family have been emerging as major transcription factors associated with defense gene activation [12]. For example, in the parsley system, PR protein gene activation is thought to be mediated in part by WRKY transcription factors [31]. In this context,

preliminary studies from our lab suggest that related sequences are activated in soybean within 1 h and peaks 1–2 h after wounding (M.Y. Graham, unpublished), consistent with a potential role in the establishment of elicitation competency, which is activated over a period of 2–4 h [18], and/or induced PR protein gene expression. More detailed studies are underway.

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