Plant Physiol. (1995) 108: 929–937

Activation of Two Osmotin-Like Protein Genes by Abiotic Stimuli and Fungal Pathogen in Transgenic Potato Plants[1]

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Osmotin-like proteins are encoded by at least six members of a multigene family in *Solanum commersonii*. A genomic clone (λpGEM2a-7) that contains two osmotin-like protein genes (OSML13 and OSML81) arranged in the same transcriptional orientation has been isolated. Restriction mapping and sequence analysis indicated that the two intronless genes correspond to the previously characterized pA13 and pA81 cDNAs. To study the transcriptional activation of OSML13 and OSML81 promoters, the 5′ flanking DNA sequence (−1078 to +35 of OSML13 and −1054 to +41 of OSML81) was fused to the β-glucuronidase (GUS) coding region, and the chimeric gene fusions were introduced into wild potato (*S. commersonii*) plants via *Agrobacterium*-mediated transformation. Analysis of the chimeric gene expression in transgenic potato plants showed that both 5′ flanking DNA sequences are sufficient to impart GUS inducibility by abscisic acid, NaCl, salicylic acid, wounding, and fungal infection. Low temperature activated both chimeric genes only slightly. Infection with *Phytophthora infestans* resulted in strong GUS expression from both chimeric genes primarily in the sites of pathogen invasion, suggesting a limited diffusion of fungal infection-mediated signals. The expression patterns of both osmotin-like protein genes implicate their dual functions in osmotic stress and plant pathogen defense.

Plants respond to adverse environmental stress and pathogen attack by expression of specific genes and synthesis of a large number of stress proteins with putative roles in stress adaptation and/or plant defense (Skriver and Mundy, 1990; Cutt and Klessig et al., 1992). Among stress proteins are osmotin and osmotin-like proteins that have also been classified as members of plant PR type-5 proteins (Singh et al., 1989; Bol et al., 1990; Zhu et al., 1993, 1995). It has been demonstrated that tobacco osmotin gene expression is activated by ABA, NaCl, wounding, viral infection, and ethylene (LaRosa et al., 1992; Nelson et al., 1992). In addition, the osmotin gene promoter exhibits specific temporal and spatial expression patterns during normal plant development and after adaptation to NaCl (Kononowicz et al., 1992). The overall expression patterns of osmotin and osmotin-like protein genes suggest their dual functions in osmotic stress and plant defense (Kononowicz et al., 1992; LaRosa et al., 1992; Nelson et al., 1992; Zhu et al., 1995). There is accumulating evidence that osmotin and osmotin-like proteins have in vitro antifungal activity toward the fungus *Phytophthora infestans*, which causes late blight in potato and tomato (Woloshuk et al., 1991), although their role in osmotic stress adaptation remains unknown. It has been recently demonstrated that overexpression of tobacco osmotin confers enhanced resistance to *P. infestans* in transgenic potato plants, providing evidence of a function for osmotin in planta (Liu et al., 1994).

Given the complexity of signal transduction pathways in plant pathogen defense, it is essential to unravel key regulatory elements mediating PR gene expression (Eyal et al., 1993; Hart et al., 1993; Hennig et al., 1993; Uknes et al., 1993; Korfhage et al., 1994). Previously, we have characterized three cDNAs encoding osmotin-like proteins, and RNA gel blot analysis with gene-specific probes indicates that *P. infestans* infection-induced accumulation of osmotin-like protein mRNAs is confined to the infected leaf tissues (Zhu et al., 1995). To gain insight into the regulation of osmotin-like protein gene expression during plant-fungal pathogen interaction, in this study we isolated and characterized two closely related genes encoding osmotin-like proteins. Using the GUS gene fusion system (Jefferson et al., 1987), we focused primarily on the transcriptional activation of two osmotin-like protein genes by fungal infection. We also examined the activation of these two genes by abiotic stimuli such as ABA, NaCl, wounding, and SA. We demonstrate that both putative promoters contain regulatory elements required for localized activation of both osmotin-like protein genes upon fungal invasion.

MATERIALS AND METHODS

Biological Materials

Plants of wild potato (*Solanum commersonii*) were propagated by cuttings and grown in vitro on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with 7 g L^{-1} agar in Magenta GA 7 vessels (Magenta Co., Chicago, IL) in a controlled-environmental

[1] This is Scientific Journal Series Paper No. 21302 of the Minnesota Agricultural Experiment Station, St. Paul.

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Abbreviations: PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance.

chamber under cool-white fluorescent lights (14-h photoperiod, 50 μmol m^{-2} s^{-1}) at 23°C. Growth of mycelia, induction of sporangia, and preparation of zoospores of *Phytophthora infestans* were according to Caten and Jinks (1967).

DNA Gel Blot Analysis

Genomic DNA of *S. commersonii* was isolated according to the method of Dellaporta et al. (1983) and digested with *Eco*RI, *Xba*I, and *Eco*RI/*Xba*I. DNA gel blot analysis followed standard procedures (Sambrook et al., 1989). A *Eco*RI/*Dra*I fragment containing the entire coding region of pA13 cDNA (Zhu et al., 1993) was labeled with [^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1984). Gene-specific riboprobes were synthesized by in vitro transcription of the 3′ noncoding regions of pA13 and pA81 cDNAs originally cloned in the *Eco*RI/*Xho*I sites of pBluescript SK− (Stratagene).

Construction of a Genomic Library and Isolation of Osmotin-Like Protein Genes

Genomic DNA was isolated from *S. commersonii* plants, partially digested with *Sau*3A, filled in with dATP and dGTP, and ligated into *Xho*I half-site arms of λGEM-11 vector (Promega). The ligated DNA was packaged in vitro using the Packagene (Promega) packaging system. Approximately 2.5 × 10^5 recombinant phages were screened by plaque hybridization (Sambrook et al., 1989) using α-^{32}P-labeled pA13 cDNA (Zhu et al., 1993). The hybridizing plaques were picked and purified by two additional rounds of hybridization, resulting in the isolation of 12 positive clones. Recombinant phage DNA was prepared following the supplier's instructions (Promega). DNA: DNA hybridization analysis with a 3′ gene-specific probe derived from pA13 cDNA identified a genomic clone (μpGEM2a-7). A 5.5-kb *Eco*RI fragment that hybridizes exclusively to the pA13 gene-specific probe was excised from λpGEM2a-7 and subcloned into the *Eco*RI site of pBluescript SK− (Stratagene). Restriction mapping of the 5.5-kb *Eco*RI fragment was performed using restriction digestion and DNA:DNA gel blot hybridization. Nested sets of exonuclease III deletions were prepared from the 5.5-kb *Eco*RI DNA subclone as described (Henikoff, 1984), and both strands of DNA were sequenced according to standard procedures (Sanger et al., 1977). Nucleotide sequence analyses were carried out using the IntelliGenetics Suite (Mountain View, CA).

Primer Extension

To determine the transcription start sites of both osmotin-like protein genes, gene-specific oligonucleotides complementary to the 5′ leader sequences of OSML13 (5′-CATGCTTGTGGAACTATATATTT-3′) and OSML81 (5′-CATGTTAATGTGTTGAAATT-3′) were synthesized (Oligo Inc., Wilsonville, OR). The oligonucleotides were labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (Sambrook et al., 1989). The ^{32}P-labeled oligonucleotides (10^5 cpm) were annealed to 20 μg of total RNA isolated from *S. commersonii* cell cultures grown in the presence or absence of ABA (75 μM) for 3 h. Experimental conditions for annealing, hybridization, and extension with reverse transcriptase followed standard procedures (Sambrook et al., 1989). Primer extension products were analyzed on an 8% denaturing polyacrylamide sequencing gel alongside a DNA sequence ladder produced from double-stranded plasmid DNA containing both OSML13 and OSML81. Gene-specific oligonucleotide primers for primer extension were used in DNA sequence determination according to the dideoxy method following the Sequenase version 2 protocol (United States Biochemical).

Construction of GUS Gene Fusions and Plant Transformation

The OSML13::GUS and OSML81::GUS chimeric gene fusions were originally constructed in pGA1072 (a gift of Dr. G. An), a pUC19 derivative containing the pBI122.1 sequence cassette (Clonetech, Palo Alto, CA), or in pBluescript II SK− (Stratagene) to facilitate high-yield plasmid DNA preparation for transient expression assay. To construct the OSML13::GUS fusion, the 5.5-kb *Eco*RI fragment subcloned in pBluescript II SK− was digested with *Dra*II, and the *Dra*II fragment was gel-purified and then digested with *Bst*XI. The 1.2-kb *Bst*XI/*Dra*II fragment designated as OSML13pro was gel purified, treated with mung bean nuclease following ligation to *Bam*HI linkers, and then inserted in the *Bam*HI site of pBluescript II SK−. The *Hin*dIII/*Xba*I fragment containing the OSML13pro was excised from pBluescript II SK− and ligated to the corresponding sites of the promoterless pBI101.1 binary vector (Clonetech), giving the OSML13::GUS chimeric gene fusion for stable transformation. To generate the OSML81::GUS gene fusion, the 5.5-kb *Eco*RI fragment in pBluescript II SK− was digested with *Pst*I, giving a 1.2-kb fragment that contains 1.1 kb of 5′ flanking sequence and 0.12 kb of coding sequence of OSML81. Digesting the 1.2-kb *Pst*I fragment with *Bst*YI resulted in a 1.1-kb fragment containing almost exclusively 5′ flanking sequences of OSML81. The *Pst*I/*Bst*YI fragment designated as OSML81pro was treated with mung bean nuclease, ligated to *Bam*HI linkers, and then inserted into the *Bam*HI site of pBluescript II SK−. To create an in-frame translation fusion with the GUS coding region, GUS coding sequence plus nopaline synthase 3′ terminator was excised from *Sma*I and *Xho*I sites of pGA1072 and ligated to the same sites of pBluescript II SK− downstream of OSML81pro to produce OSML81::GUS fusion. This plasmid was digested with *Xba*I and *Sst*I and ligated to the identical sites of pBI101.1 for stable transformation. All junction sequences of the chimeric gene fusions were verified by DNA sequencing (data not shown).

Chimeric gene constructs in the pBI101.1 binary vector (Clonetech) or pBI101.1 (control) were mobilized into the *Agrobacterium tumefaciens* strain LBA4404 by a freeze-thaw procedure (An et al., 1988) and then introduced into *S. commersonii* plants via *A. tumefaciens*-mediated leaf-disc transformation (Deblaere et al., 1987). Primary transformants were regenerated and selected for kanamycin resis-

tance. Rooted potato transformants were propagated by cuttings and maintained in Magenta boxes.

ABA, Low Temperature, NaCl, SA, Wounding, and Fungal Infection

Potato plants were grown in vitro and propagated by stimulating auxiliary bud growth. The young explants were rooted on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962). After rooting, water (control), ABA (75 μM), NaCl (100 mM), or SA (1 mM) solution with 0.01% (v/v) Tween 20 was added to each container to submerge the plants. After 2 min, solutions were decanted from the container. Plants were maintained at room temperature for 24 h and then harvested for GUS activity assays. Such treatments have been found to induce high levels of OSML13 and OSML81 mRNAs (data not shown) without inducing phytotoxic symptoms in potato plants. For low-temperature treatment, plants were cold acclimated at 4°C for 96 h. Wounding of plants was performed by cutting stems followed by incubation on moist filter paper in a Petri dish for 4 d. For whole plant fungal inoculation, plants in tissue-culture boxes were sprayed with *P. infestans* zoospores at 2500 to 5000 spores mL^{-1}. Local infection was performed by inoculating a single leaf with 5 μL of fungal spore suspensions containing 100 to 200 spores. Control plants were either treated with water or remained untreated. All inoculations were carried out under sterile conditions. After inoculation, plant materials were incubated at 15°C for 12 h to induce spore germination and then transferred to room temperature to develop disease symptoms.

Enzymatic and Histochemical Analyses for GUS Activity

Potato plant materials were collected in 1.5-mL microcentrifuge tubes, frozen in liquid nitrogen, and ground directly with a stainless-steel pestle that fits into the bottom of the tubes. Crude extracts were prepared and assayed fluorometrically for GUS activity using the substrate 4-methylumbelliferyl β-D-glucuronide as described by Jefferson et al. (1987). Protein concentrations were determined using the method of Bradford (1976) to standardize GUS activity, expressed as pmol 4-methylumbelliferone min^{-1} mg^{-1} protein. Histochemical staining analysis for GUS activity in plant materials was performed using the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuroniside cyclohexylammonium salt according to Jefferson et al. (1987). Chl was removed by incubating plant tissues in 70% ethanol.

RESULTS

Isolation and Characterization of Two Homologous Genes for Osmotin-Like Proteins

As illustrated in Figure 1, gel blot hybridization analysis of *S. commersonii* genomic DNA revealed a multigene family of osmotin-like protein genes consisting of approximately six members. A 5.5-kb *Eco*RI restriction fragment was found to hybridize to both gene-specific probes de-

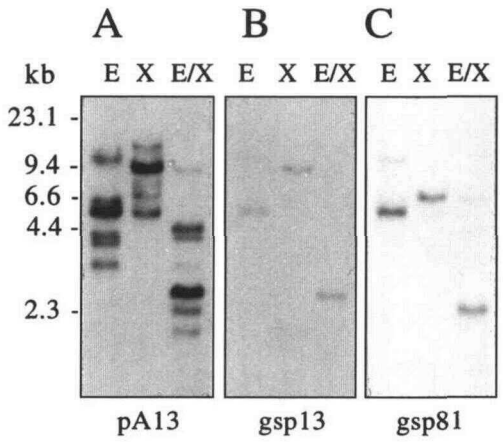

Figure 1. DNA gel blot analysis of genomic DNA isolated from *S. commersonii* plants. Ten micrograms of DNA was digested with *Eco*RI (E), *Xba*I (X), or *Eco*RI/*Xba*I (E/X) and size-fractionated on a 0.8% agarose gel. Three identical blots were hybridized with probes (pA13, gsp13, and gsp81) synthesized from the pA13 coding region (A) and the 3′ noncoding regions of pA13 (B) and pA81(C) cDNAs.

rived from the two previously characterized pA13 (Zhu et al., 1993) and pA81 (Zhu et al., 1995) cDNAs encoding osmotin-like proteins. A genomic clone (λpGEM2a-7) with a 15-kb recombinant DNA insert containing the 5.5-kb *Eco*RI fragment was isolated from a *S. commersonii* genomic library. Restriction mapping and DNA gel blot analysis confirmed that two osmotin-like protein genes (OSML13 and OSML81) corresponding to pA13 and pA81 cDNAs are located on the 5.5-kb *Eco*RI fragment in the same transcriptional orientation (Fig. 2).

Nucleotide sequences of OSML13 and OSML81 were determined according to the dideoxy chain-termination method (Sanger et al., 1977). Sequence comparison of pA13 and pA81 cDNAs with OSML13 and OSML81 revealed no introns and a 100% match in nucleotide sequences (data not shown), indicating that both genes are transcribed in vivo. The deduced amino sequences of OSML13 and OSML81 share a high degree of identity (>90%) with tobacco osmotin (Singh et al., 1989) and tobacco AP24 (Melchers et al., 1993). A detailed characterization of the osmotin-like protein gene family including OSML13 and OSML81 with gene-specific probes has been presented elsewhere (Zhu et al., 1995). Transcription start sites for both genes were determined by primer extension analysis as described (Sambrook et al., 1989) and are illustrated in Figure 3. Based on the size of the longest primer extension product, OSML13 and OSML81 were predicted to encode mRNA species with the 5′ untranslated leader sequences of 38 and 33 nucleotides in length (data not shown), respectively.

An EMBL DNA data base search with the 5′ flanking sequences and 3′ noncoding regions of both genes revealed low identity (<45%) with other PR-5 gene members, including the tobacco osmotin gene (Nelson et al., 1992). However, at least five conserved sequences found in the promoter region of tobacco osmotin gene (Nelson et al., 1992) were present in the OSML13 and OSML81 genes (Fig. 4). Both promoter regions were also searched with specific

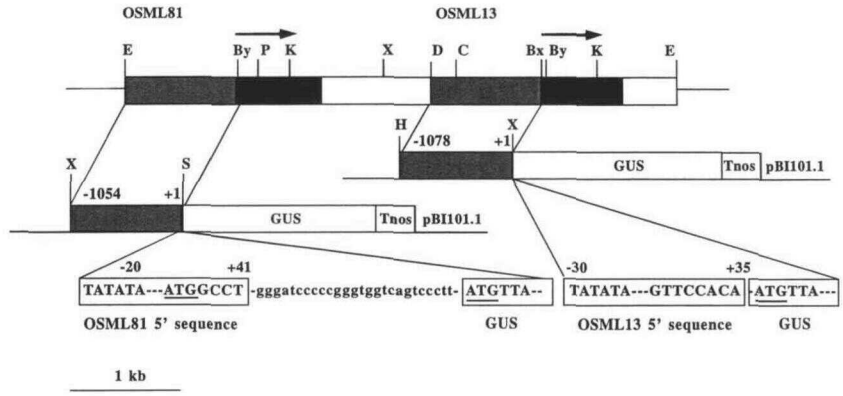

Figure 2. Restriction map and scheme for construction of chimeric gene fusions of OSML13::GUS and OSML81::GUS. The orientation of transcription is indicated by arrows. Restriction sites are denoted by Bx (*Bst*XI), By (*Bst*YI), C (*Cla*I), D (*Dra*II), E (*Eco*RI), H (*Hin*dIII), K (*Kpn*I), P (*Pst*I), S (*Sst*I), and X (*Xba*I). The 5′ sequences of OSML13 with the deleted translation start codon ATG was fused to the GUS coding sequence in *Hin*dIII/*Xba*I sites of the binary vector pBI101.1 (Jefferson et al., 1987). The 5′ sequence of OSML81 with the translation initiation codon ATG and partial coding sequence of the gene was fused in frame with the GUS coding sequence. Shaded boxes represent the isolated 5′ flanking sequences of both genes. The coding regions of both genes are shown in dark boxes. Translation initiation codons are underlined.

motifs that have been implicated in the transcriptional control of stress-inducible genes. No ABA-responsive elements were detected in these DNA sequences (Mundy et al., 1990). A 6-bp 5′-GCCGCC-3′ consensus present in certain ethylene-responsive genes encoding chitinases, glucanases, and PRB-1b (Eyal et al., 1993; Hart et al., 1993) was conserved in tobacco osmotin and potato osmotin-like protein genes on both strands (Fig. 4). TATA and CAAT boxes of known regulatory signals of eukaryotic gene expression were also identified in these genes (Fig. 4).

Chimeric GUS Gene Fusion and Plant Transformation

RNA gel blot analyses with gene-specific probes revealed similar expression patterns for OSML13 and OSML81 in response to a variety of stimuli (Zhu et al., 1995), suggesting that conserved *cis*-acting elements may be responsible for such expression patterns. To identify DNA elements involved in the regulation of both osmotin-like protein genes, we first tested the ability of both promoter regions to initiate transcription of the GUS reporter gene. Figure 2 illustrates the construction of the OSML13::GUS and OSML81::GUS chimeric gene fusions. The 5′ flanking fragment (−1078 to +35) of OSML13 was transcriptionally fused to the GUS coding sequence. The 5′ flanking fragment (−1504 to +41) of OSML81 was translationally fused immediately downstream of the ATG (+34) in frame with the GUS coding sequence, giving a fusion protein with 11 amino acid residues derived from the OSML81 coding region and the polylinker sequences of pBI101.1 (Jefferson et al., 1987).

Chimeric gene constructs in the pBI101.1 binary vector (Clonetech) or the pBI101.1 vector alone (controls) were introduced into *S. commersonii* plants via *A. tumefaciens*-mediated leaf-disc transformation (Deblaere et al., 1987). The potato transformation and regeneration procedure is comparable to that of tobacco (data not shown). Large numbers of potato transformants can be readily obtained in approximately 2 months (data not shown). DNA gel blot analysis indicated that between one and six copies of appropriate constructs were integrated into the tobacco and potato genomes (data not shown). Ten independent potato and tobacco transformants actively transcribing the OSML13::GUS or OSML81::GUS chimeric genes were chosen for further analyses. In this paper we focus primarily on the analyses of OSML13::GUS transformants, due to the similar expression patterns of both chimeric genes. Transgenic potato line 2A, carrying OSML13::GUS chimeric genes, was chosen for histochemical analysis to illustrate the overall GUS expression patterns conferred by both promoters.

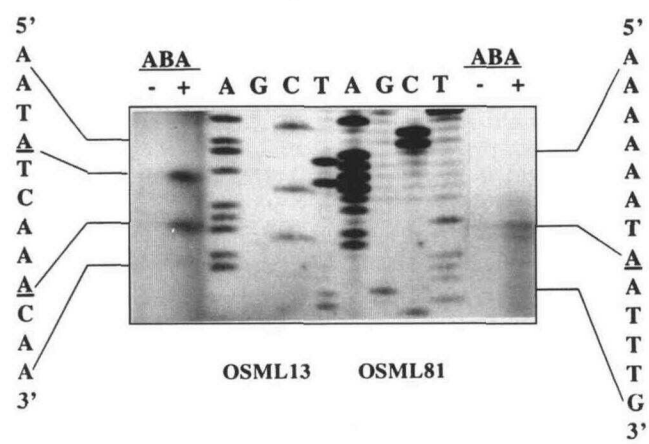

Figure 3. Primer extension mapping of transcription start sites for OSML13 and OSML81. RNAs were prepared from control (−ABA) and ABA-treated (+ABA) *S. commersonii* cell cultures. Location of the transcriptional start sites was determined based on the co-migration of the primer extension products relative to the sequence ladder of the corresponding 5′ untranslated region of OSML13 or OSML81. Primer extension products and the sequence reactions were analyzed on an 8% polyacrylamide gel. Transcription start sites are underlined.

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OSM      -235 GTCACGTTACAGAG -222
              ||||||||||||||
OSML81   -181 GTCACGTTACAGAG -168
              ||||||| | |
OSML13   -396 aTCACGTTAtAcAa -383

OSM      -182 TTCATAAAATAATTAATTATTAGGCGGCTCTTATgTTTAaGcGCCGCCTC -132
              ||||||||||||||||||||||||||||||||||| | | |
OSML81   -168 TTCATAAAATAATTAATTATTAGGCGGCTCTTActtCaAcctttaccaaC -118
              | | |  | |||||| ||||||||||||                |
OSML13   -179 TaaAgttAtggAgTAATAAgTAGGCGGCTCTTAT-aTgttGaGCCGCCTC -131

OSM      -103 TATCCGTTtATTAGTCAA -86
              ||| |||| |||||||||
OSML13   -103 TATgCGTTgATTAGTCAA -86
              |    | |||||||||
OSML81   -100 TcgtttaTaATTAGTCAA -83

OSM       -76 ATATTTaTGATTAAtATCCATAGTaCgAAAAGCCGCCATtCCCCTATATAAA -24
              |||||| ||||||| |||||||||  |||||||||| ||||||||||
OSML13    -75 ATATTTgTGATTAAAATCCATAGTttCAAAAGCCGCCAcACCCCTATATAAA -23
              ||||  | || |||||||||||  |||||||||| ||||||||||||
OSML81    -64 ATATgatTaATaAAAATCCATAGTgCCAAAAGCCGCCATACCCCTATATAAA -12

OSM       -14 ACAATTTGTCACtATATC  +2
              |||||||||||| |||||
OSML13    -15 ACAATTTGTCACAATATC  +3
              | |||||||| |||||
OSML81     -2 AtAATTTGTCctcATATa +16
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Figure 4. Comparison of the conserved 5′ flanking sequences of tobacco osmotin gene (OSM) with OSML13 and OSML81. Putative CAAT and TATA boxes are in boldface. The 6-bp 5′-GCCGCC-3′ putative ethylene responsive motif (Eyal et al., 1993; Hart et al., 1993) on the top strand is shaded. The shaded and underlined 5′-GGCGGC-3′ is complementary to the 5′-GCCGCC-3′ motif on the bottom strand.

Effects of ABA, Low Temperature, NaCl, SA, Wounding, and Fungal Infection on GUS Expression

The same set of independent transgenic potato plants carrying either chimeric gene fusion was subjected to treatments with various stimuli and analyzed for GUS activity (Fig. 5). Similar GUS expression patterns for OSML13 (Fig. 5A) and OSML81 (Fig. 5B) promoters were observed. In the untreated plants transformed with either chimeric gene construct, very low basal levels of GUS activity were present, and treatments with ABA, NaCl, and SA resulted in further increase in GUS activity. Infection with *P. infestans* caused the greatest increase in GUS activity. Low temperature activated the expression of both chimeric genes in potato only slightly. High levels of GUS activity were found in regions of cuttings during vegetative propagation (Fig. 6A) and in the wounded sites of potato stems (Fig. 6B). Little GUS activity was detected in the wild-type plants or transgenic plants carrying the pBI101.1 vector alone, and no increase in GUS activity was found in these control plants treated with different stimuli (data not shown) or infected with *P. infestans* (Fig. 5). These results suggest that GUS expression is due to the transcriptional activation of OSML13 and OSML81 promoters in response to various inducers.

Organ-Specific GUS Expression

Histochemical staining analyses indicated that the spatial expression patterns of both chimeric genes in potato transformants were quite comparable (data not shown). Basal levels of GUS activity varied in specific organs of different transformants containing either OSML13::GUS or OSML81::GUS. Such variations did not correlate with copy numbers and are presumably due to position effects. Overall, GUS activity was low in leaves and primarily localized in vascular tissues, moderate in stems, and high in root tips (Fig. 6, A, C, and D).

Localized GUS Expression by Fungal Infection

The pattern of expression of the OSML13::GUS or OSML81::GUS chimeric gene was studied in a compatible potato-*P. infestans* interaction. Histochemical staining analysis of *P. infestans*-infected potato transformants indicated that both osmotin-like protein gene promoters direct GUS expression in areas immediately adjacent to the infected sites (Fig. 6, E and F). As illustrated in Figure 7, fluorometric analyses revealed some increase in GUS activity in the infected potato leaves 12 h after *P. infestans* inoculation. A significant increase in GUS activity was detected after 48 h, consistent with the accumulation patterns of GUS (data not shown), OSML13, and OSML81 mRNAs (Zhu et al., 1995).

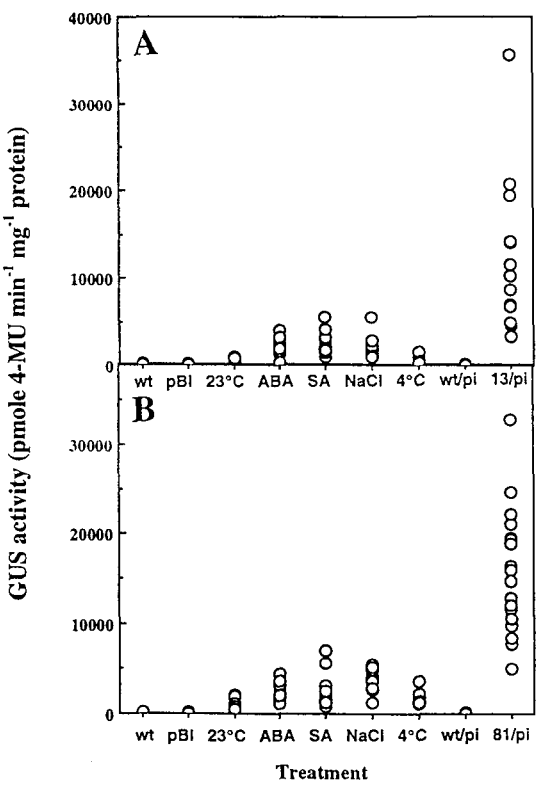

Figure 5. Fluorometric analysis of GUS activity in transgenic potato plants in response to various stimuli. A, Analysis of transgenic plants carrying the OSML13::GUS chimeric gene; B, analysis of transgenic plants carrying the OSML81::GUS chimeric gene. Potato plants propagated from 10 independent transgenic lines were grown at 23°C (23°C) for 24 h, 4°C (4°C) for 96 h, treated with 75 μM ABA (ABA), 1 mM SA (SA), or 100 mM NaCl (NaCl) for 24 h, or infected with *P. infestans* for 96 h (13/pi and 81/pi). Each circle represents the result from a plant propagated from a single transgenic line except for the fungal infection treatment 13/pi and 81/pi, which included two plants from each transgenic line. Five wild-type (wt) plants, five control plants from different pBI101.1 transgenic lines (pBI101.1), and five wild-type plants infected with *P. infestans* (wt/pi) were also assayed for GUS activity.

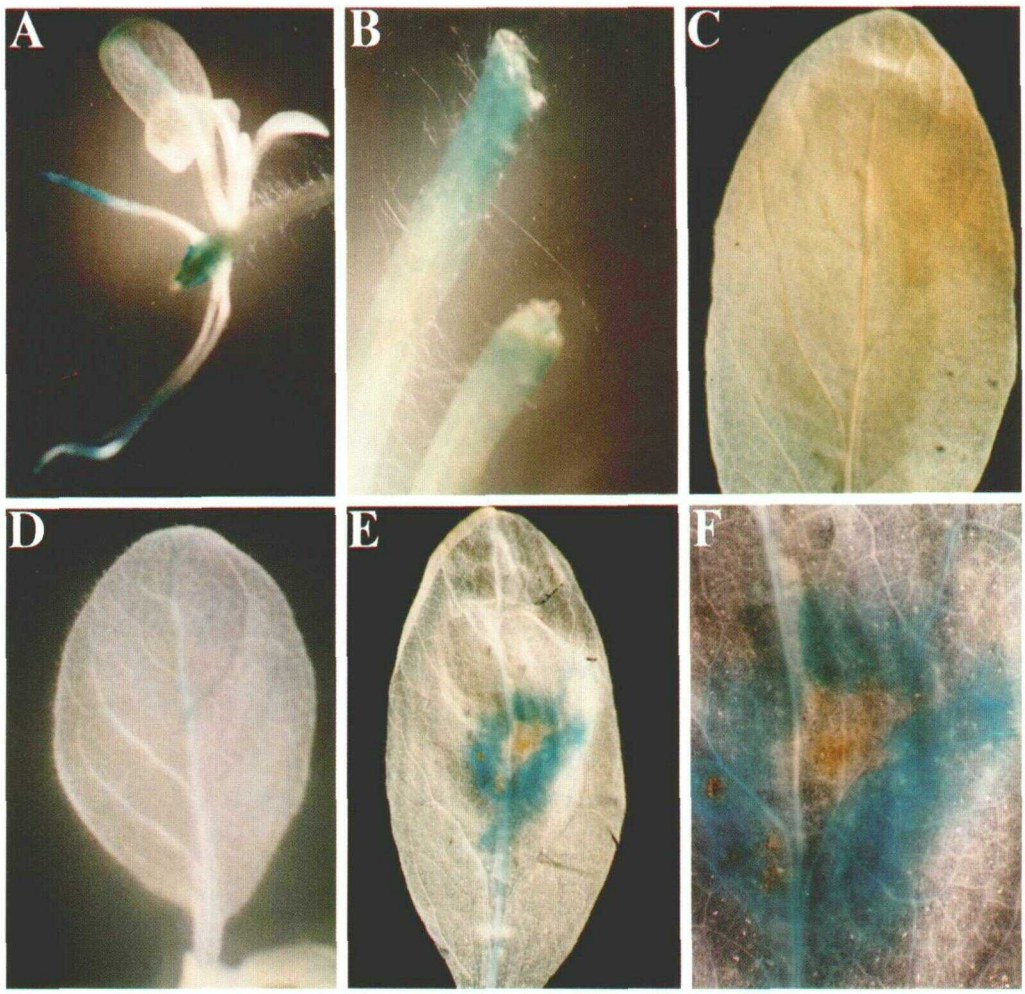

Figure 6. Histochemical detection of GUS activity in OSML13::GUS transgenic potato line 2A plants. A, Line 2A potato plant grown under normal conditions. B, Line 2A potato stems wounded for 4 d. C, Wild-type potato leaf. D, Uninfected line 2A potato leaf. E, Line 2A potato leaf infected with *P.infestans* for 2 d. F, A close view of the infection site in E.

However, little increase in GUS activity was found in leaves adjacent to the infected leaves.

DISCUSSION

Osmotin and osmotin-like proteins, members of the PR-5 family, have been shown to confer antifungal activity against *P. infestans* (Woloshuk et al., 1991; Zhu, 1993; Liu et al., 1994), a fungal pathogen that accounts for heavy losses in worldwide potato and tomato production. Although the regulation of tobacco osmotin gene expression has been studied extensively (Kononowicz et al., 1992; LaRosa et al., 1992; Nelson et al., 1992; Raghothama et al., 1993), there is a lack of information on the genomic complexity of osmotin-like protein genes in potato and their transcriptional activation by *P. infestans* infection. In this study we have demonstrated that there are at least six members of osmotin-like protein genes in potato (Fig. 1). Two of them have been isolated from a *S. commersonii* genomic library using the previously characterized pA13 cDNA as a hybridization probe (Zhu et al., 1993). Our results indicate that these two osmotin-like protein genes contain no introns and are structurally similar to the tobacco osmotin (Nelson et al., 1992) and tobacco AP24 (Melchers et al., 1993) genes. Both genes encode polypeptides characteristic of tobacco osmotin (Singh et al., 1987) and tobacco AP24 (Melchers et al., 1993), including the presence of N-terminal signal peptide and C-terminal propeptide, with the latter mediating vacuolar targeting of these osmotin-like proteins (Melchers et al., 1993). Although overexpression of one osmotin-like protein gene (OSML13) resulted in delayed development of *P. infestans* disease symptoms in *S. commersonii* (Zhu, 1993), the antifungal activity of other, closely related potato PR-5 proteins has not yet been determined.

By analysis of mRNA levels with gene-specific probes, previous studies revealed similar expression patterns for both osmotin-like protein genes during normal plant development and in response to a variety of external stimuli (Zhu et al., 1995). Thus, it is possible that the promoter regions of these two genes contain similar *cis*-acting elements. Indeed, we found that there are at least five DNA sequences conserved in these two promoters as well as in a tobacco osmotin gene promoter (Nelson et al., 1992).

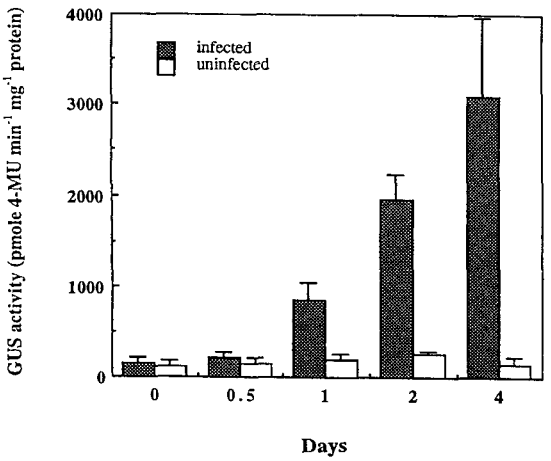

Figure 7. Localized GUS expression in transgenic potato plants by *P. infestans*. One leaf each of five individual OSML13::GUS line 2A potato plants was inoculated with *P. infestans*, and the inoculated leaf and its adjacent leaves (above and below) from each plant were sampled at 0, 0.5, 1, 2, and 4 d after inoculation. GUS activity is expressed as pmol 4-methylumbelliferone min^{-1} mg^{-1} protein. Bar = SD (n = 5).

Analysis of potato transgenic plants carrying copies of OSML13::GUS or OSML81::GUS showed comparable GUS expression patterns in response to various stimuli (Fig. 5). Whether the conserved DNA sequences are responsible for these comparable expression patterns remains to be determined by 5′ deletion and mutation analysis. Our data suggest that ABA, SA, NaCl, wounding, and fungal infection regulate the expression of osmotin-like protein genes primarily at the level of transcription. When exposed to low temperature, transgenic potato plants exhibited little increase in GUS activity, consistent with the results from RNA gel blot analyses with OSML13 and OSML81 gene-specific probes (Zhu et al., 1995). These results suggest that the observed increase in osmotin-like protein mRNA abundance during a long period of cold acclimation (Zhu et al., 1993) is due either to posttranscriptional regulation or expression from different genes. Low-temperature treatment has been demonstrated to regulate *cor* (cold regulated) gene expression through both transcriptional and posttranscriptional means (Hajela et al., 1990). Recently, a *lea* (late embryogenesis abundant)-type mRNA has been shown to be only stabilized but not induced by low temperature (Espelund et al., 1992).

Promoter sequences of both osmotin-like protein genes along with the tobacco osmotin gene were searched for specific *cis*-acting elements that may function in the activation of PR genes. A 5′-GGCGGC-3′ motif conserved in the 5′ upstream sequences of some ethylene-inducible PR genes (Eyal et al., 1993; Hart et al., 1993) was found in these three genes (Fig. 4). Our results indicate that both promoters of potato osmotin-like protein genes are wound inducible (Fig. 6, A and B). Although ethylene responsiveness of either promoter was not investigated in this study, it is possible that the wound inducibility of osmotin-like protein genes is mediated through ethylene, as demonstrated for a tobacco osmotin gene promoter (Nelson et al., 1992). Ethylene is shown to be a strong inducer for a number of PR genes (Bol et al., 1990; Linthorst, 1991; Cutt and Klessig, 1992). In addition, ABA is most likely involved in wound-induced gene activation of OSML13 and OSML81. It has been demonstrated that wounding activates the expression of wound-inducible proteinase inhibitor II genes via changes in endogenous ABA levels in potato and tomato (Peña-Cortés et al., 1989, 1991). It is noteworthy that no ABA-responsive elements were found in the promoters of osmotin-like protein genes, although both promoters have been shown to be ABA responsive, suggesting that transcriptional regulation by ABA may involve a variety of *cis*-acting elements (Mundy et al., 1990; Nelson et al., 1992).

We have demonstrated that *P. infestans* infection results in strong and localized activation of both osmotin-like protein genes during a compatible plant/fungus interaction. Given the fact that osmotin and osmotin-like proteins have in vitro (Woloshuk et al., 1991) and in vivo (Zhu, 1993; Liu et al., 1994) activity against *P. infestans*, localized activation of these genes suggests a role for these potato PR-5 genes in preventing the proximal spread of virulent *P. infestans*. Recently, Cao et al. (1994) characterized an Arabidopsis mutant, *npr1* (nonexpresser of PR genes), that is nonresponsive to inducers of SAR. When *npr1* was infected by a virulent pathogen, localized expression of PR genes was disrupted, and the disease lesion formation was less confined than in wild-type plants, suggesting the participation of PR genes in local defense against virulent pathogens. They also suggested that PR genes are induced locally and systemically through a common signal transduction mechanism that includes an increase in SA levels followed by the induction of PR gene expression and SAR (Uknes et al., 1992; Ward et al., 1991; Cao et al., 1994).

Several lines of experimental evidence suggest that SA is required for the induction of SAR (Malamy et al., 1990; Gaffney et al., 1993) but is not the translocated systemic signal (Vernooij et al., 1994). We have demonstrated that SA induces the activation of the OSML13 and OSML81 promoters. However, SA-induced GUS activity per se cannot account for the high GUS activity observed in potato plants infected by *P. infestans* (Fig. 5). Conceivably, such high GUS activity levels are a result of "super activation" of these genes by many abiotic stimuli including ABA, SA, low water potentials, and ethylene. These signals are likely produced during a plant-pathogen interaction, since we observed necrotic lesion formations and wilting in the infected plants associated with profound fungal sporulation at a later stage of infection. The regulation of these PR-5 genes by multiple abiotic stimuli including ABA, SA, low water potentials, wounding, and ethylene may act as an alternative plant defense mechanism that is independent of specific chemical elicitors derived from the pathogen (Kononowicz et al., 1992; Raghothama et al., 1993). It is also possible that these signals can act synergistically in PR gene activation (Xu et al., 1994). Besides abiotic stimuli, *P. infestans*-derived chemical elicitors such as arachidonic acid and its metabolites may also be involved in activating the potato PR-5 genes. These elicitor signals are released

from *P. infestans* zoospores and can activate plant defense genes (Ricker and Bostock, 1992).

Taken together, the mechanism that controls the expression of both potato PR-5 genes is rather complex. A mechanistic dissection of both promoters with an emphasis on the conserved DNA sequences may help us to define *cis*-acting elements specific to each signal. Although the role of osmotin-like proteins in osmotic stress has to be addressed, efforts are currently being directed toward understanding of the regulation of these potato PR-5 genes by *P. infestans*. In this study we have established an ideal pathosystem in which the host plant, wild potato (*S. commersonii*), is amenable to transformation. Large numbers of transgenic plants can be produced in a much shorter time in this species than in the cultivated potato (*Solanum tuberosum*). This will expedite the process of identifying key DNA elements that direct *P. infestans*-mediated PR-5 gene activation during a plant-pathogen interaction.

ACKNOWLEDGMENTS

We thank Drs. N.E. Olszewski, A.G. Smith, and C.P. Vance at the University of Minnesota for critical reading of the manuscript. We also thank the Central Service Laboratory of the Center for Gene Research and Biotechnology at Oregon State University for DNA sequencing. We are grateful to Dr. P.B. Hamm at Oregon State University for *P. infestans* cultures.

Received December 31, 1994; accepted March 30, 1995.

The EMBL/GenBank/DDBJ accession numbers for the nucleotide sequences reported in this article are X72927 (OSML13) and X72928 (OSML81).

LITERATURE CITED

An G, Ebert PR, Mitra A, Ha SM (1988) Binary vectors. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1–19

Bol JF, Linthorst HJM, Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopathol **28:** 113–138

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Cao H, Bowling SA, Gordon S, Dong X (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6:** 1583–1592

Caten CE, Jinks JL (1967) Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. Can J Bot **46:** 329–348

Cutt JR, Klessig DF (1992) Pathogenesis-related proteins. *In* F Meins, T Boller, eds, Plant Gene Research: Genes Involved in Plant Defense. Springer-Verlag, New York, pp 209–243

Deblaere R, Reynaerts A, Höffe H, Hernalsteens J-P, Leemans J, Montagu MV (1987) Vectors for cloning in plant cells. Methods Enzymol **153:** 277–292

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Espelund M, Sæbøe-Larssen S, Hughes DW, Galau GA, Larsen F, Jakobsen KS (1992) Late embryogenesis-abundant genes encoding proteins with different numbers of hydrophilic repeats are regulated differentially by abscisic acid and osmotic stress. Plant J **2:** 242–252

Eyal Y, Meller Y, Lev-Yadun S, Fluhr R (1993) A basic-type PR-1 promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. Plant J **4:** 225–234

Feinberg AP, Vogelstein B (1984) Addendum: a technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **137:** 266–267

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye D, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana*. Plant Physiol **93:** 1246–1252

Hart CM, Nagy F, Meins F Jr (1993) A 61 bp enhancer element of the tobacco β-1,3-glucanase B gene interacts with one or more regulated nuclear proteins. Plant Mol Biol **21:** 121–131

Henikoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:** 351–359

Hennig J, Dewey RE, Cutt JR, Klessig DF (1993) Pathogen, salicylic acid and developmental dependent expression of a β-1,3-glucanase/GUS gene fusion in transgenic tobacco plants. Plant J **4:** 481–493

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Kononowicz AK, Nelson DE, Singh NK, Hasegawa PM, Bressan RA (1992) Regulation of the osmotin gene promoter. Plant Cell **4:** 513–524

Korfhage U, Trezzini GF, Meier I, Hahlbrook K, Somssich IE (1994) Plant homoeodomain protein involved in transcriptional regulation of a pathogen defense-related gene. Plant Cell **6:** 695–708

LaRosa PC, Chen Z, Nelson DE, Singh NK, Hasegawa PM, Bressan RA (1992) Osmotin gene expression is posttranscriptionally regulated. Plant Physiol **100:** 409–415

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci **10:** 123–150

Liu D, Raghothama KG, Hasegawa PM, Bressan RA (1994) Osmotin overexpression in potato delays development of disease symptoms. Proc Natl Acad Sci USA **91:** 1888–1892

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: A likely endogenous signal in the resistance response of tobacco to tobacco mosaic virus infection. Science **250:** 1002–1004

Melchers LS, Sela-Buurlage MB, Vloemans SA, Woloshuk CP (1993) Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanase in transgenic plants. Plant Mol Biol **21:** 583–593

Mundy J, Yamaguchi-Shinozaki K, Chua N-H (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice RAB gene. Proc Natl Acad Sci USA **87:** 1406–1410

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant **15:** 473–479

Nelson DE, Raghothama KG, Singh NK, Hasegawa PM, Bressan RA (1992) Analysis of structure and transcriptional activation of an osmotin gene. Plant Mol Biol **19:** 577–588

Peña-Cortés H, Sánchez-Serrano JJ, Mertens R, Willmitzer L (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc Natl Acad Sci USA **86:** 9851–9855

Peña-Cortés H, Willmitzer L, Sánchez-Serrano JJ (1991) Abscisic acid mediates wound induction but not developmental specific expression of proteinase inhibitor II gene family. Plant Cell **3:** 963–972

Raghothama KG, Liu D, Nelson DE, Hasegawa PM, Bressan RA (1993) Analysis of an osmotically regulated pathogenesis-related osmotin gene promoter. Plant Mol Biol **23:** 1117-1128

Ricker KE, Bostock RM (1992) Evidence for release of the elicitor arachidonic acid and its metabolites from sporangia of *Phytoph-*

thora infestans during infection of potato. Physiol Mol Plant Pathol **41:** 61–72

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5468

Singh NK, Nelson DE, Kuhn D, Hasegawa PM, Bressan RA (1989) Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol **90:** 1096–1101

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512

Uknes S, Dincher S, Friedrich L, Negrotto D, Williams S, Thompson-Taylor H, Potter S, Ward E, Ryals J (1993) Regulation of pathogenesis-related protein-1a gene expression in tobacco. Plant Cell **5:** 159–169

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in Arabidopsis. Plant Cell **4:** 645–656

Vernooij B, Friedrich L, Morse A, Reist R, Kolditz-Jawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6:** 959–965

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux J-P, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094

Woloshuk CP, Meulenhoff JS, Sela-Buurlage M, van den Elzen PJM, Cornelissen BJC (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell **3:** 619–628

Xu Y, Chang P, Liu D, Narasimhan ML, Hasegawa PM, Bressan RA (1994) Plant defense genes are synergistically induced by ethylene and jasmonate. Plant Cell **6:** 1077–1085

Zhu B (1993) Isolation and characterization of abscisic acid-responsive genes during the induction of freezing tolerance in potato. PhD thesis. University of Minnesota, St. Paul

Zhu B, Chen THH, Li PH (1993) Expression of an ABA-responsive osmotin-like gene during the induction of freezing tolerance in *Solanum commersonii*. Plant Mol Biol **21:** 729–735

Zhu B, Chen THH, Li PH (1995) Expression of three osmotin-like protein genes in response to osmotic stress and fungal infection in potato. Plant Mol Biol (in press)