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Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein

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Abstract. The expression patterns of plant defense genes encoding osmotin and osmotin-like proteins imply a dual function in osmotic stress and plant pathogen defense. We have produced transgenic potato (*Solanum commersonii* Dun.) plants constitutively expressing sense or antisense RNAs from chimeric gene constructs consisting of the cauliflower mosaic virus 35S promoter and a cDNA (pA13) for an osmotin-like protein. Transgenic potato plants expressing high levels of the pA13 osmotin-like protein showed an increased tolerance to the late-blight fungus *Phytophthora infestans* at various phases of infection, with a greater resistance at an early phase of fungal infection. There was a decrease in the accumulation of osmotin-like mRNAs and proteins when antisense transformants were challenged by fungal infection, although the antisense transformants did not exhibit any alterations in disease susceptibility. Expression of pA13 sense and antisense RNAs had no effect on the development of freezing tolerance in transgenic plants when assayed under a variety of conditions including treatments with abscisic acid or low temperature. These results provide evidence of antifungal activity for a potato osmotin-like protein against the fungus *P. infestans*, but do not indicate that pA13 osmotin-like protein is a major determinant of freezing tolerance.

Key words: Late-blight disease – Osmotin-like proteins – Pathogenesis-related proteins – *Phytophthora* – Sense and antisense RNAs – *Solanum*

Introduction

Osmotin and osmotin-like proteins are encoded by a multigene family that is highly conserved in the Solanaceae. Expression of osmotin and osmotin-like protein genes is activated by developmental and environmental cues, hormonal stimuli, and microbial attack (Kononowicz et al. 1992; LaRosa et al. 1992; Zhu et al. 1993; Zhu et al. 1995a, b). Such complex expression patterns suggest that osmotin-like protein genes must have evolved to respond to specific developmental cues, with environmental stress and pathogen signals superimposed upon them. It appears that osmotin-like proteins may play important roles in normal development and plant defense against osmotic stress and pathogen infection. When assayed in vitro, osmotin and osmotin-like proteins exhibit antifungal activity toward *Phytophthora infestans* (Woloshuk et al. 1991; Melchers et al. 1993; Liu et al. 1994), the pathogen responsible for the infamous Irish potato famine in the 1840s. Similarly, a family of cysteine-rich thaumatin-like proteins sharing high identity with osmotin-like proteins also exhibits antimicrobial activity against a variety of fungi (Roberts and Selitrennikoff 1990; Heigaard et al. 1991; Vigers et al. 1991; Malehorn et al. 1994). It is likely that these proteins may share functional homology in plant defense against phytopathogenic fungi, although the mechanism of their action is not well understood.

In a recent study, Liu and coworkers demonstrated that constitutive expression of tobacco osmotin failed to induce resistance to *Phytophthora parasitica* var. *nicotianae* in transgenic tobacco plants, but resulted in delayed development of disease symptoms in transgenic potato plants challenged by *P. infestans* (Liu et al. 1994). These authors speculated that the heterologous nature of tobacco osmotin was responsible for its antifungal effect in transgenic potato. In potato (*Solanum commersonii*), the genes for osmotin-like proteins belong to a multigene family consisting of at least six members (Zhu et al. 1995b). Three closely related cDNAs corresponding to these genes have been isolated and characterized (Zhu et al. 1993; Zhu et al. 1995a). The accumulation of osmotin-like protein mRNAs was associated with the induction of freezing tolerance in *S. commersonii* cell cultures when cold-acclimated (4°C) or treated with abscisic acid (ABA). In addition, there was a large accumulation of osmotin-like mRNAs and proteins when *S. commersonii* plants were

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Abbreviations: ABA = abscisic acid; CaMV = cauliflower mosaic virus; PR = pathogenesis-related; T_0 = primary transformant

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infected with *P. infestans* (Zhu et al. 1995a). Furthermore, RNA gel blot analysis indicated that fungal infection induces non-systemic expression of at least three osmotin-like protein genes in infected potato leaves. This observation was confirmed by analyzing the expression of two chimeric gene fusions, each consisting of a osmotin-like protein gene promoter and the β-glucuronidase (GUS) coding region. Analysis of GUS activity in transgenic plants revealed that both osmotin-like protein gene promoters direct the expression of the GUS reporter gene only in sites of fungal invasion (Zhu et al. 1995b).

In this study we investigated whether *P. infestans* resistance can be altered by genetically manipulating the expression of a native potato osmotin-like protein gene in transgenic potato plants. We developed transgenic plants carrying copies of a cauliflower mosaic virus (CaMV) 35S::pA13 cDNA (Zhu et al. 1993) chimeric gene in a sense or an antisense orientation. Sense transgenic plants, showing overexpression of osmotin-like proteins, and antisense transformants, with reduced accumulation of osmotin-like proteins upon fungal infection, were assayed for fungal resistance. To determine the role of this osmotin-like protein in osmotic stress, these plants were also evaluated for their resistance to freezing stress, a common form of osmotic stress imposed by freezing-induced cellular dehydration.

Materials and methods

Biological materials. Plants of *Solanum commersonii* Dun., PI 458317 (Potato Introduction Station, Sturgeon Bay, Wis., USA), were propagated by cuttings and grown in vitro on half-strength MS medium (Murashige and Skoog 1962) solidified with 7 $g \cdot l^{-1}$ agar in Magenta GA 7 vessels (Magenta Co., Chicago, Ill., USA) in a controlled environmental chamber under cool-white fluorescent lamps (14 h photoperiod, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 23 °C. Growth of mycelia, induction of sporangia, and preparation of zoospores of *P. infestans* (Mont.) de Bary, isolate 184 (a gift of Dr. P.B. Hamm) were according to Caten and Jinks (1967). We have determined that this isolate is compatible with *S. commersonii*.

Construction of chimeric gene fusions and plant transformation. A pA13 cDNA (Zhu et al. 1993) corresponding to an osmotin-like protein gene *OSML13* (Zhu et al. 1995b) was originally cloned in *Eco*RI/*Xho*I sites of pBluescript SK − (Stratagene, La Jolla, Calif., USA). This plasmid carrying the pA13 cDNA was digested with *Dra*II, filled in to create blunt ends, and ligated to *Sac*I linkers. Double digestion with *Bam*HI and *Sac*I allows ligation of the resultant DNA fragment to the identical sites of pBI121.1 binary vector (Jefferson et al. 1987) in a sense orientation with respect to the CaMV 35S promoter. For antisense construction, the *Bam*HI site in the pBluescript SK − carrying pA13 cDNA was deleted by digesting the plasmid DNA with *Bam*HI and treated with mung-bean nuclease followed by ligation. The plasmid without *Bam*HI site was amplified in *Escherichia coli*., and purified plasmids were digested with *Dra*II, filled in to produce blunt ends, and ligated to *Bam*HI linkers. Following digesting with *Bam*HI and *Sac*I, the cDNA was cloned into the same sites of pBI121.1 in an antisense orientation.

Chimeric gene constructs in pBI121.1 binary vector or pBI101.1 vector (Clonetech Laboratories, Palo Alto, Calif., USA) alone (control) were mobilized into the *Agrobacterium tumefaciens* strain LBA4404 by a freeze-thaw procedure (An et al. 1988). Transformed *A. tumefaciens* were selected for kanamycin resistance and confirmed by DNA:DNA blot analysis for their harboring of appropriate vector plasmids. Chimeric gene constructs were then introduced into potato plants via *A. tumefaciens*-mediated transformation following a modified leaf-disc method (Deblaere et al. 1987). Primary transformants (T_0) were regenerated and selected for kanamycin resistance. Rooted T_0 potato transformants were propagated by shoot cuttings and further selected on half-strength MS medium in the presence of kanamycin in Magenta boxes. Transgenic lines analyzed in this study were all produced by vegetative propagation from independent T_0 transformants due to self-incompatibility of *S. commersonii* clones.

DNA and RNA gel blot analysis. Genomic DNA was prepared from potato plants according to the method of Dellaporta et al. (1983) and digested with *Eco*RI, which excises the chimeric fusion construct in such a way that the number of bands resolved by DNA gel blot analysis corresponds to the number of integration sites. Total RNA was isolated by a small-scale procedure (Verwoerd et al. 1989). Both DNA and RNA blot analysis followed standard protocols (Sambrook et al. 1989). The DNA or RNA was transferred to Zeta-probe GT membrane (Bio-Rad, Richmond, Calif., USA), prehybridized, and hybridized according to the instructions provided by the supplier. While DNA gel blots were hybridized to a *Hin*dIII/*Xba*I CaMV 35S promoter DNA fragment, the RNA gel blots were hybridized to either strand-non-specific DNA probes or strand-specific riboprobes synthesized from pA13 cDNA (Zhu et al. 1993). Blots were washed three times with 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), 0.1% SDS at room temperature each for 5 min; twice with 1 × SSC, 0.1% SDS at 65°C each for 20 min; and finally with 0.1 × SSC, 0.1% SDS at 65 °C for 30 min.

Synthesis of DNA and RNA probes. The coding region, an *Eco*RI/*Dra*I fragment, of pA13 cDNA (Zhu et al. 1993), and a *Hin*dIII/*Xba*I CaMV 35S promoter fragment were labeled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1984). To synthesize strand-specific pA13 RNA probes, pA13 cDNA in pBluescript SK − was transcribed in vitro using T3, or T7 RNA polymerase following the manufacturer's instructions (Stratagene). The ^{32}P-labeled RNA probes were used to detect sense or antisense RNAs in transgenic potato plants.

Immunoblot analysis. Plant materials were powdered in the presence of liquid nitrogen in 1.5-ml microcentrifuge tubes with a stainless-steel pestle that fits to the bottom of the tubes. Three volumes of Laemmli sample lysis buffer (Laemmli 1970) were added, mixed and boiled for 10 min, and centrifuged for 5 min at room temperature to eliminate cellular debris. Total protein was directly electrophoresed on 12.5% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose as described by Sambrook et al. (1989). Protein concentrations were determined according to a bicinchoninic acid procedure (Brown et al. 1989). Total protein extracted from *P. infestans*-infected potato leaves was included as a positive control. The chicken anti-osmotin antibodies were partially purified chicken immunoglobin (Ig) Y (LaRosa et al. 1992). The second antibody was anti-Chicken IgG (whole molecule) alkaline phosphatase conjugate (Sigma, St Louis, Mo., USA).

Evaluation of stress resistance of transgenic plants. Evaluation of fungal resistance was modified from the leaf-disc method of Hodgson (1961), which has been demonstrated to reveal similar relative resistance of several potato varieties when compared with field tests. Mature leaves situated approximately one-third the distance from the top of the plantlets were pooled from four-week-old plantlets propagated from independent T_0 transformant and placed on 1.5% agar in Petri dishes. These Petri dishes were then arrayed randomly prior to inoculation. Each leaf was inoculated on the adaxial surface with 5 μl fungal spore suspension containing various concentrations of fungal spores. Each assay was based on the inoculation of 30 or more leaves from 10 to 15 clonal plantlets. After inoculation, Petri dishes, covered with lids, were placed in an enclosed transparent container and maintained at 15 °C for 12 h to induce zoospore germination, which was followed by incubation at 23 °C to develop disease symptoms. The percentage of sporulating leaves was scored

randomly at 3, 5 and 10 d after inoculation. Freezing tolerance of potato plants grown at 23°C with or without ABA (75 μM), or cold acclimated at 4 °C was determined by measuring leaf electrolyte leakage after a freeze-thaw cycle (Tseng and Li 1990). Freezing tolerance was expressed as LT_{50} (temperature that results in 50% electrolyte leakage). Both disease-resistance and freezing-tolerance data were from two independent experiments.

Statistical analysis. Control and transgenic plants were classified into four groups. Two groups, including four untransformed wild-type plants regenerated from leaf discs, and three control transgenic lines carrying pBI101.1 empty vector, were negative controls for possible epigenic effect or somoclonal variation associated with transformation and regeneration processes. The other two groups included six antisense transgenic lines, and four sense transgenic lines. Each transgenic line represents one transgenic event. Analyses of variance of the fungal-resistance and freezing-tolerance data was conducted using SAS's general linear model procedure (SAS Institute Inc., Cary, N.C., USA). Percentage data were arcsin-transformed prior to analysis. The Tukey-Kramer HSD means pairwise comparison test was used to determine significant differences between means of each groups for each rating date or treatment at the 95% confidence level.

Results

Chimeric gene construction and plant transformation. A cDNA of pA13 corresponding to the *OSML13* osmotin-like protein gene (Zhu et al. 1995b) was inserted in the *Bam*HI and *Sac*I sites of binary vector plasmid pBI121.1 (Clonetech Laboratories) in either sense or antisense orientation. This cDNA retains its own poly(A)$^+$ signal and nucleotide sequences encoding an N-terminal signal peptide and a putative C-terminal propeptide. The latter has been implicated in the post-translational processing and vacuolar targeting of osmotin-like proteins (Melchers et al. 1993). Sense, antisense, and control (pBI101.1 vector alone) constructs were transferred into *S. commersonii* via *A. tumefaciens*-mediated transformation. Primary T_0 transformants that rooted on kanamycin selection medium were retained and propagated in vitro. When compared with the untransformed control plants, no visual morphological abnormalities were observed in the T_0 transgenic plants or during subsequent vegetative propagation.

Molecular analysis of transgenic plants. The DNA isolated from T_0 transformants revealed stable integration of transgenes into the potato genome, with the number of integration sites in the range of one to three in diploid *S. commersonii* (Fig. 1). Overall, we obtained 8 sense, 12 antisense, and 6 control independent *S. commersonii* transformants.

Results from probing RNA blots with strand-specific RNA probes indicated that the levels of sense and antisense RNAs corresponding to pA13 cDNA varied in both sense (Fig. 2A) and antisense (Fig. 2B) transformants. In addition, constitutive accumulation of high levels of both sense and antisense RNAs was found in all plant organs of T_0 transformants and did not alter during in-vitro propagation (data not shown). Such expression patterns are characteristic of the CaMV 35S promoter but not typical of native osmotin-like protein gene promoters (Zhu et al. 1995a, b). To examine the effect of antisense RNA on

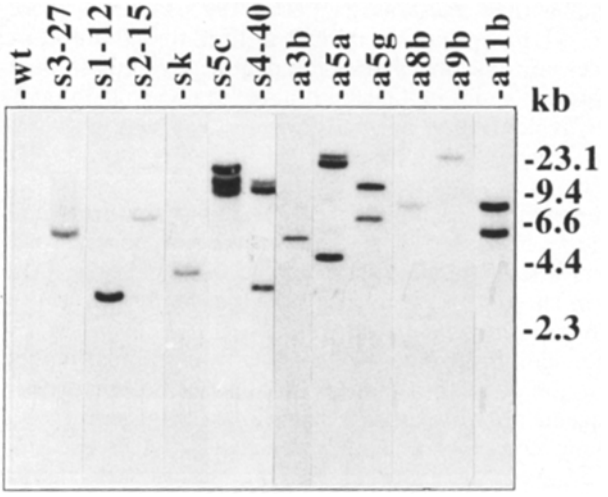

Fig. 1. DNA gel blot analysis of transgenic potato plants. Genomic DNA (10 μg) was isolated from wild-type plants (*wt*) and from sense (*s3-27, s1-12, s2-15, sk, s5c, s4-40*) and antisense (*a3b, a5a, a5g, a8b, a9b, a11b*) T_0 transgenic *S. commersonii* plants. DNA was digested with *Eco*RI and size-fractionated on 0.8% agarose gels. The DNA gel blot was hybridized to a ^{32}P-labeled *Hin*dIII-*Xba*I fragment of the CaMV-35S promoter of the pBI121.1 binary vector (Jefferson et al. 1987)

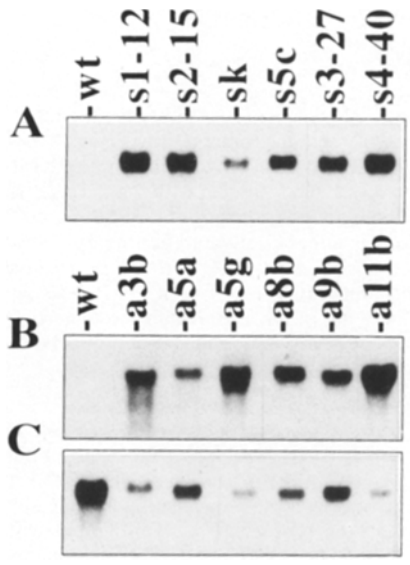

Fig. 2A–C. RNA gel blot analysis of sense and antisense RNAs corresponding to pA13 cDNA in *S. commersonii* plants. **A** Total RNA was extracted from wild-type (*wt*) and sense transgenic (*s1-12, s2-15, sk, s5c, s3-27, s4-40*) potato plants grown under normal conditions. The RNA gel blot was hybridized to a ^{32}P-labeled sense-strand-specific pA13 RNA probe. **B** Total RNA was extracted from wild-type plants (*wt*) and antisense potato transformants (*a3b, a5a, a5g, a8b, a9b, a11b*) grown under normal conditions. The RNA gel blot was hybridized to a ^{32}P-labeled antisense-strand-specific pA13 RNA probe. **C** Total RNA was extracted from the same set of plants as in **B** but infected with *P. infestans* for 4 d. Each lane contains 10 μg of total RNA. The RNA gel blots were hybridized to a ^{32}P-labeled sense-strand-specific pA13 RNA probe

fungal-infection-induced osmotin-like protein RNAs, we analyzed sense RNA levels in antisense transformants

infected with *P. infestans*. Compared with the fungal-infected wild-type control, overexpression of antisense RNA greatly inhibited fungal-infection-induced accumulation of osmotin-like RNAs (Fig. 2C).

We speculated that the incomplete inhibition of osmotin-like sense RNAs observed in Fig. 2C is due to depletion of antisense RNA by a rapid accumulation of osmotin-like protein mRNAs (Zhu et al. 1995a). To investigate the possibility of antisense RNA depletion, we analyzed the temporal accumulation of both sense and antisense RNA transcripts in a *P. infestans*-infected control and in an antisense transformant (a11b) expressing high levels of antisense RNA (Fig. 2B). Since the antisense transcript is larger than the sense transcript due to the addition of the *nos* gene terminator sequence and a 60-bp DNA fragment introduced during subcloning, strand-non-specific pA13 probes were used to detect both sense and antisense RNAs in a11b. As illustrated in Fig. 3, there was a rapid accumulation of osmotin-like protein RNAs from 1 to 4 d (Fig. 3A), coinciding with a distinct decrease in antisense RNA abundance in a11b (Fig. 3B). The sizes of both transcripts identified in Fig. 3B were also confirmed by RNA:RNA strand-specific hybridization (data not shown). This observation suggests that rapid accumulation of osmotin-like RNAs decreased endogenous antisense-RNA levels, while the expression of endogenous osmotin-like protein genes was inhibited. Our results are consistent with the hypothesis that antisense RNA inhibits gene expression at least in part by RNA:RNA duplex formation, leading to rapid RNA degradation (Mol et al. 1994).

Using chicken anti-osmotin antibodies (LaRosa et al. 1992), immunoblot analysis of proteins from sense transgenic plants revealed variable levels of expression of osmotin-like proteins of 24 kDa that co-migrated with potato osmotin-like proteins induced by fungal infection (Fig. 4A). There was little if any osmotin-like protein in wild-type plants (Fig. 4A, B) or control (pBI101.1 vector alone) transformants (data not shown). It is likely that the expressed osmotin-like protein was processed correctly and therefore targeted to the vacuoles in transgenic potato plants as demonstrated in tobacco (Melchers et al. 1993). We also detected osmotin-like proteins at reduced levels in antisense transformants infected with *P. infestans* (Fig. 4B). Such incomplete inhibition of osmotin-like protein gene expression was also evident at RNA levels (Fig. 2C).

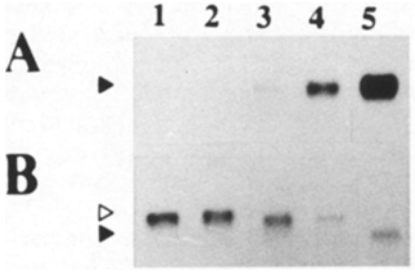

Fig. 3A, B. RNA gel blot analysis of the temporal accumulation of sense and antisense RNAs in control and antisense *S. commersonii* T_0 transgenic plants. **A** Analysis of total RNA isolated from the control (pBI101.1 vector) transgenic line. **B** Total RNA isolated from the antisense transgenic line a11b. Plants were sampled 0 (*lane 1*), 0.5 (*lane 2*), 1 (*lane 3*), 2 (*lane 4*), and 4 (*lane 5*) d after inoculation with *P. infestans*. Each lane contains 10 μg of total RNA. The RNA gel blots were hybridized to ^{32}P-labeled pA13 strand non-specific DNA probes. *Open triangle*, position where intact antisense RNA should run; *solid triangles*, position where full-length pA13 sense RNA is expected

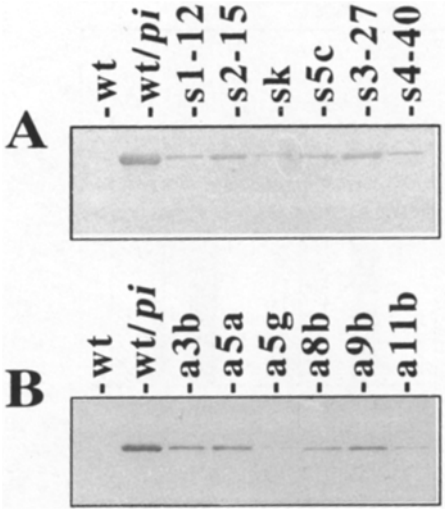

Fig. 4A, B. Immunoblot analysis of osmotin-like proteins in *S. commersonii* plants. **A** Analysis of total protein extracted from wild-type plants (*wt*), wild-type plants infected with *P. infestans* (*wt/pi*) for 4 d, and sense transgenic lines (*s1-12*, *s2-15*, *sk*, *s5c*, *s3-27*, and *s4-40*). **B** Analysis of total protein extracted from uninfected wild-type plants (*wt*), wild-type plants infected with *P. infestans* (*wt/pi*) for 4 d, and antisense transgenic lines (*a3b*, *a5a*, *a5g*, *a8b*, *a9b*, *a11b*) infected with *P. infestans* for 4 d. Each lane contains 50 μg protein

Evaluation of fungal resistance of transgenic potato plants. Osmotin and osmotin-like proteins have been demonstrated to confer antifungal activity against *P. infestans* in-vitro (Woloshuk et al. 1991). Sense transformants expressing high levels of osmotin-like protein and antisense transformants showing great inhibition in fungal-infection-induced accumulation of osmotin-like proteins were assayed for fungal resistance according to a modified leaf-disc assay (Hodgson 1961). Prior to fungal-resistance assay, we confirmed that leaves pooled from plants propagated from each independent transgenic line had levels of osmotin-like RNAs and proteins similar to those of the primary T_0 transformants (data not shown). Preliminary experiments with a range of spore concentrations on wild-type plants indicated that the percentage of sporulating leaves decreased as the concentration of inoculum was decreased below that required to cause 100% (200 spores or greater) of the leaves to show sporulation. However, spore concentrations of less than 25 per inoculum often resulted in large numbers of unsuccessful infections from assay to assay. We used fungal spore concentrations of 50 and 100 per inoculum for our analysis since these concentrations produced less than 100% sporulating leaves and were found to give more-reproducible results. Analysis of variance of fungal-resistance data within each group revealed no significant difference overall among individual control or transgenic lines at 3, 5 and

10 d for 50 and 100 spores per inoculum. We then analyzed variance of fungal-resistance data of four genotype groups with replicated transgenic lines, and found that overall differences in percentage of sporulating leaves were highly significant 3 ($P \leq 0.01$), 5 ($P \leq 0.01$), and 10 ($P \leq 0.05$) d after inoculation with 50 spores. However, there were no significant differences when leaves were inoculated with 100 spores.

Figure 5 summarizes the results of a means comparison test to determine mean percentage of sporulating leaves at both inoculation concentrations. For 50 spores per inoculation, sense transgenic lines showed a lower percentage of sporulating leaves than the wild type, control transformants pBI101.1, and antisense transgenic lines (Fig. 5A). Differences between sense transformants and others were statistically significant for all but one case (antisense at 10 d) due to an increase in the percentage of sporulating leaves in sense transformants at 10 d. For 100 spores per inoculation, there were no significant differences between the four genotypes (Fig. 5B). No significant differences were found between wild-type, control transformants, and antisense transformants in all cases (Fig. 5A, B).

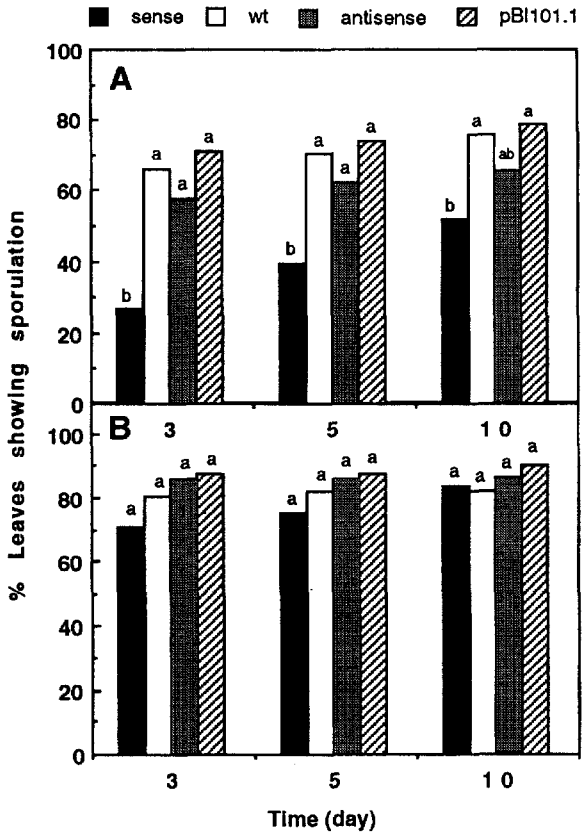

Fig. 5A, B. Evaluation of the development of *P. infestans* infection in wild-type plants (*wt*), and sense (*sense*), antisense (*antisense*), and control (*pBI101.1*) transformants. Four wild-type plants, and four sense (s1-12, s2-15, s3-27, s4-40), six antisense (a3b, a5a, a5g, a8b, a9b, and a11b), and three control (pBI101.1 vector alone) transgenic lines were analyzed. Infection efficiency, expressed as percentage of leaves showing sporulation, was determined at 3, 5, and 10 d after inoculation. Data are means of two independent experiments, each including the analysis of all independent T_0 plants from each of the four groups. **A** Inoculation with 50 *P. infestans* zoospores. **B** Inoculation with 100 *P. infestans* zoospores. Means within each day followed by the same letter are not significantly different ($P = 0.05$) based on the Tukey-Kramer HSD means comparison test

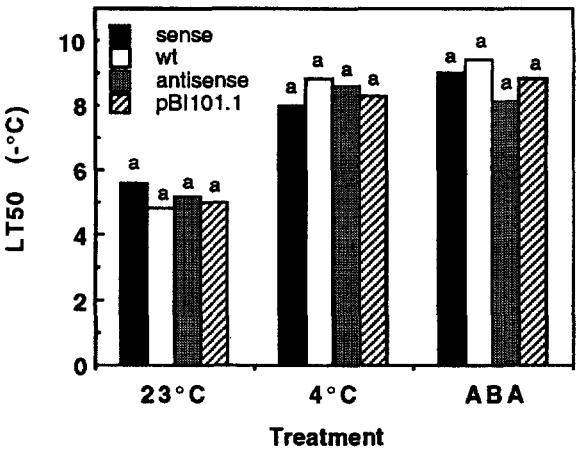

Fig. 6. Evaluation of freezing tolerance in wild-type (*wt*) plants, control pBI101.1, sense, and antisense transformants. All plants were grown at 23°C, treated with ABA (75 μM) for 5 d, or cold-acclimated at 4° for 7 d. Freezing tolerance, expressed as the temperature that results in 50% electrolyte leakage (LT_{50}), was evaluated as described by Tseng and Li (1990). Data are means of two independent experiments, each including the analysis of four *wt* plants, three pBI101.1, four sense (s1-12, s2-15, s3-27, and s4-40) and six antisense (a3b, a5a, a5g, a8b, a9b, and a11b) T_0 transgenic lines. Means within each treatment followed by the same letter are not significantly different ($P = 0.05$) based on the Tukey-Kramer HSD means comparison test

Evaluation of freezing tolerance of transgenic plants. The same groups of transgenic potato plants used for assays of *P. infestans* resistance were also evaluated for freezing tolerance. Analysis of variance and means comparison test followed the procedures described in disease-resistance data analysis. For each treatment, there were no statistical differences between individuals within each group (data not shown) or between the four genotypes. Both ABA and low temperature had similar effects on the induction of freezing tolerance in all four genotypes (Fig. 6). Treatments with ABA and low temperature resulted in little if any accumulation of osmotin-like RNAs (data not shown) in an antisense transformant (a11b) expressing high levels of antisense RNA (Fig. 3B). We have previously demonstrated that both ABA and low temperature increase the accumulation of osmotin-like protein mRNAs in wild-type potato plants (Zhu et al. 1993).

Discussion

In this study we have described the modification of the expression of a potato osmotin-like protein gene in transgenic potato plants and provided in-vivo evidence of a biological role for this protein in plant defense against an oomycete pathogen *P. infestans* that causes late-blight disease in potato and tomato. Our results, together with the finding that overexpression of tobacco osmotin delays the development of disease symptoms in transgenic potato

plants challenged by *P. infestans* but has no effect on the other oomycete pathogen *P. parasitica* var. *nicotianae* in transgenic tobacco (Liu et al. 1994), suggest these proteins confer specific antifungal activity against *P. infestans*. This notion is, however, in contrast to the assumption that the heterologous nature of tobacco osmotin accounts for its biological activity against *P. infestans* in potato (Liu et al. 1994). Their speculation is based on a co-evolution theory, suggesting that the effects of pathogenesis-related (PR) genes diminish as a result of host/pathogen co-evolution (Lamb et al. 1992).

Results from a number of studies involving overexpression of PR genes are consistent with the above conjecture (Linthorst et al. 1989; Broglie et al. 1991; Neuhaus et al. 1991; Liu et al. 1994). For example, transgenic tobacco and canola overexpressing a bean chitinase exhibited enhanced resistance to *Rhizoctonia solani* (Broglie et al. 1991); however, constitutive expression of a tobacco basic chitinase was unable to increase the resistance to the fungus *Cercospora nicotianae* in transgenic tobacco (Neuhaus et al. 1991). In addition, enhanced expression of PR-1 in transgenic tobacco did not exert any effect on infection by tobacco mosaic virus (Linthorst et al. 1989). In a separate study it was demonstrated later by Alexander et al. (1993) that constitutive expression of PR-1a was able to confer tolerance to two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae*, causing blue-mold and black-shank diseases in tobacco. The later finding, together with our results, does not support the co-evolution hypothesis but rather implies that there is a biological specificity between some PR proteins and plant pathogens, although we cannot rule out the possibility that osmotin-like proteins may also confer resistance to other pathogens which have not been tested.

We have modified the timing of a natural host defense mechanism by overexpressing an osmotin-like protein in transgenic potato plants to delay development of disease symptoms induced by a virulent fungus *P. infestans*. In a typical compatible interaction, *P. infestans* spores germinate in minutes; this is followed by cellular penetration rarely associated with cell death, leading to more or less unimpaired penetration of *P. infestans* within 24 h (Hahlbrock et al. 1989). Cell necrosis in the infected leaves with a compatible race did not occur until 48 h after inoculation in contrast to the faster hypersensitive death response evoked by an incompatible race (Hohl and Suter 1976). Conceivably, the time gained by the compatible race before host cells perceive and respond to elicitors for the activation of defense genes may provide a window crucial to the establishment of the fungus for continued colonization of the host plant. Our results showed that although the levels of osmotin-like proteins in transgenic potato plants were not as high as those accumulated in *P. infestans*-infected tissues at a later stage of fungal infection (Fig. 4A), sense transgenic plants showed delayed development of disease symptoms (Fig. 5A). Given the fact that no significant differences in disease resistance were found in untransformed wild-type plants or control transformants, somoclonal variation and epigenic effects are not relevant in our analysis. In addition, we did not observe a strong correlation between protein levels and disease development in individual sense transgenic lines. Although analysis of larger populations of both control and sense transgenic lines would facilitate the interpretation of these data, our results suggest that the timing of pA13 protein expression is more important than the absolute levels of pA13 protein once a threshold level is reached.

The above notion is further supported by results from our previous studies. We have demonstrated that activation of at least three osmotin-like protein genes and synthesis of osmotin-like proteins occurs primarily at the later stage of fungal infection (Zhu et al. 1995a), coinciding with active fungal growth and necrotic leaf lesion formation. These observations suggest that osmotin-like proteins are much less effective in inhibiting fungal growth once the virulent fungus has overgrown the natural defenses of the plant host, consistent with the results from inoculation with high spore concentrations, which failed to detect increased disease resistance in the sense transgenics (Fig. 5B).

The mode of action of osmotin-like proteins in late-blight disease tolerance of transgenic potato plants is not well understood. The PR-5 family proteins such as osmotin and osmotin-like proteins have not been shown to possess any biochemical activity although they exhibit in-vitro activity against *P. infestans* (Woloshuk et al. 1991; Liu et al. 1994). The hydrophobic nature of these proteins and their ability to permeabilize fungal cell walls have been suggested to account for their in-vitro antifungal activity (Roberts and Selitrennikoff 1990; Woloshuk et al. 1991; Liu et al. 1994). Our findings, together with those of Liu et al. (1994), suggest that the overexpressed proteins may have been processed correctly and targeted into vacuoles, where they can exert a direct fungicidal effect on hyphal growth since *P. infestans* is a necrotrophic fungal pathogen whose infection needs breaking host cells.

The antisense-RNA approach has been used to mimic mutations in prokaryotes and eukaryotes although the mechanism of antisense inhibition of endogenous gene expression is not fully understood (Mol et al. 1994). There are a number of studies dealing with the inhibition of PR gene expression using the antisense technique. For example, inhibition of the synthesis of β-1,3-glucanase by expressing antisense RNA failed to alter the susceptibility to the fungus *Cercospora nicotianae* in transgenic tobacco plants (Neuhaus et al. 1992). In our study we were not able to increase the susceptibility of transgenic potato plants to *P. infestans* although the expression of osmotin-like mRNAs and proteins was greatly suppressed. As discussed previously, rapid fungal growth and delayed activation of osmotin-like protein genes during a compatible plant-fungus interaction could be a possible reason for the failure of our antisense manipulation. In addition, expression of osmotin-like protein genes in potato is highly localized in *P. infestans*-infected leaf tissues (Zhu et al. 1995a, b). It is likely that antisense inhibition of the synthesis of osmotin-like proteins took place only in the sites where fungal infection had already been established. Taken together, analysis of the effect of antisense inhibition of PR genes on the development of systemic acquired resistance, induced either by chemical treatment or hypersensitive death (Cohen et al. 1991), would produce a better understanding of the role of PR genes in plant defense.

Alternatively, inhibition of a single PR gene may be insufficient to abolish the natural plant defense mechanism that is conferred by many PR genes working in concert. Plants synthesize at least five families of PR proteins in response to microbial attack (Cutt and Klessig 1992). Hydrolytic enzymes such as chitinase and β-1,3-glucanase have been shown to act synergistically to inhibit the growth of fungi containing chitin and β-1,3-glucan in their cell walls (Schlumbaum et al. 1986; Mauch et al. 1988; Düring 1993). Mauch et al. (1988) have demonstrated that neither chitinase nor β-1,3-glucanase is able to inhibit several species of oomycete harboring little or no chitin in their cell walls. Hence, it is unlikely that chitinase would exert any lysis activity on the fungus *P. infestans*, a member of the Oomycetes. Other PR proteins including PR-1 and PR-4 have not been shown to possess any in-vitro antimicrobial activity. However, there is in-vivo evidence that expression of PR-1a results in increased tolerance to two other oomycete pathogens (Alexander et al. 1993). Mutant analysis provides a powerful approach to unravel the role of PR genes in plant defense. Recently, Cao et al. (1994) described an *Arabidopsis* mutant *npr*1 (*n*onexpresser of *PR* genes) that is nonresponsive to inducers of systemic acquired resistance. The localized expression of several PR genes induced by a virulent *Pseudomonas* pathogen is disrupted, coinciding with much less-confined disease lesion formation compared to the wild-type plants expressing all PR genes. These results suggest that many PR genes need to work together to achieve improved efficacy in preventing proximal spread of virulent pathogens.

Osmotin was originally isolated from tobacco cell cultures adapted to high salt, and numerous studies have implicated a putative function for osmotin in osmotic stress (Kononowicz et al. 1992; LaRosa et al. 1992). We have shown that osmotin-like mRNAs accumulate in potato cell cultures when treated with ABA or low temperature, coinciding with the induction of freezing tolerance (Zhu et al. 1993). It is well known that osmotic stress occurs when plants freeze. In this study, we also attempted to study the role of osmotin-like protein genes in freezing tolerance. Our results do not reveal an appreciable role for osmotin-like proteins in freezing stress, but they are compatible with the hypothesis that the osmotic-stress inducibility of osmotin-like protein genes may act as a 'second line' of plant defense when the necrotrophic pathogens fail to release host-pathogen-specific chemical elicitors to activate plant defense genes (Kononowicz et al. 1992).

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