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Osmotin overexpression in potato delays development of disease symptoms

(osmotin gene/transgenic plants/disease responses/fungal infection)

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ABSTRACT **Transgenic potato and tobacco plants carrying the osmotin gene under the control of the cauliflower mosaic virus 35S promoter constitutively overexpressed osmotin to a level of ≈2% of total cellular protein. Leaves of transgenic potato plants exhibited delayed development of disease symptoms after inoculation with spore suspensions of *Phytophthora infestans*, which is the cause of late blight disease of potato. In contrast, transgenic tobacco plants did not display any change in the development of disease symptoms when challenged with either spore suspensions or fungal mycelia of *Phytophthora parasitica* var. *nicotianae*. Using *in vitro* assays, purified osmotin was found to be more effective against *P. infestans*. Some inhibition of *P. parasitica* also was observed *in vitro* even though no *in vivo* effect could be established.**

Osmotin is a basic 24-kDa pathogenesis-related (PR) protein that accumulates in NaCl and desiccation-adapted tobacco cells (1, 2). The gene that encodes osmotin has been cloned and the regulation of its expression has been studied extensively (3–6). The expression pattern of this gene indicates that its transcription can be activated by several factors, including NaCl, desiccation, ethylene, wounding, abscisic acid, tobacco mosaic virus, fungi, and UV light (5–8). However, in nonhypersensitive-responding genotypes the protein mainly accumulates in response to osmotic stress (5).

Using *in vitro* assays, it has been demonstrated that osmotin has antifungal activity against a variety of fungi, including *Phytophthora infestans, Candida albicans, Neurospora crassa*, and *Trichoderma reesei* (7, 9). Recent reports also indicate that not only tobacco osmotin but also osmotin-like proteins from several plant species have similar inhibitory effects on fungal pathogens and may function as plant defense proteins (10–12). We have reported that the cell, tissue, and organ-specific pattern of expression of the osmotin gene is consistent with a function in the defense against pathogen attack (6). To test the plant defense hypothesis further, we produced transgenic tobacco and potato plants that overexpress the tobacco osmotin gene in a constitutive manner and found that the resistance to fungal infection is not altered in the tobacco plants but is enhanced in the potato plants.

MATERIALS AND METHODS

***In Vitro* Antifungal Assays.** Cultures of *P. parasitica* var. *nicotianae* and *P. infestans* were maintained on V8 agar or Rye A agar and subcultured to the same fresh medium, before use in assays. After growing for 2 days, filter paper discs containing purified osmotin protein (2), water, or bovine serum albumin were added to the fungal culture plates and incubated at room temperature for another 3 days.

Construction of the Chimeric Osmotin Gene and Transformation of Tobacco and Potato Plants. A DNA fragment (−8/+1033) that contains the intact osmotin gene open reading frame and both 5′ and 3′ untranslated regions was produced from genomic clone pOG (4) by polymerase chain reaction. A *Hin*dIII site sequence was added at the 5′ end. Then this fragment was subcloned into the *Hin*dIII site of the vector pGEM-7Zf(−). The resultant plasmid was digested with *Xho* I/*Sac* I, and the excised fragment was inserted into the polylinker site between the cauliflower mosaic virus (CaMV) 35S promoter and the *rbcS* 3′ poly(A) signal sequence on the binary vector pKYLX71, a modified version of pKYLX7 (13). This chimeric osmotin gene construct, designated pKOL12 (Fig. 2*A*), was mobilized from *Escherichia coli* into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating and used to produce transgenic tobacco (*Nicotiana tabacum* L. var. Wisconsin-38) and potato (*Solanum tuberosum* L., line FL1607) plants as described by Horsch *et al.* (14) and Wenzler *et al.* (15).

Hybridization Analysis. Genomic DNAs were extracted from plant leaves according to Dellaporta's method (16) and digested with *Eco*RI. Southern analyses were performed using a *Hin*dIII/*Eco*RI fragment that contains the CaMV 35S promoter sequence from pKYLX71 as a probe. Osmotin mRNA and protein in transgenic plants were detected by Northern hybridizations and SDS/PAGE immunoblots as described by LaRosa *et al.* (5).

Fungal Infection Assays on Tobacco Plants. The fungal pathogen used for tobacco resistance assays was *P. parasitica* var. *nicotianae* (race 0), which is the cause of black shank disease. Fungus was maintained on V8 agar for hyphal growth and transferred to oatmeal agar for 2 weeks to induce sporulation, after which the fungal cultures were soaked in sterilized water for 1 week to release zoospores. Tobacco leaves taken from 5-week-old plants were inoculated with either fungal mycelia or spore suspensions. When inoculated by fungal mycelia, leaves were kept in Petri dishes (one leaf per dish), and a fungal plug with mycelia from V8 agar was placed at the center of the adaxial leaf surface. The number of infected leaves was determined every day. When inoculated by spore suspension, leaves were cut into 2.5-cm^2 discs and floated on zoospore suspensions in Petri dishes (five discs per dish). Three days after incubation with spores, the total infected area of the leaf discs was determined. All Petri dishes were kept in a growth chamber with 100% humidity and 16-hr photoperiod at 25°C.

Fungal Infection Assays on Potato Plants. The fungal pathogen used for potato plants was *P. infestans*, isolate 175A. The fungus was maintained on Rye A agar and subcultured to pea agar with 0.05 g of β-sitosterol/liter for 2 weeks to produce sporangia. The sporangia suspension was made by washing the plates with sterilized distilled water and adjusted to the required concentration before use. Five leaflets with similar size (18 ± 2 cm^2) were taken from each plant for inoculation

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Abbreviations: CaMV, cauliflower mosaic virus; PR, pathogenesis-related.

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by adding 100 μl of sporangia suspension to the center of the abaxial leaf surface. The inoculated leaflets were kept in Petri dishes and incubated in a growth chamber with 100% humidity and 16-hr photoperiod at 18°C. The development of symptoms was observed and infected leaflet areas were measured by overlaying each leaflet with a 5 mm × 5 mm transparent grid every day.

RESULTS

***In Vitro* Antifungal Assays.** Fig. 1 *A* and *B* illustrate the effects of osmotin on *in vitro* hyphal growth of *P. parasitica* var. *nicotianae* and *P. infestans*, respectively. There is only a moderate inhibitory effect of osmotin on the hyphal growth of *P. parasitica* var. *nicotianae* even at a concentration of 100 μg per disc. However, for *P. infestans*, hyphal growth can be inhibited by as little as 10 μg of osmotin per disc. These results confirm that *in vitro* antifungal activity of osmotin is variable between pathogens and illustrate that it is effective against mycelia as well as spores (7).

Induction of Osmotin and Osmotin-Like Proteins in Tobacco and Potato Plants. Tobacco leaves from 5-week-old plants were inoculated with fungal mycelia of *P. parasitica* var. *nicotianae*. Agar plugs from uninoculated plates were used as controls. Five days after inoculation, protein was extracted from leaf tissues surrounding the area that showed disease symptoms. SDS/PAGE immunoblots using chicken anti-osmotin antibodies indicated that osmotin accumulation is induced by fungal infection but does not reach the maximum level until day 4 after inoculation (Fig. 2*B*).

Similarly, in potato, leaves taken from 45-day-old plants were inoculated with spore suspensions of *P. infestans*. Water was used as a control for inoculation. The induction pattern of osmotin was similar to that seen in infected tobacco leaves (Fig. 2*C*).

Transformation of Tobacco and Potato Plants. Selection for kanamycin resistance was used to isolate 26 tobacco plants and 54 potato plants after infection with *Agrobacterium* containing pKOL12. The transformation events were confirmed by Southern hybridization using a *Hin*dIII/*Eco*RI fragment from pKYLX71 as a probe that contains the CaMV 35S promoter sequence. The results of Southern analyses on some transgenic plants are shown in Fig. 3 *A* and *B* and indicate that the number of osmotin gene copies that integrated into the plant genome ranged from one to five.

Kanamycin-resistant tobacco and potato plants were screened for overexpression of the osmotin gene using SDS/PAGE immunoblot with anti-osmotin antibodies. A considerable overproduction of osmotin protein was observed in several transformed tobacco and potato plants (Fig. 3 *C* and *D*). Using the purified osmotin to construct a standard curve, we estimated that the highest amount of osmotin protein produced in the transgenic tobacco and potato plants was 2% of total soluble protein (data not shown). This amount of osmotin is equivalent to that induced by fungal infection in either nontransformed tobacco or potato plants (data not shown). In the transgenic potato plants, the size of osmotin protein that accumulated in leaf tissue was not altered compared to that found in salt-adapted tobacco cells. This is consistent with the possibility that the precursor protein was processed correctly in the heterologous potato system, although we have not confirmed that the protein accumulated in the vacuole as it does in tobacco (2). Corresponding to the overproduction of osmotin protein, there is a large accumulation of osmotin mRNA in transgenic plants as seen by Northern hybridization using the osmotin cDNA clone as probe (Fig. 3 *E* and *F*). The overproduction of osmotin protein in transgenic plants was confirmed by an examination of the developmental and tissue-specific pattern of accumulation of the protein. High levels of osmotin were found in most tissues and at all stages of leaf development, a pattern consistent with the regulatory properties of the CaMV 35S promoter and not typical of the natural osmotin gene (data not shown) (5). Plants exhibiting the highest levels of osmotin accumulation were chosen for fungal resistance assays. There were no obvious

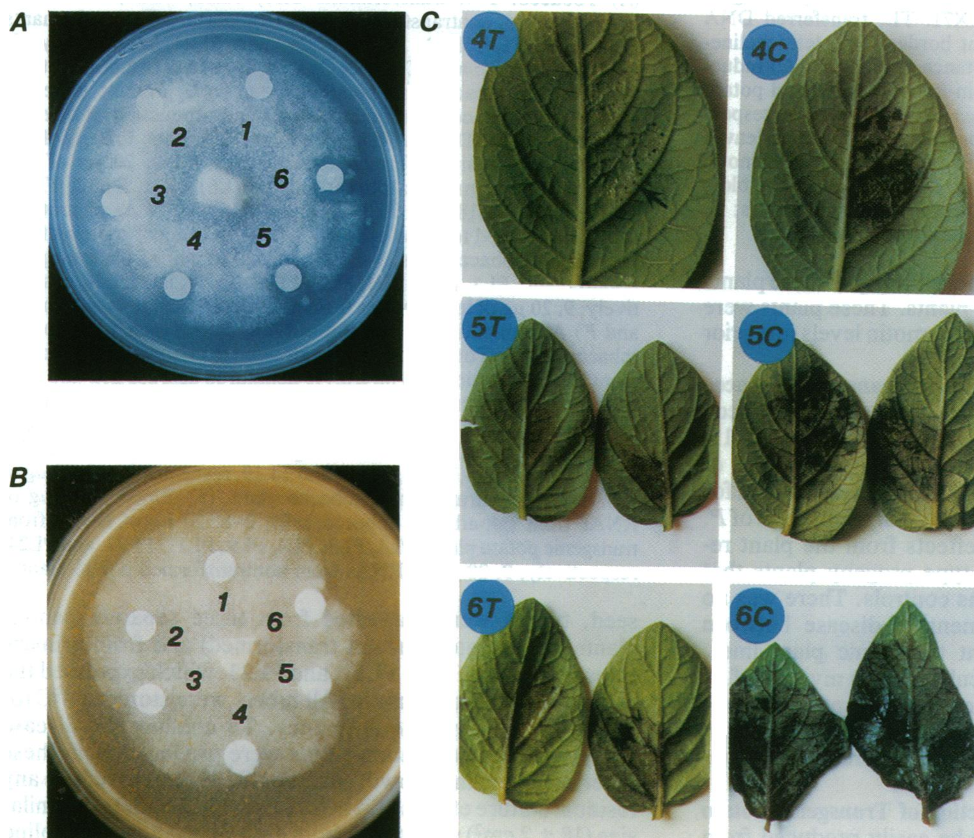

FIG. 1. Inhibitory effects of osmotin on the hyphal growth of fungal pathogens *P. parasitica* var. *nicotianae* (*A*) and *P. infestans* (*B*). Filter paper discs containing purified osmotin, bovine serum albumin, or water were placed on plates 2 days after inoculation with hyphal plugs and cultures were incubated at room temperature for another 3 days before photography. Discs: 1, water; 2, 100 μg of bovine serum albumin; 3, 10 μg of osmotin; 4, 20 μg of osmotin; 5, 50 μg of osmotin; 6, 100 μg of osmotin. (*C*) Typical development of disease symptoms in transgenic potato plant PL11 (4T, 5T, 6T) and a nontransformed control plant (4C, 5C, 6C) at 4, 5, and 6 days after inoculation.

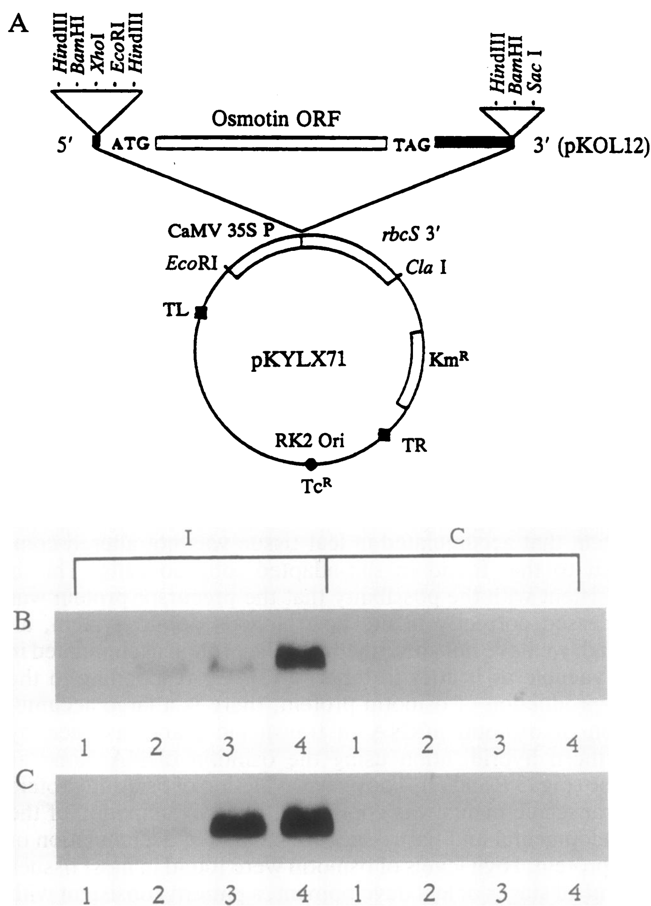

FIG. 2. (*A*) Map of the chimeric osmotin gene construct pKOL12. The intact osmotin gene open reading frame (ORF) containing both 5′ and 3′ untranslated regions was inserted into the polylinker site between the CaMV 35S promoter and the *rbcS* 3′ poly(A) signal sequence on the binary vector pKYLX71. TL, transferred DNA (T-DNA) left border; TR, T-DNA right border; Tc^R, tetracycline-resistance gene; Km^R, kanamycin-resistance gene. (*B* and *C*) Induction of osmotin and osmotin-like protein in tobacco (*B*) and potato (*C*) leaves inoculated with *P. parasitica* and *P. infestans*, respectively. Total proteins were extracted from tobacco and potato leaves and fractionated on a 12% SDS/PAGE gel. The induced osmotin protein was detected by immunoblotting. Numbers below the blots indicate the days after inoculation. I, fungal inoculated leaves; C, control leaves inoculated with an agar plug without mycelia.

morphological or developmental changes in any of these plants when compared to nontransformed plants. These plants were also examined and found to have high osmotin levels just prior to inoculation with the pathogens.

Disease Response to Fungal Infection of Transgenic Tobacco Plants. Five-week-old, 35S osmotin homozygous tobacco plants from three independent primary transformants, TL11, TL14, and TL19, which expressed the highest level of osmotin among all of the transformed plants, were chosen for blind assays of resistance to infection by fungal mycelia of *P. parasitica*. To eliminate possible effects from the plant regeneration process, azygous wild-type progeny plants that segregated from TL19 were used as controls. There was no significant difference in development of disease between control and the three independent transgenic plant lines, TL11, TL14, and TL19, when inoculated with mycelia of *P. parasitica* (Fig. 4*A*). We also did not observe any significant differences in infected leaf area between control and transgenic plants that were inoculated with spore suspensions from *P. parasitica* (Fig. 4*B*).

Disease Response to Fungal Infection of Transgenic Potato Plants. Since potato plants cannot be easily produced from

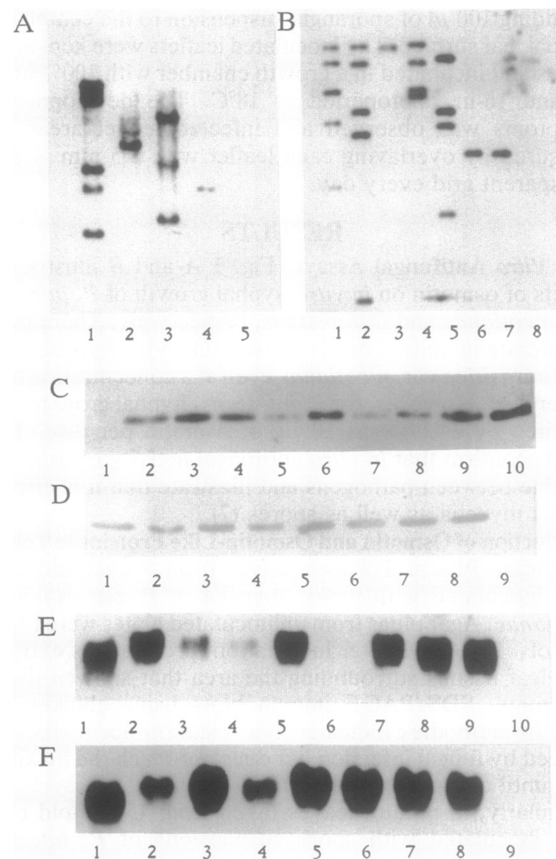

FIG. 3. (*A* and *B*) Southern analysis of transgenic tobacco and potato plants. Genomic DNA was extracted from primary transgenic plants of tobacco and potato. Ten micrograms of DNA was digested with *Eco*RI and fractionated on a 0.8% agarose gel and hybridized with a DNA fragment containing the CaMV 35S promoter sequence. (*A*) Tobacco: 1–4, transformants TL6, TL11, TL14, and TL19, respectively; 5, nontransformed plant. (*B*) Potato: 1–7, transformants PL1, PL3, PL4, PL9, PL11, PL23, and PL24, respectively; 8, nontransformed plant. (*C* and *D*) Overproduction of osmotin proteins in transgenic tobacco and potato plants. Total proteins were extracted from tobacco and potato leaves and production of osmotin was detected by SDS/PAGE immunoblots. (*C*) Blots: 1, 20 μg of leaf proteins from nontransformed tobacco plant; 2–9, 20 μg of leaf proteins from transgenic tobacco plants TL6, TL11, TL12, TL13, TL14, TL16, TL18, and TL19; respectively; 10, 10 μg of proteins from salt-adapted tobacco cells. (*D*) Blots: 1, 10 μg of proteins from salt-adapted tobacco cells; 2–8, 20 μg of leaf proteins from transgenic potato plants PL1, PL3, PL4, PL9, PL11, PL23, and PL24, respectively; 9, 20 μg of leaf proteins from nontransformed potato plant. (*E* and *F*) Accumulation of osmotin mRNAs in transgenic potato and tobacco leaves. Total RNAs were extracted from tobacco and potato plant leaves, fractionated on a 0.7% denatured agarose gel, and then transferred onto nitrocellulose and hybridized with an osmotin cDNA clone. (*E*) Blots: 1, 10 μg of RNAs from salt-adapted tobacco cells; 2–9, 20 μg of RNAs from transgenic tobacco plants TL6, TL11, TL12, TL13, TL14, TL16, TL18, and TL19, respectively; 10, 20 μg of RNAs from nontransformed tobacco plant. (*F*) Blots: 1, 10 μg of RNAs from salt-adapted tobacco cells; 2–8, 20 μg of RNAs from transgenic potato plants PL1, PL3, PL4, PL9, PL11, PL23, and PL24, respectively; 9, 20 μg of RNAs from nontransformed potato plant.

seed, tubers were harvested from three separate control plants (regenerated but not transformed) and from primary transgenic plants PL9, PL11, and PL23, which expressed the highest amounts of osmotin. All tubers were stored at 4°C for 2 months to overcome dormancy. To conduct the disease assays, second generation plants were produced from these tubers and kept in a growth chamber for 45 days to reduce any residual culture effects (17) before use. Five leaflets of similar size (18 ± 2 cm^2) were taken from each plant and used in blind

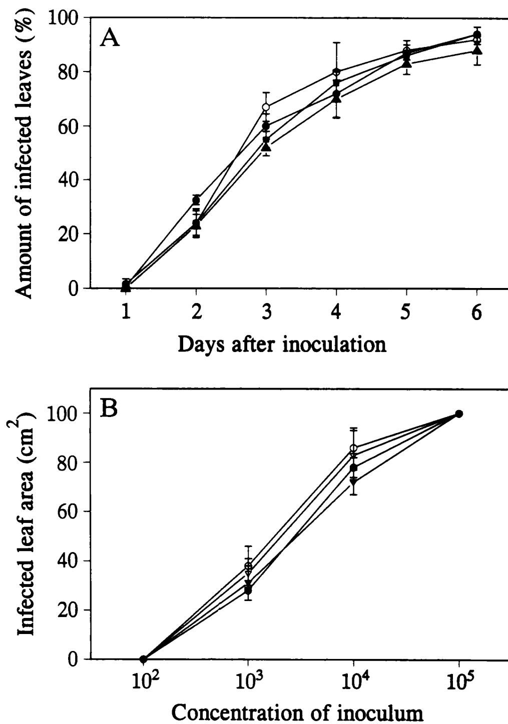

FIG. 4. Disease response of transgenic tobacco plants to fungal infection of *P. parasitica* var. *nicotianae*. (*A*) Plant leaves were inoculated with fungal mycelia. The number of infected leaves was determined every day after inoculation and expressed as a percentage of total inoculated leaves. These experiments were repeated three times each with 40 leaves taken from 40 separate plants of each genotype. Means ± standard deviations are shown. (*B*) Plant leaf discs were inoculated with fungal zoospore suspensions and total area of infected leaf tissue was determined at day 3 after inoculation. ○, Nontransformant; ●, ■, and ▼, transformed plant lines TL11, TL14, and TL19. Experiments were repeated three times using five discs from each of eight separate plants of each genotype tested. Means ± standard deviations are shown.

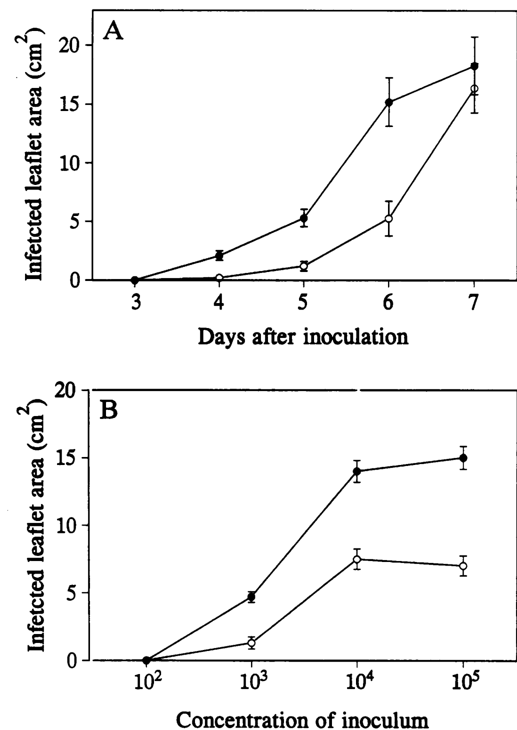

FIG. 5. Disease response of transgenic potato plants to fungal infection of *P. infestans*. (*A*) Potato plant leaflets were inoculated with sporangia suspension at a concentration of 10^5 sporangia per ml and infected leaflet areas were determined every day after inoculation. (*B*) Potato leaflets were inoculated with sporangia suspensions of different concentrations and infected leaflet areas were determined at day 6 after inoculation. ●, Nontransformed plant; ○, transformed plant PL11.

assays for resistance by inoculation with sporangia suspensions of *P. infestans*, isolate 175 A, which we had demonstrated to be sensitive to purified osmotin protein with *in vitro* growth assays (Fig. 1*B*). At day 4 after inoculation, obvious disease symptoms of black necrotic lesions were observed on leaflets in nontransformed plants, whereas there were only a few tiny necrotic spots that appeared on transgenic plants (line PL11) at this time (Fig. 1*C*). At days 4, 5, and 6, there was a statistically significant difference ($P < 0.05$) in the amount of infected leaflet area between transgenic (lines PL9, PL11, and PL23) and control plants (Fig. 5*A*). At days 4, 5, and 6, respectively, after inoculation, the infected leaflet areas (cm^2; $x \pm$ SE) were as follows for control plants: 2.12 ± 0.45, 5.33 ± 1.02, 15.07 ± 2.45; for PL9: 0.55 ± 0.43, 2.06 ± 1.01, 7.29 ± 3.21; for PL11: 0.25 ± 0.36, 1.26 ± 0.66, 5.45 ± 2.05; for PL23: 0.43 ± 0.41, 1.89 ± 0.88, 7.48 ± 2.85. At these times obvious visual differences in the degree of disease development between transgenic and nontransformed leaflets could be seen (Fig. 1*C*). In fact, the visual differences were more striking than the differences in the amount of infected leaf area because, even though infected areas of transformed plants appeared less severely damaged, we still included them as part of the infected leaf area. We also tested different concentrations of inoculum and attempted to find an inoculation condition that caused the disease only in nontransformed plants and not in transgenic plants. We could not produce these conditions. However, we did observe a consistent difference in the extent of damage after 6 days at all inoculum levels that resulted in infection (Fig. 5*B*). The values in Fig. 5 are means ± standard errors of all experiments. Three replicates of each independent transformant and each independent control were used in separate experiments that were repeated four times and the results were indistinguishable between these separate experiments.

DISCUSSION

When attacked by pathogens, plants display numerous metabolic changes, such as production of ethylene, induction of enzymes involved in propanoid synthesis, and synthesis of some antibiotic phytoalexins. One important and well-studied response of plants to pathogens is the induction of PR proteins. Initially, the PR proteins were defined as proteins that are induced by tobacco mosaic virus infection in tobacco plants. Moreover, it has been found that the expression of the genes encoding these PR proteins can be induced not only by virus infection but also by other biotic or abiotic factors, such as bacteria, fungi, ethylene, abscisic acid, wounding, etc. Our findings that the synthesis and accumulation of osmotin or osmotin-like protein can be induced in tobacco and potato plants by fungal infection further support this fact. Recently, Broglie *et al.* (18) showed that transgenic tobacco plants that overexpress the bean chitinase gene exhibited enhanced resistance to the root pathogen *Rhizoctonia solani*.

We have shown that the osmotin gene can be overexpressed in transgenic tobacco and potato plants apparently without altering the processing of the precursor protein even in heterologous potato plants. The temporal and spatial expression pattern of the introduced osmotin gene is similar to that of other genes when under the control of CaMV 35S promoter. The highest levels of osmotin production in transgenic tobacco and potato are equivalent to those induced by fungal infection in nontransformed plants (data not shown).

However, we found that constitutive accumulation of osmotin in transgenic plants led to enhanced fungal resistance only in the case of potato and not in tobacco. These experiments do indicate that some PR proteins can play a defensive role during fungal infection when they are overexpressed in a heterologous system. It is interesting to note that there is other evidence that an early or high level of accumulation of PR protein in transgenic plants of the same species from which the PR gene was obtained does not have an obvious anti-pathogen effect. For example, Linthorst *et al.* (19) made transgenic tobacco plants that overexpressed the tobacco PR-1, GRP, and PR-S genes, and Cutt *et al.* (20) produced transgenic tobacco that constitutively produced tobacco PR-1b protein, and these investigators did not find any alteration of resistance to tobacco mosaic virus infection in the transgenic plants. In addition, Neuhaus *et al.* (21, 22) produced transgenic plants of tobacco (*Nicotiana sylvestris*) where tobacco chitinase accumulation was enhanced (21) or where the tobacco β-glucanase was blocked (22), and these plants did not have altered susceptibility to the tobacco fungal pathogen *Cercospora nicotianae*. Taken together, these results reflect a specificity of particular PR gene products for activity against pathogens that are not pathogenic against the plant species that served as the source of the PR gene. This could be the result of pathogen/host coevolution, resulting in PR genes with diminished effects on coevolving pathogens as proposed by Lamb (23). However, it still should be considered that overexpression of one tobacco PR protein is not sufficient to confer the plant with a high level of resistance, because it must work with other defense factors to cause strong resistance or especially immunity. In addition, the resistance of the transgenic plants has not been tested under the variable conditions in the field with larger-scale experiments where other environmental factors may confound the genetic differences seen here.

The first evidence that osmotin and other PR family-5 proteins might have antifungal properties emerged from the discovery by Vigers *et al.* (12) that the N-terminal sequence of zeamatin, an antifungal protein from corn, was very similar to osmotin. Also, Woloshuk *et al.* (7), using a spore germination bioassay with *P. infestans*, identified from tobacco and tomato an antifungal protein that by amino acid sequence comparison was found to be osmotin. Recently, more osmotin-like proteins or cDNAs that encode these proteins have been identified in many plant species, such as tomato (7, 24, 25), potato (26), *Atriplex* (27), *Arabidopsis* (28), rice (29), etc. Some of them have been demonstrated in *in vitro* assays to be antifungal proteins. Our results, using 35S–osmotin transgenic potato plants, are consistent with the inhibitory effects of purified osmotin protein on *P. infestans* mycelia growth found by us (Fig. 1*B*) and inhibition of sporangia germination found by others (7). This report provides direct evidence that the osmotin gene can play a defensive role during fungal infection. However, the fact that the overexpression of osmotin can only delay the development of disease symptoms indicates that even greater overexpression or a redirected targeting of overexpressed osmotin from the vacuole to the extracellular matrix may be needed to further enhance resistance. Furthermore, as more antifungal gene products are identified, transgenic experiments using combinations of these genes will be of great interest.

The mechanism of the inhibitory effect of osmotin on fungal growth is not completely understood. The experiments of Woloshuk *et al.* (7) indicate that osmotin can cause sporangia lysis of *P. infestans*. A high concentration of osmotin also can cause the lysis of hyphae tips (7, 12). Moreover, many hyphae ruptured following zeamatin treatment, apparently releasing cytoplasm, and Vigers *et al.* (12) have proposed that osmotin belongs to a large class of proteins that induce fungal cell permeability and are termed permatins. Based on these data, and on sequence identity to thaumatin, which has antifungal activity (12) and whose three-dimensional structure has been determined (30), osmotin may contain structural domains that are involved in membrane receptor binding and membrane pore formation.

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