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The EREBP Gene from *Solanum tuberosum* Confers Resistance against an Oomycete and a Bacterial Pathogen in Transgenic Potato and Tobacco Plants

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ABSTRACT

Transgenic potato and tobacco lines overexpressing the *StEREBP1* gene from potato (*Solanum tuberosum* L.) were generated by *Agrobacterium*-mediated transformation. Leaves of two transgenic potato plants, overexpressing *StEREBP1* under the control of the CaMV 35S promoter, exhibited resistance to the late blight pathogen, *Phytophthora infestans*. No symptoms of late blight were observed on the leaves of the transgenic plants after infection with the oomycete pathogen, unlike the leaves of the wild type plant that showed symptoms typical of late blight. Tuber slices of these transgenic plants and another transgenic potato plant carrying the gene driven by the rd29A promoter, displayed delayed disease development. Decreased mycelial development was obtained on tuber slices from the rd29A-*StEREBP1* transgenic plant compared with growth on the tuber slices from 35S-*StEREBP1* transgenic plants. The *StEREBP1* overexpressing transgenic tobacco lines also showed delayed symptom development after infection with the oomycete pathogen *Phytophthora parasitica* var. *nicotianae*. Smaller necrotic lesions were produced on the leaves of the transgenic tobacco plants compared with those formed on the leaves of the wild type tobacco plant. Growth of *Pseudomonas syringae* pv. *tabaci* on one transgenic tobacco line was inhibited relative to the growth of the bacterial pathogen on wild type tobacco. Our results suggest that *StEREBP1* may be involved in biotic stress or defense response. The enhanced resistance of the transgenic plants to their respective pathogens may be due to the upregulation of the expression of stress-response genes encoding pathogenesis-related proteins that may have been induced by the overexpression of *StEREBP1*.

Keywords: gene overexpression, *Phytophthora infestans*, *Phytophthora parasitica* var. *nicotianae*, *Pseudomonas syringae* pv. *tabaci*
Abbreviations: CaMV, *Cauliflower mosaic virus*; **cfu**, colony forming units; **EREBP**, ethylene responsive element binding protein; **GUS**, glucuronidase; **PR**, pathogenesis-related; **St**, *Solanum tuberosum*

INTRODUCTION

Diseases caused by oomycete and bacterial pathogens are major factors constraining agricultural crop production of Solanaceae. Late blight caused by the oomycete, *P. infestans*, is the most devastating disease of potato in the world (Song *et al.* 2003). It is an extremely destructive disease attacking both tubers and foliage during all stages of crop development. The disease precipitated the Great Irish Potato Famine in the 19th century, which caused the death of more than one million people in Ireland and currently is responsible for the multibillion-dollar losses annually in both potato and tomato production (Kamoun 2001).

P. parasitica var. *nicotianae* and *Pseudomonas syringae* pv. *tabaci* are two of the most economically important pathogens of tobacco. The former causes black shank (Keller *et al*. 1999), a severe root and crown rot of all types of cultivated tobacco. The latter is a bacterial pathogen and the causal agent of the wildfire and angular spot diseases of tobacco (Knight *et al*. 1987).

Genes involved in plant defense responses may be useful in genetic engineering of cultivated plant species for resistance to diseases. Likely candidates are genes that code for ethylene responsive element binding proteins (EREBPs). EREBPs have been identified in a number of plant species including *Arabidopsis*, tobacco and tomato (Xu *et al.* 1998; Thara *et al.* 1999; Jaglo *et al.* 2001). These proteins contain a highly conserved basic DNA-binding domain (AP2/ERF domain) comprising 58 or 59 amino acids. The AP2/ERF domain recognizes and binds to *cis*-elements such as the DRE/CRT element and the GCC box (Gutterson *et al.* 2004). EREBPs are induced by a variety of stresses and pathogens, as well as by hormones associated with defense responses, such as ethylene, jasmonic acid (JA), and salicylic acid (SA).

We recently identified *StEREBP1*, a cold-inducible gene from *S. tuberosum* L. (Lee *et al.* 2007). Phylogenetic analysis indicated that *StEREBP1* may belong to the Class III subfamily of EREBP proteins, the AP2/ERF superfamily, which are believed to play important regulatory functions in plant development, as well as environmental stress and defense responses (Song *et al.* 2005). We demonstrated that constitutive overexpression of the gene in potato plants induced several GCC *cis*-element-containing stress response genes resulting in an enhanced tolerance to cold and salt

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stress. Our results suggested that *StEREBP1* is involved in the abiotic stress response in plants via transcriptional regulation of these genes. We also observed that transcript levels of *StEREBP1* was also increased by the plant defense response associated hormones salicylic acid and ethylene and also by the oomycete pathogen *P. infestsans* (Byun *et al*. 2007; Lee *et al.* 2007). This suggested that *StEREBP1* may also be involved in biotic stress or defense response in plants and raised the possibility that overexpression of the gene in potato and other plants could enhance their resistance to plant pathogens.

In this study, we investigated the effectiveness of *StEREBP1* in inducing resistance in potato and tobacco to their respective pathogens. We transformed potato and tobacco plants with the *StEREBP1* cDNA by *Agrobacterium*-mediated transformation. Our results show that *StEREBP1* overexpressing transgenic potato plants exhibited resistance to *P. infestans* while transgenic tobacco plants overexpressing *StEREBP1* displayed resistance to *P. parasitica* var. *nicotianae* and to *P. syringae* pv. *tabaci*.

MATERIALS AND METHODS

Pathogen strains, binary vector and culture media

The pathogen strains were obtained from the Korean Agricultural Culture Collection (KACC) in the Rural Development Administration, Korea. *P. infestans* KACC 40718 and *P. parasitica* var. *nicotianae* KACC 40906 were grown on V8 juice agar medium [200 ml V8 juice, 0.2% (w/v) calcium carbonate, 2.0% (w/v) agar per liter]. *P. syringae* pv. *tabac*i KACC 10388 was cultured in nutrient broth or nutrient agar (NB/NA; Difco Laboratories, Detroit, MI) at 28°C. Binary vector pBI121 (Clontech Laboratories, Inc. CA, USA) containing the CaMV (*Cauliflower mosaic virus*) 35S promoter and rd29A promoter (Yamaguchi-Shinozaki and Shinozaki 1993) were used for constructing pBI121/35S-StEREBP1 and pBI121/rd29A-StEREBP1, respectively. These constructs were mobilized into *Agrobacterium tumefaciens* LBA4404 that was also obtained from KACC. The bacterium was grown in YEP medium (1% yeast extract, 1% peptone, 0.5% NaCl, pH 7.5) at 28°C.

Construction of plant expression vectors

StEREBP1 cDNA obtained from a previously constructed cDNA library (Lee *et al.* 2007) of potato (*S. tuberosum* L. cv. 'Superior') was cloned into the *Bam*HI site of the binary plant expression vector pBI121 under the control of the CaMV 35S promoter (**Fig. 1A**). The *GUS* gene in pBI121 was replaced with the full length *StEREBP1* cDNA (GenBank Accession No. AU301558) to generate pBI121/35S-StEREBP1. The other plant expression vector used in this study, pBI121/rd29A-StEREBP1, was created by replacing the CaMV 35S promoter in pBI121 with the rd29A promoter (Yamaguchi-Shinozaki and Shinozaki 1993; Kasuga *et al.* 1999) and cloning the full length *StEREBP1* cDNA into the *Bam*HI site of pBI121 (**Fig. 1B**).

Agrobacterium transformation and generation of transgenic potato and tobacco plants

Recombinant plasmids harboring the *StEREBP1* gene were introduced into *A. tumefaciens* LBA4404 by electroporation using a MicroPulser Electroporator (BioRad CA, USA). Transgenic potato plants were obtained following the procedure of Yeo *et al.* (2000), whereas transgenic tobacco plants were generated following the transformation procedures of Kwon *et al.* (1994). The transgenic potato plants were grown in a controlled growth chamber at 23°C and then transferred to soil, grown until tuber development and used for further analysis and for determination of agronomic traits. Regenerated transgenic tobacco plants were transferred to a soil mixture and grown in the greenhouse until they produced seeds. The seeds were planted in soil to generate the T_1 generation plants that were maintained in the greenhouse and used for further analysis. Control potato and tobacco plants were obtained by transformation of the empty vector pBI121 carrying the GUS gene. Both expression vectors were used to produce the transgenic potato plants, whereas only pBI121/35S-StEREBP1 was used to create the transgenic tobacco plants.

Genomic DNA extraction and polymerase chain reaction analysis

Genomic DNA was extracted from the wild type, empty pBI121 vector transformed and transgenic potato and tobacco plants using the Nucleospin Plant kit (Macherey-Nagel, Duren, Germany). Polymerase chain reaction (PCR) amplification was performed in a Peltier thermal cycler PTC-200 (MJ Research, USA) using the following cycling parameters: denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. For PCR identification of *StEREBP1* transgenic potato and tobacco plants, the 35S-S (5′-CAAGGCTTGCTTCATAAC-3′) or the rd29A-S primer (5′-CCCAAGCTTGGGCATGATTTGATGGAGGA-3′) and the EREBP-AS1 primer (5′-CATCTCCACCAACTCCATCTTGTTC-3′) (Lee 2004) were used. PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

RNA preparation and Northern blot analysis

Total RNA was extracted from the wild type, empty pBI121 vector transformed and transgenic potato and tobacco plants using the Trizol Reagent (Molecular Research Center, Inc., Invitrogen Life Technologies, CA, USA). Samples were separated by 1.2% formaldehyde agarose gel electrophoresis, then transferred to an

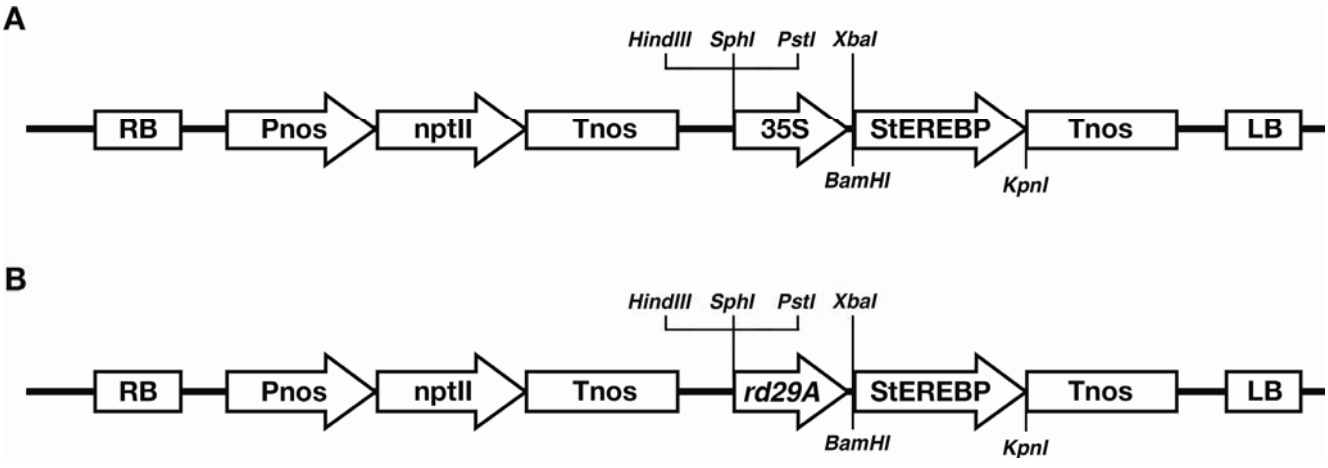

Fig. 1 Plant expression vector construction. (**A**) Binary vector pBI121 harbors the CaMV 35S promoter and the kanamycin resistance gene (*npt*II). The *StEREBP1* gene was ligated into the *Bam*HI site of the vector to generate pBI121/35S-StEREBP1. (**B**) The CaMV 35S promoter in pBI121/35S-StEREBP1 was replaced with the rd29A promoter to generate pBI121/rd29A-StEREBP1.

Immobilon membrane (Millipore, Billerica, MA, USA) and UV cross-linked. Prehybridization was performed for 15 min at 65°C in hybridization solution (1% BSA, 1 mM EDTA, 0.5M NaHPO4, 7% SDS, pH 7.2) followed by hybridization overnight at 65°C using a full-length *StEREBP1* probe (Lee 2004). The probe was labeled by the random oligonucleotide priming method (Amersham, Buckinghamshire, UK). The membranes were washed with 2x SSC/0.1% SDS for 10 min and rewashed twice with 1x SSC/0.1% SDS for 15 min at 65°C. The washed membrane was then exposed to X-ray film at -70°C for 2-3 days.

Pathogen inoculation of wild type and transgenic plants

Two methods were used to inoculate the transgenic and wild type potato (*S. tuberosum* L. cv. 'Superior') plants with *P. infestans* KACC 40718 – the detached leaf and tuber inoculation methods as adopted from the procedures of Matuszak *et al*. (1994) and Forbes *et al*. (1997), respectively. In the detached leaf method, inoculum was prepared by placing four oomycete plugs (7-mm diameter) with mycelia from a 6-day-old culture of the oomycete pathogen on the surface of V8 juice agar in Petri plates. The plugs were obtained from the oomycete plate culture using a flame sterilized cork borer. After six days of incubation at 20°C, the surface of the plates was flooded with sterile distilled water until the mycelial growth was completely covered. Sporangial production was induced by exposing the plate cultures to ultraviolet light for six hours and then to fluorescent light for 24 hours. Mycelia and sporangia were dislodged from the surface of the agar by scraping the agar surface with a rubber policeman. Sporangia were separated from the mycelia by filtration through two layers of gauze. The sporangial count of the filtrate was determined using a hemacytometer and then adjusted to 10^4 sporangia/ml.

Potato leaves were removed from 42-day-old greenhouse grown plants of the wild type and two transgenic lines harboring the CaMV 35S promoter (35S-1 EREBP and 35S-10 EREBP) and were placed in sterile Petri dishes lined with two pieces of filter paper moistened with sterile distilled water. Four young leaves of each transgenic line and the wild type were used per test. The leaves were inoculated individually by making three pricks on both sides of the midrib (adaxial surface) with the sharp end of a toothpick and pipetting 20 µl of the sporangial suspension on the pricked area. The inoculated leaves were incubated in a growth chamber under 12 h of light per day at 20°C.

For tuber inoculation, tubers of wild type potato and three transgenic lines, namely, 35S-1 EREBP, 35S-10 EREBP (both harboring the CaMV 35S promoter), and EREBP rd29A-2 (bearing the rd29A promoter) were washed thoroughly in running water and cut into 0.5 cm slices. The tuber slices were placed in sterile Petri dishes and an oomycete plug with mycelia from a 7-day-old culture of the oomycete pathogen was placed at the center of each potato tuber slice. The plates were incubated in a growth chamber set at 20°C and 90% relative humidity in the absence of light. After 24 hours the potato tuber slices were carefully inverted and the plates were further incubated until disease symptoms appeared.

The detached leaf method as described by Liu *et al.* (1994) with some modifications was used to inoculate wild type tobacco (*Nicotiana tabacum* L. var. 'Xanthi') and three transgenic tobacco lines (EREBP-6, EREBP-9, and EREBP-11) with *P. parasitica* var. *nicotianae*. Leaves of 3-week-old wild type and transgenic tobacco plants were placed in moist filter paper lined Petri dishes and were individually inoculated by placing an oomycete plug (9-mm diameter) containing mycelia from a 2-week-old culture of the oomycete pathogen on the midrib or on one side of the midrib (adaxial surface). The inoculated leaves were incubated at room temperature until the appearance of disease symptoms. The diameter (cm) of the lesions produced on the leaves was measured three days after inoculation.

Bacterial suspensions of *P. syringae* pv. *tabaci* were prepared by growing the bacterium in nutrient broth (Difco Laboratories, Detroit, MI) for 24 hours at 28°C with vigorous shaking. The cells were collected by centrifugation at 6,000 rpm for 5 min at 20°C. The pellet was resuspended in sterile distilled water and the bacterial suspension was adjusted to an optical density of $A_{600nm} = 0.2$. Wild type tobacco and the transgenic tobacco plant EREBP-6 were inoculated by infiltrating 3 ml of the bacterial suspension on the abaxial leaf surface using a needleless plastic syringe. Inoculated plants were incubated in the greenhouse. Bacterial growth in leaf tissues was monitored immediately following inoculation and daily until the fourth day post inoculation. Three leaves per plant were collected and homogenized separately with 5 ml sterile distilled water and 1 g sterile sea sand using mortars and pestles. The homogenate was serially diluted in sterile distilled water then plated in duplicate onto nutrient agar. Bacterial colonies were counted after 2-3 days of incubation.

RESULTS

Generation of *StEREBP1* overexpressing transgenic potato and tobacco plants

The *StEREBP1* gene was introduced into potato and tobacco plants by *Agrobacterium*-mediated transformation. Two different promoters were used for expression of the gene in the transgenic potato plants, the constitutive CaMV 35S promoter and the stress-inducible rd29A promoter. Only the former was used to express the gene in transgenic tobacco plants. To confirm the integration of the *StEREBP1* gene into the potato and tobacco genomes, kanamycin-resistant putative transgenic potato and tobacco plants were subjected to PCR analysis using primers designed to the 35S-S or rd29A-S and the EREBP-AS1 sequences. PCR using these primers amplifies a 720-bp fragment that corresponds to the *StEREBP1* gene.

Ten putative transgenic potato plants under the control of the CaMV 35S promoter and two putative transgenic potato plants driven by the rd29A promoter were obtained after *Agrobacterium* transformation. PCR analysis detected the presence of the expected 720-bp band in all the transgenic potato plants confirming the presence of the *StEREBP1* gene in the genome of these transgenic plants (**Fig. 2A, 2B**). However, the band intensities varied among the CaMV 35S driven transgenic plants (**Fig. 2A**). Intense bands were observed from lines 1, 2, 4, 6, 7, and 10 and faint bands from lines 3, 5, 8, and 9. No amplification products were obtained with the genome of the wild type and the empty pBI121 vector transformed plants.

Ten putative transgenic tobacco plants harboring the CaMV 35S promoter were likewise generated after *Agrobacterium* transformation. However, amplification of the 720-bp fragment, indicating the presence of the *StEREBP1* gene in the genome, was only detected with six of the putative transgenic tobacco plants (lines 5, 6, 8, 9, 10, and 11) (**Fig. 2C**). Band intensities also differed among the transgenic tobacco plants with line 6 showing the most intense band. A band was not observed with the wild type and the empty pBI121 vector transformed tobacco plants.

The twelve T_o generation transgenic potato plants and the six transgenic tobacco plants harboring the *StEREBP1* gene were selected for further analysis.

StEREBP1 expression in transgenic potato and tobacco plants

The expression of *StEREBP1* in the transgenic potato and tobacco plants was detected by Northern blot analysis. As expected, expression of the gene was not detected in the wild type and the empty pBI121 vector transformed potato plants (**Figs. 3A, 3B**). However, varying levels of expression of the *StEREBP1* under the control of the 35S CaMV promoter were observed with the ten transgenic potato plants. Lines 1 and 10 exhibited the strongest expression, while lines 2, 3, 8, and 9 showed weaker expression levels (**Fig. 3A**). A very faint band was seen from the rest of the transgenic plants indicating very weak expression of the gene. The two transgenic potato plants carrying the rd29A promoter also exhibited differing expression levels. Line 2 displayed stronger expression of the *StEREBP1* gene than line 1 (**Fig. 3B**). The transgenic potato plants exhibiting high *StEREBP1* expression levels – lines 1 and 10 (bearing the

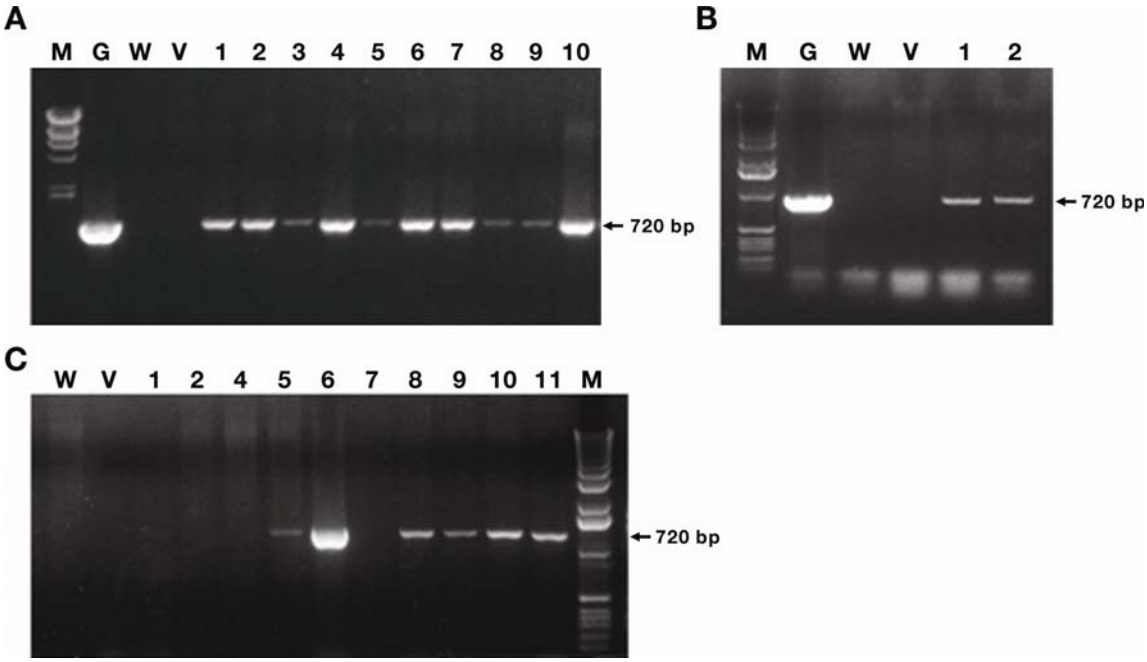

Fig. 2 PCR analysis of *StEREBP1* transgenic potato and tobacco plants. The expected 720-bp band was amplified from the genomes of the ten transgenic potato plants under the control of the CaMV 35S promoter (**A**, 1-10), the two potato transgenic plants driven by the rd29A promoter (**B**, 1 and 2), and six transgenic tobacco plants under the control of the CaMV 35S promoter (**C**, 5, 6, 8-11), but not from the wild type and the empty pBI121 vector transformed plants. W, wild type; V, control plant transformed with the empty pBI121 vector containing the *GUS* gene only, G, plant overexpression vector DNA, M, molecular marker.

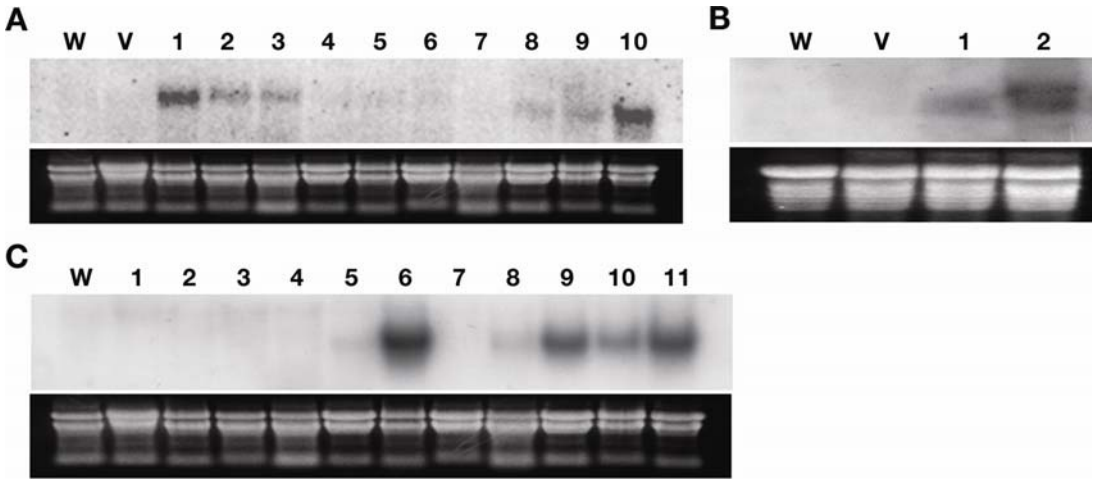

Fig. 3 Northern blot analysis for detection of expression of *StEREBP1*. *Upper panel* Hybridization signal with a [α-^{32}P]-labeled *StEREBP1* probe detected from transgenic potato (**A** and **B**) and tobacco (**C**) plants. *Lower panel* The same gel was stained with ethidium bromide to confirm equal loading.

CaMV 35S promoter) and line 2 (carrying the rd29-A promoter) were selected for the assay for resistance to *P. infestans*. These plants were designated as 35S-1 EREBP, 35S-10 EREBP and EREBP rd29A-2, respectively.

Among the six transgenic tobacco lines, lines 6, 9 and 11 displayed high accumulations of *StEREBP1* mRNA, line 10 exhibited lower mRNA accumulations and lines 5 and 8 showed very low transcription levels (**Fig. 3C**). Like the control potato plants, no transcription of *StEREBP1* was observed in the wild type and the empty pBI121 vector transformed tobacco plants. Transgenic tobacco lines 6, 9, and 11 were chosen for screening for resistance against *P. parasitica* var. *nicotianae* and were designated EREBP-6, EREBP-9, and EREBP-11, respectively. Only EREBP-6 was tested for resistance to *P. syringae* pv. *tabaci*.

Evaluation of resistance of transgenic potato and tobacco lines to pathogens

The detached leaf and tuber inoculation methods were employed to assess the resistance of the transgenic potato plants to *P. infestans*. Both methods were employed to confirm the expression of the *StEREBP1* gene in the leaves and in the tubers of the wild type and the transgenic potato plants. With the detached leaf method, typical late blight symptoms were seen on the wild type leaf six days after inoculation. The lesions appeared circular with dark brown and purplish-black necrotic spots with a pale green to yellow border. In contrast, only browning of the pricked area where the sporangial suspension was applied was observed on the leaves of the two transgenic potato plants (35S-1 EREBP and 35S-10 EREBP) six days after inoculation. The browning of the pricked area could have been caused by injury alone resulting from pricking the leaves prior to application of the sporangial suspension and not due to patho-

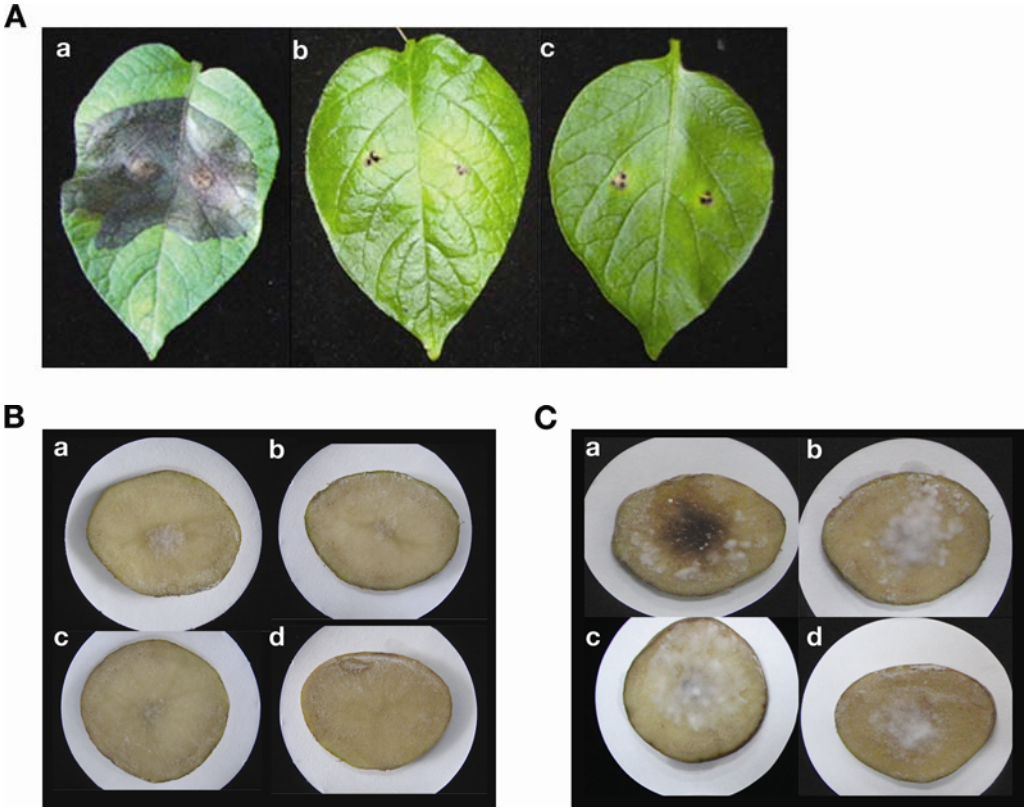

Fig. 4 Disease evaluation in *StEREBP1* transgenic potato plants. (**A**) Irregularly shaped and purplish-black expanded late blight lesions observed with the wild type potato leaf (a) and browning of the pricked area observed with the transgenic potato leaves (b and c). (**B**) Mycelia formation observed three days after inoculation with *P. infestans* on tuber slices of wild type potato (a) and transgenic potato plants under the control of the CaMV 35S promoter (b and c), but not on the tuber slice of the transgenic potato plant driven by the rd29A promoter (d). (**C**) Rotting of the tuber slice observed five days after inoculation with *P. infestans* on wild type potato (a), but not on tuber slices from the three transgenic potato (b, c, and d). a, wild type potato; b, 35S-1 EREBP; c, 35S-10 EREBP; and d, EREBP rd29A-2 transgenic potato.

gen infection. Control leaves, that were prepared from the same transgenic plants that were treated with sterile distilled water only, also showed browning of the pricked area (data not shown). With further incubation, the lesions on the wild type leaf expanded and became irregularly shaped and more purplish black and contained mycelia and sporangia (**Fig. 4A**). The browning on the sporangial suspension treated leaves from the transgenic plants, however, did not expand with further incubation and no mycelia and sporangia were observed.

In the potato tuber inoculation, the two transgenic potato lines bearing the CaMV 35S promoter (35S-1 EREBP and 35S-10 EREBP) and another transgenic potato line carrying the rd29A promoter (EREBP rd29A-2) were tested for resistance to *P. infestans* alongside the wild type potato plant. Visual comparison of differences in the amount of mycelial growth on inoculated tuber slices was done. At three days post inoculation, mycelial growth was observed at the center of the tuber slice of the wild type and the tuber slices from the two transgenic plants carrying the gene under the control of the CaMV 35S promoter, while no mycelial growth was observed on the tuber slice of the transgenic plant with *StEREBP1* driven by the rd29A promoter (**Fig. 4B**). More abundant mycelia were produced on the tuber slice of the wild type than on those of the transgenic plants 35S-1 EREBP and 35S-10 EREBP. Five days after inoculation, more severe infection was observed on the tuber slice from the wild type potato, which included rotting with reddish brown to dark grey patches (**Fig. 4C**). On the other hand, the tuber slices of the transgenic potato lines only exhibited proliferation of mycelia with no rotting. Less mycelial growth was observed on the tuber slice of EREBP rd29A-2 than on those of the two transgenic lines carrying the CaMV 35S promoter.

The wild type and the three transgenic tobacco lines (6, 9, and 11) were tested for resistance to *P. parasitica* var. *nicotianae* by the detached leaf method using oomycete plugs with mycelial growth of the oomycete pathogen instead of sporangial suspension of the pathogen as inoculum. Distinct necrosis of the inoculated area on the leaves was visible in both transgenic and wild type tobacco plants three days after inoculation (**Fig. 5A**). Smaller necrotic lesions were formed on the leaves of the three transgenic tobacco lines relative to those produced on the leaves of the wild type (**Fig. 5B**). Line 9 exhibited the smallest lesions among the three tobacco transgenic lines. This indicates that the overexpression of *StEREBP1* in the transgenic tobacco lines enhanced their resistance to *P. parasitica* var. *nicotianae*.

Only transgenic potato line 6 (designated as EREBP-6), which showed the highest expression level of *StEREBP1* by Northern blot analysis was evaluated for resistance to the bacterial pathogen, *P. syringae* pv. *tabaci*. Leaves of the wild type and EREBP-6 were infiltrated with bacterial suspensions and the growth of the bacterium within the leaves was monitored immediately after infiltration and daily until the fourth day post infiltration. Growth of the bacterium was slightly faster on the transgenic plant than on the wild type plant one day following infiltration. A maximum density of 6×10^7 colony forming units (cfu)/ml was obtained, but the numbers abruptly declined after the second day (**Fig. 5C**). Bacterial numbers on the wild type plant continued to increase and peaked on the third day, reaching a maximum of 1.2×10^8 cfu/ml. However, a decline in the numbers was observed on the fourth day. This implies that the transgenic tobacco plant was more resistant to *P. syringae* pv. *tabaci* than the wild type tobacco plant.

The responses of the transgenic potato and tobacco lines to inoculation with their respective pathogens are summarized in **Table 1**.

Table 1 Expression of the *StEREBP1* gene in the transgenic potato and tobacco lines and their response to pathogen inoculation

Transgenic line	Expression of *StEREBP1* gene[a]	Response to pathogen inoculation			
		P. infestans		*P. parasitica* var. *nicotianae*	*P. syringae* pv. *tabaci*
		Leaf	Tuber	Leaf	Leaf
Potato					
35S-1 EREBP	Strong	NDS	RMG		
35S-10 EREBP	Strong	NDS	RMG		
EREBP rd29A-2	Strong	NT	MRMG		
Tobacco					
6	Strong			RLS	RBG
9	Moderate			MRLS	NT
11	Moderate			SRLS	NT

[a] Based on the results of the Northern blot analysis; the *StEREBP1* gene was under the control of the CaMV 35S promoter in all the transgenic plants except EREBP rd29A-2 which harbored the rd29A promoter

NDS, no disease symptom; RMG, reduced mycelial growth; MRMG, more reduced mycelial growth; RLS, reduced lesion size; MRLS, more reduced lesion size; SRLS, slightly reduced lesion size; RBG, reduced bacterial growth; NT, not tested

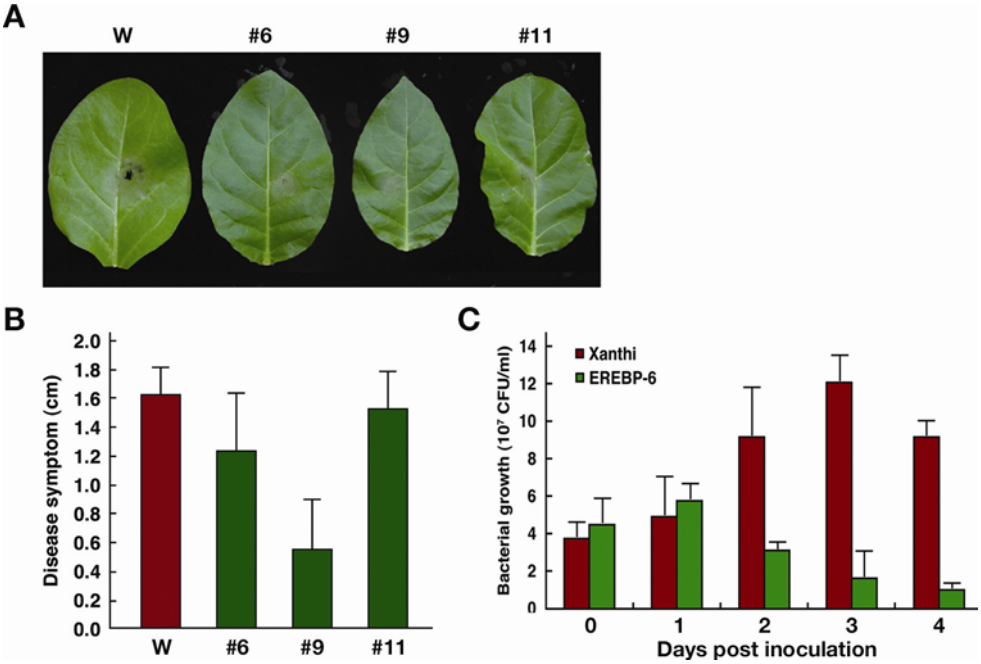

Fig. 5 Disease evaluation in *StEREBP1* transgenic tobacco plants. (**A**) Necrosis observed on the leaves of the wild type and transgenic tobacco lines inoculated with *P. parasitica* var. *nicotianae* three days after inoculation. (**B**) Diameter of lesions (in centimeters) produced by *P. parasitica* var. *nicotianae* on wild type and transgenic tobacco lines three days after inoculation. Data represent the mean ± standard error of three replications. (**C**) Growth of *P. syringae* pv. *tabaci* infiltrated into leaves of the wild type tobacco and the transgenic tobacco line EREBP-6. Data represent the mean ± standard error of three replications.

DISCUSSION

We generated *StEREBP1* overexpressing potato and tobacco plants by introducing the gene into wild type potato and tobacco plants by *Agrobacterium*-mediated transformation. Transgenic plants exhibiting the highest level of StEREBP1 were chosen for pathogen resistance assay.

The two *StEREBP1* overexpressing transgenic potato lines obtained under the control of the CaMV 35S promoter exhibited resistance to the late blight pathogen *P. infestans*. Late blight symptoms did not develop on the leaves of both transgenic lines after infection with the oomycete pathogen, unlike the leaves of the wild type plant that showed typical late blight lesions containing mycelia and sporangia of the oomycete pathogen within six days post inoculation. In addition, delayed disease development was observed on tuber slices of the three *StEREBP1* transgenic potato lines inoculated with *P. infestans*. Within three days after infection with *P. infestans*, mycelial growth was visible on the wild type potato tuber slice and those from the two transgenic potato lines harboring the CaMV 35S promoter. However, less mycelia were produced on the transgenic potato tuber slices than on that of the wild type potato. At this time, no mycelia were seen on the tuber slice from the transgenic line bearing the stress-inducible rd29A promoter. The delayed mycelia production on the rd29A-*StEREBP1* transgenic line relative to the two 35S-*StEREBP1* transgenic lines suggests stronger expression of the *StEREBP1* gene in the former than in the latter. The rd29A promoter is from the stress-inducible *rd29A* gene from *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994; Kasuga *et al*. 1999). It bears a *cis*-acting element (TACCGACAT) termed the dehydration-responsive element (DRE) that is essential for regulation of dehydration-responsive gene expression and is found in the promoter regions of dehydration and cold-stress inducible genes (Wang *et al.* 1995; Iwasaki *et al.* 1997). Being stress-inducible, the rd29A promoter may have induced a higher level of expression of *StEREBP1* than the constitutive CaMV 35S promoter. The potato tubers used in the assay had been in storage for more than a year and therefore had been under stress prior to the assay. Five days after infection, the disease symptoms became more severe on the wild type potato tuber slice and rotting ensued, whereas only mycelial proliferation was observed on the tuber slices from the three transgenic potato lines with no sign of rotting. This suggests that overexpression of the *StEREBP1* gene in the transgenic potato plants was effective in delaying disease development on potato tuber.

The transgenic tobacco plants overexpressing *StEREBP1* showed resistance to the oomycete pathogen *P. parasitica*

var. *nicotianae* and the bacterial pathogen *P. syringae* pv. *tabaci*. Lesions produced on the leaves of the three transgenic tobacco lines inoculated with *P. parasitica* var. *nicotianae* were smaller compared with those formed on the leaves of the wild type tobacco plant indicating delayed symptom development in the transgenic tobacco lines. Overexpression of *StEREBP1* in the transgenic tobacco line EREBP-6 inhibited the growth of *P. syringae* pv. *tabaci*. Maximum population density of the bacterium on EREBP-6 was significantly lower than that on the wild type. Moreover, bacterial growth declined earlier in the transgenic tobacco line than in the wild type tobacco plant.

The resistance to plant pathogens exhibited by the *StEREBP1*-overexpressing potato and tobacco lines could be due to the increased expression of genes that are involved in biotic stress or defense response. We have previously demonstrated by microarray and RT-PCR analyses that the expression of 153 genes was up-regulated in *StEREBP1*-overexpressing transgenic potato plants (Lee *et al.* 2007). Among these were genes encoding pathogenesis-related (PR) proteins – peroxidase, StPR1, and an osmotin-like protein, all of which are related to biotic or abiotic stress responses. PR proteins are known to be induced and accumulate in host plants as a result of pathogen infection or abiotic stress conditions (Kim and Hwang 2000). Peroxidases are known to play a part in increasing a plant's defenses against pathogens, although the exact mechanisms have yet to be elucidated (Karthikeyan *et al.* 2005). Peroxidase is involved with the assembly of lignin and influences several reactions involved in cell wall formation (Lee and Lin 1996). Specific members of the tobacco and tomato PR-1 families have been reported to have anti-oomycete activity (Sarowar *et al.* 2005). Tomato PR-1 strongly inhibited germination of *P. infestans* zoospores as well as the development of symptoms on the surface of leaf disks infected with the oomycete pathogen (Niderman *et al.* 1995). Osmotin has also been demonstrated to have antifungal activity against a variety of fungi including *P. infestans*, *Candida albicans*, *Neurospora crassa*, and *Trichoderma reesei* (Woloshuk *et al.* 1991; Vigers *et al.* 1992). Osmotin-like proteins or cDNAs that encode these proteins have been identified in many plant species such as tomato (King *et al.* 1988; Rodrigo *et al.* 1991; Woloshuk *et al.* 1991), potato (Pierpoint *et al.* 1990), *Atriplex* (Casas *et al.* 1992), *Arabidopsis* (Uknes *et al.* 1992), rice (Reimmann and Dudler 1993), and other plants. Some of these proteins have been reported to exhibit inhibitory effects on fungal pathogens and may function as plant defense proteins (Hejgaard *et al.* 1991; Vigers *et al.* 1991).

Several studies have also shown that overexpression of PR proteins in plants enhanced their resistance to a number of pathogens; e.g., bean chitinase in tobacco against *Rhizoctonia solani* (Brogue *et al.* 1991); PR-1a in tobacco against *P. parasitica* var. *nicotianae* and *Peronospora tabacina* (Alexander *et al.* 1993); osmotin in potato against *P. infestans* (Liu *et al.* 1994); thaumatin-like protein in rice against *R. solani* (Datta *et al.* 1999), tomato PR-5 in orange against *Phytophthora citrophthora* (Fagoaga *et al.* 2001) and pepper basic PR-1 in tobacco against *P. nicotianae*, *Ralstonia solanacearum* and *P. syringae* pv. *tabaci* (Sarowar *et al.* 2005).

In summary, our results suggest that *StEREBP1* also plays a role in biotic stress or defense response. We have demonstrated that overexpression of the *StEREBP1* gene induced resistance of potato to the late blight pathogen *P. infestans*. We have also shown that overexpression of the gene also improved resistance of tobacco to *P. parasitica* var. *nicotianae* and *P. syringae* pv. *tabaci* indicating that it can also play a defensive role in a heterologous system. Field testing of the potato and tobacco transgenic lines will be conducted to determine if their resistance to their respective pathogens will hold out under the variable conditions in the field.

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