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Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco

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Abstract

Osmotin promoter binding protein 1 (OPBP1), an AP2/EREBP-like transcription factor of tobacco (*Nicotiana tabacum*), was isolated using a yeast one-hybrid system. RNA gel blot analysis indicated that expression of the *OPBP1* gene was induced by elicitor cryptogein, NaCl, ethephon, methyl jasmonate, as well as cycloheximide. Transient expression analysis using an *OPBP1-eGFP* fusion gene in onion epidermal cells revealed that the OPBP1 protein was targeted to the nuclear. Further, electrophoretic mobility shift assays demonstrated that the recombinant OPBP1 protein could bind to an oligonucleotide containing the GCC-box *cis* element. Transgenic tobacco plants with an over expression of the *OPBP1* gene accumulated high levels of *PR-1a* and *PR-5d* genes and exhibited enhanced resistance to infection by *Pseudomonas syringae* pv *tabaci* and *Phytophthora parasitica* var *nicotianae* pathogens. They also exhibited increased tolerance to salt stress. These results suggest that OPBP1 might be a transcriptional regulator capable of regulating expression in sets of stress-related genes.

Introduction

Plants activate a battery of defense responses to protect themselves from pathogen attacks, including accumulation of pathogenesis-related (PR) proteins. The accumulation of these PR proteins can also be induced by chemical stimuli, such as salicylic acid, jasmonic acid, and ethylene treatments. An important approach to understanding *PR* gene regulation is the identification of defense-responsive promoter regions, then isolating their cognate DNA binding proteins. Several transcription factors have been isolated using this method, including a WRKY transcription factor (Rushton *et al.*, 1996) and ethylene-responsive element binding proteins (EREBPs, Ohme-Takagi and Shinshi, 1995). Analyses of promoters of several basic *PR* genes indicate that an 11-bp consensus sequence (TAAGAGCCGCC with the core sequence of AGCCGCC known as GCC box) is required for the ethylene responses (Ohme-Takagi and Shinshi, 1995; Yang *et al.*, 1997). These ethylene-responsive transcription factors have a conserved basic 58 or 59-amino acid DNA-binding domain and function as regulators of the GCC box mediated transcription of genes (Ohme-Takagi and Shinshi, 1995; Liu *et al.*, 1998). Plant specific *AP2/EREBP* genes, containing a sequence homologous to the DNA-binding domain of AP2/EREBPs are widely distributed in plants and form a super family of genes. For example, the *Arabidopsis* genome sequencing project revealed 124 putative AP2/EREBP proteins in the genome (Riechmann *et al.*, 2000).

Members of the AP2/EREBP superfamily are believed to play important roles in a variety of biological processes. For example, TINY and APETALA2 are involved in developmental and growth processes (Wilson *et al.*, 1996; Okamuro

et al., 1997). ORCA3 is a master regulator of primary and secondary metabolism in *Catharanthus roseus*, regulating jasmonate-responsive genes via direct interaction with the jasmonate- and elicitor-responsive element in the promoter of *Strictosidine synthase* gene in the terpenoid indole alkaloid biosynthetic pathway (van der Fits and Memelink, 2001). Pti4, Pti5, and Pti6, three members of the EREBP family in tomato have been shown to interact with disease resistance gene products of Pto (Zhou *et al*., 1997). Gu *et al*. (2000) demonstrated that phosphorylation of Pti4 by Pto kinase enhances the ability of Pti4 to bind with the GCC box at least *in vitro*. Three other EREBPs, CBF1, DREB1A and DREB2A, bind to a CRT/DRE (C-repeat/dehydration-responsive element) that is involved in gene expression in response to drought and cold stress (Stockinger *et al*., 1997; Liu *et al*., 1998). Further, ectopic expression of genes such as *CBF1* or *DREB1A* has been shown to improve dehydration stress tolerance in *Arabidopsis* (Kirsten *et al*., 1998; Kasuga *et al*., 1999).

Osmotin, also known as pathogenesis-related protein 5 (PR-5), is strongly induced by several biotic and abiotic stresses, such as pathogen infection, NaCl, ethylene, and ABA treatments (Xu *et al*., 1994; Raghothama *et al*., 1997) has been used as a molecular marker in studying the effects of plant hormones and environmental stresses. Several other PR proteins, such as peroxidase, glucanase, and chitinase can also be induced by dehydration stress (Ingram and Bartels, 1996; Zhu *et al*., 1997; Gong *et al*., 2001). However, the role of these gene products under dehydration stress is obscure. Similarly, many salt-regulated genes are induced by other biotic or abiotic perturbations, indicating that these stresses have common etiologies (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu *et al*., 1997; Hasegawa *et al*., 2000; Gong *et al*., 2001).

Osmotin promoter binding protein 1 (OPBP1) is an AP2/EREBP DNA binding protein isolated from tobacco using the yeast one-hybrid system through its binding to the promoter (−248 to −108 region) of the *osmotin* gene (Xu *et al*., 1998). In this study, we generated *OPBP1* transgenic tobacco plants and found the transgenic progenies of overexpression *OPBP1* gene exhibited enhanced disease resistance against pathogens and tolerance to salt. The results indicate that OPBP1 functions as a positive regulator of the *PR* genes and regulate plants' responses to both pathogen infection and salt stresses.

Materials and methods

Construction of plasmids and tobacco transformation

To overexpress *OPBP1* gene in tobacco, we generated a plasmid for sense expression of the *OPBP1* gene by cloning it into the *Sal* I site at CHF3, a pPZP211-based plant expression vector carrying a cauliflower mosaic virus 35S (CaMV35S) promoter and a pea ribulose 1,5-bisphosphate carboxylase/oxygenase terminator. Enzyme digestion was used to determine the sense orientation of the OPBP1 gene relative to the CaMV35S promoter in the recombinant plasmid, with the plasmid labeled as CHF3-35S::OPBP1. The CHF3-35S::OPBP1 and CHF3 (vector only) plasmids were first introduced into *Agrobacterium tumefaciens* EHA 105, then into tobacco (*Nicotiana tabacum* cv Samsum nn) using the *Agrobacterium*-mediated transformation method as described by (Hoekema *et al*., 1983). Kanamycin-resistant plants were grown in a greenhouse and allowed to self-fertilize. The T1 seeds were germinated in a 1/2 MS (Murashige and Skoog, 1962) medium containing 150 mg/l kanamycin (Km), and the selected seedlings grown in a greenhouse. The positive plants of the T2 progeny were confirmed by PCR analysis using primers of the 35S promoter (P35S: 5′-cacaatcccactatccttcgc-3′) and the *OPBP1* gene (P454: 5′-ggagctcccaccatcaccaaaa tttatac-3′).

Subcellular localization of OPBP1

The termination codon of the *OPBP1* cDNA was removed after PCR using primer pairs of OBP1BI: 5′-attggatccacgaggaaaaacaaaatggattct-3′ and OBP1Nco:5′-tcataccatggaaacaaagagatggaattcc cctat-3′. The resulting fragment was cloned onto a pUCm-T vector (Sangon, China) and sequenced to ensure that no PCR-induced errors were introduced into the *OPBP1* gene. After digestion with *Nco* I and *Bam*H I, the *OPBP1* fragment was fused in frame to the coding region of green fluorescent protein (eGFP), prior to construction of the chimeric gene under the control of the CaMV35S promoter. The

resulting CHF3-35S::OPBP1-eGFP plasmid was then used for transient expression of the GFP fusion construct as described by Varagona *et al*. (1992). The inner epidermal peels of white onions were placed inside-up on a MS medium containing 100 mg/l ampicillin. The onion peels were bombarded with DNA coated golden particles (Bio-Rad, USA) using a PDS-1000/He system at 1100 psi. The particles were coated with 2 μg of DNA onto 3 μg of water-washed golden particles with 50 μl of 2.5 M $CaCl_2$ and 20 μl of 0.1 M spermidine, then washed with 70% ethanol and resuspended in 30 μl of 100% ethanol. Approximately 10 μl of particles was placed on each delivery disc.

Recombinant protein and gel mobility shift assay

For expression of the OPBP1 protein, the coding sequence was amplified by PCR, introducing a *Sal* I site in front of the ATG codon and *Not* I site immediately before the stop codon. Both OBPSal: 5′-attgtcgacaaatggattcttcttcttgttcttctc-3′ and OBP-Not: 5′-ttgcggccgcaaagaacaaagagatggaattcc-3′ were used as primers for the PCR. The *OPBP1* fragment released by the *Sal* I and *Not* I digestions was cloned onto a pET-32a expression vector digested with the same enzymes (Novagen, USA). The resulting pET-OPBP1 plasmid was then sequenced to confirm that no PCR-induced errors were introduced into the *OPBP1* gene prior to transformation into *Escherichia coli* BL21 for protein expression. The expression of the his fusion protein was induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 h at 37 °C. Bacteria were pelleted after the induction, and suspended in lysis buffer (100 mM Na_2HPO_4, pH 7.2, 300 mM NaCl, 10% glycerol, and 1% Triton X-100), and subjected for sonication. Bacterial lysates were then centrifuged, and the supernatant was used for gel mobility shift assays.

The GCC box oligonucleotide was 5′-ttgactaag-agccgccattgaccaca-3′, and that of the mutated mGCC oligonucleotide was 5′-ttgactaag**aa**ccacc-attgaccaca-3′ (the mutated nucleotides in bold). The DRE/CTR box oligonucleotide was 5′-gatcc atttcatggccgacctgcttttaagctttta-3′. Electrophoretic mobility shift assays were performed in a 20 μl volume that contained 3 μg crude OPBP1 recombinant protein, 0.4 pmol of the oligonucleotide end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and DNA binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 0.025 g/l Poly(dI-dC), 10 mM Tris–HCl, pH 7.5). In the competition experiments, the appropriate amount (see Figure 2 legend) of competitor (cold GCC) was included in the reaction prior to the addition of the protein. Once the protein was added to the reaction, the mixture was incubated at room temperature for 15 min and electrophoresed on a 10% polyacryamide gel. Radioactivity was detected by autoradiography on a Typhoon system.

Pathogen challenge

The pathogen which causes tobacco black shank disease (*Phytophthora parasitica* var *nicotianae*) was grown on potato dextrose agar plates (20% potato, 2% glucose and 2% agar, w/v). After the fungal mycelia had spread throughout the plate, a plug of medium containing the fungal mycelia was taken by excision with a 3 mm cork borer. Healthy leaves (the fourth from the top) from transgenic plants (both the *35S::OPBP1* and the control plants) were detached and inoculated with the mycelium-agar plugs with the mycelium side contacting the leaf surface at sites subject to toothpick wounding (three sites per leaf). At least four leaves were used for each transgenic line and the challenged leaves were kept in a plastic dish with water soaked filter paper at the bottom at 28 °C under a 16 h light, 8 h dark regimen. Disease severity was evaluated after inoculation at designed times. If one of the three inoculated sites on a leaf showed disease symptoms, the leaf was considered to be susceptible. The disease rating for the transgenic plants were scored as an average of the inoculated leaves of the line, where "+" indicated a disease lesion smaller than 0.5 cm in size, + + for lesions between 0.5–1.0 cm, + + + for lesions between 1.0–1.5 cm, and + + + + for lesions larger than 1.5 cm. If there was no disease symptom, no "+"was used. Leaves were photographed 60 h after inoculation.

Pseudomonas syringae pv *tabaci*, which causes tobacco wildfire disease, was grown in King's medium B and infiltrated according to Li *et al*. (2000) at concentration of 10^7 colony forming units/ml in 10 mM $MgCl_2$. Bacteria were infiltrated into the 4th upper leaves using a 10 ml plastic syringe without a needle. Seven days after

inoculation, six leaf discs (two discs at each infiltration site) were taken by excision with a cover of 1.5 ml centrifuge tube beside the infiltration site. The bacterial population inside the leaf discs was released by grinding the tissue in a centrifuge tube in 10 mM $MgCl_2$ and determined based on the numbers of colonies formed on King's B plates according to Bertoni and Mills (1987).

Determination of chlorophyll fluorescence

Leaf discs were taken by excision with a 1 cm cork borer and floated on 10 mM MES (4-morpholineethanesulfonic acid) solution containing 400 mM NaCl in a plastic dish. The dishes were kept in 16 h light and 8 h dark at 25 °C. Measurement of chlorophyll fluorescence was performed according to Aono *et al*. (1993).

Elicitor and chemical treatments

Fully expanded leaves from 6-week-old tobacco plants (the 3rd and 4th from the top) were detached and sliced to ~1 cm widths, floated on a MES buffer in a plastic dish, and incubated either with or without 50 nM cryptogein, 200 mM NaCl, 0.2 mM ethephon (2-chloroethylphosphonic acid), 0.1 mM methyl jasmonate (MJA), and 0.1 mM salicylic acid (SA) at 25 °C in 16 h light and 8 h dark. The leaf slices were sampled at designed times for RNA isolation. For cycloheximde (CHX) experiments, the leaves were treated with 20 nM CHX 20 min prior to the addition of cryptogein.

To determine the effect of salt on seed germination and seedling growth, seeds of the *35S::OPBP1* transgenic plants (T1 progeny) were sterilized with 5% NaClO and plated onto 1/2MS medium containing 150 mg/l Km with or without 200 mM NaCl. Seeds from the control plants (harboring CHF3 empty vector) received the same treatments. Both the seeds germinated and seedlings grown were counted every week two weeks after sowing. The ratio of seedlings germinated in the presence of NaCl to that in the absence was used to evaluate tolerance to salt stress.

RNA isolation and Northern blot

Total RNA was isolated from 6-week-old leaves using the Trizol Reagent kit according to manufacturer's instructions. An aliquot of 20 μg total RNA was fractionated in a 1.0% denaturing agarose/formaldehyde gel and subsequently transferred onto nylon membranes (Hybond-N^+, Amersham). Filters were pre-hybridized for 4 h at 60 °C in 6 × SSC, 5 × Denhardt's regent (0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrolidone), 7% SDS and 100 mg/l salmon sperm DNA. Hybridizations were performed with α^{32} P-labeled DNA probe generated using a random primer synthesis kit, and the membranes washed according to Sambrook *et al*. (1989). The membranes were dried and exposed to x-ray film or visualized directly with a Typhoon system.

Plasmid carrying tobacco *PR-1a* sequence was obtained according to Shen *et al*. (2000). A probe of tobacco neutral *PR-5d* (accession number D76437, Kitajima *et al*., 1998) was obtained by PCR using primers of npr52: 5′-tcgagcgaggtcaaagctggt-3′ and npr54: 5′-ggaccacaaggtcctgtgtgca-3′ design based on the sequence in the database.

Results

Sequence analysis of OPBP1

OPBP1 was cloned by the yeast one-hybrid system (Xu *et al*., 1998) to bind a 141-bp promoter fragment of *PR-5* (accession number S40046). The sequence of OPBP1 predicts a protein of 277 amino acids with a calculated molecular weight of 31.6 kDa and isoelectric point of 6.06. Database searches reveal that the OPBP1 amino acid sequence contains a single conserved DNA binding domain from the AP2/EREBP super family, extending from amino acid 89–149. In addition, the predicted OPBP1 polypeptide contains a segment rich in acidic amino acids just ahead of the AP2/EREBP domain, and two potential nuclear localization signals. Cluster analyses of 12 AP2/EREBP proteins from five different plant species indicated that the predicted OPBP1 amino acid sequence was highly similar to that of tomato LeTSRF1 (GenBank accession number AF494201) with 80.0% identity (Figure 1) and with 51.4% identical to *Arabidopsis* ERF1 (Solano *et al*., 1998; Lorenzo *et al*., 2003). These results further suggest that OPBP1 might function as a transcription regulator.

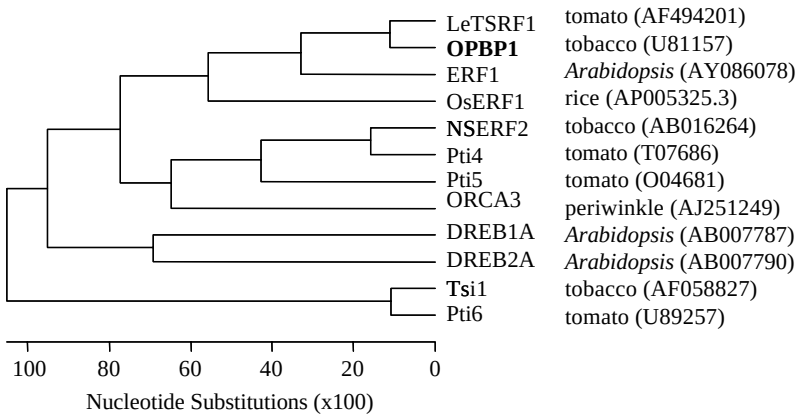

Figure 1. Phylogenetic tree for the amino acid sequence of OPBP1. AP2/EREBP proteins were aligned using the CLUSTAL W program, and the phylogenetic tree was drawn by the TREEVIEW program. Plant sources and GenBank accession numbers are indicated.

OPBP1 protein binds specifically to the GCC box

AP2/EREBP proteins are reported to bind to GCC and/or DRE/CRT box *cis* elements (Stockinger *et al*., 1997; Liu *et al*., 1998). To examine the possible interaction between OPBP1 and the GCC box, the expressed OPBP1 was used in an electrophoretic mobility shift assay. As shown in Figure 2, the recombinant OPBP1 protein did bind to the GCC box containing DNA fragment, as confirmed by competition experiments. Addition of a 100-fold excess of unlabeled GCC box oligonucleotide to the binding assay completely inhibited OPBP1 interaction with the ^{32}P-labeled GCC box DNA fragment. The addition of a mutated GCC box (mGCC, containing two G-to-A substitutions in the GCC core sequence), on the other hand, did not interfere with the binding of the OPBP1 protein with GCC box. We also investigated the interaction between the OPBP1 fusion protein and the DRE/CRT *cis* element present in drought and cold regulated genes. However, the binding of OPBP1 to a DRE/CRT box containing DNA fragments was not observed.

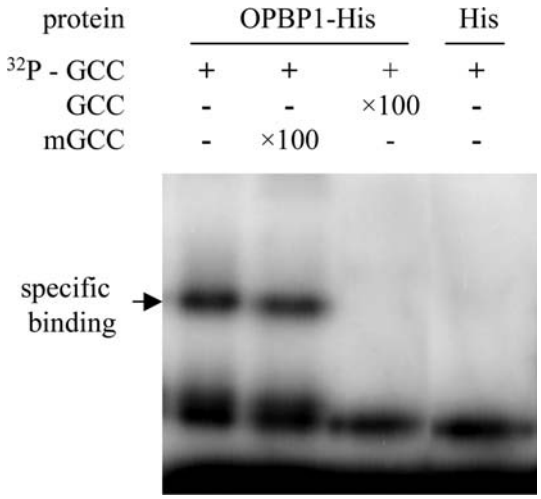

Figure 2. OPBP1 specifically binds the GCC box sequence. A gel mobility shift assay was performed using OPBP1-His fusion protein mixed with ^{32}P-labeled wild-type GCC-box or mGCC-box oligonucleotides. The notations 100×, indicates that the amount of unlabeled wild-type GCC-box oligonucleotide added to these reactions was 100-fold of the labeled GCC-box nucleotide. The (−) signs indicate that no competitor was added to these reactions.

Nuclear localization of OPBP1 protein

The OPBP1 protein contains two putative nuclear localization signals of RRRP and KKRH amino acids as predicted by PSORT analysis (Nakai and Kanehisa, 1992). To investigate the cellular distribution of OPBP1, we performed an *in vivo* targeting experiment using an OPBP1-fused green fluorescent protein (eGFP) as a fluorescent marker. The coding region of *OPBP1* was fused in frame with *eGFP* and the chimeric gene put under the control of CaMV35S promoter. The resulting plasmid was introduced by bombardment into onion epidermal cells. Localization of the OPBP1-eGFP fusion protein was visualized exclusively in the nucleus, whereas the control GFP (CaMV35S::eGFP) was

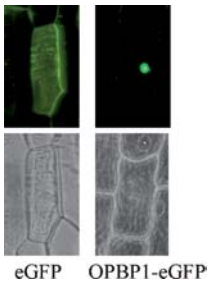

Figure 3. Nuclear localization of OPBP1-eGFP fusion protein in onion cells. GFP fluorescence (top panel) and differential interference contrast images (bottom panel) of onion epidermal cells were compared to show the subcellular localization of eGFP (cytoplasmic and nuclear) and OPBP1-eGFP (nuclear).

distributed throughout the cell (Figure 3). These results suggest that OPBP1 is a nuclear protein and that no additional post-translational modification was required for the OPBP1 protein to be targeted to the nucleus. However, we cannot rule out the possible role of factors present in the onion cells in the targeting of OPBP1 to the nucleus.

Expression of OPBP1 under various stimuli

The expression of the *OPBP1* gene under various stress conditions was studied using RNA gel blot analysis. We first chose cryptogein, a proteinous elicitor secreted from *Phytophthora cryptogea*, as an inducer for the expression of *OPBP1* gene. Cryptogein can induce a number of defense responses in tobacco, including the oxidative burst, protein phosphorylation, Ca^{2+} influx, and expression of *PR* genes (Lecourieux-Ouaked *et al*., 2000; Lebrun-Garcia *et al*., 2002). As shown in Figure 4, expression of the *OPBP1* gene in sliced tobacco leaves was induced rapidly and strongly by cryptogein (50 nM) in 1 h, and the mRNA accumulation of *OPBP1* gene was increased up to 24 h post the treatment. Expression of *OPBP1* gene was also induced by the treatment of NaCl, methyl jasmonate, and ethephon, an ethylene release reagent. However, the accumulation of *OPBP1* mRNA was not significant under the SA treatment. Further, the same membranes were used for examining the expression of *PR-5d* and

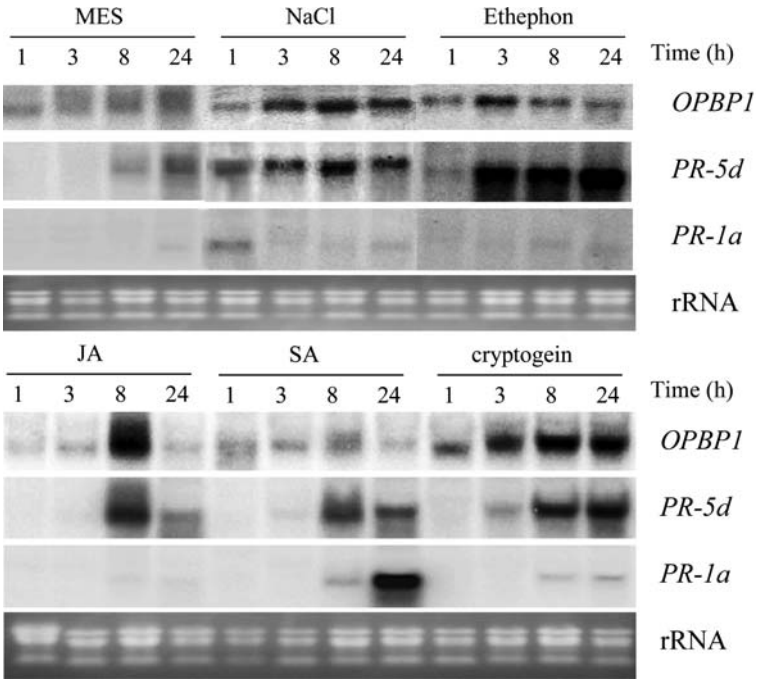

Figure 4. Expression of *OPBP1* gene in response to abiotic stress and elicitor treatment. Fully expanded leaves from 6-week-old tobacco plants were detached and sliced in about 1 cm wide, and treated with MES buffer (10 mM, pH 5.8, containing 0.01% Triton X-100) or MES buffer containing 200 mM NaCl, 0.2 mM ethephon (top panel), 0.1 mM methyl jasmonate (MJA), 0.1 mM salicylic acid (SA), and 50 nM cryptogein (bottom panel) at 25 °C. The leaf slices were sampled at the designed time for total RNA isolation. Ethidium bromide staining for the RNA gel was used to show equal loading.

PR-1a genes as marker genes. Expression of the *PR-5d* gene was significantly induced by treatment with ethephon and cryptogein, and was likely co-ordinate with the expression of *OPBP1*. The amount of *PR-1a* mRNA accumulated was high under the SA treatment 24 h post treatment. Meanwhile, the expression of the *PR-1a* gene was induced slightly by treatment of cryptogein and NaCl in the sliced leaves. There was also a weak induction of the *OPBP1* gene in the control of the MES buffer treatment, indicating that the induction may have been due to the wounding caused by slicing the leaves.

To determine whether the elicitor-inducible transcription of *OPBP1* gene required any protein synthesis *de novo*, we analyzed the mRNA accumulation of *OPBP1* gene after treatment with cryptogein in the presence of cycloheximide (CHX), an inhibitor of protein synthesis. As illustrated in Figure 5, the *OPBP1* gene was induced by CHX alone, a characteristic feature often observed in early genes.

Overexpression of OPBP1 induced expression of PR genes

Overexpression of *AP2/EREBP* genes has been shown to induce a number of genes, such as *PR* genes, with or without the GCC box *cis* element in their upstream promoters (Gu *et al*., 2000; Park *et al*., 2001; Lorenzo *et al*., 2003). To investigate the possible biological function of the *OPBP1* gene in plants, we generated transgenic tobacco plants carrying the *35S::OPBP1* construct and control plants transformed with only the CHF3 vector sequence. Seeds from the T1 progeny were selected and placed on a 1/2 MS medium containing Km, and the surviving seedlings transferred to the soil. After PCR confirmation, the transgenic plants were used for additional experiments. As shown in Figure 6, the accumulated levels of *OPBP1* mRNA were higher in the *35S::OPBP1* transgenic lines than in the control plants. Expression of *PR-1a* and *PR-5d* genes was increased in the *35S::OPBP1* plants compared to those in the control under normal growth conditions. Some correlation was found between the levels of mRNA accumulated in the *PR*s and the *OPBP1* transcription in the *35S::OPBP1* transgenic plants of the T2 progeny, suggesting that expression of the *OPBP1* gene might regulate expression of genes related to plant defense responses.

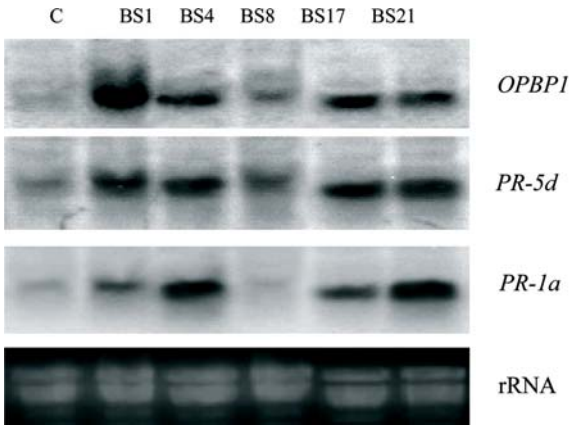

Figure 6. Overexpression of *OPBP1* gene induced expression of *PR* genes in tobacco transgenic plants. Total RNA was isolated from the leaves of transgenic plants of T2 progeny under normal growth conditions. RNA gel blotting was conducted using ^{32}P labeled probes of *OPBP1*, *PR-1a*, and *PR-5d*. BS- indicates individual *35S::OPBP1* plant; C indicates the control plant (vector only). Ethidium bromide staining for the RNA gel was used to show equal loading.

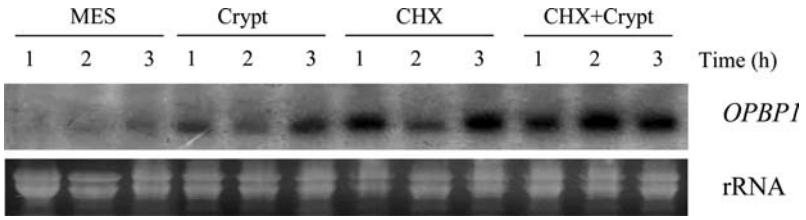

Figure 5. Effect of cycloheximide on expression of *OPBP1* gene. Fully expanded leaves from 6-week-old tobacco plants were detached and sliced in about 1 cm wide for analysis of *OPBP1* gene expression. The leaves were treated with MES buffer (10 mM, pH 5.8, containing 0.01% Triton X-100) or MES buffer containing 20 nM cycloheximde (CHX), then 50 nM cryptogein was added 20 min after the CHX treatment. The leaf slices were sampled at the designed time for total RNA isolation. Ethidium bromide staining for the RNA gel was used to show equal loading.

Overexpression of OPBP1 enhances salt tolerance and disease resistance

Induction of *PR* gene expression has been observed under various stimulus conditions, including pathogen attack and dehydration stress (Xu *et al*., 1994; Gong *et al*., 2001). Up regulation of *PR* gene expression also has been observed in transgenic tobacco plants over expressing the *OPBP1* gene. These data imply that transgenic plants with over expression of *OPBP1* gene might be capable of altering their responses to both biotic and abiotic stresses. To test the idea, we studied salt tolerance of the transgenic plants by examination of salt-induced senescence. Leaf discs from sense expressing *OPBP1* and the control transgenic plants were floated in a 400 mM NaCl solution and their chlorophyll content measured. During treatment, we found that the discs of the control plants bleached faster than that of the sense transgenic plants. The phenotypic differences among the transgenic plants are shown in Figure 7a. Measurement of the chlorophyll content in these plants further supported the observed phenotypic differences in the salt treated discs (Figure 7a).

In addition, we also investigated the effect of *OPBP1* transgenic plants on seed germination and seedling growth was investigated. The T1 seeds of each sense transformant as well as the control transgenic plant were selected on Km plates in the presence or absence of 200 mM NaCl. The seed sets from the *35S::OPBP1* construct germinated in the presence of 200 mM NaCl, although there was discrepancy among individual transgenic lines. However, the seeds of the control plants had extreme difficulty germinating in the presence of the 200 mM NaCl. The phenotypic differences after 3 weeks of treatments are shown in Figure 7b. Seeds from some of the transgenic lines, such as BS11 and BS21 required a longer time to adapt to the salt stress during the germination comparing with those of BS4 and BS 17 (Figure 7b). Once the seeds germinated, however, the seedlings were able to grow under the salt stress condition. The results indicate that the seeds of the *OPBP1* transgenic plants were able to adapt to the salt stress during germination, and also provide evidence for a positive relationship between the expression of *OPBP1* gene and salt tolerance.

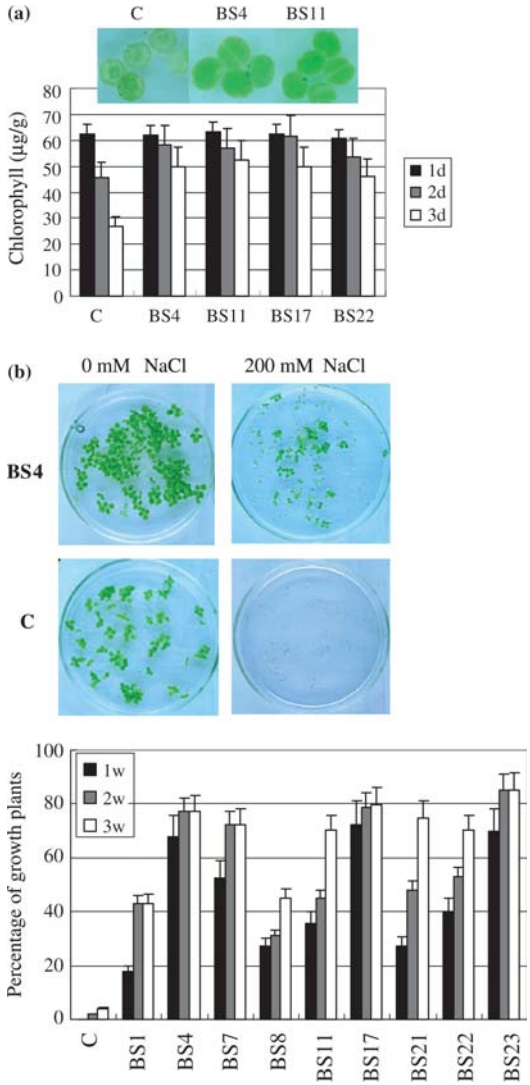

Figure 7. Analysis of salt tolerance in *OPBP1* transgenic plants. (a) Leaf discs from the transgenic plants carrying the sense (BS-) orientation of *OPBP1* gene and vector only (C) were floated on 400 mM NaCl solution in a plastic dish (top panel). The dishes were kept in 16 h light and 8 h dark at 25 °C. Chlorophyll contents (mg/g fresh weight) were measured every 24 h for 3 days (bottom panel). Values are means of three different experiments, and bars are standard errors. The photograph in the inset was taken 3 days after salt treatment. (b) Seeds of *35S::OPBP1* and the control transgenic plants (T1 progeny) were sterilized, and plated onto 1/2MS medium containing 150 mg/l Km with or without 200 mM NaCl. The seedlings germinated and grown were counted every week 2 weeks after sowing (top panel). Ratio of the seedlings germinated in the presence of NaCl to that of in the absence of NaCl was used to evaluate the tolerance to salt stress. Values are means of three different experiments, and bars are standard errors. The photograph in the inset was taken 3 weeks after sowing (bottom panel).

To examine whether the *OPBP1* gene was also involved in disease resistance, the transgenic plants were inoculated with *Pseudomonas syringae* pv *tabaci*, a virulent bacterial pathogen. Bacterial growth around the inoculation sites was determined 7 days after inoculation. As illustrated in Figure 8, the growth of *P. s. tabaci* was inhibited by one to two orders of magnitude in the T2 progeny of the *35S::OPBP1* transgenic lines compared to the control. The exception was line BS8, which was associated with a low expression level of the transferred gene (Figure 6).

Similarly, we studied the disease resistance against *Phytophthora parasitica* var *nicotianae*, a fungal pathogen causing tobacco black shank disease. The disease symptoms developed very fast on the leaves of the control plants. As shown in Figure 9, the *P. p. nicotianae* inoculated half-leaves displayed water soaked wilt within 3 days. On the other hand, most of the transgenic plants expressing the *OPBP1* gene exhibited no symptoms or the development of the disease was significantly delayed (Figure 9 and Table 1). Taken together, the data indicate that over expression of the *OPBP1* gene in tobacco plants enhances the disease resistance and salt tolerance.

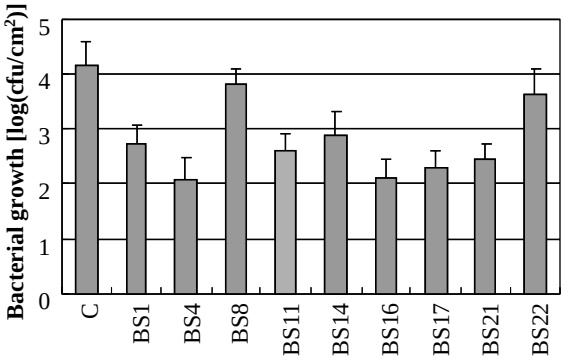

Figure 8. Analysis of resistance to *Pseudomonas syringae tabaci* in *OPBP1* transgenic plants. Bacterial *P. s. tabaci* (10^7 colony forming units/ml) was inoculated into the fourth upper leaves using a 10 ml plastic syringe without a needle. Seven days after the inoculation, the infected leaves were collected and the bacterial populations were determined. C indicates the vector only plant, and BS- indicates independent sense OPBP1 transgenic line. Values are means of three different experiments, and bars are standard errors. The photograph in the inset was taken 7 days post-inoculation.

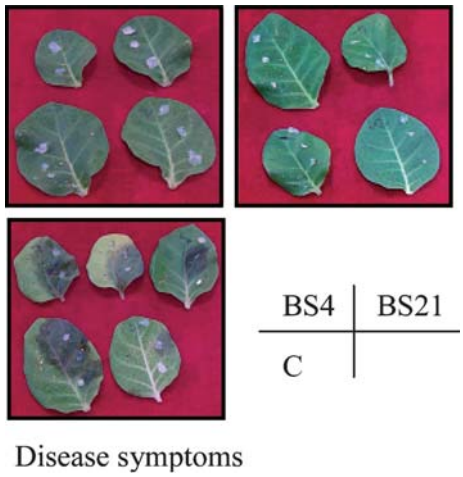

Figure 9. Analysis of resistance to fungal pathogen *Phytophthora parasitica nicotianae* in OPBP1 transgenic plants. A plug of medium containing the fungal mycelia of P. p. nicotiana was inoculated onto the detached health leaves (the fourth leaf from top) from the transgenic plants with the mycelium side contacting the leaf surface of toothpick wounding sites (three sites per leaf). The challenged leaves were kept in a plastic dish with water soaked filter paper at the bottom and set at 28 °C with 16 light and 8 h dark. C indicates the vector only plant,

Table 1. Disease resistance against *Phytophthora parasitica nicotianae* in *OPBP1* transgenic plants of T2 progeny.

Plant	Time post-inoculation (h)		
	36	48	60
C	+ +	+ + + +	+ + + +
BS4			
BS6		+	+ +
BS7		+ +	+ + +
BS8		+ +	+ + + +
BS11		+ +	+ +
BS14			
BS15			
BS16	+	+	+ +
BS17			+ +
BS20			
BS21			
BS23			

The fourth leaves from the top of *35S::OPBP1* and the control transgenic plants were inoculated with mycelium-agar plugs of *Phytophthora parasitica nicotianae*. Each leaf was inoculated at three sites, and four or five leaves for each individual line. The disease rating of a transgenic plant was scored as an average of the inoculated leaves of the line, following as + for the disease lesion smaller than 0.5 cm in size, + + for 0.5–1.0 cm, + + + for 1.0–1.5 cm, + + + + for larger than 1.5 cm. If there was no disease symptom, no "+" symbol was used. The experiments were repeated three times.

Discussion

OPBP1 genes belong to the AP2/EREBP family of plant transcription factors (Xu *et al*., 1998). A variety of genes belonging to this family have been cloned and shown to play significant roles in the plant life cycle. APETALA2 of *Arabidopsis*, the first protein identified to contain the AP2/EREBP domain, functions in plant development (Jofuku *et al*., 1994). Sequence comparison and phylogenetic studies have shown that the OPBP1 protein is homologous to *Arabidopsis* ERF1, which has been studied in detail.

The EREBPs, Ptis, and AtEBP have been shown to bind specifically to the GCC box sequence, which is present in the promoter region of a large number of ethylene-inducible genes encoding pathogenesis-related proteins (Ohme-Takagi and Shinshi, 1995). Gel retardation experiments have shown the OPBP1 recombinant protein bound specifically to the GCC box *cis* element. This result implies that OPBP1 might induce the expression of the GCC box containing genes in their promoters. Over expression of the *OPBP1* gene in tobacco plants exhibited some constitutive expression of the *PR-1a* and *PR-5d* genes, indicating that the OPBP1 protein also plays a regulatory role *in vivo*. The dehydration stress-related EREBPs, such as DREB1A, DREB2A, and CBF1 proteins, bind specifically to the DRE/CRT box with the core sequence of C/GCCGAC, which resembles the GCC box. However, the binding activity of OPBP1 to the DRE/CTR box was not observed in this study. Tsi1, a tobacco AP2/EREBP protein has been demonstrated to bind specifically to both the GCC box and the DRE/CTR box, although the binding activity of Tsi1 to the DRE/CTR box is weaker than that to the GCC box (Park *et al*., 2001). In the case of OPBP1, more experiments are needed to clarify the differences in OPBP1 binding to the two *cis* elements.

Expression of the *OPBP1* gene was strongly induced by cryptogein, MJA, ethephon, and NaCl, but not significantly affected by treatment with SA. SA and ethylene/JA are typically considered to regulate two different signal transduction pathways in plant disease resistance, impling that OPBP1 is possibly involved in the ethylene/JA-dependent signaling pathway. Cryptogein, an elicitor secreted from *P. cryptogea*, acts as a trigger to activate a number of tobacco defense responses (Lecourieux-Ouaked *et al*., 2000; Lebrun-Garcia *et al*., 2002). The induction of the *OPBP1* gene by cryptogein indicates that AP2/EREBP transcription factors are important components in the regulatory events of the elicitor signal transduction pathways. This is also supported by the observation of constitutive induction of the *OPBP1* gene in transgenic plants with overexpressed *cryptogein* genes in tobacco plants (Jiang *et al*., 2002). Furthermore, expression of the *OPBP1* gene is also likely regulated by a component of the salt-stress related signaling pathway, since the induction of the *OPBP1* gene expression was observed early on after NaCl treatment. The induction of *OPBP1* expression by both biotic and abiotic stresses reveals that both biotic and abiotic signal pathways may interact to regulate expression of genes for plant adaptation to stimuli.

Activation of OPBP1 transcription by CHX treatment alone is similar to that of *PS-IAA4/5* and *PS-IAA6* (Koshiba *et al*., 1995), the wound inducible *ERFs* (Suziki *et al*., 1998), and *WRKY*s (Cormack *et al*., 2002). The induction mode is explained as the prevention of synthesis activation of a short-lived transcriptional repressor (Koshiba *et al*., 1995), or the influence of mRNA stability (Hill and Treisman, 1995). Either way, the stimulus-dependent activation of immediate early genes appears not to require *de novo* protein synthesis. The cryptogein-induced activation of *OPBP1* expression must therefore be mediated by preformed transcription factors acting positively or negatively on the gene.

Transgenic plants with *OPBP1* gene overexpression showed a high tolerance to salt stress. At the same time, the *35S::OPBP1* plants exhibited enhanced resistance against both bacterial (*P. s. tabaci*) and fungal (*P. p. nicotianae*) pathogen attacks. The enhanced tolerance to salt and resistance against pathogen attack may be due to the increased expression of stress-inducible genes by over expression of the *OPBP1* gene in the sense transgenic plants. Several reports have demonstrated that overexpression of *EREBP* genes enhances the transgenic plants ability to withstand environmental stress. Ectopic expression of *CBF1/DREB1* genes activated cold responsive genes and enhanced the freezing as well as the osmotic tolerance of non-acclimated *Arabidopsis* plants. *DREB2*, on the other hand, was found to be

involved in drought-responsive gene expression (Kirstern *et al*., 1998; Liu *et al*., 1998; Kasuga *et al*., 1999). The similarity of the amino acid sequences between OPBP1 and Tsi1 was quite low, suggesting the complexity of the regulatory system in tobacco plants in response to environment stimuli. Conflicts may exist between the biotic and abiotic signal transduction pathways.

Expression of *PR* genes is induced by a variety of stimuli, including pathogen infection, SA, ethylene, JA, ABA, and other chemical treatments. However, there is not a GCC box in the promoter region of *PR-1a* gene. Nevertheless, the constitutive induction of *PR1* expression also has been observed in tobacco *Tsi1*, *Arabidopsis ERF1*, and tomato *Pti*4 transgenic lines (Gu *et al*., 2000; Park *et al*., 2001; Lorenzo *et al*., 2003). These results suggest that induction of *PR-1a* mRNA accumulation in *35S::OPBP1* transgenic plants was probably not by direct influence of the OPBP1 on the *PR-1a* promoter. ERF1 is the closest gene to OPBP1 in *Arabidopsis*, with a 51.4% amino acid identity and similar serine-rich and acidic domains in the N- terminals of their proteins. This implies that OPBP1 may also play significant roles in the defense responses in tobacco plants. Whether or not OPBP1 is a switcher of biotic and abiotic stresses is yet to be understood.

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