Ethylene-Induced Gene Expression of Osmotin-Like Protein, a Neutral Isoform of Tobacco PR-5, is Mediated by the AGCCGCC cis-Sequence

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Osmotin-like protein (OLP) is a neutral isoform in the group 5 pathogenesis-related (PR) tobacco proteins. The OLP gene, like the basic PR protein genes, is constitutively expressed in tobacco roots and cultured cells. OLP is not naturally present in intact healthy leaves, but ethylene treatment induces a high accumulation there. To study the mechanism of OLP gene expression as induced by ethylene, we cloned the gene from *Nicotiana sylvestris*, an ancestor of *N. tabacum*. Sequence analysis showed that it has no intron and that its promoter region contains two AGCCGCC sequences that are conserved in most basic PR-protein genes. The function of the AGCCGCC sequences in transgenic tobacco plants that harbor the wild and mutated OLP promoter::GUS fusion genes was analyzed. Mutation in the AGCCGCC sequences clearly inhibited the GUS expression induced by ethylene, indicative that the AGCCGCC sequence(s) is a DNA element(s) responsive to ethylene. An EREBP2 protein, isolated as one of the proteins binding to the AGCCGCC sequence of the tobacco β-1,3-glucanase gene, also was found to bind to the AGCCGCC sequence(s) of OLP gene. These results suggest that the ethylene-induced expression of OLP is regulated by a trans-acting factor(s) common to basic PR-proteins.

Key words: Ethylene — Neutral PR-5 — Osmotin-like protein — Tobacco (*Nicotiana sylvestris*) — Transgenic tobacco

The tobacco osmotin-like protein (OLP) is a protein that accumulates markedly in cultured tobacco cells (Takeda et al. 1990). It is a third subclass of pathogenesis-related type 5 (PR-5) proteins; a neutral isoform (called “PR-5d”) by van Loon et al. 1994). The deduced amino acid sequence of the isolated cDNA of tobacco OLP shares 76% identity with tobacco osmotin (a basic isoform of PR-5, Takeda et al. 1991). PR-5 proteins recently have been shown to have antifungal activities against a variety of fungi (Roberts et al. 1990, Vigers et al. 1990, Heijgaard et al. 1991, Woloshuk et al. 1991, Liu et al. 1994, Koiwa et al. in preparation).

Osmotin first was isolated as a protein that accumulates markedly in salt-adapted tobacco cells (Singh et al. 1985, 1987). Recent studies of the regulatory mechanism of the gene expression of tobacco osmotin and potato osmotin-like proteins have shown that their expressions are controlled both by fungal infection and related signals including those of ethylene and various developmental and environmental stimuli, such as salt stress (Kononowicz et al. 1992, Zhu et al. 1995). IEF-immunoblotting analysis, however, clearly showed that tobacco OLP is the major PR-5 protein present in roots and cultured cells of tobacco (Koiwa et al. 1994). PR-5 proteins are not found in any other parts of healthy intact tobacco plants, but ethylene induces markedly accumulation of OLP as well as osmotin in leaves (Koiwa et al. 1994).

To clarify the mechanism of tobacco OLP gene expression, we have cloned and characterized the OLP gene of *Nicotiana sylvestris*, an ancestral species of *N. tabacum*. We also report the regulation of ethylene-responsive expression of OLP gene by AGCCGCC sequence(s), which are conserved in many basic PR protein genes (Hari et al. 1993), and the interaction of AGCCGCC sequence(s) of OLP gene with EREBPs, which were isolated as a DNA-binding protein that recognizes the sequences of β-1,3-glucanase gene containing AGCCGCC (Ohme-Takagi and Shinshi 1995).

Materials and Methods

Plant material—Shoot cultures of the wild type and transgenic tobacco (*N. tabacum* cv. Samsun NN) were maintained in AGRIPOTs (Kirin, Tokyo, Japan) on a half concentration of Linsmaier-Skoog media (Linsmaier and Skoog 1965) containing 1.5% sucrose and 0.8% agar under continuous illumination (3,000 lux) at 25±2°C. Healthy leaves were used for *Agrobacterium*-mediated transformation and genomic DNA extraction.

DNA isolation and southern blot analysis of the OLP gene—DNA was manipulated by the procedures described by Sambrook et al. (1989) and Ausubel et al. (1987) unless otherwise indicated. The genomic DNAs of *N. sylvestris* and *N. tabacum* (cv. Samsun NN) were isolated from healthy green leaves by the CTAB method (Rogers and Bendich 1985). Ten or twenty micrograms of these DNAs was digested with *HindIII* or *XbaI* then separated on a 0.7% agarose gel by electrophoresis. The separated DNAs were transferred to a GeneScreen plus nylon membrane filter (DuPont,
DE, U.S.A.) by capillary transfer then fixed by UV crosslinking. The filter was incubated at 55°C in a solution containing 6 x SSC (1 x SSC; 0.15 M NaCl and 15 mM sodium citrate, pH 7.0); 5 x Denhardt solution (1 x Denhardt solution; 0.02% polyvinylpyrrolidone, 0.02% ficoll 400 and 0.02% bovine serum albumin); 0.5% SDS; 0.1 mg/ml salmon sperm DNA and 10% dextran sulfate, then re-incubated in the same solution supplemented with the 32P-labeled probe. The probe DNAs were prepared by PCR amplification with M13 forward and reverse primers from pTOL1 that carried the OLP cDNA, and were labeled with 32P using a random primed-DNA-labeling kit (Boehringer Mannheim, Germany). Lastly the filter was washed with 1 x SSC and 0.1% SDS at 65°C then autoradiographed.

Cloning and sequencing of OLP gene from N. sylvestris—To prepare the genomic library, the total genomic DNAs of N. sylvestris were partially digested with MboI and introduced into the BamHI site of the ßEMBL3 vector. A total of 1 x 106 clones were screened by plaque hybridization with the 32P-labeled OLP cDNA of tobacco as the probe. The filters were incubated and washed under the conditions described for southern analysis, except that the washing was done at 60°C. Positive clones were isolated, and the insert DNAs subcloned into pUC or pbuScript II (Stratagene, CA, U.S.A.) plasmids. Nucleotide sequences were determined by the dideoxy chain termination method on a single- or double-stranded DNA template using a BoaBEST dideoxy sequencing kit (Takara Shuzo Co., Kyoto, Japan) or an AutoRead sequencing kit (Pharmacia, Uppsala, Sweden) and an A.L.F. DNA sequencer (Pharmacia). DNA sequences were determined in both orientations. Sequence alignment analysis was done with the Gene Works program (IntelliGenetics, Inc., Mountain View, CA, U.S.A.).

Construction of the OLP promoter::GUS fusion genes—A BamHI site was introduced +23 bp downstream of the putative transcription start site of the OLP gene by PCR-mediated site-directed mutagenesis. The EcoRI site of pBI221 (Clontech, CA, U.S.A.) was changed to the KpnI site by linker ligation (pBI221K). The 5' flanking region (about -2.1 kb) upstream of the promoter sequence at -46 to -52 in pOL1099 was mutated to ACTCGAG by linker ligation. The remaining AGCCGCC sequences of the OLP gene, was amplified by PCR using mutagenic sequences of the OLP gene, was amplified by PCR using mutagenic primers that generated the HindIII-BamHI site upstream of ß-glucuronidase (GUS) gene of pBI221K. This generated pOL2.1, which was digested with EcoRI to remove the region from -1,099 bp to -2.1 kb then circularized to generate the construct bearing the +23 to -1,099 promoter sequence (pOL1099). The DNA fragment coding +23 to -1,099 was removed from pOL1099 by HindIII and BamHI digestion then inserted into the HindIII-BamHI site of the binary vector pBI101 (Clontech) to generate pBOL1099. The AGCCGCC sequence at -161 to -167 in pOL1099 was mutated to ACTCGAG by PCR-mediated disruption. The BamHI-AatII DNA fragment of the PCR product then was substituted for the BamHI-AatII fragment of pBOL1099 in order to generate the binary plasmid pBOL1099m5 which had only disrupted AGCCGCC sequences. All the fragments amplified by PCR were sequenced, the possibility of the misincorporation of nucleotide during PCR being eliminated.

Plant transformation—The binary plasmid was introduced to Agrobacterium tumefaciens LBA4404 by electroporation as described for the transformation of E. coli (Ausubel et al. 1987), except that culture was at 25°C. Tobacco leaf disk was infected as described by Horsh et al. (1985).

Ethylene treatment—Ethylene at the concentration of 80 µl liter−1 was injected to the AGRIPOTs containing the tobacco plantlets, and the containers sealed for 48 h. The plantlets in the sealed AGRIPOTs that had not been injected with ethylene were used as the negative controls.

Fluorometric GUS assay—A fluorometric GUS assay with 4-methylumbelliferonyl-ß-D-glucuronide (MUG; Sigma, St. Louis, MO, U.S.A.) as the substrate was done by the method of Jefferson et al. (1987). Leaves were homogenized in GUS assay buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na3EDTA, 0.1% [w/v] sodium laurylsarcosine, 0.2% [w/v] Triton X-100) then centrifuged at 4°C and 10,000 rpm for 5 min. The supernatants were frozen in liquid nitrogen and stored at -70°C. The reaction took place at 37°C for 60 min in 150 µl of GUS assay buffer containing 0.9 mM MUG and was stopped by transferring 40 µl portions of the reaction solution to 960 µl of 0.2 M Na2CO3. The amount of 4-methylumbelliferone (4-MU), the product of GUS reaction, was determined with a fluoroscence spectrophotometer (excitation at 365 nm, emission at 455 nm; model F-2000, Hitachi, Japan). The protein concentration was determined by the procedure of Bradford (1976) with protein assay reagent (BioRad, CA, U.S.A.).

Preparation of recombinant EREBP—Tobacco EREBP2 cDNA was inserted in the expression vector pGEX4T3 (Pharmacia) in the frame of the C-terminal of the glutathione S-transferase (GST) gene, and the gene introduced to E. coli BL21 (Omote-Takagi and Shinshi 1995). Production of EREBP2 was induced by the addition of isopropyl ß-D-thiogalactoside (final concentration 0.1 mM) to the liquid culture of E. coli. After being cultured for one hour, the cells were harvested and resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 and 1.4 mM KH2PO4, pH 7.3) then lysed by sonication. Protein was made soluble by the addition of Triton X-100 (final concentration 1%). Insoluble materials were removed by centrifugation. Extracts were mixed with glycerol (final concentration 10%) and stored at -70°C. Protein concentrations were determined as described above.

Southwestern analysis—Twenty micrograms of soluble protein was suspended in SDS loading buffer (final 50 mM Tris-HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS, 0.1% bromphenol blue and 10% glycerol) then loaded on an SDS-polyacrylamide gel (8% polyacrylamide). After electrophoresis, the proteins in the transfer buffer (25 mM Tris, 0.2 M glycine and 10% methanol) were transferred to nitrocellulose filters by electroblotting, denatured, then reacted on the filters using guanidine-HCl according to Ausubel et al. (1987). The filters were blocked with BLOTTO (5% skim milk, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) then incubated at room temperature for 1 h in binding buffer (40 mM MCl, 25 mM HEPES-KOH, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol and 10% glycerol) supplemented with 0.25% skim milk, 20 ng ml−1 of poly(dA-dT) (da-dT) (Pharmacia), 50 mg ml−1 of heat-denatured salmon sperm DNA, and 8 ng ml−1 of the 32P-labeled probe DNA. They were then washed twice for 10 min in binding buffer supplemented with 0.25% of skim milk and autoradiographed. Probe DNAs were prepared by PCR with pBOL1099 or pBOL1099m5 plasmid as the template. The promoter region between -18 and -195, which contains both the two wild type or mutated AGCCGCC sequences of the OLP gene, was amplified by PCR using mutagenic primers that generated the XbaI site at the downstream end and the MboI site at the upstream end of the PCR product. PCR products were digested with XbaI and labeled with 32P using Klenow DNA polymerase.
Results

Southern analysis of the OLP gene—Southern blot analysis of the genomic DNAs isolated from common tobacco (N. tabacum) and N. sylvestris, an ancestral species of N. tabacum, showed that the OLP cDNA of tobacco, which contains neither of HindIII nor Xbal sites, hybridized intensely to one band (N. sylvestris) or two bands (N. tabacum) of the HindIII-digested DNAs. The OLP cDNA also bound to two bands of the Xbal-digested DNAs of N. sylvestris and tobacco (Fig. 1). Furthermore, independent clones isolated from the N. sylvestris genomic library were shown to be identical (see below). These findings suggest that a gene highly homologous to tobacco OLP cDNA is present as a single-copy gene in the N. sylvestris genome and as two-copy genes in the tobacco genome. The less intense bands seen in each lane may be due to small differences between the two DNA sequences.

![Fig. 1 Southern blot hybridization of genomic DNAs isolated from Nicotiana sylvestris and N. tabacum.](image)

Genomic DNAs of N. sylvestris (lanes 1 and 3) and N. tabacum (lanes 2 and 4) were digested with HindIII (lanes 1 and 2) or Xbal (lanes 3 and 4), separated in an agarose gel then transferred to a nylon membrane. The membrane was incubated at 60°C with the 32P-labeled OLP cDNA of tobacco, then washed at 65°C in 1 x SSC/0.1% SDS. The OLP cDNA hybridized intensely to one band of the A'ftal-digested genomic DNAs of N. sylvestris and tobacco (Fig. 1). Furthermore, cDNA also bound to two bands of the Xbal-digested genomic DNAs. The OLP cDNA is present as a single-copy gene in the N. sylvestris genome and as two-copy genes in the tobacco genome. The less intense bands seen in each lane may be due to small differences between the two DNA sequences.

Fig. 2 Nucleotide and deduced amino acid sequences of the Nicotiana sylvestris OLP gene. The purine-pyrimidine repeat and AGCCGCC motif are shaded. Tandem repeats are shown by arrows. The unique A/T-rich region not present at the corresponding site in the osmotin gene is underlined. The putative TATA box and transcription start sites are double underlined. The putative processing site of the precursor protein is marked by an arrowhead. DDBJ, EMBL and GenBank DNA databases accession number is D76437.
rived from some other PR-5 gene, e.g., osmotin.

Nucleotide and deduced amino acid sequences of the N. sylvestris OLP gene—Four independent λ clones that hybridize to tobacco OLP cDNA were isolated from the N. sylvestris genomic library of 1 × 10⁶ clones. Restriction enzyme mapping of these clones showed them to be identical, therefore a single clone was used for further subcloning. Sequencing of the insert subcloned indicated that the gene has an open reading frame composed of 251 amino acids and no intron (Fig. 2).

Because the deduced amino acid sequence of the gene obtained was highly homologous to that of tobacco OLP cDNA (98% identity in the amino acid sequence), we concluded that the isolated gene was the OLP of N. sylvestris. The putative glycosylation site and PEST (proline-glutamate-serine-threonine-rich) sequence of the tobacco OLP also were present in the N. sylvestris OLP. The untranslated 5'-GTCCACTAAA-3' region of the tobacco OLP cDNA was conserved in the N. sylvestris OLP gene, whereas the sequence similarity for the 3' untranslated region of the tobacco OLP cDNA and that of the N. sylvestris OLP gene was low.

Sequence alignment of the N. sylvestris OLP and tobacco osmotin (Nelson et al. 1992) genes disclosed that the nucleotide sequence from the translation start site to about 0.25 kb upstream of the OLP gene is highly homologous to that of the osmotin gene, except for the A/T-rich sequence, 5'-ATATAAATAATATA-3' which is found only in the OLP gene at −114 to −99 from the putative transcription start site (Fig. 3, see below). In the osmotin gene, the transcription start sites were determined to be 34 and 38 bp upstream of the translation start site by primer extension and S1 nuclease protection analyses. On the basis of the analogy of sequence, the adenine of the 34 bp upstream from the translation start site in the OLP gene was numbered −1. The putative TATA and CAAT boxes respectively are at −31 to −38 and −87 to −90.

AGCCGCC sequences are highly conserved in the promoters of basic PR genes with ethylene-inducible expressions (Hart et al. 1993) and are thought to constitute an ethylene-responsive cis element. AGCCGCC sequences were found at −46 to −52 and −161 to −167. There was no repeated sequence (−968 to −903) composed of purine-pyrimidine dinucleotide and the tandem repeats 5'-CATCTCAAATTCGA-3' (−785 to −771 and −747 to −733) in the osmotin gene.

Induction of GUS activity by ethylene treatment of transgenic tobacco that has the wild type and mutated OLP promoter::GUS fusion genes—GUS fusion genes bearing the wild (pBOL1099) or AGCCGCC-mutated (pBOL1099m5) promoter of the OLP gene (1,099 bp) were introduced to tobacco by Agrobacterium-mediated transformation (Fig. 4). Three independent transgenic tobacco lines were analyzed for each construct.

GUS activity in normal healthy leaves was low when leaves were not treated with ethylene. When transgenic plants were treated with 80 μl liter⁻¹ ethylene for 48 h, transgenic tobacco bearing the wild type OLP promoter (pBOL1099) showed a several fold increase in GUS activity (Fig. 5, left panel); whereas, transgenic tobacco with the

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Fig. 3 Nucleotide sequence comparison of the 5' regions of the N. sylvestris OLP and tobacco osmotin genes. Homologous regions were aligned using the Gene Works program. The AGCCGCC motif in the OLP gene and corresponding sites in the osmotin gene are shaded. The transcription start sites of the osmotin gene are double underlined.
mutated AGCCGCC promoter (pBOL1099m5) had very low GUS activity in both untreated and ethylene-treated leaves (Fig. 5, right panel). This clearly indicates that complete AGCCGCC sequences are necessary for ethylene-induced expression of the OLP gene.

Binding of EREBP to AGCCGCC sequences of the OLP promoter—EREBPs which are DNA-binding proteins that recognize the ethylene-responsive AGCCGCC cis-element of the tobacco β-1,3-glucanase gene now have been cloned (Ohme-Takagi and Shinshi 1995). Because complete AGCCGCC sequences are necessary for full in-
duction of the OLP gene by ethylene, it is postulated that these EREBP2s interact with AGCCGCC sequences of the OLP gene thereby regulating the expression of this gene in tobacco. To investigate this possibility, we used the southwester assay to measure the binding activity of EREBP2, a cloned EREBP, with AGCCGCC sequences of the OLP gene. EREBP2 was fused to the C-terminal of glutathione-S-transferase (GST) then expressed in E. coli. The GST::EREBP2 fusion protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated in the presence of 32P-labeled DNA fragments of the OLP gene bearing the wild or mutated AGCCGCC sequences (Fig. 6A). The DNA fragment with the wild AGCCGCC-bound specifically to the GST::EREBP2 protein but there was no binding to the control GST protein (Fig. 6B, lanes 1 and 2). The binding of the DNA fragment bearing mutated AGCCGCC sequences to GST::EREBP2 was very low (Fig. 6B, lane 3). These results indicate that EREBP2 binds specifically both to the AGCCGCC sequence(s) of the β-1,3-glucanase gene and that(those) of the OLP gene.

**Discussion**

OLP, a neutral isoform of tobacco PR-5, accumulates constitutively in tobacco roots and cultured cells, and marked accumulation in tobacco leaves is induced by ethylene treatment (Koiva et al. 1994). The accumulation pattern is very similar to that of the basic PR proteins seen in roots and cultured tobacco cells, and that produced by ethylene in the leaves. In contrast, acidic tobacco PR protein in the leaves is induced by salicylic acid rather than by ethylene. To study the mechanism of OLP gene expression, we cloned the OLP gene of *N. sylvestris*.

Southern blotting analysis and characterization of independent genomic clones isolated suggested that *N. sylvestris* has one copy and tobacco two copies of the OLP gene. This indicates that one of tobacco OLP genes is derived from *N. sylvestris* and the other from *N. tomentosiformis*, both ancestors of tobacco. Acidic (PR-S, van Kan et al. 1989) and basic (osmotin, Singh et al. 1989) PR-5 genes also are encoded by the two copies of each gene in tobacco.

The deduced amino acid sequence of the OLP gene of *N. sylvestris* shows high identity (98%) with that of tobacco OLP cDNA (Takeda et al. 1991). The low identity of the genes also are encoded by the two copies of each gene in *N. sylvestris* dependent genomic clones isolated suggested that marked accumulation in tobacco leaves is induced by ethylene. A recent study showed that a small fragment bearing two AGCCGCC sequences of tobacco basic β-1,3-glucanase fused to the minimal CaMV 35S promoter provided ethylene-responsivity in transgenic tobacco and that disruption of the AGCCGCC sequences dramatically reduced this responsivity (Ohme-Takagi and Shinshi 1995). Our transgenic tobacco that bears the wild and mutated OLP promoter::GUS fusion provides further evidence that the AGCCGCC sequences are crucial for the ethylene-response in the OLP gene.

EREBPs (EREBP1-4) are DNA-binding proteins which recognize DNA bearing the AGCCGCC sequences of the tobacco β-1,3-glucanase gene. There is no homology between the flanking region of AGCCGCC sequences of OLP and β-1,3-glucanase genes. Southwestern analysis with the GST::EREBP2 fusion protein provided additional evidence that the AGCCGCC sequences of the OLP gene interact with an EREBP (EREBP2). This result suggests that flanking sequence of AGCCGCC are not necessary for binding of EREBP2 and EREBP3s are involved in the general regulation of gene expression in many basic PR proteins through interaction with the AGCCGCC sequences.

Many of the basic PR protein genes have at least two AGCCGCC sequences in the promoter. Although it is not clear whether two AGCCGCC sequences are necessary for ethylene induction, a mutated promoter of the OLP gene bearing only the intact upstream AGCCGCC sequence (POL1099m1, see materials and methods) has considerably reduced ethylene-responsivity in tobacco leaves transiently transformed by microprojectile bombardment (data not shown).

Expression of the osmotin gene is similar to that of the OLP gene. The osmotin gene also has several AGCCGCC sequences; a complete AGCCGCC (from −50 to −44), a slightly modified CGCCGCC (from −144 to −138), and an AGCCGCC sequence in reverse orientation (from −162 to −156). The OLP gene, however, has the tandem repeat sequence 5'-CATCTC(AATAATC)−771 and −747 to −733) and purine-pyrimidine dinucleotide repeat (TA)2(TG)2(TA(TG)2−3' (from −968 to −903) which do not exist in the osmotin gene. The variations in AGCCGCC sequences, A/T-rich sequences and far upstream region, where there is no homology, may account for the differences in the expression of the osmotin and
OLP genes, e.g., lower expression of osmotin gene in root.

The function of these sequences in the OLP gene is not clear, but A/T-rich DNA sequences are reported that used microprojectile bombardment and a promoter region of the promoter or the hot spot for recombination (Rich et al. 1984, An 1987). A transient expression assay that used microprojectile bombardment and a promoter::GUS fusion gene lacking the purine-pyrimidine dinucleotide repeat sequence had no effect on GUS expression in cultured N. sylvestris cells (data not shown), indicative that this purine-pyrimidine dinucleotide repeat sequence is not a general enhancer or silencer in the OLP gene.

We have shown that AGCCGCC sequence(s) constitute the ethylene-responsive cis-element in the gene expression of OLP. As shown, OLP accumulates not only in ethylene-treated tobacco leaves, but in roots and cultured cells as well. Further analysis has indicated that the AGCCGCC sequence(s) of the OLP gene also is involved in the developmental regulation of the gene’s expression (Kita-jima et al. unpublished data). Because EREBP3s are encoded by four genes, and one gene (EREBP3) is highly expressed in cultured tobacco cells and roots (Ohme-Takagi and Shinshi 1995), the tissue-specific expressions of OLP and many of the basic PR protein genes may be regulated by EREBP as well as in ethylene-induced expression.

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