

A Nitrilase-Like Protein Interacts with GCC Box DNA-Binding Proteins Involved in Ethylene and Defense Responses¹

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Ethylene-responsive element-binding proteins (EREBPs) of tobacco (*Nicotiana tabacum* L.) bind to the GCC box of many pathogenesis-related (PR) gene promoters, including osmotin (PR-5). The two GCC boxes on the osmotin promoter are known to be required, but not sufficient, for maximal ethylene responsiveness. EREBPs participate in the signal transduction pathway leading from exogenous ethylene application and pathogen infection to PR gene induction. In this study EREBP3 was used as bait in a yeast two-hybrid interaction trap with a tobacco cDNA library as prey to isolate signal transduction pathway intermediates that interact with EREBPs. One of the strongest interactors was found to encode a nitrilase-like protein (NLP). Nitrilase is an enzyme involved in auxin biosynthesis. NLP interacted with other EREBP family members, namely tobacco EREBP2 and tomato (*Lycopersicon esculentum* L.) Pti4/5/6. The EREBP2-EREBP3 interaction with NLP required part of the DNA-binding domain. The specificity of interaction was further confirmed by protein-binding studies in solution. We propose that the EREBP-NLP interaction serves to regulate PR gene expression by sequestration of EREBPs in the cytoplasm.

The plant hormone ethylene influences several aspects of plant growth and development and is also associated with response to stresses such as drought, wounding, and pathogen infection (Abeles et al., 1992). Invasion of plants by pathogens or application of pathogen-derived elicitors results in rapid evolution of ethylene (Yang and Hoffman, 1984) and is followed by the increased expression of genes encoding PR proteins such as PR-1, β -1,3-glucanase, chitinase, and osmotin (PR-5) (Liu et al., 1994; Yang et al., 1997). These PR genes are also induced by exogenous application of ethylene (Ward et al., 1991; Raghothama et al., 1997;

Yang et al., 1997; Yun et al., 1997). Ethylene, therefore, is considered to be a component of the signal transduction pathway leading from pathogen infection to defense responses. However, ethylene-insensitive mutants of Arabidopsis are not compromised for certain disease-resistance responses (Bent et al., 1992; Lawton et al., 1994), indicating that signal transduction pathways from exogenous ethylene and pathogen infection share overlapping components but are not identical.

A consensus 11-bp promoter sequence (TAAGAGC-CGCC), known as the GCC box, is required for the ethylene responsiveness of several basic PR protein genes (Ohme-Takagi and Shinshi, 1995; Raghothama et al., 1997; Yang et al., 1997). The GCC box, which has a role in signal transduction from exogenous ethylene to the induction of basic PR genes, is distinct from ethylene-responsive elements involved in ripening or senescence (Raghothama et al., 1991; Coupe and Deikman, 1997).

DNA-binding proteins specific for the GCC box were originally identified in tobacco (*Nicotiana tabacum* L.) (Ohme-Takagi and Shinshi, 1995). The four EREBPs have a single, conserved, basic 59-amino acid DNA-binding domain. Proteins containing this DNA-binding domain include, in order of similarity, the tomato (*Lycopersicon esculentum*) proteins Pti4/5/6 (Zhou et al., 1997), and Arabidopsis AtEBP (Büttner and Singh, 1997), TINY (Wilson et al., 1996), AP2 (Jofuku et al., 1994), ANT (Elliott et al., 1996; Klucher et al., 1996), and CBF1 (Stockinger et al., 1997). This DNA-binding domain is also found in other proteins or expressed sequence tags encoding proteins of unknown function. The genes *TINY*, *AP2*, and *ANT* are involved in plant and flower development and were isolated by mutant analysis. They are assumed to encode transcription factors, but their target genes and binding sequences are unknown (Jofuku et al., 1994; Weigel, 1995; Elliott et al., 1996; Klucher et al., 1996). *CBF1* encodes a dehydration and low-temperature-responsive promoter-element-binding protein. *AtEBP* encodes an ethylene-responsive EREBP-like DNA-binding protein that can bind

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Abbreviations: EREBP, ethylene-responsive element-binding protein; GST, glutathione S-transferase; IAN, indole-3-acetonitrile; NLP, nitrilase-like protein; PR, pathogenesis-related.

to the GCC box on the PR-1b promoter (Büttner and Singh, 1997). It was cloned in a screen for proteins interacting with a basic Leu-zipper protein. The EREBP-like DNA-binding domain has also been identified in tomato proteins Pti4/5/6, which are also GCC-box specific and participate in the signaling pathway leading from pathogen infection to disease resistance. *Pti4/5/6* were isolated using a yeast two-hybrid interaction trap with the tomato resistance gene *Pto* as bait (Zhou et al., 1997). *Pto* encodes a protein kinase and confers resistance to *Pseudomonas syringae* strains carrying the *avrPto* gene. It is becoming apparent, therefore, that EREBPs participate in signal transduction via a variety of protein-protein interactions.

The osmotin gene (PR-5) is induced by fungal infection (Liu et al., 1994), as well as by numerous developmental, hormonal, and environmental signals (Kononowicz et al., 1994). A 140-bp minimal promoter fragment that was responsive to the same signals as the full-length osmotin promoter was found to contain two GCC boxes that were required for ethylene responsiveness but were not adequate for maximal ethylene response (Raghothama et al., 1997). Superinduction of the osmotin gene by combinations of inducers has been reported (Xu et al., 1994; Chang et al., 1997). Therefore, to further investigate the signal transduction pathways that converge on these GCC boxes, we first determined that EREBPs bind to the osmotin promoter and then determined their interaction partners using a yeast two-hybrid interaction trap. One of the strongest interaction partners was found to encode NLP. Nitrilase is an enzyme involved in auxin biosynthesis.

MATERIALS AND METHODS

Plant Materials

Tobacco (*Nicotiana tabacum* cv Wisconsin 38) suspension-cultured cells adapted to growth on 428 mM NaCl (S25) were maintained as described (Binzel et al., 1985). S25 cells were harvested 15 d after subculture.

Plasmids

An *EcoRI*-tagged primer (5'-CCGAATTCTATCAACCA-ATTTCGAC-3') and a *SalI*-tagged primer (5'-AAGTCGACTTAAGTACTGACTAATAGCTG-3') were used to amplify EREBP2 (Ohme-Takagi and Shinshi, 1995; accession no. D38126) by reverse-transcription PCR from total RNA isolated from S25 cells (Sambrook et al., 1989). EREBP3 (accession no. D38124) was amplified in a similar manner using the *EcoRI*- and *SalI*-tagged primer pair 5'-CCGAATTC-GCTGTCAAAAATAAGG-3' and 5'-AAGTCGACTCAA-AATTCCATAGGTG-3'. The PCR products were cloned into the *EcoRV* site in pBluescript SK(-) (Stratagene) and the fidelity of amplification was checked by DNA sequencing (Sequenase version II, United States Biochemical). The inserts were then released by digestion with *EcoRI* and *SalI* and inserted into the corresponding polylinker sites in the yeast two-hybrid bait plasmid pBD-GAL4 (HybriZAP two-hybrid vector kit, Stratagene) to yield pBD-EBP2 and pBD-EBP3, respectively, or into the corresponding polylinker

sites in pGEX4T-1 (Pharmacia) for protein expression. Except for pBD-EBP3Δ174-225, all other in-frame deletion constructs of EREBP2 and EREBP3 in the bait plasmid pBD-GAL4 were made by generating the appropriate 5' *EcoRI*- and 3' *SalI*-tagged inserts by PCR from pBD-EBP2 and pBD-EBP3 and cloning these into pBD-GAL4 or pGEX4T-1, as described above. pBD-EBP3Δ174-225 was made by ligating an *EcoRI*-*StuI* fragment of pBD-EBP3 between the *EcoRI* and *SmaI* sites of pBD-GAL4 and pGEX4T-1.

The full-length *TNIT4A* gene was isolated by reverse-transcription PCR from S25 tobacco cell total RNA with the *NcoI*-tagged primer 5'-AAGAATTCATGGCTTTGGTCCCAACC-3 and the *SalI*-tagged primer 5'-CCGTCGACAGAAACAACCAAGACAACAG-3 and then cloned into the corresponding sites in pET28(a) (Novagen, Madison, WI). For measurement of interaction of NLP with Pto and Pti4/5/6, the truncated NLP gene *yeb4* was excised from pAD-yeb4 with *EcoRI* and *XhoI* and cloned in frame into the corresponding sites in the bait vector pEG202 and the prey vector pJG4-5 (Zhou et al., 1997).

Gel Mobility-Shift Assays

The oligonucleotides used in the gel mobility-shift assays were chemically synthesized (Integrated DNA Technologies, Coralville, IA). ³²P-labeled tetramers of the oligonucleotides were isolated essentially as described previously (Liu et al., 1995). The standard binding reaction (15 μL) contained 10,000 cpm of ³²P-labeled DNA, 2 μg of poly(dA-dT), and 5 μg of test protein in 1× binding buffer (25 mM Hepes-KOH, pH 7.5, 40 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol). The binding reaction was allowed to proceed at 30°C for 20 min. Reaction mixtures were electrophoresed on 5% polyacrylamide gels (acrylamide:bis-acrylamide, 29:1) in 0.5× TBE (1× TBE is 89 mM Tris base, 89 mM disodium borate, and 2 mM EDTA, pH 8.0). Gels were run at 10 V/cm for 1 h, fixed with 5% glacial acetic acid in 5% methanol for 30 min, dried, and exposed to x-ray film at -80°C for 12 h.

Protein Expression

EREBP2, EREBP3, and their deletion constructs, cloned in pGEX4T-1, were expressed as GST-fusion proteins in the *Escherichia coli* strain BL21. Expression of fusion protein was induced with 1 mM isopropylthio-β-galactoside for 16 h at 15°C. All subsequent steps were conducted at 4°C. Cells were harvested by centrifugation and lysed by sonication in PBST (16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl chloride). Insoluble materials were removed by centrifugation (10,000g, 20 min). The supernatant was incubated with PBST-equilibrated glutathione-agarose beads for 2 h with shaking. Non-GST proteins were removed by five washes with PBST. Bound GST proteins were used for protein-interaction assays in solution. For use in gel mobility-shift assays, the bound GST proteins were eluted with 20 mM GSH and the eluates dialyzed extensively against 1× bind-

ing buffer. The GST proteins were quantitated by SDS-PAGE.

Protein-Interaction Assays in Solution

Labeled full-length NLP was generated by in vitro transcription/translation from pET28-NLP (TNT-coupled reticulocyte lysate system, Promega) using T7 RNA polymerase and labeling with 60 μCi [^{35}S]Met/50 μL reticulocyte lysate. Incorporation of label was measured by TCA precipitation. For measurement of the interaction with [^{35}S]NLP, equal amounts of glutathione-agarose-bound GST-tagged test proteins were mixed with glutathione-agarose beads blocked with *E. coli* strain BL21(DE3) cell-free extract to yield a packed bead volume of 30 μL . The beads were then blocked by gentle mixing with PBST (200 μL) containing BSA (1.5 mg/mL) for 30 min at 4°C. Reticulocyte lysate (2 μL) was added to each sample, and binding was allowed to proceed for 16 h at 4°C with gentle mixing. After five washes with 1 mL of PBST the complexes were dissociated by boiling in 20 μL of sample buffer and separated by 12% SDS-PAGE. The gel was enhanced with 1 M sodium salicylate for 30 min, dried, and exposed to x-ray film at -80°C for 12 h.

Protein Interactions in Yeast

The HybriZAP two-hybrid vector kit (Stratagene) was used to detect interacting proteins with pBD-EBP3 as bait. An S25 tobacco cell cDNA library was constructed in the HybriZAP GAL4-activation-domain vector to generate the primary lambda phage library. After amplification and mass excision to a phagemid library, DNA encoding the library proteins (pAD-library DNA insert) and the bait protein (pBD-EBP3) was transformed and co-expressed in the yeast strain YRG2, according to the manufacturer's protocols. Positive interactions were scored on synthetic dropout plates supplemented with 15 mM 3-aminotriazole under Leu⁻, Trp⁻, His⁻-selective conditions. Positive interactions were verified by secondary screening and by β -galactosidase assays. The interaction between EREBP2 (which had a high background) and yeb4 was scored by comparing the growth of 0.6-mL liquid cultures under Leu⁻, Trp⁻, and Leu⁻, Trp⁻, His⁻-selective conditions. Overnight cultures grown in synthetic dropout medium under Leu⁻, Trp⁻-selective conditions were washed with water and diluted to an A_{600} of 0.04 into Leu⁻, Trp⁻-selective synthetic dropout medium and Leu⁻, Trp⁻, His⁻-selective synthetic dropout medium containing 35 mM 3-aminotriazole. They were grown with shaking at 30°C for 20 to 22 h, and the A_{600} reached was determined with appropriate dilutions. Yeast cells were grown in the appropriate minimal medium and harvested at the mid-log growth phase (A_{600} approximately 0.5) for β -galactosidase assays. Interaction of NLP with Pto and Pti4/5/6 was measured in yeast as described previously (Zhou et al., 1997). Quantitative assays of β -galactosidase activity were performed essentially as described by Reynolds and Lundblad (1989). β -Galactosidase activities were expressed as Miller's units ($A_{420} \times 1000 \text{ mg}^{-1} \text{ protein min}^{-1}$).

RESULTS

EREbps Can Bind to Osmotin Promoter Sequences

EREbps were originally cloned by their ability to bind the consensus GCC box in the context of the tobacco β -1,3-glucanase (*glu2*) promoter (Ohme-Takagi and Shinshi, 1995). To establish their ability to bind to osmotin promoter sequences, EREBP2 and EREBP3 were cloned by reverse-transcription PCR from total RNA isolated from 428 mM NaCl-adapted tobacco cells (S25) and expressed as GST-fusion proteins in *E. coli*. Both fusion proteins bound specifically to the osmotin GCC box in an electrophoretic mobility-shift assay; disruption of the element by substitution of TA for the central CG abolished binding (Fig. 1).

Screening of the S25 Two-Hybrid Library

For the yeast two-hybrid screen, the entire open-reading frame of EREBP3 was cloned into the yeast vector pBD-GAL4 to produce a protein fusion with the GAL4 DNA-binding domain (PBD-EBP3). A lambda phage library of tobacco S25 cDNA was constructed in the HybriZAP GAL4-activation-domain vector, and subsequently converted to a library in the yeast vector pAD-GAL4. pBD-EBP3 and the GAL4-activation-domain-S25 cDNA library fusion plasmids were co-transformed into the yeast strain YRG2 containing two reporter genes, *HIS3* and *lacZ*. Interactions between EREBP3 and S25 proteins were identified from 5×10^5 co-transformants by His prototrophy and then confirmed by β -galactosidase activity. One of the strongest *lacZ* positive clones, designated pAD-yeb4, was further characterized in this study.

Secondary screening of pAD-yeb4 involved recovery of the plasmid DNA and retransformation into the yeast strain YRG2, along with appropriate test plasmids. The

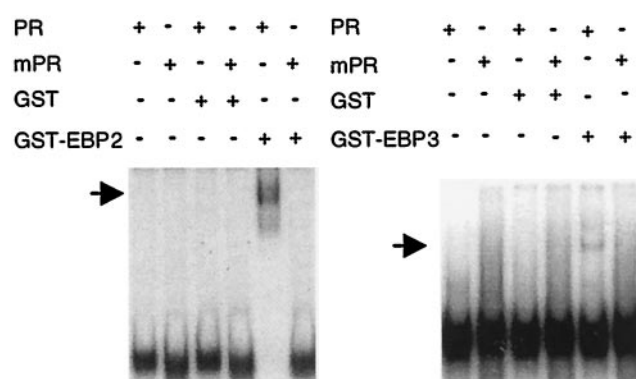


Figure 1. Gel-mobility shift analysis of the binding of EREbps to the osmotin promoter GCC box. The synthetic oligonucleotides were: PR: TACGTATTAGGCGGCTCTTATGTT (-168 to -149), ATAATCGCCGAGAATACAAATGC, mPR: TACGTATTAGGTAGCTCTTATGTT (-168 to -149), and ATAATCCATCGAGAATACAAATGC. Gel-purified, end-labeled 4-mers of the oligonucleotides were used. GST (from empty vector), GST-EREBP2, and GST-EREBP3 were expressed in *E. coli* and purified on glutathione-agarose columns. Test proteins (5 μg) and an oligonucleotide probe (10,000 cpm) were included in the mobility-shift assays in the indicated combinations. The positions of the bound probe are shown (arrows).

specificity of the interaction was tested in several independent transformants co-transformed with (a) pAD-yeb4 and pBD-EBP3; (b) pAD-yeb4 and pBD-EBP3 deletions that abolished DNA binding or the putative acidic transactivation domain; (c) pAD-yeb4 and pBD-GAL4; (d) pBD-EBP3 and pAD-random tobacco cDNA, as shown in Figure 2; and (e) pBD-EBP3 with pAD-GAL4 (not shown). As shown in Figure 2, b and c, activation of both reporter genes was dependent on the simultaneous presence of pAD-yeb4 and pBD-EBP3. The interaction was unaffected by the C-terminal deletions in pBD-EBP3 Δ 90-225 and pBD-EBP3 Δ 174-225 but was disrupted by the N-terminal deletion in pBD-EBP3 Δ 2-45, which removed part of the DNA-binding region (Ohme-

Takagi and Shinshi, 1995). Therefore, specific sequences in EREBP3 were required for interaction with yeb4 protein in yeast.

Sequence Analysis for pAD-yeb4

Sequence analysis of the pAD-yeb4 clone revealed that the insert encoded the partial sequence of a nitrilase (EC 3.5.5.1) fused in frame to the GAL4-activation domain in the vector. At the nucleotide level, the sequence shared 98% identity with tobacco mRNA for nitrilase *TNIT4A* (accession no. D63331) over the entire open-reading frame, 93% identity with tobacco mature-leaf mRNA for nitrilase *TNIT4B* (accession no. D83078), 73% identity with Arabidopsis *NIT4* (accession no. U09961), and 62% to 67% identity with Arabidopsis *NIT1* (accession no. U38845), *NIT2* (accession no. U09958), and *NIT3* (accession no. U09959). However, the pAD-yeb4 insert was missing the first 23 nucleotides of the open-reading frame of tobacco nitrilase *TNIT4* (D63331). The full-length tobacco nitrilase (*TNIT4*) was subsequently cloned by reverse-transcriptase PCR and was found to interact specifically with EREBP3, in the same manner as pAD-yeb4 (data not shown).

The truncated and full-length proteins encoded by *yeb4* and *TNIT4A*, respectively, clearly belong to a family of enzymes involved in the hydrolysis of C-N bonds in organic nitrogen compounds that include amidases, cyanide hydratase, and β -Ala synthetase (Bork and Koonin, 1994). They contain a conserved Cys, which has been shown by mutagenesis to be involved in the active site of very different nitrilases. Near the N terminus there is an invariant glutamate, which follows a conserved hydrophobic β strand. They also share conserved blocks of amino acids all along their sequence. Arabidopsis *NIT1*, *NIT2*, *NIT3*, and *NIT4* possess nitrilase activity in vitro and in vivo (Bartel and Fink, 1994; Schmidt et al., 1996; Normanly et al., 1997). The clone pAD-yeb4 was presumed to encode a NLP, because we were never able to demonstrate nitrilase activity of the full-length insert using a bacterial expression system and IAN as the substrate.

Interaction of EREBP2 with Nitrilase in Yeast

The four EREBPs are inducible by ethylene (Ohme-Takagi and Shinshi, 1995). EREBP1 is induced by pathogen infection and this induction precedes induction of several basic PR protein genes (Zhou et al., 1997). On this basis it was reasoned that if the interaction between EREBP3 and NLP was physiologically significant, it would be evident with several EREBP family members, because they respond to the same signals and probably share similar functions. If this was indeed the case, it was also predicted that the interaction would involve common motifs or amino acid sequences. The only conserved sequence between the deduced amino acid sequences of the four EREBPs is the conserved DNA-binding region (Ohme-Takagi and Shinshi, 1995). To test the validity of these predictions, the interaction of yeb4 with EREBP2 was studied in the yeast two-hybrid system. pBD-EBP2 by itself was found to activate both *HIS3* and *lacZ* expression slightly but signifi-

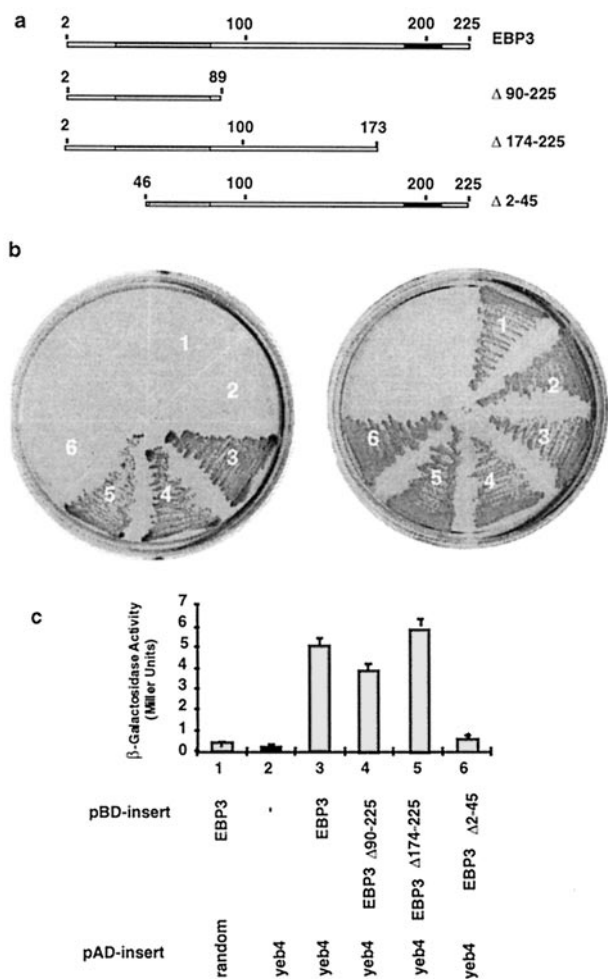


Figure 2. Interaction of EREBP3 with yeb4 in yeast. **a**, Deletion constructs of pBD-EBP3. The putative acidic transactivation domain (black box) and the DNA-binding domain (gray box) are shown. **b**, Activation of the *HIS3* reporter gene. *Saccharomyces cerevisiae* YRG2 containing combinations (1–6) of the GAL4-binding-domain (BD) and GAL4-activation-domain (AD) plasmids indicated in **c** were streaked under Leu⁻, Trp⁻, His⁻-selective conditions (left) and under Leu⁻, Trp⁻-selective conditions (right) to demonstrate *HIS3* gene activation. The His⁻-selective plates were supplemented with 15 mM 3-aminotriazole. **c**, Activation of the *lacZ* reporter gene. Quantitative β -galactosidase assays were performed on strain YRG2 containing the indicated combinations (1–6) of BD and AD plasmids. The values represent the average of three determinations \pm SD.

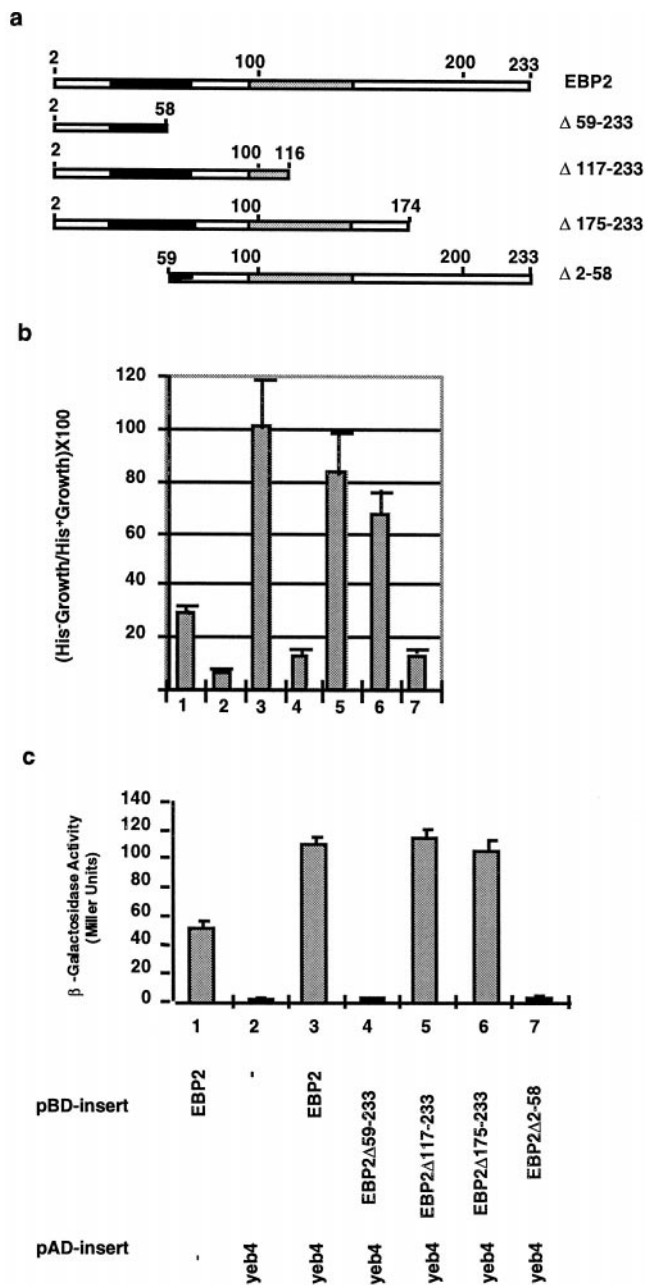


Figure 3. Interaction of EREBP2 with yeb4 in yeast. **a**, Deletion constructs of pBD-EBP2. The putative acidic transactivation domain (black box) and the DNA-binding domain (gray box) are shown. **b**, Activation of the *HIS3* reporter gene. Growth of *S. cerevisiae* YRG2 containing combinations (1–7) of the GAL4-binding-domain (BD) and GAL4-activation-domain (AD) plasmids indicated in **c** under Leu[−], Trp[−]-selective conditions and Leu⁺, Trp[−], His[−]-selective conditions were compared in liquid cultures. **c**, Activation of the *lacZ* reporter gene. Quantitative β -galactosidase assays were performed on strain YRG2 containing the indicated combinations (1–7) of BD and AD plasmids. The values represent the average of three determinations \pm SD.

cantly (Fig. 3, b and c). However, in the presence of pAD-yeb4, activation of both reporter genes was significantly greater, showing interaction between EREBP2 and yeb4.

pAD-yeb4 by itself did not activate the reporter genes. The N-terminal 58-amino acid deletion in pBD-EBP2 Δ 2-58 resulted in the loss of its ability to activate both reporter genes. This 58-amino acid sequence expressed in pBD-EBP2 Δ 59-233 failed to activate both reporter genes, showing that these sequences in EREBP2 were required but not sufficient for interaction with NLP. The N-terminal 116-amino acid fragment in pBD-EBP2 Δ 117-233 was sufficient for activation of both reporter genes.

In keeping with the predictions, and as shown in Figure 4, among the common sequences between the smallest fragments of EREBP3 and EREBP2 that were found to interact with yeb4 in these limited-deletion analyses was part of their DNA-binding regions. Two sequence motifs referred to as YRG and RAYD are conserved between a number of AP2- and EREBP-like proteins (Okamuro et al., 1997). The common feature in the two EREBP sequences that participate in interaction with NLP is a Y/FAAEIRD motif in the YRG domain that is unique to EREBP- and not AP2-like proteins (Okamuro et al., 1997), which is juxtaposed to an acidic motif. The acidic motifs occur in the putative transactivation domain EREBP2 and in an amphipathic α -helix in the RAYD domain of EREBP3 (Fig. 4; Okamuro et al., 1997). Involvement of DNA-binding regions in protein-to-protein interactions has been observed before (Mizukami et al., 1996).

Protein-Interaction Assays in Solution

The ability of NLP to interact with EREBP2/3 outside of the environment of the yeast cell was tested by protein-interaction assays in solution. GST-tagged EREBP3, EREBP3 Δ 174-225, EREBP3 Δ 2-45, EREBP2, and the GST tag alone were expressed in bacteria (Fig. 5a) and purified by immobilization on a glutathione-agarose matrix. As shown in Figure 5b, [³⁵S]NLP generated by in vitro transcription/translation was able to interact specifically with immobilized GST-tagged EREBP2/3. No interaction was detected with GST alone. Furthermore, EREBP3 Δ 2-45, which failed to interact with NLP in yeast, and EREBP3 Δ 174-225, which was able to interact with NLP in yeast, exhibited similar interactions with [³⁵S]NLP in solution. Thus, the specificity of the interaction between NLP and EREBP3 in yeast and solution was the same.

Interaction of Tobacco Nitrilase with Tomato Homologs of EREBPs

The tomato homologs of EREBPs, designated as Pti4/5/6, participate in a signal transduction pathway leading from *P. syringae* (*avrPto*) infection to PR-5 induction and disease resistance in tomato plants carrying the corresponding resistance gene, *Pto* (Zhou et al., 1997). Pti4 is closely related to EREBP1/2 and is 71% to 78% identical with these proteins over the entire open-reading frame. Pti5 is more distantly related (43%–48% identical). Pti6 and EREBP3/4 are unrelated to EREBP1/2 and each other outside of the DNA-binding region. It was postulated that if the interaction between NLPs and EREBPs was physiologically significant, it was likely to be conserved between

Figure 4. Alignment of the deduced amino acid sequences of EREBP2 Δ 117-233 and EREBP3 Δ 90-225. The YRG motif (underline), RAYD motif (bold underline), YAAEIRD motif (bold overline), amphipathic α -helix (overline), and clusters of acidic (overbar) amino acid residues are indicated. Gaps (dots) were introduced in the sequence to facilitate alignment. Identical amino acids in the two sequences are indicated in boldface.

EBP2 Δ 117-233	MYQPISTELP PTFSSSLMPC LTTDTWGDLP LKVDDSELMVI YGLLSDALTA	50
EBP3 Δ 90-225MAV KNKVSNG...	13
EBP2 Δ 117-233	GWTFFNLST EIKAEPR EEPATIPVPSV APPAETTTAQ AVVPKGRHYR	100
EBP3 Δ 90-225NLKGG NVKTDGVKEVHYR	30
EBP2 Δ 117-233	GVRQRPWGKF AAEIRD.....	116
EBP3 Δ 90-225	GVRKRPWGRY AAEIRDPGKK SRVWLGTFTD AEEAAKAYDT AAREFRGPKA	78
EBP2 Δ 117-233	116
EBP3 Δ 90-225	KTINFPSPTEN Q	89

species. Therefore, possible interaction between the Pto kinase and NLP, as well as between Pti4/5/6 and NLP, was evaluated in the yeast two-hybrid system of Zhou et al. (1997). As shown in Figure 6a, when an in-frame fusion of yeb4 with the LexA-binding domain was used as bait, interaction with the B42-activation-domain-fused Pti4/5/6 prey was detected as Leu prototrophy upon co-transformation with the two plasmids. Interaction was specific for the yeb4 insert and was not detected with the empty vector or with the Bicoid gene insert in the bait plasmid. Quantitative assays of β -galactosidase activity showed that the interaction between NLP and Pti4 was about 10-fold stronger than the interaction between Pti5/6 and NLP (Fig. 6c). No interaction was observed between

Pto and NLP with the LEU2 reporter (Fig. 6b) or the lacZ reporter (Fig. 6c).

DISCUSSION

Role of Nitrilase in Auxin Biosynthesis

In Arabidopsis nitrilase is involved in the biosynthesis of the auxin IAA, since IAN appears to be the direct precursor

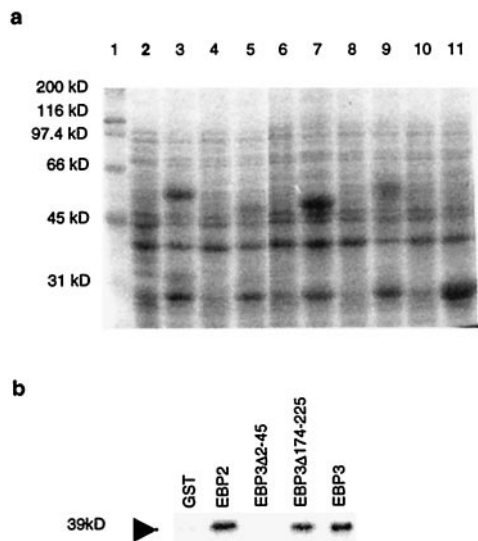


Figure 5. Interaction of EREBPs with NLP in solution. a, Bacterial expression of GST fusion proteins. Expression of GST-EREBP3 (lanes 2 and 3), GST-EREBP3 Δ 174-225 (lanes 4 and 5), GST-EREBP3 Δ 2-45 (lanes 6 and 7), GST-EREBP2 (lanes 8 and 9), and GST (lanes 10 and 11) in uninduced (lanes 2, 4, 6, 8, and 10) and isopropylthio- β -galactoside-induced (lanes 3, 5, 7, 9, and 11) cultures was analyzed by SDS-PAGE followed by Coomassie blue staining. The GST fusion proteins are indicated (arrowheads). There were 10 μ g of protein per lane. b, Interaction of immobilized GST fusion proteins with [35 S]NLP. In vitro-translated [35 S]Met-labeled NLP was incubated with the glutathione-agarose-bound GST (left lane) or GST-fused EREBPs (other lanes). After extensive washing, bound proteins were separated by SDS-PAGE and detected by autoradiography.

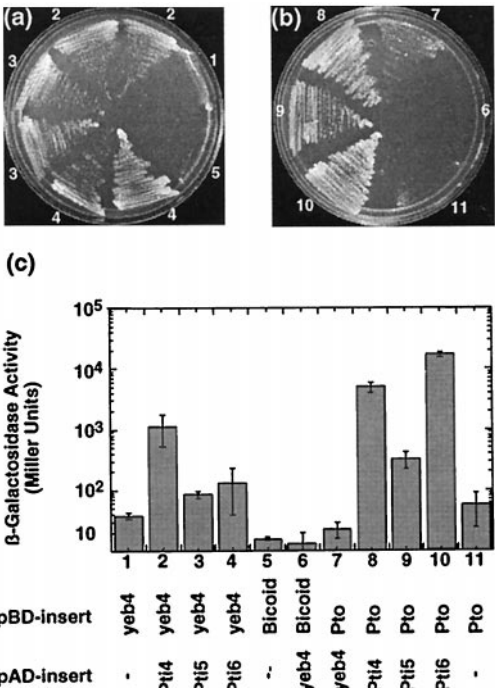


Figure 6. Interaction of Pto and Pti4/5/6 with yeb4 in yeast. a and b, Activation of the LEU2 reporter gene. EGY48 yeast cells co-expressing in-frame B42-activation-domain (AD) fusions with Pti4 (2 and 8), Pti5 (3 and 9), Pti6 (4 and 10), or yeb4 (6 and 7) from the prey plasmid pJG4-5, and LexA-binding-domain (BD) fusions with yeb4 (1–4), Pto (7–11), or Bicoid (5 and 6) from the bait plasmid pEG202 were grown on Gal⁺,Leu[−] synthetic dropout medium. The plates were incubated at 30°C for 3 d. Positive interactions were scored as Gal-dependent Leu prototrophy. c, Activation of the lacZ reporter gene. Quantitative β -galactosidase assays were performed on EGY48 yeast cells containing the indicated combinations (1–11) of BD and AD plasmids grown in Gal⁺,Leu[−] synthetic dropout medium. The values shown represent averages of six assays (two independent colonies assayed for activity in triplicate). Bars represent \pm SE.

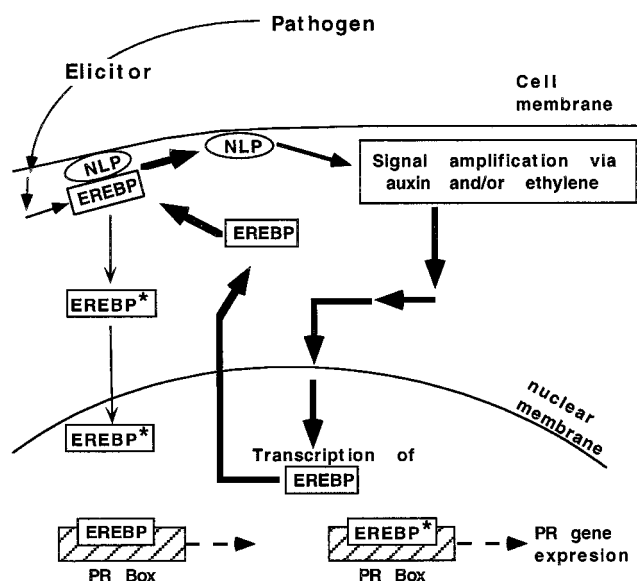


Figure 7. Hypothetical model of the role of the NLP-EREBP interaction. The asterisk (*) represents signal-mediated modification of EREBP that results in dissociation of NLP (Nit). The proposed signal-amplification cycle is represented by bold arrows. Increased cytoplasmic levels of EREBPs could participate both in increasing PR gene expression and in turning off the amplification cycle by interacting with NLP. It is not clear if PR gene expression requires modification of EREBP.

of IAA (Schmidt et al., 1996). Four genes, NIT1/2/3/4, encoding nitrilases appear to be expressed in a tissue-specific manner, with NIT4 (most closely related to yeb4) being expressed mainly in green tissues. NIT2 was specifically found to be induced by pathogen attack (Bartel and Fink, 1994).

Transgenic *Arabidopsis* overexpressing the four nitrilases had no growth defects, but NIT2 overexpression increased sensitivity to IAN. A mutant defective in NIT1 had no growth defects but was resistant to IAN (Normanly et al., 1997). The K_m of the *Arabidopsis* nitrilases for IAN is high (Bartling et al., 1994). These data show that although nitrilases can function in auxin biosynthesis in *Arabidopsis* (and in other Brassicaceae species), they are clearly not the primary or only mechanism for this purpose. The role of nitrilase in auxin biosynthesis in tobacco is unclear. It was not possible to detect nitrilase activity in tobacco leaf discs, as measured by the ability to convert [^{13}C]IAN to [^{13}C]IAA or to detect nitrilase protein on immunoblots with nitrilase II antibodies (Schmidt et al., 1996).

Physiological Role for NLP-EREBP Interaction

Defense gene expression is induced in plants by several factors, including pathogens, elicitors produced by pathogens, and ethylene (Hammond-Kosack and Jones, 1996; Yang et al., 1997; Yun et al., 1997). Although it is not clear that the signal transduction pathways from pathogens or elicitors and exogenous ethylene are the same, ethylene (Ward et al., 1991; Hammond-Kosack and Jones, 1996; Yang et al., 1997; Yun et al., 1997) and EREBPs and EREBP-

like proteins (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997) appear to be involved in these pathways. There are also some reports that auxin (IAA) is involved in pathogenesis- and elicitor-induced defense gene expression, probably by inducing ethylene synthesis (Hughes and Dickerson, 1991). These observations and the ability of nitrilase to interact specifically with EREBPs and Pti4/5/6 (Figs. 2, 3, and 6) suggest a physiological role for the interaction between NLP and EREBPs in relation to defense gene expression.

Nitrilases are soluble cytoplasmic proteins loosely associated with the plasma membrane (Bartling et al., 1994). Therefore, it is proposed here that the interaction between NLP and EREBPs serves to sequester EREBPs in the cytoplasm. Signaling by a pathogen would then result in dissociation of the two proteins. The dissociated EREBPs would be free to be translocated to the nucleus and induce transcription of defense-related genes. Signal-mediated translocation of EREBPs to the nucleus has been suggested before (Zhou et al., 1997). The tomato Pto kinase, which is involved in resistance to *P. syringae* strains carrying the *avrPto* gene, was found to interact with EREBP analogs Pti4/5/6 and also with EREBP2. Although it was not demonstrated that Pto kinase phosphorylates EREBPs, it was proposed that signaling by the pathogen resulted in activation of Pto kinase in the cytoplasm and that phosphorylation by Pto was the signal for translocation of EREBPs to the nucleus. This model is supported by other reports of signal-mediated translocation of transcription factors to the nucleus as a mechanism for the regulation of gene expression in plants (Harter et al., 1994; von Arnim and Deng, 1994). Release of NLP from its interaction with EREBP by interaction with an upstream component of the signal transduction pathway is consistent with the observation that the association of EREBPs with nitrilase or Pto kinase does not require posttranslational modification of EREBPs such as by phosphorylation and can be observed with bacterially expressed protein.

It has been proposed that the initial plant-pathogen interaction signal is later amplified by the plant, most likely by a mechanism involving ethylene biosynthetic enzymes such as ACC synthase (Hammond-Kosack and Jones, 1996). The interaction between an enzyme involved in C-N bond hydrolysis and EREBPs suggests that EREBPs participate in the signal-amplification step of plant-defense responses by as-yet-unidentified mechanisms. In our model (Fig. 7) it is envisaged that the extent of amplification is regulated by the abundance of cytoplasmic EREBPs, which are ethylene- and pathogen-inducible proteins (Ohme-Takagi and Shinshi, 1995; Büttner and Singh, 1997; Zhou et al., 1997). Induction of EREBP would increase the level of NLP-free EREBP by overtitrating the cytosolic level of NLP, or perhaps by transcription of an EREBP family member that lacks interaction with nitrilase.

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