Expression analysis of five tobacco EIN3 family members in relation to tissue-specific ethylene responses

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Abstract

Ethylene induces different sets of genes in different tissues and at different stages of development. To investigate whether these differential responses are caused by differential expression of members of the EIN3 family transcription factors, five tobacco family members were isolated. They can be divided into three subgroups, which is probably due to the amphidiploid nature of tobacco. In phylogenetic analysis, each of the subgroups clustered with one of the three tomato EIL proteins and all NtEILs proved to be most homologous to Arabidopsis EIN3 and EIL1.

Although organ-specific ethylene responses have been observed before, northern blot analysis showed that all NtEILs were expressed in all organs. To study differential NtEIL expression at the cellular level, in situ hybridization was used on the tobacco ovary. It was found that different ovary tissues displayed variable ethylene-induced expression of two ethylene-responsive marker genes. By contrast, no differences were found in expression level or tissue-specificity for any of the NtEILs in the ovary, before or after ethylene treatment. This indicates that the organ and tissue-specific ethylene responses are not caused by differential expression of NtEIL family members. These results support a model in which the developmental signals that regulate the tissue-specific responses are integrated with the ethylene signal downstream of a common ethylene-signalling pathway.

Key words: Ethylene, ETHYLENE-INSENSITIVE3 (EIN3), ETHYLENE-INSENSITIVE3-LIKE (EIL), Nicotiana tabacum, signal-transduction pathway, tissue-specific response.

Introduction

The plant hormone ethylene regulates a variety of processes, such as the ‘triple response’ of germinating seedlings, root hair formation, leaf expansion, fruit ripening, and petal senescence after fertilization. In addition, ethylene is known to induce defence responses in reaction to biotic and abiotic stresses (Abeles et al., 1992).

Initiation of these processes involves complex regulation of ethylene biosynthesis and depends on the ability of cells to respond in an appropriate manner. Over the last decades considerable progress has been made towards the elucidation of both the ethylene-biosynthesis pathway and the ethylene-response pathway. In higher plants, ethylene is synthesized from methionine via the formation of S-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylate (ACC). The individual reactions of this biosynthetic pathway are catalysed by the enzymes AdoMet synthase, ACC synthase and ACC oxidase, respectively (Yang and Hoffman, 1984; Kende, 1993). Analysis of Arabidopsis thaliana mutants with an aberrant triple response, either absent or constitutive, has led to the identification of a number of components of the primary ethylene-signalling pathway (reviewed by Johnson and Ecker, 1998). Ethylene is perceived by a family of integral membrane receptors that are similar to bacterial two-component histidine kinase receptors and includes ETR1, ERS1, EIN4, ETR2, and ERS2 (Bleecker et al., 1988; Chang et al., 1993; Hua et al., 1995, 1998; Sakai et al., 1998). A Raf-like protein kinase called CTR1 acts downstream of the receptors (Kieber et al., 1993). The receptors and CTR1 actively repress the downstream pathway in the absence of ethylene (Kieber et al., 1993; Hua and Meyerowitz, 1998). The ethylene signal is propagated via a MAPK pathway (Ouaked et al., 2003) to EIN2, which is a membrane protein with homology to...
Nramp metal-ion transporters (Alonso et al., 1999). The unknown gene products of EIN5, EIN6 and EIN7 also act downstream of CTR1 (Roman et al., 1995). Finally, the signal is transmitted to the EIN3 family of transcription factors. Out of this six-member family, only for EIN3 and EIN3-LIKE1 (EIL1) was a function in the ethylene-signalling pathway demonstrated conclusively (Chao et al., 1997; Alonso et al., 2003). EIN3 has been shown to act as a transcriptional activator and bind to the primary ethylene-response element present in the promoter of the ethylene-responsive ERF1 gene (Chao et al., 1997; Solano et al., 1998).

Ethylene induces different sets of genes in different tissues, and the response also depends on the developmental stage of the tissue (Lincoln and Fischer, 1988; Raghothama et al., 1997; Ruperti et al., 2002; Verlinden et al., 2002). Currently, it is not known how developmental information of the cells is integrated with the ethylene signal to obtain these tissue-specific ethylene responses. One possibility is that the developmental signal acts upstream of the ethylene-signalling pathway and regulates its composition. A potential target for such a regulatory mechanism is represented by EIN3, which is the last upstream of the ethylene-signalling pathway and regulates its composition. A potential target for such a regulatory mechanism is represented by EIN3, which is the last component of the primary signalling pathway. Because the EIN3 family members may each act on specific target genes, differential expression of the EIN3 genes could be responsible for the tissue-specific ethylene responses. Although recent silencing experiments suggest redundancy within the tomato (Lycopersicon esculentum) EIN3 family (Tieman et al., 2001), a detailed analysis of gene expression has not been reported so far.

In order to investigate the role of the EIN3 family in the ethylene-signalling pathway in tobacco (Nicotiana tabacum), the family members have been isolated, designated NtEILs, and their expression pattern studied at the organ and cellular levels. Although there are organ-specific ethylene responses in tobacco, all NtEILs were found to be expressed in all organs. In addition, using two ethylene-response marker genes, tissue-specific ethylene responses were identified within the ovary. However, mRNA of all NtEILs was detected in all cells of the ovary. Together, these data suggest that developmental cues that cause the tissue-specific ethylene responses are integrated downstream of the primary ethylene-signalling pathway.

**Materials and methods**

**Plant material and treatments**

Seeds of Nicotiana tabacum L. cv. Petit Havana SR1 were derived from the Solanaceae germplasm collection of the Botanical garden of the University of Nijmegen, The Netherlands (http://www-bgard.sci.kun.nl). Plants were grown under standard greenhouse conditions with additional lighting. Plant tissues were harvested, frozen in liquid nitrogen and stored at -80 °C. For experiments with ethylene treatments, flowers at stage 9 (according to Koltunow et al., 1990) were harvested, placed in a desiccator with the petioles submerged in water and incubated for 24 h with air or with air containing 1 μl l-1 ethylene. The ethylene concentration was confirmed by gas chromatography at the beginning and at the end of the treatment.

**Isolation of NtEIL cDNAs**

A tobacco ovary cDNA library was constructed from poly(A)+ RNA purified from ovaries at stage 1 to stage 6. cDNA synthesis and cloning was preformed by using the ZAP Express cDNA synthesis and cloning kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions.

A partial cDNA was amplified from this cDNA library by PCR, using the following degenerated primers: GTMYARRGHCAGAGYGG and GMHGTCTATTTVTCCTGTY. The resulting fragment was used to screen the same cDNA library under low stringency conditions according to the manufacturer's instructions. The cDNA clones were sequenced using vector and gene-specific synthetic primers. The 5'-end of the NtEIL1 cDNA was extended with 254 bp by PCR amplification of a 648 bp fragment from the cDNA library, using an upstream primer based on the highly homologous NtEIL2 sequence (GGGAAGGCATCTAAATTTGAG) and a downstream primer specific for NtEIL1 (GAAGTCCTGCAAAAGT). To extend the 5'-end of the NtEIL4 cDNA with 110 bp, a 1038 bp fragment was amplified with a primer based on the highly homologous NtEIL5 sequence (GGAGATTGGTGATTATCGTG) and an NtEIL4-specific primer (ACCCAGCTGAGGACAA-GGC). The large overlapping part of the resulting fragments was used to ensure that the fragments belonged to the desired cDNA clone. All five NtEILs were sequenced completely at least twice. The presence of the stop codon in NtEIL3 was confirmed by sequencing a clone obtained independently by RT-PCR. Genbank accession numbers are AY248903 (NtEIL1), AY248904 (NtEIL2), AY248905 (NtEIL3), AY248906 (NtEIL4), and AY248907 (NtEIL5).

**Phylogenetic analysis**

Protein sequences were aligned using the ClustalW program (version 1.82 at http://www.ebi.ac.uk/clustalw/index.html; Thompson et al., 1994). The Phylib package (version 3.5c at http://bioweb.pasteur.fr/seqanal/phylodyen/phylib.uk.html; Felsenstein, 1989) was used to calculate a distance matrix and to generate a tree using the bio-Neighbor-Joining method (Gascuel, 1997). An unrooted tree was plotted using the drawtree program of the package.

**RNA analysis**

Total RNAs were extracted using the method of van Eldik et al. (1995). 10 μg of total RNA was separated on a formaldehyde agarose gel and blotted on Hybond-N membrane (Amersham Biosciences, Buckinghamshire, UK). Northern hybridization was carried out according to the instructions for Hybond-N. Washing was done with a 1× SSC buffer at 57 °C. NtEIL probes were prepared from restriction fragments corresponding to bp 2004–2392 of NtEIL1 (EcoRV/Scal), bp 1987–2277 of NtEIL3 (BglII/MscI) and bp 1907–2241 of NtEIL5 (EcoRV/HindIII).

**In situ hybridization studies** were performed as described by Cox and Goldberg (1988) with minor modifications. Immediately after ethylene-treatment the ovaries were excised and fixed overnight at 4 °C in 1% glutaraldehyde, 4% paraformaldehyde solution prepared in 0.1 M phosphate buffer (pH 7.0), dehydrated, cleared, and embedded in paraffin. Eight-micrometer-thick sections were hybridized to 32P-labelled antisense RNA probes at a specific activity of 4–5 × 108 dpm μg−1. Probes were transcribed from a tobacco ACC-oxidase cDNA clone (Weterings et al., 2002), a cloned PCR fragment corresponding to bp 1753–2068 of the tobacco ERS homologue (Terajima et al., 2001) and from clones of the NtEIL...
fragments described above. Hybridization with sense probes was used to determine the background signal. After hybridization and emulsion development, digital photographs were taken with a CoolSNAP CCD camera (Roper Scientific, USA) using bright-field illumination on a Leitz Orthoplan (LSM, Wetzlar, Germany). The images were enhanced using Adobe Photoshop 5.0 (San Jose, CA).

Results

The tobacco EIN3 family

To identify tobacco EIN3 family members, a PCR-based approach was taken using degenerate oligonucleotides based on the sequence of the four Arabidopsis EIN3 family proteins (Chao et al., 1997). A 570 bp fragment was amplified from a cDNA library made from tobacco ovaries. This fragment was 97% homologous to TEIL, a recently identified tobacco EIN3 family member (Kosugi and Ohashi, 2000) and was used to screen the same tobacco cDNA library under low stringency. The 17 positive clones that resulted from this screening corresponded to five different EIN3-like cDNAs, which were named NtEIL1 (Nicotiana tabacum EIN3-LIKE1, previously TEIL) to NtEIL5. The cDNAs of NtEIL1 and NtEIL4 were extended to obtain full-length clones (see Materials and methods). The sequence identity between the NtEIL cDNAs ranges from 48% to 96%, with NtEIL1/2 and NtEIL3/4 being highly homologous pairs. Analysis of the NtEIL cDNAs revealed that they share predicted amino acid repeats of asparagine and glutamine present in the C-terminus of Arabidopsis EIN3 and EIL1, respectively, are absent in the NtEILs. As in AtEIN3, the amino acids between the acidic N-terminus and the basic domain II are predicted to form a coil (Lupas, 1996). To examine the relationship between the EIN3-like proteins from tobacco, tomato and Arabidopsis, a phylogenetic analysis was performed. Figure 1B shows that each branch containing a tomato EIL also contained one or two of the tobacco EILs. Furthermore, all tobacco and tomato proteins proved to be more related to Arabidopsis EIN3 and EIL1 than to the other EILs.

Accumulation of NtEIL mRNA in cells with different ethylene responses

Accumulation of NtEIL mRNA in plant organs was determined by northern analysis. Because of the high homology between the cDNAs of NtEIL1 and NtEIL2 (96%) and between the cDNAs of NtEIL3 and NtEIL4 (96%) the probes used in this experiment did not discriminate between these related NtEILs, but they did discriminate between the other NtEILs as confirmed by dot-blot analysis (data not shown). The probes recognized a single fragment of about 2.6 kb. Figure 2A shows that mRNA of the NtEILs was present at almost similar levels in all plant organs examined, although the NtEIL1/2 signal was somewhat higher in root and the NtEIL3/4 and NtEIL5 signals somewhat lower in seeds.

To be able to compare the expression of the NtEILs between cells that have different ethylene responses, in situ hybridization analysis of two genes that were known to be ethylene-inducible, the tobacco ERS gene and ACC-oxidase (ACO) gene (Terajima et al., 2001; Rieu et al., 2003), was performed first. Figure 2B shows that after treatment with ethylene, the cells of the vascular bundles...
and placental epidermis gave a strongly increased ERS and ACO hybridization signal. By contrast, the cells of the ovules showed a strong increase in ERS hybridization signal and only a weak increase in ACO signal and the cells of the carpel wall did not show an effect of ethylene on the hybridization signal for any of the two marker genes. To see if these different responses could be related to expression patterns of specific NtEILs, sections from the same ovaries were hybridized with probes against NtEIL1/2, NtEIL3/4 and NtEIL5. As shown in Fig. 2C, all NtEILs were expressed throughout the ovary, albeit at a low level. The level and pattern of NtEIL expression were not influenced by ethylene-treatment (data not shown). Taken together, these results show that, although different cells within the ovary respond differently to ethylene, the NtEILs do not show differential expression.

Discussion

Five different NtEIL cDNAs from tobacco that encode deduced proteins with high similarity to the Arabidopsis EIN3 protein have been isolated (Fig. 1A). The highest homology was found between the N-termini, which contain the DNA-binding domain and the dimerization domain (Kosugi and Ohashi, 2000; Solano et al., 1998). When compared with tomato, a closely related solanaceous species, one or two close homologues for each of the three LeEILs were found (Fig. 1B), which is probably due to the amphidiploid nature of tobacco. The fact that the LeEILs are functionally similar to Arabidopsis EIN3 (Tieman et al., 2001) suggests that the NtEILs fulfill the EIN3 function in tobacco. Indeed, it has been shown that over-expression of NtEIL1 in Arabidopsis causes a constitutive
triple response phenotype (Kosugi and Ohashi, 2000). The introduction of an early stop codon in the *NtEIL3* gene seems to be a very recent event, as the remainder of the open reading frame on the cDNA is still intact and would generate a protein with 96% identity to NtEIL4. So, although partial transcription factors can have a function as negative regulators (for example, CAPRICE; Lee and Schiefelbein, 2002), this is probably not the case for NtEIL3.

From genetic studies in *Arabidopsis* it is not clear whether the EIN3 family members are functionally redundant or have distinct functions. Recently, analysis of *ein3/eil1* double mutants showed that EIN3 and EIL1 are fully responsible for ethylene-signal transmission during the triple-response of seedlings and act redundantly during this process (Alonso et al., 2003). The inability of the remaining family members to compensate for EIN3/EIL1 deficiency may reflect functions in other tissues or during other stages of development or functions other than in ethylene signalling. In tomato, silencing experiments pointed towards functional redundancy within the EIN3 family of this species (Tieman et al., 2001). If tissue-specificity of ethylene responses is regulated at the level of the EIN3 component, the different EIN3 family members need to be expressed or activated in a tissue-specific manner. It has been observed before that different plant organs react differently to ethylene. In addition, it was demonstrated that even within an organ, different tissues can react differently to ethylene, using the *ERS* and *ACO* genes as markers for the ethylene response in the tobacco ovary (Fig. 2B). However, it is shown that all *NtEILs* are expressed in all organs and in all ovary cells (Fig. 2A, C). Therefore, these results imply that the differences in ethylene response are not due to differences in composition of the primary ethylene-signalling pathway at the level of EIN3. Although it should be noted that almost nothing is known about the accumulation and activity of the EIN3 proteins, all data collected so far on ethylene signalling and responses fit in a model in which the primary signalling pathway is similar in all cells, and in which the unique developmental signals and the ethylene signal are integrated downstream of this primary signalling pathway. Combinatorial control of the expression of ethylene-responsive genes such as those from the Ethylene Response Factor (ERF) family (Solano et al., 1998; Fujimoto et al., 2000) may well be the actual point of regulation.

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