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A two-component gene (*NTHK1*) encoding a putative ethylene-receptor homolog is both developmentally and stress regulated in tobacco

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Abstract The full-length of a two-component gene *NTHK1* (*Nicotiana tabacum* histidine kinase-l) was isolated from tobacco (*N. tabacum* var. Xanthi) using a previously obtained *NTHK1* cDNA fragment as a probe. Sequence analysis revealed that *NTHK1* shared high homology with *LeETR4* from tomato and encoded an ethylene-receptor homolog. The predicted NTHK1 protein had a putative signal peptide, three transmembrane domains, a histidine kinase domain and a receiver domain. The putative autophosphorylation site at His378 and the phosphate receiver site at Asp689 were also identified. By using the in situ hybridization technique, *NTHK1* mRNA was detected during flower organ development. It is also highly expressed in the processes of pollen formation and embryo development. The expression of *NTHK1* in response to wounding and other stresses was investigated using competitive RT-PCR. The results demonstrated that *NTHK1* was inducible upon wounding (cutting). Floating of the cut leaf pieces in 0.5× MS, with shaking, led to a relatively rapid and strong expression. This phenomenon was confirmed by the in situ hybridization results. In addition to the up-regulation by wounding, *NTHK1* expression was also induced following NaCl and PEG treatment, indicating a possible role for NTHK1 in multiple stress responses.

Keywords Ethylene-receptor homolog · Stress · *Nicotiana tabacum*

Introduction

Living cells respond rapidly to the surrounding stimuli to adapt to changes in their environment. One of the mechanisms for the perception and transduction of these stimuli is known as the 'two-component' regulatory system. In bacteria, many adaptive responses are mediated by two-component systems, including osmoregulation, chemotaxis, microbial pathogenesis, photosensitivity, nutrient availability and stress-induced differentiation, such as sporulation and fruiting-body formation (Parkinson and Kofoid 1992). The typical two-component pathway is comprised of two proteins: a His protein kinase (sensor kinase) and a response regulator. The N-terminal region of the His protein kinase can detect the changes of the environment and transfer the signal to its transmitter domain at the C-terminal end. A conserved His residue in the transmitter domain is then autophosphorylated. The response regulator has a receiver domain at its N-terminal end, where a conserved Asp residue can receive the phosphoryl group from the His of the sensor kinase. The phosphorylation state of the receiver regulates the activity of the output domain in the response regulator, leading to a cellular response.

Similar two-component systems have been found in eukaryotic organisms (Chang and Stewart 1998). In the yeast *Saccharomyces cerevisiae*, the Sln1p-Ypd1p-Ssk1p pathway is the best-characterized eukaryotic two-component system that functions in the osmoregulation process. It contains three components and involves a four-step phosphorelay (Posas et al. 1996). In the fungus *Neurospora*, the two-component gene *Nik1* has been found to act during hyphal growth (Alex et al. 1996); while in the slime mold *Dictyostelium*, the two-component genes *Dhk1* and *Dok1* are reported to play important roles in development and osmoregulation respectively (Schuster et al. 1996; Wang et al. 1996).

In the higher plant *Arabidopsis*, two-component genes have been found to be involved in the signal-transduction pathway of two plant hormones, cytokinin and ethylene, with the pathway of ethylene being extensively studied (Kakimoto 1996; Hua et al. 1998; Sakai et al. 1998). Ethylene is a simple gaseous hormone that functions in different developmental processes and various stress responses in plants. Mutant analyses of ethylene perception result in the isolation of five ethylene receptor-related genes: *ETR1*, *ETR2*, *EIN4*, *ERS1* and *ERS2* (Chang et al. 1993; Hua et al. 1995, 1998; Sakai et al. 1998). Among them, the first three encode proteins with a putative His kinase domain plus a receiver domain, whereas the last two encode proteins with only a kinase domain. In vitro studies have demonstrated His kinase activity in ETR1 (Gamble et al. 1998). High-affinity binding of ethylene to the

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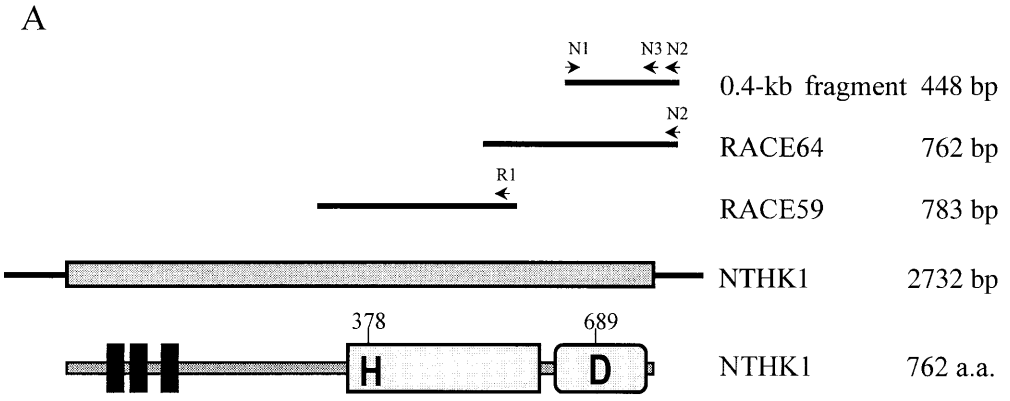


Fig. 1 **A** Schematic representation of tobacco *NTHK1* cDNA and its encoded protein. The 0.4-kb fragment (448 bp) was isolated previously (Zhang et al. 1999b). RACE64 and RACE59 were obtained using the RACE method (See Materials and methods). *N2* and *R1* were the specific primers used in RACE. Primers *N1* and *N2* were used in the competitive RT-PCR assay for the amplification of the target cDNA and the competitor DNA at the same time. *N1* and *N3* (contained the sequence of *N2*) were used for the cloning of the competitor DNA. The *shaded box* and the *lines* in the *NTHK1* gene indicate the open reading frame and flanking regions respectively. The deduced NTHK1 protein had 762 amino acids (*a.a.*). The three *filled boxes* represent the three transmembrane regions. The *rectangular box* containing the autophosphorylation site *H* (His) at position 378 represents the His kinase domain (amino acids 368–610). The *oval box* containing the phosphate receiver *D* (Asp) at position 689 represents the receiver domain (amino acids 638–756). The *bars* outside these regions indicate other amino-acid sequences in NTHK1. **B** Alignment of the NTHK1 amino-acid sequence with those of other known proteins. *LeETR4* and *LeETR5* are tomato sequences (Tieman and Klee 1999). *AtEIN4*, *AtETR2* and *AtETR1* are *Arabidopsis* sequences (Chang et al. 1993; Hua et al. 1998; Sakai et al. 1998). *Shaded areas* indicate the identity of amino acids. *Regions I, II and III* represent the three transmembrane segments. *Region IV* represents the putative signal peptide. The regions corresponding to the five boxes *H, N, G1, F* and *G2* from the His kinase domain of AtETR1 were *overlined*. The *asterisks* in the receiver domain indicate the typical conserved residues and Asp689 represents a putative receiver of the phosphate. *Dashes* indicate the gaps added for maximum alignment. The accession number for *NTHK1* is AF026267

ETR1 protein has also been proven and mutations in the transmembrane domain reduce the binding, indicating that ETR1 functions as an ethylene-receptor (Schaller and Bleecker 1995). Homologs of the ethylene receptor gene have been isolated from other plants, e.g. *TAE1*/*LeETR1*, *LeETR2* and *NR* from tomato (Wilkinson et al. 1995; Zhou et al. 1996; Lashbrook et al. 1998), *RP-ERS1* from *Rumex palustris* (Vriezen et al. 1997), and *Cm-ETR1* and *Cm-ERS1* from *Cucumis melo* (Sato-Nara et al. 1999). Recently, novel members of the two-component system genes have been reported in plants and they may encode new ethylene receptors and osmosensors (Tieman and Klee 1999; Urao et al. 1999).

Wounding is one of the environmental stresses that plants must cope with, and is generally caused by mechanical injury and pathogen or herbivore attack. In response to wounding, plants activate a series of gene expressions that are involved in wound healing, tissue repair and defense action. A number of factors such as ethylene, jasmonic acid, salicylic acid, systemin and MAPK etc. regulate the transcription of the wound-responsive genes, and may be the components of the wound signal-transduction pathway (Seo et al. 1995; O'Donnell et al. 1996; Rickauer et al. 1997; Ryan and Pearce 1998). However, the possible components that may play roles in the very early stage of the wounding response remain unclear.

In the present study, a full-length cDNA corresponding to a two-component gene *NTHK1* was isolated and characterized. Sequence analysis indicated that *NTHK1* encoded an ethylene-receptor homolog. The expression patterns of *NTHK1* were investigated during development and under conditions of stress. Its possible functions in these processes are also discussed.

Materials and methods

Plant material and treatments

Tobacco (*Nicotiana tabacum* L. var. Xanthi) plants were grown in the greenhouse with a temperature ranging from 20°C to 27°C. Plants at the vegetative stage (40–50 cm in height, 15–20 leaves) were used for the wounding experiments. Young expanding leaves were wounded by cutting the lamina into pieces of 1–2 cm^2 and then incubated in a box (maintained at 80% relative humidity) or floated in 0.5× MS solution with shaking, for the indicated times. Salt and drought stresses were made by watering tobacco seedlings at the five-leaf stage with soil-saturating volumes of different concentrations of NaCl and PEG 6000 (w/v) for the indicated times. All the materials harvested were frozen in liquid N_2 and stored at –70°C for DNA or RNA isolation.

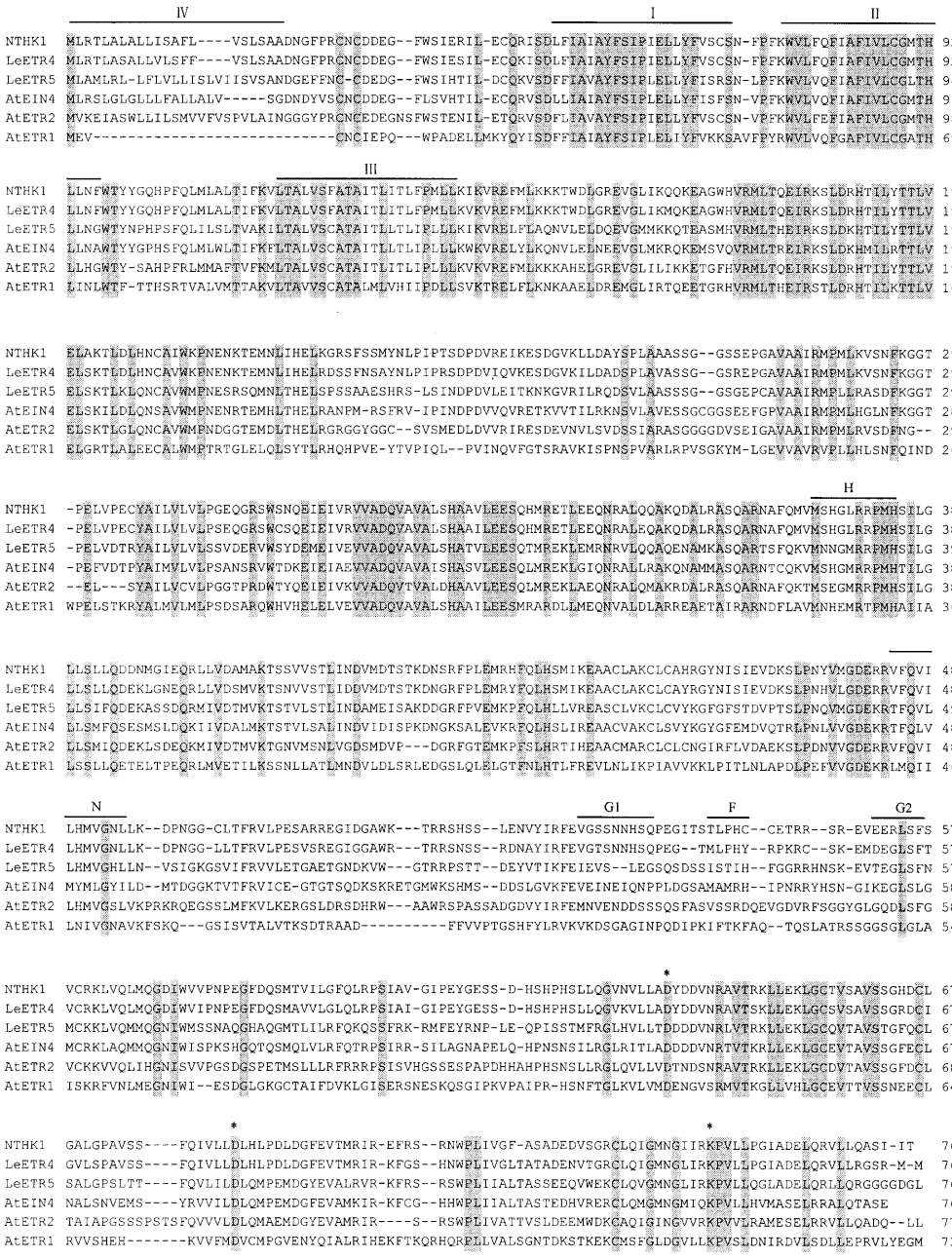
DNA, RNA isolation and hybridization

DNA extraction followed the method of Chen et al. (1991). Genomic DNA (8 µg) was digested overnight with restriction enzymes, fractionated on 0.8% agarose gel and transferred onto Hybond-N^+ nylon membranes for Southern-blot analysis. The hybridization and washing procedures followed standard protocols. Total RNA isolation was according to the description by Zhang et al. (1996). RNA (30 µg) was separated on a 1.0% agarose gel containing formaldehyde, blotted onto Hybond-N^+ nylon membranes and hybridized according to standard protocols. Equal loading of RNA samples was confirmed by examination of ribosomal RNA bands after staining with ethidium bromide, and from the equal intensity of 18 s rRNA hybridization bands. The probe was prepared by the random-priming method. Washing was performed with 2× and 1× SSC plus 0.1% SDS at 60°C, each for 20 min. The hybridization signals were quantitated using a Imaging Densitometer (BioRad).

For the rapid amplification of the 5'-cDNA end (RACE) of the *NTHK1* cDNA fragment (Zhang et al 1999b), 4 µg of total RNA from wounded leaf pieces was used to synthesize the 1st-strand cDNAs with an Oligo(dT) primer (with a *Not*I site). The 2nd-strand cDNAs were made using a cDNA synthesis kit (Promega). *Eco*RI adapters were added to the ends of 2nd-strand cDNAs and the cDNAs were digested with *Not*I to eliminate the *Eco*RI adapter at the 3'-ends. PCR was then performed at 94°C for

Fig. 1 Continued

B



3 min, and with 35 cycles of 94°C, 50 s; 54°C, 1 min 30 s; 72°C, 1 min 30 s with an adapter primer (5'-TAGAATTCCGTT GCTGTCGTCG-3') and a *NTHK1*-specific primer (N2: 5'-AG-CAATTTTGTTTGCTTAGC-3'). The amplified DNA fragment was cloned into the T-easy vector (Promega) and sequenced. One clone, RACE64, was obtained (see Fig. 1A). To obtain more 5'- sequence of the *NTHK1* gene, the same PCR procedure was conducted with the adaptor primer and a new primer (R1: 5'- GCAATGTGGATGTTATGCC-3') designed according to the sequence of RACE64. A new clone, RACE59, resulted and was sequenced in this round (see Fig. 1A).

Construction and screening of a cDNA library

Using an oligotex mRNA purification kit (Qiagen), poly(A^+) RNA was isolated from wounded leaf pieces that were floated in 0.5× MS, with shaking, for 30 min. Two micrograms of poly(A^+) RNA was used to synthesize the 1st- and 2nd-strand cDNAs using a cDNA synthesis kit (Promega). cDNA was cloned into the *Eco*RI site of λExCell (Amersham) using an *Eco*RI adaptor. About 500000 recombinants were packaged in vitro using a packaging extract (Promega). Approximately 400000 plaques were screened with RACE59 and RACE64 as probes. Six positive plaques were obtained from the third-round screening. These plaques were excised in vivo into pExCell plasmids, following instructions, and the plasmid with the longest insert was subjected to sequencing analysis.

Quantitation of mRNA by competitive RT-PCR

Competitive RT-PCR for the quantitation of *NTHK1* gene expression was conducted according to the method described by Gilliland et

al. (1990). The target *NTHK1* gene-fragment selected in this assay was a 408-bp sequence that contained 324 bp of a 3'-coding region and 84 bp of 3'-noncoding region. Two primers, N1, 5'-CTACTTGAAAAATTAGGATGC-3', and N2, 5'-AGCAATT TTGTTTGCTTAGC-3', were used to amplify this target 408-bp fragment (Fig. 1A). For competitor DNA preparation, primers N1 and N3, 5'-AGCAATTTTTGTTGGCTTAGCTCATCAGCAATT CCAGGC-3', were used to amplify a 307-bp fragment which has the same sequence as the target cDNA but lack 101-bp from its 3'-end. Primer N3 contained the sequence of primer N2. The PCR condition was 94°C for 3 min, with 30 cycles of 94°C, 50 s; 54°C, 1 min 30 s; 72°C, 1 min 30 s, and a final extension of 10 min at 72°C. The amplified 307-bp DNA fragment was cloned into T-easy vector (Promega) and used as a competitor plasmid after sequencing.

Total RNA (4 µg) was reverse-transcribed using MMLV (Gibco) at 37°C for 60 min. After heating for 5 min at 94°C, onetwentieth of the mixture was used for PCR amplification with primers N1 and N2. Amplification was carried out in a final volume of 30 µl containing PCR buffer, 0.2 mM of dNTPs, 0.67 µM of each primer, 0.5 pg or 2.5 pg of competitor plasmid DNA and 1 unit of *Taq* DNA polymerase for 30 cycles of 94°C, 50 s; 54°C, 1 min 30 s; 72°C, 1 min 30 s. For the samples that have *NTHK1* expression, competitive RT-PCR assay with the same set of primers, N1 and N2, produced two bands of the sizes corresponding to the target and competitor DNA species. In one case, the two bands were cloned and sequenced, and corresponded to the target and competitor DNA respectively. A titration experiment was also performed. A single concentration (one-twentieth) of the 1st-strand cDNA from 4 µg of total RNA of wounded leaf pieces (floated in 0.5× MS with shaking for 30 min) was co-amplified with 0.5, 5.0, 50 and 500 pg of the competitor plasmid DNA. The intensity of the competitor bands increased from 2.16 arbitrary units to 3.15, 10.96 and 16.17, whereas the intensity of the target DNAs decreased from 11.18 to 6.66, 4.08 and 2.50 respectively. A plot of the ratio of competitor to target band intensity versus the known concentrations of input competitor is linear when plotted on a log-log scale (data not shown), indicating the effectiveness of the competition.

Products were separated on a 1.2% agarose gel containing ethidium bromide and photographed, and the intensity of the bands was quantitated by scanning the negative on an Imaging Densitometer (BioRad). The ratio of target to competitor represented the relative level of *NTHK1* gene expression. Control experiments in which reverse-transcriptase or RNA was excluded gave no signals after amplification, indicating the absence of contamination. All the quantitations were performed at two competitor concentrations. Each experiment was repeated at least twice with similar results, and only one was presented.

RNA in situ hybridization

Tobacco samples were fixed, embedded and 10-µm sections were prepared on a micotome (Leica2135). Plasmid RACE64 (see Fig. 1A) was linearized and used as a template for the synthesis of Dig-labeled antisense and sense RNA probes with SP6 or T7 RNA polymerase following the instructions of the kit (Boeringer). The RNA probes were partially hydrolyzed by incubation in 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 10.2) at 60°C for 70 min. The hybridization was performed as described by Xie et al. (1999). The signals were revealed by NBT/BCIP, and the purple color in cells or tissues indicated a positive signal. The sections were also stained with alcian blue for better observation of the morphology of the cells or tissues.

Sequencing and data analysis

Nucleotide sequences of the cDNAs in the plasmid were determined on a DNA sequencer (ABI377) and compared with those in the Genbank database using the GAPPED BLAST program.

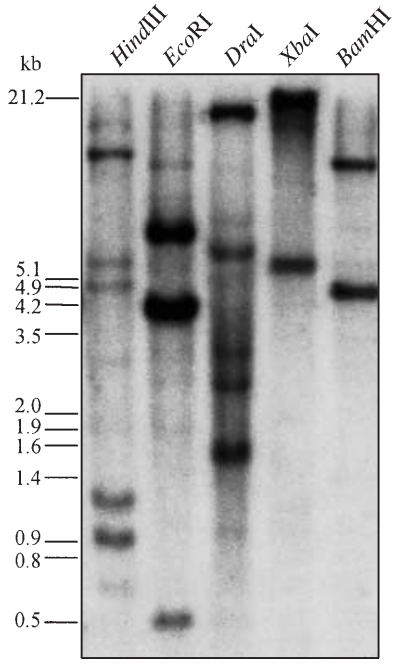


Fig. 2 Southern analysis of the tobacco *NTHK1* gene. Genomic DNA was digested with restriction enzymes, electophoresed on a 0.8% agarose gel and transferred to a nylon membrane. The hybridization was performed with the probe prepared using full-length *NTHK1* cDNA. The membrane was washed with 2× and 1× SSC plus 0.1% SDS at 60°C, each for 20 min. Numbers on the left indicate the molecular-size markers (kb)

Results

Cloning of the tobacco *NTHK1* gene

A 448-bp cDNA fragment that showed homology to bacterial two-component genes has been identified from tobacco in a previous PCR experiment, and its corresponding gene was designated *NTHK1* (*Nicotiana tabacum* His kinase-like) (Zhang et al. 1999b). PCR amplification (RACE) was performed to obtain the 5'-part of the 448-bp cDNA fragment (see Materials and methods). Two clones, RACE64 and RACE59, were isolated, which were still part of the *NTHK1* gene (Fig. 1A). These two clones were then used to screen a cDNA library constructed from the wounded leaf pieces. Several positive clones were obtained and the one with the longest insert was sequenced and found to contain the full-length cDNA of the *NTHK1* gene (Fig. 1A). The *NTHK1* cDNA has a length of 2732 bp and encodes a protein of 762 amino acids with a molecular mass of 85.3 kDa and a pI of 6.7. In the 5'- non-coding region, *NTHK1* has a sequence of 246 bp. In the 3'- non-coding region, *NTHK1* has a sequence of 187-bp together with a 13-bp polyA sequence.

Structural analysis of the tobacco *NTHK1*

The deduced amino-acid sequence of NTHK1 was compared with those of other known proteins (Fig. 1B).

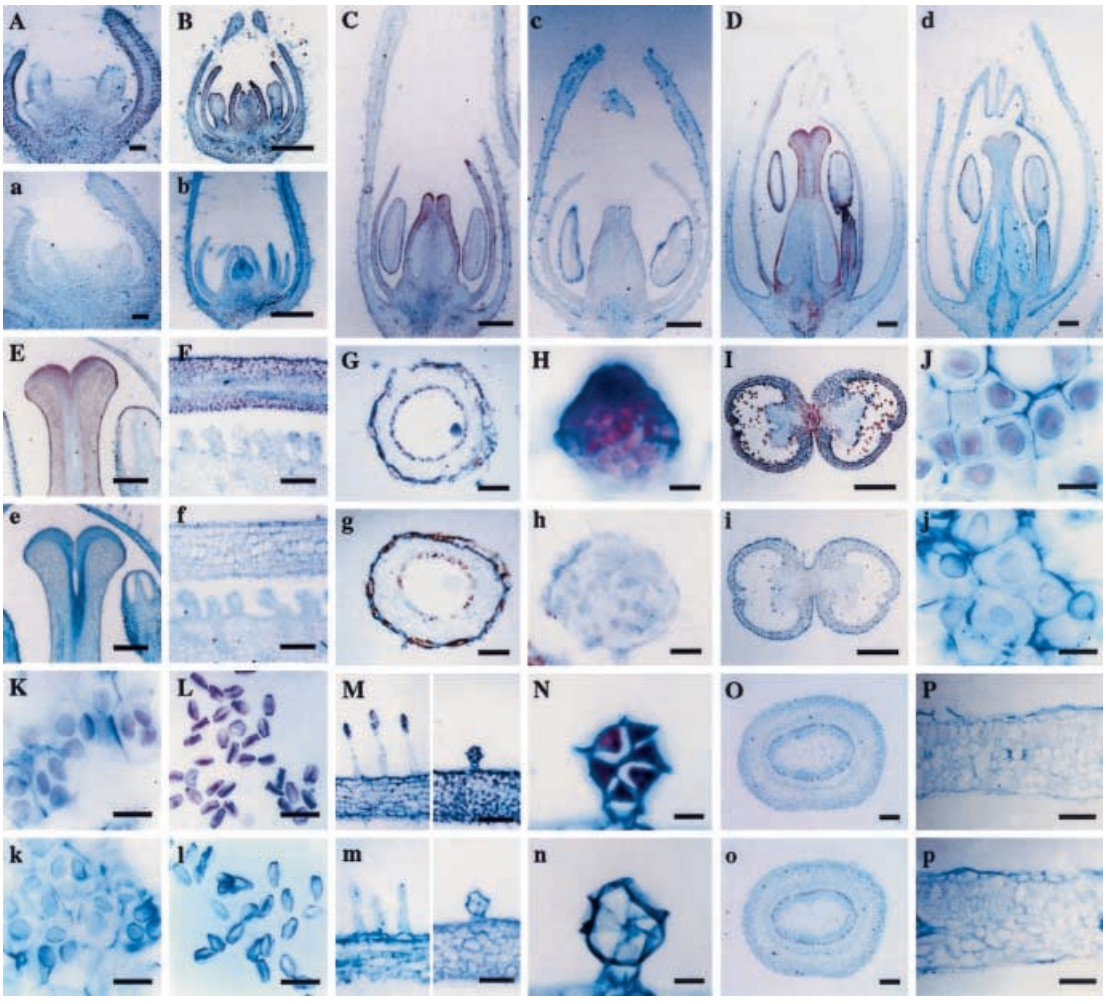


Fig. 3 In situ localization of *NTHK1* transcripts in tobacco tissues. (**A**), (**B**), (**C**), (**D**), (**E**), (**F**), (**G**), (**H**), (**I**), (**J**), (**K**), (**L**), (**M**), (**N**), (**O**) and (**P**) indicate sections hybridized with antisense RNA probe. (**a**), (**b**), (**c**), (**d**), (**e**), (**f**), (**g**), (**h**), (**i**), (**j**), (**k**), (**l**), (**m**), (**n**), (**o**) and (**p**) represent sections hybridized with sense RNA probe. A *purple color* indicates a positive signal. *Bars* in (H), (h), (N) and (n) represent 10 µm. *Bars* in (B), (b), (C), (c), (D), (d), (E), (e), (I), (i), (O) and (o) represent 500 µm. *Bars* in all the other figures represent 100 µm. The stages of the flower buds were determined according to the description by Mandal et al. (1992). (**A**) and (**a**) Longitudinal section of a stage-5 flower. (**B**) and (**b**) Longitudinal section of a stage-7 flower. (**C**) and (**c**) Longitudinal section of a stage-8 flower. (**D**) and (**d**) Longitudinal section of a stage-11 flower. (**E**) and (**e**) Longitudinal section of stigma and style from a stage-11 flower. (**F**) and (**f**) Longitudinal section of a ovary with developing ovules from a stage-12 flower. (**G**) and (**g**) Ovule with a developing embryo. (**H**) and (**h**) Enlargement of the developing embryo from (**G**) and (**g**) respectively. (**I**) and (**i**) Cross-section of an anther from a stage-15 flower. (**J**) and (**j**) Pollen mother cells from an anther of a stage-7 flower. (**K**) and (**k**) Tetrads from an anther of a stage-8 flower. (**L**) and (**l**) Pollen grains from an anther of a stage-15 flower. (**M**) and (**m**) *Left panel*, trichomes on petal; *right panel*, a trichome on ovary. (**N**) and (**n**) Enlargement of the trichome from the right panel of (**M**) and (**m**) respectively. (**O**) and (**o**) Cross-section of a stem, 5 cm below the shoot apex. (**P**) and (**p**) Cross-section of an expanding leaf

NTHK1 has homology with many hybrid two-component proteins in plants, e.g. 87% identity to LeETR4, 57% identity to LeETR5, 54% identity to ETR2, 54% identity to EIN4, and 35% identity to ETR1 (Chang et al. 1993; Hua et al. 1998; Sakai et al. 1998; Tieman and Klee 1999). Since LeETR4 and the other compared proteins were ethylene receptors or their homologs, it is speculated that NTHK1 was also an ethylene-receptor homolog in tobacco.

By comparison with ETR1 (Chang et al. 1993), the specific domains in NTHK1 were identified using the SMART program (Schultz et al. 1998). In the N- terminal end of NTHK1, four hydrophobic regions named I to IV were identified (Fig. 1B). Three of them (regions I, II and III) were putative transmembrane domains. The fourth hydrophobic region was a putative signal peptide according to the prediction method of Nielsen et al. (1997), and the possible cleavage site was between position 21 and position 22. At positions 28 and 30, NTHK1 had two Cys residues that were conserved among the proteins compared (Fig. 1B). These two Cys residues have been demonstrated to be involved in the dimerization of the ETR1 protein (Schaller et al. 1995). Another two domains featuring the hybrid two-component proteins were the His kinase domain and

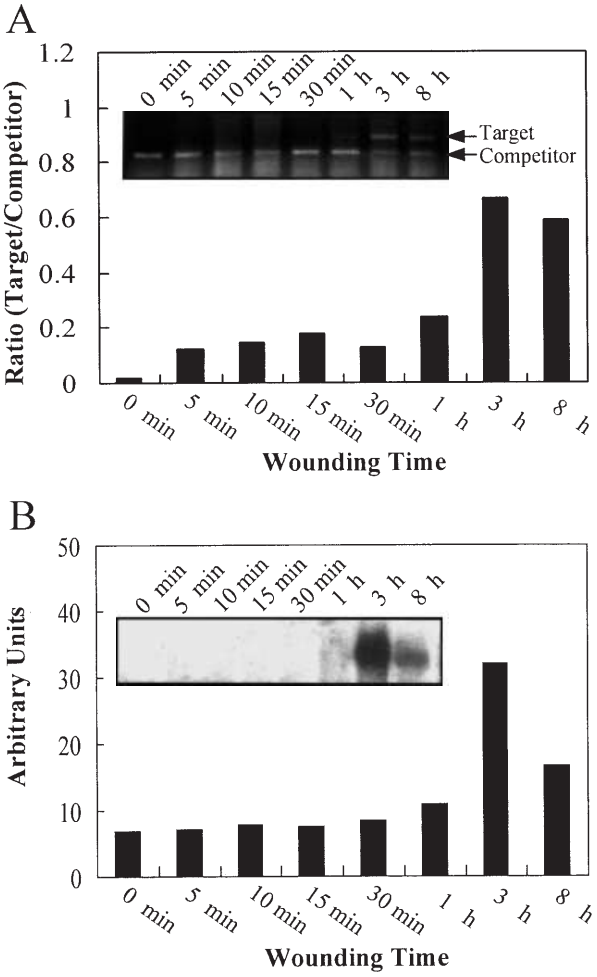


Fig. 4A, B *NTHK1* expression in leaves upon wounding. **A** *NTHK1* expression revealed by competitive RT-PCR assay. Wounding was performed by cutting the detached leaves into pieces of 1–2 cm^2 and incubated in a box (maintained at 80% relative humidity) for different times. Total RNA (4 µg) from each sample was reverse-transcribed into 1st-strand cDNAs. One-twentieth of the cDNAs was used for the competitive RT-PCR assay. The concentration of competitor plasmid DNA was 0.5 pg per 30 µl of the PCR reaction mixture. The intensity of the bands was quantitated using an Imaging Densitometer (BioRad). The ratios of the target band (408 bp) intensity to the competitor band (307 bp) intensity represented the relative levels of *NTHK1* expression. **B** Northern analysis of *NTHK1* expression. Total RNA (30 µg) from the samples in **A** was subjected to Northern hybridization with a probe prepared using the 3' 0.4-kb *NTHK1* cDNA fragment (Fig. 1A). Washing was performed in 2× and 1× SSC plus 0.1% SDS at 60°C, each for 20 min. The signals on the film were quantitated using an Imaging Densitometer (BioRad)

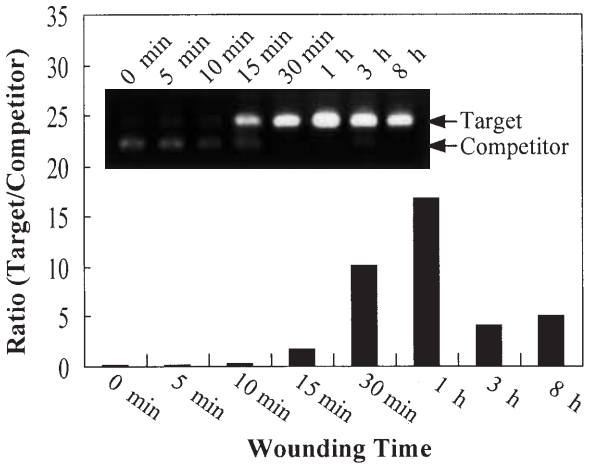


Fig. 5 *NTHK1* expression in leaves upon wounding and floating in solution. Detached leaves were cut into pieces of1–2 cm^2 and immediately floated in 0.5× MS for the indicated times with shaking at 150 rpm. Total RNA was isolated and subjected to competitive RT-PCR assay as in Fig. 4A

the receiver domain. These two domains were identified in NTHK1 (Fig. 1A, B). The His kinase domain contained five boxes, namely H, N, G1, F and G2. H and N boxes were substantially conserved among the six proteins compared (Fig. 1B), while the other three boxes were highly divergent among the proteins. The putative autophosphorylated residue His was present at position 378 of NTHK1. The receiver domain of NTHK1 was also conserved and the Asp residue at position 689 was the putative phosphate receiver (Fig. 1B). Although the homologous His and Asp are identified, whether these amino acids are actually phosphorylated is unknown.

Southern-hybridization analysis was performed to investigate the genomic organization of the *NTHK1* gene. The result is shown in Fig. 2 where it can be seen that 2–5 apparent hybridization signals were identified in each lane. Considering the occurrence of one *Hind*III site and one *Eco*RI site in the *NTHK1* cDNA, it is likely that at least two copies of the *NTHK1* gene were present in the genome of amphidiphoid tobacco although the interpretation of such data is not simple. The weaker signals may represent other homologous genes.

Spatial and temporal expression of the *NTHK1* gene in tobacco

RNA in situ hybridization was conducted to examine the expression of the *NTHK1* gene. Basically, *NTHK1* was expressed in the reproductive organs but not in the vegetative organs tested (Fig. 3). In the stage-5 flower bud, *NTHK1* expression was observed in the sepals (Fig. 3A, a), whereas in stage-7 and stage-8 flowers the expression can be seen in the petals and was very strong in the carpels (Fig. 3B, b, C, c). In stage 11 or a later flower, *NTHK1* was mainly expressed in the stamens, stigma and the wall of the ovary, but not in the developing ovules (Fig. 3D, d, E, e, F, f). It is also strongly expressed in the developing embryo (Fig. 3G, g, H, h). During anther development, *NTHK1* was observed in vascular tissue, pollen mother cells, tetrads and pollen grains (Fig. 3I, i, 3J, j, K, k, L, l). It is also expressed in the trichomes of the petal and ovary (Fig. 3M, m, N, n). In the stem and leaf, *NTHK1* expression was not detected (Fig. 3O, o, P, p).

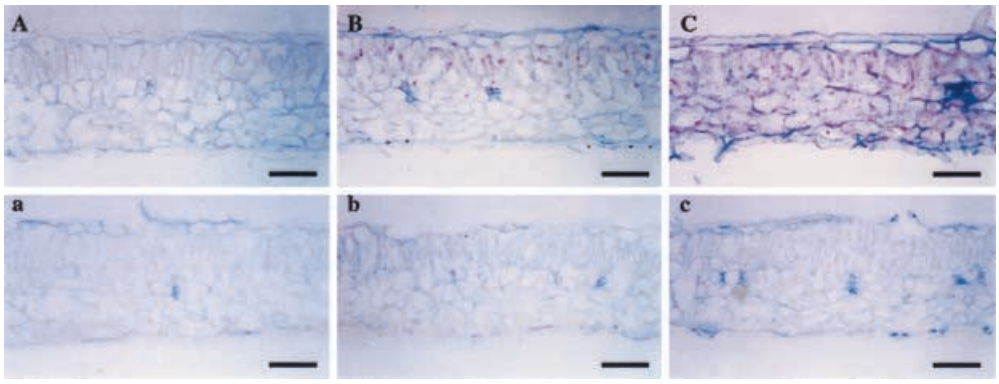


Fig. 6 In situ localization of *NTHK1* expression in wounded tobacco leaf tissues. (**A**), (**B**) and (**C**) indicate sections hybridized with an antisense RNA probe. (**a**), (**b**) and (**c**) indicate sections hybridized with a sense RNA probe. The purple color indicates a positive signal. *Bars* represent 100 µm. (**A**) and (**a**) Cross-section of a untreated control leaf. (**B**) and (**b**) Cross-section of one of the leaf pieces that have been cut and incubated for 1 h as treated in Fig. 4A (**C**) and (**c**) Cross-section of one of the leaf pieces that had been cut and floated in 0.5× MS for 1 h, with shaking at 150 rpm

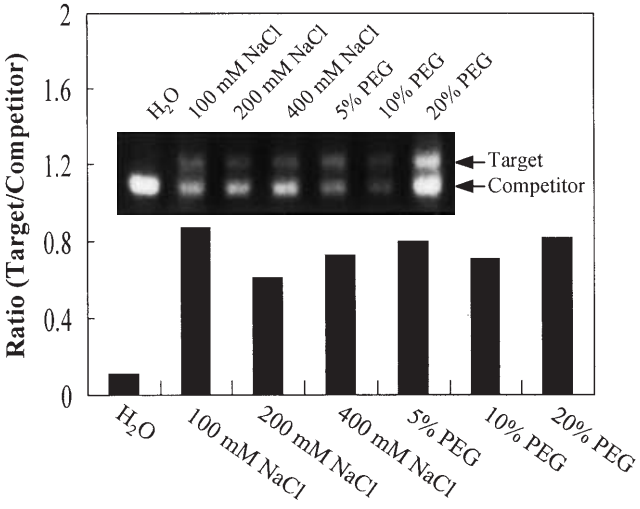


Fig. 7 Effects of salt and drought stress on *NTHK1* expression in tobacco seedlings. Tobacco seedlings at the five-leaf stage grown in pots containing vermiculite were irrigated twice with soil-saturating volumes of water or different concentrations of NaCl and PEG 6000 (w/v) at 24 h, and the second irrigation was performed 5 h before the harvest. Total RNA was isolated and subjected to competitive RT-PCR. The concentration of competitor was 0.5 pg per 30 µl of the PCR reaction mixture; others were the same as in Fig. 4A

NTHK1 expression was inducible upon wounding and other stresses

Ethylene plays important roles in stress responses in addition to its functions in development (Abeles et al. 1992; Morgan and Drew 1997). Since *NTHK1* encoded a putative ethylene-receptor homolog, its expression in response to wounding and other stresses was investigated using a competitive RT-PCR assay. In this assay, a 408-bp target fragment from samples at the 3'-end of the *NTHK1* cDNA was co-amplified with a 307-bp competitor DNA using the same set of primers. The ratio of the target-band intensity to competitor-band intensity represented the relative levels of *NTHK1* expression. It can be seen from Fig. 4A that *NTHK1* expression appeared 1 h after the wounding (cutting) was initiated and reached higher levels after 3-h and 8-h treatment. A Northern-hybridization analysis was also performed and a similar induction of *NTHK1* expression was observed (Fig. 4B). This result indicated that the competitive RT-PCR assay reported relative levels that are comparable to those detected by Northern hybridization. When the cut leaf pieces were floated in 0.5× MS solution, with shaking, *NTHK1* was induced within 15 min and reached a peak 30 min to 1 h after the treatment was started (Fig. 5). Thereafter, *NTHK1* expression tended to decrease but was still at a high level. Compared with wounding without floating in solution, the induction in this treatment was faster and stronger. The cut leaf pieces with or without floating were also subjected to RNA in situ hybridization analysis. The results are presented in Fig. 6 where it is shown that there was no *NTHK1* expression in the untreated leaf (Fig. 6A, a). In cut-leaf pieces incubated without floating, *NTHK1* was expressed at a low but significant level (Fig. 6B, b), and the expression was mainly localized in the upper palisade parenchyma cells. By comparison in cut-leaf pieces floated in solution, *NTHK1* expression spread almost all over the cells of the leaf including both the palisade and the spongy parenchyma (Fig. 6C, c). The expression levels revealed by in situ hybridization (Fig. 6) were consistent with the results from competitive RT-PCR (Figs. 4A, 5).

Because ethylene has been reported to be involved in salt and drought stresses (Morgan and Drew 1997), the expression of *NTHK1* was investigated in tobacco seedlings in response to these treatments. It can be seen from Fig. 7 that *NTHK1* expression was induced in all the NaCl and PEG treatments but not in the water-control treatment, suggesting that it may be involved in the plants responses to salinity and drought stresses.

Discussion

In the present study, a hybrid two-component gene *NTHK1* was isolated from tobacco. Its predicted amino-

acid sequence showed similarity to many plant ethylene-receptor homologs, especially the LeETR4 protein from tomato (Tieman and Klee 1999). Considering the high homology (87%) of NTHK1 with LeETR4, but less homology with others, the two proteins may represent a novel class of ethylene receptor in addition to the five members reported in *Arabidopsis* (Chang et al. 1993; Hua et al. 1998; Sakai et al. 1998). Structurally, NTHK1 contained three domains, namely the N-terminal domain, the His kinase domain and the receiver domain. The N-terminal domain had four hydrophobic regions. The first three (regions I, II and III) were putative transmembrane segments highly conserved among the proteins compared (Fig. 1B) and were essential for ethylene-binding as demonstrated in ETR1 (Schaller and Bleecker 1995). By expressing the NTHK1, with or without the transmembrane segments, in an insect cell line, and with a GFP protein ligated to the C-terminal of the NTHK1, we have observed the plasma-membrane location of NTHK1 on a confocal microscope (Xie C, Zhang JS and Chen SY, unpublished data). The fourth hydrophobic region in NTHK1 is probably a signal peptide based on the prediction method of Nielsen et al. (1997). Similar regions were also found in LeETR4, LeETR5, EIN4 and ETR2, but not ETR1. This signal peptide may lead NTHK1 and other ethylene-receptor homologs to different parts of the cell, and hence function under conditions that are different from that of ETR1. The specificity of the signal sequences has been suggested by Zheng and Gierasch (1996). In the His kinase domain of NTHK1, a putative phosphorylation site was identified in the H box at residue His378, which was conserved among LeETR4, EIN4 and ETR1, but not in LeETR5 and ETR2 (Fig. 1B). In the other four boxes, the N box showed limited similarity with the corresponding part of the six proteins compared, while the G1, F and G2 boxes were more divergent among the different proteins. These boxes may be responsible for ATP-binding as in the case of the bacterial chemosensor CheA and osmosensor EnvZ (Tanaka et al. 1998; Bilwes et al. 1999). In the receiver domain of N THK1, a conserved Asp689 residue was identified, which may accept the phosphoryl group from His378. The exact functions of these predicted domains in NTHK1 remain to be investigated.

NTHK1 expression was studied during flower organ development. Its transcripts appeared first in the sepals, and then the signals disappeared from the sepals and went into the inner whorls such as the petals, stamens and carpels. Later, *NTHK1* expression in the petals also disappeared and only those in the stamens and carpels were maintained. The reason for this inner-whorl movement of *NTHK1* expression is not known. It is possible that NTHK1 may function in cell elongation of the flower organ but not the initiation process. In addition, *NTHK1* may play important roles in pollen formation and embryo development since its expression was observed in both of these processes. No *NTHK1* expression was seen in stems and leaves. Other ethylene-receptor homolog genes have also been reported to be expressed in the reproductive organs. *LeETR4* and *LeETR5* from tomato were found to have high levels of transcripts in reproductive tissues, and low levels in vegetative tissues (Tieman and Klee 1999). In *Arabidopsis*, *ETR1* showed expression in floral-organ primordia, the locules of the anthers, the developing carpels, and the ovules and funiculi in late carpel development. Its expression in etiolated seedlings and leaves was at a low level. Similar expression patterns were observed for *ETR2* and *EIN4* (Hua et al. 1998; Sakai et al. 1998). Higher expressions of *NTHK1* and other ethylene-receptor homologs in reproductive organs may be consistent with the roles played by ethylene in these organs because ethylene has been reported to be involved in flowering, pollination and fruit ripening (Tsao 1988; Abeles et al. 1992).

Wounding enhanced ethylene production (Boller and Kende 1980). Ethylene has been reported to mediate the wounding responses (O'Donnell et al. 1996). In the present study, wounding by cutting induced *NTHK1* expression. It is possible that wounding induced ethylene production and *NTHK1* expression at the same time, and that the interaction of ethylene with NTHK1 regulated the downstream event, e.g. the MAPK cascade, and finally led to the stress responses. MAPK has been identified in tobacco and other plants and was rapidly induced by wounding (Seo et al. 1995; Bogre et al. 1997; Zhang and Klessig 1998). A link between two-component systems and the MAPK cascade has been established in the yeast osmosensing pathway (Posas et al. 1996) and has been suggested in the *Arabidopsis* ethylene signal-transduction pathway (Chang and Stewart 1998).

Floating of the cut leaf pieces in 0.5× MS solution (or phosphate buffer, water), with shaking, activated a relatively rapid and strong *NTHK1* expression when compared with the control treatments without floating (with or without shaking) (Figs. 4, 5 and data not shown). This phenomenon was confirmed by the in situ hybridization results (Fig. 6). It is speculated that, upon wounding, one or several unidentified factors may be immediately released from the wound site, and these factors then triggered *NTHK1* induction in adjacent cells. In air, this process could be slow, whereas in a shaking solution it may be fast due to the close contact of these factors with the cells. The palisade parenchyma cells in leaf tissues may be the first batch of cells that perceive the signals and then spread the expression signal to the spongy cells (Fig. 6B, C). Although the *NTHK1* mRNA was up-regulated by wounding, whether or not the amount of the NTHK1 protein and its activity were changed remains to be investigated.

While NTHK1 may function in the mechanical-wounding responses, other ethylene receptors may act in pathogen responses. Lund et al. (1998) found that the tomato ethylene-receptor mutant Nr was healthier and tolerated the infection of virulent bacterial and fungal pathogens in comparison to the wild-type plant. In contrast, transgenic tobacco harboring the dominant *Arabidopsis* ethylene-receptor allele *etr1–1* showed increased susceptibility to infection by two bacterial and one fungal species (Knoester et al. 1998).

Besides wounding, plants experienced other stresses like salt and drought stress. In response to these stresses,

lots of genes were induced and some of them have been isolated and characterized in our laboratory (Zhang et al. 1996, 1999a; Li et al. 1999; Li and Chen 2000). However, most of the studied genes were functional, but not regulatory, genes. In the present study, the ethylene-receptor homolog gene *NTHK1* was found to be inducible in tobacco seedlings when treated with NaCl and PEG, indicating a possible role for NTHK1 in the signal-transduction pathway of salt and drought responses. Considering its up-regulation by wounding, *NTHK1* may function in multiple stress responses. Recently, Hua and Meyerowitz (1998) have demonstrated that the ethylene-receptor family negatively regulates the ethylene responses. Under normal conditions (no ethylene), the ethylene responses are repressed by the receptors. When ethylene is present, the receptors are inactivated, and the ethylene responses result. Because many stresses lead to responses similar to the ethylene responses, it may be beneficial to the plants to produce more receptors upon stress, and therefore the stress responses are not easily induced due to the fact that higher ethylene concentrations are needed to inactivate the receptors. Further investigations will focus on the transgenic plants that over- or under-express NTHK1 in order to study its functions.

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