Ethylene receptor is the first component of ethylene signaling that regulates plant growth, development and stress responses. Previously, we have demonstrated that tobacco subfamily 2 ethylene receptor NTHK1 had Ser/Thr kinase activity, and overexpression of NTHK1 caused large rosette, reduced ethylene sensitivity, and increased salt sensitivity in transgenic Arabidopsis plants. Here we found that N-box mutation in the NTHK1 kinase domain abolished the kinase activity and led to disruption of NTHK1 roles in conferring reduced ethylene sensitivity and salt sensitive response in transgenic Arabidopsis plants. However, N-box mutation had partial effects on NTHK1 regulation of rosette growth and expression of salt- and ethylene-responsive genes AtNAC2, AtERF1 and AtCor6.6. Mutation of conserved residues in the H box did not affect kinase activity, seedling growth, ethylene sensitivity or salt-induced epinasty in transgenic plants but did influence NTHK1 function in control of specific salt- and ethylene-responsive gene expression. Compared with NTHK1, the tobacco subfamily 1 ethylene receptor NtETR1 had His kinase activity and played a weak role in regulation of rosette growth, triple response and salt response. Mutation of the conserved His residue in the NtETR1 H box eliminated phosphorylation and altered the effect of Ntetr1-1 on reporter gene activity. These results imply that the Ser/Thr kinase activity of NTHK1 is differentially required for various responses, and NTHK1 plays a larger role than NtETR1.

**Keywords:** Ethylene receptor • Mutation • Ser/Thr kinase activity • Salt stress response • Signaling.

**Abbreviations:** ACC, 1-aminocyclopropane-1-caboylic acid; GST, glutathione S-transferase.

**Introduction**

As a gaseous plant hormone, ethylene is an unsaturated two-carbon hydrocarbon which modulates many physiological and developmental processes in the whole life cycle of plants. It also regulates plant adaptations to environmental stresses such as chilling, wounding and pathogen attack (Abeles et al. 1992). Etiolated seedlings exhibit a typical triple response in the presence of ethylene. By using this phenotypic change, a series of mutants have been identified that have altered ethylene responses. Further analysis of these mutants leads to the identification of major components of the ethylene signaling pathway, including ethylene receptors, a MAPKKK protein CTR1, a central membrane protein EIN2 and a transcription factor EIN3, followed by a transcriptional cascade (Chang et al. 1993, Kieber et al. 1993, Hua et al. 1995, Chao et al. 1997, Hua et al. 1998, Sakai et al. 1998, Alonso et al. 1999, Bleecker and Kende 2000, Wang et al. 2002, Huang et al. 2003, Guo and Ecker 2004, Chen et al. 2005). EIN3 is quickly degraded in the absence of ethylene through an ubiquitin/proteasome pathway mediated by two F-box proteins, EBF1 and EBF2 (Guo and Ecker 2003, Potschak et al. 2003, Gagne et al. 2004, Binder et al. 2007). The EBF1 and EBF2 mRNA levels can be regulated indirectly by exoribonuclease XRN4/EIN5 (Olmedo et al. 2006, Potschak et al. 2006). In a recent study, it was discovered that ethylene inactivates CTR1 to activate the...
MKK9–MPK3/6 pathway and promote EIN3-mediated transcription in ethylene signaling (Yoo et al. 2008). Two F-box proteins ETP1 and ETP2 were also found to affect EIN2 stability (Qiao et al. 2009).

Ethylene receptors are the first components of ethylene signaling. In Arabidopsis, five members of the ethylene receptor family, including ETR1, ETR2, ERS1, ERS2 and EIN4, have been identified and these proteins are similar to the two-component histidine kinases in bacteria and yeast (Chang et al. 1993, Hua et al. 1995, Hua et al. 1998, Sakai et al. 1998, Guo and Ecker 2004, Chen et al. 2005, Hall et al. 2007). Based on structural features and sequence similarity, ethylene receptors can be divided into two subfamilies. Subfamily 1 includes ETR1 and ERS1, both of which have three transmembrane domains and highly conserved boxes in the kinase domain. Subfamily 2 includes ETR2, ERS2 and EIN4, and has putative signal sequences before three transmembrane domains plus diverged boxes in the kinase domain. Homologous genes of ethylene receptors have been identified from tomato, tobacco, rice, soybean and many other plants (Zhang et al. 2001, Klee 2004, Yau et al. 2004, Chen et al. 2005, Xie et al. 2007). Ethylene binding has been found for ethylene receptors from Arabidopsis and tomato (Schaller and Bleecker 1995, O’Malley et al. 2005, Wang et al. 2006), and ethylene negatively regulates receptor function (Hua and Meyerowitz 1998). Ethylene receptors are mainly localized in the membrane systems (Chen et al. 2002, Xie et al. 2003, Ma et al. 2006, Dong et al. 2008). Arabidopsis ETR1 receptor function can be regulated by a membrane protein RTE1 (Resnick et al. 2006, Resnick et al. 2008, Zhou et al. 2007, Dong et al. 2008). Recently, two studies have shown that ethylene receptor degradation represents a mechanism for regulation of ethylene responses (Chen et al. 2007, Kevany et al. 2007).

Arabidopsis ethylene receptor ETR1 has histidine kinase activity whereas ETR2, EIN4 and ERS2 have Ser/Thr kinase activity (Gamble et al. 1998, Moussatche and Klee 2004). Subfamily 2 ethylene receptor NTHK1 from tobacco and OsETR2 from rice also have Ser/Thr kinase activity (Xie et al. 2003, Wuriyanghan et al. 2009). However, Arabidopsis ERS1 and tobacco NTHK2 can have Ser/Thr kinase activity or histidine kinase activity under different ionic conditions (Moussatche and Klee 2004, Zhang et al. 2004). The histidine kinase activity in ETR1 appears to have no role or have subtle roles in regulation of the ethylene response and seedling growth recovery although the histidine kinase domain of ETR1 is required for signal transduction (Wang et al. 2003, Binder et al. 2004, Qu and Schaller 2004, Cho and Yoo 2007).

Bacterial two-component histidine kinases catalyze the transfer of phosphate from ATP to a specific histidine residue, and all the histidine kinases have a conserved ATP-binding catalytic domain required for kinase activity. The catalytic domain and a dimerization domain form the kinase domain. The dimerization domain contains an H box harboring the site of autophosphorylation. The catalytic domain has four conserved motifs, the N, G1, F and G2 boxes, which are involved in ATP binding, and probably also in catalysis and phosphotransfer (Wolanin et al. 2002). Residues from the G1, F and other boxes provide the principal contacts with the adenosine moiety, whereas the N and G2 boxes contact both the adenosine moiety and Mg2+ phosphates. The ATP lid (a loop that closes over the bound ATP) interacts exclusively with the phosphates and the divalent cation (Marina et al. 2001).

Arabidopsis ethylene receptor ETR1 resembles bacterial histidine kinases and has typical motifs including H, N, G1, F and G2 boxes. Mutation of the conserved H box, G1 or G2 box leads to complete and/or partial loss of histidine kinase activity (Gamble et al. 1998, Moussatche and Klee 2004). Other ethylene receptors including subfamily 2 members have divergent motifs and mainly possess Ser/Thr kinase activity. Mutation of the H box does not affect the Ser/Thr kinase activity whereas mutation of the G1 or G2 box results in partial loss of the activity. N-box mutation appears to cause more loss of Ser/Thr kinase activity, suggesting the importance of this box in phosphorylation (Xie et al. 2003, Moussatche and Klee 2004, Zhang et al. 2004).

Tobacco subfamily 2 ethylene receptor gene NTHK1 can be induced by salt and other stresses (Zhang et al. 1999, Zhang et al. 2001). Transgenic plants overexpressing this gene have large rosette or seedlings but exhibit salt sensitivity, and ethylene precursor ACC suppresses the salt sensitivity (Cao et al. 2006, Cao et al. 2007). Ethylene receptor signaling modulates stress response through regulation of downstream genes (He et al. 2005, Cao et al. 2007). The kinase domain of NTHK1 is required for plant growth and stress response (Zhou et al. 2006). NTHK1 has Ser/Thr kinase activity and mutation of the conserved H378 in the H box does not affect the kinase activity. Removal of the ATP domain eliminates the kinase activity (Xie et al. 2003). However, mutations affecting the NTHK1 kinase activity have not been identified, and whether these mutations will alter plant growth and stress response remains unclear. In this study, we identified mutations in the N box that resulted in the complete loss of Ser/Thr kinase activity in NTHK1, and found that kinase-inactive NTHK1 fails to cause the salt sensitivity and reduced ethylene sensitivity that can be seen in NTHK1-overexpressing Arabidopsis plants. However, kinase-inactive NTHK1 still has a partial role in promotion of rosette growth and regulation of gene expression. H-box mutation did not affect the kinase activity or most of the responses. Tobacco subfamily 1 receptor NtETR1 was also characterized and we found that NtETR1 has His kinase activity. Compared with NtETR1, NTHK1 appears to have a larger role in the regulation of plant growth and stress responses.
Results

N-box mutation abolishes the kinase activity of NTHK1

Tobacco ethylene receptor NTHK1 has four transmembrane domains, a GAF (for cyclic GMP, adenyl cyclase, E1A) domain, a kinase domain containing H box, N box and other diverged boxes, and a putative receiver domain (RD) (Fig. 1A). Ser/Thr kinase activity has been identified in NTHK1 and mutation of the presumptive phosphorylation site H378 does not affect the kinase activity. However, removal of the ATP-binding domain eliminates this activity (Xie et al. 2003). We further investigated whether the other highly conserved motifs in the H box or N box of the kinase domain had any effects on NTHK1 kinase activity through alteration of dimerization or ATP binding. The highly conserved residues in these boxes were selected and mutations were introduced into the gene fragment encoding the GAF plus kinase domain (KD, amino acids 145–636), and mutant proteins were expressed (Fig. 1A). Multi-site mutations were generated because previous analysis found that mutations in the ATP-binding domain of ethylene receptor kinases cause only partial loss of their kinase activity (Moussatche and Klee 2004, Zhang et al. 2004). The mutant protein KD-mH contains five mutations including M376A, R381Q, P383A, M384A and H385Q in the H box. The KD-mN mutant contains mutations including R484Q, F486A, Q487H, M492A and G494A in the N box. These proteins were assayed for their autophosphorylation ability. Compared with the phosphorylation signal from KD, KD-mH exhibited no significant difference in signal intensity (Fig. 1B). However, KD-mN showed no 32P incorporation, suggesting that the original five residues in the N box are the most important for autophosphorylation (Fig. 1B). Inclusion of EDTA in the reaction removed Mn2+ and thus affected the ability of KD phosphorylation. Substrate phosphorylation was also examined. The kinase domain of NTHK1 can phosphorylate myelin basic protein (MBP) (Xie et al. 2003; Fig. 1B). Mutations of the H box do not affect MBP phosphorylation in KD-mH. However, mutations in the N box of KD-mN resulted in the loss of substrate phosphorylation (Fig. 1B). These results indicate that mutations in the N box disrupt NTHK1 autophosphorylation and substrate phosphorylation.

Effects of NTHK1 mutations on seedling growth

Overexpression of NTHK1 leads to large rosette or seedlings in both the transgenic tobacco and Arabidopsis plants (Cao et al. 2006, Cao et al. 2007, Zhou et al. 2006). We examined whether the NTHK1 mutations leading to kinase disruption would affect plant growth. Mutant NTHK1 genes, whose products (mN and mH) harbored N-box or H-box mutations as in KD-mN and KD-mH proteins, respectively, were introduced into Arabidopsis plants (Fig. 2A). Around 90% of the NTHK1-overexpressing lines (e.g. S10 line) exhibited rosettes larger than the Col wild type (Fig. 2B). Similarly, >80% of the mH-transgenic lines also showed large rosettes, indicating that H-box mutations did not significantly affect the roles of NTHK1 in promotion of seedling growth (Fig. 2B). On the contrary, N-box mutations in mN apparently reduced the percentage of transgenic lines that showed large rosettes, and only 22% of the lines showed rosettes slightly larger than those of Col (Fig. 2B). The remaining lines resembled the Col phenotype. This phenomenon suggests that the N box is at least partially required for rosette growth. Representative lines harboring each construct were compared for transgene expression (Fig. 2C). The NTHK1-transgenic line S10, the two mH-transgenic lines (L1 and L11) and the two
Fig. 2 Phenotype of transgenic plants harboring NTHK1 or its mutants. (A) NTHK1 and mutant versions used for plant transformation. (B) Percentage of transgenic lines that showed large rosette compared with Col. (C) Transgene expression in various transgenic lines. Real-time quantitative PCR was used for this analysis. Each datum is the average of three experiments and each experiment has three replicates. (D) Rosette phenotype of the transgenic lines and Col. S10 is a representative line of NTHK1-overexpressing plants, and the other transgenic lines are derived from the same name construct as in (A). For each construct, the first representative transgenic line in (C) is shown and this statement is also suitable for other figures where only the construct name is indicated. Totally, 11–40 transgenic lines were examined for each construct. Upper panel: comparison of rosette phenotype. Lower panel: comparison of the fifth true leaf from various transgenic lines. Four-week-old plants were used for analysis. (E) Length of the fifth true leaves of different transgenic lines. (F) Rosette size of different transgenic lines. (G) Scanning electron micrograph of the epidermal cells of the adaxial surface of the fifth leaves from the transgenic lines. For (E) and (F), the letters above each column indicate significant difference between compared pairs ($P < 0.05$). Each datum is derived from 20 plants.
mN-transgenic lines (L9 and L10) had comparable levels of expression for the corresponding transgene (Fig. 2C). The phenotype of these representative lines was also examined. The NTHK1-overexpressing line S10 and the mH-transgenic lines exhibited significantly large leaf and rosette compared with Col (Fig. 2D, E, F). The mN-transgenic plants harboring the kinase-inactive NTHK1 had smaller leaf and rosette size than the S10 plants containing the NTHK1 gene. However, the mN-transgenic plants were still significantly larger than the Col plants (Fig. 2F). These results suggest that the kinase activity of NTHK1 may play a partial role in regulation of rosette growth. The large leaf and rosette size in S10 and mH-transgenic plants were substantially correlated with the large epidermal cells of leaf as observed from the scanning electron micrograph (Fig. 2G). All these analyses indicate that N-box mutation significantly disrupts NTHK1 roles in the promotion of plant growth. However, the mutant version still has residual ability for regulation of rosette size.

N-box mutation in NTHK1 altered ethylene sensitivity and salt stress response in transgenic Arabidopsis plants

Overexpression of NTHK1 conferred reduced sensitivity to ethylene in transgenic plants (Xie et al. 2002, Zhou et al. 2006, Cao et al. 2007). We then examined whether the mutations in NTHK1 had any effect on ethylene sensitivity of transgenic Arabidopsis plants. Ethylene biosynthesis precursor ACC was used for the analysis. Similar to the NTHK1-transgenic plant S10, mH-transgenic lines (mH-L1, mH-L11) also showed reduced sensitivity to ACC (Fig. 3). However, the mN-transgenic lines (mN-L9, mN-L10) harboring NTHK1 with N-box mutation had ACC sensitivity comparable to that of Col (Fig. 3). These results indicate that N-box mutation leads to loss of reduced ACC sensitivity in transgenic plants.

Ethylene signaling affects plant salt stress response (Achard et al. 2006, Cao et al. 2006, Cao et al. 2007, Zhou et al. 2006, Wang et al. 2007, Yoo et al. 2008). Transgenic Arabidopsis plants overexpressing NTHK1 are sensitive to salt stress at seedling stage (Zhou et al. 2006, Cao et al. 2007). The effects of H-box and N-box mutations in regulation of salt stress response were investigated. Compared with the NTHK1-transgenic line S10, mutation in the H box did not affect the salt-sensitive epinasty response in mH-transgenic lines (L1, L11) (Fig. 4A, B). However, N-box mutation drastically reduced the percentage of salt-sensitive epinasty (Fig. 4B). These results indicate that N-box mutation in NTHK1 almost removed the salt sensitivity of the transgenic plants, implying that NTHK1 kinase activity is most likely to be essential for the salt-sensitive response in plants.

H-box and N-box mutations in NTHK1 differentially regulate gene expression

Ethylene receptor signaling suppresses salt-induced AtNAC2 expression but promotes salt-induced AtCor6.6 expression (He et al. 2005, Zhou et al. 2006, Cao et al. 2007). Ethylene signaling also regulates AtERF1 gene expression (Solano et al. 1998). We examined whether the H-box and N-box mutations in NTHK1 have any effects on expression of these genes in transgenic plants. In the S10 line, the intensity of AtNAC2 salt induction was reduced compared with the induction in wild-type Col. In the mH-transgenic plants, salt induction of AtNAC2 was further reduced. However, salt induction of
AtNAC2 was significantly recovered in the mN-transgenic plants, compared with induction in the S10 line (Fig. 5A, top panel). For ACC induction of the AtNAC2 gene, it was apparently reduced in S10 and slightly further reduced at 6-h treatment in the mH-transgenic plants (Fig. 5A, top panel). In the mN-transgenic plants, ACC induction of the AtNAC2 gene was largely recovered. For AtCor6.6 expression, both salt and ACC induction was moderately enhanced in the S10 line and further slightly increased in the mH-transgenic plants (Fig. 5A, middle upper panel). In the mN-transgenic plants, salt induction of AtCor6.6 was not significantly affected whereas ACC induction of this gene was reduced at the 12-h treatment compared with the corresponding induction patterns in the NTHK1-transgenic S10 line. For AtERF1 expression, salt induction was transient in Col but not transient in the S10 line, the mH-transgenic line or the mN-transgenic line (Fig. 5A, middle lower panel).

Effects of tobacco ethylene receptor mutations

Tobacco subfamily 1 ethylene receptor NtETR1 is a histidine kinase

To compare the signaling behavior between subfamily 1 and subfamily 2 members, we cloned the subfamily 1 ethylene receptor gene NtETR1 from tobacco, expressed the NtETR1 protein and its mutant versions mHis and mG2, and investigated their biochemical property (Fig. 6A, B, C). For NtETR1 protein, two versions, GKD and KD, were made, with GKD containing the GAF plus the kinase domain whereas KD contains only the kinase domain (Supplementary Fig. 1), implying that hypo-osmotic conditions also change gene expression. However, these changes should not affect gene expression during hyperosmotic salt stress and/or specific ACC treatment. All these results suggest that NTHK1 Ser/Thr kinase activity is likely required for suppression of salt and ACC induction of the AtNAC2 gene, for suppression of ACC induction of AtERF1 and for promotion of ACC induction of AtCor6.6. However, this activity appears not to be required for the promotion of salt induction of AtCor6.6. The H-box mutation may tend to strengthen NTHK1 effects on gene expression.

Arabidopsis protoplast assay was further adopted to examine the effects of H-box and N-box mutations on NTHK1 regulation of gene expression. Three effector plasmids harboring NTHK1, NTHK1 with H-box mutation (mH) and NTHK1 with N-box mutation (mN) were constructed. Three reporter plasmids containing the AtNAC2, AtERF1 or AtCor6.6 promoter-driven $\text{LUC}$ gene were also made (Fig. 5B). Fig. 5C showed that NTHK1 inhibited AtNAC2 and AtERF1 promoter activity compared with the control. H-box mutation of NTHK1 (mH) appeared not to significantly affect the activity of the two gene promoters. However, N-box mutation of NTHK1 (mN) significantly increased activity of the two promoters in comparison with NTHK1, suggesting that NTHK1 Ser/Thr kinase activity is at least partially required for inhibition of AtNAC2 and AtERF1 promoter activity. H-box and N-box mutations in NTHK1 seemed to have no effect on AtCor6.6 promoter activity in the assay (Fig. 5C).


---

**Fig. 4** Phenotype of the transgenic lines overexpressing NTHK1 or its mutants under salt stress. (A) Comparison of the transgenic plants under normal and NaCl treatment. Five-day-old seedlings were transferred onto MS medium or MS plus 130 mM NaCl. The pictures were taken after treatment for 2 weeks. The NTHK1-transgenic line S10 and the mH-transgenic line showed a severe salt-sensitive epinasty phenotype. (B) The percentage of the transgenic plants in each transgenic line that showed salt-sensitive epinasty phenotype. Values are the average of three experiments and each experiment has 30 plants.
The GK version had autophosphorylation ability in the presence of Mn$^{2+}$, but not in the presence of Mg$^{2+}$ or Ca$^{2+}$ (Fig. 6B). GST was not phosphorylated by GKD in the presence of Mn$^{2+}$ or the other two ions. Neither mHis nor mG2 proteins had any autophosphorylation ability, suggesting that NtETR1 is a histidine kinase and conserved G547 and G549 are essential for phosphorylation (Fig. 6B, C). A short version, KD, without the GAF domain also had autophosphorylation ability in the presence of Mn$^{2+}$, and may have weak ability for autophosphorylation in the presence of Mg$^{2+}$ or Ca$^{2+}$ (Fig. 6C). Because the mG2 protein cannot autophosphorylate, this protein was further incubated with KD to see whether the H351 phosphorylation site of mG2 can be phosphorylated by KD. 

Fig. 5 Effects of H-box and N-box mutations on NTHK1-regulated gene expression. (A) Regulation of AtNAC2, AtCor6.6 and AtERF1 gene expression by different versions of NTHK1 in response to salt and ACC treatments. Col, the NTHK1-transgenic line S10, the mH- and mN-transgenic lines were treated with NaCl and ACC, and total RNAs were subjected to Northern analysis. Total RNAs were stained by methylthionine chloride to confirm equal loading. (B) Schematic diagram of reporter plasmids and effector plasmids used for transient expression assay in Arabidopsis protoplasts. (C) Effects of NTHK1 mutations on gene expression as revealed by the relative LUC activity. The protoplasts were co-transfected with the reporter and effector plasmids, and the relative LUC activity indicates the effects of NTHK1 or its mutants on the promoter activity of each gene compared with the control. The values are the averages of three replicates and bars indicate SD. The experiments have been repeated independently three times and the results were consistent. Results from one experiment are presented.
showed that mG2 can not be phosphorylated by KD, suggesting that the histidine phosphorylation in NtETR1 is through an intramolecular, not an intermolecular, mechanism. mHis also can not be phosphorylated by KD (Fig. 6B).

Comparison of signaling behavior between subfamily 2 member NTHK1 and subfamily 1 member NtETR1 in plant growth, stress response and gene activation

To compare the strength of the roles of the subfamily 1 member NtETR1 and subfamily 2 member NTHK1, the NtETR1 gene was introduced into transgenic Arabidopsis plants under the control of the 35S promoter and two lines (R1 and R2) overexpressing NtETR1 were used for comparison with the S10 line overexpressing NTHK1. These lines had similar levels of gene expression for the corresponding transgene (Fig. 7A, lower panel). The two NtETR1 transgenic lines had rosette sizes similar to or slightly larger than the Col plants, but apparently smaller than the S10 line (Fig. 7A). Under salt stress, >90% of the S10 plants had salt-sensitive epinastic phenotype whereas only approximately 20% of the R1 and R2 plants with NtETR1 overexpression showed salt-sensitive epinastity (Fig. 4A, B, 7B). Ethylene sensitivity was not significantly affected in the two NtETR1 transgenic lines compared with Col whereas the S10 line had reduced sensitivity to ethylene (Fig. 7C). These results suggest that the subfamily 1 member NtETR1 may have a weaker role than the subfamily 2 member NTHK1 in effects on plant rosette growth, salt stress response and ethylene sensitivity.

Because Arabidopsis mutant etr1-1 represents the gain-of-function of subfamily 1 receptor ETR1 and has a strong phenotype including large rosette, ethylene insensitivity and salt sensitivity, it is possible that a mutation at the same position of NtETR1 may lead to a gain-of-function mutation. We then generated the Ntetr1-1 mutant protein, which has Tyr encoded by TAT at position 64 instead of the original Cys encoded by TGT, and compared the intensity of the signal output in regulation of the ATERF1 promoter activity using a protoplast assay (Fig. 7D). While NtETR1 had no effect on ATERF1 promoter activity, the mutant version Ntetr1-1 had the strongest inhibition on the reporter gene activity, which was even stronger than the NTHK1 role, suggesting that Ntetr1-1 is a gain-of-function mutation (Fig. 7E). Seven mutations were further generated in the Ntetr1-1 background to examine the effects of these mutations on signal output of the receptor (Fig. 7D). Mutation in m-mG2, which disrupts histidine kinase activity (Fig. 6), did not significantly affect Ntetr1-1 activity, suggesting that histidine kinase activity is dispensable for regulation of reporter gene activity (Fig. 7F). Mutations in m-mAlpha3 or m-mRD did not alter the receptor output either. However, mutation in m-mHis leading to the disruption of histidine phosphorylation (Fig. 6), together with the other three mutations in
Fig. 7 Comparison of the roles of NtETR1 and NTHK1 in regulation of rosette size, stress response and gene expression. (A) Comparison of the rosette of the NtETR1-transgenic lines with Col and NTHK1-transgenic line S10. Different letters above each column indicate significant difference between compared pairs (P < 0.05). Bars indicate SD and each datum is derived from 20 plants. The lower panel shows transgene expression in different transgenic plants by RT–PCR analysis. (B) The percentage of the salt-sensitive epinasty phenotype in the NtETR1-transgenic plants, S10 and Col. (C) ACC dose–response curves of the etiolated seedling length of Col and transgenic lines. (D) Schematic diagram of the reporter plasmid AtERF1-LUC and effector plasmids used for transient expression assay in Arabidopsis protoplast. ‘∗’ indicates mutation in Ntetr1-1. ‘♦’ indicates corresponding mutations in each construct in the Ntetr1-1 background. (E) Relative LUC activity driven by the AtERF1 promoter in protoplasts overexpressing control plasmid, NtETR1, NTHK1 or Ntetr1-1. (F) Roles of various mutations in Ntetr1-1 in regulation of AtERF1 promoter activity. For (E) and (F), the values are averages of three replicates and bars indicate SD. The experiments have been repeated independently three times and the results were consistent. Results from one experiment are presented.
Ser/Thr kinase activity has been identified in tobacco subfamily 2 receptors NTHK1 and NTHK2, and the four Arabidopsis ethylene receptors except ETR1 (Xie et al. 2003, Moussatche and Klee 2004, Zhang et al. 2004). However, mutations in these receptors only partially reduced but did not completely abolish the activity. In NTHK2, six-residue mutations in the N box led to an approximately 60% reduction in Ser/Thr kinase activity whereas four-residue mutations in the G2 box resulted in an approximately 24% reduction in kinase activity (Zhang et al. 2004). In ERS1, mutations in the N box caused more loss of kinase activity than the G-box mutations, and N-box mutations in ERS2 partially decreased the kinase activity (Moussatche and Klee 2004). Mutation of the conserved H384 in NTHK2, and conserved His residues in the H box of ERS1, ETR2 or EIN4 had no effects on the Ser/Thr kinase activity of these receptors (Moussatche and Klee 2004, Zhang et al. 2004). In NTHK1, mutation of the conserved H378 also did not affect kinase activity (Xie et al. 2003). In the present study, mutations of five residues in the H box of NTHK1 also exerted no effects on its phosphorylation ability. However, mutation of five residues in the N box completely abolished the Ser/Thr kinase activity of NTHK1, indicating that these residues are essential for phosphorylation. In addition, this result also demonstrates that Ser/Thr kinase activity is intrinsic to the NTHK1 protein and is not derived from other contaminating proteins. Our results are consistent with a recent report showing that mutations in the N box of rice ethylene receptor OsETR2 abolished its Ser/Thr kinase activity (Wuriyanghan et al. 2009).

The present study and our previous analysis revealed that NTHK1-overexpressing Arabidopsis plants had large rosettes, reduced ethylene sensitivity and increased salt sensitivity (Cao et al. 2007, Zhou et al. 2006). However, when N-box mutations were introduced and kinase activity was abolished in NTHK1, the transgenic plants harboring the mutant mN gene almost lost the phenotypes involving reduced ethylene sensitivity and salt sensitivity. Disruption of the Ser/Thr kinase activity also substantially suppressed the NTHK1 roles in regulation of salt- and ACC-induced AtNAC2 expression, and ACC-induced AtERF1 expression (Fig. 5). These results suggest that the Ser/Thr kinase activity of the NTHK1 ethylene receptor is most likely required for these alterations. Regarding rosette size, NTHK1 kinase activity is probably partially required because the transgenic lines harboring the kinase-inactive form of NTHK1 still have a larger rosette (Fig. 2F). Alternatively, residual kinase activity may be present in the mN mutant protein and contributed to the slight larger rosette of the mN-transgenic plants (Fig. 2F). Moreover, other domains in the mN mutant may also have roles in partial promotion of rosette growth. How the Ser/Thr kinase activity of NTHK1 affects these responses is unclear. It is possible that NTHK1 may phosphorylate the interacting protein(s) for signal transduction. Arabidopsis ETR1, with His kinase activity, can interact with CTR1 (Clark et al. 1998, Gao et al. 2003). However, the His kinase activity of ETR1 is not required for its association with CTR1 (Gao et al. 2003). Subfamily 2 ethylene receptors with Ser/Thr kinase activities may associate with other components to regulate these responses independently of CTR1 function, considering that subfamily 2 receptors appear to have weak interactions with CTR1 (Hall et al. 2007).

Although the roles of NTHK1 kinase activity can be speculated in control of seedling growth and stress response through mutation analysis, the possibility exists that the altered response in mN-transgenic plants, in comparison with those in the NTHK1-overexpressing line S10, was due not to the loss of NTHK1 kinase activity but to the loss of interaction between NTHK1 and its interacting protein(s). However, yeast two-hybrid assay disclosed that N-box mutations of NTHK1 did not significantly affect its interactions with a few interacting proteins (Y.R. Cao, S.Y. Chen and J.S. Zhang, unpublished results). This fact may imply that at least for a subset of interacting proteins, N-box mutation does not affect their interactions with NTHK1. However, the possibility can not be excluded that interaction of other proteins with NTHK1 was influenced by N-box mutation. Other mechanisms may also be involved. One of these involves the possible dimerization of NTHK1 with endogenous wild-type Arabidopsis receptors. Recently, Gao et al. (2008) have found that ethylene receptors from Arabidopsis interacted with each other through non-covalent interactions. Similar interactions with tobacco receptor may affect the phenotypic change and need to be further investigated. It should be mentioned that the phenotypes from ectopic overexpression may not reflect normal gene function. However, this possibility may be less likely in the present study since NTHK1 can cause large seedlings and salt sensitivity in both tobacco and Arabidopsis transgenic plants (Cao et al. 2006, Zhou et al. 2006, Cao et al. 2007). Introduction of the mutant NTHK1 gene in tobacco plants should reveal the exact roles of NTHK1 kinase activity in regulation of plant growth, stress response and gene expression.

Mutations in the H box of NTHK1 did not affect its roles in regulation of rosette growth, ethylene sensitivity and salt sensitivity. This result indicates that either the H box is substantially not involved in the regulation of these phenotypic changes or non-essential residues have been mutated in the
H box. However, this mutation seems to enhance the NTHK1 role in suppression of salt- and ACC-induced AtNAC2 or ACC-induced AtERF1, and promotion of the AtCor6.6 gene.

This phenomenon indicates that the H box may play an inhibitory role toward the signal output of NTHK1. Because H-box mutation did not change the kinase activity, the inhibitory role of the H box on NTHK1 signal output may not be through the regulation of kinase activity but probably through other mechanisms, e.g. dimerization. The H box-harboring domain in a sensor histidine kinase PhoQ has been found to mediate the interaction with its partner and undergo dimerization (Castelli et al. 2003).

Inhibition of ACC induction of the AtNAC2 and AtERF1 genes by NTHK1 is consistent with the negative regulation between ethylene and its receptors. However, the fact that the intensity of the ACC induction of AtCor6.6 was further enhanced seems to be in contradiction with the negative regulatory relation between ethylene and its receptors. This phenomenon may be due either to the special features of the AtCor6.6 gene or to specific mechanisms for ethylene and receptor signaling. From gene expression analysis, it appears that the ethylene receptor function is stronger in regulation of ethylene response of the marker genes than the regulation of the salt response of these genes.

Two sets of ethylene receptors have been studied for their biochemical properties, with one set from tobacco including NtETr1, NTHK1 and NTHK2 and another set from Arabidopsis plants. Subfamily 1 members ETR1 and NtETr1 have His kinase activity whereas subfamily 2 members NTHK1, NTHK2 and the remaining Arabidopsis members mainly have Ser/Thr kinase activity. Different kinase activities may imply different signaling pathways and/or differential signal output for these receptors in regulation of plant growth and stress response. In the present study, Ser/Thr kinase activity of NTHK1 may play a larger role than His kinase activity of NtETr1 in suppression of AtERF1 promoter activity (Fig. 7E). Additionally, NTHK1 appears to play a larger role than NtETr1 in promotion of rosette size, generation of salt-sensitive epinasty response and reduction of ethylene sensitivity. In line with this analysis, tobacco subfamily 2 members LeETR4 and LeETR6 also have strong roles in regulation of the ethylene response when their expression was reduced in transgenic plants (Tieman et al. 2002). In contrast, Arabidopsis subfamily 1 receptors seems to play more important roles than subfamily 2 receptors in determining ethylene response as judged from the rescue of the etr1ers1 double mutant and the subfamily 2 receptor triple mutant by subfamily 2 and subfamily 1 members, respectively (Wang et al. 2003). The discrepancy in the roles of subfamily 1 and subfamily 2 members may reflect the divergence of the ethylene signaling pathway at different levels, and different plant species may have adopted different mechanisms for delicate regulation of ethylene signaling.

In the background of Ntetr1-1, which harbors a gain-of-function mutation and causes repression of the AtERF1 reporter gene, abolition of H351 phosphorylation in m-mHis significantly further reduced the reporter gene activity (Fig. 7F), indicating that H351 phosphorylation regulates receptor signal output. This observation appears to be consistent with the fact that mutation of the H box in NTHK1 also altered NTHK1-regulated gene expression (Fig. 5A), suggesting that modification of the H box in ethylene receptors affects signaling intensity.

Taken together, we have identified the N-box mutation that disrupts the Ser/Thr kinase activity of the tobacco ethylene receptor NTHK1, and demonstrated that this mutation, and possibly the inactivated kinase activity, differentially regulated NTHK1 roles in plant growth, salt response and gene expression. Compared with the subfamily 1 member NtETr1 with His kinase activity, NTHK1 with Ser/Thr kinase activity may play a larger role in the responses tested. Further studies should reveal more about the role of kinase activity in ethylene receptor signaling.

Materials and Methods

Plant materials and treatments

Seeds of Arabidopsis (Arabidopsis thaliana, ecotype Col-0) and all of the transgenic plants used in this study were sterilized, stratified and germinated in plates or grown in pots at 23°C under continuous light following previous description (Zhou et al. 2006, Cao et al. 2007).

For salt stress treatment, 5-day-old seedlings from wild-type Arabidopsis (Col) and the transgenic lines were transferred onto Murashige and Skoog (MS) medium plus 130 mM NaCl. Each plate was divided into several equal regions to grow the seedlings. After around 7 d, the salt-sensitive epinasty phenotype in NTHK1-transgenic line S10 was observed (Cao et al. 2007). The occurrence of this phenotype in other compared lines was also examined and the percentage was calculated after salt treatment for 2 weeks. To examine gene expression, 12-day-old seedlings were immersed in water, 130 mM NaCl or 10 µM ACC for various times, collected and stored at –80°C for total RNA isolation.

For the triple-response test, the seeds were sown on MS medium containing various concentrations of ACC. The plates were placed at 4°C for 3 d, exposed to light for 6 h and incubated in the dark for 5 d at 23°C. The total length of the seedlings, including the hypocotyls and roots, was measured and calculated. At least 30 seedlings were measured for each data point.

Construction of vectors and generation of Arabidopsis transgenic plants

To express different mutated versions of tobacco ethylene receptor gene NTHK1 in transgenic Arabidopsis, DNA
fragments encoding NTHK1 with mutation in the H box (mH), NTHK1 with mutation in the N box (mN), were amplified from the original NTHK1 gene (Zhang et al. 2001). The mutant version mH of NTHK1 has multi-site mutations at the H box, with Ala(GCG), Gln(CAA), Ala(GGC), Ala(GCG) and Gln(CAG) at positions 376, 381, 383, 384 and 385 instead of the original Met(ATG), Arg(AGA), Pro(CCC), Met(ATG) and His(CAC), respectively. The mN mutant has multi-site mutations at the N box, with Gln(CAA), Ala(GCT), His(CAT), Ala(GGC) and Ala(GCC) at positions 484, 486, 487, 492 and 494 instead of the original Arg(AGA), Phe(XXX), Gln(CAA), Met(ATG) and Gly(GGG), respectively. The two sets of mutations were generated by using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and confirmed by sequencing. Tobacco NtETR1 gene (EU620576) was cloned based on a previous sequence (ETR1 homolog, AF022727) using the primers 5′-GCCGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCATGCTGAAAAAGTTCTGCCTCTTGG-3′. The present NtETR1 has 22 residues different from the published sequence (AF022727) and is closer to Arabidopsis ETR1. All of the DNA fragments were digested with BamHI and KpnI, and inserted into the BamHI–KpnI site of the pBIN438 binary vector. These constructs were transformed into Arabidopsis (Col) according to previous descriptions (Cao et al. 2007). Homozygous lines (T3 or later) with higher transgene expression were used for further analysis.

The epidermal cells were observed by scanning electron microscope based on the previous method (Cao et al. 2007).

RNA isolation, northern hybridization and reverse transcription–PCR

Total RNA isolation and hybridization were performed following the description by Zhang et al. (1999). Probes were labeled with [α-32P]dCTP by the random-priming method. The signal was detected by Typhoon TRIO Variable Mode Imager (GE Healthcare Life Sciences). The gene-specific templates were amplified by PCR with primers 5′-ATCTTCTTATG-3′ and 5′-TGCAAGAACCTCGCAAATCC-3′ for AtActin2, 5′-ACATCAAAAACGATTTTAC-3′ and 5′-GAACTTAAACTAGATTTTGTTG-3′ for AtCor6.6 and 5′-CCCCATCTCCGGCTCTTCTCAC-3′ and 5′-CACCAGTCCCCACTATTTTACC-3′ for AtERF1. These primers were also used for reverse-transcription (RT)–PCR analysis of water treatment samples in Supplementary Fig. 1. The AtActin2 gene was amplified as a control with primers ATACTIN2F (5′-TGTTGCTGAGAGATCCAG-3′) and ATACTIN2R (5′-TGTTAAGCGATCCTTGAC-3′). PCR was performed at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s for 32–35 cycles. For the RT–PCR analysis in Fig. 7A, 5′-ATGGATTGAATCTGGTTGAT-3′ and 5′-CTAGGAGTAAA GAATAACTCC-3′ were used for examination of NtETR1; 5′-TGTTGCTTGGTCTCTG-3′ and 5′-TCAGGAATTCG-3′ were used for NTHK1. Two biological replicates were performed for expression analysis and the results were consistent. One set of the results was presented.

Real-time quantitative PCR

Total RNA was reverse-transcribed into cDNA with MMLV (Promega). The cDNAs were used as templates in real-time PCR for examination of NTHK1 and other truncated NTHK1 genes in different transgenic lines. The specific primers for NTHK1 or its truncated form were 5′-TGGTGCTCTTGGA TCTCGCT-3′ and 5′-TCAGCAATTCGGGCAGCAG-3′. AtActin primers were 5′-GGAAAAAGGCTCTTGAGTAACT-3′ and 5′-TGTAACGATTCTGGCAC-3′. Real-time PCR was performed on a Chromo 4 Real-time PCR Detector (Bio-Rad). The total volume of the PCR reaction was 25 µl, containing 1× PCR buffer, 0.5 µl SYBR GREEN I, 0.2 mM dNTPs, 2 µl cDNAs, 0.2 µM of each primer and 1 U Taq polymerase. The PCR mixtures were preheated at 94°C for 5 min, followed by 40 cycles of amplification (94°C 30 s, 58°C 30 s, 72°C 30 s, 80°C plate read). A final extension step was performed at 72°C for 5 min. The real-time PCR results were analyzed using Opticon Monitor™ analysis software 3.1 (Bio-Rad). Each data point was the average from three experiments and each experiment had three replicates.

Expression of NTHK1 and NtETR1 proteins

For expression of different truncated versions of NTHK1 and NtETR1 as GST fusions in yeast (Schizosaccharomyces pombe), the pEsp2 vector (Stratagene, La Jolla, CA) was used. DNA fragments corresponding to the NTHK1 GAF plus kinase domain (NTHK1-GKD, amino acids 145–636), NtETR1 GAF plus kinase domain (NtETR1-GKD, amino acids 105–611), NtETR1 kinase domain (NtETR1-KD, amino acids 316–611) were amplified from the NTHK1, NTHK1-mH, NTHK1-mN and NtETR1 plasmids, respectively. The primers 5′-AGGGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′ were used for NTHK1-KD, NTHK1-KD-mH, NTHK1-KD-mN and NtETR1, respectively. The primers 5′-AGGGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′ were used for NTHK1-KD, NTHK1-KD-mH, NTHK1-KD-mN and NtETR1-KD, respectively. The primers 5′-AGGGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′ were used for NTHK1-KD, NTHK1-KD-mH, NTHK1-KD-mN and NtETR1-KD, respectively. The primers 5′-AGGGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′ were used for NTHK1-KD, NTHK1-KD-mH, NTHK1-KD-mN and NtETR1-KD, respectively. The primers 5′-AGGGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′ were used for NTHK1-KD, NTHK1-KD-mH, NTHK1-KD-mN and NtETR1-KD, respectively.

Two mutant versions (NtETR1-mHis, NtETR1-mG2) of NtETR1 as GST fusions in yeast were generated by using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and confirmed by sequencing. Tobacco NtETR1 gene (EU620576) was cloned based on a previous sequence (ETR1 homolog, AF022727) using the primers 5′-GCCGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′. The present NtETR1 has 22 residues different from the published sequence (AF022727) and is closer to Arabidopsis ETR1. All of the DNA fragments were digested with BamHI and KpnI, and inserted into the BamHI–KpnI site of the pBIN438 binary vector. These constructs were transformed into Arabidopsis (Col) according to previous descriptions (Cao et al. 2007). Homozygous lines (T3 or later) with higher transgene expression were used for further analysis.

The epidermal cells were observed by scanning electron microscope based on the previous method (Cao et al. 2007).

RNA isolation, northern hybridization and reverse transcription–PCR

Total RNA isolation and hybridization were performed following the description by Zhang et al. (1999). Probes were labeled with [α-32P]dCTP by the random-priming method. The signal was detected by Typhoon TRIO Variable Mode Imager (GE Healthcare Life Sciences). The gene-specific templates were amplified by PCR with primers 5′-GCCGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′. The present NtETR1 has 22 residues different from the published sequence (AF022727) and is closer to Arabidopsis ETR1. All of the DNA fragments were digested with BamHI and KpnI, and inserted into the BamHI–KpnI site of the pBIN438 binary vector. These constructs were transformed into Arabidopsis (Col) according to previous descriptions (Cao et al. 2007). Homozygous lines (T3 or later) with higher transgene expression were used for further analysis.

The epidermal cells were observed by scanning electron microscope based on the previous method (Cao et al. 2007).

RNA isolation, northern hybridization and reverse transcription–PCR

Total RNA isolation and hybridization were performed following the description by Zhang et al. (1999). Probes were labeled with [α-32P]dCTP by the random-priming method. The signal was detected by Typhoon TRIO Variable Mode Imager (GE Healthcare Life Sciences). The gene-specific templates were amplified by PCR with primers 5′-GCCGGATCCATGGATTGAAGAAGGAAAGGATCTGTACGGTAAC-3′ and 5′-GCCGGATCCCTAGGATTGAAGAAAGGAAAGGATCTGTACGGTAAC-3′. The present NtETR1 has 22 residues different from the published sequence (AF022727) and is closer to Arabidopsis ETR1. All of the DNA fragments were digested with BamHI and KpnI, and inserted into the BamHI–KpnI site of the pBIN438 binary vector. These constructs were transformed into Arabidopsis (Col) according to previous descriptions (Cao et al. 2007). Homozygous lines (T3 or later) with higher transgene expression were used for further analysis.

The epidermal cells were observed by scanning electron microscope based on the previous method (Cao et al. 2007).
Mutagenesis kit and confirmed by sequencing. Finally, the PCR products for NTHK1-KD, NTHK1-KD-mH, NTHK1-KD-mN, NtERTR1-GKD, NtERTR1-mHis, NtERTR1-mG2 and NtERTR1-KD were digested with BamHI and Nhel, cloned into the yeast expression vector pEsp2, and confirmed by sequencing. The recombinant plasmids were transformed into S. pombe SP-Q01 yeast strain (Stratagene). The fusion proteins were expressed and purified as described (Xie et al. 2003, Zhang et al. 2004).

**Phosphorylation assay**

Phosphorylation was performed in a 25 µl kinase assay buffer [50 mM Tris–HCl pH 7.6, 50 mM KCl, 2 mM DTT, 10% (v/v) glycerol] containing 0.5–1 µg GST fusion proteins in the presence of 5 mM MnCl₂, MgCl₂, or CaCl₂ (Xie et al. 2003, Zhang et al. 2004). Phosphorylation was initiated by adding 25 µCi of [γ-32P]ATP (30 Ci mmol⁻¹), incubated at 22°C for 40 min and terminated by the addition of EDTA to a final concentration of 10 mM. NTHK1-KD, NTHK1-KD-mH or NTHK1-KD-mN was also incubated under phosphorylating conditions with MBP to test substrate phosphorylation.

**Transient assay of the ethylene receptor activity in Arabidopsis protoplast system**

Arabidopsis protoplast isolation and transfection were based on our previous descriptions (Liao et al. 2008). For co-transfection assays, we used 6 µg of reporter plasmid containing the firefly luciferase (LUC) gene and 6 µg of effector plasmid for each PEG transfection. For normalization values, 0.5 µg of pPTRL that contained 35S::Renilla LUC for each PEG transfection. We used 6 µg of reporter plasmid containing pPTRL as internal controls. After culturing for 16 h, luciferase assays were performed with the Promega Dual-luciferase reporter assay system and the GloMax™ 20-20 luminometer. The ratio of the reporter firefly LUC activity to Renilla LUC activity was used as the relative activity of the effector gene.

Reporter plasmids AtNAC2-LUC, AtERF1-LUC and AtCor6.6-LUC were made from the original LUC construct (Ohta et al. 2000). The promoter regions of AtNAC2, AtERF1 and AtCor6.6 were amplified by PCR, digested by PstI and Sall, and inserted into the PstI–Sall site of the LUC vector. For AtNAC2, the primers are 5'-CGCGTTCAGATCTTTGCTC GTTTTAAGTCAGA-3' and 5'-GGGCAGACTTATCCCTA ATAGGTTTCTAAAAA-3'. For AtERF1, the primers are 5'-CGCGTTCAGATCTTTGCTC GTTTTAAGTCAGA-3' and 5'-CGCGTTCAGATCTTTGCTC GTTTTAAGTCAGA-3'.

To construct the effector plasmids, DNA fragments encoding NTHK1, NTHK1 with multi-site mutation in the H box (mH), NTHK1 with multi-site mutation in the N box (mN) and NtERTR1 were amplified from the original NTHK1, NTHK1-mH, NTHK1-mN and NtERTR1 plasmids, respectively, and inserted into the pUC plasmid. All these genes were driven by the 35S promoter. The primers 5'-CGCGTTCAGATCTTTGCTC GTTTTAAGTCAGA-3' and 5'-CGCGTTCAGATCTTTGCTC GTTTTAAGTCAGA-3' were used for NTHK1, NTHK1-mH and NTHK1-mN. For NtERTR1, 5'-CGCGTTCAGATCTTTGCTC GTTTTAAGTCAGA-3' and 5'-CGCGTTCAGATCTTTGCTC GTTTTAAGTCAGA-3' were used.

A mutant version (Ntetr1-1) of NtERTR1, which has Tyr (encoded by TAT) at position 64 instead of the original Cys (encoded by TGT), was generated by using the QuickChange Site-Directed Mutagenesis kit and confirmed by sequencing. In the background of Ntetr1-1, other mutations were further introduced. The m-mDimer has Gly-Asn-Gly (encoded by GGGCTTGAGCC) at positions 3–5 instead of the original Cys-Asn-Cys (encoded by GTGACTGCG). The m-mAlpha3 has Phe-Ile-Met-Pro-Pro (encoded by TTTAAAATGCCCA-CCA) at positions 145–149 instead of the original Val-Arg-Met-Leu-Thr (encoded by GTGAGATGCTAACA). The m-mBeta1 has Phe-Trp-Lys-Pro-Pro (encoded by TTTGTGAAGCCCTCCA) at positions 161–165 instead of the original Ile-Leu-Lys-Thr-Thr (encoded by ATTTGAAGACTACA). The m-mAlpha5 has Glu-Pro-His-Pro-Glu (encoded by GAACCCCATCCGCC) at positions 297–301 instead of the original Val-Ala-Asp-Gln-Val (encoded by GTAGCCGATCGTAA). The m-mHis has a Gln (CAG) at position 351 instead of the original His (CAT). The m-mG2 has Ala-Leu-Ala (encoded by GGGCTTGCC) at positions 547–549 instead of the original Gly-Leu-Gly (encoded by GGGCTTGCC). The m-mRd has Pro-Arg-Phe-Asp-Glu (encoded by CCACCGTT CGATGA) at positions 634–638 instead of the original Leu-Gly-Cys-Asp-Val (encoded by CTAGGGTGCGATGA).

**Statistical analysis**

The data were analyzed by one-way ANOVA with the SPSS program (version 10.0).

**Supplementary data**

Supplementary data are available at PCP online.

**Acknowledgments**

This work was supported by the National Natural Science Foundation of China (90717005), the National Basic Research Program of China (2006CB100102), the project from Chinese Academy of Sciences (KSCXZ-YW-N-010) and the National High Tech Project (2006AA10A111).

**References**


(Received April 28, 2009; Accepted July 14, 2009)