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RESEARCH PAPER

AtTRP1 encodes a novel TPR protein that interacts with the ethylene receptor ERS1 and modulates development in *Arabidopsis*

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Abstract

***Arabidopsis* AtTRP1 is an orthologue of SlTPR1, a tomato tetratricopeptide repeat protein that interacts with the tomato ethylene receptors LeETR1 and NR in yeast 2-hybrid assays and *in vitro*, and modulates plant development. AtTRP1 is encoded by a single copy gene in the *Arabidopsis* genome, and is related to TCC1, a human protein that competes with Raf-1 for Ras binding, and distantly related to the immunophilin-like FK-binding proteins TWD1 and PAS1. The former is involved in auxin transport and the latter is translocated to the nucleus in response to auxin. AtTRP1 interacted preferentially with the *Arabidopsis* ethylene receptor ERS1 in yeast two-hybrid assays. This association was confirmed by *in vivo* co-immunoprecipitation. AtTRP1 promoter–GUS was highly expressed in vascular tissue, mature anthers, the abscission zone, and was induced by ACC. Overexpression of AtTRP1 in wild-type *Arabidopsis* resulted in dwarf plants with reduced fertility, altered leaf/silique morphology, and enhanced expression of the ethylene responsive gene *AtChitB*. Exogenous GA did not reverse the dwarf habit. Etiolated transgenic seedlings overexpressing AtTRP1 displayed enhanced sensitivity to low ACC and this was correlated with the transgene expression. Seedlings overexpressing AtTRP1 at high levels exhibited shortened and swollen hypocotyls, inhibited root growth, and an altered apical hook. Plants overexpressing AtTRP1 also showed a reduced response to exogenous IAA and altered expression of a subset of auxin early responsive genes. These results indicated that overexpression of AtTRP1 affects cross-talk between ethylene and auxin signalling and enhances some ethylene responses and alters some auxin responses. A model for AtTRP1 action is proposed.**

Key words: *Arabidopsis*AtTRP1, auxin response, development, ethylene signalling, tetratricopeptide repeat protein.

Introduction

Ethylene regulates many aspects of plant growth and development, including flower development and sex determination, fruit ripening, abscission, senescence, and responses to biotic and abiotic stresses (Abeles *et al.*, 1992). Ethylene has dramatic effects on plant growth habit, such as the classic triple response of exaggerated apical hook, swollen hypocotyl, and inhibited root growth displayed by etiolated seedlings (Guzman and Ecker, 1990). Ethylene biosynthesis occurs via the Yang pathway (Yang and Hoffmann, 1984) involving two key biosynthetic enzymes, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO), encoded by differentially expressed multigene families (Holdsworth *et al.*, 1987; Kende, 1993; Zarembinski and Theologis, 1994; Barry *et al.*, 1996; Tsuchisaka and Theologis, 2004). The amount of ethylene produced by plant cells varies with cell type and developmental stage, and is regulated by controlling both mRNA synthesis and enzyme activity (Smith *et al.*, 1986;

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Hamilton *et al.*, 1991; Llop-Tous *et al.*, 2002; Yamagami *et al.*, 2003). Both *ACS* and *ACO* genes appear to be transcriptionally controlled by homeotic proteins (Ito *et al.*, 2008; Lin *et al.*, 2008*a*). The activity of ethylene biosynthetic enzymes is also regulated by phytohormones and ubiquitin/26S proteasome degradation (Chae and Kieber, 2005; Christians *et al.*, 2008).

In *Arabidopsis*, ethylene is perceived by a family of five receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) that possess sequence similarity with bacterial two-component His kinases (Bleecker *et al.*, 1988; Chang and Shockey, 1999; Wang *et al.*, 2002). Two subfamilies of receptors are recognized with ETR1 and ERS1 in subfamily 1 and ETR2, ERS2, and EIN4 in subfamily 2. The receptors form homo- or heterodimers (Gao *et al.*, 2008) and have been located in several membranes including the endoplasmic reticulum (ER), the plasma membrane, the nuclear envelope, and the Golgi apparatus (Xie *et al.*, 2003; Ma *et al.*, 2006; Dong *et al.*, 2008; Zhong *et al.*, 2008; Lin *et al.*, 2008*b*). Ethylene binding to the membrane-bound N-terminal region of the receptors involves a copper ion and results in the inactivation of receptor signalling to CTR1, a negative regulator with similarity to Raf-like protein kinases that interacts with the receptors in the ER (Kieber *et al.*, 1993; Clark *et al.*, 1998; Gao *et al.*, 2003). The receptors appear to act as redundant negative regulators of ethylene signalling to suppress ethylene responses (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003). A single loss-of-function receptor mutant does not produce phenotypic alteration, but multiple receptor loss-of-function mutants show enhanced ethylene responses, grow slowly, have small organ size, and are infertile (Hua and Meyerowitz, 1998). Recently, it has been shown that null mutations in either ETR1 or ERS1 result in increased sensitivity to ethylene and double null mutations show strong constitutive ethylene-response phenotypes (Qu *et al.*, 2007), suggesting that subfamily 1 receptors are absolutely required to suppress ethylene responses in *Arabidopsis*, and their functions cannot be replaced by subfamily 2 receptors. In tomato, the ERS1 type ethylene receptor NR is highly expressed in flowers at anthesis and during tomato ripening and the melon CmERS1 is important throughout fruit development (Payton *et al.*, 1996; Lashbrook *et al.*, 1998; Takahashi *et al.*, 2002). In addition, reduction in the levels of the subfamily 2 ethylene receptors LeETR4 and LeETR6 causes an early-ripening phenotype and it therefore appears that different receptors are important at different stages of development. Furthermore, exposure of immature fruits to ethylene causes a reduction in the amount of receptor protein leading to earlier ripening, suggesting that ethylene receptor degradation controls the timing of tomato ripening (Kevany *et al.*, 2007). This indicates that receptor inactivation and degradation may also play important roles in ethylene signalling.

Ethylene receptors are individually regulated by different proteins. The ETR1 receptor is positively regulated by the membrane protein REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1), and the function of RTE1 is primarily dependent on ETR1 and can be independent of the other receptors (Resnick *et al.*, 2006; Zhou *et al.*, 2007; Dong *et al.*, 2008). ETR1 and RTE1 are co-localized in the ER and the Golgi apparatus (Dong *et al.*, 2008). Recently, a TPR protein SlTPR1 has been shown to interact with the tomato ethylene receptors LeETR1 and NR in a yeast two-hybrid system and *in vitro*, and transgenic plants that overexpressed SlTPR1 displayed pleiotropic phenotypes related to ethylene and auxin (Lin *et al.*, 2008*b*). The characterization of the *Arabidopsis* orthologue of SlTPR1, AtTRP1 (Tetratricopeptide Repeat Protein1) is reported here. AtTRP1 interacts preferentially with the *Arabidopsis* ethylene receptor ERS1 in the yeast two-hybrid system. The interaction of AtTRP1 with ERS1 was confirmed by *in vivo* immunoprecipitation pull-down assays. Overexpression of the full-length AtTRP1 in wild-type *Arabidopsis* resulted in dwarfed plants with reduced fertility and altered leaf/silique morphology, and enhanced expression of *AtChiB*. Expression of the same construct in the dominant ethylene insensitive receptor ETR1 mutant *etr1-1* altered aspects of the mutant phenotypes, such as stature and leaf/silique morphology, but did not affect the dominant ethylene insensitivity of the *etr1-1* mutants. The results suggested that AtTRP1 affects some ethylene responses, possibly via its association with the ethylene receptor ERS1, and that overexpression of AtTRP1 may affect cross-talk between ethylene and auxin signalling.

Materials and methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype *Columbia* and the *etr1-1* mutant) were grown from homozygous lines. All plants were grown in a regulated growth room with a 16 h photoperiod (250 μmol m^{-2} s^{-1} photosynthetic photon flux) at 23 °C in the light and 22 °C in the dark unless otherwise specified.

Generation of constructs and transgenic plants

All molecular cloning procedures were carried out using standard methods (Sambrook *et al.*, 1989). The full-length coding sequence or partial cDNA of AtTRP1 were PCR amplified and cloned into the pENTR/D-TOPO vector (Invitrogen) and confirmed by sequencing. The pENTR-AtTRP1 constructs were recombined with the Gateway binary vector pK7FWG2. The resulting constructs were sequenced and introduced into competent *Agrobacterium tumefaciens* C58 cells and used to transform *Arabidopsis* by the floral dip method (Clough and Bent, 1998).

Hormone treatment

Wild-type and transgenic *Arabidopsis* seeds were surface-sterilized and 20–25 seeds from each line were grown on 120×120 mm plates containing Murashige and Skoog (MS) medium (2.2 g MS salts, 8 g bacterial agar per litre) with or

without ACC or IAA or GA_3 at concentrations described in the text at 22 °C in a vertical position in the dark or in the light. After a period of growth as described in the text, the growth habit of the seedlings was examined under an Olympus microscope and photographed.

RNA isolation and Northern analysis

Total RNA was isolated using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. Probes were synthesized using the Amersham Rediprime™ II random prime labelling system following the manufacturer's instructions (GE Healthcare). Pre-hybridization and hybridization was carried out for 16 h at 42 °C in buffer containing 1% (w/v) SDS, 50% (v/v) deionized formamide, 5× SSC, 50 mM sodium phosphate pH 6.8, 0.1% (w/v) sodium pyrophosphate, 10% (w/v) dextran sulphate, and 50 μg ml^{-1} salmon sperm DNA. Hybridized membranes were finally washed in 0.2× SSC, 0.1% SDS and the signal was detected by autoradiography.

RT-PCR

2 μg of total RNA was used for reverse-transcription in a reaction volume of 20 μl using SuperScript™ II Reverse Transcriptase (Invitrogen). 2 μl of this RT mixture was then used for PCR using primers AtTRP1F/R (Fig. 1).

Yeast two-hybrid analysis

The LexA-based Interaction Trap system described by Golemis and Brent (1997) was used in this study. All plasmids and *S. cerevisiae* strain EGY48 were kindly supplied by R Brent, Massachusetts General Hospital, Boston. 'Bait' protein constructs consisting of partial ethylene receptor sequences of ERS1 (nt: 921–1839), ETR1 (nt: 1050–2214), NR (nt 351–1905), and LeETR1 (nt 396–2262 and nt 1092–1941) were constructed by insertion of cDNA sequences into the *Eco*RI/*Xho*I or *Bam*HI/*Xho*I restriction sites of plasmid pEG202, downstream of and in frame with the bacterial LexA DNA-binding domain sequence (DB). The AtTRP1 cDNA (nt 1–834) was inserted into the *Eco*RI/*Xho*I restriction site of prey vector pJG4-5. All the constructs were confirmed by sequencing. The homeodomain of bicoid protein fused to the LexA DNA-binding domain, encoded in plasmid pRFHM1, was used as a negative control, while pSH17-4, encoding the LexA DNA-binding domain upstream of the Gal4 activation domain, was used as a positive control.

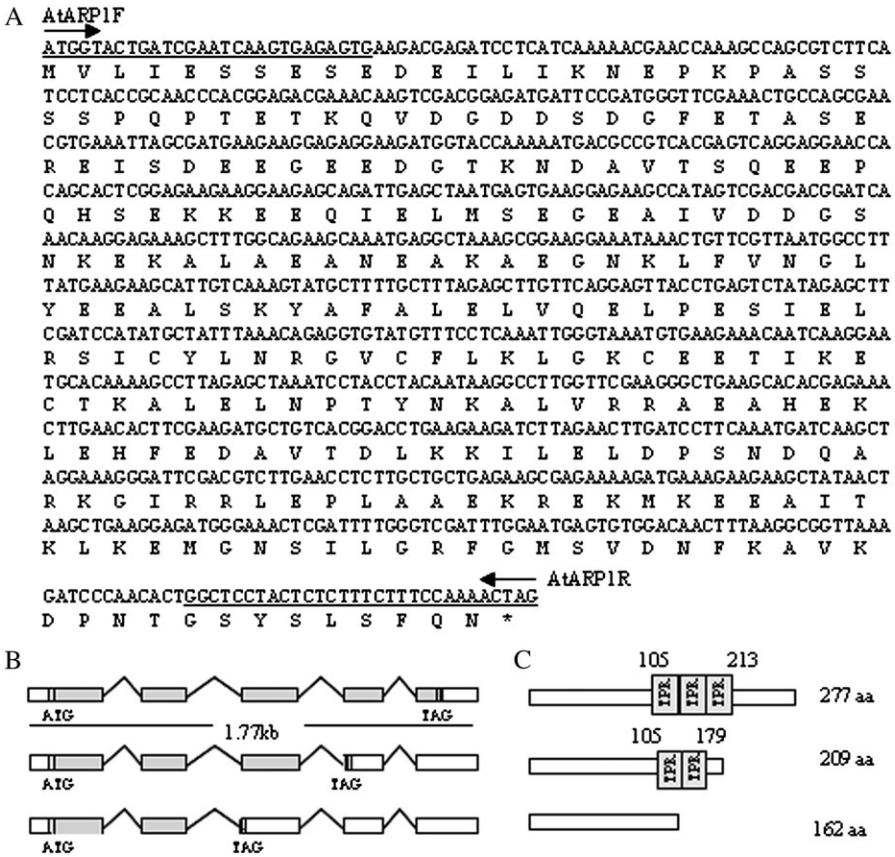

Fig. 1. Sequence and structure analysis of AtTRP1. (A) The nucleotide and amino acid sequences of AtTRP1 used for this study. The primers AtTRP1F/AtTRP1R used for RT-PCR to isolate the AtTRP1 coding sequence are underlined. (B) The 1.77 kb *AtTRP1* gene has three predicted splicing variants according to the databases (AtEnsembl). (C) The corresponding putative proteins with TPR motifs and corresponding amino acid numbers indicated.

Microsomal membrane isolation and protein blot analysis

Total proteins were isolated from seedlings with a homogenization buffer containing 30 mM TRIS (pH 8.0), 150 mM NaCl, 10 mM EDTA, and 20% v/v glycerol with protease inhibitors (1 mM PMSF and 1 μg ml^{-1} leupeptin), as described previously (Schaller *et al.*, 1995). Tissue was homogenized at 4 °C and then centrifuged at 8500 *g* for 20 min. The supernatant was strained through cheesecloth, and then centrifuged at 100 000 *g* for 30 min. The subsequent membrane pellet was resuspended in ice-cold membrane resuspension buffer [10 mM TRIS (pH 7.5), 5 mM EDTA, and 10% (w/w) sucrose with protease inhibitors (1 mM PMSF and 1 μg ml^{-1} leupeptin)]. Protein concentrations were determined using the Bio-Rad protein assay reagent (http://www.bio-rad.com/).

Immunoprecipitation

Membrane proteins extracted from plants were resuspended in membrane resuspension buffer as described above. 5 μg of anti-GFP antibody (Invitrogen) was incubated with 100 μl protein A-magnetic beads (Invitrogen) in 0.5 ml PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 KH_2PO_4 per litre, pH 7.4) for 2 h with rotation at 4 °C, and washed three times with PBS buffer. 100 μg membrane proteins were added to the anti-GFP antibody/protein A-magnetic beads and incubated in 0.5 ml immunoprecipitation buffer containing 5% Normal Goat Serum overnight at 4 °C with gentle agitation. The magnetic beads were pelletted on a magnetic rack for 1 min and washed five times with ice-cold PBS. The samples were then dissolved in 2× SDS buffer, treated for 20 min at 37 °C, 15 min at 50 °C, and 15 min at 65 °C prior to electrophoresis by 10% SDS PAGE and western detection assays as described below.

Western blot analysis

Protein samples were separated on a 10% polyacrylamide TRIS-HCl gel, and transferred to a nitrocellulose membrane. Membranes were blocked in 10% non-fat milk/TBS at room temperature for 40 min and then incubated with anti-GFP antibody (1:5000; Invitrogen, www.invitrogen.com) or anti-NR antibody (1:1000) in TBS overnight at 4 °C. Membranes were subsequently washed three times for 5 min in TBS, and then incubated with IgG-conjugated anti-rabbit or anti-goat (1:5000 in TBS) secondary antibody for 1 h at room temperature. Membranes were finally washed three times for 5 min in TBS and developed with SIGMA FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets (Sigma, http://www.sigmaaldrich.com) (one tablet in 20 ml carbonate buffer, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8).

Florescence microscopy

All subcellular images were obtained by using a Leica TCS SP2 AOBS confocal scanning microscope. GFP was excited using a 488 nm laser and emissions were collected from 495 nm to 550 nm.

Results

AtTRP1 is a single copy gene in Arabidopsis

AtTRP1 (At4g30480) was identified by its homology to tomato SlTPR1, a TPR motif-containing protein that interacts with some tomato ethylene receptors and modulates both ethylene and auxin responses during development (Lin *et al.*, 2008*b*). The AtTRP1 coding sequence was isolated by RT-PCR using primers AtTPR1F/R (Fig. 1A). The *AtTRP1* gene is 1766 bp in length and contains five exons (Fig. 1B) that encode a putative 277 aa protein with three tetratricopeptide repeat (TPR) motifs (Fig. 1C). At the amino acid level AtTRP1 has overall 64% identity and 72% similarity to SlTPR1 (data not shown; Lin *et al.*, 2008*b*). There are also two splicing variants of *AtTRP1* with early stop codons resulting in two truncated putative proteins of 209 aa and 162 aa respectively, which eliminate one or all TPR motifs (Fig. 1C).

AtTRP1 is a single copy gene in the *Arabidopsis* genome, although a large number of genes encode proteins containing TPR1 motifs. A phylogenetic tree analysis using the full-length protein sequences of 91 TPR genes from the *Arabidopsis* genome as entry indicated AtTRP1 is distantly related to a group of immunophilin FK506-binding proteins (FKBPs), such as TWISTED DWARF1 (TWD1), PASTICCINO1 (PAS1), ROF1 and 2 (Geisler *et al.*, 2003; Smyczynski *et al.*, 2006) (see Supplementary Fig. S1 at *JXB* online, boxed). In addition to the C-terminal TPRs, FKBPs have N-terminal FKBP domains that are absent in the AtTRP1 protein. FKBPs belong to the superfamily of peptidyl-prolyl *cis-trans* isomerases (PPIases) and catalyse the *cis-trans* isomerization of *cis*-prolyl bonds. Mammalian FKBPs have been identified as targets of immunosuppressant drugs and are therefore classified as immunophilins. AtTRP1 has most similarity to TWD1 and PAS1 (Fig. 2A). TWD1 contains one FKBP domain in its N-terminus, three TPRs from aa 170–288, and a transmembrane domain in the C-terminal, which is absent in the AtTRP1 protein (Fig. 2B). AtTRP1 shares 48% identity with TWD1 at the DNA level (see Supplementary Fig. S2 at *JXB* online) and 47% similarity at the amino acid level over the C-terminus that contains the TPR motifs (Fig. 2B, C). TWD1 is plasma membrane-anchored and has been shown to interact physically with the multidrug resistance/P-glycoprotein ATP-binding cassette (ABC) transporters PGP1 and PGP19, and to control PGP-mediated auxin transport (Bouchard *et al.*, 2006). Mutation in this gene resulted in twisted dwarf plants (Bouchard *et al.*, 2006). PAS1 contains triple FKBP domains (Fig. 2B) and is involved in the control of cell proliferation and differentiation during plant development. Mutations in the C-terminal region of PAS1 result in severe developmental defects. The C-terminal region of PAS1 controls the subcellular distribution of the protein and is required for interaction with FAN (FKBP-associated NAC), a new member of the plant-specific

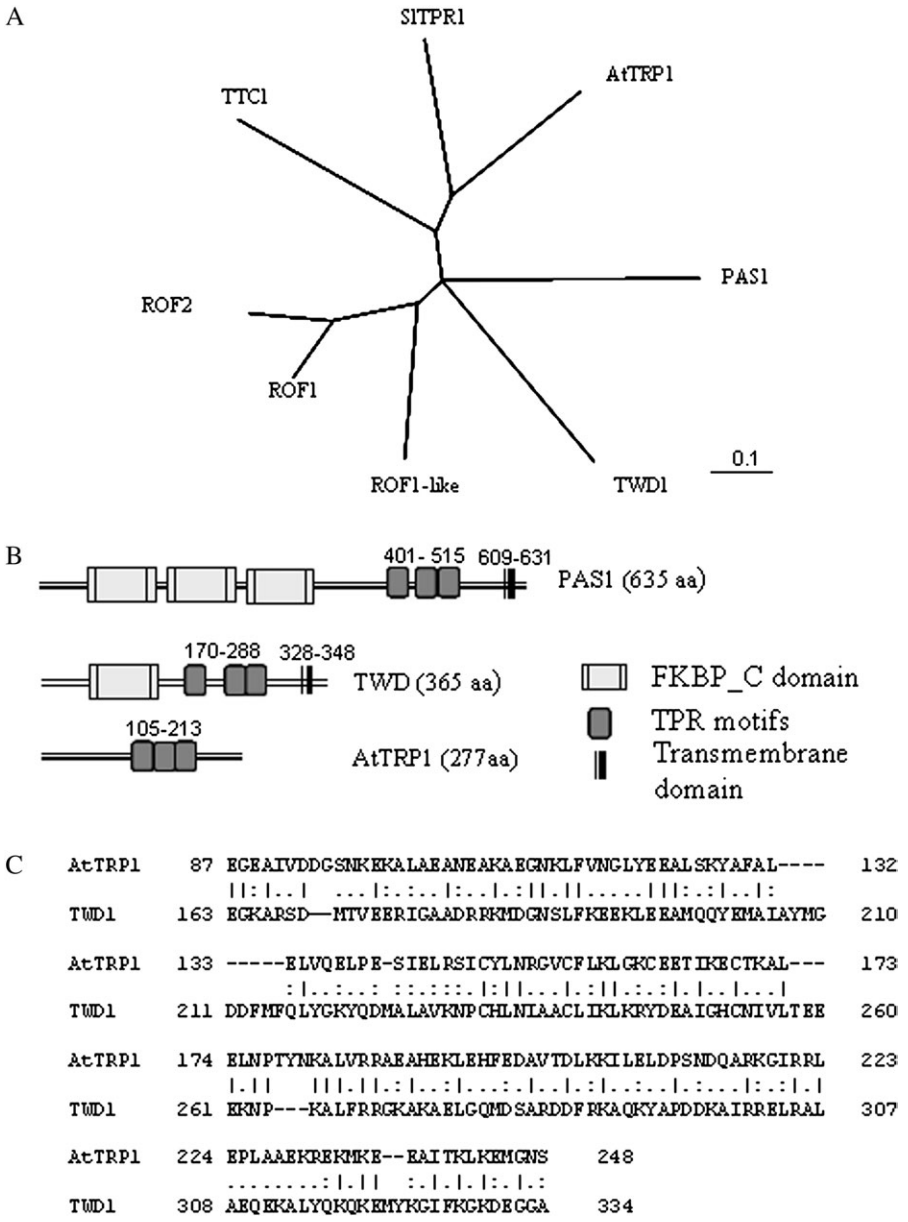

Fig. 2. Comparison of AtTRP1 and related genes. (A) Phylogenetic tree analysis using the protein sequences of three orthologues AtTRP1, SlTPR1 and TTC1, and *Arabidopsis* immunophilin-like proteins TWD1, PAS1, ROF1, ROF2, and ROF1-like. The tree was produced in ClustalW2 (http://www.ebi.ac.uk). (B) Structures of AtTRP1, TWD1, and PAS1, which was produced in SMART programme (Simple Modular Architecture Research Tool), with features and amino acid numbers indicated. (C) Sequence alignment of the C-termini of AtTRP1 and TWD1. Vertical lines indicate identity, and double dots represent similarity.

family of NAC transcription factors. PAS1 and FAN are translocated into the nucleus upon auxin treatment in plant seedlings (Smyczynski *et al.*, 2006). AtTRP1, like SlTPR1 (Lin *et al.*, 2008*b*), is also closely related to the human protein TTC1 (Fig. 2), which links G-proteins and Ras signalling in mammalian cells and competes with Raf-1 for Ras-binding (Marty *et al.*, 2003).

AtTRP1 *mRNA was highly expressed in leaves, stems, and flowers*

Northern analysis using the full-length *AtTRP1* cDNA as probe indicated that *AtTRP1* mRNA was consistently highly expressed in all stages of developing flowers, i.e. unopened, early-opened, fully-opened, and senescing flowers, stems, and leaves (including emerging, mature, and senescing leaves), but was absent or present at a low level in siliques (Fig. 3).

AtTRP1 promoter–GUS was highly expressed in vascular tissue, anther and pollen, abscission zone, and induced by exogenous ACC

In order to understand the regulation of the *AtTRP1* gene, the *AtTRP1* promoter (–1 to –600) was fused to the β-glucuronidase (GUS) reporter gene and transformed to

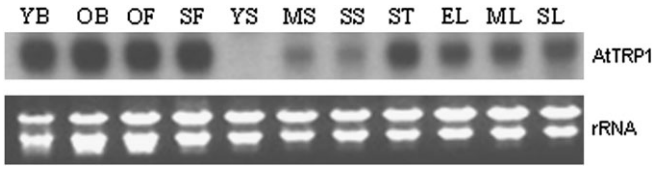

Fig. 3. Expression pattern of AtTRP1 by Northern analysis. RNA samples were isolated from a range of tissues at different developmental stages from wild-type plants (*Columbia*). YB, unopened flower buds; OB, flower buds starting to open; OF, fully opened flowers; SF, senescing flowers; YS, young siliques; MS, mature siliques; SS, senescing siliques; ST, stems; EL, emerging leaves; ML, fully expanded leaves; SL, senescing leaves. 10 μg total RNA was used for the electrophoresis and blotting and the full-length AtTRP1 cDNA was used as probe. The ethidium bromide-stained rRNA gel below indicates the sample loading.

Arabidopsis (Materials and methods). Histochemical analysis of the T_1 plants revealed that the *AtTRP1* promoter-GUS construct was predominantly expressed in the vascular tissue (Fig. 4A, B), mature anther (Fig. 4C, D), and pollen (Fig. 4E, arrow), the abscission zone (Fig. 3F), and funiculus of mature seeds (Fig. 3G). AtTRP1 promoter-GUS was induced in seedlings grown in medium containing ACC (Fig. 3H, I), but was not affected by exogenous IAA (data not shown).

Cis-element analysis of the *AtTRP1* promoter sequence indicated putative binding sites for known mammalian transcription factors with high score and frequency (see Supplementary Table S1 at *JXB* online), including SRY (sex-determining region Y gene product) and CdxA (chicken homeobox transcription factor) (Pontiggia *et al.*, 1994; Margalit *et al.*, 1993). SRY is a member of the high mobility group, which initiates male sex determination in mammals (Lovell-Badge, 1993); whereas *CdxA* is expressed in the epiblast and the early endodermal lineage and may play an important role during the early steps of organogenesis (Frumkin *et al.*, 1991).

Overexpression of the full-length AtTRP1 *in* Arabidopsis *resulted in dramatic developmental abnormality in plant morphology and reproduction*

To examine whether or not *AtTRP1* possessed similar functions to the tomato orthologue SlTPR1 *in planta,* two constructs for overexpression of *AtTRP1*, named as AtTRP1**F**–GFP and AtTRP1**N**–GFP, respectively, were made using the full-length coding cDNA (nt 1–831) and a partial cDNA (nt 1–648) encoding the N-terminal protein (equivalent to the splicing variant 2; Fig. 1) under the control of the CaMV 35S promoter, each with a downstream GFP tag in vector pK7FWG2 (Karimi *et al.*, 2002) (Fig. 5A; see Supplementary Fig. S3A at *JXB* online). This would be expected to lead to overexpression in cells and tissues that normally produced AtTRP1 mRNA (Fig. 3), plus ectopic expression in other cells. The two constructs, after confirmation by sequencing, were independently transformed into *Arabidopsis* ecotype *Columbia*. Twenty-four independent transgenic lines overexpressing the partial *AtTRP1* cDNA (the AtTRP1**N**–GFP construct) were selected on kanamycin medium and grown to maturity. Northern analysis and subcellular localization of the AtTRP1N-GFP fusion protein confirmed the transgene expression (see Supplementary Fig. S3B at *JXB* online), but none of these lines displayed any phenotypic alteration under normal growth conditions (data not shown).

To investigate whether silencing AtTRP1 caused phenotypic affects, a T-DNA insertion line (NASC ID: N449203), which has an insertion in the beginning of the third exon of AtTRP1 was screened, but no phenotypic changes were observed in normal growth conditions (data not shown).

Six independent transgenic lines overexpressing the full-length AtTRP1 sequence fused to GFP (AtTRP1**F**-GFP) were selected on kanamycin and Northern analysis confirmed expression of the transgene (Fig. 5B). All lines (T_1 generation) exhibited noticeably reduced stature, and appeared bushy with small rounded leaves and reduced flower numbers. The severity of the phenotype was related to the levels of the transgene expression (Fig. 5B, C). Overexpression of AtTRP1F-GFP at high levels *in planta* affected fertility, with reduced size and number of inflorescences and flowers. The flowers were often asymmetrically arranged and smaller compared to the wild type (Fig. 5F, G). Siliques were wider, shorter, and misshapen with reduced numbers of seeds, and often had elongated narrow regions at their proximal and distal ends (Fig. 5F, H). Line 2 with the strongest transgene expression was tiny, senesced earlier, and produced only a few seeds (Fig. 5B, D; see Supplementary Fig. S4 at *JXB* online). Line 3 (also with strong transgene expression) was only able to produce heterozygous and not homozygous seeds (see Supplementary Fig. S4 at *JXB* online; data not shown), and other lines displayed intermediate phenotypes (Fig. 5; see Supplementary Fig. S4 at *JXB* online).

AtTRP1 overexpressers displayed enhanced responses to exogenous ACC

The ethylene responses of the transgenic seedlings that overexpressed AtTRP1F-GFP were investigated. Seeds from lines 2, 3, 4, 5, and 6 (Fig. 5B, C) were grown on MS medium with or without 0.5 μM ACC in the dark or light (Fig. 6). Line 4 did not germinate well. In the dark, lines 2 and 3 displayed great growth inhibition, with shortened and swollen hypocotyls (2–3 mm versus 9 mm in the wild type; Table 1), inhibited root growth, and altered apical hook in the absence of ACC, although lines 5 and 6 displayed normal growth (Fig. 6A, B). Etiolated seedlings from all lines exhibited enhanced responses to 0.5 μM ACC, with shortened roots and hypocotyls and altered apical hook, and hypocotyls of lines 2 and 3 were swollen (Fig. 6A, C, which shows an enlarged image of line 3 in response to 0.5 μM ACC; Table 1). In the light, seedlings of all transgenic lines exhibited a reduction in cotyledon expansion and greening in the absence of ACC, and enhanced responses to 0.5 μM ACC, with a great reduction in root

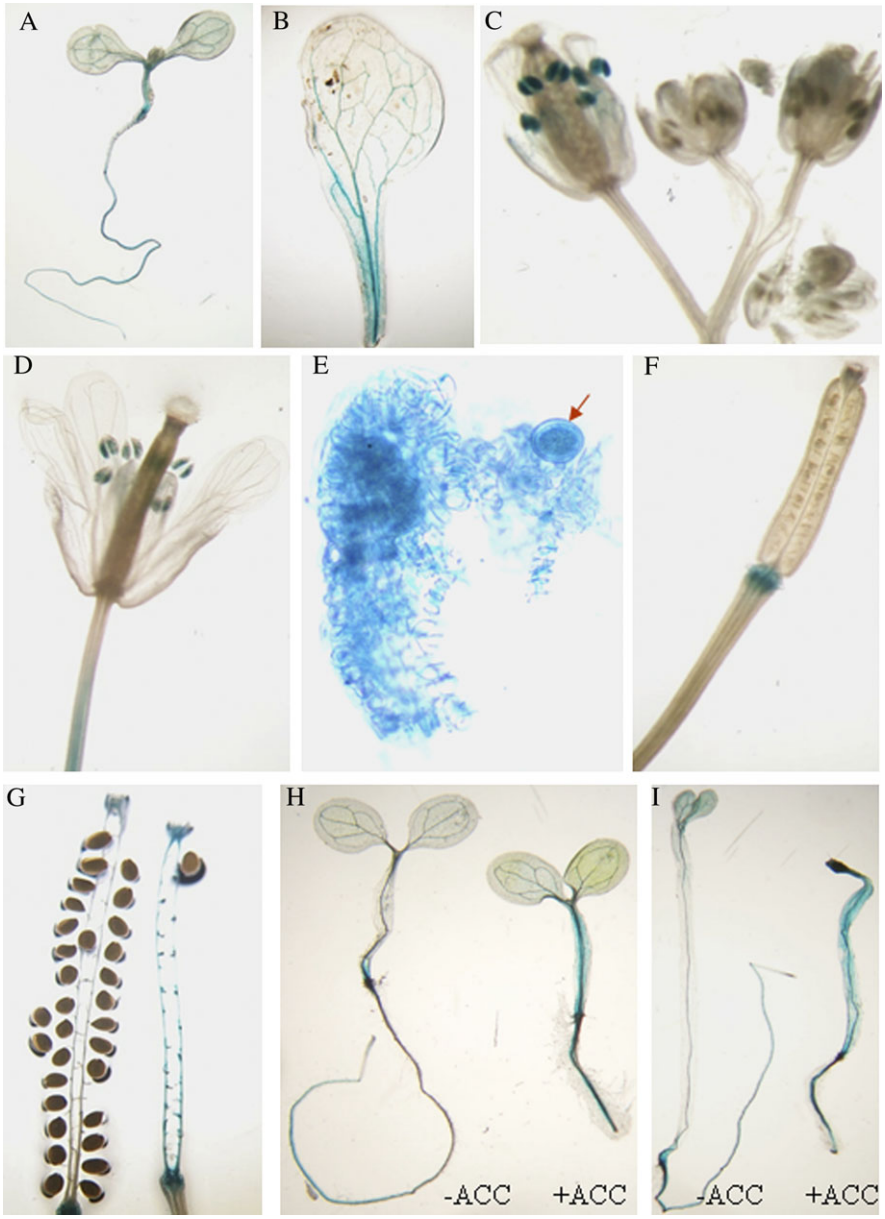

Fig. 4. AtTRP1 promoter–GUS reporter analysis. AtTRP1 promoter–GUS was expressed in vascular tissue of 12-d-old light-grown seedlings (A), senescent leaves (B), and in the anthers of mature flowers (C, D). (E) A high magnification image of a squashed mature anther, showing pollen. (F) AtTRP1 promoter–GUS was expressed in the abscission zone of a developing silique during sepal and petal senescence (fallen sepals and petals not shown). (G) Comparison of AtTRP1 promoter–GUS expression in a mature (left) and a ripe silique (right), the latter showing strong GUS expression in the abscission zones. (H, I) AtTRP1 promoter–GUS was expressed more strongly in seedlings grown in medium containing ACC (H) in the light (10-d-old) and (I) in the dark (3-d-old).

length compared to the wild type (Fig. 6D; Table 2). Taken together, both dark- and light-grown AtTRP1 overexpressing seedlings showed enhanced responses to low concentration of exogenous ACC, although the seedlings did not always display swollen hypocotyls or exaggerated apical hooks.

The response to ACC of the transgenic plants overexpressing the AtTRP1 N-terminal construct was also examined, although, as described above, they showed no visible phenotypic changes in the normal growth condition (i.e they were not dwarfed and showed no morphological changes). Seeds from lines 5, 6, 8, 16, and 18 were grown on MS medium with or without 0.5 μM ACC in the dark. Transgenic seedlings from all lines displayed enhanced responses to ACC to some extent, with shortened hypocotyls and roots, and an exaggerated apical hook, although in the absence of ACC they showed no difference to the wild type (see Supplementary Fig. S8 at *JXB* online).

To clarify whether growth inhibition displayed by the transgenic lines was due to the overproduction of ethylene or the disruption of signalling, the expression of ethylene biosynthesis genes *ACS5* and *9* and the ethylene responsive gene *AtChiB* was examined by Northern analysis. The results showed no detectable alteration in the levels of

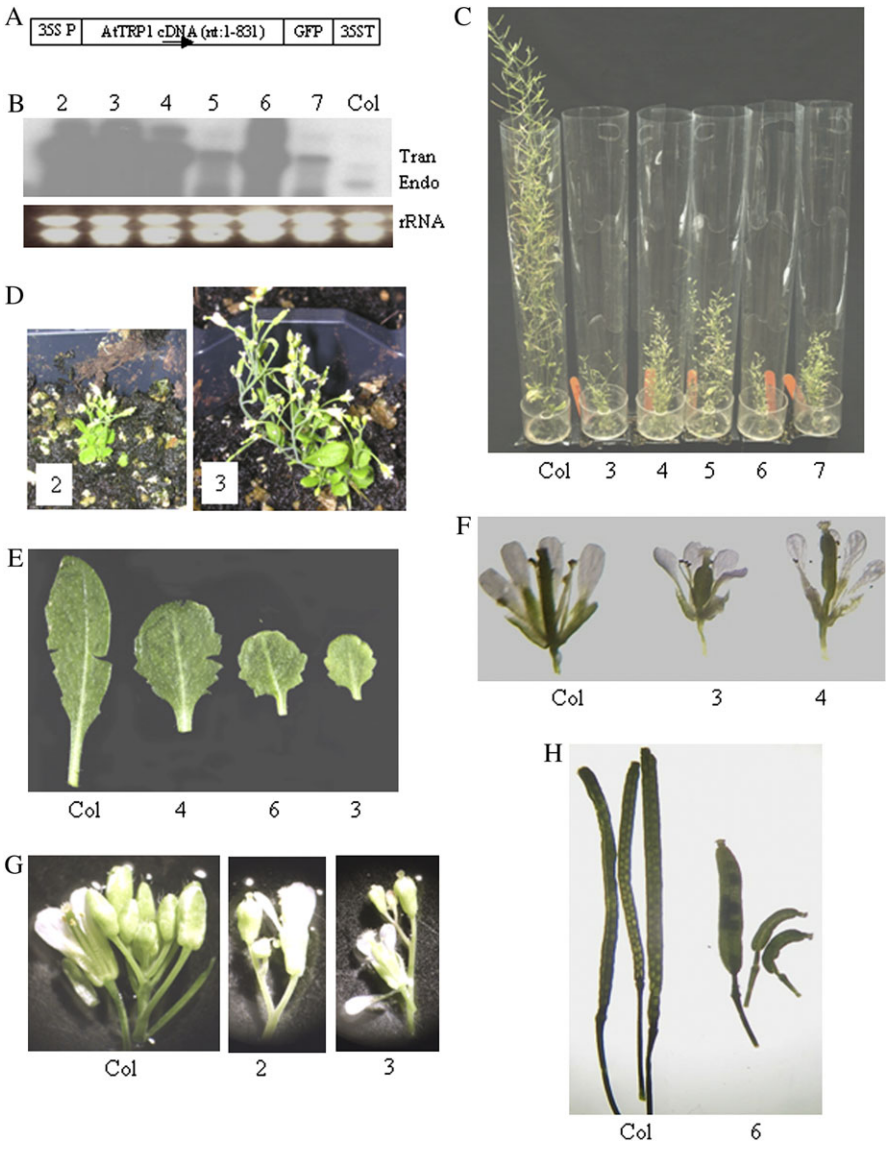

Fig. 5. Characterization of *Arabidopsis* wild-type *(Columbia*) plants overexpressing AtTRP1. (A) The construct used for generating transgenic plants. (B) Characterization of transgene expression in plants transformed with the AtTRP1F–GFP construct by Northern analysis. 10 μg total RNA was fractionated, blotted, and the full-length AtTRP1 cDNA was used as probe. Tran, AtTRP1 transgene transcript; Endo, endogenous AtTRP1 mRNA. rRNA stained with ethidium bromide indicates sample loading. (C) Phenotypes of the mature transgenic plants in comparison with the control (Col), showing great reduction in size. (D) A closer picture of lines 2 and 3, showing bushy-like appearance. (E) Leaf morphology of the transgenic plants in comparison with the control. Leaves from the transgenic lines were smaller and rounder with shorter pedicels. (F) Flower morphology: flowers from the transgenic plants produced twisted, shorter and wider siliques, and an elongated zone at the base was clearly seen. (G) Phenotypes of inflorescences: the transgenic stalks (2, 3) produced fewer flowers compared with the wild type (Col). (H) Morphology of mature siliques. Siliques from the transgenic plants were much shorter and wider with fewer seeds and an elongated structure between the pedicel and the fruit.

ACS5 and *9* mRNA, but increased expression of *AtChitB* in the transgenic lines compared to the wild type (see Supplementary Fig. S5 at *JXB* online). The *AtChitB* mRNA has been shown to be induced by ethephon, a compound that is converted to ethylene by plants (Samac *et al.*, 1990), and to be up-regulated in the constitutive ethylene response mutant *ctr1* (Kieber *et al.*, 1993). Measurement of ethylene production by either light-grown or dark-grown seedlings also indicated no significant differences between the transgenic lines and the wild type (data not shown). Furthermore, the effects of the ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG) on the constitutive ethylene response phenotype of lines 2 and 3 (Fig. 6A, B) were also examined. Etiolated seedlings grown on 0.2 μM AVG still exhibited the reduced growth phenotype (Fig. 7A). Silver nitrate, which inhibits ethylene receptor action, also had no effect on the phenotype of the etiolated seedlings compared with seedlings grown on MS medium without the inhibitors (Fig. 7B), whereas the same concentrations of the inhibitor abolished the growth inhibition of the wild-type

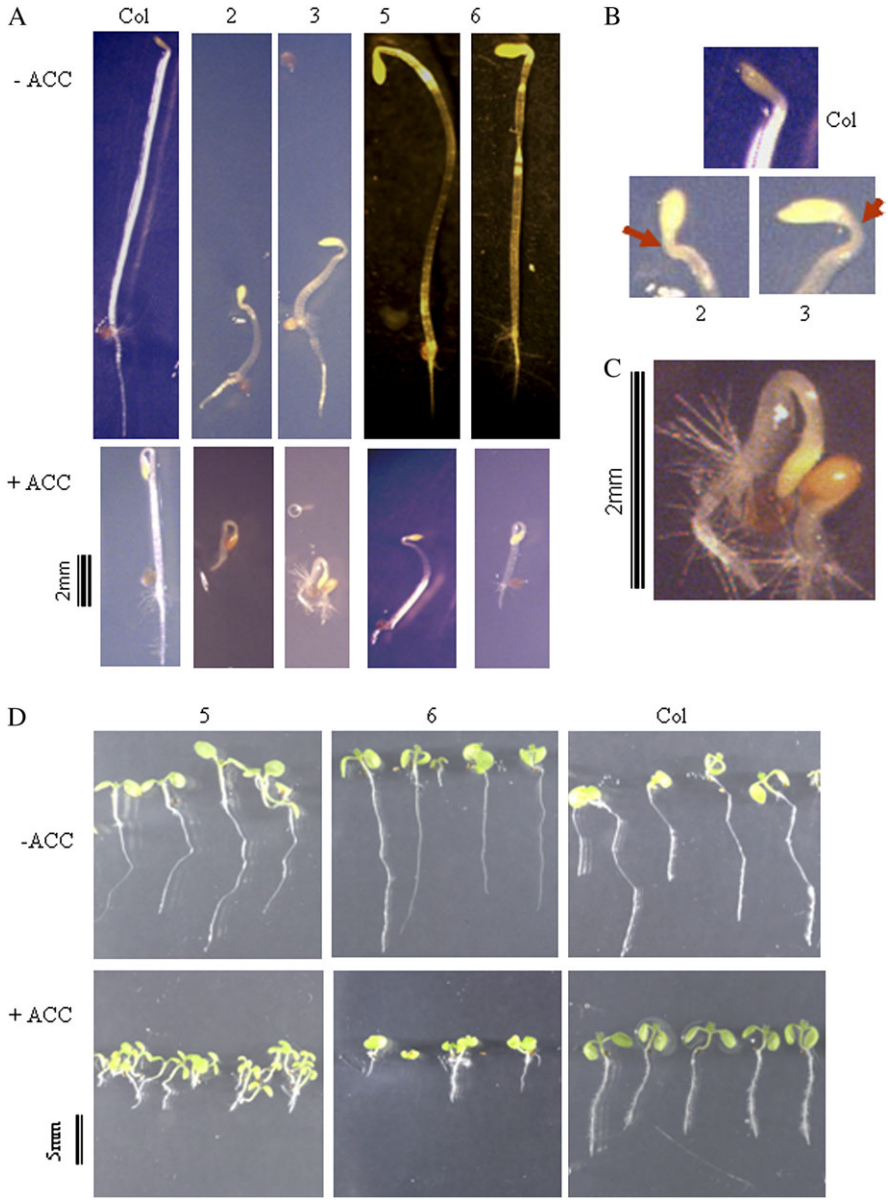

Fig. 6. Characterization of the ethylene responses of transgenic plants overexpressing AtTRP1F–GFP. (A) Responses of etiolated seedlings to ACC. Wild-type and transgenic seedlings were grown on MS medium with or without 0.5 μM ACC in the dark and photographed in white light under an Olympus microscope at 3-d-old. (B) Enlarged images of the apical hooks of wild type (Col) and lines 2 and 3 from (A) grown in the absence of ACC. (C) Enlarged image of line 3 in response to ACC from (A), showing shortened and swollen hypocotyls and inhibited root growth. (D) Responses of light-grown seedlings to ACC. Wild-type and transgenic seedlings were grown on MS medium with or without 0.5 μM ACC in the light and photographed at 7-d-old.

etiolated seedlings in the presence of ACC (Fig. 7C). These results suggested that altered growth responses caused by overexpression of AtTRP1 *in planta* were not related to enhanced ethylene synthesis but resulted from the disruption of signalling downstream of the ethylene receptors.

Exogenous gibberellic acid (GA) did not alter the dwarfed phenotype of AtTRP1 overexpressors

To clarify whether the retarded growth of the transgenic plants overexpressing AtTRP1 resulted from defects in GA production, the responses to GA of the transgenic plants were investigated. Seeds from three dwarfed transgenic lines 3, 4, and 6 and the wild type were grown on MS medium in the absence or in the presence of 10 μM GA for 8 d and were then transferred to soil with continuous spraying and watering with 100 μM GA twice a week for 18 d in normal growth condition (see Materials and methods), and the phenotypic characteristics of the plants, including bolting time, height, flower numbers, side shoots, and leaf size, were measured. The results showed that GA did not reverse the dwarfed phenotypes of the transgenic plants overexpressing AtTRP1 (see Supplementary Fig. S6A, B at *JXB* online). This indicated that the growth retardation caused by

Table 1. Etiolated seedlings in response to exogenous ACC

Twenty to twenty-five seeds from each line were surface-sterilized and grown on 120×120 mm plates containing MS with or without ACC (0.5 μM) at 22 °C in the dark for 3 d, and the hypocotyl length was measured. The data represent the mean of two experiments. The error bars represent the standard error.

Lines	Hypocotyl length (mm)	
	–ACC	+ACC
Col	8.7±1.7	5.4±1.2
2	2.4±1.2	1.2±0.2
3	2.6±1.5	1.2±0.2
5	9.4±1.8	3.4±1.1
6	8.7±1.8	2.2±1.1

Table 2. Light-grown seedlings in response to exogenous ACC

Twenty to twenty-five seeds from each line were surface-sterilized and grown on 120×120 mm plates containing MS with or without ACC (0.5 μM) at 22 °C in the light for 7 d, and the root length of the seedlings were measured. The data represent the mean of two experiments. The error bars represent the standard error.

Lines	Root length (mm)	
	–ACC	+ACC
Col	12.8±3.8	6.7±1.2
3	8.7±5.4	2.2±2.4
5	10.4±5.2	4.1±2.1
6	8.9±5.4	3.1±2.1

overexpression of AtTRP1 in plants did not result from a deficiency in GA production.

Transgenic plants that overexpressed AtTRP1 displayed altered responses to IAA

In a previous study, it has been shown that overexpression of SlTPR1 in tomato resulted in auxin-related phenotypes and altered expression of some auxin early responsive genes (Lin *et al.*, 2008*b*). The altered morphology of leaves and siliques and the bushy habit of the AtTRP1-overexpressing plants in the present experiments also suggested the possibility that AtTRP1 overexpression led to altered auxin responses. Expression of auxin early response genes *IAA1*, *IAA5*, and *SAUR-AC1* was analysed by Northern analysis and showed a decrease in *SAUR-AC1* and an increase in *IAA5* (see Supplementary Fig. S7A at *JXB* online). Responses of the transgenic seedlings to exogenous IAA treatment were tested. Seeds from the transgenic lines 3, 4, 5, and 6 were grown on MS medium containing 0.01, 0.03, and 0.1 μM IAA in the light. After 6 d, both the transgenic and wild-type seedlings exhibited no significant growth inhibition on 0.01 μM, but strong inhibition at 0.1 μM IAA (data not shown). AtTRP1 overexpressing seedlings, however, were less sensitive to intermediate levels (0.03 μM) of IAA compared to the wild type (see Supplementary Fig. S7 and Table S2 at *JXB* online).

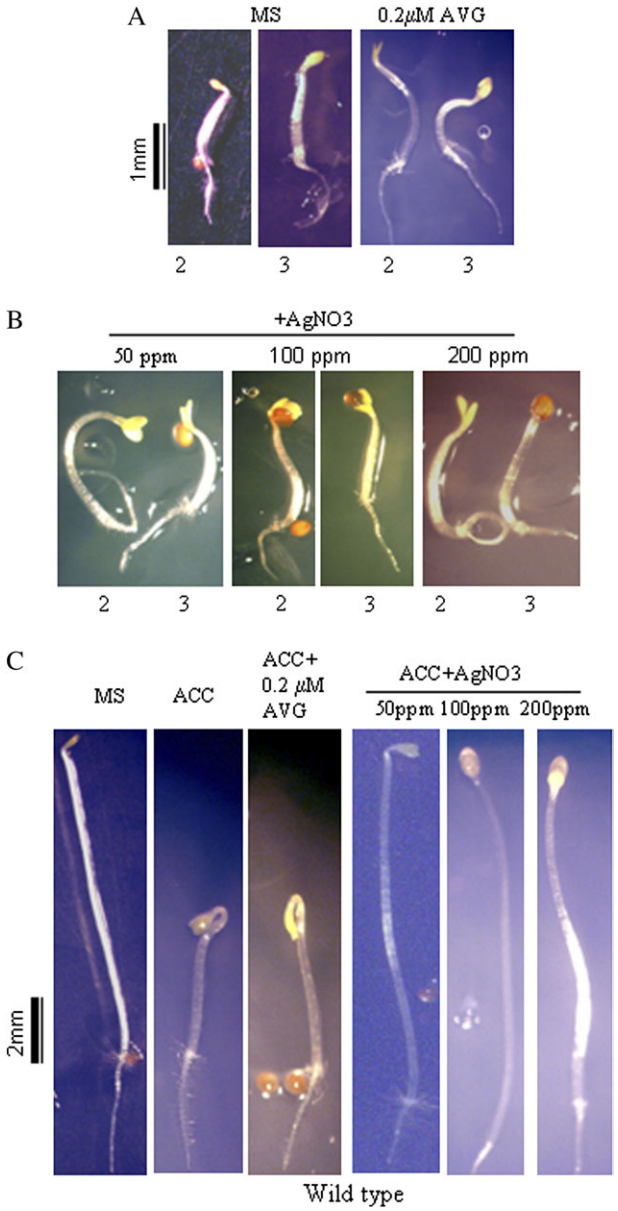

Fig. 7. Response to ethylene inhibitors AVG and silver nitrate. (A) Etiolated seedlings of lines 2 and 3 grown on MS with or without the ethylene biosynthesis inhibitor AVG (A) and the ethylene action inhibitor silver nitrate (B). Photographs of 3-d-old seedlings were taken under an Olympus microscope. (C) Etiolated wild-type seedlings were grown on MS, MS+0.5 μM ACC, and MS+0.5 μM ACC with ethylene inhibitor 0.2 μM AVG, or silver nitrate (50, 100, or 200 ppm), and photographed under an Olympus microscope at 3-d-old.

Overexpressing AtTRP1 in the etr1-1 *mutant resulted in alterations in leaf and silique morphology, but did not change the ethylene insensitivity of the mutant*

To investigate the role of AtTRP1 in ethylene signalling and its relationship with the ethylene receptor AtETR1, the AtTRP1**F**–GFP construct was introduced into the dominant ETR1 receptor mutant *etr1-1* (Bleecker *et al.*, 1988; Chang *et al.*, 1993) (referred to here as AtTRP1-in-*etr1-1*).

Ten independent positive transgenic lines were obtained from two separate transformation events, and three lines (2, 4, and 5) were grown to maturity to produce seeds. Northern analysis confirmed expression of the transgene (Fig. 8A), and microscopic examination confirmed the AtTRP1–GFP fusion protein expression (data not shown). All three lines had smaller stature (T_1 generation) (Fig. 8B), fewer rosette leaves (10 versus 13 at 22-d-old), and altered morphology of the rosette leaves and siliques compared with the *etr1-1* mutant (Fig. 8B, C, D).

Ethylene sensitivity of AtTRP1-in-*etr1-1* transgenic plants was examined. Seeds from the above transgenic lines and *etr1-1* mutants were grown on MS medium with or without 5 μM ACC in the dark and light. The morphology of both etiolated *etr1-1* and AtTRP1-in-*etr-1.1* seedlings was not affected by 5 μM ACC, but AtTRP1-in-*etr-1.1* seedlings were slightly shorter (Fig. 8E), which suggested that AtTRP1 has an ethylene-independent affect on cell elongation. Light-grown seedlings did not exhibit excessive root hair formation in the presence of ACC, a phenomenon stimulated by ethylene (data not shown). The results indicated that overexpression of AtTRP1 in the *etr1-1* mutant did not change the ethylene insensitivity of the *etr1-1* mutant although it altered the growth habit of the mutant in response to light, including reduced stature and leaf numbers and altered morphology of leaves and siliques (Fig. 8), suggesting that the dominant receptor mutation might mask AtTRP1 function related to ethylene sensitivity or the AtTRP1 protein might function independently or downstream of the ETR1 receptor.

AtTRP1 preferentially interacted with the ethylene receptor ERS1 in yeast

It was previously reported that SlTPR1 interacts with the tomato ethylene receptors NR and to a lesser extent to LeETR1 in a yeast two-hybrid system (Lin *et al.*, 2008*a*). To determine whether or not AtTRP1 functions in the same way as SlTPR1, the interactions of AtTRP1 with ERS1 and ETR1, the two *Arabidopsis* orthologues of the tomato ethylene receptors NR and LeETR1, were tested using the LexA-based yeast two hybrid system (Lin *et al.*, 2008*b*). The

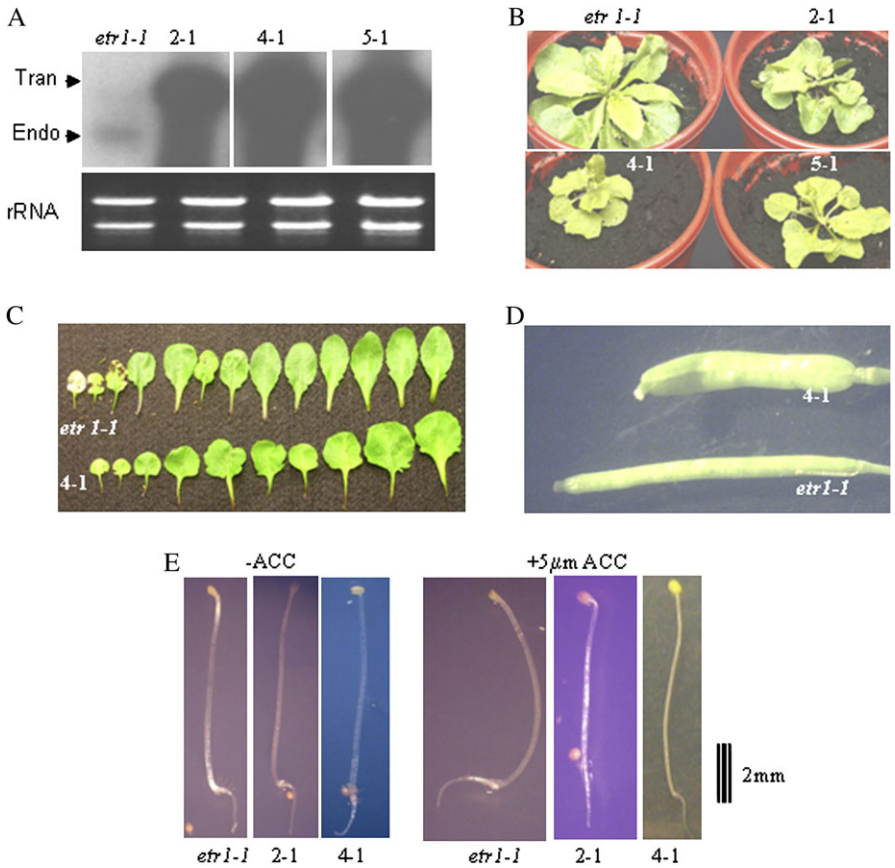

Fig. 8. Characterization of the *etr1-1* mutant plants overexpressing AtTRP1. (A) Transgene expression in *etr1-1* by Northern analysis. 10 μg total RNA was used and the full-length AtTRP1 cDNA was used as probe. Tran, AtTRP1 transgene transcript; Endo, endogenous AtTRP1 mRNA. rRNA stained with ethidium bromide indicates sample loading. (B) Phenotypic changes of *etr1-1* overexpressing AtTRP1, showing smaller stature, and fewer and smaller rosette leaves. Plant were grown in soil under long-day conditions and photographed at 22-d-old. (C) Leaf morphology. Total rosette leaves were excised from the transgenic and wild-type plants at 22-d-old and photographed. Line 4 (lower) had 10, whereas *etr1-1* (upper) had 13 leaves. (D) Silique morphology of the transgenic line (upper) and the wild-type (lower). (E) Lack of response of etiolated seedlings to ACC. *Etr1-1* mutant and transgenic seedlings were grown on MS medium with or without 5 μM ACC in the dark and photographed under an Olympus microscope at 3-d-old.

cDNAs encoding ERS1 or ETR1, without the transmembrane domain and the GAF domain, were inserted into the bait vector pEG202 ($ERS1^{307-613}$, $ETR1^{350-738}$), and the coding sequence of AtTRP1 (nt: 1–830) was cloned in the prey vector pJG4-5 (Fig. 8A, B). All the constructs were confirmed by sequencing prior to transforming into yeast. Each bait construct was transformed into yeast strain EGY48 containing the LacZ reporter plasmid pSH18-34 and genetically integrated LEU2 reporter (Materials and methods). The suitability of the bait constructs was examined by testing their synthesis in yeast and the activation of the LacZ and LEU2 reporters prior to interaction assays (data not shown) (Lin *et al.*, 2008*c*), and the prey construct AD-TRP1 and the prey vector pJG4-5 (as a negative control) were transformed with yeast containing each receptor construct. The interaction assays showed that AtTRP1 interacted with ERS1 and not with ETR1, whereas the prey vector pJG4-5 caused no interaction with either receptor (Fig. 9C). The interactions of AtTPR1 with the tomato ethylene receptors NR and LeETR1 were also examined. The NR partial cDNA encoding the NR protein without the transmembrane domain ($NR^{117-635}$), together with the two partial cDNAs of LeETR1 encoding either the LeETR1 protein lacking the transmembrane domain ($LeETR1^{132-754}$) or the histidine kinase domain alone ($LeETR1^{364-647}$) were cloned into the bait vector pEG202 downstream of the LexA DNA-binding domain (DB) (Fig. 9B) (Lin *et al.*, 2008*c*). All three tomato receptor constructs were shown to interact with AtTRP1 in yeast (Fig. 9C). The interactions of AtTRP1 with the ethylene receptors did not occur when yeast containing the combination of bait/prey constructs was grown on medium in the presence of glucose, which suppresses the expression of the prey protein AD-AtTRP1 (Fig. 9D).

The association of AtTRP1 with the *Arabidopsis* ERS1 ethylene receptor *in vivo* was determined using the transgenic plants that overexpressed AtTRP1F-GFP. Membrane proteins were isolated from 15-d-old seedlings of the transgenic lines 3, 4, and 6 by ultracentrifugation at 100 000 *g*. The presence of the AtTRP1F–GFP fusion in membrane proteins from the transgenic plants was verified by western blotting using anti-GFP antibody, which detected a band corresponding to the AtTPR1–GFP fusion protein in all transgenic lines, but not in the wild type (Col) (Fig. 9E, top panel). In total membrane protein extracts, two NR antibody-reacting proteins were detected, with approximate molecular weights of 130 kDa and 68 kDa, indicating that the NR antibody recognized the ERS1 protein. These proteins corresponded to the known sizes of the homodimer and monomer of ERS1 (Fig. 9E, lower panel). The ERS1 protein has been shown to migrate as both homodimer and monomer proteins in SDS-PAGE without treatment with dithiothreitol (Hall *et al.*, 2000). For co-immunoprecipitation analysis, the membrane proteins were pooled from all three transgenic lines and immunoprecipitated with anti-GFP antibody-protein A-magnetic beads (Invitrogen, Materials and methods). The protein complex from immunoprecipitation was bound to protein A-magnetic beads, washed, and fractionated by SDS-PAGE, and probed with anti-GFP antibody (Fig. 9E, upper panel). When the immunoprecipitated samples were challenged with the anti-NR antibody, the results showed that the ERS1 protein was co-immunoprecipitated with the AtTPR1–GFP protein as homodimers and no monomers were detected (Fig. 9E, lower panel), which is consistent with AtTRP1 being associated with the ERS1 receptor in cell membranes *in vivo*.

AtTRP1 fluorescent tagged protein was localized in cell membranes and cytoplasm

The subcellular localization of AtTRP1 *in planta* was examined by confocal microscopy of leaf tissue from plants expressing AtTRP1**F**–GFP. The confocal images indicated that the fusion protein appeared to localize in the cell membranes, including plasma and nuclear membranes (Fig. 10A, C), which was consistent with the detection of the AtTRP1–GFP fusion protein in the membrane fractions (Fig. 9E, upper panel). The subcellular expression pattern of AtTRP1–GFP appeared different from an ER-targeted GFP protein. Plants expressing the ER-targeted GFP construct (kindly provided by Dr Ranjan Swarup from the University of Nottingham) produced a more diffuse pattern, with fluorescent images spread in the ER and possibly the plasma membrane (Fig. 10E), whereas plants expressing the AtTRP1–GFP construct produced a more pronounced fluorescence surrounding the nucleus and from the plasma membranes, suggesting that AtTRP1–GFP was likely to be located in these membranes (Fig. 10A, C), although the broad fluorescent areas surrounding the plasma and nuclear membranes suggested that the fusion protein might be localized in the cytoplasm.

To verify whether the AtTRP1–GFP was also located in the cytosol, the membrane and soluble proteins were separated by ultracentrifugation of the total proteins extracted from the AtTRP1 overeexpressing line and the wild type, and were challenged with the anti-GFP antibody. The results indicated that the AtTRP1–GFP fusion protein was also detected in the soluble phase in addition to its detection in the membrane fraction (Fig. 10G) in the transgenic line. Therefore, the AtTRP1 protein was localized in the plasma and nuclear membranes as well as the cytoplasm, similar subcellular locations that were found for SlTPR1-GFP when the construct was transiently co-expressed with the NR receptor in the onion epidermal cells (Lin *et al.*, 2008*b*).

Discussion

AtTRP1, an orthologue of the tomato TPR protein SlTPR1 that interacts with the tomato ethylene receptors NR and LeETR1 in yeast two-hybrid and *in vitro* pull-down assays and that modulates plant development, has been characterized (Lin *et al.*, 2008*b*). The designation of *AtTRP1* is proposed for the *Arabidopsis* gene because *AtTPR1* is already in use. AtTRP1, like SlTPR1, is closely related to

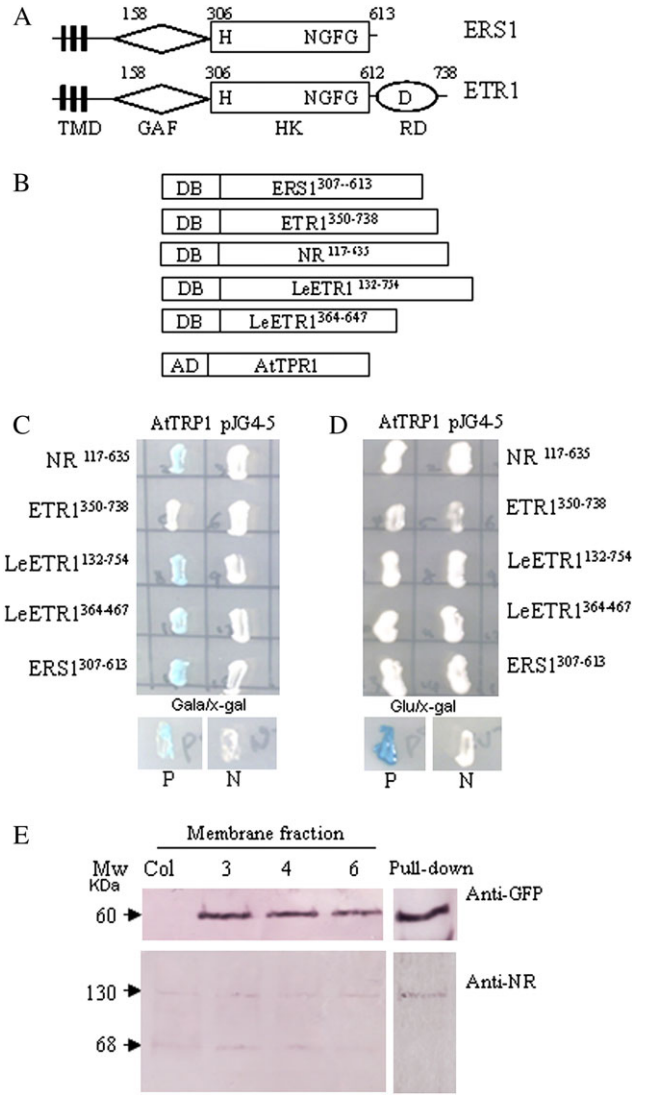

Fig. 9. Interaction assays of AtTRP1 with the ethylene receptors ERS1 and ETR1 in the yeast two-hybrid system and *in planta*. (A) Structure of ERS1 and ETR1 ethylene receptors. TMD, transmembrane domain; GAF, GAF domain; HK, histidine kinase domain; RD, receiver domain. Amino acid positions are numbered. (B) Domains used for interaction assays in the yeast two-hybrid system. DB, LexA DNA-binding-domain; AD, activation domain. Amino acids are numbered. (C) Activation analysis of the reporter gene LacZ to the bait/prey combination: Yeast transformed with DB-ERS1$^{307-613}$/AD-AtTRP1, DB-NR$^{117-635}$/AD-AtTRP1, DB-LeETR1$^{132-754}$/AD-AtTRP1, and DB-LeETR1$^{364-467}$/AD-AtTRP1 generated blue colour in 3 d when grown on minimal medium containing galactose and X-gal (Gala/x-gal), whereas yeast transformed with DB-ETR1$^{350-738}$/AD-AtTRP1 remained white when grown on the same plate, indicating no interaction. (D) All the recombinants remained white when grown on medium containing glucose and X-gal (Glu/x-gal). P, positive control; N, negative control. (E) Top panel: western blot using anti-GFP to detect AtTRP1–GFP fusion protein in the total membrane protein fraction extracted from the transgenic (lines 3, 5, 6) and wild-type (Col) seedlings (15-d-old grown in the light in soil) or after immunoprecipitation with anti-GFP antibody using total membrane proteins from the transgenic seedlings (pull-down) prior to electrophoresis. One band was detected with size 60 kDa corresponding to AtTRP1–GFP protein. Lower panel: western blot using anti-NR antibody to detect ERS1 in the same samples as the top panel. In the total membrane protein fraction two bands of 130 and 68 kDa corresponding to the homodimer or monomer of the ERS1 receptor were detected. After immunoprecipitation prior to electrophoresis only the larger band (130 kDa) corresponding to the size of the ERS1 homodimer was detected in the pull-dwon pellet, suggesting that AtTRP1 forms a complex with ERS1 dimers and not monomers.

TTC1, a human TPR protein (Fig. 2A) which links G-proteins and Ras signalling in mammalian cells and competes with Raf-1 for Ras-binding (Marty *et al.*, 2003). AtTRP1 shares overall 72% similarity to SlTPR1 and 52% to TTC1 at the protein sequence level. This sequence similarity between AtTRP1/SlTPR1 and TTC1 suggests that they may possess similar functions. Although no Ras-like proteins are found in plants, the interaction of AtTRP1 with the ethylene receptor may compete for receptor binding with CTR1, a Raf-like protein, and modulate ethylene signalling (Fig. 11).

A BLAST search of the *Arabidopsis* genome using the AtTRP1 cDNA sequence indicated that it is a single copy gene in *Arabidopsis.* Phylogenetic tree analysis using 91 *Arabidopsis* TPR proteins indicated that AtTRP1 is most closely related to TWD1 and PAS1, two immunophilin FKBPs. TWD1 is a plasma membrane protein that interacts with ABC transporters PGP1 and PGP19 to control PGP-mediated auxin transport (Bouchard *et al.*, 2006), and PAS1, together with the interacting protein FAN, is translocated into the nucleus in response to auxin treatment (Smyczynski *et al.*, 2006). AtTRP1 does not contain FKBP domains, which excludes it as an immunophilin FKBP, but the C-terminal region of AtTRP1 that contains TPR motifs shares 47% similarity with those of both TWD1 (Fig. 2C) and PAS1 (data not shown), and these structural similarities may signify a common mechanisms of action.

Expression analysis of the *AtTRP1* promoter fused to the GUS reporter gene demonstrated that the gene was highly expressed in the vascular tissue, anthers and pollen, and abscission zone, and accumulated at elevated levels in response to exogenous ethylene, supplied as ACC (Fig. 4). These results were consistent with the Northern results (Fig. 3), but revealed more detail. The high level expression of AtTRP1 in those tissues and organs is consistent with this gene playing a role in a range of developmental processes. The induction of the *AtTRP1* promoter–GUS, particularly in the abscission zone and by exogenous ACC, highlighted a positive role of *AtTRP1* in response to ethylene, as did the enhanced accumulation of *AtChitB* mRNA (see Supplementary Fig. S5 at *JXB* online). *Cis*-element analysis of the *AtTRP1* promoter identified putative biding sites of transcription factors SRY (Pontiggia *et al.*, 1994) and CdxA (Margalit *et al.*, 1993), which are involved in sex determination and organogenesis in animals (see Supplementary

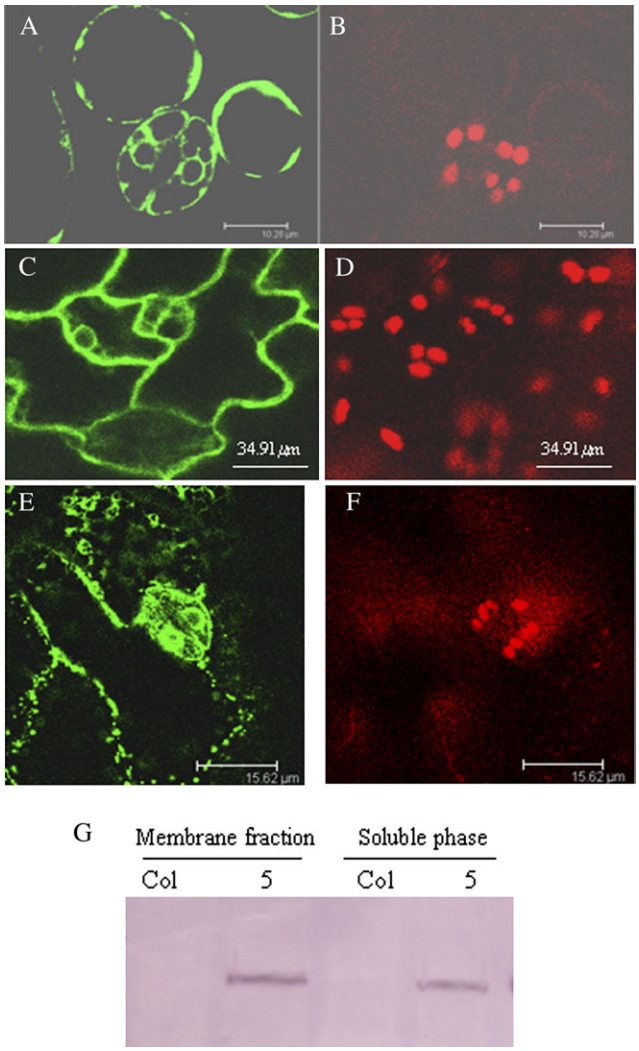

Fig. 10. Subcellular location of the AtTRP1F–GFP fusion protein. (A–F) Young leaves from 2-week-old transgenic plants over-expressing AtTRP1F–GFP (A–D) or 35S–GFP (E, F) were examined under a confocal microscope. The AtTRP1–GFP fusion protein fluorescence was localized in the plasma and nuclear membranes, and probably cytoplasm as well (A shows an image of two mesophyll cells and two GARD cells; C shows the epidermal cells), while the ER-targeted GFP was more diffused in the ER and endomembrane system (E). (B, D, F) The fluorescence from chloroplasts of the same cells as (A), (C), and (E), respectively. (G) Western blot showed that AtTRP1–GFP fusion protein was detected in the membrane fraction and the soluble phase of the transgenic line 5 by anti-GFP antibody, but was not detected in the wild-type control. Col, wild-type control; 5, transgenic line 5.

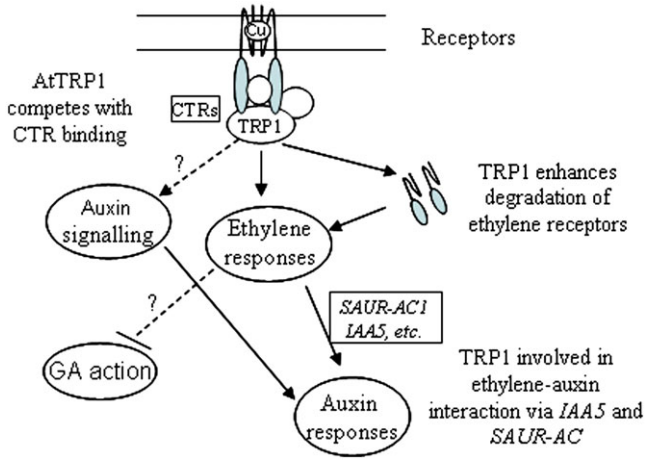

Fig. 11. A model of AtTRP1 action. AtTRP1 functions as a positive regulator to modulate ethylene signalling possibly through its interaction with one or more ethylene receptors the ethylene receptors. The exact mechanisms of AtTRP1 function remain to be elucidated, but it may compete with CTR1 for receptor binding, leading to increased ethylene responses; or it may function as an adaptor to bring a receptor for degradation. Increased ethylene responses caused by constitutive overexpression of AtTRP1 led to cross-talk with auxin via SAUR-AC1 and IAA5 at least, and possible also with GA signalling. AtTRP1 may directly interact with auxin signalling components as well.

Table S1 at *JXB* online). Overexpression of AtTRP1 to high levels *in vivo* indeed caused developmental abnormalities related to fertility and morphogenesis (Figs 5, 7). The results suggest that the *AtTRP1* gene may be transcriptionally regulated by transcription factors with similarity to those involved in sex determination or with homeotic roles. This is intriguing since the ethylene biosynthetic genes *LeACO1* and *LeACS2* are both reported to be transcriptionally regulated by homeotic proteins (Lin *et al.*, 2008*a*; Ito *et al.*, 2008), and ethylene can affect sex development in plants (Trebitsh *et al.*, 1997; Boualem *et al.*, 2008).

The *AtTRP1* gene has three predicted splicing variants in the databases (Fig. 1B, C). The full-length AtTRP1 (nt 1–831) encoding a 277 aa protein (corresponding to the splicing variant 2 from the databases) and a partial cDNA (nt 1–648) encoding the AtTRP1 N-terminal 216 aa protein (corresponding to the variant 1 in the database) in *Arabidopsis* were overexpressed. Overexpression of the N-terminal protein, which is equivalent to the splicing variant 2 with two TPR motifs, did not cause visible phenotypic effects in the normal growth condition, but etiolated transgenic seedlings exhibited enhanced responses to 0.5 μM ACC, with shortened hypocotyls and roots and an exaggerated apical hook (see Supplementary Fig. S8 at *JXB* online), indicating increased ethylene sensitivity.

Overexpression of the full-length AtTRP1 in the wild-type background resulted in pleiotropic phenotypes, including greatly reduced stature, altered leaf shape, reduced fertility, and altered morphology of siliques (Fig. 5). The severity of the phenotypes was related to the level of the transgene expression: the stronger the expression, the greater the severity of the phenotypic effects. Line 2, with strongest transgene expression, was tiny, senesced early, and produced only a few seeds (Fig. 5; see Supplementary Fig. S4 at *JXB* online). Line 3, also with strong transgene expression, was unable to produce homozygous progeny, and other lines with lower transgene expression gave intermediate phenotypes (Fig. 5; see Supplementary Fig. S4

at *JXB* online). Exogenous GA did not reverse the dwarf phenotypes of the AtTRP1 overeexpressor (see Supplementary Fig. S6 at *JXB* online), as also found in our previous study of SlTPR1 tomato transgenic plants (Lin *et al.*, 2008*b*). Achard *et al.* (2003) showed that ethylene increased the stability of the DELLA proteins to suppress GA action in *Arabidopsis* roots. Whether or not GA signalling was affected by overexpression of AtTRP1 in plants, however, requires further investigation. Etiolated transgenic seedlings overexpressing the full-length AtTRP1 were either smaller or displayed enhanced sensitivity to 0.5 μM ACC compared to the wild type (Fig. 6), and this was correlated with transgene expression. In the absence of ACC, etiolated seedlings of lines 2 and 3 displayed an enhanced ethylene response, with shortened and swollen hypocotyls and inhibited root growth, whereas lines 5 and 6, with intermediate transgene expression, were of normal size without ACC but produced shortened hypocotyls and roots in response to a low concentration of ACC. Light-grown seedlings overexpressing AtTRP1 also exhibited enhanced responses to 0.5 μM ACC compared to the wild type (Fig. 6). These results indicated that overexpression of AtTRP1 in plants resulted in enhanced ethylene sensitivity, although the transgenic seedlings did not always show swollen hypocotyls or exaggerated apical hooks.

Etiolated transgenic seedlings overexpressing AtTRP1 sometimes failed to form a typically exaggerated apical hook in response to ACC (Figs 6, 7). A possible explanation is that AtTRP1 also affects auxin responses. Apical hook formation is known to be regulated by both ethylene and auxin. Wild-type *Arabidopsis* seedlings grown in the presence of auxin or the auxin transport inhibitor 1-naphthylphthalamic acid display no hook and the auxin transport mutant *aux1* also disrupts hook formation (Roman *et al.*, 1995). The '*hookless*' mutant is caused by mutation in *HOOKLESS1* (*HLS1*), and the expression of this gene was increased by ethylene treatment, and decreased in the ethylene-insensitive mutant *ein2* (Lehman *et al.*, 1996). Other phenotypes caused by overexpression of AtTRP1, including altered morphology of leaves and siliques, were also consistent with altered auxin signalling and responses. Indeed, the AtTRP1 overexpressors displayed less sensitivity to 0.3 μM IAA treatment and the expression of a subset of early auxin responsive genes was also altered (see Supplementary Fig. S7 at *JXB* online). Microarray experiments confirmed changes to several ethylene- and auxin-related mRNAs, including *AtChitB*, *IAA5*, *SAUR-AC1* (data not shown). This could result from altered auxin transport, sensitivity, or signalling (Fig. 11). Whitelaw *et al.* (2002) has shown that auxin movement in tomato plants is affected by the reduction in *LeETR1* transcript levels, indicating that cross-talk between ethylene signalling and auxin transport involves ethylene receptors.

The e*tr1-1* mutation, which changes Cys^{65} to Tyr, abolishes ethylene binding to the receptor and causes dominant ethylene insensitivity (Bleecker *et al.*, 1988; Chang *et al.*, 1993). Overexpression of the full-length AtTRP1 in the *etr1-1* mutant resulted in some phenotypic alterations of the mutant, including reduced numbers of rosette leaves and altered morphology of rosette leaves and siliques (Fig. 8). In contrast to overexpression of the same construct in the wild-type background, overexpression of AtTRP1 in *etr1-1* did not cause dwarfed phenotypes, nor change the ethylene insensitivity of the *etr1-1* mutation (Fig. 8E), suggesting that the mutant etr1-1 receptor may mask AtTRP1 function related to ethylene sensitivity.

Protein–protein interaction analysis of AtTRP1 with ETR1 and ERS1 demonstrated that AtTRP1 was preferentially associated with ERS1 not ETR1 in yeast two-hybrid assays (Fig. 9). The association of AtTRP1 with the ERS1 receptor *in vivo* was confirmed by co-immunoprecipitation of the AtTRP1-GFP-ERS1 complex from cell membrane proteins with anti-GFP antibody and anti-NR antibody (Fig. 9E). This is consistent with the suggestion that AtTRP1 affects ethylene signalling through interaction with ERS1. However, specific antibodies against ERS1 and the other *Arabidopsis* ethylene receptors are required in order to test the specificity of the interaction *in vivo*. Although the NR antibody recognized the ERS1 monomer and dimer, only the dimer was co-immunoprecipitated with AtTRP1–GFP (Fig. 9E). These results suggest that the ERS1 receptor may function as a homodimer *in vivo* in terms of its association with AtTRP1, although at present the possibility of ERS1–ETR1 heterodimer formation can not be excluded (Gao *et al.*, 2008). This association may also bring AtTRP1 to cell membranes (Fig. 9), since there is no recognized membrane localization sequence in the AtTRP1 protein.

The physical association of AtTRP1 with ERS1, and phenotypic alterations caused by overexpression of AtTRP1, including growth retardation, reduced fertility, early senescence of line 2, enhanced responses to ACC, and increased accumulation of *AtChitB* mRNA, are all consistent with altered ethylene signalling. In addition, plants overexpressing AtTRP1 also show some auxin-related responses. It is proposed that overexpression of AtTRP1 *in vivo* results in inactivation or degradation of ERS1, or its disassociation from downstream interacting partners such as CTR1. This, in turn, would, either partially or completely, depending on the expression level, release the suppression of the ethylene responses (Fig. 11). It is known that the null mutation in ERS1 results in increased sensitivity to ethylene and that subfamily I receptors (ERS1 and ETR1) are absolutely required to suppress ethylene responses in *Arabidopsis* (Qu *et al.*, 2007). The degradation of ethylene receptors by the 26S proteasome/ubiquitin system, caused by ethylene binding, has been reported in tomato and *Arabidopsis* (Chen *et al.*, 2007; Kevany *et al.*, 2007). AtTRP1/SlTPR1 could function as adaptors causing receptor degradation, resulting in enhanced ethylene responses, as proposed previously by Lin *et al.* (2008*b*). It is also possible that this has downstream effects on auxin and GA signalling (Fig. 11). Ethylene is known to affect the stability of DELLA proteins and to reduce bioactive GA levels (Achard *et al.*, 2003, 2007), and reduced ethylene receptor mRNA can alter auxin movement (Whitelaw *et al.*,

2002). Any model for AtTRP1 action should take into account the binding to ERS1 and modulation of some, but not all ethylene, auxin, and GA-related responses. The fact that AtTRP1 binds only ERS1 may explain some of the results, since it is now becoming clear that different ethylene receptors may have different functions (Resnick *et al.*, 2006; Kevany *et al.*, 2007). AtTRP1 might also interact directly with auxin signalling components or transporters (Fig. 11). This possibility is suggested by the similarity of AtTRP1 with TWD1 and PAS1, which are involved in auxin transport or trafficking in responses to auxin (Bouchard *et al.*, 2006; Smyczynski *et al.*, 2006). However, it is not clear whether AtTRP1 acts solely through ERS1 or in concert with other proteins, and the exact function of SlTPR1/AtTRP1 remains to be elucidated.

Subcellular studies of AtTRP1–GFP fusion protein by confocal microscopy and immunodetection indicated that the protein was localized in the plasma and nuclear membranes, and cytoplasm (Fig. 10), a similar localization to that found for the tomato orthologue SlTPR1. This is intriguing since various subcellular localizations of ethylene receptors have been reported, including the ER, the Golgi apparatus, and the plasma and nuclear membranes (Xie *et al.*, 2003; Ma *et al.*, 2006; Dong *et al.*, 2008; Lin *et al.*, 2008*b*; Zhong *et al.*, 2008). This may suggest that the ethylene receptors are involved in movement or trafficking in response to diverse developmental and environmental cues. Although our immunoprecipitation experiments showed that the ERS1 receptor was pulled-down with AtTRP1 in the membrane fraction, this does not exclude the possibility that protein interactions may occur in the cytosol, since the melon CmERS1 ethylene receptor is topologically anchored at the ER membrane via its N-terminal transmembrane domains, but its C-terminal domains face the cytosol, and could interact with proteins located in the cytoplasm (Ma *et al.*, 2006). Further studies are required to unveil the mechanisms whereby AtTRP1/SlTPR1 binds to specific ethylene receptors and perhaps other proteins, and modulates hormone effects and signalling, as a step towards understanding the complexity of hormone interactions.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Putative *cis*-elements present in the AtTRP1 promoter sequence.

Supplementary Table S2. Responses of light-grown seedlings to exogenous IAA.

Supplementary Fig. S1. Phylogenetic tree analysis to determine the relationship between AtTRP1 and other TPR proteins.

Supplementary Fig. S2. DNA sequence alignment of AtTRP1 and TWD1.

Supplementary Fig. S3. (A) Construct to overexpress the N-terminal region of AtTRP1 used for this study. (B) Verification of the AtTRP1N-GFP expression in plants.

Supplementary Fig. S4. Phenotypes of the progeny of the AtTRP1 overexpressors.

Supplementary Fig. S5. Northern analysis to determine the expression of ethylene biosynthesis and response genes in AtTRP1-overexpressing *Arabidopsis* plants.

Supplementary Fig. S6. Characterization of the transgenic plants overexpressing AtTRP1F-GFP in response to GA.

Supplementary Fig. S7. Characterization of the auxin responses of transgenic plants overexpressing AtTRP1F-GFP.

Supplementary Fig. S8. Characterization of transgenic plants overexpressing the AtTRP1 N-terminal construct.

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