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RESEARCH PAPER

Modification of tomato growth by expression of truncated ERECTA protein from *Arabidopsis thaliana*

Hector Villagarcia[1], Anne-Claire Morin[1,3], Elena D. Shpak[2,*] and Mariya V. Khodakovskaya[1,*]

[1] Department of Applied Science, University of Arkansas at Little Rock, Little Rock, AR 72204, USA
[2] Department of Biochemistry, Cellular & Molecular Biology, University of Tennessee, Knoxville, TN 37996, USA
[3] École d'Ingénieurs du Cesi – Pôle d'excellence Ingénieurs et Enseignement Supérieur, 86 route de Breuty, 16400, La Couronne, France

* To whom correspondence should be addressed. E-mail: mvkhodakovsk@ualr.edu or eshpak@utk.edu

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Abstract

***ERECTA* family genes encode leucine-rich repeat receptor-like kinases that control multiple aspects of plant development such as elongation of aboveground organs, leaf initiation, development of flowers, and epidermis differentiation. These receptors have also been implicated in responses to biotic and abiotic stress, probably as a consequence of their involvement in regulation of plant architecture. Here, ERECTA signalling in tomatoes (*Solanum lycopersicum*) was manipulated by expressing truncated ERECTA protein (At∆Kinase) from *Arabidopsis* using two different promoters. In *Arabidopsis*, this protein functions in a dominant-negative manner, disrupting signalling of the whole *ERECTA* gene family. Expression of *At∆Kinase* under a constitutive 35S promoter dramatically reduced vegetative growth and led to the formation of fruits with a reduced seed set. Similarly, expression of *At∆Kinase* under its own promoter resulted in transgenic tomato plants with diminished growth, a reduced number of leaves, changed flowering time, and slightly increased stomata density. The transgenic plants also exhibited increased tolerance to water deficit stress, at least partially due to their diminished surface area. These phenotypes of the transgenic plants were the result of ERECTA signalling disruption at the protein level, as the expression of two endogenous tomato *ERECTA* family genes was not suppressed. These results demonstrate the significance of *ERECTA* family genes for development and stress responses in tomato and suggest that truncated ERECTA can be used to manipulate the growth of crop species.**

Key words: ERECTA, receptor-like kinase, *Solanum lycopersicum*, vegetative development, water deficit stress.

Introduction

The development and growth of multicellular organisms relies on the control of the number of cell divisions and on the temporal and spatial accuracy of cell differentiation. The precise regulation and coordination of these processes is especially important in plants, where the cell wall prevents cell migration. Together with phytohormones, receptor-like kinases (RLKs) are central regulators of cell proliferation and differentiation (Morris and Walker, 2003; Morillo and Tax, 2006; De Smet *et al*., 2009). Structurally, a typical receptor kinase contains an extracellular ligand-binding domain, a single-span transmembrane domain, and an intracellular Ser/Thr kinase domain with two flanking regulatory sequences: the juxtamembrane and the C-terminal regions. RLKs with a variable number of leucine-rich repeats (LRRs) in the extracellular domain constitute the largest subfamily, with approximately 200–350 members in angiosperms (Dievart *et al*., 2011).

ERECTA family LRR-RLKs are important regulators of multiple aspects of plant development. In *Arabidopsis*, this family is composed of three genes: *ERECTA* (*ER*), *ERECTA-LIKE 1* (*ERL1*) and *ERECTA-LIKE 2* (*ERL2*). Together, they regulate

Abbreviations: LRR, leucine-rich repeat; RLK, receptor-like kinase; SGN, Solanaceae Genomics Network.

elongation along the proximodistal axis in all aboveground organs, with the triple *er erl1 erl2* mutant being a dwarf with small, round leaves, stunted inflorescence growth, short petioles, pedicels and siliques, and small sterile flowers that have a reduced number or fused organs (Shpak *et al.*, 2004). During flower development, *ERECTA* family genes have been shown to promote integument growth (Pillitteri *et al.*, 2007) and to regulate anther differentiation (Hord *et al.*, 2008). The regulation of organ elongation by *ERECTA* family genes is attributed to their function in the phloem, where they perceive signals from the endodermis (Uchida *et al.*, 2012). These signals are the small secreted peptides EPFL4 and EPFL6/CHALLAH (Abrash *et al.*, 2011; Uchida *et al.*, 2012). While the *ERECTA* gene has been linked previously with vascular development, with the *er* mutation resulting in premature differentiation of vascular bundles (Douglas and Riggs, 2005) and radial expansion of xylem (Ragni *et al.*, 2011), the exact nature of its function in the phloem is not known.

In addition to the phloem, *ERECTA* family genes are expressed in the epidermis, where they control stomatal development (Shpak *et al.*, 2005). Differentiation of a stoma begins with asymmetric division in a subset of protodermal cells called meristemoid mother cells, giving rise to small, triangular meristemoid cells and larger sister cells. In *Arabidopsis*, a meristemoid undergoes one to three rounds of asymmetric division before it differentiates into a round guard mother cell. The guard mother cell divides symmetrically to produce a pair of guard cells. All three *ERECTA* family genes inhibit differentiation of protodermal cells into meristemoid mother cells. In addition, ERL1 and, to a lesser extent, ERL2 promote asymmetric cell divisions in meristemoids, preventing their differentiation into guard mother cells. Among the ligands of the ERECTA family receptors in the epidermis are the two peptides EPIDERMAL PATTERNING FACTOR 1 and 2 (EPF1 and EPF2), which activate receptors inhibiting development of stomata (Hara *et al.*, 2007, 2009; Hunt and Gray, 2009; Lee *et al.*, 2012). Due to their role in stomatal development, *ERECTA* family genes have a significant impact on plant responses to water stress. These genes decrease stomata density on the leaf surface, and as a result reduce overall stomata conductance leading to lower water loss (Masle *et al.*, 2005). Furthermore, signalling via ERECTA family receptors coordinates transpiration with the photosynthetic capacity of a leaf, as mesophyll cells produce the small signal peptide STOMAGEN, which inhibits receptor activity and promotes stomatal development (Kondo *et al.*, 2010; Sugano *et al.*, 2010). The involvement of ERECTA in leaf development affects both transpiration and photosynthetic capacity, resulting in improved transpiration efficiency (Masle *et al.*, 2005).

Considering the importance of the *ERECTA* gene family for plant morphology and water stress tolerance, it is surprising how little this gene family has been studied in agronomically important crop species. In this study, we explored the function of the *ERECTA* signalling pathway in tomato (*Solanum lycopersicum*), because of the importance of this plant for agriculture and its amiability to genetic manipulations. Tomato is a model organism for the family *Solanaceae*, which consists of around 2000–3000 species including such crops as potato, eggplant, and peppers. To downregulate *ERECTA* gene family signalling in tomato, we used truncated ERECTA protein from *Arabidopsis* (Shpak *et al.*, 2003). Truncated ERECTA (ΔKinase) lacks the cytoplasmic domain, which includes the kinase domain and two flanking regulatory sequences. This truncation drastically increases the accumulation of ΔKinase in *Arabidopsis* (Shpak *et al.*, 2003). As ΔKinase binds the ligands (Lee *et al.*, 2012) but cannot transmit the signal, it functions in a dominant-negative manner and decreases signalling of the ERECTA family receptors (Shpak *et al.*, 2003). Detailed morphological analysis of established *AtERpro:ΔKinase* transgenic tomato lines showed that the truncated *Arabidopsis* ERECTA protein interacts with the endogenous *ERECTA* gene family signalling, negatively regulating the vegetative growth of tomato plants. One of the aspects of reduced vegetative growth was decreased leaf size and number, which significantly reduced plant surface area. We assume that the decrease in surface area is the cause of the increased drought tolerance observed in the transgenic tomatoes. While the *AtERpro:ΔKinase* tomato lines exhibited a change in flowering/fruiting time, neither fruit yield nor fruit size was affected. These data demonstrated that truncated ERECTA protein can be used successfully to modify ERECTA family signalling in other plant species. In addition, they highlight the importance of ERECTA for plant growth and transition to flowering in tomatoes.

Materials and methods

Comparative sequence and phylogenetic analysis

DNA and amino acid sequences of *ERECTA* family genes from grape (*Vitis vinifera*), rice (*Oryza sativa* L. ssp. *japonica*), sorghum (*Sorghum bicolor*), soybeans (*Glycine max*), castor oil plant (*Ricinus communis*), barley (*Hordeum vulgar*e), purple false brome (*Brachypodium distachyon*), the spike moss *Selaginella moellendorffii*, and the moss *Physcomitrella patens* were obtain by BLAST searches against the NCBI database (http://www.ncbi.nlm.nih.gov/). DNA and amino acid sequences of *ERECTA* family genes from tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum phureja*) were obtained by BLAST searches against the Solanaceae Genomics Network (SGN) database (http://solgenomics.net/). Protein sequences of *ERECTA* family genes were aligned using MAFFT v6.859b (http://mafft.cbrc.jp/alignment/software/). A phylogenetic tree with aligned sequences was constructed using the RaxmlGUI 1.0 program (http://sourceforge.net/projects/raxmlgui/) with 1000 bootstrap replicas.

Generation of transgenic tomato plants

The miniature tomato cultivar Micro-Tom (*Solanum lycopersicum* cv. Micro-Tom) was used to generate transgenic plants carrying the *AtΔKinase* gene. A genomic sequence from *Arabidopsis* encoding truncated ERECTA protein was cloned under the control of the cauliflower mosaic virus 35S dual promoter (pESH454) or its native 1.7 kb promoter region (pESH 456b). Both plasmids are binary vectors based on pBin20 and contain the endogenous 1.9 kb *ERECTA* terminator region. The cloned 4196 bp genomic sequence of ERECTA encodes the N-terminal part of the *ERECTA* gene with a stop codon introduced after the codon for S614.

The engineered plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101/pMP90. Tomato plants (*Solanum lycopersicum* cv. Micro-Tom) were transformed by agrobacterial transformation of cotyledon explants according to Cortina and Culianez-Macia (2004). Kanamycin-resistant plants (generation T_0)

were analysed by PCR for integration of the transgene using forward (5'-GGACTTGTCCTACAATCAGCTAACT-3') and reverse (5'-TTGAACCAGTCAGCTTGTTACTGTG-3') primers. T1 and T2 seeds of transgenic plants transformed with pESH456b were germinated on Murashige and Skoog medium containing 2.5% (w/v) sucrose and 0.8% (w/v) agar supplemented with 100 mg l^{-1} of kanamycin, and the presence of the transgene was confirmed by PCR (Supplementary Fig. S2A at *JXB* online). Expression of the transgene in transgenic lines was confirmed by RT-PCR using primers for a 100 bp fragment of the *At*Δ*Kinase* gene (Supplementary Fig. S2B). Finally, two independent homozygous lines (L1 and L2) were selected in the T_3 generation for further analysis. Total genomic DNA for the PCR analysis was extracted using a MasterPure Plant Leaf DNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturer's protocol.

Real-time RT-PCR

Total RNA was isolated from the apex and young leaves of 55-d-old tomato plants and flowers and green fruits of 77-d-old plants using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). To minimize contamination with genomic DNA, RNA was treated with an RNase-Free DNase Set (Qiagen). cDNA was generated from 1 µg of total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) with a dT_{16} oligonucleotide as primer according to the manufacturer's protocol. cDNA samples were diluted ten times and 4 µl of each was used for real-time quantitative PCR analysis with SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA, USA) in an iCycler iQ Multicolor Real Time PCR detection system (Bio-Rad, Hercules, CA, USA). The total volume of the reaction was 20 µl and the following primers were used: 5'-AAAGGTCCAATCCCGGTTGAG CTA-3' and 5'-AAAGTCGCCTGGAACTACACCAGT-3' to amplify *At*Δ*Kinase*; 5'-TACTTGGTATTGCTCTGGGTGGCT-3' and 5'-ATGAAA GGTGCAGGTTTCTGTGGC-3' to amplify *SlER* (Solyc08g061560.2.1); 5'-CCCGATCGTTTTGGCTCTCACC-3' and 5'-CACAACACGCC TCTCCATGAGC-3' to amplify *SlERL* (Solyc03g007050.2.1); and AGGTATTGTGTTGGACTCTGGTGAT-3' and 5'-ACGGAGAATGGC ATGTGGAA-3' to amplify *Tom41*, a tomato actin gene used as a housekeeping control to confirm the consistency of tissue collection. Three independent biological replicates were used in the analysis. For each biological replica, we performed three technical replicas. The real-time PCR data were generated and analysed by the 'comparative count' method to obtain relative mRNA expression of each tissue as described in the iCycle manual (Bio-Rad). Initial RNA samples were run as negative controls to exclude the possibility of DNA contamination.

Plant growth conditions and morphometric analysis of transgenic and wild-type plants

Seedlings (10-d-old) of wild-type and L1 and L2 transgenic tomato lines were transferred into pots containing sterile Sun Gro Redi-earth Plug and Seedling Mix (Sun Gro Horticulture, Bellevue, WA, USA). All plants were grown in a growth chamber under conditions of 12 h light (28 °C) and 12 h dark (22 °C), 45% humidity, and 500 µmol m^{-2} s^{-1} light intensity, and were watered once a day. Eight mature plants from each experimental group (wild type, L1, and L2) were analysed, and the following data were recorded: length of leaves at 28-d-old, total number of leaves in 45-d-old plants, and number of mature fruits and size of fruits in 119-d-old plants. Student's *t*-test was used to assess the significance of differences in the data between groups of plants. In a separate experiment, plant height was recorded every other day starting from 35-d-old plants, up to 71 d of plant cultivation. Total leaf surface area was measured in 45-d-old plants by scanning all leaves removed from a plant and then analysing the area using the ImageJ software (http://rsb.info.nih.gov/ij/). To determine total dry biomass, 45-d-old plants from each group were oven dried at 80 °C for 4 d. The roots were weighed separately from the shoots to evaluate the shoot-to-root ratio. Stomata density was calculated in the adaxial side of fully expanded leaves of 56-d-old plants.

Water deficit stress experiments

Eight-week-old tomato plants were grown in soil and watered evenly. The soil was then allowed to dry by withholding watering until plants showed severe drought stress symptoms (wilting and visible loss of turgor). For water loss studies, fully expanded leaves of comparable size, weight, and development were excised from well-hydrated plants and incubated at room temperature under light and 30% humidity for 5 h. Leaf water loss in detached leaves was determined as a percentage of total initial weight for each leaf. In all of the drought tolerance and water loss studies, plants or detached leaves were kept under 500 µmol m^{-2} s^{-1} light intensity, 12 h light (28 °C) and 12 h dark (22 °C), and 45% humidity. During drought stress experiments, the moisture in the soil was quantified by measuring the volumetric water content value of each pot using a VH400 Vegetronix Moisture Sensor Probe. Prior to sample measurements, the probe was calibrated (Supplementary Fig. S4 at *JXB* online). During the drought stress experiment, stomatal conductance values were measured using a portable Licor 6400 photosynthesis system (LI-6400; Li-Cor, Lincoln NE, USA) according to the manufacturer's protocol.

Accession numbers

The sequence data for *Arabidopsis thaliana* genes can be found in The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/) under the accession numbers At2g26330 (*AtER*), At5g62230 (*AtERL1*), At5g07180 (*AtERL2*), and At1g31420 (*AtFEI1*). The sequence data for tomato genes can be found in the SGN database under accession numbers Solyc08g061560.2 (*SlER*) and Solyc03g007050.2 (*SlERL*). The sequence data for potato genes can be found in the Potato Genome Sequencing Consortium database (http://potatogenomics.plantbiology.msu.edu/blast.html) under accession numbers PGSC0003DMP400032803 (*StER*) and PGSC0003DMC400054040 (*StERL*).

The sequence data for other genes can be found on the NCBI website under the following accession numbers: XP_002280069 (*VvER*), XP_002269540 (*VvERL*), NP_001057087 (*OsER1*), AFJ14786 (*OsER2*), BAD44800 (*OsERL*), XP_002438023 (*SbER1*), AAL68842 (*SbER2*), XP_002436407 (*SbERL*), XP_003544548 (*GmER1*), XP_003522304 (*GmER2*), NP_001237639 (*GmERL1*), NP_001235330 (*GmERL2*), XP_003534036 (*GmERL3*), XP_002516144 (*RcER*), XP_002510897 (*RcERL*), BAK07025 (*HvER*), BAJ89970 (*HvERL*), BAK07025 (*BdER*), XP_003561134 (*BdERL*), XP_002975149 (*SmERL1*), XP_002981099 (*SmERL1*), XP_001783127 (*PpERL1a*), XP_001755734 (*PpERL1b*), XP_001770079 (*PpERL1c*), XP_001755991 (*PpERL1d*), XP_001763775 (*PpERL2a*), XP_001770421 (*PpERL2b*) and U60480.1 (*Tom41* actin).

Results

In tomato, the ERECTA gene family consists of SlER and SlERL, which are expressed in young developing aboveground tissues

The complete tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum* L.) genomes have been published recently (Xu *et al*., 2011; Sato *et al*., 2012). Our analysis of these genomes uncovered two genes belonging to the *ERECTA* gene family in each species (Supplementary Fig. S1 at *JXB* online). The tomato and potato ERECTA proteins (SlER and StER) share 98% identity and 99% similarity with each other, and 80% identity and 89% similarity with *Arabidopsis* ERECTA. The tomato and potato ERECTA-like proteins (SlERL and StERL) share 97% identity and 98% similarity with each other, and 75% identity and 85% similarity with *Arabidopsis* ERL1 and 73% identity and 84 % similarity with *Arabidopsis* ERL2. As expected,

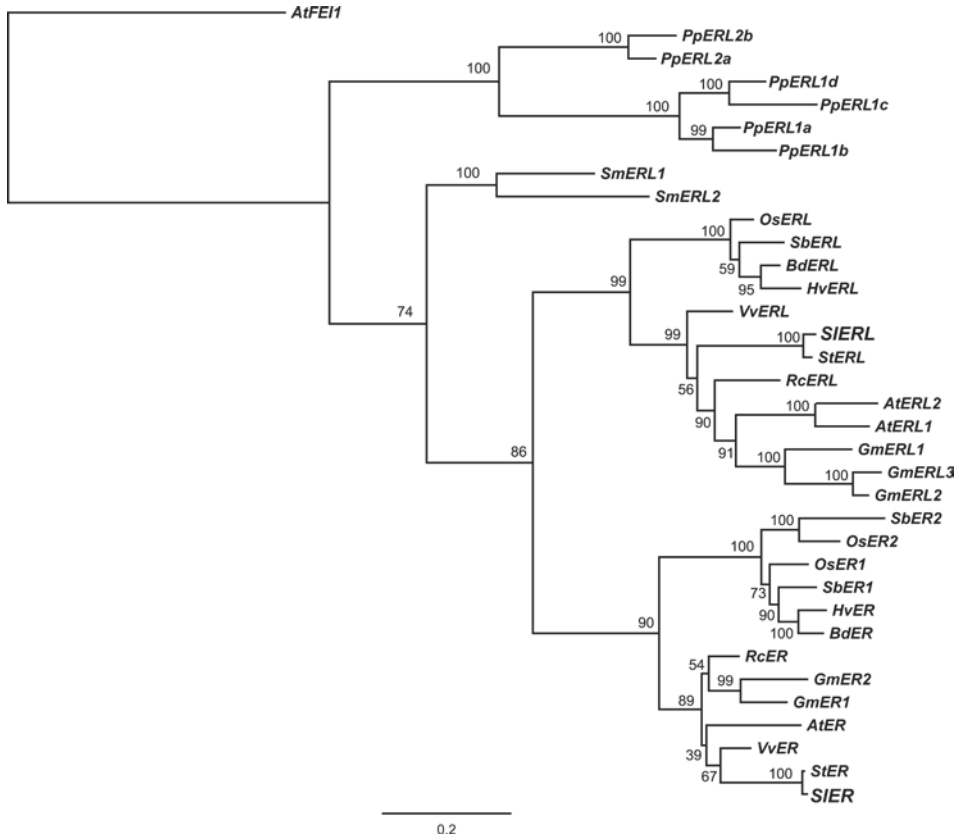

Fig. 1. Phylogenetic tree of *ERECTA* family genes from a variety of species, based on homology of full-length protein sequences minus the signal sequence. The tree was constructed using maximum likelihood-based phylogenetic analyses with *AtFEI1* as an outgroup. Bar, 0.2 amino acid substitutions per site. Bootstrap values are indicated next to corresponding nodes. *At*, *Arabidopsis thaliana*; *Vv*, *Vitis vinifera*; *Sl*, *Solanum lycopersicum*; *St*, *Solanum tuberosum phureja*; *Gm*, *Glycine max*; *Rc*, *Ricinus communis*; *Os*, *Oryza sativa* L. ssp. *japonica*; *Sb*, *Sorghum bicolor*; *Hv*, *Hordeum vulgare*; *Bd*, *Brachypodium distachyon*; *Sm*, *Selaginella moellendorffii*; *Pp*, *Physcomitrella patens*.

the least conservation of sequence was observed in the signal sequence, transmembrane, and C-terminal regions. Analysis of the *ERECTA* family phylogenetic tree (Fig. 1) suggested that this group of genes appeared early during land-plant evolution in the bryophytes. Some time before the appearance of dicots and monocots, the gene family split into two main groups, *ER* and *ERL*. Later on, several duplication events occurred in selected groups of species creating multiple *ER* (e.g. in monocots and in soybeans) or *ERL* (e.g. in *Arabidopsis* and in soybeans) genes, while in a range of species, including tomato, potato, grapes, and castor oil plants, this gene family is still represented by only two genes.

Next, we investigated *SlER* and *SlERL* expression in aboveground tomato organs by quantitative RT-PCR (Fig. 2). Both genes were very highly expressed in the apices of tomato plants, which resembled the high expression of *ERECTA* family genes in the shoot apical meristem and developing leaves of *Arabidopsis* (Yokoyama *et al.*, 1998; Shpak *et al.*, 2004). *SlER* gene expression was also high in green fruits (65% of the expression level in the apex). Both genes were expressed in fully expanded leaves and in opened flowers but at considerably lower levels compared with the apex. These data suggested that both genes function in young developing aboveground organs and that *SlER* might have a unique role in fruit development.

To explore the function of the *ERECTA* gene family in tomato, we used a dominant-negative approach with the goal of disrupting the signalling of the whole gene family.

Establishment of transgenic tomato plants carrying AtERpro:AtΔKinase and 35Spro:AtΔKinase constructs

In *Arabidopsis*, the *ERECTA* family genes are partially redundant (Shpak *et al.*, 2004). To overcome the problem of potential redundancy of this gene family in tomatoes, we used a dominant-negative approach and expressed a truncated version of ERECTA (ΔKinase), which was successful previously in disrupting *ERECTA* gene family signalling in *Arabidopsis* (Shpak *et al.*, 2003). To ensure that truncated ERECTA was expressed at a high level, we used a genomic version, as introns are essential for *ERECTA* mRNA accumulation (Karve *et al.*, 2011). This dominant-negative approach, using truncated receptor kinases consisting of only extracellular and transmembrane domains, is commonly used for dissecting the function of animal receptor kinases and, among others, has been used to study the

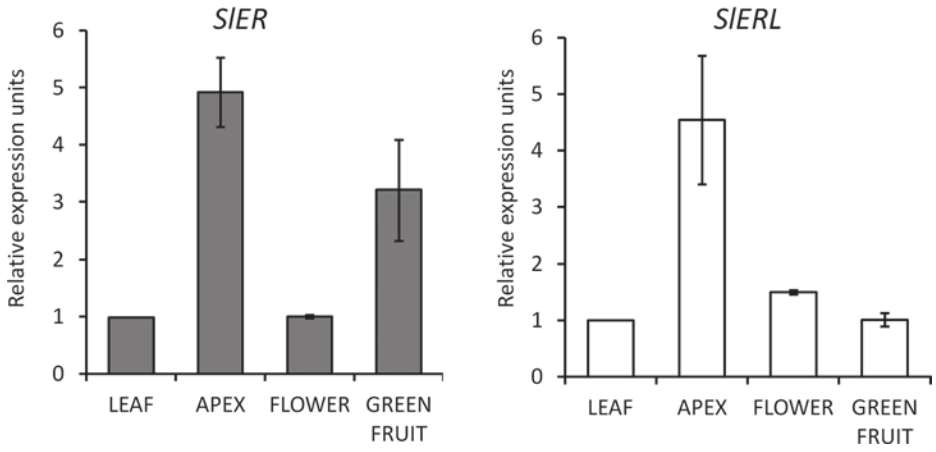

Fig. 2. Analysis of *SlER* and *SlERL* expression in different organs of tomato plants by quantitative RT-PCR with tomato actin (*Tom41*) as an internal control. The apex is the most distal part of a shoot consisting of an apical meristem with surrounding leaf primordia and adjacent stem tissue. Samples of leaves and apices were collected from 55-d-old plants and of flowers and green fruits from 77-d-old plants. Vertical bars indicate means ±SE of three biological replicates.

signalling of platelet-derived growth factor β, fibroblast growth factor, activin, BMP4, and Sek-1 receptors (Amaya *et al.*, 1991; Ueno *et al.*, 1991; Hemmati-Brivanlou and Melton, 1992; Graff *et al.*, 1994; Xu *et al.*, 1995). Ligand binding to the extracellular domain of a receptor kinase usually leads to activation of its intracellular catalytic domain (Lemmon and Schlessinger, 2010). Overexpression of a truncated receptor disrupts the signalling, as this protein forms heterodimers with endogenous receptors and binds the ligands but cannot activate downstream targets (Ueno *et al.*, 1991). In *Arabidopsis*, the truncated form of ERECTA is expressed at a considerably higher level compared with the endogenous receptor (Shpak *et al.*, 2003). As truncated ERECTA can bind ligands (Lee *et al.*, 2012) and is likely to form heterodimers with endogenous ERECTA family receptors, we predicted that, in transgenic plants expressing ΔKinase, the formation of functional receptor complexes should be strongly reduced.

We used *At*Δ*Kinase* to suppress *ERECTA* gene family signalling in tomato plants (cv. Micro-Tom) by expressing it under the control of an endogenous *Arabidopsis ERECTA* promoter or a constitutive 35S promoter. Twelve transgenic lines were obtained for the *AtERpro:At*Δ*Kinase* construct and five for *35Spro:At*Δ*Kinase*. PCR analysis confirmed integration of constructs into the genome of all lines (see data for selected *AtERpro:At*Δ*Kinase* lines in Supplementary Fig. S2A). We also confirmed expression of the *At*Δ*Kinase* gene by RT-PCR in selected *AtERpro:At*Δ*Kinase* lines (Supplementary Fig. S2B).

T_0 transgenic plants expressing *At*Δ*Kinase* under the constitutive 35S promoter and grown *in vitro* had a strong dwarf phenotype, exhibited dramatically deceased vegetative development, and instead of leaves produced a flower that formed a sterile fruit (Fig. 3B). We were not able to establish *35Spro:At*Δ*Kinase* plants suitable for cultivation in soil or producing seeds, and thus did not obtain the next T_1 generation.

In contrast, *AtERpro:At*Δ*Kinase* transformants did not show such a drastic dwarf phenotype, and we were able to obtain the next generation (Fig. 3A). Two independent homozygous *AtERpro:At*Δ*Kinase* lines (L1 and L2) were chosen for detailed

Fig. 3. Transgenic tomato plants expressing *At*Δ*Kinase* under native *AtER* (A) and under constitutive 35S (B) promoters show extreme morphological differences compared with wild-type tomato plants. All plants were grown on Murashige and Skoog medium with agar in Magenta boxes. Ten-day-old wild-type and transgenic T_1 *AtERpro:At*Δ*Kinase* seedlings (lines L1 and L2), and 1-month-old T_0 *35Spro:At*Δ*Kinase* plants were removed from the medium and photographed. Bar, 1 cm.

analysis. Examination of *At*Δ*Kinase* expression in L1 and L2 revealed that the highest expression of the transgene occurred in the apices (Fig. 4A). A much lower level of *At*Δ*Kinase* expression was detected in leaves, flowers, and green fruits of transgenic lines (Fig. 4A). As expected, *At*Δ*Kinase* gene expression was not observed in wild-type tomato plants. Interestingly, the expression of endogenous *SlER* and *SlERL* genes was elevated in both transgenic lines and in all analysed organs compared with the wild type (Fig. 4B, C). Expression was especially

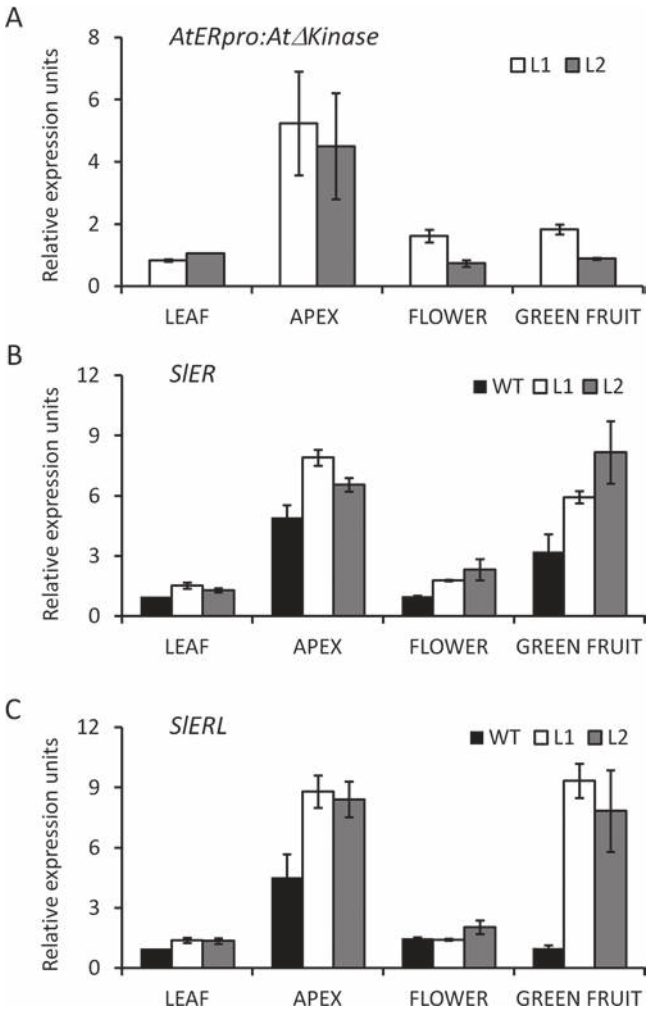

Fig. 4. In transgenic tomato plants *AtΔKinase* is expressed in a pattern similar to endogenous *ERECTA* family genes and leads to their upregulation. A quantitative RT-PCR analysis of *AtΔKinase* (A) and of endogenous *SlER* (B) and *SlERL* (C) gene expression was performed in different organs of wild-type plants and of transgenic L1 and L2 *AtERpro:AtΔKinase* lines. Tomato actin (*Tom41*) was used as an internal control. Samples of leaves and apices were collected from 55-d-old plants and of flowers and green fruits from 77-d-old plants. Results are shown as means ±SE of three biological replicates.

strongly upregulated in the apices and green fruits (Fig. 4B, C). Previously, it has been noted that in *Arabidopsis* a decrease in the number of functional *ERECTA* family genes results in upregulation of the remaining genes of that family (Pillitteri *et al.*, 2007). Similarly, it is likely that the downregulation of the ERECTA signalling pathway by *AtΔKinase* induced compensation and increased expression of endogenous *SlER* and *SlERL* genes.

Stem growth and leaf formation are reduced in AtERpro:AtΔKinase tomato plants

From the beginning, we observed that *AtERpro:AtΔKinase* tomato plants grew more slowly than the wild type. Ten-day-old T_1 *AtERpro:AtΔKinase* seedlings of both analysed lines (L1 and L2) had considerably shorter stems and primary roots when grown in the medium (Fig. 3A). During long-term cultivation of homozygous T_3 and T_4 plants in soil, we again observed slower growth of the transgenic lines (Fig. 5A, B). After 24 d growing in soil, the transgenic plants were markedly shorter than the wild type (Fig. 5A). Temporal analysis of *AtERpro:AtΔKinase* plant growth demonstrated that these lines grew more slowly compared with the wild type but ultimately reached the same size (Fig. 5B). The duration of growth in transgenic lines was extended by ~7–10 d (Fig. 5C). The cultivar Micro-Tom is a determinate variety of tomatoes. Our data suggested that, while the growth rate of tomato plants was reduced by the *AtERpro:AtΔKinase* construct, the transition to flowering occurred at the same plant height as in the wild type.

Further analysis of shoot development detected differences in leaf initiation and growth between the *AtERpro:AtΔKinase* lines and the wild type. The terminal leaflets of the oldest leaves that had reached their final size were consistently shorter in transgenic plants than in the wild type (Fig. 6A). In addition, a dramatically smaller number of total leaves was formed by *AtERpro:AtΔKinase* plants (Fig. 6B). As leaves were smaller and fewer of them formed, the total leaf area per plant and the dry weight of shoots were significantly decreased in transgenic plants compared with the wild type (Fig. 6B, C). The dry weight of total root biomass was also significantly reduced in the L2 *AtERpro:AtΔKinase* line but not in L1 (Fig. 6C).

These data suggested that the expression of *Arabidopsis ΔKinase* in tomatoes changes their development by inhibiting vegetative growth. The effect was most dramatic in aboveground organs, stems, and leaves, but reduced root growth was also observed.

The transition to flowering occurs at an earlier developmental time in AtERpro:AtΔKinase plants, but the size and number of fruits are not changed

In most plant species, including tomato, there is a strong correlation between the number of leaves formed and the transition to flowering (Lifschitz *et al.*, 2006). The *AtERpro:AtΔKinase* transgenic tomato plants grew more slowly and flowered approximately 6 d later (Fig. 7A, B). At the same time, flower development occurred when fewer leaves were formed compared with the wild type, suggesting an earlier developmental transition to flowering (Fig 6B). As a result of the timing delay in flowering, the production of fruits in the transgenic plants was also postponed (Fig. 7B). However, the mean number of fruits per plant and the fruit size were not changed in the transgenic lines (Fig. 7C). In *Arabidopsis*, *ERECTA* family genes are known to regulate the size of siliques (Torii *et al.*, 1996; Shpak *et al.*, 2003, 2004). Based on the high expression level of *SlER* in the wild-type tomato fruits, one might expect that this gene is also involved in the regulation of fruit development and size. One possibility is that upregulation of *SlER* and *SlERL* in transgenic plants can overcome downregulation of the pathway by *AtΔKinase*. Alternatively, the function of the *ERECTA* family genes in fruits of *Arabidopsis* and tomato might be somewhat distinct.

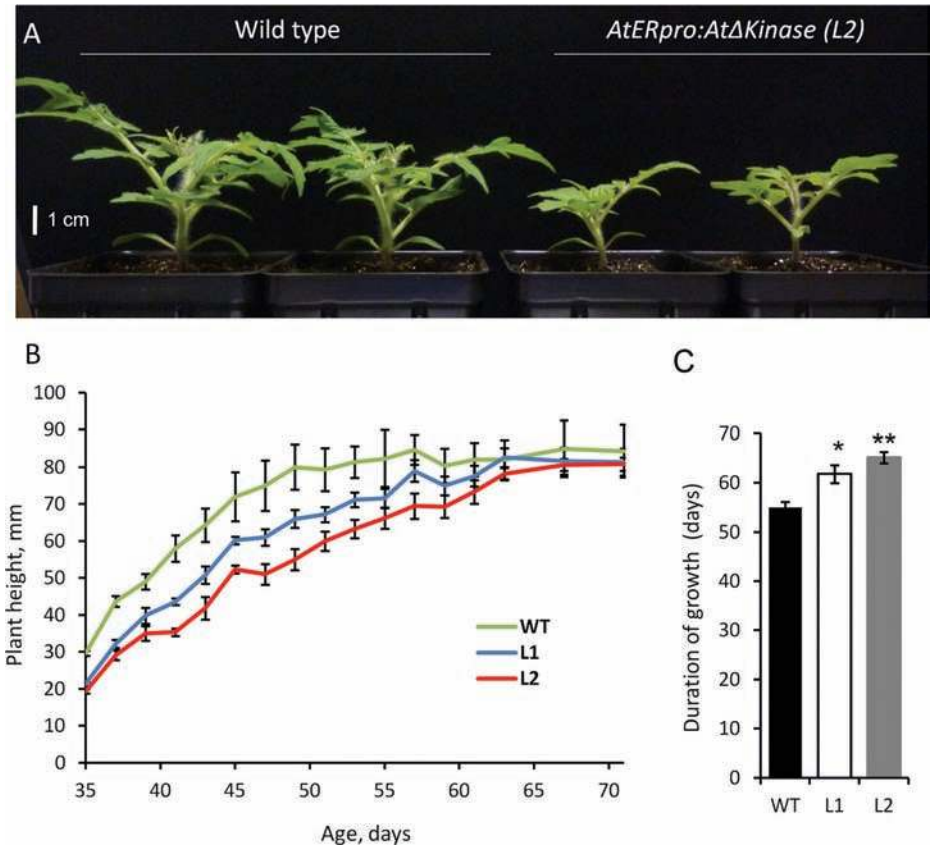

Fig. 5. *AtERpro:AtΔKinase* transgenic plants grow more slowly compared with wild-type plants but reach a similar size at maturity. (A) Comparison of 24-d-old wild-type plants and *AtERpro:AtΔKinase* plants (T_3 generation of L2). (B) Changes in height of wild-type and two *AtERpro:AtΔKinase* transgenic lines (L1 and L2; generation T_4) during long-term cultivation in a growth chamber (n=5). (C) Comparison of the duration of plant growth in wild-type and transgenic lines (generation T_4). Results are shown as means ±SE (n=5; *P <0.05, **P <0.01).

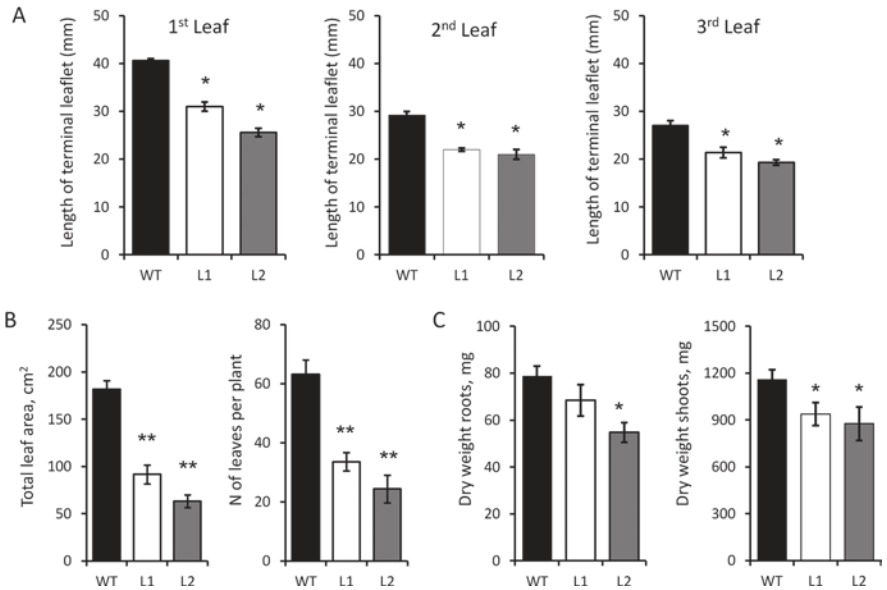

Fig. 6. *AtERpro:AtΔKinase* transgenic plants (generation T_3) have smaller leaves that form more slowly. (A) Length of the terminal leaflet of the three oldest leaves from 28-d-old wild-type and *AtERpro:AtΔKinase* transgenic lines L1 and L2 (n=5; *P <0.01). (B) Total leaf area per plant and total number of leaves in 45-d-old wild-type and transgenic lines (n=5; **P <0.01). (C) Mean dry weight of roots and shoots of 45-d-old tomato wild-type and transgenic lines (n=9; *P <0.05). Results are shown as means ±SE.

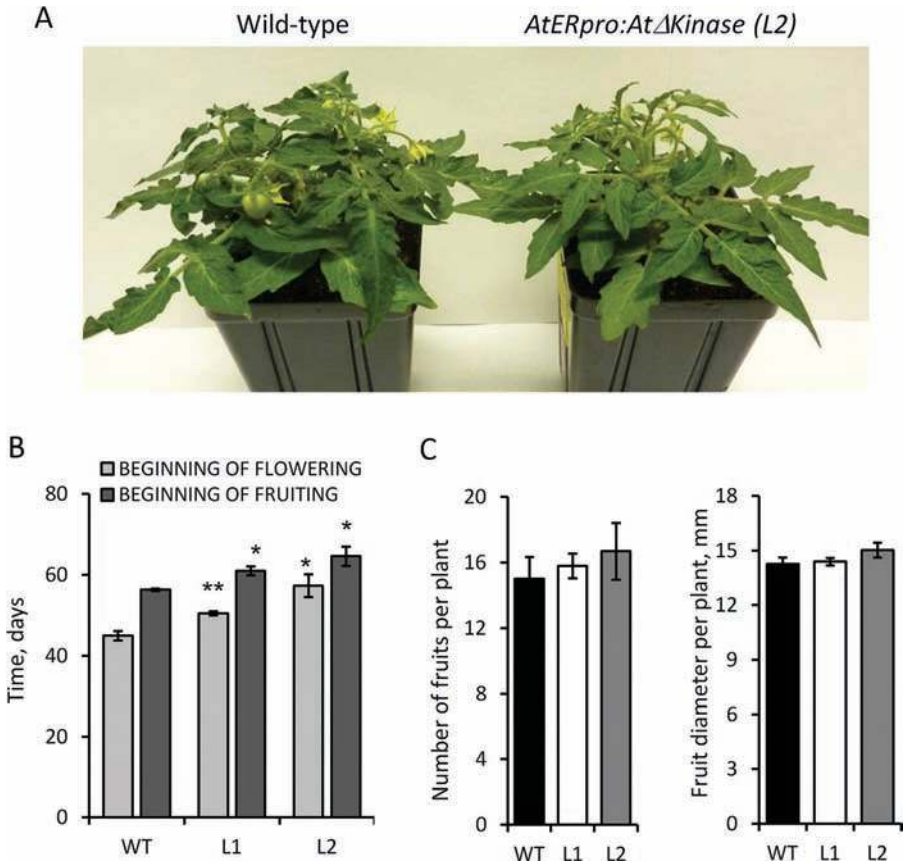

Fig. 7. Transgenic *AtERpro:AtΔKinase* tomato plants (L1 and L2) exhibit a delay in transition to flowering but produce an equal number of similar-sized fruits compared with wild type. (A) At d 56, formation of fruit was observed in wild-type plants but not in *AtERpro:AtΔKinase* lines. (B) Transition to flowering and formation of a first fruit in wild-type and *AtERpro:AtΔKinase* plants (n= 5; $^{*}P$ <0.05, $^{**}P$ <0.01). (C) Number and size of fruits per plant in 17-week-old wild-type and transgenic lines (n=14). Results are shown as means ±SE.

Expression of AtΔKinase alters the response to water deficit stress in transgenic tomato plants

Any modifications to growth rate and morphology of crop plants have to take into account the consequence of these changes to biotic and abiotic stress tolerance. The *ERECTA* gene family downregulates stomata density in the epidermis and controls transpiration efficiency (Masle *et al.*, 2005; Shpak *et al.*, 2005). As a result, these genes should be important for drought tolerance. To understand how disruption of *ERECTA* gene family signalling by AtΔKinase changes the response of tomato plants to drought, we exposed *AtERpro:ΔKinase* transgenic lines to water deficit stress by terminating watering at 56 d. After 9 d of water deficit, the wild-type plants exhibited typical symptoms of stress such as wilting and rolling of leaves, while the *AtERpro:AtΔKinase* transgenic lines appeared unstressed (Fig. 8A). Visible symptoms of drought stress were observed in transgenic plants only after 12 d of stress. This was an unanticipated result, as one would expect that disruption of the *ERECTA* gene family signalling would lead to increased stomatal density and as a result to increased water loss.

To better understand the connection between the resistance to drought stress and water balance, we measured stomatal conductance during the water deficit stress experiment. During the first 2 d of water deficit stress, no significant difference in stomatal conductance was found between leaves of transgenic lines and the wild type (Fig. 8C). On d 5, as the wild-type plants started to lose their ability to close stomata efficiently, their stomatal conductance increased. Due to wilting, we were not able to measure stomatal conductance in the wild-type plants after d 5. In transgenic lines, on d 7 and d 8 the stomatal conductance decreased and no wilting was observed. Next, we analysed the rate of water loss from detached leaves of well-watered plants. The detached leaves from both *AtERpro:ΔKinase* transgenic lines lost water faster than leaves from the wild-type plants (Supplementary Fig. S3 at *JXB* online), which was probably a consequence of the higher stomata density in leaves of transgenic lines compared with the wild type (Fig. 8B). Thus, just as in *Arabidopsis*, disruption of *ERECTA* gene family signalling in tomato increases the number of stomata in an individual leaf, which increases the transpiration rate. This suggests that the increased drought resistance of *AtERpro:ΔKinase* lines is probably unrelated to the role of the *ERECTA* gene family in epidermis development.

Besides stomatal index, the drought resistance of a plant depends on evaporating surface area. In the transgenic plants, the total leaf area was reduced by a factor of at least two due to the

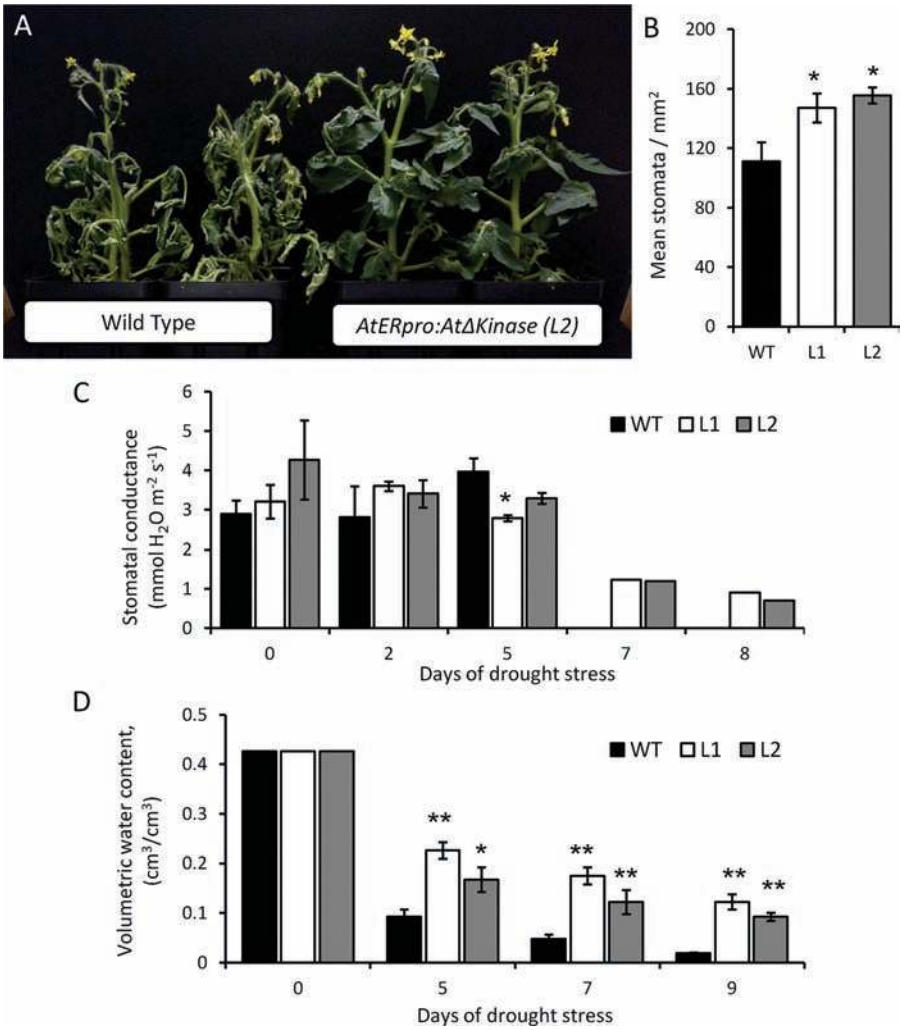

Fig. 8. *AtERpro:AtΔKinase* transgenic plants are more tolerant to water deficit stress. (A) Fifty-six-day-old *AtERpro:AtΔKinase* plants (L2) exhibit fewer visible stress symptoms after 9 d without watering. (B) Stomata index on the abaxial side of leaves of 56-d-old wild-type and transgenic (L1 and L2) plants. (C) Stomatal conductance (mean ±SE; *n*=3) of wild-type and transgenic plants (L1 and L2) during the drought stress experiment (**P* <0.05). (D) Soil moisture (mean ±SE; *n*=7) in pots with wild-type and transgenic lines (L1 and L2) expressed as volumetric water content during drought stress (**P* <0.05, ***P* <0.01).

smaller number of leaves and the decrease in leaf size (Fig. 6B). This decrease in leaf surface area should lead to decreased total loss of water by transgenic plants. To clarify whether the transgenic lines consumed less water during water deficit stress, we measured soil moisture by calculating the volumetric water content of pots (Fig. 8D and Supplementary Fig. S4). The soil water content in pots with transgenic lines was significantly higher than in pots with wild-type plants starting from d 5 of water stress. Based on these data, we hypothesize that the increased water deficit stress tolerance of the transgenic lines is related to their smaller surface area and consequent lower water loss due to evaporation.

Discussion

The *ERECTA* family of genes belongs to an ancient group of RLKs that are probably present in all land plants. These receptors regulate multiple aspects of development including aboveground organ elongation and stomata formation (Torii *et al.*, 1996; Shpak *et al.*, 2004, 2005; van Zanten *et al.*, 2009). In *Solanaceae*, the family consists of two genes: *SlER* and *SlERL*, which share over 80% similarity with their *Arabidopsis* orthologues. Analysis of their expression suggests a pattern similar to that of *Arabidopsis* in young developing aboveground tissues, and especially high expression in the shoot apex.

In this study, we demonstrated that the dominant-negative approach using ΔKinase allows manipulation of the ERECTA signalling pathway not only in *Arabidopsis* but also in tomato and thus, potentially, in other crops. Using either the 35S promoter or the *ERECTA* promoter, we observed that ΔKinase inhibited aboveground vegetative tomato growth. When ΔKinase was expressed under the strong constitutive 35S promoter, the effect on vegetative development was especially robust, with transgenic tomato explants almost directly transitioning to flowering instead of developing leaves and stems. The use of the *ERECTA* promoter

allowed us to obtain several homozygous transgenic lines expressing ΔKinase. Although the phenotype was weaker than that produced under the 35S promoter, we again observed decreased stem elongation and smaller leaves. These phenotypic characteristics of transgenic ΔKinase tomato plants are reminiscent of those observed in mutants of *ERECTA* family genes in *Arabidopsis* and in transgenic *Arabidopsis* plants expressing ΔKinase. The *er* mutant of *Arabidopsis* exhibits decreased inflorescence stem growth and shorter, rounder leaves (Torii *et al.*, 1996; Uchida *et al.*, 2012); these defects are further enhanced by the *erl1* and *erl2* mutations (Shpak *et al.*, 2004). The reduced inflorescence elongation is one of the most obvious defects of *Arabidopsis* plants expressing ΔKinase (Shpak *et al.*, 2003). Thus, notwithstanding the differences in plant architecture, with tomato plants having compound leaves and sympodial growth while in *Arabidopsis* leaves are simple and growth is monopodial, the *ERECTA* family genes regulate elongation of stems and leaves in both.

Another interesting characteristic of our transgenic tomato plants was the reduced rate of leaf initiation, with the total leaf number being decreased by approximately half. An involvement of ERECTA in regulation of total leaf number in *Arabidopsis* has been suggested by a quantitative trait loci analysis (El Lithy *et al.*, 2004, 2010). Due to their relatively large shoot apical meristems, tomato plants have been used extensively to study leaf initiation (Reinhardt *et al.*, 2000, 2005; Stieger *et al.*, 2002). Further analysis of leaf initiation in ΔKinase tomato plants might be useful in advancing our understanding of the role that *ERECTA* family genes play in those processes.

Because tomato is a crop grown for its fruits, we were interested in the impact that the manipulation of *ERECTA* family genes would have on the transition to flowering and fruit development. Domesticated tomatoes are autonomous flowering plants that transition to flowering generally independently of environmental conditions, in contrast to *Arabidopsis*, which is a long-day vernalization-dependent plant. However, the basic mechanisms regulating flowering in tomatoes and *Arabidopsis* are similar (Samach and Lotan, 2007). The suppression of *ERECTA* gene family signalling in tomatoes by ΔKinase delayed flowering time. However, a typical characteristic of late-flowering mutants is an increased number of leaves (Koornneef *et al.*, 1991). In contrast, the transgenic tomatoes flowered with a significantly smaller number of leaves, suggesting that flowering occurred at an earlier developmental stage. We observed a similar delay in flowering in *Arabidopsis er* and *er erl1 erl2* mutants grown in short days, but again this did not correlate with increased leaf number at transition (unpublished data). We hypothesize that in both species the impact of *ERECTA* family receptors on flowering is a side effect of their involvement in regulation of vegetative development. While the ΔKinase construct had an impact on the transition to flowering, we did not detect any changes in the number of fruits formed or in their size. This is somewhat surprising considering that *ERECTA* family genes are highly expressed in fruits and that these genes are known to regulate fruit size in *Arabidopsis* (Torii *et al.*, 1996; Shpak *et al.*, 2004). One possibility is that the observed upregulation of *SlER* and *SlERL*, which was especially high in fruits of the obtained transgenic plants, overcomes the dominant-negative effects of ΔKinase. Alternatively, the function of *ERECTA* family genes in *Arabidopsis* and tomato might differ. Alteration of this gene family expression in fruits and analysis of *SlER* and *SlERL* expression patterns in that organ might be helpful in establishing the role that these genes play in fruit development. Interestingly, while our transgenic plants had considerably decreased total leaf area, the fruit yield was not changed, suggesting suboptimal genetic yield potential in the tomato cultivar used. While the yield of a crop plant is dependent on plant morphology and its photosynthetic efficiency, it is not directly proportional to the leaf area. The optimal plant architecture ensures uniform distribution of light through the canopy, which maximizes the total solar energy absorbed by the foliage per unit of ground (Zhu *et al.*, 2010).

Any modification to crop architecture and productivity has to be regarded in the context of prevalent environmental conditions, including biotic and abiotic stresses. Drought is the most frequently encountered abiotic stress, and tolerance to water deficit stress is often critical for cost-efficient cultivation of a crop. ERECTA family receptors are of special interest for crop modifications as they regulate both plant morphology (Shpak *et al.*, 2004) and transpiration efficiency (Masle *et al.*, 2005; Shpak *et al.*, 2005). However, the effect of ERECTA family receptors on transpirational water losses is rather complex. On one hand, these receptors inhibit stomata development (Shpak *et al.*, 2005), which decreases stomata density and consequently reduces leaf stomatal conductance (Masle *et al.*, 2005). On the other hand, the receptors increase leaf size and leaf number, enlarging the plant surface area and leading to higher water losses. Inhibition of ERECTA family receptor signalling by ΔKinase in transgenic tomatoes increased stomata density and water loss by an individual leaf, but as the number of leaves decreased, the total water loss also decreased and the plants exhibited a higher drought tolerance. This result demonstrates that deliberate modification of crop growth and development through the *ERECTA* gene family signalling pathway might necessitate the specific control of its function in different tissues and organs.

In summary, this is the first example, to our knowledge, in which ERECTA signalling has been successfully manipulated in a plant other than *Arabidopsis*. In spite of the fact that *Arabidopsis* and tomato belong to two different clades of eudicots that diverged more than 100 million years ago (Yang *et al.*, 1999), this study suggests a similarity of *ERECTA* family gene structure and function in these two clades. This work has also demonstrated that *Arabidopsis* ΔKinase can be used directly to manipulate this signalling pathway in a heterologous system. Similarly, *Arabidopsis* C-REPEAT BINDING FACTOR (CBF) transcription factors have been used previously to modify abiotic stress tolerance in multiple other crop species (reviewed by Zhang *et al.*, 2004). Another important conclusion of this study is that selection of a promoter is critical for more targeted modifications of plant development through ERECTA family receptors, and that such modification can impact the abiotic stress tolerance in an unpredictable way.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. MAFFT alignment of the predicted amino acid sequences for *ERECTA* family genes from

Arabidopsis thaliana (At), *Solanum lycopersicum* (Sl), and *Solanum tuberosum phureja* (St). Residues labelled with an asterisk are conserved, those labelled with a colon have conservation between groups of strongly similar properties, and those labelled with a period have conservation between groups of weakly similar properties.

Supplementary Fig. S2. Confirmation of *At*Δ*Kinase* integration (A) and of transgene expression (B) in established *AtERpro:At*Δ*Kinase* tomato lines. M, molecular marker; Pl, positive control (a plasmid containing *AtERECTA* gene); WT, wild type; L1–L5, transgenic tomato lines. The size of the amplified fragment is 500 bp in (A) and 100 bp in (B).

Supplementary Fig. S3. Detached leaves of *At*Δ*Kinase* plants (L1 and L2) lose water faster than detached leaves of wild-type plants. The weight of detached leaves was recorded over time to calculate the percentage of transpirational water loss. All data represent means ±SE (n=6).

Supplementary Fig. S4. Plot of soil-specific calibration data. The soil-specific calibration equation is shown in the upper left corner of the graph.

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