Synergistic interaction of three ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower development by promoting cell proliferation

Elena D. Shpak[1], Chris T. Berthiaume[1], Emi J. Hill[1] and Keiko U. Torii[1,2,*]

[1]Department of Biology, University of Washington, Seattle, WA 98195, USA
[2]CREST, Japan Science and Technology Corporation, Saitama 332-0012, Japan
*Author for correspondence (e-mail: ktorii@u.washington.edu)

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Summary

Growth of plant organs relies on coordinated cell proliferation followed by cell growth, but the nature of the cell-cell signal that specifies organ size remains elusive. The *Arabidopsis* receptor-like kinase (RLK) ERECTA regulates inflorescence architecture. Our previous study using a dominant-negative fragment of ERECTA revealed the presence of redundancy in the ERECTA-mediated signal transduction pathway. Here, we report that *Arabidopsis* ERL1 and ERL2, two functional paralogs of ERECTA, play redundant but unique roles in a part of the ERECTA signaling pathway, and that synergistic interaction of three ERECTA-family RLKs define aerial organ size. Although *erl1* and *erl2* mutations conferred no detectable phenotype, they enhanced *erecta* defects in a unique manner. Overlapping but distinct roles of ERL1 and ERL2 can be ascribed largely to their intricate expression patterns rather than their functions as receptor kinases. Loss of the entire *ERECTA* family genes led to striking dwarfism, reduced lateral organ size and abnormal flower development, including defects in petal polar expansion, carpel elongation, and anther and ovule differentiation. These defects are due to severely reduced cell proliferation. Our findings place ERECTA-family RLKs as redundant receptors that link cell proliferation to organ growth and patterning.

Supplemental data available online

Key words: *Arabidopsis*, Receptor-like kinase, Cell proliferation, Organ size, Flower development, Functional redundancy

Introduction

The control of body and organ size of multicellular organism remains one of the unresolved questions in developmental biology. In plants and animals, increasing evidence supports the view that, although cell proliferation and cellular growth are an instrumental process of organ growth, the final size and form of organs is governed by the intrinsic mechanisms that monitor and balance the number and size of cells within the context of developmental programs (Conlon and Raff, 1999; Day and Lawrence, 2000; Mizukami, 2001; Nijhout, 2003; Potter and Xu, 2001). Studies found that experimental manipulation of cell cycle regulators, for example, does not always lead to altered organ size, as defects in cell number are compensated by alteration of cell size (Hemerly et al., 1995; Neufeld et al., 1998). Similarly, alteration of cellular growth in *Drosophila* by manipulation of Myc expression, as well as promotion of cell expansion in tobacco by induced expression of the *Arabidopsis* auxin-binding protein 1 (ABP1), conferred limited impact on overall organ size because of cell number compensation (Johnston et al., 1999; Jones et al., 1998).

In higher plants, lateral organs are generated reiteratively by the continual activity of the shoot apical meristem (SAM). Because plant cells are encapsulated by cell walls, organogenesis occurs in the absence of cell migration or the removal of overproduced cells. As such, unraveling developmental programs that coordinate cell proliferation and expansion during organ growth is key for understanding plant organ size control. The molecular links that integrate regulation of cell proliferation and organ size/shape as a unit in plants have just begun to emerge. One such regulator is the *Arabidopsis AINTEGUMENTA* (*ANT*) gene, which encodes an AP2-domain transcription factor (Elliott et al., 1996; Klucher et al., 1996). *ANT* acts to prolong the duration of cell proliferation by sustaining the expression of D-type cyclins (Mizukami, 2001; Mizukami and Fischer, 2000). Consequently, overexpression of *ANT* confers hyperplasia. The recent finding that the auxin-inducible gene *ARGOS* acts upstream of *ANT* suggests a role for the phytohormone auxin in regulating inherent organ size (Hu et al., 2003).

We aim to uncover the molecular basis of cell-cell communication that fine tunes coordinated cell proliferation during plant organ growth. The *Arabidopsis ERECTA* gene is a candidate for this. *ERECTA* is highly expressed in the SAM and developing lateral organs, where cells are actively dividing (Yokoyama et al., 1998). Loss-of-function *erecta* mutations confer compact inflorescence with short lateral organs and internodes, and these phenotypes are largely attributable to reduced cell numbers in the cortex cell files (Shpak et al., 2003;

Torii et al., 2003; Torii et al., 1996). The shortened *erecta* pedicels (floral stems) are associated with an increase in 4C cells, suggesting a possible aberration in cell cycle progression (Shpak et al., 2003). *ERECTA* encodes a leucine-rich receptor-like serine/threonine kinase (LRR-RLK) (Torii et al., 1996), a prevalent subfamily of signaling receptors in plants (Shiu and Bleecker, 2001; Shiu and Bleecker, 2003). LRR-RLKs regulate a wide-variety of signaling processes, including development of the SAM and microspores, brassinosteroid perception, floral abscission, pathogen recognition and symbiosis (for reviews, see Becraft, 2002; Carles and Fletcher, 2003; Kistner and Parniske, 2002; Li, 2003; Torii et al., 2004). The structure, expression patterns, and cellular and developmental phenotypes all support the notion that ERECTA mediates cell-cell signals that sense and coordinate organ growth.

We have previously shown that expression of a dominant-negative form of ERECTA (a ΔKinase fragment) enhances the growth defects of the null *erecta* plants, suggesting redundancy in the ERECTA signaling pathway (Shpak et al., 2003). To reach a full understanding of how ERECTA controls inherent plant size, it is imperative to identify its redundant receptors.

Here, we report the identification and functional characterization of ERL1 and ERL2, two redundant, paralogous ERECTA-like receptors that play a role in a part of ERECTA signaling pathway. Although loss-of-function mutations in *ERL1* and *ERL2* loci gave no detectable phenotype, they each enhanced *erecta* defects in a unique manner. Strikingly, loss of the entire ERECTA-family LRR-RLKs conferred extreme dwarfism and abnormal flower development. Molecular and cellular analysis revealed that ERECTA-family RLKs link cell proliferation to organ growth and patterning via a novel mechanism.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis* ecotype Columbia (Col) was used as a wild type. The T-DNA knockout seed population that contains *erl1-2* and *erl2-1* mutants was obtained from the *Arabidopsis* Biological Resource Center. All mutant lines were backcrossed three times to Col wild-type plants prior to any phenotypic analysis. The conditions for plant growth were as described by Shpak et al. (Shpak et al., 2003).

Oligo DNA sequences

Lists of oligo DNA sequences used for all experiments are available as supplementary data at http://dev.biologists.org/supplemental/.

Cloning of *ERL1* and *ERL2*

RT-PCR was performed using wild-type cDNA as a template, and using primer pairs: ERL1.14coding and ERL1g6054rc for *ERL1*; and ERL2.3coding and ERL2g5352rc for *ERL2*. The 5′ ends of mRNA were recovered by a rapid amplification of cDNA ends (RACE) using FirstChoice™ RLM RACE kit (Ambion, Austin, TX). Elk1-300rc or Ekl2-300.rc was used as the outer primer, and Elk1-185rc or Elk2-185rc was used as the nested primer for *ERL1* and *ERL2*, respectively. The amplified fragments were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced.

Reverse transcriptase-mediated (RT) PCR

RNA isolation, cDNA synthesis and RT-PCR were performed as described by Shpak et al. (Shpak et al., 2003) with various cycles. Primer pairs used are as follows:

ERECTA, ERg4359 and ERg5757rc;
ERL1, ERL1g2846 and ERL1g4411rc;
ERL2, ERL2g3085 and ERL2g4254rc;
ANT, 5′ant-1 and ANT1600rc;
STM, STM781 and STM2354rc;
WUS, U3WUS5 and U34WUS3rc;
KNAT1, BP681 and BP3100rc;
CYCLIN D2, CycD2.501 and CycD2.801rc;
CYCLIN D3, CycD3.501 and CycD3.935rc; and
ACTIN, ACT2-1 and ACT2-2.

Complementation of *erecta* by *ERL1* and *ERL2*

Full-length genomic coding regions for *ERL1* and *ERL2* were cloned into the *ERECTA* promoter-terminator cassette by the following procedure. PCR was performed using the wild-type Col genomic template and primer pairs: ERL1g3036 and ERL1-3endrc for *ERL1*; and ERL2g2166 and ERL2-3endrc for *ERL2*. The amplified fragments were digested with *Spe*I and *Xba*I, and inserted into *Spe*I-digested pKUT522 to generate pESH208A and pESH209A for *ERL1* and *ERL2*, respectively. Subsequently, PCR was performed using primer pairs: ERL1-5end and ERL1g4411rc for *ERL1*; and ERL2-5end and ERL2g3182rc for *ERL2*. The amplified fragments were digested with *Spe*I and *Xba*I, and inserted into *Spe*I-digested pESH208A and pESH209A, respectively, to generate pESH208 (*ER::ERL1*) and pESH209 (*ER::ERL2*). The plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101/pMP90 by electroporation, and into *Arabidopsis erecta-105* plants by vacuum infiltration.

ERECTA::GUS, *ERL1::GUS* and *ERL2::GUS* transgenic plants

For construction of *ERECTA::GUS*, the *GUS* gene was inserted as a *Spe*I fragment into pKUT522 between the *ERECTA* promoter and terminator. The plasmid was named pNI101. To make *ERL1::GUS* and *ERL2::GUS* constructs, the *Eco*RI/*Pst*I fragment of pRT2-GUS was cloned into pZP222 (Hajdukiewics et al., 1994). The plasmid was named pESH244. The *ERL1* promoter region was amplified with primers ERL1g-3680link and ERL1g403linkrc, using the MMI1 BAC clone as a template. The *ERL2* promoter region was amplified with primers ERL2g-4364link and ERL2g4linkrc, using the T28J14 BAC clone as a template. The amplified fragments were digested with *Eco*RI and inserted into pESH244. The plasmids were named pESH245 (*ERL1::GUS*) and pESH246 (*ERL2::GUS*). pNI101, pESH245 and pESH246 were introduced into *Arabidopsis* wild type as described above. GUS histochemical analysis was performed according to Sessions et al. (Sessions et al., 1999).

Screening and isolation of the Arabidopsis T-DNA insertion mutants

Screening and isolation of T-DNA insertion lines were performed as described by the *Arabidopsis* KO Facility (http://www.biotech.wisc.edu/*Arabidopsis*/). The *erl1-2* was isolated from an α population (vector pD991, kanamycin resistance), and *erl2-1* was isolated from a β population (vector pROK2, basta resistance), using gene-specific primers and JL-202 T-DNA left border primer. The gene-specific PCR primers were: ERLK765 and ERLK6137rc for *ERL1*; and ERTJ70 and ERTJ5855rc for *ERL2*. Precise locations of the insertions were determined by sequencing the PCR fragments. Both *erl1-2* and *erl2-1* were backcrossed three times. The B3F2 populations of *erl1-2* and *erl2-1* exhibited a 3:1 ratio of kanamycin resistance and Basta resistance, respectively (*erl1-2*, KanR:KanS=163:58, χ^2=0.183, *P*=0.669; *erl2-1*, BastaR:BastaS=203:76, χ^2=0.747, *P*=0.388), indicating a single T-DNA insertion. PCR-based genotyping confirmed that these single insertions disrupt the *ERL* loci.

Generation of double- and triple-knockout plants

To generate *erecta erl1* and *erecta erl2* double mutants, *erl1-2* and *erl2-1* plants were crossed with *erecta-105* plants. To generate *erl1*

erl2 double mutants, *erl1-2* plants were crossed with plants of the genotype *erecta-105/erecta-105 erl1-2/erl1-2 erl2-1/+*. Plants of a correct genotype were isolated from the F2 populations. *erecta-105/erecta-105 erl1-2/+ erl2-1/erl2-1* plants were self-fertilized to obtain the *erecta er11 erl2* triple mutants. The T-DNA insertion that disrupts the *ERL1* locus in *erl1-2*, and the *ERL2* locus in *erl2-1*, conferred resistance to kanamycin and Basta, respectively. Thus, progenies of each cross were first tested for resistance, then subsequently the genotype of individual plants, whether they were heterozygous or homozygous, was determined by PCR using gene-specific primer pairs, and a combination of T-DNA- (JL-202) and gene-specific primers. The presence of the *erecta-105* mutation was determined by PCR using the primer pairs: ERg2248 and er-105 (Torii et al., 2003).

Light and scanning electron microscopy

Fixation, and embedding and sectioning, of tissues for light microscopy using Olympus BX40, as well as preparation of samples for scanning electron microscopy using JOEL 840A, were performed as described by Shpak et al. (Shpak et al., 2003).

Cell number measurement

Light microscopy images of four regions of sectioned wild-type, *erecta-105*, *erecta-105 erl1-2* and *erecta-105 erl2-1* pedicels were taken, and the number of cells in a middle longitudinal cortex row was determined. This number was used to calculate the total number of cells in the cortex row of an average length pedicel. The number of cells was counted in three sectioned *erecta-105 erl1-2 erl2-1* pedicels and the average was determined.

Accession numbers

The GenBank accession numbers for the *ERL1* and *ERL2* sequences reported in this paper are AY244745 and AY244746, respectively.

Results

ERL1 and ERL2, two ERECTA-like LRR-RLKs in *Arabidopsis*

To identify candidate RLKs that act in parallel pathways with ERECTA, we surveyed the *Arabidopsis* genome and found two *ERECTA-LIKE* genes, *ERL1* (At5g62230.1) and *ERL2* (At5g07180.1). We subsequently isolated full-length cDNA clones for *ERL1* and *ERL2* by a combination of RT-PCR and 5′ RACE-PCR (Fig. 1A). Among the 223 *Arabidopsis* genes encoding LRR-RLKs (Shiu and Bleecker, 2003), *ERECTA* possesses an unusual, characteristic exon-intron structure with 26 introns (Torii et al., 1996). A comparison of genomic and cDNA sequences reveals that *ERL1* and *ERL2* also contain 26 introns, all of which are located at identical positions to the introns of *ERECTA*. The predicted ERL1 and ERL2 proteins share high overall sequence identity to ERECTA (60% identity, 72% similarity), and even higher sequence identity between each other (78% identity and 83% similarity). The LRR region and the kinase domain possess the highest degree of sequence conservation (Fig. 1A). The juxtamembrane domain and the extracellular paired cysteine regions adjacent to the LRR region have relatively high sequence identity, whereas the N-terminal signal sequence and the C-terminal tail region are poorly conserved (Fig. 1A). The phylogenetic, parsimony analysis suggests that *ERL1* and *ERL2* have evolved by recent duplication, and that they are immediate paralogs of *ERECTA* (Fig. 1B). The result is consistent with the neighbor-jointing analysis of the LRR-RLK phylogeny reported by Shiu and Bleecker (Shiu and Bleecker, 2001), as well as by Yin et al. (Yin et al., 2002). The finding that ERL1, ERL2 and ERECTA constitute a subfamily of LRR-RLKs opens the possibility that the two ERLs may have functions related to ERECTA.

ERL1 and *ERL2* rescue the *erecta* phenotype when expressed under the *ERECTA* promoter and terminator

To investigate whether *ERL1* and *ERL2* genes are functional homologs of *ERECTA*, *ERL1* and *ERL2* were expressed in the null allele *erecta-105* under the control of the native *ERECTA* promoter and terminator. Both constructs rescued the *erecta* defects. Transgenic *erecta-105* plants expressing *ERECTA::ERL1* or *ERECTA::ERL2* displayed phenotypes, such as elongated inflorescence and pedicels, nearly identical to the wild-type plants (Fig. 1C). Therefore, both ERL1 and ERL2 can substitute for ERECTA function when expressed in the tissue and cell types that normally express *ERECTA*, suggesting that the two ERLs are capable of perceiving and transducing the same signal as ERECTA.

ERECTA, *ERL1* and *ERL2* display overlapping, but unique expression patterns

The inability of *ERL1* and *ERL2* to complement *erecta* mutants when expressed under their endogenous promoter (Fig. 1C) suggests differences in expression patterns. At the same time, if ERL1 and ERL2 are the RLKs whose function is inhibited by the dominant-negative ERECTA fragment expressed under the control of the *ERECTA* promoter, we should expect them to be expressed, at least in part, in an overlapping manner with *ERECTA*. To clarify these points, we next analyzed the developmental expression of *ERL1* and *ERL2*.

RT-PCR analysis showed that, similar to *ERECTA*, expression levels of the two *ERLs* were higher in developing organs, including bud clusters, flowers, siliques and young rosettes, lower in mature aboveground organs, such as leaves, stems and pedicels, and barely detectable in roots (Fig. 2). The expression levels of *ERL1* and *ERL2* in mature organs were much lower than those of *ERECTA*.

To examine the organ- and tissue-specific expression patterns of *ERL1* and *ERL2* in detail, promoter fragments of *ERL1* (4.1 kb) and *ERL2* (4.4 kb) were fused transcriptionally to the *GUS* gene and introduced in *Arabidopsis* wild-type plants. The expression patterns of *ERECTA::GUS*, *ERL1::GUS* and *ERL2::GUS* mark the actively-proliferating organs (Fig. 3). At the vegetative stage, both *ERL1::GUS* and *ERL2::GUS* were strongly expressed in the shoot meristem, leaf primordia and juvenile leaves (Fig. 3A-C). At the reproductive stage, GUS expression was detected in the young developing flowers up to stage 12 for *ERECTA* and *ERL2*, and up to stage 14 for *ERL1* (Fig. 3D-I). *ERECTA::GUS* and *ERL1::GUS* were detected in inflorescence meristem and were visibly upregulated during flower initiation and formation of flower organs (Fig. 3J-L). *GUS* expression was also detected in cells that will differentiate into pedicels (Fig. 3J-L). In developing flowers, the expression of *ERECTA*, *ERL1* and *ERL2* was in the actively growing region of the floral organs, and thus altered dynamically as the developmental stages of the floral organs progressed (Fig. 3G-I). At the early stages, all three genes were expressed in an overlapping manner in all flower organs (Fig. 3D-L, and data not shown). Later, their expression became confined to different subsets of proliferating tissues.

For example, at flower stage 11, *ERECTA::GUS* was expressed largely in the mesocarp, and to a lesser degree in ovules, whereas *ERL1::GUS* was expressed predominantly in ovules, and *ERL2::GUS* in style and ovules (Fig. 3M-O). The finding that *ERL1* and *ERL2* display overlapping but unique expression patterns suggests their roles to be parallel to, or a part of, the ERECTA signaling pathway.

Isolation of the null alleles of *ERL1* and *ERL2*

To investigate the roles of the two ERLs in *Arabidopsis* growth and development, we next identified T-DNA-tagged, loss-of-function alleles of *ERL1* and *ERL2*. Locations of the insertions are shown in Fig. 4. *erl1-2* has a T-DNA insertion at nucleotide +3410 from the translation initiation codon, within exon 18, which encodes the sixteenth LRR (Fig. 4A). *erl2-1* has a T-DNA insertion at nucleotide +2454 from the translation initiation codon, within exon 14, which encodes the twelfth LRR (Fig. 4B). The T-DNA insertions in *erl1-2* and *erl2-1* are associated with a deletion of 59 and 76 nucleotides, respectively, suggesting that they represent the knockout (null) alleles. Indeed, no detectable *ERL1* transcripts were observed in *erl1-2* and no *ERL2* transcripts were observed in *erl2-1* by RT-PCR (Fig. 4C,D).

The absence of either *ERL1* or *ERL2* transcripts had no

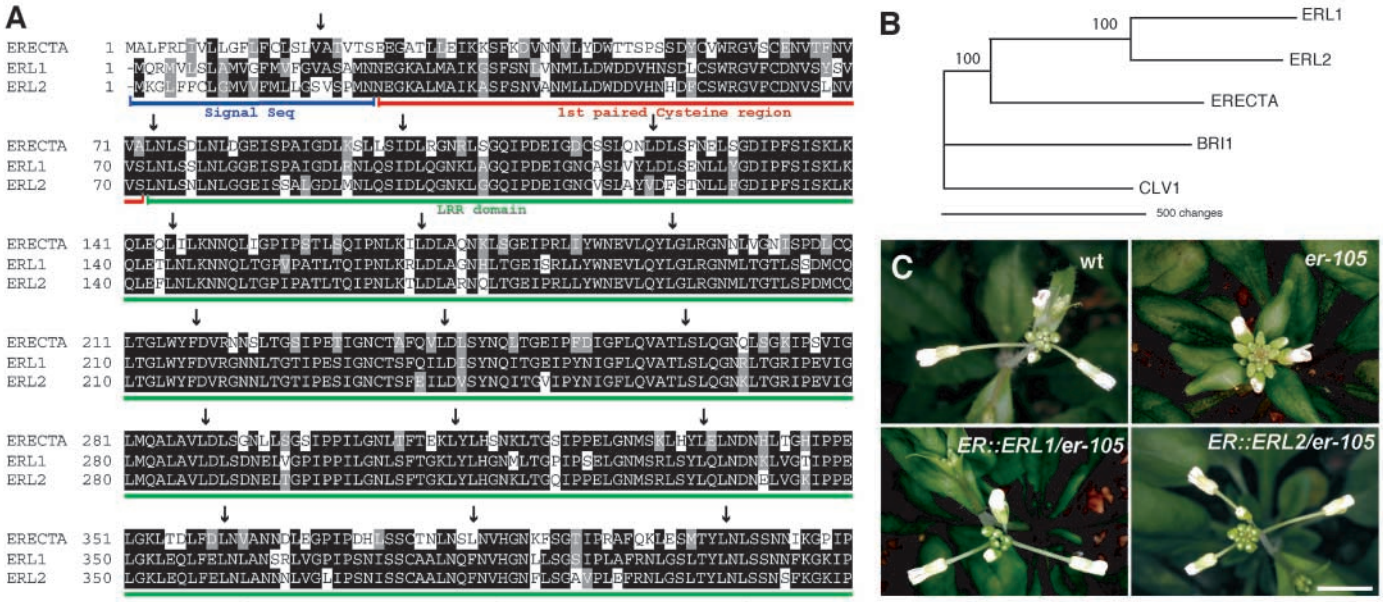


Fig. 1. *ERECTA-LIKE* genes *ERL1* and *ERL2* are immediate, functional paralogs of *ERECTA*. (A) Amino-acid sequence alignment of ERECTA and its paralogs. The alignment was generated by the CLUSTAL X program. Identical amino acids and similar amino acids are highlighted by black and gray backgrounds, respectively. Arrows indicate the positions of introns. Respective domains are highlighted with colored underlines: N-terminal signal sequence, blue; extracellular paired cysteine regions, red; the LRR-domain, green; the transmembrane domain, black; the juxtamembrane domain, purple; the kinase domain, pink; and a C-terminal tail region, cyan. (B) A phylogenetic tree of ERECTA, ERL1 and ERL2. The single most parsimonious tree generated using heuristic searches (PAUP*) based on the kinase domain sequence is shown. BRI1 and CLV1 were used as outgroups. Branch lengths are proportional to the number of amino-acid substitutions, and the numbers at the branch points indicate bootstrap values of 500 replications. (C) *ERL1* and *ERL2* genes rescue the *erecta* phenotype when expressed under the *ERECTA* promoter and terminator. Shown are the inflorescence apices from wild type, *erecta-105*, *erecta-105* plants expressing *ERECTA::ERL1* and *erecta-105* plants expressing *ERECTA::ERL2*. Scale bar: 5 mm.

effect on *ERECTA* expression levels (Fig. 4C,D). Similarly, the expression levels of *ERL1* and *ERL2* were not altered in *erecta-105* plants, which do not express any *ERECTA* transcripts (Fig. 4E). The lack of up- or downregulation among the three ERECTA-family LRR-RLKs implies that their signaling pathways do not constitute an interconnected feedback loop.

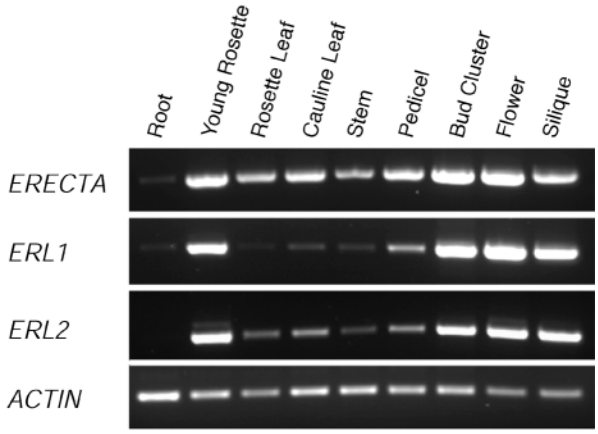
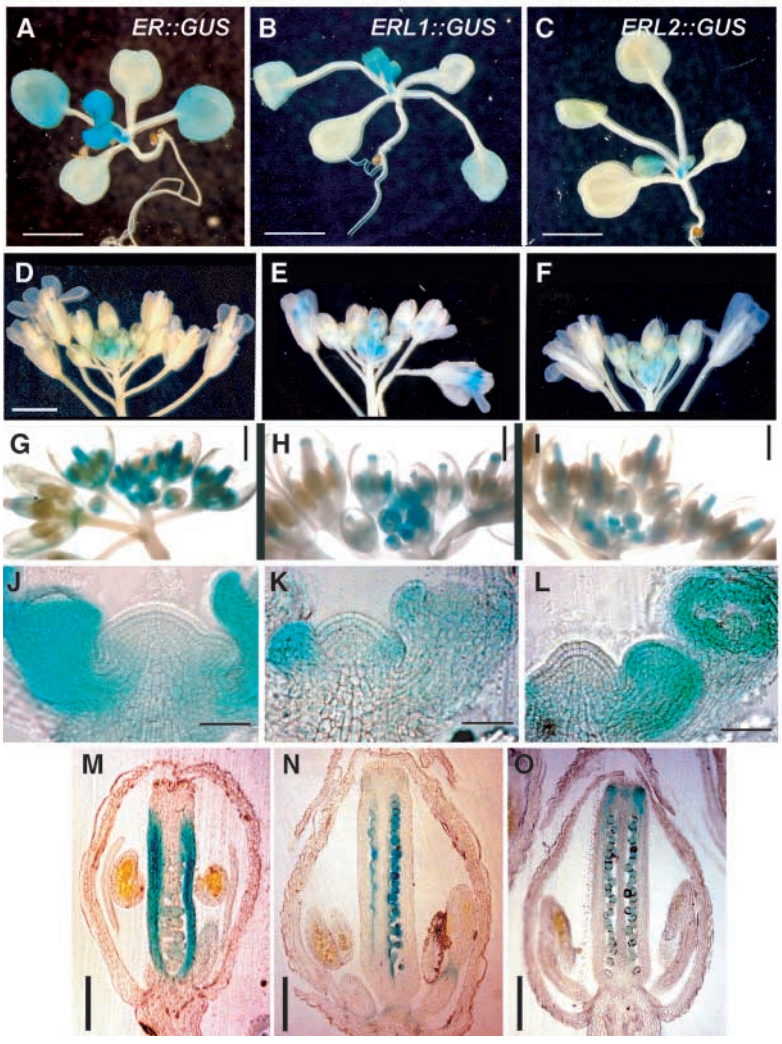


Fig. 2. Semi-quantitative RT-PCR analysis of *ERECTA*, *ERL1* and *ERL2* transcripts in different tissues. The actin fragment was amplified simultaneously as a control. *ERECTA*, *ERL1* and *ERL2* were amplified for 32 cycles; the control actin was amplified for 30 cycles.

Both *ERL1* and *ERL2* are redundant

erl1-2 and *erl2-1* were subjected to further phenotypic characterization. *erl1-2* and *erl2-1* single mutant plants were indistinguishable from wild-type plants (Fig. 5A-D). Their inflorescence undergoes elongation of the internodes between individual flowers, and they all displayed normal length petioles, stems (Fig. 5A), pedicels (Fig. 5B,D) and siliques (Fig. 5C,D). The lack of any visible phenotype suggests that *ERL1* and *ERL2* are redundant.

As *ERL1* and *ERL2* appear to have undergone recent gene duplication (Fig. 1B), it may be necessary to remove both gene products in order to reveal their biological functions. To test this hypothesis, we generated an *erl1-2 erl2-1* double mutant. *erl1-2 erl2-1* plants did not exhibit any visible phenotype (Fig. 5A-D). Although *ERL1* and *ERL2* are capable of rescuing the growth defects of *erecta-105* (Fig. 1C), *erl1* and *erl2* single mutants, as well as the *erl1 erl2* double mutant failed to confer any developmental phenotype. This finding suggests that loss-of-function of *ERL1* and *ERL2* is masked by the presence of the functional *ERECTA* gene.



erl1 and *erl2* enhance a subset of *erecta* defects in a unique manner

To uncover the developmental role of *ERL1* and *ERL2* in the absence of functional *ERECTA*, *erl1* and *erl2* mutations were introduced into *erecta-105* plants (Torii et al., 1996; Torii et al., 2003). Both *erl1* and *erl2* enhanced the *erecta* defects in a unique manner. The *erl1-2* mutation notably exaggerated the silique and pedicel elongation defects of *erecta-105*. *erecta-105 erl1-2* double mutant plants developed very short, blunt siliques and short pedicels (Fig. 5B-D), both of which are reminiscent of part of the phenotype conferred by the dominant-negative *ΔKinase* (Shpak et al., 2003). The presence of the *erl1-2* mutation did not significantly affect the height of *erecta-105* plants (Fig. 5A).

By contrast, the *erl2-1* mutation primarily enhanced the internodal elongation defects of *erecta*. *erecta-105 erl2-1* double mutant plants were much shorter than *erecta-105*, and developed very compact inflorescence with tightly clustered flowers and flower buds at the tip (Fig. 5A,E). The architecture of *erecta-105 erl2-1* inflorescence resembles that of the transgenic *erecta-105* expressing *ΔKinase* (Shpak et

Fig. 3. *ERECTA*, *ERL1* and *ERL2* are expressed in young proliferating tissues in an overlapping but unique manner. Expression of *ERECTA::GUS* (A,D,G,J,M), *ERL1::GUS* (B,E,H,K,N) and *ERL2::GUS* (C,F,I,L,O) reporter genes in: a 14-day-old seedling (A-C); inflorescence apices at lower (D-F) and higher (G-I) magnifications; inflorescence meristem (J-L); and a stage 11 flower (M-O). Scale bars: 3 mm for A-C; 2 mm for D-F; 0.5 mm for G-I; 50 μm for J-L; 0.2 mm for M-O.

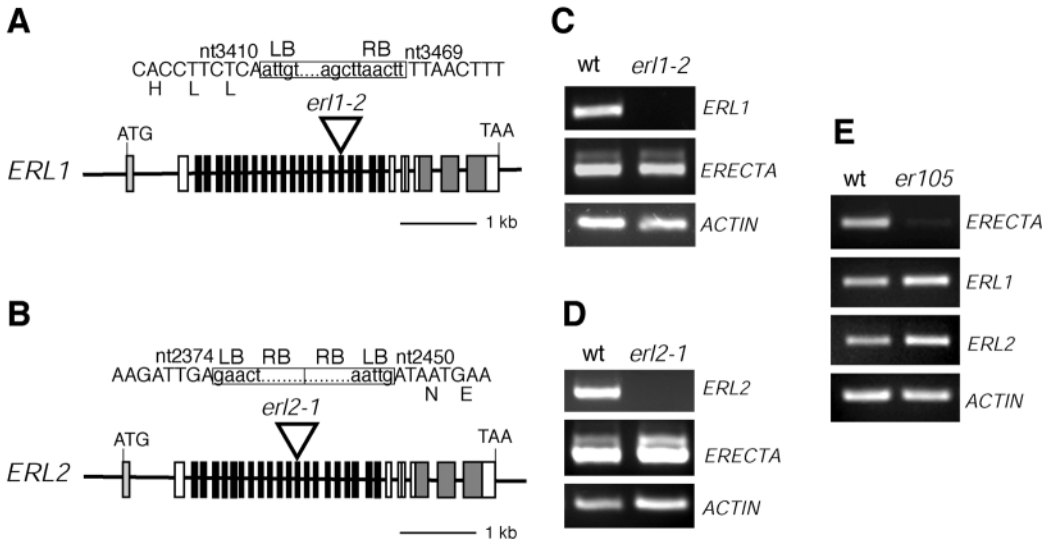


Fig. 4. *erl1-2* and *erl2-1* are null alleles. (A,B) Structure of *ERL1* (A) and *ERL2* (B) genes and T-DNA insertion sites. The insertion in *erl2-1* consists of two T-DNAs with inverse orientation. The sequence of *ERL1* and *ERL2* adjacent to the insertion is shown in upper case. The T-DNA sequence is in lower case. (C-E) Semi-quantitative RT-PCR analysis of *ERECTA*, *ERL1* and *ERL2* transcripts in *erl1-2* (C), *erl2-1* (D) and *erecta-105* (E) mutant backgrounds. *erl1-2*, *erl2-1* and *erecta-105* plants do not have detectable levels of *ERL1*, *ERL2* and *ERECTA* mRNA, respectively. None of these null mutations affect expression levels of other members of ERECTA-family. The actin fragment was amplified simultaneously as a control. *ERECTA*, *ERL1* and *ERL2* were amplified for 35 (C,D) or 32 (E) cycles; the control actin was amplified for 30 cycles.

al., 2003). In addition, the *erecta-105 erl2-1* siliques were slightly shorter than those of *erecta-105* (Fig. 5C,D).

The morphology of the silique tip was analyzed in detail. The *erecta-105* silique tip has a blunt appearance due to a wide style that protrudes less from the valves than does the wild type. Both *erl1* and *erl2* mutations exaggerated this characteristic *erecta* silique phenotype, with even wider valves and shorter, broader styles (Fig. 5F). This indicates that the enhancement of the silique phenotype by *erl1-2* and *erl2-1* is not due to general elongation defects unrelated to the ERECTA pathway. From this, we conclude that ERL1 and ERL2 act in an overlapping but distinct part of the ERECTA signaling pathway in regulating inflorescence architecture and organ shape.

Synergistic interaction of *ERECTA*, *ERL1* and *ERL2* in promoting organ growth and flower development

To understand the biological function of the ERECTA-family LRR-RLK as a whole, we next generated an *erecta-105 erl1-2 erl2-1* triple mutant. For this purpose, F2 plants that were homozygous for *erecta* and *erl2*, but heterozygous for *erl1*, were self-fertilized. A subsequent F3 population segregated extremely dwarf, sterile plants at an ~25% ratio (dwarf plants/total=74/315, χ^2=0.382, *P*=0.537), suggesting that they may be the triple mutant. To test this hypothesis, genotypes of 86 F3 plants were analyzed. Among 63 compact, fertile plants, 40 were heterozygous for *erl1*, 23 were wild type for *ERL1* and none were homozygous for *erl1*, consistent with the expected 2:1 ratio (χ^2=0.286, *P*=0.593). By contrast, all 23 extremely dwarf, sterile plants were homozygous for *erl1* and thus carried *erecta-105 erl1-2 erl2-1* triple mutations. Furthermore, progeny of the F3 siblings with a genotype *erecta-105 ERL1 erl2-1* failed to segregate extremely dwarf plants (0/227 scored). These results provide statistical evidence that the triple mutations confer severe growth defects (Fisher's exact test, *P*<0.00000001).

We subsequently analyzed a phenotype of *erecta-105 erl1-2 erl2-1* triple mutant plants during postembryonic development. The striking effects of *erecta-105 erl1-2 erl2-1* mutations on organ growth can be seen in all aboveground organs (Fig. 6A-E) and are evident soon after germination, at a time when cells start to divide. Decreased cotyledon growth is notable in 4-day-old *erecta-105 erl1-2 erl2-1* seedlings, and it is more striking in 12-day-old seedlings, which have small, misshaped cotyledons with very short petioles (Fig. 6A). Growth of primary leaves is strongly diminished in the triple mutant seedlings (Fig. 6A), whereas leaf primordia are forming on a flank of the SAM (data not shown). Interestingly, the triple mutations do not affect hypocotyl elongation, which occurs solely because of cell elongation (Gendreau et al., 1997). At a later stage of vegetative development, *erecta-105 erl1-2 erl2-1* plants form a small rosette with small, round leaves that lack petiole elongation (Fig. 6B). Transition to flowering occurs approximately at the same time in wild-type, *erecta-105* and *erecta-105 erl1-2 erl2-1* plants, suggesting that mutations in the three *ERECTA*-family genes do not affect phase transition (Fig. 6B, and data not shown).

The phenotypes of triple mutant plants at the reproductive stage are variable. Although the main inflorescence stem always exhibits severe elongation defects, axillary branches occasionally show various degrees of phenotypic rescue (Fig. 6C). A variable level of phenotypic rescue was also noticeable in flowers and pedicels at a later stage of axillary inflorescence development. Flowers with stronger phenotypes have a reduced number of organs with an occasional fusion of organs, and their pedicels are either absent or too short to be detected (Fig. 6C). Those with weaker phenotypes have all

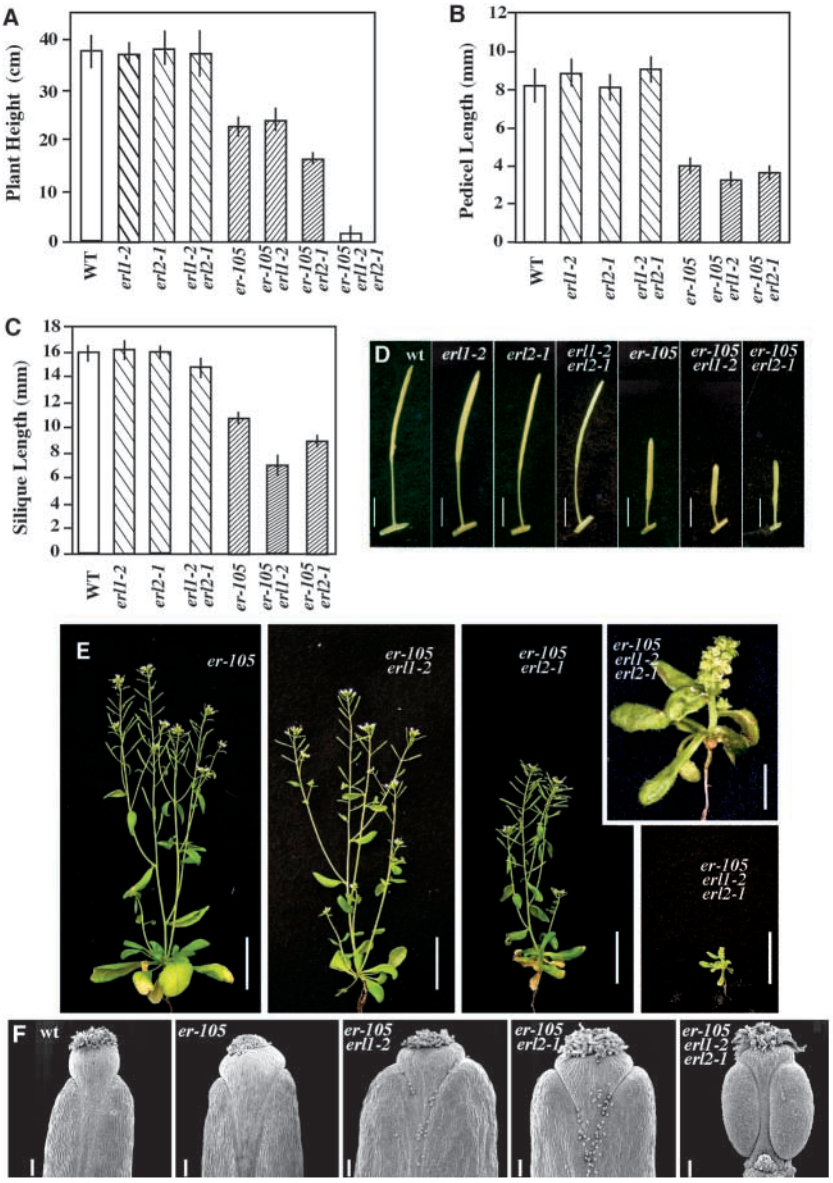


Fig. 5. *ERL1* and *ERL2* genes are partially redundant and show synergistic interaction with *ERECTA*. (A-C) Morphometric analysis of fully-grown eight-week-old wild-type, *erl1-2*, *erl2-1*, *erl1-2 erl2-1*, *erecta-105*, *erecta-105 erl1-2*, *erecta-105 erl2-1* and *erecta-105 erl1-2 erl2-1* plants. (A) Plant (inflorescence) height (n=20). (B,C) Lengths of mature pedicels (B) and siliques (C) on the main stem (n=40; eight measurements per stem). Bars represent the average; error bars represent s.d. (D) Mature siliques and attached pedicels of wild-type, *erl1-2*, *erl2-1*, *erl1-2 erl2-1*, *erecta-105*, *erecta-105 erl1-2* and *erecta-105 erl2-1*. Scale bars: 5 mm. (E) Six-week-old plants of *erecta-105*, *erecta-105 erl1-2*, *erecta-105 erl2-1* and *erecta-105 erl1-2 erl2-1*. Scale bars: 3 cm. The top right insert is an image of an *erecta-105 erl1-2 erl2-1* plant at higher magnification (scale bar: 5 mm). (F) Scanning electron micrographs of silique tips from wild type, *erecta-105*, *erecta-105 erl1-2 and erecta-105 erl2-1*, and a silique from *erecta-105 erl1-2 erl2-1*. Scale bar: 100 μm

four organs formed, but they are smaller in size and are incompletely developed (Fig. 6E). Such flowers have extremely short, but recognizable pedicels (Fig. 6E). Unlike *erecta-105*, the triple mutant flowers develop cylindrical, needle-like petals that lack polar expansion, very short gynoecium, and small anthers that are incompletely differentiated, all of which are visible in stage 9 flowers as well as in mature flowers (Fig. 6D,E,H). Ovule development is either absent or aborted at a very early stage (Fig. 6H), and this is consistent with the overlapping expression of *ERECTA*, *ERL1* and *ERL2* in developing ovules (Fig. 3M-O). These phenotypes are much more severe than *erecta-105* plants expressing *ΔKinase*, suggesting that the dominant-negative interference previously shown by Shapk et al. (Shapk et al., 2003) was not complete. The results demonstrate that the *ERECTA*, *ERL1* and *ERL2* genes interact synergistically, and that these three ERECTA-family LRR-RLKs, as a whole, specify the proper growth and differentiation of all aboveground organs.

erecta-105 erl1-2 erl2-1 triple mutants are defective in cell proliferation

To unravel the cellular basis of reduced organ growth, we examined cellular morphology in petals and pedicels. *Arabidopsis* petals have a simple cell layer structure with epidermal cells that are uniform in size and shape (Bowman, 1993). Although petals of *erecta-105 erl1-2 erl2-1* plants are very small and filamentous in shape (Fig. 6E), their abaxial epidermis cells are slightly larger than in *erecta-105* petals (Fig. 6F).

As reported previously, *erecta-105* pedicels have a reduced number of expanded cortex cells (Shpak et al., 2003). Similar to *erecta-105*, *erecta-105 erl1* and *erecta-105 erl2* double mutations, and *erecta-105 erl1-2 erl2-1* triple mutations, confer reduced cell numbers associated with enlarged and irregular cell shape in the cortex (Fig. 6G). Interestingly, *erecta-105 erl1-2* and *erecta-105 erl1-2 erl2-1* mutations lead to disorganized cell growth in the cortex. Cells are irregular in size and shape, and have gaps in between. This phenotype is similar to transgenic *erecta-105* plants expressing *ΔKinase* (Shpak et al., 2003). Cell numbers in a longitudinal cortex file are severely reduced in the mutants, with a concomitant decrease in the final pedicel length (Fig. 5B, Fig. 6I). *erecta-105* pedicel has three times fewer cells per longitudinal row, and *erecta-105 erl1-2 erl2-1* has 11 times fewer cells, compared with the wild type (Fig. 6I). These results demonstrate that organ growth defects of *erecta erl1 erl2* are largely due to a decrease in cell number, and suggest that *ERECTA*-family genes promote cell proliferation during organ growth.

Molecular analysis of *erecta erl1 erl2* inflorescence suggests a novel mechanism for organ growth regulation

To understand the molecular basis of organ growth/cell number

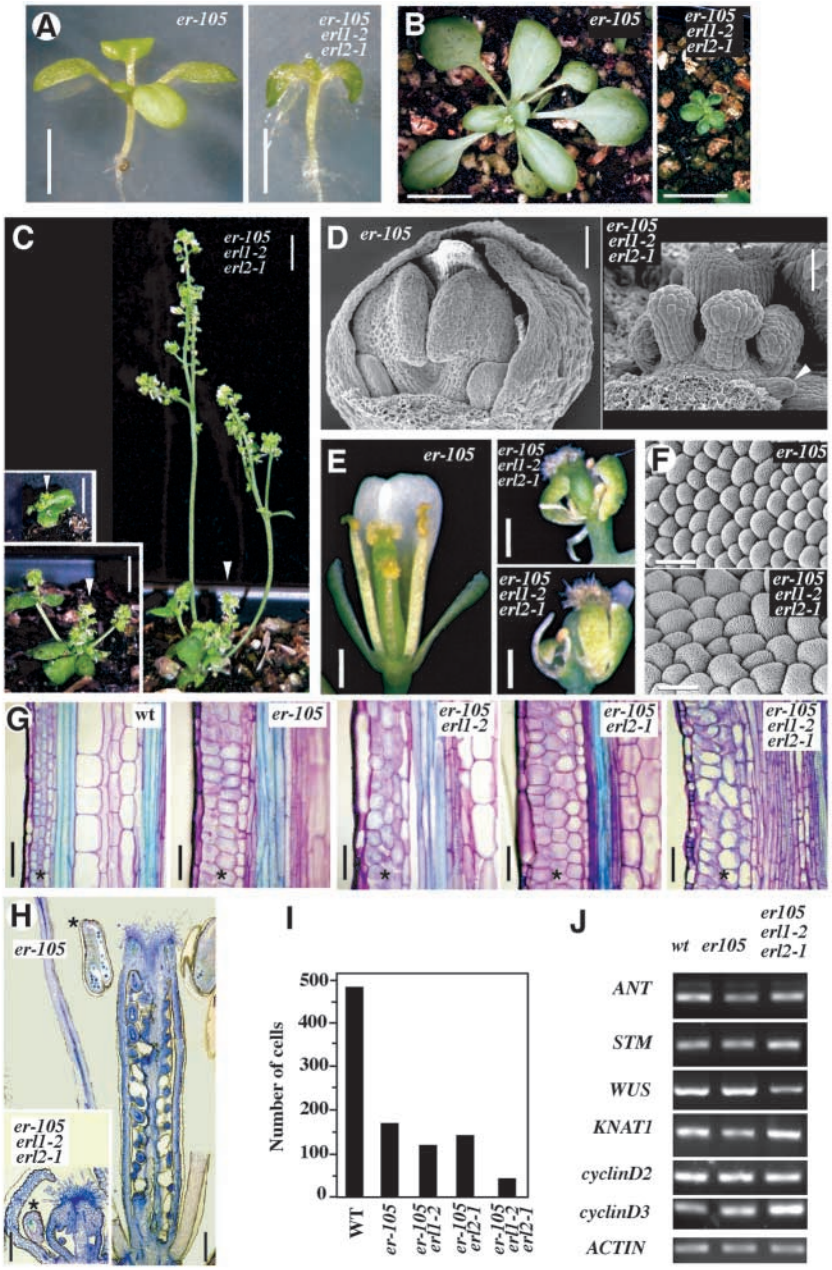


Fig. 6. *erecta-105 erl1-2 erl2-1* triple mutations lead to dramatically reduced plant growth, aberrant flower development, and reduced cell numbers in petals and pedicels. (A) 12-day-old seedlings of wild type and *erecta-105 erl1-2 erl2-1*. Scale bars: 3 mm. (B) 24-day-old plants of *erecta-105* and *erecta-105 erl1-2 erl2-1*. Scale bars: 1 cm. (C) Phenotypic variation of seven-week-old *erecta-105 erl1-2 erl2-1* plants. Although the main inflorescence stem (arrowheads) always exhibits severe elongation defects, axillary branches occasionally show various degrees of phenotypic rescue. Scale bars: 5 mm. (D) Scanning electron micrographs of *erecta-105* and *erecta-105 erl1-2 erl2-1* flowers at stage 9. Note that one sepal was removed from the *erecta-105* flower and several sepals were removed from *erecta-105 erl1-2 erl2-1*. Anther development and polar expansion of petals (arrowhead) are severely defective in the *erecta-105 erl1-2 erl2-1* flower. Scale bars: 40 μm. (E) Flowers of *erecta-105* and *erecta-105 erl1-2 erl2-1* with mild phenotype. One sepal and two petals were removed from the *erecta-105* flower to expose the inner organs. Note that none of the floral organs were removed from the *erecta-105 erl1-2 erl2-1* flowers. The triple mutant flowers have reduced numbers of organs and develop characteristic needle-like petals. Scale bars: 0.5 mm. (F) Scanning electron micrographs of petal abaxial epidermis cells of *erecta-105* and *erecta-105 erl1-2 erl2-1*. The images are taken at the same magnifications. Scale bars: 10 μm. (G) Longitudinal sections of mature pedicels from wild type, *erecta-105*, *erecta-105 erl1-2*, *erecta-105 erl2-1* and *erecta-105 erl1-2 erl2-1* plants. All combinations of mutants shown have defects in shape, size and number of cells in the cortex cell file (asterisks). Scale bars: 50 μm. (H) Longitudinal section of the mature flower of *erecta-105* and *erecta-105 erl1-2 erl2-1*. The lack of proper ovule and anther (asterisk) differentiation is visible in *erecta-105 erl1-2 erl2-1*. The images are taken under the same magnifications. Scale bars: 0.2 mm. (I) Number of cells in the longitudinal cortex file of a mature pedicel of wild type, *erecta-105*, *erecta-105 erl1-2*, *erecta-105 erl2-1* and *erecta-105 erl1-2 erl2-1* plants. (J) Effect of *erecta-105* and *erecta-105 erl1-2 erl2-1* mutations on RNA levels of genes regulating meristem (*STM*, *WUS*) or organ (*ANT*, *KNAT1*, D-type cyclins) growth. Semi-quantative RT-PCR analysis was performed. An actin fragment was amplified simultaneously as a control. The number of PCR cycles were as follows: *ANT*, 28 cycles; *STM*, 28 cycles; *WUS*, 29 cycles; *KNAT1*, 32 cycles; *CYCD2;1*, 32 cycles; *CYCD3;1*, 29 cycles; actin, 24 cycles.

defects conferred by the triple mutations, we analyzed the expression levels of four transcription factor genes that regulate shoot and floral organ size (Fig. 6J). *ANT* acts to prolong duration of cell proliferation during lateral organ development, and its loss of function confers reduced organ size (Elliott et al., 1996; Mizukami and Fischer, 2000). Loss-of-function mutations in *SHOOTMERISTEMLESS* (*STM*) and *WUSCHEL* (*WUS*) homeobox genes cause a decrease in the number of meristem cells and growth defects of lateral organs (Laux et al., 1996; Long et al., 1996). The *BREVIPEDICELLUS* (*BP*) locus encoded by the *KNAT1* homeobox gene interacts synergistically with *ERECTA* in promoting internodal elongation and floral organ size (Douglas et al., 2002). Semi-quantitative RT-PCR analysis of flower and bud clusters reveals that *erecta-105 erl1-2 erl2-1* triple mutations do not affect expression levels of *ANT*, *STM* or *KNAT1* (Fig. 6J). *WUS* expression was slightly reduced in the triple mutant background (Fig. 6J). However, such a slight reduction is not likely to account for severe defects in shoot and floral organ growth, and internodal elongation in the triple mutants.

It is known that *ANT* leads to prolonged expression of D-type cyclins, which control the entry to cell cycle progression at the G1 stage (Cockcroft et al., 2000; Dewitte and Murray, 2003; Mizukami and Fischer, 2000). Transcript levels of two

D-type cyclins, *CYCD2;1* and *CYCD3;1*, were not significantly altered by the triple mutations (Fig. 6J). This is consistent with the notion that the control of organ size by ERECTA-family RLKs involves mechanisms other than the pathway mediated by ANT. Taken together, the results suggest that the three ERECTA-family LRR-RLKs promote cell proliferation via a novel mechanism.

Discussion

Our previous study of a dominant-negative ERECTA action led us to predict that the ERECTA signaling pathway is redundant and shared by multiple receptors (Shpak et al., 2003). Here, we present direct evidence that two paralogous ERECTA-LIKE LRR-RLKs play a role in a subset of the ERECTA signaling pathway and that synergistic interaction of these three family members as a whole specifies aerial organ size by promoting cell proliferation.

Functional redundancy and synergistic interaction among three ERECTA-family LRR-RLKs

Duplications of developmental regulatory genes followed by subsequent mutation and selection are thought to have driven morphological diversity in multicellular organisms. Acquisition of novel gene functions occurs by alteration of protein function or of gene expression patterns. The fact that ERL1 and ERL2 are capable of substituting for ERECTA activity when driven by the *ERECTA* promoter and terminator (Fig. 1C) indicates that specificity among *ERECTA*, *ERL1* and *ERL2* lies largely in their cis-regulatory elements rather than in their protein-coding regions. Consistently, specific sites of enhancement of the *erecta* phenotype by either *erl1* or *erl2* mutation appear to correspond to the expression domains of these two LRR-RLKs, which are weaker and confined to a subset of *ERECTA* expression domains (Figs 2, 3). The dominance of cis-regulatory sequences over protein-coding regions in functional specification among closely-related multigene families has been documented for transcription factors regulating development, such as: Hox genes in mouse development; the Myb genes *WER* and *GL1* in *Arabidopsis* epidermal patterning; and *AGAMOUS*-family MADS-box genes in *Arabidopsis* ovule development (Greer et al., 2000; Lee and Schiefelbein, 2001; Pinyopich et al., 2003).

Because *ERECTA*-family genes encode putative receptor kinases, their functional equivalence indicates that ERECTA, ERL1 and ERL2 are capable of perceiving the same ligand(s) and eliciting the same downstream response(s). This raises a novel view on how the extent of organ growth is monitored by cell-cell signaling in *Arabidopsis*. The prevalent model based upon *Drosophila* wing development is that final organ size is determined by the steepness of morphogen gradients (Day and Lawrence, 2000). According to this model, concentration gradients of ligands, such as Dpp or Wg, dictate where and when cells proliferate. By contrast, we hypothesize that tissue-specific and redundant expression of functionally equivalent receptors plays a regulatory-role in coordinating *Arabidopsis* aerial organ growth. In the organ primordium, where cells are proliferating ubiquitously, uniform expression of all three ERECTA-family LRR-RLKs maximizes the organ growth. As the organ matures, localized and non-redundant expression of each RLK fine-tunes local, subtle growth for elaboration of final form and size. Transient, non-overlapping expression of *ERECTA*, *ERL1* and *ERL2* in a developing gynoecium (Fig. 3M-O) reflects such intricate local growth patterns, as growth and differentiation of distinct tissues, such as stigma, style valves and ovules, must occur concomitantly during carpel development (Ferrandiz et al., 1999). This view is in accordance with previous findings that strength of the ERECTA pathway specifies final organ size in a quantitative manner (Lease et al., 2001; Torii et al., 2003; Torii et al., 1996). Future identification of the ligands shared by these three receptors will address this hypothesis.

A recent molecular evolutionary study implies that the RLK superfamily underwent radical expansion within the plant lineage. The existence of more than 600 RLK-coding genes in the *Arabidopsis* genome is in sharp contrast with the small numbers of their counterparts (Pelle/IRAK family) in animals: three in mice and four in humans (Shiu and Bleecker, 2003). Consistently, gene duplication events among RLK sub-families have been documented (Baudino et al., 2001; Nishimura et al., 2002; Searle et al., 2003; Shiu and Bleecker, 2003; Yamamoto and Knap, 2001; Yin et al., 2002), but their biological significance is not fully understood. Our finding confirms the effectiveness of the dominant-negative approach, and further provides a framework for understanding functional redundancy among recently duplicated plant RLK gene families.

ERECTA family genes control plant organ size through coordination of cell proliferation

The most prominent feature of *erecta* single and *erecta erl* double and triple mutations is a reduction in aerial organ size due to reduced cell numbers. In theory, cell numbers in lateral organs can be regulated by affecting the number of SAM cells available for recruitment to organ primordia, by promotion of cell proliferation, or by prolonging the duration (window) of cell proliferation during organ growth. Our results suggest that *ERECTA*-family genes are most likely to function in the promotion of cell proliferation. The triple mutations are not likely to disturb SAM function; even a strikingly tiny leaf of the triple mutant initiates and increases in size with the same timing as wild type. Consistently, *WUS* and *STM* expression levels are not significantly altered by the mutation (Fig. 6J). Furthermore, expression of *CYCLIN D2*, whose overexpression confers an increase in growth rate by accelerating primordia initiation in the SAM (Cockcroft et al., 2000), is not affected in the triple mutant background. It is also unlikely that *ERECTA*-family genes prolong duration of cell proliferation, as *erecta erl1 erl2* mutations do not lead to early cessation of organ growth. Consistently, expression of *ANT*, which promotes the meristematic competency of developing organs through prolonged expression of *CYCLIN D* (Mizukami and Fischer, 2000), is not downregulated by the triple mutations.

In addition to growth defects, *erecta erl1 erl2* plants exhibit aberrant floral organ differentiation, notably in anthers and ovules. This may be due to inhibited primordia growth, which results in a diminished supply of progenitor cells for tissues that differentiate at later stages of flower development. Alternatively, *ERECTA*-family genes as a whole may play some specific roles in flower organ differentiation. In this regard, it is interesting that *ANT*, which also specifies organ size but via a distinct mechanism, is known to be required for

proper ovule differentiation and floral organ identity (Elliott et al., 1996; Klucher et al., 1996; Krizek et al., 2000).

In contrast to the main inflorescence, axillary branches of *erecta erl1 erl2* plants displayed various degrees of phenotypic rescue (Fig. 6C). One possibility that explains such rescue could be that the indirect effects caused by premature termination of the SAM (the main inflorescence) relieves the growth of axillary branches via ERECTA-independent mechanisms (Leyser, 2003). Alternatively, control of axillary branch development may involve factors that possess partially redundant function with ERECTA-family receptor-like kinases. Such factors might be more distantly related receptor-like kinases and/or gene products with no primary sequence similarity to ERECTA. It is noteworthy that ERECTA, ERL1 and ERL2 belong to the LRR-XIII family with four additional, distantly-related members (Shiu and Bleecker, 2001). The biological functions of these four members are not understood.

The increase in cell size in *erecta* single and *erecta erl* double and triple mutants is likely to be secondary to reduction in cell number. When cell proliferation is decreased, the total mass checkpoint often leads to decreased inhibition of cell growth, resulting in increased cell size (Conlon and Raff, 1999; Day and Lawrence, 2000; Mizukami, 2001; Nijhout, 2003; Potter and Xu, 2001). The expression of *ERECTA*, *ERL1* and *ERL2* in actively dividing tissues correlates well with their proposed function in cell proliferations. Interestingly, a striking decrease in cortex cell numbers occurs only at the vertical cell files, whereas compensatory cell expansion is much more notable along the radial axis. As a consequence, *erecta* single and *erecta erl* double mutants develop organs with characteristic shapes that are shorter but thicker. Therefore, ERECTA-family RLKs may respond to elusive signals that determine the longitudinal dimension of organ growth. Alternatively, it is possible that ERECTA-family RLKs possess specific roles in regulation of cell shape and polarity in addition to cell division.

Remarkably, the cortex cells in *erecta-105 erl1-2* and *erecta-105 erl1-2 erl2-1* pedicels are disorganized, with erratic shape and uneven size (Fig. 6G). The cellular phenotype suggests that ERECTA-family RLKs play a fundamental role in coordinating cell proliferation within tissues. In this respect, ERECTA-family RLKs are distinct from the receptor for peptide-hormone phytosulfokine (PSK), which also encodes an LRR-RLK (Matsubayashi et al., 2002). Although the PSK-receptor stimulates rapid, unorganized cell proliferation in culture cells, ERECTA-family RLKs mediate cell proliferation in the context of whole organism. Consistent with this hypothesis, *ERECTA*-family genes are not highly expressed in *Arabidopsis* culture cells (C.T.B., E.D.S. and K.U.T., unpublished).

Although recent studies brought significant insight into the functions of core cell-cycle regulators in plant growth and development (Dewitte and Murray, 2003; Mironov et al., 1999; Pardee, 1989), how neighboring cells coordinate proliferation remains unclear. Future identification of the ligands and downstream targets shared by ERECTA-family LRR-RLKs may unravel the complete picture of the signaling mechanism coordinating cell proliferation during plant organ morphogenesis.

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