

Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*

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Cell elongation in plants is controlled by environmental cues such as light and internal growth regulators including plant steroid hormones, brassinosteroids (BRs). In this study, we found that 3 related receptor-like kinases (RLKs), HERCULES1, THESEUS1, and FERONIA, are transcriptionally induced by BRs and are down-regulated in the loss-of-function BR mutant *bri1* and up-regulated in the constitutive BR-response mutant *bes1-D*. These RLKs belong to the CrRLK family that has 17 members in *Arabidopsis*. We hypothesize that these RLKs are involved in BR-regulated processes. Although 2 of the RLKs were recently found to mediate male-female interaction during pollen tube reception (FERONIA) and to sense cell wall integrity (THESEUS1), our genetic studies demonstrated that they are required for cell elongation during vegetative growth as *herk1 the1* double and *fer* RNAi mutants displayed striking dwarf phenotypes. The *herk1 the1* double mutant enhances the dwarf phenotype of *bri1* and partially suppresses *bes1-D* phenotype, supporting a role of HERK1/THE1 in BR-mediated cell elongation. Microarray experiments demonstrated that these RLKs control the expression of a unique set of genes including those implicated in cell elongation and 16% of the genes affected in *herk1 the1* are regulated by BRs. Our results, therefore, identify a previously unknown pathway that functions cooperatively with, but largely independent of the BR pathway to regulate cell elongation. The work establishes a platform to identify other signaling components in this important pathway for plant growth and provides a paradigm to study the coordination of independent pathways in the regulation of a common biological process.

brassinosteroid | FERONIA | HERCULES1 | THESEUS1

Understanding signaling mechanisms and gene networks regulating growth and development is a central issue in developmental biology. Plant growth and development are controlled by intrinsic growth regulators or hormones and environmental cues through interconnected signal transduction pathways (1). A single hormone can regulate many different processes and likewise different hormones can cooperate to control the same cellular process. Mechanisms underlying the cross-talk between different signaling pathways have been described recently (2, 3). Plant steroid hormones, brassinosteroids (BRs), regulate many growth and developmental processes such as cell elongation, senescence, vascular development, reproduction, and various stress responses (4–7). BRs signal through a membrane-localized, leucine-rich repeat (LRR) receptor kinase BRI1 and coreceptor BAK1 to regulate several intermediate signaling components (4, 8–10). BR signaling eventually controls BES1 and BZR1 family transcription factors, which mediate the expression of many genes for various BR responses (11–14). Consistent with the main function of BRs in cell elongation, BRs promote the expression of a number of cell wall remodeling enzymes required for cell elongation (15–17). In addition, BRs also regulate a variety of other genes implicated in signal transduction including several receptor-like kinases (RLKs), which may be involved in communicating BR signals to other pathways. In this study, we report on the characterization of 3 related genes, HERCULES Receptor Kinase 1 (*HERK1*,

At3g46290), *THESEUS1* (*THE1*, At5g54380) and *FERONIA* (*FER*, At3g51550), all of which are induced by BRs.

These 3 RLKs belong to CrRLK family that has 17 members in *Arabidopsis* (18, 19). During the course of characterizing these genes, *THE1* and *FER* were reported to repress cell elongation in various systems (18). Loss-of-function mutations in *THE1* were shown to suppress the hypocotyl elongation defect of a cellulose-deficient mutant *cesA6* in dark-growing seedlings. Therefore, *THE1* was proposed to be a sensor of cell wall integrity - inhibiting cell elongation when the cell wall is damaged (20). However, *FER* was found to be involved in male-female interaction during pollen tube reception in *Arabidopsis* (21). More specifically, *FER*, which is expressed in synergid cells of female gametophytes, was proposed to receive a signal from incoming pollen tubes and to inhibit pollen tube elongation, leading to eruption of the pollen tip and release of sperm cells to fertilize the egg cell (21, 22). In complete loss-of-function mutants of *FER*, *feronia* (*fer*) and *sirene* (*sir*), pollen tubes continued to grow without fertilizing the egg cell (21, 23, 24).

The broad expression patterns of *FER*, *THE1*, and *HERK1*, especially during vegetative growth (18), imply additional functions for these RLKs. Our genetic studies demonstrated that the HERK1/THE1/*FER* pathway is required for optimal cell elongation and interacts with the BR pathway. Microarray experiments indicated that most of the genes affected in the mutants are not BR target genes. We therefore conclude that HERK1/THE1/*FER* are components of a newly recognized pathway that functions cooperatively, but largely independent of the BR pathway to promote cell elongation.

Results

HERK1, THE1, and FER Are Induced by BRs and Modulated in BR mutants. In our effort to identify and characterize BR regulated genes, we found that *HERK1*, *THE1* and *FER* are induced by BRs as revealed by both published (15) and publicly available microarray data (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). *HERK1*, *THE1*, and *FER* are the most highly induced genes by BR and, therefore, were chosen for further study. Our quantitative RT-PCR experiments confirmed that brassinolide (BL), the most active BR, up-regulated these RLKs by ≈20–80% in seedlings and/or adult plants (Fig. 1A). The expression changes are significant as demonstrated by student's *t* test ($P < 0.01$). In addition, the expression levels of the RLK genes were reduced in the loss-of-function mutant *bri1* and increased in the constitutive BR-response mutant *bes1-D* (Fig. 1B). The close relationship of these 3 RLKs in the phylogenetic tree, especially between *HERK1* and *THE1* (Fig. S1) and their induction by BRs suggest an involvement in BR-regulated processes.

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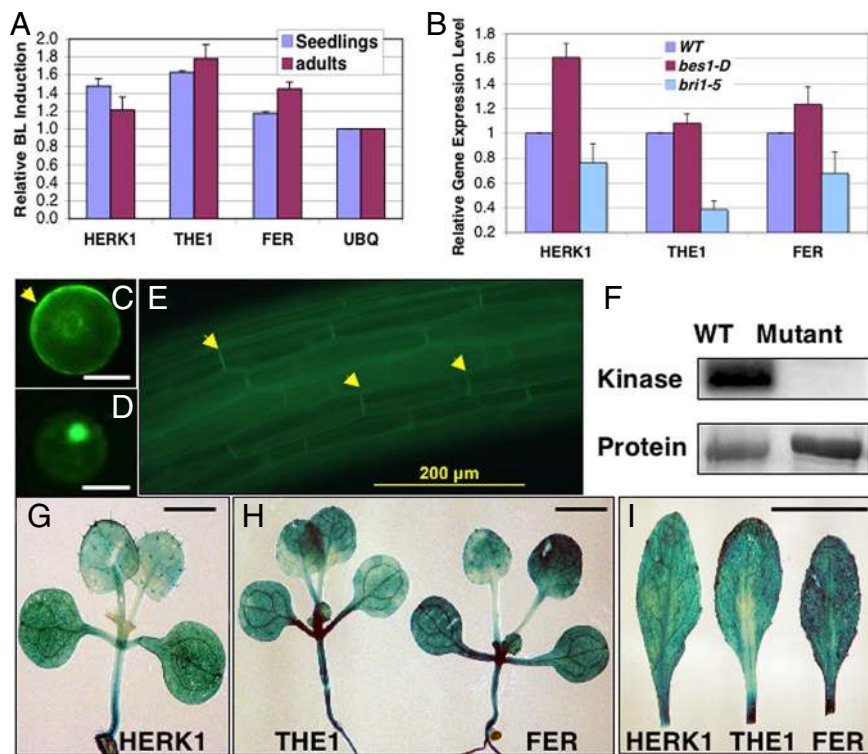


Fig. 1. HERK1, THE1, and FER are related receptor-like kinases induced by BRs. (A) Expression of HERK1, THE1, and FER is induced by BL treatment in 10-day-old seedlings and 4-week-old adult plants. Triplicate biological samples were used to prepare RNA for quantitative RT-PCR. The relative expression levels compared with *UBQ5* gene were used to determine the BL-induction levels of each gene. The averages and standard deviations from 3 biological repeats are shown. (B) *HERK1*, *THE1*, and *FER* are up-regulated in *bes1-D* and down-regulated in *bri1-5*. Ten-day-old seedlings were used to prepare RNA for qRT-PCR as described in A. (C–E) HERK1 cellular localization: A HERK1-GFP (C) or BES1-D-GFP (D) construct was introduced into protoplasts or transgenic plants (E). HERK1 is mostly localized at the plasma membrane (C and E), whereas BES1-D is primarily in the nucleus (D). (F) HERK1 displayed kinase activity. Recombinant proteins fused with MBP representing the WT HERK1 kinase domain (WT), and a mutant version with disrupted kinase activity (mutant), were used in kinase assay. Autophosphorylation was detected by phosphorimaging, and the proteins were detected by SYPRO RUBY staining. (G–I) Expression patterns of HERK1, THE1, and FER in 10-day-old seedlings (G and H) or 3-week-old adult leaves (I) as revealed by GUS reporter gene. (Scale bars: C and D, 10 μ m; G and H, 2.5 mm; I, 10 mm.)

We first determined the cellular localization of HERK1 by fluorescence microscopy. Similar to FER (21) and THE1 (20), the HERK1-GFP fusion localized to the plasma membrane when expressed in *Arabidopsis* protoplasts and plants (Fig. 1 C and E). The kinase activity of HERK1 was tested with recombinant proteins expressed and purified from *Escherichia coli*. The WT kinase domain of HERK1 was autophosphorylated, whereas a mutant form, in which a conserved lysine was mutated to arginine (K513R), lost its kinase activity in vitro (Fig. 1 F). Taken together, these results demonstrate that HERK1, like its homologs THE1 and FER, is a plasma membrane-localized receptor kinase.

HERK1, *THE1* and *FER* are universally expressed in most vegetative tissues, including leaves, stems and roots as revealed by our promoter-GUS (β -glucuronidase) reporter gene studies (Fig. 1 G–I) and by studies carried out by others (20, 21). The expression is particularly strong in the regions undergoing cell elongation, such as in hypocotyls and leaf petioles. Because *HERK1*, *THE1*, and *FER* are regulated by BRs and modulated in BR mutants, we hypothesize that HERK1, THE1, and FER have a more general role in cell elongation processes, in addition to the function of *THE1* in sensing cell wall integrity and *FER* in pollen tube and female gametophyte interactions.

HERK1, THE1, and FER Are Required for Cell Elongation During Vegetative Growth. T-DNA insertion mutants were identified for both *HERK1* and *THE1*, designated as *herk1-1* and *the1-4*, which had the T-DNA insertions in the kinase domains in both genes. Both mutants are null because transcripts were not detected in mutant plants (see Fig. 4). The close homology and overlapping expression patterns of HERK1 and THE1 prompted us to test whether these two genes function redundantly. Toward that end, we constructed a *herk1-1 the1-4* double mutant, referred to hereafter as *herk1 the1*. Because there are no T-DNA insertion mutants available for *FER* and the null allele of *fer* was embryonic lethal due to failed fertilization, we used an artificial microRNA, or amiRNA (25) to knockdown the *FER* gene to

determine its function during vegetative growth. An amiRNA specifically targeting *FER* was cloned into an expression vector driven by the strong, constitutive *BRI1* gene promoter (26). More than 80% of the transgenic lines displayed a reduced growth phenotype (Fig. 2 B). Two transgenic lines, designated as *fer1* and *fer2*, with different degrees of *FER* reduction, were chosen for further studies.

We first determined the BR response of the mutants by measuring hypocotyl lengths in the absence or presence of BL. BRs stimulate cell elongation in the light-grown seedlings, and hypocotyl elongation has been widely used as a measure BR activity. *herk1*, *the1*, and *herk1 the1* double mutants have somewhat shorter hypocotyls compared with WT without BL treatment, whereas *fer1* and *fer2* have shorter hypocotyls than WT with or without BL treatment (Fig. 2 A). Interestingly, the BR response was largely unchanged in the mutants (Fig. 2 A). We therefore concluded that HERK1, THE1 and particularly, FER, are required for hypocotyl elongation mostly in a BR-independent manner at the seedling stage.

At the adult stage, although *herk1* and *the1* single mutants are indistinguishable from WT, *herk1 the1* double mutants were clearly stunted in growth (Fig. 2 B). The elongation of both leaves and leaf petioles was affected (Fig. 2 C and D). We focused on leaf petioles that were most severely affected in the mutants. Although the elongation of petioles was slightly affected in *herk1* and *the1* single mutants, the petioles were reduced to half the length of the WT in *herk1 the1* double mutants (Fig. 2 C and D). To determine the basis for the stunted growth phenotypes, we examined the cells in the leaf petioles. Most cells in the mutant leaf petioles were shorter than the corresponding cell types in WT (Fig. 2 E and F).

To confirm that the mutant phenotype is caused by T-DNA knockout, a genomic clone of *HERK1* under the control of its native promoter was transformed back into the *herk1 the1* double mutant. From >10 transgenic lines recovered, approximately half of them showed clear rescue of the mutant phenotype (Fig. S2 A–C). The level of the HERK1 transcript was higher in the

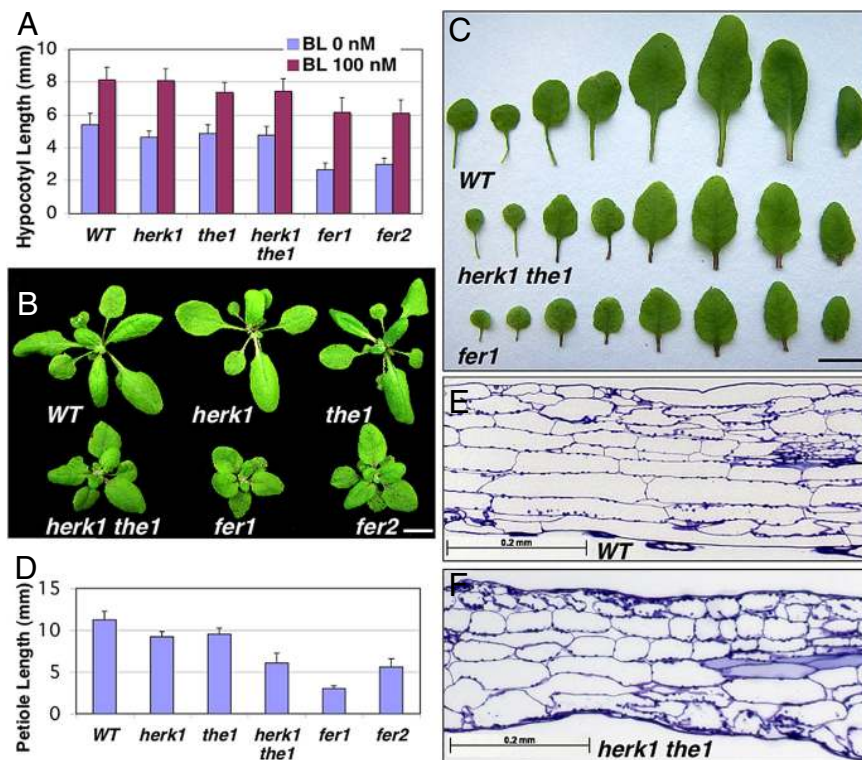


Fig. 2. HERK1, THE1 and FER are required for cell elongation. (A) BR responses of the *herk1*, *the1*, and *fer* mutants at the seedling stage. The seeds were germinated in 1/2MS media containing the indicated concentrations of brassinolide (BL) and grown under the light for 10 days. Twenty to 30 hypocotyls were measured, and the average hypocotyl lengths and standard deviations are presented. (B) Shoot phenotypes of 24-day-old adult plants. (Scale bar: 10 mm.) (C) The leaves of the WT, *herk1 the1* double mutants, and *fer1* mutants, showing the reduced lengths in leaf blades and leaf petioles. (Scale bar: 10 mm.) (D) Quantification of petiole lengths of the 6th leaf in WT and mutants. Averages and standard deviations from 10 plants are shown. (E and F) The *herk1 the1* double mutant plants have reduced cell elongation. The petioles of the WT (E) and *herk1 the1* double mutant (F) plants were fixed, stained with toluidine blue, and embedded. Longitudinal sections were examined under a bright-field light microscope and photographed. The sections shown are midway along the length of the petioles and cut through the center (traces of vascular tissues can be seen on both WT, and mutant sections can be seen on the right side).

transgenic plants with rescued phenotype (Fig. S2D). We conclude that HERK1 and THE1 function redundantly to promote cell elongation, especially in adult plants.

Both *fer1* and *fer2* adult plants have almost the same phenotypes as the *herk1 the1* double mutant, with a more severe cell elongation defect in *fer1* (Fig. 2 B–D). The fertility in both lines was also reduced, presumably because of impaired FER function in pollen tube-female gametophyte interaction. The extent of both vegetative and reproductive phenotypes correlated well with the reduction in expression of the *FER* gene (Fig. 2 B–D, Fig. 4C).

To determine the gain-of-function phenotype for the RLKs, we identified several transgenic plant lines overexpressing the *HERK1* gene under its own promoter, aided by CAMV 35S enhancer elements. Of these, we characterized 1 representative line that accumulated ≈ 10 times more *HERK1* RNA than WT (Fig. S3). Overexpression of *HERK1* appeared to increase petiole length by ≈ 15 –20% (Fig. S3A). Consistent with the phenotype, several *HERK1*/*THE1* regulated genes (see next) were up-regulated by ≈ 20 –70% (Fig. S3B). Taken together, our results demonstrate that, in addition to their established roles in sensing cell wall-integrity (20) and pollen and female gametophyte interaction (21), *HERK1*/*THE1*/*FER* RLKs play a major role in promoting cell elongation during vegetative growth.

Genetic Interactions Between BR and HERK Pathways. Triple mutants of *bri1-5 herk1 the1* and *bes1-D herk1 the1* were constructed to investigate the genetic interactions between BR and HERK signaling (Fig. 3). *bri1-5* is a weak loss-of-function allele of the BR receptor gene, *BRI1*, that displays a semidwarf phenotype (27). The *herk1 the1* double mutant enhanced the *bri1-5* dwarf phenotype (Fig. 3 A–C). However, *bes1-D* is a gain-of-function mutant that displays constitutive BR responses including excessive cell elongation (13). The *herk1 the1* double mutant partially suppressed the cell elongation phenotype of *bes1-D* (Fig. 3 D and E). These genetic studies suggest that *HERK1* and *THE1*

cooperate with the BR pathway and mediate part of BR-regulated cell elongation.

Most of the *HERK1*/*THE1*/*FER* Affected Genes Are Not Regulated By BRs. Despite similar defects in cell elongation, the *herk1 the1* double or *fer* mutants differ from BR mutants. BR mutants have epinastic and dark green leaves that were not observed in *herk1 the1* double or *fer* mutants (Fig. 2). We asked whether the cell elongation phenotype of *herk1 the1* double mutant was due to changes in BR-regulated gene expression. To that end, the global gene expression patterns of *herk1 the1* double mutant, in the absence and presence of BL during the adult stages, were first determined by microarray analysis (Fig. 4 A and B; Table 1; and Table S1, Table S2, Table S3, and Table S4). In adult plants, although BL induces and represses the expression of 650 and 520



Fig. 3. *herk1 the1* double mutant enhances *bri1-5* and suppresses *bes1-D* mutant phenotypes. (A) Two-week-old seedlings. (B) Adult plants (≈ 35 -day-old). (C) Fifth and sixth leaves. (D) Nine-day-old seedlings. (E) Adult plants (≈ 24 -day-old). Note that one of the leaves from the *bes1-D herk1 the1* plant was removed for genotyping.

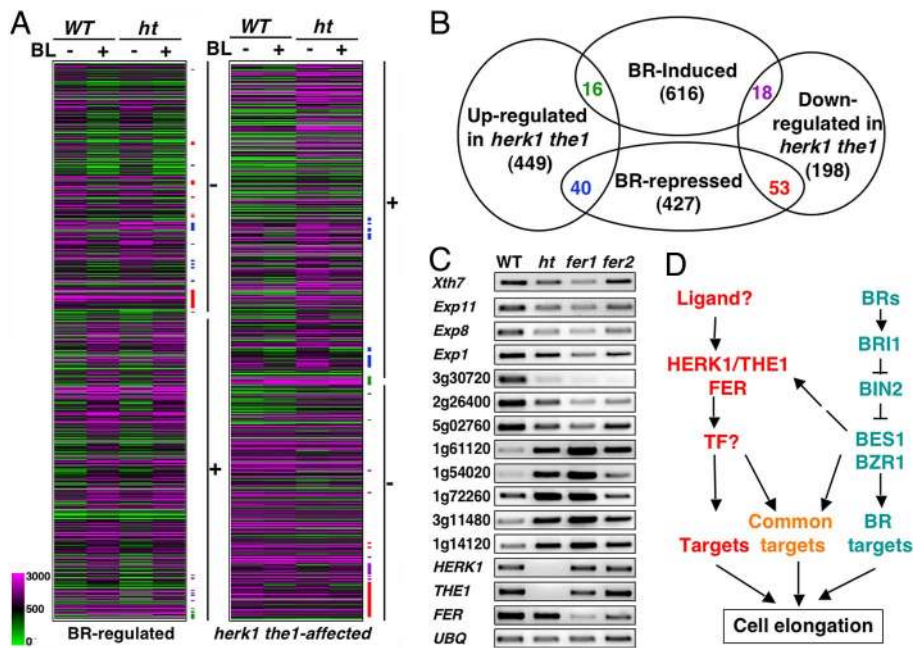


Fig. 4. HERK1/THE1/FER and BR pathways affect independent genes with some overlap. (A) Cluster analysis of BR-regulated genes in WT and mutant plants. The majority of BL-repressed genes (marked by $-$) and BL-induced genes (marked by $+$) are not affected in *herk1 the1* double mutant (*ht*). Likewise, the majority of genes down-regulated ($-$) or up-regulated ($+$) in *herk1 the1* double mutant are not regulated by BRs. The overlap between BR- and HERK1/THE1-affected genes are indicated by colored numbers corresponding to the colored numbers in the overlaps shown in Fig. 4B. (B) A diagram showing the overlap of BR- and HERK1/THE1-regulated genes. Lists of the genes represented in this diagram are presented in Table S1, Table S2, Table S3, and Table S4. (C) Semicquantitative PCR with primers from indicated genes to confirm the microarray data in the *herk1 the1* double mutant (*ht*) and to examine the expression levels in *fer1* and *fer2* mutants. Genes either down- or up-regulated in the *herk1 the1* double mutant and the 3 receptor genes were tested. (D) A model for the BR- and HERK1/THE1-pathways in the regulation of cell elongation. See Discussion for details. TF stands for putative transcription factor.

genes respectively, 269 and 505 genes were down- or up-regulated, respectively, in *herk1 the1* double mutant compared with WT control. Using GeneSpring (www.chem.agilent.com/enUS/Products/software/lifesciencesinformatics/genespringgx/Pages/default.aspx), we performed cluster analysis of BR- and HERK1/THE1-regulated genes and found that the majority of BR-regulated genes were not affected in the mutant, and likewise, the majority of HERK1/THE1 regulated genes were not clearly regulated by BL (Fig. 4A and B). It appears that only $\approx 10\%$ of BR-regulated genes were affected in the *herk1 the1* double mutant or 16% genes affected in *herk1 the1* double mutant are regulated by BRs (Fig. 4B). These results suggest that

the HERK pathway is largely independent of the BR pathway with a subset of overlapping genes.

Consistent with the similar phenotypes between *fer1*, *fer2* and *herk1 the1* double mutant, 12 of 16 randomly chosen genes affected in *herk1 the1* double mutant are similarly affected in *fer1* and *fer2* mutants (Fig. 4C). The gene expression changes correlate well with the mutant phenotypes, i.e., the genes are reduced or increased more in *fer1* than in *fer2* and *herk1 the1* double mutant. The results support the conclusion that HERK1/THE1 and FER likely affect a similar set of genes and, therefore, function in a common pathway.

To gain a better understanding of how the BR and HERK/

Table 1. The regulation of genes implicated in cell elongation by BRs and HERK1/THE1

Treatment/genotype	Gene no.	WT-BL	WT + BL	<i>herk1 the1</i> -BL	<i>herk1 the1</i> + BL	Annotation	
BL	AT5G57560	2277	7344	2728	7941	<i>XTH22</i>	
	AT1G10550	47	223	48	191	<i>XTH33</i>	
	AT1G11545	453	1178	357	1249	<i>XTH8</i>	
	At2g06850	7247	13455	5096	11194	<i>XTH4</i>	
	At4g30290	153	297	162	259	<i>XTH19</i>	
	At1g65310	137	214	95	302	<i>XTH17</i>	
	At3g23730	559	819	636	1069	<i>XTH16</i>	
	At3g07010	427	939	325	829	<i>PLL20</i>	
	At1g04680	2466	3402	1982	2932	<i>PLL26</i>	
	At4g38400	188	433	151	381	<i>EXPL2</i>	
	At3g45970	581	1339	529	970	<i>EXPL1</i>	
	At2g37640	384	641	340	594	<i>EXP3</i>	
	At3g45960	24	92	18	44	<i>EXPL3</i>	
	At1g20190	141	395	35	225	<i>EXP11</i>	
	BL/ <i>herk1 the1</i>	At2g40610	1006	2407	437	1586	<i>EXP8</i>
		At1g69530	6798	8465	3595	6462	<i>EXP1</i>
<i>herk1 the1</i>	At2g20750	295	198	161	157	<i>EXPB1</i>	
	At2g28950	3287	2524	2074	1919	<i>EXP6</i>	
	At3g29030	2323	2112	1523	1848	<i>EXP5</i>	
	At4g37800	2899	3419	1831	2864	<i>XTH7</i>	

Affymetrix *Arabidopsis* genomic arrays were used to detect gene expression. BL-regulated or *herk1 the1*-affected genes implicated in cell elongation are included. Average of the gene expression level from 3 biological replicates are shown. Note that several BL-induced genes appeared to be reduced in *herk1 the1* double mutant, but did not pass the statistical test.

THE pathways function to regulate similar processes, we further examined genes implicated in cell elongation (Table 1). It is well established that BRs promote cell elongation by inducing the expression of the genes involved in cell wall-loosening enzymes, such as xyloglucan endotransglycosylase/hydrolase (*XTH*) and Pectin Lyase-like (*PLL*) as well as expansins (*EXP*) (28–30). Consistent with its major function in promoting cell elongation, BRs induce the expression of 7 *XTH*s, 2 *PLL*s and 7 *EXP*s in adult plants. Similarly, HERK1/THE1 is also required for the expression of 6 cell elongation genes, including 1 *XTH*, and 5 *EXP*s. Interestingly, 2 genes (*EXP1* and *EXP8*) are induced by both pathways and are likely to be common targets. These results indicated that although BR and HERK induce different sets of genes for cell elongation, there are some common target genes that may integrate signals from both pathways. Because only 2 of 16 BR-induced genes implicated in cell elongation are reduced in *herk1 the1* double mutant, it seems unlikely that the mutant phenotype is solely due to the changes in BR target gene expression.

Discussion

Based on our results, we propose a working model for the HERK1/THE1/FER pathway and its relationship with the BR pathway in the regulation of cell elongation (Fig. 4D). Although BRs function through BRI1 and BIN2 to regulate the BES1/BZR1 family transcription factors and, therefore, modulate the expression of target genes including those required for cell elongation, HERK1, THE1, and FER function in an independent pathway to regulate different genes including those implicated in cell elongation. The regulation of common targets may represent cross-talk between these 2 pathways.

Our study, therefore, identified an important pathway required for plant growth. Plant cell elongation is regulated by environmental cues such as light and several plant hormones, including BRs, auxin and gibberellin (31, 32). Our qRT-PCR information and the public microarray data revealed that although BRs induce the expression of all 3 RLKs (Fig. 1 and Fig. S4A), other hormones, including auxin, gibberellin, ethylene, and cytokinin, which are known to regulate cell elongation or division, modulate individual RLKs (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). For example, our qRT-PCR analysis indicated that GA3 up-regulates the expression of *HERK1*, *THE1*, and *FER* whereas IAA appears to induce *HERK1* (Fig. S4B). However, the *herk1 the1* double mutant responds normally to gibberellin in hypocotyl elongation assays and to auxin in root elongation assays (Fig. S4C and D), implying that HERK/THE1/FER RLKs are unlikely involved in gibberellin or auxin perception. It is more likely that different hormones can modulate the expression of *HERK1/THE1/FER* to regulate plant growth. By comparison to other hormone-regulated genes (33), we found that among 774 *herk1 the1* affected genes, 61, 7, 41, and 37 genes are known to be regulated by auxin (789 genes), gibberellin (122 genes), ethylene (532 genes), and cytokinin (491 genes), respectively. The observation further supports the notion that HERK1/THE1 and FER represent another pathway required for optimal cell elongation. The dwarf mutant phenotypes of *HERK1/THE1* and *FER* at adult stage should greatly facilitate the identification of other signaling components for this important family of RLKs.

What is the relationship of HERK1, THE1 and FER? The fact that *fer1* and *fer2* showed almost identical phenotypes and affected similar genes to the *herk1 the1* double mutant suggests that HERK1/THE1 and FER function in the same pathway. For example, FER could serve as a coreceptor for HERK1/THE1 such that a knockdown of *FER* has the same effect as a knockout of both *HERK1* and *THE1*. It's worth noting that several other members in the family, such as At2g39360, At5g24010, At1g30570, At5g38990/At5g39000, and At5g39020 are slightly induced by BL (Fig. S4A). They may function in other processes

including cell elongation. It's possible that different combinations of members regulate growth in different tissues or organs at different developmental stages.

HERK1/THE1/FER signaling apparently interacts with the BR pathway. First, BRs induce the expression of *HERK1*, *THE1* and *FER* through BRI1 and BES1 (Fig. 1A and B). Second, *herk1 the1* double mutants enhance *bri1* and suppress *bes1-D* mutant phenotypes (Fig. 3). Third, the 2 pathways regulate a common set of 127 target genes, including those involved in cell elongation (Fig. 4 and Table 1). It's possible that functional cooperation between effectors in each pathway (i.e., BES1 and its counterpart in the RLK pathway) accounts for the expression of the common target genes. Identification of downstream effector protein(s) in the HERK1/THE1/FER pathway can help test the hypothesis.

Our study provides significant insight into the function of CrRLK family of RLKs. HERK1, THE1, and FER are members of the CrRLK family RLKs in *Arabidopsis*. Two members of the family, FER and THE, were found recently to inhibit cell elongation in two different contexts (18). In contrast, we observed that knockout of both the *HERK1* and *THE1* genes or the reduction of *FER* led to reduced cell elongation. Meanwhile, overexpression of *HERK1* resulted in up-regulation of some HERK target genes and slightly enhanced petiole elongations (Fig. S3). Therefore, our results suggest a role of the RLK family in promoting cell elongation. Several possibilities can explain the seemingly opposite functions. First, the pathways may have different outputs in different tissues/organs at different developmental stages. Second, like many signaling pathways, different signaling intensities can lead to opposite biological effects. For example, although exogenously applied BRs promote hypocotyl elongation in light-grown seedlings, they inhibit hypocotyl growth in the dark where cell elongation is already very active.

Finally, our results extend the role of FER from female: male interaction to vegetative growth. Interestingly, BRs, which are well established for their role in vegetative growth, function in reproduction as well. BRs are required for pollen tube growth, as demonstrated by the fact that BR mutants are defective in pollen tube elongation and have reduced fertility (17, 34). Because FER functions to block further pollen tube growth once the tube reaches synergid cells, it is apparent that although BRs and FER have similar functions in vegetative tissue, they have opposite roles in pollen tube growth. The identification of other signaling components is needed to fully understand the functional divergence of the HERK1/THE1/FER pathway.

In summary, this study has identified signaling components that regulate an important plant growth process, cell elongation. The established phenotypes and target genes will help identify the ligand(s) and other signaling components. In addition, the apparent interactions between BR and HERK pathways provide a paradigm for the way in which different signaling pathways contribute to the regulation of a similar process, a general theme for growth and development.

Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis thaliana* ecotype Col-0 was the WT. Seeds were germinated and grown on ½ MS medium with 1% sucrose under 15 h light/9 h dark cycle at 22 °C. Two-week-old seedlings were usually transferred to soil and grown in growth rooms under same conditions.

HERK1 Localization and Kinase Activity. A HERK1-GFP or BES1-GFP construct was introduced to protoplasts derived from 5-day-old seedlings grown in liquid culture by PEG-mediated transfection and culture for 16 h (35). The BRI1::HERK1-GFP was also introduced into *Arabidopsis* plants by floral dip method (36). Transgenic plants were identified by screening T1 seeds in ½MS containing 50 mg/L Kanamycin. Protoplasts or hypocotyls cells from 3-day-old transgenic lines (T2) grown in the dark were observed under an Olympus fluorescence microscope with a filter for GFP visualization. For the in vitro kinase assay, WT HERK1 kinase domain (WT), and a mutant version (K513R),

were cloned into a MBP (Maltose Binding Protein) fusion vector and expressed in *E. coli*. Recombinant proteins were purified using amylose resin (New England Biolabs) and used in kinase assay in 50 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM ATP (pH 7.5), plus 2 μ L of ³²P- γ -ATP (Perkin Emer, 3,000 Ci/mmol), room temperature, for 0.5 h. Autophosphorylation was detected after protein gel electrophoresis and phosphorimaging with a Typhoon 9400 (GE Healthcare). Protein was visualized by SYPRO RUBY protein gel stain (Invitrogen).

Transgenic Studies. The promoters of *HERK1* (-2114/-19 relative to the translational start site) and *FER* (-1193/-27) were amplified from BAC DNA, and *THE1* (-2222/-43) was amplified from genomic DNA, and cloned to the pBI101 with the β -glucuronidase (GUS) gene (Clontech). A 5.3-kb *HERK1* genomic fragment was cloned into pMN20 that harbors 4 copies of CaMV 35S enhancer (37). The constructs were transfected into *Agrobacterium* strain GV3101, which were used to transform *Arabidopsis*. T2 or T3 transgenic plants were used to detect reporter gene expression by GUS assay (38) or phenotypic analysis.

Identification of T-DNA Knockout Mutants and Creation of Artificial microRNA (amiRNA) Mutants. The T-DNA knockout seeds, *herk1* (SALK_008043) and *the1-4* (CS829966), were obtained from the *Arabidopsis* Biological Resource Center (www.arabidopsis.org). Homozygous plants were identified by PCR genotyping with primers listed in Table S5. Double mutants were constructed by cross single homozygous mutants and F2 plants were genotyped for double homozygous plants. The amiRNA system was used to knockdown *FER*. A set of 4 primers (Table S5) were designed using the tools found at <http://wmd.weigelworld.org/cgi-bin/mirnatools.pl> and used to amplify an amiRNA fragment (25), which was cloned into a binary vector under the control of BR11 promoter.

Characterization of Growth Phenotype. For BR response experiments, brassinolide (BL, from Wako Chemicals USA Inc, Richmond VA) was added to the medium after autoclaving and cooling to 50 °C. The seeds were treated at 4 °C for 4 days and germinated and grown for 10 days before measuring hypocotyl lengths. For adult phenotypes, WT and mutant plants were grown side-by-side in a growth flat. The 24-day-old mature plants were examined for the growth phenotype and photographed. The petiole lengths of the 6th leaves, from 10 independent plants, were measured. The average and standard deviations were calculated. To examine the cell lengths, leaf petioles were fixed with 2% glutaraldehyde (wt/vol) and 2% paraformaldehyde (wt/vol) in 0.1M cacody-

late buffer (pH 7.2) for 48 h at 4 °C. Samples were rinsed in 0.1M cacodylate buffer and dehydrated in a graded ethanol series, infiltrated and embedded using LR White resin (Electron Microscopy Sciences). Resin blocks were polymerized for 48 h at 60 °C. Thick sections were made using a Reichert UC6 ultramicrotome (Leeds Precision Instruments). Sections were stained with 1% toluidine blue in 1% borax and images were taken using a Zeiss Axioplan II compound microscope with a MRC digital camera and Axiovision software (Carl Zeiss).

Gene Expression Studies. Microarray experiments were performed with 24-day-old adult plants. The whole plants were sprayed with either water or 1 μ M BL and incubate for 2.5 h. The plants were pooled into 3 groups as triplicates (8 plants per group). Total RNA was prepared with RNeasy Plant Mini Kit (QIAGEN) and used to make probes for microarray experiments with Affymetrix *Arabidopsis* Genomic arrays. The probe labeling, hybridization, and scanning were performed according to manufacturer's instructions. Microarray data were normalized by the MAS 5.0 method implemented in the R package *affy* and the linear model was applied to 2 sample comparison (samples with and without BL treatment or WT compared with mutant), using the *limma* package (39, 40). Genes with an adjusted $P \leq 0.01$ were considered to be differentially expressed. The BR-induced genes, *HERK1*/*THE* regulated genes were used for clustering analysis with the GENESPRING program (Silicon Genetics), using Pearson correlation.

For reverse transcription-PCR (RT-PCR), 2 μ g of total RNA was reverse-transcribed to cDNA by SuperScript II Reverse Transcriptase (Invitrogen). For real-time quantitative RT-PCR, the primers were designed so that the products are between 200 and 300 bp. The SYBR Green PCR Master mix (Applied Biosystems) was used and the PCRs were run on the Mx4000 multiplex quantitative PCR system (Stratagene). Two or 3 biological replicates were each analyzed with 2–3 RT-PCRs. For semiquantitative RT-PCR, the same amount of cDNA was used for each PCR. The primers used are listed in Table S5, and the *UBQ5* gene was the control. PCRs were stopped in the linear range of the amplification and were repeated for 2 times. Very similar results were obtained with both sets of samples.

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