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Molecular Cloning and Characterization of a Gene Coding for a Putative Receptor-like Protein Kinase with a Leucine-rich Repeat Expressed in Inflorescence and Root Apices from *Arabidopsis*

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Abstract

We isolated a novel receptor-like kinase with leucine-rich repeat by a differential screening for genes that were specifically expressed in inflorescence shoot apices of *Arabidopsis*, and named it *IRK* (*inflorescence and root apices receptor-like kinase*) after its expression pattern and structural features. IRK contains sequentially from the amino- to carboxy-terminus: a potential signal peptide for membrane targeting, a leucine-rich repeat between two conserved cystein motifs, a transmembrane domain, and a serine/threonine kinase domain. The kinase domain expressed in *E. coli* showed an autophosphorylation activity *in vitro*, indicating that *IRK* encodes an active protein kinase. The IRK protein fused to green fluorescence protein was targeted to plasma membrane, indicating that IRK is a membrane-localized receptor kinase. *IRK* was expressed not only at shoot apices but also in root tips and developing flowers. Thus, IRK seems to be a receptor-like kinase with a relatively general function in inflorescence and root apices for an unknown signaling mechanism.

Keywords: *Arabidopsis*, inflorescence, leucine-rich repeat, Receptor-like kinase, root apex.

Accession number: AB076907, AB076906

Abbreviations

cRT-PCR, competitive reverse transcription-polymerase chain reaction; IRK, inflorescence and root apices receptor-like kinase; LRR, leucine-rich repeat; RLK, recepor-like kinase.

Introduction

Sessile plants have to respond to environmental changes to survive. In higher plants with multicellular organization, intercellular signaling is required to coordinate different cells of the whole plant so it can adapt adequately to its environment throughout its life cycle. Cell to cell communication plays a crucial role in shoot apices during development and morphogenesis. For example, surgical removal of leaf primordia in *Dryopteris* affects phyllotaxy (Wardlaw, 1949), indicating that intercellular communication mechanisms via unidentified signals released from leaf primordia must function for proper organogenesis.

Comparative genomics among multicellular eukaryotes for which whole sequences are known such as *Arabidopsis, C. elegans*, and *Drosophila* indicates that different systems for intercellular signaling are adopted in phylogenetically distinct species (Arabidopsis Genome Initiative, 2000). For example, none of the counterparts widely adopted for signaling in vertebrates, insects, and worms such as Wingless/Wnt, Hedgehog, Notch/lin12, Jak/STAT and TGF-b/SMAD is found in *Arabidopsis*. However, it was estimated that the *Arabidopsis* genome is encoding at least 340 genes for receptor-like protein kinases (RLKs) with serine and threonine specificity (Arabidopsis Genome Initiative, 2000). Indeed, many important genes involved in signal transduction in various physiological and morphological processes were recently described in plants (van den Berg *et al.*, 1997; Innes, 2001). One important component is the group of RLKs (Irish and Jenik, 2001). RLK is a transmembrane factor

that transmits extracellular information to cytoplasm. The extracellular information in the form of a ligand received by the receptor domain in apoplast is transmitted to the cytoplasmic kinase domain to promote kinase activity. RLKs in plants have been mainly classified into five types based on the structure of the extracellular domains: the S-domain related to self-incompatibility in stigma-pollen interaction (Nasrallah and Nasrallah, 1993), the leucine-rich repeat (LRR) domain, the EGF domain (Massague, 1992), the lectin-like domain (Herve *et al.*, 1999), and the CR4-like domain (Becraft *et al.*, 1996). The RLK protein with the LRR domain is a dominant class in higher plants. It is estimated that there are more than 170 genes of this group of receptor-like kinase in the *Arabidopsis* genome. The function of the LRR motif is generally is to provide protein-protein interaction related to hormone signaling, cell adhesion, binding of glycoprotein in the extracellular matrix, and signal perception of a number of tyrosine kinase receptors. In higher plants, important members of RLK family with extracellular LRR have been reported for *Xa21* (Song *et al.*, 1995), *ER* (Torii *et al.*, 1996), *CLV1* (Clark *et al.*, 1997), *BRI1* (Li *et al.*, 1997), and *HAESA* (Jinn *et al.*, 2000), which play a role in disease resistance, morphogenesis, meristem maintenance, brassinosteroid signaling, and organ abscission, respectively. However, the function of most LRR kinase members deduced from the genome analysis in *Arabidopsis* remains to be investigated.

The activity of the apical meristem is required for the continuous production of plant bodies after embryogenesis. To understand the meristematic function and the underlying molecular mechanisms in plant development, systematic differential hybridization was conducted to isolate genes that were expressed in inflorescence shoot apices. Here we report the cloning and the characterization of a novel RLK gene, *IRK*, expressed in apices, and discuss the intercellular communication and signal transduction.

Materials and Methods

Plant materials

Arabidopsis thaliana (ecotype Columbia) was used as the wild type strain. The plants were grown under continuous fluorescent illumination at 22 ℃. For RNA preparation and GUS staining, aerial parts were harvested from plants grown on soil and roots were on MS agar plates.

Cloning and sequencing

The genomic clones and cDNA clones were isolated from the Columbia genomic library in Lambda FixII, and from the *Ler* flower cDNA library in Lambda ZAPII, respectively. 5' RACE against Columbia RNA was performed to obtain a full-length cDNA following the manufacturer's instruction (Marathon cDNA amplification kit, Clontech, Palo Alto, CA, U.S.A.). The nucleotide sequences were determined using a DNA sequencer (ABI373S, Applied Biosystems, Forstercity, CA, U.S.A.).

Northern blot analysis

Northern blot hybridization using poly(A)$^+$ RNA (2 μg) was performed as described (Takemura *et al.*, 1999).

Autophosphorylation assay

For the expression of the kinase domain of IRK, the region containing the kinase domain of IRK was obtained by PCR with primers 5'-GGGTCGACGAACCTGATTTCAGCACTGG-3' and 5'-CCGCGGCCGCCAAACCGGATTCCTCTTCGC-3' which had a *Sal*I and a *Not*I restriction sites, respectively. The amplified DNA fragment digested with *Sal*I and *Not*I was ligated into corresponding cloning sites of pGEX4T-3 (Amersham Pharmacia, Buckingham, UK). The IRK kinase domain fused to GST was expressed in *E. coli*, and purified with glutathione-Sepharose 4B according to the manufacturer's protocol (Amersham Pharmacia). Two micrograms of fusion protein was used for the autophosphorylation assays. The assay mixture contained 50 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM $MnCl_2$, 20 μM ATP, 10 μCi [γ-^{32}P]ATP (3,000 Ci mmol^{-1}) in a volume of 10 μl. The mixture was incubated for 1 h at 26 ℃. The reaction products were resolved by SDS-PAGE and visualized by autoradiography.

Subcellular localization analysis with the GFP reporter gene

The construct to express the IRK protein fused to GFP under the control of the CaMV 35S promoter was generated by PCR based cloning. Primers 5'-CCGGTACCACTAGTCCATGGACAAAGCACTGATT-3' and 5'-CCGGATCCGGGCCCAAACTTGAACCCAACTCATCT-3' which included an *Nco*I and an *Apa*I restriction sites, respectively, were used for cloning into the pTH2XA vector. In pTH2XA, five glycine residues were introduced at the site of junction with GFP (Takemura, unpublished). The construct was introduced into onion epidermal cells by particle bomberdment as described (Nishii *et al.*, 2000).

After overnight incubation at 22 ℃ in the dark, fluorescence was observed by confocal laser mi-

croscopy (LSM510, ZEISS, Oberkochen, Germany). Plasmolysis was performed by a 10% glycerol treatment.

Histochemical GUS assays

The *IRK* promoter region containing a 2.6 kb fragment upstream from the initiation codon was amplified by PCR from genomic DNA template (ecotype Columbia) with 5'-CCGTCGACCTCCTCCATTGACGATAAAC-3' and 5'-CCGGATCCCAGTGCTTTGTACATCTTTCC-3' primers. The amplified fragment was digested with *Bam*HI and *Sal*I, and cloned into pBI101.1 (Clontech). *Arabidopsis* thaliana (ecotype Columbia) plants were transformed with the above construct by the vacuum infiltration method (Bechtold and Pelletier, 1998). Ten independent transgenic lines were examined in T2 generation and no differences in GUS expression patterns were detected. Whole-mount GUS staining was performed at different developmental stages according to method of the Topping *et al.* (1991) modified by the use of a buffer containing 100 mM $NaPO_4$, 10 mM EDTA, 0.1% (v/v) TritonX-100, 0.5 mM $K_4[Fe(CN)_6]$, 0.5 mM $K_3[Fe(CN)_6]\cdot 3H_2O$, and 20% methanol, at pH 7.0. Incubation times were at least 45 min and at most overnight, depending on the penetration capacity of the organ assayed. GUS staining was viewed with a dark field stereoscopic microscope (Stemi SV11, ZEISS). For histochemical analysis, stained samples were dehydrated sequentially with ethanol, and embedded in Technovit 7100 resin (Heraeus, Wehrheim, Germany) according to the manufacturer's protocol. GUS staining was observed with a dark field microscope (Axioscop, ZEISS).

Competitive RT-PCR

Total RNA from different *Arabidopsis* tissues were prepared by using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), then purified by LiCl precipitation. For the first strand cDNA synthesis, one microgram of total RNA was used according to the manufacturer's protocol (the First strand synthesis kit, Amersham Pharmacia). One-microliter aliquots of the 15 μl reaction mixture of cDNA and 1 μl of each concentration of DNA competitors were submitted to PCR. A gradient series from 10^4 to 10^9 copies of DNA competitors was used for the analysis. To prevent amplification from contaminating genomic DNA, 5'-GTTCCCTTCGGTCTTTGATCC-3' and 5'-GCAAATTCCCGGGTTTCCTG-3' primers, which flank the intron 2 of *IRK* gene, were used. The DNA competitor of the *IRK* gene was designed to amplify a DNA fragment that is longer than the endogenous *IRK* transcript by 160 bp. The PCR reactions were performed with one denaturation cycle of 2 min at 95 ℃ and 35 cycles of 30 s at 95 ℃, 30 s at 55 ℃, and 30 s at 72 ℃.

Results

Identification and isolation of a novel receptor-like kinase gene, IRK, expressed in the inflorescence apex

To reveal the underlying molecular mechanism of organ initiation in plant development, 384 genes were identified by the systematic differential screening for genes expressed in shoot apices in reproductive phase in *Arabidopsis* (Takemura *et al.*, 1999). The nucleotide sequences of all the clones were determined from the 5'-ends (Takemura *et al.*, 1999). Of these, the clone 3-37 showed a sequence homology to RLK, which is an important protein for the perception of intercellular communication signals in multicellular organisms. We named it *IRK* for Inflorescence and Root apices Kinase, based on its structure and expression specificity as described later.

To confirm that the differential screening of *IRK* was successful, northern-blot analysis was performed (**Fig. 1**). Since expression levels were expected to be low in our strategy, poly(A)$^+$ RNA was prepared from various tissues. A single band at 3.8 kb was detected in the RNAs from flowers and shoot apices containing buds, while no signal was detected in the RNAs from leaves and stems. The expression pattern of *IRK* detected by northern hybridization analysis indicated that our differential hybridization worked successfully.

IRK encodes RLK with LRR

To deduce entire structural information of *IRK*, cDNA clones and genomic clones were isolated.

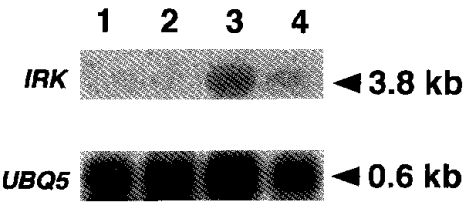

Fig. 1 Northern-blot analysis of *IRK*
Poly (A)$^+$ RNA was extracted from leaves, stems, shoot apices with flower primordium before stage 5 of flower development, and flower tissues of 5-week-old *Arabidopsis*. Two micrograms of poly(A)$^+$ RNA were applied in each lane. The *UBQ5* gene was used as a probe for loading control. Lane 1; rosette leaves, lane 2; stems, lane 3; shoot apices with flower primordium, lane 4; flowers.

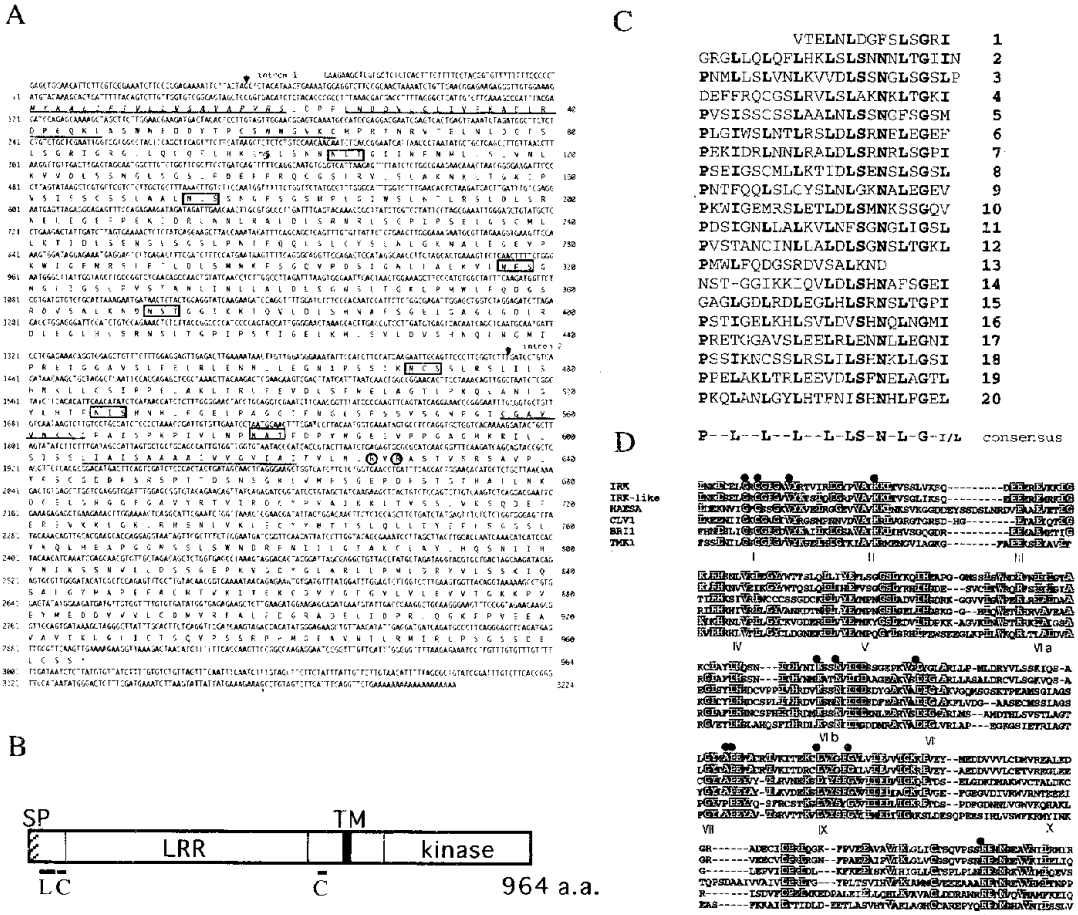

Fig. 2 Nucleotide sequence and structural features of *IRK*

(A) cDNA and deduced amino acid sequences of *IRK*. The positions of introns are indicated by arrows. The regions for a possible signal peptide (1–20 a.a.), a leucine zipper motif (25–46 a.a.), two spaced cysteine motifs(57–64, 557–565 a.a.), and a transmembrane domain (605–621 a.a.) are underlined. Positively charged amino acid residues for 'positive inside' rule are indicated by open circle. Possible N-glycosylation sites [NX(S/T)] are indicated by open squares. (B) Schematic diagram of the domain structure of the IRK protein. SP, signal peptide; L, leucine zipper motif; C, spaced cysteine motif; TM, transmembrane domain. (C) LRR domain of predicted amino acid sequences of IRK. The consensus sequence of the LRR in IRK is shown at the bottom. (D) Comparison of the kinase domain of plant receptor-like kinases. Alignment of the kinase domains of IRK and other plant LRR receptor kinases (IRK-like, HAESA, CLV1, BRI1, TMK1) is shown. Residues that are conserved among at least four of the compared sequences are in white-blanked-letters. Invariant amino acids in all kinase domains are indicated as dots. The alignment was generated by clustalW. Conserved kinase subdomains are indicated as I to XI.

Since even the longest cDNA clone isolated by conventional cDNA library screening was partial with a 1.8 kbp insert, full length cDNA was synthesized from RNA prepared from flower buds by using 5'-RACE. We sequenced the full-length cDNA of 3.8 kb that matched the estimated transcript size by northern analysis. Genomic Southern-blot for *IRK* indicated that *IRK* is a single copy gene (data not shown), and the corresponding genomic clones containing the IRK gene were also isolated and sequenced. IRK had two introns in the 5' untranslated region and in the coding region (**Fig. 2A**).

The *IRK* gene encodes a protein of 964 amino acid residues with a molecular mass of 103 kD (**Fig. 2A**). A schematic representation of the domain structure is shown in **Fig. 2B**. The presence of two hydrophobic domains was predicted by a hydropathy analysis. The first hydrophobic domain in the N-terminal region is a potential N-terminal signal sequence, and the site of cleavage is predicted to be between the 20th and the 21st amino acid residue by

PSORT program (Nakai and Horton, 1999). The second hydrophobic domain is a possible transmembrane segment that separates the extracellular receptor domain from the cytoplasmic kinase domain. The transmembrane domain is followed by positively charged amino acid residues which are assumed to hold the transmembrane segment in an orientation such that the positive charges remain inside the cytoplasm (Andersson, 1994).

The potential signal peptide is followed by a 3-heptad leucine-zipper motif that might be involved in the formation of homo- or heterodimers. IRK contains 20 imperfect repeats of an LRR motif with 24 amino acid residues in the potential extracellular region. The LRR domain of IRK is flanked by pairs of spaced cysteine motifs on both sides, which are often observed in adhesive proteins or receptors (Kobe and Deisenhofer, 1994). In the LRR of IRK, leucine and other hydrophobic amino acid residues are highly conserved in each repeating unit (**Fig. 2C**). There are 7 possible target sites for N-linked glycosylation, NX(S/T), in the potential extracellular region, suggesting that IRK may be a protein glycosylated in the extracellular region.

Carboxy-terminus of the IRK protein has a putative serine- and threonine-specific protein kinase domain composed of 11 subdomains (Hanks and Hunter, 1995). This domain of IRK is mostly related to the kinase domain of putative plant receptor kinases containing LRRs. The amino acid sequences of the kinase domain of IRK shared 34%, 35%, 33% and 32% amino acid sequence identities with those of HAESA (Jinn *et al.*, 2000), CLV1 (Clark *et al.*, 1997), TMK1 (Chang *et al.*, 1992), and BRI1 (Li *et al.*, 1997), respectively (**Fig. 2D**).

Autophosphorylation of the IRK kinase domain

To clarify whether IRK is a functional receptor kinase, the kinase domain of IRK was expressed in *E. coli*, and autophosphorylation assay was performed. We observed a phosphorylated band with an apparent molecular mass of 59 kD, which matched the molecular mass of IRK as a GST fusion protein (**Fig. 3**). This indicates that IRK is an active protein kinase with autophosphorylation activity.

IRK is localized to plasma membrane

The presence of a putative signal peptide and a transmembrane domain followed by a 'stop transfer' suggested that the IRK protein translocalizes to plasma membrane. To investigate the localization of the IRK protein in plant cells, we constructed a plasmid for the transient expression of the IRK protein fused to GFP (IRK:GFP). The construct was introduced into onion bulb epidermal cells by bomberdment (**Fig. 4**). Fluorescence was only detected in the cell surface regions, no fluorescence signal was detected in the cytoplasm and nucleus. We confirmed the plasma membrane localization of the IRK:GFP protein by plasmolysis (**Fig. 4A**). The fluorescence of control GFP was detected in the nucleus and the cytoplasm by confocal laser microscopy (**Fig. 4B**). The signal peptide and the transmembrane domain of IRK constitute a functional domain for the localization of the IRK protein to the plasma membrane. This localization feature of IRK together with the autophosphorylation activity described above indicates that IRK is a receptor kinase with a single membrane spanning configuration.

IRK gene is expressed at the inflorescence and the root apices

Since the timing and the levels of gene expression govern cellular development and physiology, we investigated the expression pattern of *IRK* in detail. We analyzed the transgenic plants expressing the GUS reporter under the control of the *IRK* promoter to examine the *IRK* expression pattern (Jefferson *et al.*, 1987). Plants in different stages were stained for GUS (**Fig. 5**). Several independent transformant lines were analyzed to deduce common patterns of expression. GUS staining reporting the activity of the *IRK* promoter was analyzed in 6-day-old seedlings (**Fig. 5A**). In root tissues, the GUS activity was very strong in the apex (**Fig. 5B**). The GUS staining was also detected in the lateral root primordium and newly emerging lateral root apices (**Fig. 5C**). Such GUS stainings in roots were always observed during plant growth. In 12-day-old plants, the *IRK* gene expression was also detected weakly in the vascular tissue of leaves after longer incubation (**Fig. 5D**).

In the reproductive phase, the expression patterns of the *IRK* gene in inflorescence, shoot apical

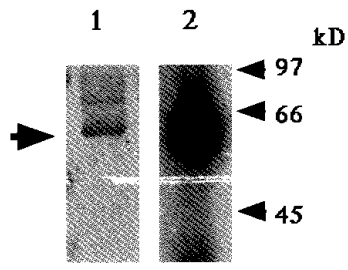

Fig. 3 Autophosphorylation assay of the IRK kinase domain expressed in *E. coli*

The kinase domain fused to GST (59 kD) was examined for autophosphorylation. The numbers on the right side of lane 2 indicate the sizes of the protein makers. SDS-PAGE (lane 1), and autoradiography (lane 2).

meristem and floral bud tissues were analyzed in detail by sectioning. The GUS expression was detected in shoot apical tissues, carpel primordium and sepals (**Fig. 5E**), and in the carpels and the sepals of immature flowers (**Fig. 5F**). The GUS expression was detected in flower cluster (**Fig. 5G**). In filament tissues, the GUS activity appeared in elongated filaments (stage 14) of flower development (Smyth *et al.*, 1990), but not in unelongated filaments (stage 11) (**Fig. 5H**). The expression of GUS was also observed in immature seeds (**Fig. 5I**). No GUS staining was detected in stems or in cauline leaves.

To confirm the endogenous expression pattern of *IRK*, we performed a competitive RT-PCR (cRT-PCR) as a quantitative RNA analysis (**Fig. 6A**). In all the stages we examined, mRNA levels of *IRK* gene were low in the rosette leaves and in the stems.

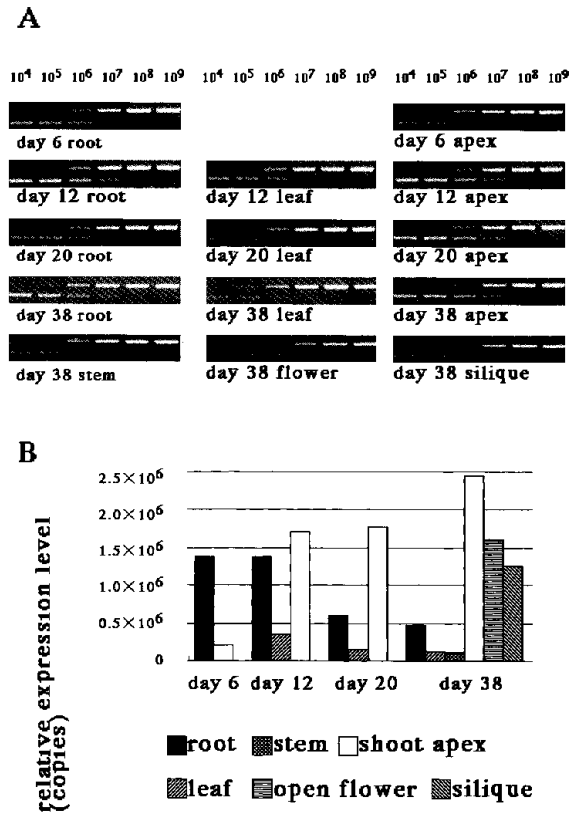

Fig. 6 Competitive RT-PCR analysis of IRK mRNA expression in plant development(A) Gel images of the competitive PCR products. Bands at 500 bp and 340 bp are derived from competitor DNA and IRK mRNA, respectively. The copy numbers of the competitors in each reaction tube are shown at the top of the sequential gel images. Each RNA sample used in reverse transcription reactions was purified from plants on days 6, 12, 20 and 38 after germination. Note that sample from shoot apex on day 6 includes cotyledon and shoot apical meristem. (B) Relative expression levels of the IRK mRNA.11

On the contrary, expression of *IRK* was relatively high in the inflorescence apex and in the root. In shoot apical tissues including flower buds on days 12, 20 and 38 after germination, the *IRK* expression levels were gradually increased (**Fig. 6B**). The high expression of *IRK* in siliques reflected the GUS staining in the seeds. The expression levels of *IRK* were increased as reproductive growth proceeded. The gain of the *IRK* expression levels in the reproductive phase suggests that *IRK* may have additional functions in the reproductive growth.

In roots, the cRT-PCR analysis shows that the abundance of the *IRK* mRNA decreased as plants grew (**Fig. 6B**). The result of cRT-PCR analysis apparently seemed to be inconsistent with the constant expression of GUS in root tips. Taking into account the fact that the proportion of root tips in root samples decreased during growth, however, the time-dependent reduction of the *IRK* expression levels in the cRT-PCR analysis was reasonable.

Discussion

Intercellular communication mediated by cell surface RLK is generally important for plant development. By a differential screening of genes expressed in shoot apices during the reproductive phase in *Arabidopsis*, in this study, we isolated and characterized a gene for a novel RLK, *IRK*, encoding a putative signal sequence, a characteristic extracellular domain, a transmembrane domain, and a kinase domain. We demonstrate that IRK has an autophosphorylation activity in the kinase domain and that it is localized to the plasma membrane. A characteristic feature of IRK is that it contains an LRR motif in the extracellular receptor domain. The LRR motif is expected to be involved in protein-protein interaction. In particular, LRR with a cysteine motif is known to be related to signal perception and cell adhesion (Kobe and Deisenhofer, 1994). For example, human LH-CG receptor for luteinizing hormone and chorionic gonadotropin recognition (Braun *et al.*, 1991), as well as Trk for nerve growth factor, are involved in signal transduction pathways of peptide hormones (Nakagawara, 2001), while connectin mediates cell-cell adhesion in vitro (Raghavan and White, 1997). Since these flanking cysteine motifs are also found in the LRR domain of IRK, IRK may be involved in cell-to-cell communication through the receptor domain containing LRR.

RLKs with the LRR motif show versatile functions in plant development. In *Arabidopsis*, 174 members of RLKs containing LRR are encoded in the genome, while the only a limited number of

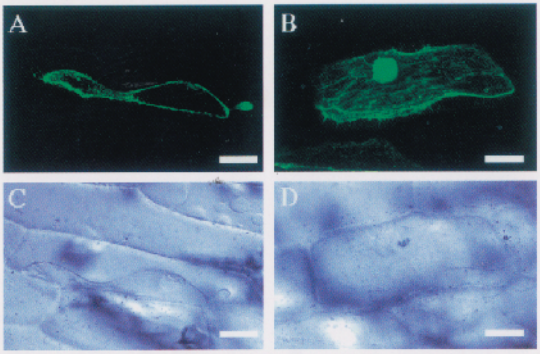

Fig. 4 Cellular localization of the IRK protein
Onion epidermal cells expressing 35S::*IRK*::*GFP* (A, C) and 35S::*GFP* (B, D) were observed after plasmolysis with 10% glycerol. Confocal laser microscopy (A, B), and light field microscopy (C, D). Bars=50 μm

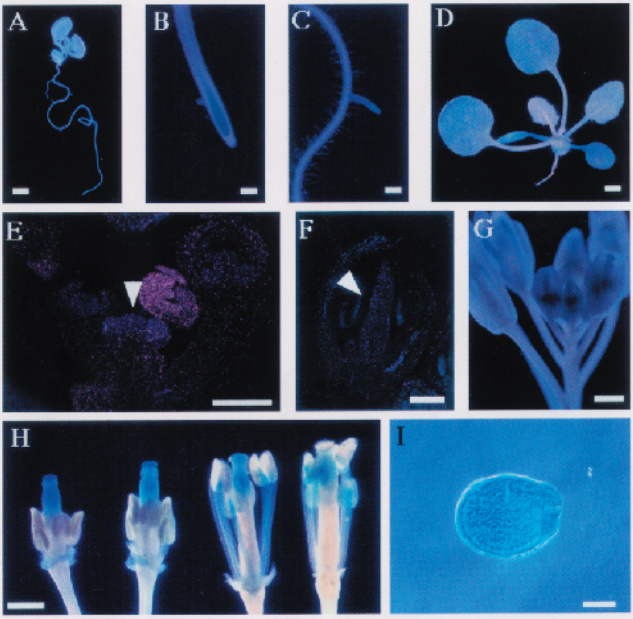

Fig. 5 Promoter activity of *IRK* during plant development
(A) 8-day-old seedling. (B) root apex of 6-day-old seedling. (C) emerging lateral root of 6-day-old plant. (D) 12-day-old plant. (E) inflorescence meristem observed under a dark field microscope; the arrowhead indicates a shoot apical meristem. (F) flower buds a under dark field microscope; the arrowhead indicates a carpel. (G) flower cluster. (H) GUS staining of stamen and carpel; development of flower from stage 11 to stage 14. (I) seed. GUS staining was performed for 45 min. (A-C), for 14h. (D-H), and for 3h. (I). In (A) and (D), bars=1.0mm; in (B), (C), (E), (F), and (I), bars=100 μm; in (G) and (H), bars=0.5 mm.

genes have been characterized so far: *CLAVATA1*, *ERECTA* and *HAESA* for developmental processes, *FLS2* in transducing pathogen signals, and *BRI* for brassinosteroid signaling pathway, etc. Generally, the LRR domain is composed of conserved and variable amino acid residues in each repeat. The sequence variations within LRR affect the specificity of ligand binding (Braun *et al.*, 1991). Various functions of RLKs are partly due to different ligand specificity. In LRR-containing RLKs of plants, only a few examples of ligand molecules which interact with receptor have been reported; e.g. CLV3 for CLV1 (Trotochaud *et al.*, 1999) and flg22 for FLS2 (Gomez-Gomez *et al.*, 2001). Since our knowledge on the combinations between ligands and receptors is limited, individual studies on RLKs are required. Ligand screening for the IRK using the isolated gene remains to be performed.

The spatial and temporal patterns as well as the levels of gene expression have a profound relation with the function of the protein encoded. The *IRK* gene is shown to express in root apex and inflorescence by the promoter-GUS and cRT-PCR analyses. Since auxin plays a critical role in root development and cell elongation (Doerner, 2000), we examined the effect of auxin treatment on the *IRK* gene expression. No induction of the *IRK* expression, however, was observed within 2 h after auxin treatment (data not shown). So far, we could not find any condition affecting the *IRK* expression exogenously; *IRK* might have an autonomous role in root development. The expression of *IRK* was detected in root tips, immature seeds, and vascular tissues. Similar expression patterns have been previously reported in *CycA2;1* (Burssens *et al.*, 2000) and *cyc1At* (Ferreira *et al.*, 1994), which are associated with the cell cycle, their expression tending to be present in mitotically active tissues. However, the *IRK* gene was not expressed necessarily in mitotic cells only, but also in tissues with low mitotic activity such as elongated filaments. This suggests that *IRK* has a tissue-specific function rather than a general function in cell division.

Analysis of loss-of-function mutants is very powerful to identify the role in plant development. Although knock-out mutants with T-DNA insertions in the LRR domain of *IRK* have been identified, they did not show any significant phenotype in roots and inflorescences (Kanamoto *et al.*, unpublished results). This indicates either that the function of *IRK* is not essential or that *IRK* is functionally redundant with other genes. We discovered that there is a gene that is highly homologous to *IRK* (T48210) in the *Arabidopsis* genome by a BLAST search. The value of the identity between IRK and the IRK homologue was 68% in the kinase domains and 51% in the receptor domains at amino acid sequence level (**Fig. 2D**). *IRK* encodes a protein which contains 964 amino acid residues, while the *IRK* homologue protein contains 967 amino acid residues. The presence of an intron at exactly the same position in *IRK* and the *IRK* homologue indicates that they may have evolved by gene duplication. Consideration of the redundancy in the RLK gene family would be needed for functional analysis.

Many RLK genes are supposedly involved in various physiological and developmental processes in higher plants. However, identified examples of signal transduction mediated by RLKs are limited. Systematic but well-focused approaches to uncover the functions of RLKs are desirable in functional genomics. The functional differences between RLKs must be determined by the combinations of three factors including ligand binding specificity, target protein for phosphorylation, and expression pattern of individual genes. Here we have shown that the expression of *IRK* is regulated in a tissue-dependent manner, suggesting that the function of *IRK* might be related to organ initiation or development. Further investigation of ligand or target molecules of IRK, as well as a phenotypic analysis with sophisticated constructs such as dominant-negative or constitutively active gene designs (Chang *et al.*, 1994) should reveal the function of IRK. The molecular cloning and the initial characterization of *IRK* described here provides an essential basis to analyze fundamental signaling pathways in plants.

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References

Andersson, H., von Heijne, G., 1994. Membrane protein topology: effects of $\Delta\mu H^+$ on the translocation of charged residues explain the 'positive inside' rule. EMBO J., **13**: 2267-2272.

Arabidopsis Genome Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis*

thaliana. Nature, **408**: 796-815.

Bechtold, N., Pelletier, G., 1998. In planta Agrobacterium-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol. Biol., **82**: 259-266.

Becraft, P. W., Stinard, P. S., McCarty, D. R., 1996. CRINKLY4: A TNFR-like receptor kinase involved in maize epidermal differentiation. Science, **273**: 1406-1409.

Braun, T., Schofield, P. R., Sprengel, R., 1991. Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. EMBO J, **10**: 1885-1890.

Burssens, S., de Almeida Engler, J., Beeckman, T., Richard, C., Shaul, O., Ferreira, P., Van Montagu, M., Inze, D., 2000. Developmental expression of the *Arabidopsis thaliana* CycA2;1 gene. Planta, **211**: 623-631.

Chang, C., Schaller, G. E., Patterson, S. E., Kwok, S. F., Meyerowitz, E. M., Bleecker, A. B., 1992. The TMK1 gene from Arabidopsis codes for a protein with structural and biochemical characteristics of a receptor protein kinase. Plant Cell, **4**: 1263-1271.

Chang, P. Y., Benecke, H., Le Marchand-Brustel, Y., Lawitts, J., Moller, D. E., 1994. Expression of a dominant-negative mutant human insulin receptor in the muscle of transgenic mice. J. Biol. Chem., **269**: 16034-16040.

Clark, S. E., Williams, R. W., Meyerowitz, E. M., 1997. The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell, **89**: 575-585.

Doerner, P., 2000. Root patterning: does auxin provide positional cues? Curr. Biol., **10**: 201-203.

Ferreira, P. C., Hemerly, A. S., Engler, J. D., van Montagu, M., Engler, G., Inze, D., 1994. Developmental expression of the arabidopsis cyclin gene cyc1At. Plant Cell, **6**: 1763-1774.

Gomez-Gomez, L., Bauer, Z., Boller, T., 2001. Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in Arabidopsis. Plant Cell, **13**: 1155-1163.

Hanks, S. K., Hunter, T., 1995. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J., **9**: 576-596.

Herve, C., Serres, J., Dabos, P., Canut, H., Barre, A., Rouge, P., Lescure, B., 1999. Characterization of the Arabidopsis lecRK-a genes: members of a superfamily encoding putative receptors with an extracellular domain homologous to legume lectins. Plant Mol. Biol., **39**: 671-682.

Innes, R. W., 2001. Mapping out the roles of MAP kinases in plant defense. Trends Plant Sci., **6**: 392-394.

Irish, V. F., Jenik, P. D., 2001. Cell lineage, cell signaling and the control of plant morphogenesis. Curr. Opin. Genet. Dev., **11**: 424-430.

Jefferson, R. A., Kavanagh, T. A., Bevan, M. W., 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., **6**: 3901-3907.

Jinn, T. L., Stone, J. M., Walker, J. C., 2000. HAESA, an Arabidopsis leucine-rich repeat receptor kinase, controls floral organ abscission. Genes Dev., **14**: 108-117.

Kobe, B., Deisenhofer, J., 1994. The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci., **19**: 415-421.

Li, J., Chory, J., 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Plant Cell, **90**: 929-938.

Massague, J., 1992. Receptors for the TGF-β family. Cell, **69**: 1067-1070.

Nakagawara, A., 2001. Trk receptor tyrosine kinases: a bridge between cancer and neural development. Cancer Lett., **169**: 107-114.

Nakai, K., Horton, P., 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci., **24**: 34-36.

Nasrallah, J., Nasrallah, M., 1993. Pollen-stigma signalling in the sporophytic self-incompatibility response. Plant Cell, **5**: 1325-1335.

Nishii, A., Takemura, M., Fujita, H., Shikata, M., Yokota, A., Kohchi, T., 2000. Characterization of a novel gene encoding a putative single zinc-finger protein, ZIM, expressed during the reproductive phase in *Arabidopsis thaliana*. Biosci. Biotechnol. Biochem., **64**: 1402-1409.

Raghavan, S., White, R. A., 1997. Connectin mediates adhesion in Drosophila. Neuron, **18**: 873-80.

Smyth, D. R., Bowman, J. L., Meyerowitz, E. M., 1990. Early Flower Development in *Arabidopsis*. Plant Cell, **2**: 755-767.

Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., Gardner, J., Wang, B., Zhai, W. X., Zhu, L. H., 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science, **270**: 1804-1806.

Takemura, M., Fujishige, K., Hyodo, H., Ohashi, Y., Kami, C., Nishii, A., Ohyama, K., Kohchi, T., 1999. Systematic isolation of genes expressed at low levels in inflorescence apices of *Arabidopsis thaliana*. DNA Res., **6**: 275-282.

Topping, J. F., Wei, W., Lindsey, K., 1991. Functional tagging of regulatory elements in the plant genome. Development, **112**: 1009-1019.

Torii, K. U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R. F., Komeda, Y., 1996. The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell, **8**: 735-746.

Trotochaud, A. E., Hao, T., Wu, G., Yang, Z., Clark, S. E., 1999. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a rho-related protein. Plant Cell, **11**: 393-406.

van den Berg, C. Willemsen, V., Hendriks, G., Weisbeek, P., Scheres, B., 1997. Short-range control of cell differentiation in the Arabidopsis root meristem. Nature, **390**: 287-289.

Wardlaw, C.W., 1949. Expression on organogenesis in ferns. Growth, **13**: 93-131.