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.

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Abbreviations

cRT-PCR, competitive reverse transcription-polymerase chain reaction; IRK, inflorescence and root apices receptor-like kinase; LRR, leucine-rich repeat; RLK, receptor-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 C-terminal-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 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°C. 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, Forster City, 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’-GGGTGCAG-GAACCTGATTCTCAGCTGG-3’ and 5’-CC-GCGCCGGCACAACGGATCCTCTTCGCG-3’ which had a SauII and a NotI restriction sites, respectively. The amplified DNA fragment digested with SauII and NotI 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 MnCl₂, 20 μM ATP, 10 μCi [γ-³²P]ATP (3,000 Ci mmol⁻¹) in a volume of 10 μl. The mixture was incubated for 1 h at 26°C. 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’-CCGGTACAATGTCATGGCAAAAGCAGTAGTT-3’ and 5’-CCGGATCCGGCGCCCAACCGTGAACCTACTCCTCCTTCGG-3’ which included an NcoI and an ApaI 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 bombardment as described (Nishii et al., 2000).

After overnight incubation at 22°C in the dark, fluorescence was observed by confocal laser mi-
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'-CCGTCAGCCTCTCCATTGACGATAAAC-3' and 5'-CCGGATCCAGTGCTTGTACATTTTCCC-3' primers. The amplified fragment was digested with BamHI and SalI, 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 NaPO₄, 10 mM EDTA, 0.1% (v/v) TritonX-100, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆]-3H₂O, 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 (Axioskop, ZEISS).

Competitive RT-PCR

Total RNA from different Arabidopsis tissues were prepared by using the RNasy 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 10⁴ to 10⁸ copies of DNA competitors was used for the analysis. To prevent amplification from contaminating genomic DNA, 5'-GTTCCCTCGGTCTTTTGA-TCC-3' and 5'-GCAAAATCCCAGGCTTCTTG-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 °C and 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C.

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.
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 hydrophy 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
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 (Jim 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 bombardment (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

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**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 markers. 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.

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 (Nakagawa, 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. 1. Cellular localization of the BBE protein
Ovule epidermal cells expressing BBE:GFP (A, C) and BBE::GFP (B, D)
were observed after plastid cal with 10% glycerol. Confocal laser microscopy
(A, B) and light field microscopy (C, D). Bars = 50 μm.

Fig. 3. Promoter activity of BBE during plant development
(A) 8-day-old seedling, (B) root cap of 8-day-old seedling, (C) emerging lateral root of 9-day
old plant, (D) 22-day-old plant, (E) freeze-dried mature seed observed under a dark field epi-
scopic microscope, the arrowheads indicate a capsulium. (F) Flowers (callicarpa) in stage 9. (G)
Stage 10 flower. (H) O. sativa stage 10 flower and carpel development of flower from stage 11 to stage 15. (I) BB and GUS staining was performed for
BB::GUS (A-G) or GUS (H-I), and (J, K, L, M, N, O, P) in (A) and (B). Bars = 100 μm in (J, K, L, M, N, O, P) and (B). Bars = 5 mm.
genes have been characterized so far: **CLAVATA1**, **ERECTA** and **HAESA** for developmental processes, **FLS2** in transducing pathogen signals, and **BRI1** 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 (Trotchaud et al., 1999) and **fgr2** 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 **cycA1** (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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