Identification of Three Clones Which Commonly Interact with the Kinase Domains of Highly Homologous Two Receptor-Like Kinases, RLK902 and RKL1

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We have previously reported the characterization of highly homologous two leucine-rich repeat (LRR)–receptor-like kinase (RLK) genes, RLK902 and RKL1, which showed 75% identity at the amino acid sequence level. To investigate the RLK902 and RKL1 mediated signal transduction pathways, we performed yeast two-hybrid screening using the kinase domains of RLK902 and RKL1 as baits. Three clones, Y-1, 2, and 3, were found to interact commonly with the kinase domain of RLK902 and RKL1 and not to interact with the kinase domain of BRI1, a member of LRR–RLKs. This result suggests that RLK902 and RKL1 may have common biochemical functions, especially in their downstream signal transduction. Furthermore, the detailed analysis of their responsiveness to various conditions suggests their involvement in such stress conditions as mechanical wounding, treatment with salicylic acid, and pathogen infection.

Key words: receptor-like kinase; W-box; wounding; salicylic acid; pathogen

Plant receptor-like kinase (RLK) genes have been identified from a variety of plant species. In Arabidopsis, more than 400 genes are predicted to encode RLKs.1,2) Functional studies using transgenic approaches or mutant analyses have shown that RLKs play diverse physiological roles, including control of shoot meristem maintenance by CLAVATA1 of Arabidopsis,3) mediation of the brassinosteroid signal transduction pathway by BRI1/BAK1 of Arabidopsis,4–6) recognition of bacterial pathogens by Xa21 of rice,7) and control of the female determinant of self-incompatibility by SRK of Brassica,8) but very little is known about the signal transduction pathways of most RLKs.

To understand the signal transduction cascade mediated by RLK, it is essential to identify its downstream components. The yeast two-hybrid system has been used successfully to identify cytosolic proteins that interact with the kinase domain of several RLKs. For example, THL1, THL2 (Thioredoxin-h-like 1 and 2) and ARC1 (ARM REPEAT CONTAINING1) have been identified as proteins which interact with the kinase domain of SRK.9,10) THL1 and THL2 have been shown to act as negative regulators of SRK, since interaction of these proteins inhibits the kinase activity of SRK.11) ARC1 might be a positive effector of self-incompatibility signaling, because reducing levels of ARC1 transcripts showed a partial breakdown of self-incompatibility.12)

Previously we reported the characterization of the two RLKs, RLK902 and RKL1, which are most similar to each other in Arabidopsis.13) RLK902 and RKL1 showed 75% amino acid sequence identity over their entire regions. The kinase domain was the most highly conserved between these two RLKs, exhibiting 82% identity. RLK902 and RKL1 belong to the LRR III subfamily. Using the promoter::GUS transgenic lines, RLK902 was expressed strongly in root tips, lateral root primordia, stipules, and floral organ abscission zones, while RKL1 was expressed dominantly in the stomata cells, hydathodes, and trichomes of young rosette leaves, and floral organ abscission zones. Since neither the rlk902 mutant, the rkl1 mutant nor the rlk902/rkl1 double mutant showed any obvious phenotypes under normal growth conditions, it is hard to speculate as to biological functions of RLK902 and RKL1.

To gain a better understanding of RLK902 and RKL1 mediated signal transduction pathways and to examine the functional redundancy of these proteins, the yeast two-hybrid system was used to isolate proteins interacting with the kinase domains of these two RLKs. In this report, we describe the isolation and characterization of Y-1, 2, and 3, which commonly bind to the kinase domains of RLK902 and RKL1.

Abbreviations: LRR, leucine-rich repeat; Psm, Pseudomonas syringae pv. maculicola; RLK, receptor-like kinase; RT-PCR, reverse transcription-polymerase chain reaction; SA, salicylic acid

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Materials and Methods

Plant materials. Arabidopsis thaliana ecotype Columbia was used for all the experiments described in this report. The plants were grown under continuous light at 23°C. The aerial parts were obtained from plants grown on rock wool and roots from plants grown on an MS-agar (0.8%) medium. The wounding treatment on the rosette leaves was done by making 15–20 holes per leaf with a pin. Salicylic acid (SA) treatment was performed by spraying 3-week-old plants with 2 mM SA dissolved with a pin. Salicylic acid (SA) treatment was performed by spraying 3-week-old plants with 2 mM SA dissolved in water and adjusted to pH 6.5 with KOH. The experiments with pathogen infection involved infecting Arabidopsis leaves with the *Pseudomonas syringae* pv. *maculola* (Psm) strain or Psm strain expressing the avirulent gene (Rpt2), according to the method of Yoshioka et al.14) For all the experiments with the above stress conditions, rosette leaves were used to prepare total RNA.

Autophosphorylation assay. The kinase domains of RLK902 and RKL1 were amplified by PCR using primers as follows; sense for RLK902, 5'-GGAGATCT-(BglII)GAGAGAACAAGGGCCATTGACC-3'; antisense for RLK902, 5'-GGGAATTCC(EcoRI)TTACCC-ACCCGATCTGCACC-3'; sense for RKL1, 5'-GGGGATCC(BamHI)GCTGTTGATATCTCAAC-3'; antisense for RKL1, 5'-GGGAATTCC(EcoRI)ATCAGC-TCGTCAC-3'. The amplified fragments were cloned into the EcoRV site of pBluescript SK(+). After the clones with no sequence error were selected, the fragments of kinase domain of RLK902 excised with BglII and EcoRI and RKL1 excised with BamHI and EcoRI were cloned into BamHI/EcoRI sites of pGEX4T-2 (Amersham Biosciences). The kinase domains of RLK902 and RKL1 were amplified by PCR using primers as follows: sense for RLK902, 5'-GGGAATTCC(EcoRI)TTACCC-ACCCGATCTGCACC-3'; sense for RKL1, 5'-GGGGATCC(BamHI)GCTGTTGATATCTCAAC-3'; antisense for RKL1, 5'-GGGAATTCC(EcoRI)ATCAGC-TCGTCAC-3'. The amplified fragments were cloned into the EcoRV site of pBluescript SK(+). After the clones with no sequence error were selected, the fragments of kinase domain of RLK902 excised with BglII and EcoRI and RKL1 excised with BamHI and EcoRI were cloned into BamHI/EcoRI sites of pGEX4T-2 (Amersham Biosciences). The kinase domain of RLK902 and RKL1 fused to GST were expressed in *E. coli* (Amersham Biosciences). The clones with no sequence error were selected, the fragments of kinase domain of RLK902 excised with BglII and EcoRI and RKL1 excised with BamHI and EcoRI were cloned into BamHI/EcoRI sites of pGEX4T-2 (Amersham Biosciences). The kinase domain of RLK902 and RKL1 fused to GST was expressed in *E. coli* and purified with glutathione-Sepharose 4B according to the manufacturer’s protocol (Amersham). Autophosphorylation activity was assayed by incubating 7.5 µg of fusion protein in a 15-µl reaction mixture containing 50 mM Hepes, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, and 10 µCi [γ-³²P]ATP for 2 h at room temperature. The reaction products were resolved by SDS–PAGE and visualized by autoradiography.

Yeast two-hybrid screening. To produce bait constructs, the kinase domains of RLK902, RKL1, and BR1 were amplified by PCR using primers as follows: sense for RLK902, 5'-GGGAATTCC(EcoRI)GAGAGAACAAGGGCCATTGACC-3'; antisense for RLK902, 5'-GACCTCGAG(XhoI)TTACCCACCCGATCTGCACC-3'; sense for RKL1: 5'-GGGAATTCC(EcoRI)GCTGTTGATATCTCAAC-3'; antisense for RKL1: 5'-AATTCGAG(SalI)TTAATCAGGTTGATATCTCAAC-3'; sense for BR1: 5'-GGGAATTCC(EcoRI)ATGTTAGGCGAAGCATGGAAC-3'; antisense for BR1: 5'-GGTTGCGAC(SalI)TCAATAATTCCCTTCAGGAC-3'. The amplified fragments were cloned into the EcoRV site of pBluescript SK(+). After those clones with no sequence error were selected, the fragments of RLK902 excised with EcoRI and XhoI, RKL1 and BR1 excised with EcoRI and SalI were cloned into the EcoRI/SalI sites of the GAL4-binding domain vector pBD-GAL4 Cam (Stratagene), giving plasmid pBD-902, pBD-001, and pBD-BRI1 respectively. An Arabidopsis cDNA library was provided by the Arabidopsis Biological Resource Centre. The yeast strain AH109 (Clontech) was used. Yeast transformation was carried out according to a modified lithium acetate method. Yeast transformants that produced interacting proteins were selected by plating on synthetic dropout medium without Leu, Trp, or His. Assay for α-galactosidase activity was performed according to the manufacturer’s protocol (Matchmaker Gal4 two-hybrid system 3 and libraries; Clontech Laboratory).

Expression analysis of RLK902, RKL1, Y-1, 2, and 3 by RT-PCR. RT-PCR was run on single-strand cDNA that had been synthesized by reverse transcription with AMV reverse transcriptase (Promega) and 1 µg of DNase I-treated total RNA according to the manufacturer’s protocol. PCR reactions with the RT mixture were carried out under the following conditions: 1 cycle of denaturing at 94°C for 5 min, 28 cycles for RLK902, 27 cycles for RKL1, 33 or 31 cycles for Y-1, 27 cycles for Y-2, 30 cycles for Y-3, and 25 cycles for AAC1 of denaturing at 94°C for 12 s, annealing at 55°C for 12 s and extension at 72°C for 15 s. The primers used for the RT-PCR analysis were as follows: sense for RLK902, 5'-GGAATGAGGTTTTTGACTCGGAGC-3'; antisense for RLK902, 5'-TTACCCCAACCGATCTGCACC-3'; sense for RKL1, 5'-GGAGAGAAGTTTTCGACTCGGAG-3'; antisense for RKL1, 5'-GGCCCGGGTTAAT-CAGCAGTTCGTCAC-3'; sense for Y-1, 5'-GAGGATC-CAATGGACGAGCAGACGTC-3'; antisense for Y-1, 5'-CTCAAGATACACAAATGTCACATTAC-3'; sense for Y-2, 5'-GGCCGATCTGTTGATCTGCACC-3'; antisense for Y-2, 5'-GGCCCGGGTATGGTTGCTTC-3'; antisense for Y-3, 5'-GTCTGCGATCTGCTTCACCACCGCTGC-3'; for AAC1, 5'-TCCATAATGAACTGTTGATG-3'; and antisense for AAC1, 5'-GGACCTGACTGCT-CATACTC-3'.

Results

Autophosphorylation of the kinase domains of RLK902 and RKL1

To clarify whether RLK902 and RKL1 are functional receptor kinases, their kinase activities were examined. Kinase domains of RLK902 and RKL1 fused to glutathione S-transferase were expressed in *E. coli*, and the affinity-purified proteins were incubated with radiolabeled-ATP. Phosphorylated radioactive bands,
with molecular masses of GST:RLK902 and GST:RKL1, were observed (Fig. 1). This result indicated that RLK902 and RKL1 are functional protein kinases capable of autophosphorylation.

**Screening of proteins that interact with the kinase domain of RLK902 or RKL1**

To identify proteins that interact with the RLK902 kinase domain (RLK902-KD) or the RKL1 kinase domain (RKL1-KD) and thus may be involved in the downstream events of the RLK902 or RKL1 mediated signaling pathways, yeast two-hybrid screening of the Arabidopsis cDNA library was performed using RLK902-KD or RKL1-KD as a bait. The N-terminal amino acid of the region used as a bait was 7- and 9-amino acids downstream from the C-terminal end of the transmembrane domain for RLK902 and RKL1 respectively, and about 70-amino acids upstream of the conserved kinase domains for both RLKs. Thus the bait fragments covered almost the entire cytoplasmic regions. By the first screening, using the His-requirement index, about 800 and 450 colonies were obtained using RLK902-KD and RKL1-KD respectively from about $3.5 \times 10^6$ independent yeast clones. Of the selected colonies, about 500 and 200 clones survived from the second screening by detecting $\alpha$-Gal activity. Reproduction of plasmids from 160 and 48 clones into yeasts gave 133 and 34 reproducible positive results for the assays using RLK902-KD and RKL1-KD respectively. By sequencing 40 clones and 34 clones of the respective selected clones, five independent clones (At5g05190, At3g27210, At3g51310, and At5g19140) and four independent clones (At5g05190, At3g27210, At3g17950, and At2g37480) were identified as in-frame fused to the GAL1 activation domain in the right direction. Since we have been interested in the common biochemical functions of RLK902 and RKL1 based on the high homology of their kinase domains (82% amino acid identity), we focused on three clones obtained by both screenings; Y-1 (At5g05190, 4 and 10 independent clones were obtained using RLK902-KD and RKL1-KD respectively), Y-2 (At3g27210, 10 and 3 independent clones were obtained using RLK902-KD and RKL1-KD respectively), and Y-3 (At5g17950, 5 and 2 independent clones were obtained using RLK902-KD and RKL1-KD respectively). To demonstrate the specific interaction of these protein fragments with RLK902 and RKL1, the similar region (most of the cytoplasmic region) of BRI1, a brassinosteroid receptor belonging to the LRR X subfamily, was used as a negative control. These clones showed activation of both reporter genes ($HIS3$ and $MEL1$) in the presence of RLK902-KD or RKL1-KD, but not of BRI1-KD, indicating common but specific interaction with RLK902-KD and RKL1-KD (Fig. 2). These results suggest that Y-1, 2, and 3 might be common components involved in the signal transduction pathways of RLK902 or RKL1.

**Fig. 1.** Autophosphorylation Assay of the Kinase Domains of RLK902 and RKL1.

The kinase domains fused to GST (GST:RLK902 and GST:RKL1) purified from E. coli cell extract were examined for autophosphorylation activities. Arrows indicate the molecular sizes corresponding to GST:RLK902 (lane 1) and GST:RKL1 (lane 2).

**Fig. 2.** Isolation of Proteins Interacting with the Kinase Domains of RLK902 and RKL1.

Y-1, 2, and 3 were tested for interactions with the kinase domains of RLK902, RKL1, and BRI1. Interactions between proteins were assayed by growth on selective plates lacking histidine (activation of the $HIS3$ reporter gene) and $\alpha$-galactosidase activity (activation of the $MEL1$ reporter gene).
Y-1 encoded a 615-amino acid peptide without any annotation. The protein sequence showed no special property, including any signal sequences and apparent motifs. Y-2 was a 234-amino acid peptide containing a N-terminal myristoylation site, a Ser-rich region, and a putative nuclear localization signal. Y-3 encoded a 211-amino acid peptide containing a putative nuclear localization signal. A blast search, however, gave no information on the physiological functions of either Y-2 or Y-3.

Expression patterns of Y-1, 2, and 3
Since two proteins must be expressed in the same places for interaction, the gene expression patterns of Y-1, 2, and 3 were compared with those of RLK902 and RKL1. As shown in Fig. 3, expression of Y-1 was detected in inflorescence stems and rosette leaves, weakly in inflorescence, but not in the roots. Expression of Y-2 was detected in inflorescence stems, rosette leaves, and roots, and weakly in inflorescence. Expression of Y-3 was detected in inflorescence, inflorescence stems, and rosette leaves, and weakly in roots. Since RLK902 and RKL1 were expressed highly in inflorescence, moderately in roots, and at low level in rosette leaves and inflorescence stems,13) these results suggest that Y-1 might not be the main component of the RLK902 or RKL1 mediated pathway, and that Y-2 might interact with RLK902 or RKL1 mainly in roots, and Y-3 mainly in inflorescence. Since the spatial patterns of promoter activities were quite different between those of RLK902 and RKL1 although their rough expression patterns, shown by Northern hybridization, were similar, detailed analysis of the expression sites of Y-1, 2, and 3 is necessary to determine in which signaling pathways Y-1, 2, and 3 are involved, the RLK902 mediated pathway, the RKL1 mediated pathway, or both.

Transcriptional regulation of RLK902, RKL1, and Y-1, 2, and 3 by environmental stress
To speculate as to the possible environmental factors which regulate the transcription of RLK902, RKL1, and Y-1, 2, and 3, cis-elements were searched and the W-box sequence (TGACC/T) was identified in the promoter region of these genes (Fig. 4). This W-box sequence was recognized by WRKY transcription factors that have been implicated in the regulation of such plant processes as wound response, pathogen defense, and senescence.15–20) Since a single W-box in a promoter region is sometimes sufficient to mediate WRKY-dependent gene expression, we analyzed the response to mechanical wounding, salicylic acid, and pathogen attack by RT-PCR. To examine the response to mechanical wounding, 3-week-old Arabidopsis rosette leaves were wounded by making holes with a pin. As shown in Fig. 5A, the expression of RLK902 increased during the first 30 min, decreased up to 1 h, and then increased again up to 3 h, the expression of Y-1 and 2 increased during the first 30 min and then decreased to the original level within 3 h, and the expression of RKL1 and Y-3 decreased during the first 30 min and then increased to the original level within 3 h. When rosette leaves were treated with 2 mM SA, the expression of RLK902, RKL1, and Y-3 in rosette leaves decreased during the first 30 min and then recovered to the original level within 3 h, while the expression of Y-1 and 2 first increased and then decreased to the original level (Fig. 5B). To examine their responses to pathogen infection, 3-week-old Arabidopsis plants were infected with a virulent Pseudomonas syringae pv. maculicola (Psm) strain or with a Psm strain expressing the
avirulent \textit{avrRpt2} gene (Rpt2) that triggers a hypersensitive reaction (HR). After infection by these pathogens, the expression of RLK902, RKL1, and Y-3 in rosette leaves decreased, while Y-1 and 2 increased during the first 8 h and subsequently recovered, regardless of the presence or absence of the avirulent gene (Fig. 5C). This result indicates that the transient change in the message levels was caused by the infection process in an HR-independent manner. In summary of the stress responses, the expression of Y-1 and Y-2 was transiently and positively regulated by all the stress responses, the expression of Y-1 was increased by wounding and decreased by SA treatment and pathogen attack. If it is assumed that these responses are regulated by the single W-box in the RLK902 promoter region and by binding of the WRKY factors to the element, this result can be explained by two types of WRKY factor, one induced by wounding, pathogen infection, and treatment with a pathogen elicitor or SA. Although in many cases WRKY factors act as positive transcriptional regulators, a negative function for WRKY factors has been reported. The increased expression of Y-1 and Y-2 by wounding, SA treatment, and pathogen attack suggests that WRKY factor(s) might positively regulate the expression of Y-1 and 2 through binding to the W-box, and the decreased expression of RKL1 and Y-3 suggests that WRKY factor(s) might negatively regulate the expression of RKL1 and Y-3. On the other hand, the expression of RLK902 was increased by wounding and decreased by SA treatment and pathogen attack. If it is assumed that these responses are regulated by the single W-box in the RLK902 promoter region and by binding of the WRKY factors to the element, this result can be explained by two types of WRKY factor, one induced by wounding, which acts as a positive regulator and the other induced by an SA treatment or pathogen attack, which acts as a negative regulator for the expression of RLK902. A promoter analysis with mutated W-boxes should reveal their involvement in the stress response.

Since Y-2 and Y-3 contained a putative nuclear localization signal, we examined whether the subcellular localizations of Y-2 and Y-3 were regulated by RLK902 or RKL1. Constructs expressing fusion proteins with GFP under the control of CaMV 35S promoter were introduced into onion epidermal cells or Arabidopsis rosette leaf cells by particle bombardment. With or without co-expression with RLK902 or RKL1, Y-2::GFP was distributed both in the nucleus and the cytoplasm, while the localization of Y-3::GFP was inconsistent, in some cases localized in the nucleus and in others distributed both in the nucleus and the cytoplasm (data not shown). These results suggest that the localization of

**Discussion**

Receptor-like kinases (RLKs) play important roles in detecting and transducing extracellular signals in plant development, but little is known about the molecular mechanisms of RLK signaling pathways. To understand the signaling mechanisms, we investigated the components of RLK902- and RKL1-mediated signal transduction pathways using the yeast two-hybrid system and obtained three clones, Y-1, 2, and 3, which interacted commonly with the kinase domains of RLK902 and RKL1.

By expression analysis, Y-2 showed a clear overlap of expression sites with RLK902 and RKL1 in roots and Y-3 in inflorescence, although Y-1 did not show clear overlaps. These results suggest that Y-2 mediates signals from RLK902 or RKL1 mainly in roots, and Y-3 in inflorescence. Since Y-1, 2, and 3 were also expressed in rosette leaves and inflorescence stems where RLK902 and RKL1 were very weakly expressed, they might not be the only components of RLK902- and RKL1-mediated signaling pathways.

W-box sequences, which could be recognized by WRKY transcriptional factors, were found in the promoter regions of all the genes focused on in this study, RLK902, RKL1, and Y-1, 2 and 3. A number of genes encoding WRKY factors have been isolated from several plants, including some that were rapidly induced by wounding, pathogen infection, and treatment with a pathogen elicitor or SA. Although in many cases WRKY factors act as positive transcriptional regulators, a negative function for WRKY factors has been reported. The increased expression of Y-1 and Y-2 by wounding, SA treatment, and pathogen attack suggests that WRKY factor(s) might positively regulate the expression of Y-1 and 2 through binding to the W-box, and the decreased expression of RKL1 and Y-3 suggests that WRKY factor(s) might negatively regulate the expression of RKL1 and Y-3. On the other hand, the expression of RLK902 was increased by wounding and decreased by SA treatment and pathogen attack. If it is assumed that these responses are regulated by the single W-box in the RLK902 promoter region and by binding of the WRKY factors to the element, this result can be explained by two types of WRKY factor, one induced by wounding, which acts as a positive regulator and the other induced by an SA treatment or pathogen attack, which acts as a negative regulator for the expression of RLK902. A promoter analysis with mutated W-boxes should reveal their involvement in the stress response.
Y-2 and Y-3 is distinctly regulated by factors other than or in addition to RLK902 or RLK1. The inconsistent result as to Y-3 localization may indicate that its localization is affected by subtle environmental differences from experiment to experiment. Subcellular localization analysis using stable transformants expressing Y-2:GFP and Y-3:GFP with a background of wild-type and rlk902/rlkl double knockout mutant might be helpful to examine the relationships of these proteins under various growth conditions.

Although confirming data have not been obtained for the localization of Y-3, the result that two RLKs have no effect on the localization of Y-2 and Y-3 suggests that Y-2 and Y-3 are not the substrates of those kinases. In addition, we have negative results that the recombinant RLKs which showed autophosphorylation activity (Fig. 1) did not phosphorylate Y-1, 2, or 3, and that the kinase-dead mutant protein of RLK902-KD (K393E) showed interaction with Y-1, 2, and 3 in a yeast two-hybrid system (data not shown). These results support the possibility that these proteins regulate RLK-mediated signal transductions either negatively or positively as a component of the signaling complex. Taking into account that the transcript levels of Y1, 2, and 3 change under various stress treatments, regulation might be controlled at the transcriptional level. Further characterization of each gene, including detailed expression properties and identification of the phosphorylation substrates, will be needed to examine this possibility.

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References


