Two Distinct Forms of M-Locus Protein Kinase Localize to the Plasma Membrane and Interact Directly with S-Locus Receptor Kinase to Transduce Self-Incompatibility Signaling in *Brassica rapa* \(^\text{a)**}

Mitsuru Kakita,a,1 Kohji Murase,a,1,2 Megumi Iwano,a Tomohito Matsumoto,a,3 Masao Watanabe,b Hiroshi Shiba,a Akira Isogai,a and Seiji Takayamaa,4

a Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0192, Japan
b Graduate School of Life Sciences, Tohoku University, Aoba 980-8577, Japan

Many flowering plants possess systems of self-incompatibility (SI) to prevent inbreeding. In *Brassica*, SI recognition is controlled by the multiallelic gene complex (S-haplotypes) at the S-locus, which encodes both the male determinant S-locus protein 11 (SP11/SCR) and the female determinant S-receptor kinase (SRK). Upon self-pollination, the S-hapalotype–specific interaction between the pollen-borne SP11 and the cognate stigmatic SRK receptor induces SI signaling in the stigmatic papilla cell and results in rejection of the self-pollen. Our genetic analysis of a self-compatible mutant revealed the involvement of a cytoplasmic protein kinase, M-locus protein kinase (MLPK), in the SI signaling, but its exact physiological function remains unknown. In this study, we identified two different MLPK transcripts, MLPKf1 and MLPKf2, which are produced using alternative transcriptional initiation sites and encode two isoforms that differ only at the N termini. While MLPKf1 and MLPKf2 exhibited distinct expression profiles, both were expressed in papilla cells. MLPKf1 localizes to the plasma membrane through its N-terminal myristoylation motif, while MLPKf2 localizes to the plasma membrane through its N-terminal hydrophobic region. Although both MLPKf1 and MLPKf2 could independently complement the mlpk/mlpk mutation, their mutant forms that lack the plasma membrane localization motifs failed to complement the mutation. Furthermore, a bimolecular fluorescence complementation assay revealed direct interactions between SRK and the MLPK isoforms in planta. These results suggest that MLPK isoforms localize to the papilla cell membrane and interact directly with SRK to transduce SI signaling.

INTRODUCTION

Self-incompatibility (SI) is a genetic system present in flowering plants that promotes outbreeding by rejecting self-pollen (de Nettancourt, 2001; Takayama and Isogai, 2005). SI is based on the discrimination of self and non-self between pollen and pistil, followed by selective inhibition of self-pollen germination and/or growth. In *Brassica*, SI recognition is controlled by a multiallelic gene complex (termed the S-haploptype; S1, S2, - , Sn) at a single locus, termed the S-locus. More than 100 S-haplotypes are thought to be present in certain *Brassica* species (Nou et al., 1993).

Genomic analyses of the *Brassica* S-locus revealed the presence of three multiallelic genes: S-receptor kinase (SRK) (Stein et al., 1991), S-locus protein 11 (SP11; also called S-locus Cys rich [SCR]) (Schopfer et al., 1999; Suzuki et al., 1999), and S-locus glycoprotein (SLG) (Nasrallah et al., 1987; Takayama et al., 1987). SRK encodes a membrane-spanning Ser/Thr receptor kinase that localizes to the papilla cell membrane and functions as the sole determinant of the SI phenotype of the stigma (Takasaki et al., 2000). SP11 encodes a small Cys-rich protein that localizes to the pollen coat and functions as the sole determinant of the SI phenotype of the pollen (Schopfer et al., 1999; Takayama et al., 2000; Shiba et al., 2001). SLG encodes a secreted glycoprotein that shares significant sequence similarity with the extracellular domain of SRK and is abundant in the papilla cell wall. Transformation experiments suggested that SLG is not involved directly in SI phenotype determination, and its physiological role remains controversial (Takasaki et al., 2000; Silva et al., 2001). Biochemical studies have clearly shown that SP11 is a ligand for SRK, and the specificity of the SI response can now be explained by the S-hapalotype–specific interactions between the SP11 ligand and the SRK receptor (Kachroo et al., 2001; Takayama et al., 2001). During a self-pollination event, the pollen-borne SP11 ligand binds specifically to the extracellular domain of its cognate self SRK and activates its kinase domain...
Identification of Alternative Transcriptional Isoforms of MLPK

In our previous work (Murase et al., 2004), we obtained the MLPK cDNA sequence, which covers the entire coding region, by RT-PCR using primers based on the cDNA sequence of APK1b, a putative Arabidopsis ortholog of MLPK (Hirayama and Oka, 1992). To further characterize the MLPK transcript, we performed 5' rapid amplification of the cDNA ends (5' RACE) with stigmatic cDNA. The 5' RACE analysis revealed two isoforms of the MLPK gene product expressed in the stigma (Figure 1). One, designated MLPK form 1 (MLPKf1), is the transcript previously reported (Murase et al., 2004), which consists of eight exons encoding a protein of 404 amino acids (Figures 1A and 1B). The other, designated MLPK form 2 (MLPKf2), is an alternative transcript produced using an alternative transcription initiation site located in the third intron of the MLPKf1 gene. The third intron also contains a potential translation start codon followed by a nucleotide sequence encoding an alternative N-terminal 23–amino acid sequence of MLPKf2 fused in frame to the codon for Gly-18 of MLPKf1 (Figure 1A). Thus, the MLPKf2 transcript encodes a protein of 410 amino acids that differs from the MLPKf1 isoform only at the N terminus.

In the 5' RACE analysis using stigmatic cDNA, MLPKf2 was the major amplification product (MLPKf2, 26 clones; MLPKf1, 2 clones). To confirm that MLPKf2 was the major transcript expressed in the stigma, we screened stigmatic cDNA libraries by plaque or colony hybridization. Of the 10 independent cDNA clones we obtained, four clones contained the short fragments common to both isoforms, but the other six contained the longer fragments corresponding to MLPKf2 isoform. These data suggest that MLPKf2, rather than MLPKf1, is the major isoform transcribed in the stigma.

Tissue-Specific Expression of MLPK Isoforms

Previous RNA gel blot analyses suggested that MLPK was predominantly expressed in the stigma but that low expression levels were observed in other tissues (Murase et al., 2004). Although we could not distinguish the two MLPK isoforms by RNA gel blot analysis because they have similar molecular sizes, the 5' RACE analysis suggested that the major isoform expressed in the stigma was MLPKf2. To confirm this finding, we performed RT-PCR analysis using isoform-specific primers. As expected, strong and specific expression of MLPKf2 was observed in the stigma (Figure 2A). On the other hand, weak and divergent expression of the MLPKf1 isoform was observed in stigma, stem, and leaf but not in anther. To compare the expression levels of these transcripts in more detail, we performed real-time RT-PCR analysis on stigmas collected at various developmental stages (Figure 2B). The expression levels of both MLPKf2 and MLPKf1 transcripts increased with stigma development and reached maximum levels at anthesis. However, the MLPKf2 transcript is ~10-fold more abundant than that of MLPKf1 throughout the various developmental stages of the stigma, while MLPKf2 was less abundant than MLPKf1 (~25%) in leaf tissue.

RESULTS

Identification of Alternative Transcriptional Isoforms of MLPK

We have recently identified another positive mediator of SI signaling by genetically analyzing a spontaneous self-compatible mutant of Brassica rapa var Yellow Sarson (Murase et al., 2004). Yellow Sarcon has a recessive mutation in the modifier (m) gene, which completely eliminates the SI response in the stigma. Positional cloning of the m gene revealed that it encodes a cytoplasmic Ser/Thr protein kinase, designated M-locus protein kinase (MLPK). The mutant form of mlpk is a newly identified transcript produced by an alternative transcription initiation site located in the third intron of the MLPKf1 gene, which completely eliminates the SI response in the stigma. Although both MLPK isoforms localized to the stigma, leaf, and stem. Although both MLPK isoforms localized to the plasma membrane, the localization of MLPKf1 is myristoylation dependent, while that of MLPKf2 is dependent upon its specific N-terminal hydrophobic domain. Transient expression of MLPKf2, as well as that of MLPKf1, complemented the mlpk/mlpk mutation and restored the SI phenotype in mutated papilla cells, but the MLPK mutants that lack the plasma membrane localization motifs failed to complement the mlpk/mlpk mutation. Furthermore, a bimolecular fluorescence complementation (BiFC) assay in plant protoplasts revealed a direct interaction between SRK and the MLPK isoforms. These results suggested that MLPK isoforms localize to the papilla cell membrane and interact directly with SRK to transduce SI signaling.
To further confirm that these isoforms are expressed in the papilla cell of stigma, where the SI recognition event occurs, we conducted an in situ hybridization analysis (Figure 2C). The MLPKf2 antisense oligo probe gave an intense signal in the papilla cells and the transmitting tissue. Weak but significant signal was also observed in the papilla cells by the MLPKf1 antisense oligo probe. These results suggested that both MLPKf2 and MLPKf1 are actually expressed in the stigmatic papilla cells, where SI signaling is transduced.

**Figure 1.** Gene Structures of Brassica MLPK and Arabidopsis APK1b.

(A) Nucleotide and deduced amino acid sequences of the alternative transcription region of the MLPK gene. Genome sequence was obtained by sequencing the BAC251-22 clone (Murase et al., 2004). Exon/intron structure was deduced by comparing the genome sequence and the cDNA sequence. The 5’ end of MLPKf2 was determined by sequencing MLPKf2 clones obtained from a stigmatic full-length cDNA library. The 5’ end of MLPKf1 was predicted by two independent 5’ RACE reactions. Putative TATA boxes are denoted by double underlined letters. The predicted N-terminal amino acid sequences of MLPKf1 and MLPKf2 are indicated by arrows, and the nucleotide sequences of the MLPKf1 and MLPKf2 transcripts are shaded in dark and light gray, respectively, and their common nucleotide sequence is underlined. The putative initiation sites (ATG) are shaded in black.

(B) Exon-intron structures of MLPKf1 and MLPKf2.

(C) Exon-intron structures of APK1bf1 and APK1bf2. The exons are indicated by thick horizontal lines, and the introns are indicated by dips. Coding regions are indicated by boxes, and the putative initiation sites are indicated by ATG.

To further confirm that these isoforms are expressed in the papilla cell of stigma, where the SI recognition event occurs, we conducted an in situ hybridization analysis (Figure 2C). The MLPKf2 antisense oligo probe gave an intense signal in the papilla cells and the transmitting tissue. Weak but significant signal was also observed in the papilla cells by the MLPKf1 antisense oligo probe. These results suggested that both MLPKf2 and MLPKf1 are actually expressed in the stigmatic papilla cells, where SI signaling is transduced.

APK1b is thought to be an Arabidopsis ortholog of MLPK based on the highest sequence similarity (76% amino acid identity) and genomic synteny between their loci. APK1b has a genomic structure similar to MLPK, and the protein-coding region was predicted to be contained within six exons as MLPKf1 (named APK1bf1). The genomic sequence analysis suggested that the first intron of APK1bf1 contains an MLPK-like potential translation start codon, suggesting the presence of an alternative transcript like MLPKf2 (named APK1bf2). To test this possibility,
we performed RT-PCR analysis and found that both isoforms are transcribed (Figure 1C). RT-PCR analysis with isoform-specific primers suggested that the APK1bf2 transcript was predominantly expressed in stigma, as found for MLPKf2 (Figure 2A), and also suggested that APK1bf1 was widely expressed in various tissues, similar to MLPKf1. These results suggested that the molecular mechanisms that produce these two alternative MLPK (or APK1b) transcripts and regulate their expression patterns are conserved in the genera of Brassica and Arabidopsis.

**The MLPK Isoforms Localize to the Plasma Membrane by Different Mechanisms**

Previous biochemical studies using a polyclonal antibody raised against the full-length recombinant MLPKf1 protein have shown that native MLPK protein is localized to the plasma membrane fraction but not to the soluble fraction of stigma extracts (Murase et al., 2004). Considering that two MLPK isoforms are expressed in the stigma, these previous studies suggested that both MLPK isoforms are localized to the plasma membrane.

MLPKf1 has a typical plant myristoylation consensus sequence, M-G-X(not EDFKRVWY)-X-X-[STACFGRV]-X(not DE), at its N terminus (Figure 3A) (Hanks and Quinn, 1991; Boisson et al., 2003). This motif was also found in the APK1bf1 isoform and in closely related RLCKVII subfamily members, including APK1a, APK2a, and NAK (Figure 3B). By contrast, MLPKf2 and APK1bf2 lack the typical myristoylation motif, in which the third Phe and sixth Lys residues do not match the consensus sequence (Figure 3A). However, the second Gly, the attachment site of the myristoyl residue, is retained (Figure 3A).

To test whether the motif in MLPKf1 actually functions as a myristoylation site and that the motif in MLPKf2 does not, in vitro transcription and translation experiments were performed in the presence of 3H-labeled myristic acid (Figure 3C). Consistent with the prediction, myristic acid was incorporated into MLPKf1 but not into MLPKf2 (Figure 3C). Furthermore, when we translated the mutant form of MLPKf1G2A, in which the second Gly of MLPKf1 was changed to Ala, the incorporation of myristic acid was not observed (Figure 3C). These results clearly indicate that MLPKf1, but not MLPKf2, is myristoylated at its N-terminal Gly site.

To analyze the relationship between myristoylation and subcellular localization, we transiently expressed green fluorescent protein (GFP) fusions of these MLPK isoforms in protoplasts...
prepared from tobacco (Nicotiana tabacum) BY-2 cells (Figure 4). Consistent with our previous observations, the fluorescent signal of the MLPKf1-GFP fusion protein was detected predominantly at the plasma membrane, as was that of the SRK-GFP fusion protein. However, the fusion protein of the MLPKf1G2A mutant form (MLPKf1G2A-GFP) localized around the nucleus and cytosol similar to free GFP (control). These results suggested that MLPKf1 localizes to the plasma membrane in a myristoylation-dependent manner. The fusion of the MLPKf2 isoform (MLPKf2-GFP) also exhibited the plasma membrane localization as with MLPKf1 isoform, but its localization was not affected by the mutation at the second Gly, and the fluorescent signal of MLPKf2G2A-GFP was detected at the plasma membrane. These results suggested that MLPKf2 also localizes to the plasma membrane but in a myristoylation-independent manner.

The plasma membrane localization of MLPKf2 must depend on its specific N-terminal amino acid sequence. Although no typical membrane localization motif was found in MLPKf2, its hydropathy profile indicates hydrophobicity in the N terminus (Figure 3D). The hydrophobic region was predicted not to be a signal peptide by a signal peptide–predicting algorithm (SignalP; http://www.cbs.dtu.dk/services/SignalP-2.0/) and predicted not to be a transmembrane helix by the transmembrane hidden Markov model (TMHMM) program (http://www.cbs.dtu.dk/services/TMHMM/). Thus, we hypothesized that the hydrophobic region of MLPKf2 might mediate its monotopic membrane interaction. To determine whether the hydrophobic region is involved in the localization to the plasma membrane, we expressed a GFP fusion protein of mutated MLPKf2 (MLPKf2DH) lacking the hydrophobic region (from Val-11 to Ile-23) and monitored its subcellular localization (Figure 4). MLPKf2DH-GFP failed to localize to the plasma membrane and was instead distributed in the cytosol, suggesting that the hydrophobic region was necessary for the plasma membrane localization of MLPKf2.

Both MLPK Isoforms Complement the Self-Compatible Phenotype of the mlpk Mutation

We have previously shown that the transient expression of MLPKf1 complemented the mlpk/mlpk mutation and restored the SI phenotype in mutated papilla cells (Murase et al., 2004). To test whether the MLPKf2 isoform also complements the mlpk/mlpk mutation, we adapted the transient assay. MLPKf2 and red fluorescent protein (RFP) genes were cointroduced into the papilla cells of mlpk/mlpk plants (S8S8 or S9S9) by particle bombardment. After confirming gene introduction by RFP fluorescence, self- or cross-pollen (S8S8 or S9S9) was attached to the RFP-expressing papilla cells and we monitored pollen tube growth microscopically. In control experiments in which only the RFP gene was introduced, 71% of self-pollen grains germinated and penetrated into papilla cells (Table 1). When RFP and MLPKf1

mock reaction. Reaction products were separated by SDS-PAGE and the gel was analyzed by fluorography.

Hydropathy profiles of MLPKf1 and MLPKf2. The profiles were obtained according to the method by Hopp and Woods (1981).
genes were cointroduced, the percentage of penetrated self-pollen tubes was significantly reduced (to 38%), confirming our previous results (Murase et al., 2004). When the MLPK2 gene was cointroduced, the penetration of self-pollen tubes was also inhibited (to 36%), similar to MLPK1. This inhibition was specific for self-pollen but not cross-pollen, suggesting the restoration of the SI phenotype of mlpk/mlpk plants. These results suggest that both MLPK isoforms can independently transduce SI signaling in papilla cells.

Membrane Localization Is Essential for MLPK Isoforms to Function in SI Signaling

Both MLPK isoforms seemed to localize to the plasma membrane, although the localization mechanisms differed. This suggests that MLPK may function in the vicinity of SRK and that the plasma membrane localization is important for MLPK to function in SI signaling. To address this issue, we transiently expressed mutated forms of MLPK lacking membrane localization signals in papilla cells and monitored the complementation of the mlpk/mlpk mutation (Table 1). The MLPK1G2A mutant form lacking the myristoylation site failed to complement the mlpk/mlpk mutation and failed to restore the SI phenotype. The MLPK2ΔHR mutant form, which lacks the N-terminal hydrophobic region and loses its plasma membrane localization (Figure 4), also failed to complement the mlpk/mlpk mutation. These results suggested that membrane localization is crucial for MLPK to function in SI signaling.

MLPK Isoforms Directly Interact with SRK in the Plasma Membrane

The prerequisite for plasma membrane localization of MLPK isoforms during SI signaling supported the view that MLPKs functions in the vicinity of SRK. To examine the possibility that MLPKs directly interact with SRK on the plasma membrane in plant cells, we used a BIFC assay (Hu et al., 2002). MLPK1 and MLPK2 were fused to N-terminal yellow fluorescent protein (YN), and SRK was fused to C-terminal yellow fluorescent protein (YC), and the fusion proteins were used for BIFC assays (Figure 5A). Distantly related stigmatically expressed Leu-rich repeat receptor kinases, RLK1 (At1g09970) and RLK2 (At5g25930), which localized to the plasma membrane (Figure 4), were fused to YC and used as controls. When the fused MLPK1-YN or MLPK2-YN were coexpressed with SRK-YC in tobacco BY-2 cell protoplasts, a strong fluorescent signal was reproducibly
Table 1. Effect of the Expression of MLPK Isoforms and Their Mutant Forms in mlpk/mlpk Papilla Cells

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Pollen</th>
<th>Number of Penetrated Pollen Tubes/Tests (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFP</td>
<td>Self</td>
<td>29/41 (71)</td>
</tr>
<tr>
<td></td>
<td>Cross</td>
<td>26/32 (81)</td>
</tr>
<tr>
<td>RFP + MLPKf1</td>
<td>Self</td>
<td>15/40 (38)*</td>
</tr>
<tr>
<td></td>
<td>Cross</td>
<td>30/39 (77)</td>
</tr>
<tr>
<td>RFP + MLPKf1^{G2A}</td>
<td>Self</td>
<td>50/69 (72)</td>
</tr>
<tr>
<td></td>
<td>Cross</td>
<td>46/66 (70)</td>
</tr>
<tr>
<td>RFP + MLPKf2</td>
<td>Self</td>
<td>12/33 (36)*</td>
</tr>
<tr>
<td></td>
<td>Cross</td>
<td>20/25 (80)</td>
</tr>
<tr>
<td>RFP + MLPKf2^{ΔHR}</td>
<td>Self</td>
<td>32/49 (65)</td>
</tr>
<tr>
<td></td>
<td>Cross</td>
<td>33/42 (78)</td>
</tr>
</tbody>
</table>

Each MLPK gene was delivered together with RFP marker gene into papilla cells of S_{2}S_{3}mm plants by particle bombardment. After 18 h, RFP-expressing cells were selected and pollinated with either self (S_{2}S_{2}) or cross (S_{2}S_{3}) pollen and then microscopically examined for the penetration of pollen tubes into the papilla cells.

*Self-pollinated cointransformed RFP and MLPK isoforms (experimental) compared with self-pollinated with only RFP introduced (control): MLPKf1, χ² = 9.01, P < 0.01; MLPKf2, χ² = 8.74, P < 0.01.

detected at the plasma membrane. The fluorescence emission spectral analysis revealed that these fluorescent signals were derived from the reconstituted YFP fluorophore (Figure 5B). The YFP signal was never detected when MLPKf1-YN or MLPKf2-YN were coexpressed with nonfused YC, RLK1-YC, or RLK2-YC (Figure 5A). The YFP signal was also not detected when MLPKf1-YN or MLPKf2-YN were coexpressed with SRK-YC, suggesting that the membrane localization of MLPK isoforms is a prerequisite for the direct interaction with SRK. In the cells transformed with the membrane-localized form of MLPKf1-YN (or MLPKf2-YN) and SRK-YC constructs, we consistently detected reconstituted YFP signals in all of the cells expressing enhanced cyan fluorescent protein (eCFP), which was coexpressed with the BiFC constructs as a transformation marker. The reconstitution of the YFP fluorophore was not dependent on SP11 ligand, and we observed no obvious difference in the expression of YFP signals when the protoplasts were incubated with or without SP11 protein. These results suggested that both MLPKf1 and MLPKf2 spontaneously interact with SRK on the plasma membrane of stigmatic papilla cells to transduce SI signaling.

**DISCUSSION**

Here, we describe an alternative MLPK isoform, MLPKf2, which is transcribed from an alternative transcription start site located in the third intron of the MLPKf1 gene. The resulting MLPKf2 isoform encodes a protein sequence identical to the MLPKf1 isoform, excepting the N terminus. Such expression systems, called alternative first exons (AFEs), in which the first exon of one splice variant of a gene is located within an intronic region of another variant, have been well documented in mammals and are suggested to contribute to the diversification of gene expression (Suzuki et al., 2001; Zavolan et al., 2002). Although some AFEs merely change the 5’-untranslated region and lead to no protein differences, many AFEs alter the N termini of the translated proteins, as is the case with MLPK. A very similar example of AFE was reported in c-Abl, a mammalian cytoplasmic Tyr kinase that is closely related to Src family kinases (Hantschel et al., 2003). Like MLPK, c-Abl is expressed by AFE in two isoforms, 1a and 1b, which differ only at the N termini. The 1b form contains a myristoylation signal and is expressed in divergent tissues, and the 1a form contains hydrophobic residues that are absent in 1b and is expressed in some tissues in a more specific manner (Renshaw et al., 1988; Nagar et al., 2003). When expressed under β-actin promoter, both isoforms could independently rescue most defects caused by c-Abl gene knockout (Hardin et al., 1996); thus, the functional difference of two isoforms and the biological significance of AFE remain unknown.

Very few studies have looked at AFE transcripts in plants. Arabidopsis SYN1, a RAD21-like gene essential for meiosis, is one of the few reported AFE transcripts (Bai et al., 1999). Alternative promoters and splicing produced two SYN1 transcripts encoding similar-sized proteins differing only at their N termini. One transcript (BP2) was expressed at low levels in most tissues, whereas the other (BP5) was expressed only in prebolting buds. The low levels of BP2 expression throughout the plant suggested a potential role in processes other than meiosis, but its physiological significance remains unknown. Another example of AFE transcripts reported in plants is the SBE gene, which encodes a starch-branching enzyme (Hamada et al., 2002; Kitagawa et al., 2005). One shorter SBE transcript was expressed constitutively, while a longer transcript was expressed in limited tissues, including developing seeds. It was suggested that the differences between the N termini of SBE affect its enzymatic properties and subcellular localization. Thus, AFEs may play a role in the coupling of promoter selection (tissue-specific expression) and protein diversity (Kitagawa et al., 2005).

In MLPK AFE transcripts, MLPKf1 is expressed divergently and weakly in most tissues, while MLPKf2 is predominantly expressed in stigma. This AFE system and the expression pattern of each transcript are conserved in APK1b, the Arabidopsis MLPK ortholog. This finding implies that the expression system has been conserved for more than 23 million years, since the genera Arabidopsis diverged from Brassica (Koch et al., 2000), suggesting a potential physiological implication for this expression system. Arabidopsis is a selfing plant that has lost functional SP11 and SRK alleles, but the transformation experiments of these genes suggested that the rest of the genes required to express the pollen rejection response remain intact (Nasrallah et al., 2002; Shimizu et al., 2004). MLPKf2 and APK1bf1, the major isoforms expressed in the stigma, must be the primary signal transducers functioning (or previously functioning) in SI signal transduction. By contrast, MLPKf1 and APK1bf1 isoforms are expressed in most tissues, including the stigma. Because MLPKf1 can complement the mlpk/mlpk mutation, it must also function in SI signal transduction in the stigma. However, the divergent expression profiles of these isoforms and the fact that a functional APK1b gene has been retained in Arabidopsis since the plant lost functional SP11 and SRK genes...
suggest the possibility that these isoforms play different roles in other tissues.

To test for biological functions of \textit{APK1b} aside from SI signal transduction, we analyzed a T-DNA knockout line for \textit{APK1b}; we failed, however, to detect any phenotypic alterations including fertility (see Supplemental Figure 1 online). \textit{APK1b} belongs to the subfamily RLCK VII, which comprises 46 members in \textit{Arabidopsis} (Shiu and Bleecker, 2001), and some of these members are closely related to \textit{APK1b}. For example, \textit{APK1a} and \textit{NAK} have 85 and 68% amino acid similarity with \textit{APK1bf1}, respectively, and both of these genes are suggested to be divergently expressed in most tissues (http://www.cbs.umn.edu/Arabidopsis/; Figure 2A). Redundancy with regard to RLCK VII kinases might explain the lack of aberrant phenotype in knockout mutants of \textit{APK1b}.

Further studies are needed to conclude whether \textit{APK1b} plays any other role(s) in addition to SI signal transduction.

The plasma membrane localization of both GFP fusion proteins of MLPK1 and MLPK2 is consistent with the previous observation that native MLPK protein in stigma separated into the plasma membrane fraction during differential centrifugation and aqueous two-phase partitioning experiments (Murase et al., 2004). Interestingly, the two MLPK isoforms are targeted to the plasma membrane by different mechanisms. The membrane localization of MLPK1 is myristoylation dependent, but that of MLPK2 is dependent upon its specific N-terminal hydrophobic region. MLPK1 has a typical plant myristoylation motif at its N terminus and incorporated myristic acid in an in vitro translation experiment. Proteins singly modified by N-myristoylation tend to

---

**Figure 5. BiFC Analysis between MLPK Isoforms and Receptor Kinases.**

(A) N-terminal YFP (YN), YN-fused MLPK isoforms (MLPK1-YN and MLPK2-YN), and YN-fused MLPK mutant forms (MLPK1\(^{G2A}\)-YN and MLPK2\(^{ΔHR}\)-YN) were coexpressed with C-terminal YFP (YC) and YC-fused receptor kinases (SRK-YC, RLK1-YC, and RLK2-YC) in tobacco BY-2 protoplasts. Bright-field (left), reconstructed YFP fluorescent (middle), and cointroduced eCFP fluorescent (left) images were obtained using fluorescence confocal microscopy. Bars = 10 μm.

(B) Fluorescence emission spectral analysis. The representative fluorescence spectra taken from the plasma membrane area of MLPK1-YN (or MLPK2-YN) and SRK-YC coexpressing protoplasts were compared with the spectrum of the YFP fluorophore.
be only loosely coupled to membranes, and in many cases, membrane association was further strengthened by the subsequent palmitoylation that often occurs at Cys residues near the N terminus (Thompson and Okuyama, 2000). The fourth Cys residue of ML PKf1 is highly conserved among the members of the RLCK VII subfamily (e.g., APK1bf1, APK1a, APK2a, and NAK; Figure 3B) and can serve as a possible palmitoylation site, though this should be confirmed in future studies.

Myristoylation is an irreversible posttranslational protein modification found in animals, plants, fungi, and viruses. Despite the fact that a vast number of plant proteins contain consensus signature sequences for myristoylation and could theoretically be myristoylated (Podell and Gribskov, 2004), our knowledge is very limited as to whether they are in fact myristoylated and whether such a modification is important for their function. Calcium-dependent protein kinases (CDPKs) form a multigene family and are involved in various signaling pathways in plants (Raices et al., 2003; Gargantini et al., 2006). CDPKs exhibit a variety of subcellular localizations, and myristoylation was shown to be a prerequisite for the membrane localization of certain CDPK members, but the biological significance of such localization remains unclear. Myristoylation was shown to be essential for the function of a Ca²⁺ sensor protein, SOS3 (for salt overly sensitive 3), in plant salt tolerance (Ishitani et al., 2000). Why myristoylation is necessary for SOS3 function, however, remains a mystery, as no significant difference was observed between the membrane association of wild-type SOS3 and a nonmyristoylated SOS3 mutant. The tomato (Solanum lycopersicum) R gene product Pto confers race-specific resistance to Pseudomonas syringae pv tomato bacteria carrying avrPto or avrPtoB, and myristoylation was shown to be required for the induction of defense responses, including the hypersensitive responses (Andriotis and Rathjen, 2006; de Vries et al., 2006). The N-myristoylation of Pto did not affect its subcellular localization; rather, the N-myristoyl moiety was suggested to be involved in the interactions with downstream signaling components and/or the autoinhibition of its kinase activity. In contrast with these examples, clear relationships were observed for ML PKf1 between N-myristoylation, plasma membrane localization, and the biological function of SI signaling. This observation suggested that the membrane localization of ML PK is essential for SI signal transduction. This hypothesis was further strengthened by the observations that functional ML PKf2 also localized to the plasma membrane via a different mechanism using its specific hydrophobic amino acid region and that the deletion of this region resulted in both the loss of plasma membrane localization and the loss of biological function.

The prerequisite for plasma membrane localization of ML PK during SI signaling could be explained by our BiFC assays, suggesting that ML PK directly interacts with SRK on the plasma membrane. In our previous study using a conventional yeast two-hybrid system and a split-ubiquitin membrane-based yeast two-hybrid system, we failed to detect a direct interaction between SRK and ML PK (Kakita et al., 2007). We showed that the recombinant SRK kinase domain could efficiently phosphorylate recombinant ML PK in vitro, suggesting that the interaction between SRK and ML PK should be rather weak or transient, as observed for certain interactions between mammalian kinases and their substrates (Miyazawa et al., 2002; Waas and Dalby, 2002). Thus, in this study, we used a BiFC assay system to detect a possible SRK-ML PK interaction because this system is known to stabilize weak or transient interactions (Bhat et al., 2006). The BiFC assays clearly suggested that both ML PKf1 and ML PKf2 specifically interact with SRK on the plasma membrane in plant protoplasts. The interaction was observed in the absence of SP11, as in the case of a previous interaction analysis between Brassinosteroid Insensitive1 (BRI1) and BRI1-associated receptor kinase using fluorescence life time imaging microscopy (Russinova et al., 2004). These results suggested that SRK and ML PK spontaneously interact on the stigmatic papilla cell membrane.

Because the mlpk/mlpk mutants exhibit a complete self-compatible phenotype, ML PK must be a primary component of SI signaling. Only one other molecule that is suggested to function in the SI signaling pathway is a U-box motif-containing protein, ARC1 (Stone et al., 1999). ARC1 interacts directly with the kinase domain of SRK in a phosphorylation-dependent manner via a C-terminal arm repeat domain. ARC1 has U-box-dependent E3 ubiquitin ligase activity and is thought to be involved in the degradation of unknown compatibility factors in the pistil, leading in turn to pollen rejection (Stone et al., 2003). If ARC1 is also a primary component of SRK signaling, the SRK signal transferred to ML PK must return to SRK because ARC1 is a direct downstream effector of SRK. In animal cells, Src family kinases, which are myristoylated membrane-anchored Tyr kinases, are known to interact with activated receptor Tyr kinases and mutually stimulate the other’s activity, thereby strengthening and prolonging the signal (Thomas and Brugge, 1997). ML PK acting as a coreceptor within the SRK receptor complex should be an attractive model for future studies of SI signaling.

METHODS

Plant Materials

Self-incompatible Brassica rapa S₁ and S₂ homozygotes and the self-compatible mlpk/mlpk mutant line (S₁S₁mm) have been described previously (Murase et al., 2004). Tobacco (Nicotiana tabacum) BY-2 culture cells were maintained according to established procedures (Tse et al., 2006).

RT-PCR and RACE

Total RNA was isolated from various tissues using ISOGEN (Nippon Gene) according to the manufacturer’s protocol. First-strand cDNA (cDNA) was synthesized from 1 μg of DNase I-treated total RNA using Superscript II RNaseH reverse transcriptase (Invitrogen). Target genes were amplified using the following primers: ML PKf1, MF1F (5'-ATGGGGATT TTTGCATGAGTGT-3') and Race6 (5'-TTTCATCCTTCAAGCAGTAA CCGAT-3'); ML PKf2, MF2F (5'-ATGGGTTTTGTAAGTTATGG-3') and Race6; ubiquitin (control) gene, Ubif (5'-ATGCAGATCTTTTGTGAA- CGT-3') and Race6; ubiquitin (control) gene, Ubir (5'-ACACACCGAGGACCGGAG-3') and Ubir (5'-ACACACCGAGGAGACGAGGAG-3'). RT-PCR was conducted using the following profiles: 5 min denaturation at 94°C, followed by 28 cycles for ML PKf1, 25 cycles for ML PKf2, and 21 cycles for ubiquitin of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min. A final incubation was for 5 min at 72°C. After the PCR products were separated on 1% agarose gels and stained with ethidium bromide, gel images were captured using a UV illuminator.
The 5' RACE was performed using a GeneRacer kit (Invitrogen). First and nested PCR were performed with the MLPK gene-specific reverse primer M5race1 (5'-TGCTTAGTCGAAAATCTCA-3') and M5race3 (5'-TTGCAGATCCTCTCTTCCG-3'), respectively. The PCR products were cloned into pGEM T-Easy (Promega) and sequenced.

cDNA Screening

A stigma cDNA library from an S₈ homozygote was constructed in lambda ZapII (Stratagene), and a stigma cDNA library from an S₀ homozygote was constructed in pBluescript II SK+ (Stratagene) using a cDNA synthesis kit (Takara). A stigma full-length cDNA library of the S₀ homozygote was constructed by Hitachi High-Technologies using the vector-capping method (Kato et al., 2005). An alkaliphosphatase-labeled MLPK cDNA probe was prepared using Alkphos Direct (GE Healthcare), and the stigma cDNA libraries were screened by plaque or colony hybridization. Two, one, and seven positive clones were obtained from 100,000 clones of the S₀ stigma cDNA library, 40,000 clones of the S₈ stigma cDNA library, and 200,000 clones of the S₉ stigma full-length cDNA library, respectively.

Real-Time RT-PCR

Total RNA samples from B. rapa tissues were purified using ISOGEN (Nippongene) according to the manufacturer’s protocols and treated with RNase-free DNaseI (Qiagen). The concentration and purity of total plant RNA was determined by spectrophotometric analysis. Expression profiles of MLPKf1 and MLPKf2 were analyzed by one-step real-time RT-PCR analyses using the QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer’s protocol. All reactions contained 100 ng of total RNA, 1× QuantiTect SYBR Green RT-PCR Master Mix, 1 μM forward primer, 1 μM reverse primer, and 0.2 μL QuantiTect RT mix in a 20 μL total volume. The MLPKf1-specific forward primer was 5'-ATGGGGATTTGCATGAGTGTTCAGGTTAAAG-3', and the MLPKf2-specific forward primer was 5'-ATGGGTGTGGTTTGAAAAAAGTGCAAACAGACAGAAAG-3'. The reaction was monitored using an ABI PRISM 7700 sequence detection system (Applied Biosystems). Reaction mixtures were incubated for 30 min at 50°C for reverse transcription and 15 min at 95°C, followed by 40 amplification cycles of 15 s at 94°C/30 s at 55°C/30 s at 60°C. To estimate absolute mRNA levels of unknown samples, a standard curve of a six-dilution series (1 × 10⁻², 1 × 10⁻¹, 1 × 10⁻¹, 1 × 10⁻¹, and 1 × 10⁻² fg) was constructed from plasmid DNA quantified by a spectrophotometer.

In Situ Hybridization

The pistils of B. rapa S₀S₀ homozygote were collected 1 d before anthesis. The digoxigenin-labeled antisense and sense oligo-DNA probes were purchased from Nihon Gene Research Laboratory. The cDNA sequences used for antisense and sense probes were as follows: 5'-GATCCGCGTCTCAAAAATCATGGGGATTTGCATGAGTGTTCAGGTTAAAG-3' for MLPKf1 and 5'-GACATGTGCAAGATCTCTCCTTACAAAATCTTCATCTTC-3' for MLPKf2. In situ hybridization was performed with 10-μm-thick Paraplast (Sigma-Aldrich) sections of formaldehyde-fixed pistils was performed as previously described (Shiba et al., 2002).

In Vitro Myristoylation Assay

For in vitro transcription and translation analyses, the pCMVTrT vector (Promega) was converted to a Gateway-compatible destination vector (pCMVTrT/gw) using the Gateway vector conversion system (Invitrogen). The following forward primers were used for PCR amplification from the cDNA: MLPKf1, 5'-CAGCAATGGTTGCTGATGAGTTCCAG-3', MLPKf2, 5'-CAGCATGATGTTGCTGCTGATGAGTTCCAG-3', MLPKf1G2A, 5'-CACATGGCAGTTTTGCAATGATGAGTTCCAG-3', and MLPKf2G2A, 5'-CACATGGCAGTTTTGCAATGATGAGTTCCAG-3'. The common reverse primer was 5'-TCAGACAAACAGACAGCTAGCACGAG-3'. The amplified fragments were cloned into the pENTR/D-TOPO cloning vector (Invitrogen) and recombined into pCMVTrT/gw using Gateway LR clonase mix (Invitrogen).

In vitro transcription and translation reactions were performed using the TNT-coupled wheat germ extract system (Promega). MLPKf1, MLPKf2, MLPKf1G2A, and MLPKf2G2A were transcribed and translated in TNT quick master mix containing wheat germ extract (Promega) at 30°C for 120 min. Radiolabeling was performed with 5 μCi L-[³⁵S]Met (1000 Ci/mM; GE Healthcare) in the presence of 20 μM amino acids (minus Met) or 50 μCi [9, 10 (α-³H)] myristic acid (54 Ci/mM; GE Healthcare). Aliquots of reaction products were separated by SDS-PAGE, gels were dried, and autoradiograms were captured by a BAS2500 IP Reader (FujiFilm).

Transient Expression of the MLPK-GFP Fusion Protein

The previously described plasmid 35S:GFPPBI221 (Murase et al., 2004) was converted to a Gateway-compatible destination vector (pGFPP/gw) using the Gateway vector conversion system. The entry vectors for the expression of MLPKf1, MLPKf2, MLPKf1G2A, and MLPKf2G2A were prepared by PCR using the same forward primers used in the in vitro transcription/translation analyses. For the expression of MLPKf2ΔHR, the following forward primer was used: 5'-CAGCATGATGTTGCTGCTGATGAGTTCCAG-3', and the common reverse primer was 5'-GACAACACAGAGCACAGACAGCAGG-3'. Full-length S₈-SRK (D88193) was amplified from stigmatic cDNA of B. rapa S₈ homozygote using the specific primers 5'-CAGCATGATGTTGCTGCTGATGAGTTCCAG-3' and 5'-GACAACACAGAGCACAGACAGCAGG-3'. The common reverse primer was 5'-GACAACACAGAGCACAGACAGCAGG-3'. Full-length RLK1 (At1g09970) and RLK2 (At5g25930) were amplified from Arabidopsis thaliana stigmatic cDNA using the following primer pairs: RLK1, 5'-5'-CACATGGGATTTGCTGCTGATGAGTTCCAG-3' and 5'-ACCTTTCCATTCCGCCAG-3' and RLK2, 5'-CAGCATGATGTTGCTGCTGATGAGTTCCAG-3' and 5'-GACAACACAGAGCACAGACAGCAGG-3'. All amplified fragments were cloned into the pENTR/D-TOPO cloning vector and then recombined into pGFPP/gw.

Tobacco BY-2 culture cells were incubated in an enzyme solution (1% [w/v] of cellulase Onozuka RS [Yakult], 0.1% [w/v] pectolyase Y-23 [Kikkoman], 0.4 M mannitol, and 5 mM MES-KOH, pH 5.8) for 2 h at room temperature. BY-2 protoplasts were collected by centrifugation (5 min at 100g) and washed twice in wash buffer (0.4 M mannitol, 70 mM CaCl₂, and 5 mM MES-KOH, pH 5.8). Polyethylene glycol-mediated transient transformation of protoplasts was performed as described (Abel and Theologis, 1994). GFP-expressing cells were analyzed by fluorescence confocal microscopy.

Complementation Experiments

The RFP expression vector, pSLG-RFP, has been previously described (Murase et al., 2004). For the expression of MLPK-related genes, the pSLG9 plasmid containing the 3.2-kb 5'-upstream region of the SLG₃ gene (Murase et al., 2004) was converted to a Gateway-compatible destination vector (pSLG/gw) using the Gateway vector conversion system. The MLPKf1, MLPKf2, MLPKf1G2A, and MLPKf2ΔHR genes were amplified by PCR using the above-described forward primers and the common reverse primer 5'-TCAGACAAACAGACAGCTAGCACGAG-3'. All fragments were cloned into the pENTR/D-TOPO cloning vector and then recombined into pSLG/gw. The procedure of transiently expressing the RFP and MLPK-related genes in mpk/mpk papilla cells was performed as described previously (Murase et al., 2004). Pistils were collected from B. rapa S₃S₃ gynoecium flower buds 1 d prior to flowering and...
placed onto Murashige and Skoog nutrient agar medium containing Gamborg B5 vitamins and 3% sucrose. Stigmata were bombarded twice with 100 μg of gold particles (1 μm diameter) coated with 2 μg of each plasmid using the PDS-1000/He system (Bio-Rad) fitted with 900-p.s.i. rupture discs. After incubating 18 h at 22°C, self (S₀S₀) - or cross (S₁S₂)-pollen grains were attached onto RFP-expressing papilla cells using a micromanipulator. After 2 h, the pollen grains were examined microscopically for pollen tube penetration into papilla cells.

**BiFC Assay**

A hemagglutinin (HA) epitope tag fragment from pGADT7 (Clontech) was inserted into the EcoRI site of pCMV-script (Invitrogen) and designated pCMV-HA. The sequences encoding the N-terminal fragment comprised of residues 1 to 154 (YN), as well as the C-terminal fragment comprised of residues 155 to 238 (YC) of the Venus YFP variant (Nagai et al., 2002; Shyu et al., 2006), were amplified using the following primer pairs: YN, 5′-AATAAAGCTTATGGTACAGCCAGGAAGCGAGGA-3′ and 5′-TATTCCT-CAGTATGTTAATAGCGTTGCGGAC-3′; and YC, 5′-AATAAAGCTTGCCAGCAGACAGGAAGCGAGGA-3′ and 5′-AATTCTCAGTAGTCTGTAACGAGGAAGCGAGGA-3′. The amplified YN and YC sequences were integrated into the HindIII/Xhol site of pCMV-HA. The obtained fusion sequences, HA-VN and HA-VC, were inserted into the BamHI/Sacl site of pBII221 vector and converted to the Gateway-compatible destination vectors (pVN/gw and pVC/gw, respectively) using the Gateway conversion system. The MLPKf1, MLPKf2, MLPKf2 (G2A), and MLPKf2 (HR) entry vectors were recombined into pVN/gw using Gateway LR clonase mix. SRK, RLK1, and RLK2 entry vectors were recombined into pVC/gw. The plasmid DNA containing eCFP, eCFP/pRSETb, was provided by A. Miyawaki (RIKEN, Saitama, Japan) for providing the tobacco BY-2 cells, Tetsuyuki Entani (Nara Institute of Science and Technology) for helpful discussions, and Hitomi Ichikawa, Satsuki Takahashi, Aya Ota, Mami Ohue, Maki Matsumura, Yoshie Ohnishi, and Humiyo Yamamoto for their technical assistance. This work was supported by Grants-in-Aid for Creative Scientific Research (16GS0316 to A.I.), by Grants-in-Aid for Scientific Research (16380072, 18380069, and 18075008 to S.T), and by the global Centers of Excellence Program to Nara Institute of Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Received December 25, 2006; revised November 13, 2007; accepted November 15, 2007; published December 7, 2007.

**REFERENCES**


Miyazaki, K., Shinozaki, M., Hara, T., Furuya, T., and Miyazono, K. (2002). Two major Smad pathways in TGF-


Two Distinct Forms of M-Locus Protein Kinase Localize to the Plasma Membrane and Interact Directly with S-Locus Receptor Kinase to Transduce Self-Incompatibility Signaling in Brassica rapa

Mitsuru Kakita, Kohji Murase, Megumi Iwano, Tomohito Matsumoto, Masao Watanabe, Hiroshi Shiba, Akira Isogai and Seiji Takayama

Plant Cell 2007;19;3961-3973; originally published online December 7, 2007; DOI 10.1105/tpc.106.049999

This information is current as of July 12, 2017

Supplemental Data
/content/suppl/2007/11/16/tpc.106.049999.DC1.html

References
This article cites 57 articles, 30 of which can be accessed free at:
/content/19/12/3961.full.html#ref-list-1

Permissions

eTOCs
Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain

CiteTrack Alerts
Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain

Subscription Information
Subscription Information for The Plant Cell and Plant Physiology is available at:
http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY