Intercellular signaling mediated by receptor-like kinases (RLKs) is important for diverse processes in plant development, although downstream intracellular signaling pathways remain poorly understood. Proteins interacting directly with RLK were screened for by yeast two-hybrid assay with the kinase domain as bait. A MADS-box protein, AGL24, was identified as a candidate substrate of MRLK (Meristematic Receptor-Like Kinase), which was named for its spatial expression in shoot and root apical meristems in Arabidopsis. The AGL24 protein specifically interacted with, and was phosphorylated by, the MRLK kinase domain in vitro assays. The simultaneous expression of AGL24 and MRLK in shoot apices during floral transition suggested that the interaction occurs in plants. Using plants constitutively expressing a fusion protein of AGL24 and green fluorescent protein, the subcellular localization of AGL24 protein was observed exclusively in the nucleus in apical tissues where MRLK was expressed, while AGL24 was localized in both the cytoplasm and the nucleus in tissues where no MRLK expression was detectable. These results suggest that MRLK signaling promotes translocation of AGL24 from the cytoplasm to the nucleus. We propose that the RLK signaling pathway involves phosphorylation of a MADS-box transcription factor.

Keywords: Arabidopsis thaliana — MADS-box gene — Protein phosphorylation — Protein–protein interaction — Receptor-like kinase.

Abbreviations: DAS, day after sowing; GFP, green fluorescent protein; GST, glutathione S-transferase; KAPP, kinase-associated protein phosphatase; LRR, leucine-rich repeat; RLK, receptor-like kinase; RTK, receptor tyrosine kinase.

Introduction

Intercellular signaling is an essential process in plant development. Receptor-like kinases (RLKs), which receive extracellular signals and transduce them to intracellular downstream factors by protein phosphorylation, play important roles in intracellular communication (Becraft et al. 1996, Takasaki et al. 2000). Completion of the Arabidopsis genome project has revealed that over 400 genes are predicted to encode RLKs in the genome and that LRR (leucine-rich repeat)-typed RLKs are the largest class, containing over 200 genes (Arabidopsis Genome Initiative 2000, Shiu and Bleecker 2001). Indeed, biological functions have been identified, mainly by molecular genetic approaches. For example, Arabidopsis CLV1 (Clark et al. 1997), Arabidopsis BRI1 (Li and Chory 1997) and rice Xa21 (Song et al. 1995) were shown to function in shoot meristem maintenance, hormone perception and disease resistance, respectively. Despite extensive genetic studies, however, functions for most RLKs remain to be elucidated, especially with regard to their downstream components. In addition to mutant analysis, molecular and biochemical analyses of signal transduction pathways for RLKs will help in the understanding of their genetic function in plant development.

Studies using genetic and biochemical approaches have been undertaken to explore signaling pathways for some RLKs in plants. For example, in the CLAVATA signaling pathway that regulates maintenance of shoot apical meristem, three CLAVATA genes, CLV1, CLV2 and CLV3, encode an RLK (Clark et al. 1997), a receptor-like protein (Jeong et al. 1999), and a secreted polypeptide (Fletcher et al. 1999), respectively. The CLV3 polypeptide, which probably acts as a ligand, binds the receptor domain of CLV1, resulting in activation of the CLV1 kinase domain (Trotochaud et al. 2000). Kinase-associated protein phosphatase (KAPP), which was originally identified as a cytoplasmic interacting factor with the kinase domain of RLK5 (HAESA) (Stone et al. 1994), also interacts with the kinase domain of CLV1 (Stone et al. 1998). Apparently KAPP is not a substrate for CLV1 kinase activity, although KAPP may function as a signaling component by dephosphorylation. Association of Rop (Rho-like small GTPase) in the CLV1 receptor complex has been reported (Trotochaud et al. 1999), but information on the RLK signal transduction system is limited in plants, particularly with regard to downstream targets.

Recent comparative genome analysis suggested that signal transduction pathways have evolved independently in plants and animals (Arabidopsis Genome Initiative 2000). For example, a large number of receptor tyrosine kinases (RTKs) have been identified in animals, and the Ras superfamily of monomeric small GTPase play important roles in RTK signal transduction pathways (van der Geer and Hunter 1994). In plants, however, all known receptor-like kinases have serine/
threonine kinase consensus sequences, and no RAS-like proteins have been identified (Arabidopsis Genome Initiative 2000). Plant RLKs have a variety of functions mediated through diverse signaling pathways (Becraft 2002). It is largely unknown how a great number of different RLKs regulate their specific downstream signaling pathways in plants.

Identification of downstream components that directly interact with, and are phosphorylated by, RLK kinases is important in the understanding of the underlying process of signal transduction. The yeast two-hybrid system has been widely used to screen for direct targets for RLKs. For example, \( \text{THL1, THL2 (Thioredoxin-h-like 1 and 2), and ARC1 (ARM REPEAT CONTAINING1)} \) were found to interact with an SRK, that is a receptor kinase involved in self-incompatibility in \( \text{Brassica} \) (Bower et al. 1996, Gu et al. 1998). Repression of \( \text{ARC1} \) reduces the potential for self-incompatibility and may thus function as a positive downstream effector of \( \text{SRK} \) (Stone et al. 1999).

\( \text{MRLK (Meristematic Receptor-Like Kinase)} \) was isolated from an equalized cDNA library of \( \text{Arabidopsis inflorescence} \) apices by differential screening (Takemura et al. 1999). \( \text{MRLK} \) encodes a receptor-like kinase with LRRs in its extracellular domain (\text{At3g56100}). Although \( \text{MRLK} \) is predicted to function in meristems based on its expression profile, its precise function based on knock-out has not yet been phenotypically clear (M. Takemura et al., unpublished work). To investigate the \( \text{MRLK} \)-mediated signal transduction pathway and its function in meristems, the yeast two-hybrid system was used to isolate factors interacting with the kinase domain of \( \text{MRLK} \). In this report, we describe the identification of AGL24, which binds directly to the kinase domain of \( \text{MRLK} \) and is specifically phosphorylated by the \( \text{MRLK} \) kinase domain. \( \text{AGL24} \) encodes a MADS-box transcription factor which has been suggested to regulate floral transition (Yu et al. 2002, Michaels et al. 2003, M. Takemura et al. unpublished observation). Here we propose a novel pathway in plants, in which a receptor-like kinase transduces a signal directly to a transcription factor by phosphorylation.

### Results

**Screening for proteins that interact with the MRLK kinase domain**

RLK cytoplasmic kinase domains are known to transduce signals by phosphorylation of substrate proteins. Identification of the substrate for the MRLK kinase domain (\( \text{MRLK-KD} \)) may lead to an understanding of the signal transduction pathway for \( \text{MRLK} \), as well as a more general view of RLK-mediated signaling in plants. To determine substrates that interact directly with the catalytic domain of \( \text{MRLK} \), yeast two-hybrid screening was performed using \( \text{MRLK-KD} \) as bait (Fig. 1A). By screening approximately \( 2^{17} \times 10^6 \) independent cDNA clones, we isolated 33 colonies that could proliferate in the absence of histidine and activate the \( \text{lacZ} \) reporter gene. Sequence analysis of these clones revealed that three genes were recovered in the screening. Thirty out of 33 clones encoded the cDNA for RNA polymerase subunit 6-like protein (\text{At2g04630}), one encoded a MADS-box gene that was registered as \( \text{AGL24 (At4g24540)} \) and two encoded a functionally unknown gene (\text{At3g14180}). Specific interactions between bait \( \text{MRLK-KD} \) and each gene product in yeast was confirmed, as yeast transformants examined are shown in the left diagram. Yeast growth phenotypes (upper) and filter lift assays (lower) are shown.

### Table 1 Interaction of the isolated proteins with the kinase domain of MRLK in yeast

<table>
<thead>
<tr>
<th>GAL4BD fusion</th>
<th>GAL4AD fusion</th>
<th>β-Gal activity (MU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{MRLK-KD} )</td>
<td>( \text{AGL24} )</td>
<td>7.08±0.57</td>
</tr>
<tr>
<td>( \text{MRLK-KD} )</td>
<td>( \text{At2g04630} )</td>
<td>2.02±0.05</td>
</tr>
<tr>
<td>( \text{MRLK-KD} )</td>
<td>( \text{At3g14180} )</td>
<td>1.35±0.04</td>
</tr>
<tr>
<td>( \text{p53} )</td>
<td>( \text{SV40} )</td>
<td>0.73±0.07</td>
</tr>
</tbody>
</table>

*β-Galactosidase activity is in Miller Units (Miller 1972).
Fig. 2  In vitro binding and phosphorylation assays for identified proteins with the MRLK kinase domain. (A) Pull-down assay of AGL24, At2g04630, and At3g14180 in vitro. Each protein was translated and labeled with [35S]Met in vitro. The MRLK kinase domain (MRLK-KD) was purified as a fusion protein with GST (GST-MRLK-KD). After incubating each labeled protein with beads containing GST-MRLK-KD or GST proteins, complexes were analyzed on SDS-PAGE. Lanes 1, 4 and 7, 35S-labeled protein translated in vitro. Twenty percent of each input protein used for binding assay was loaded. Lanes 2, 5 and 8, samples recovered after incubation with GST-MRLK-KD. Lanes 3, 6 and 9, samples recovered after incubation with GST. (B) Phosphorylation assay of AGL24, At3g14180 and GST protein by MRLK kinase. Lanes 1, 4 and 7, reaction mixtures containing only MRLK-KD. Lanes 2, 5 and 8, reaction mixtures containing only substrate proteins, AGL24, At3g14180 and GST, respectively. Lanes 3, 6 and 9, reaction mixtures containing MRLK-KD and substrate proteins, AGL24, At3g14180 and GST, respectively. Black arrows indicate the phosphorylated MRLK-KD protein. White arrows indicate substrate proteins. Numerals under kDa indicate molecular mass of marker proteins.

The AGL24 protein specifically binds to and is phosphorylated by the MRLK kinase domain in vitro

To confirm interaction of MRLK-KD with the proteins that were isolated by yeast two-hybrid assay, in vitro binding assays were performed. Full-length cDNAs for individual genes were isolated, since the original two-hybrid clones only contained partial cDNAs fused to the GAL4 DNA binding domain. The AGL24 cDNA contained an open reading frame of 220 amino acid residues with a predicted molecular mass of approximately 25 kDa (data not shown). Annotations of At2g04630 and At3g14180 in the GenBank database were also confirmed by cDNA cloning. At2g04630 and At3g14180 encoded open reading frames with predicted molecular masses of approximately 19 kDa and 48 kDa, respectively (data not shown). 35S-labeled proteins prepared by an in vitro translation system were incubated with MRLK-KD protein fused to glutathione S-transferase (GST) (Fig. 2A). An interaction was detected between AGL24 and the MRLK-KD protein (Fig. 2A, left), indicating that interactions between AGL24 and MRLK-KD in yeast were specific. In this pull-down assay, the At2g04630 protein did not show any in vitro binding to MRLK-KD (Fig. 2A, middle). The At3g14180 protein not only bound to MRLK-KD fused to GST, but also to GST itself, indicating that the interaction between At3g14180 protein and MRLK-KD is less specific (Fig. 2A, right). Thus, At2g04630 was excluded from further analysis as a putative substrate for MRLK.

Signals transmitted by RLKs are believed to involve protein phosphorylation. To determine whether the interaction between MRLK-KD and AGL24 or At3g14180 led to the phosphorylation of these substrates, in vitro phosphorylation assays of AGL24 or At3g14180 were performed with the MRLK-KD protein, which has kinase activity (Fig. 2B). Phosphorylated proteins were observed for MRLK-KD fused to GST (66 kDa) and AGL24 (30 kDa) (Fig. 2B, left). In contrast, phosphorylation of At3g14180 protein was not detected in vitro (Fig. 2B, middle). As the GST protein was not phosphorylated by MRLK-KD (Fig. 2B, right), the in vitro kinase assay is specific to the protein. In these assays, AGL24 specifically interacted with and was phosphorylated by MRLK kinase, suggesting that AGL24 is a substrate of MRLK-KD.

AGL24 gene is preferentially expressed in shoot apices in Arabidopsis

As signaling through interaction and protein phosphorylation should occur in cells where both genes are expressed, the gene expression patterns of AGL24 were compared with those of MRLK. AGL24 transcripts were detected at significant levels in shoot apices from the late vegetative phase (14 d after sowing (DAS)) towards the early reproductive phase (20 DAS) (Fig. 3). Expression of AGL24 was also analyzed by in situ hybridization for shoot apical meristems (18 DAS), indicating that AGL24 was expressed throughout in shoot apical meristems (Fig. 4A). When plants bolted (35 DAS), AGL24 transcript levels were relatively decreased, but still detectable in both inflorescence apices and stems. In a later developmental stage (50 DAS), AGL24 was weakly expressed in inflorescences, stems and leaves. AGL24 transcripts were not detected in roots, open flowers or siliques (Fig. 3). MRLK was consistently expressed in shoot apical meristems throughout the life cycle (Fig. 3). Thus, MRLK and AGL24 may interact in shoot and inflorescence meristems, especially during the phase transition between vegetative and reproductive growth, although these analyses also suggested that the regulation of AGL24 expression is independent of MRLK expression.

The K domain and C region of AGL24 are required for interaction with the MRLK kinase domain

AGL24 encodes a MADS-box protein composed of a MADS domain, 1 region, K domain and C region. We attempted to identify domains responsible for interaction and
proteins function in protein–protein interactions (Riechmann et al. in preparation). AGL24 protein fused to GFP (AGL24-GFP) (Fig. 5). Promoter analysis using a GUS reporter gene transgenously expressing AGL24 fused to green fluorescent protein (GFP) (Fig. 5). To further define the domains required for interaction, a construct containing the second and third \( \alpha \)-helix regions (AGL24-KCA1) was analyzed by yeast two-hybrid assay. As no evidence for interaction by means of \( \beta \)-galactosidase activity was detected for AGL24-KCA1, the second and third \( \alpha \)-helix regions may not be sufficient for interacting with MRLK-KD. These results suggest that KC regions are required for interaction with MRLK-KD, assuming that the levels of protein expression for all constructs were equal in yeast. Although the in vitro binding experiment showed that full-length AGL24 protein bound to the MRLK kinase domain, little \( \beta \)-galactosidase activity was found between AGL24-MIKC and MRLK-KD in the yeast two-hybrid system. One possible explanation is that full-length AGL24 protein somehow was not properly expressed in yeast, causing a failure to detect binding with MRLK-KD. Alternatively, intact AGL24 protein may be phosphorylated by the MRLK kinase domain in yeast, as shown by the in vitro phosphorylation assay, and as a result it is spontaneously released from complex with the MRLK-KD. To test this hypothesis, it will be necessary to identify sites of interaction and the phosphorylated amino acid residues in AGL24 for more detailed analyses.

**Table 2** Determination of the MRLK-KD interacting region in AGL24

<table>
<thead>
<tr>
<th>Prey</th>
<th>Position (^a)</th>
<th>Activity (MU) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGL24-MIKC</td>
<td>1–220</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td>AGL24-MIK</td>
<td>1–157</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>AGL24-IKC</td>
<td>58–220</td>
<td>2.38±0.52</td>
</tr>
<tr>
<td>AGL24-K</td>
<td>91–157</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>AGL24-KC</td>
<td>91–220</td>
<td>6.01±0.17</td>
</tr>
<tr>
<td>AGL24-C</td>
<td>158–220</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>AGL24-KCA1</td>
<td>117–220</td>
<td>0.28</td>
</tr>
<tr>
<td>pGAD (^c)</td>
<td></td>
<td>0.09±0.01</td>
</tr>
</tbody>
</table>

\(^a\) Amino acid positions.

\(^b\) \( \beta \)-Galactosidase activities are shown in Miller Units.

\(^c\) pGAD vector only as a negative control.

Subcellular localization of the AGL24 protein is potentially regulated by MRLK-dependent phosphorylation

AGL24 is a MADS-box protein that may function in the nucleus for transcription. On the other hand, MRLK is a receptor-like kinase with a cytoplasmic kinase domain that is anchored in the plasma membrane. Therefore, the AGL24 protein should contact the MRLK kinase domain in the cytoplasm for interaction and signal transduction. One of our working hypotheses is that MRLK phosphorylates AGL24 in the cytoplasm and releases phosphorylated AGL24 protein to function in the nucleus. To test this hypothesis, subcellular localization of AGL24 was analyzed using transgenic *Arabidopsis* ubiquitously expressing AGL24 fused to green fluorescent protein (GFP) (Fig. 5). Promoter analysis using a GUS reporter gene revealed that MRLK is expressed preferentially in root and shoot apical meristems but not in hypocotyls or the elongation zone of the roots throughout the plant life cycle (M. Takemura et al. in preparation). AGL24 protein fused to GFP (AGL24-
Interaction of AGL24 and receptor-like kinase

GFP) was localized largely in the nucleus of cells in shoot and root apical meristems where MRLK is expressed (Fig. 5A, D). In contrast, AGL24-GFP protein was distributed in the cytoplasm, as well as in the nucleus, in hypocotyls and elongated root cells where MRLK is not expressed (Fig. 5G, J). These results suggest that AGL24 is intrinsically present in cytoplasm and may be translocated from the cytoplasm to the nucleus by MRLK-dependent phosphorylation. Identification and disruption of phosphorylation sites in AGL24 is necessary to further evaluate this hypothesis.

Discussion

Receptor-like kinases (RLKs) play important roles in plant development and environmental responses via intercellular communication. However, the molecular mechanisms underlying RLK signaling pathways are largely unknown. In order to identify the downstream signaling pathway, we investigated the substrate for the kinase domain of an RLK gene, MRLK, which is preferentially expressed in shoot and root apical meristems. Using yeast two-hybrid screening, in vitro binding and phosphorylation assays, we showed that a MADS-box transcriptional factor, AGL24, could interact with and be phosphorylated by the kinase domain of MRLK. To our knowledge, this is the first report of a transcription factor directly interacting with the kinase domain of RLKs in plants.

AGL24 expression was detected in shoot apical meristem from the late vegetative phase to the reproductive phase, suggesting that AGL24 may interact with and be phosphorylated by the MRLK kinase in the shoot apical meristem at the transition from vegetative to reproductive growth. This raises the possibility that AGL24 is post-translationally modified by MRLK as well as transcriptionally regulated. In contrast, MRLK was expressed in root apical meristem where AGL24 transcripts were not detectable. This suggests that AGL24 is not the only substrate for MRLK, and that MRLK may have a different substrate in roots. Divergent combinations between RLKs and substrate proteins might have evolved in plants.

The majority of plant MADS-box genes have been classed as "type II" MADS-box genes, which encoded a MADS domain, I region, K domain and C region (Alvarez-Buylla et al. 2000). The MADS domain is involved in DNA binding and dimerization with MADS proteins. The K domain and C region contain α-helix regions that are generally believed to be involved in protein–protein interactions (Riechmann and Meyerowitz 1997, Theissen et al. 2000, Alvarez-Buylla et al. 2000). The I region and K domain are suggested to mediate specific dimerization. The C region is not required for dimerization, but is needed for the formation of ternary complexes (Egea-Cortines et al. 1999, Honma and Goto 2001). The yeast two-hybrid studies indicated that both the K domain and C region in AGL24 were required for interaction with the MRLK kinase domain, suggesting that the regions required for interaction between AGL24 and MRLK-KD are potentially important for the formation of the MADS protein complex containing AGL24.

The MADS-box proteins are transcription factors that function in the nucleus. However, there have been reports that some MADS-box proteins are not localized in the nucleus by themselves. For example, either APETALLA3 (AP3) or PISTILLATA (PI) alone are localized in the cytoplasm, but are translocated to the nucleus when they interact to form AP3/PI heterodimers (McGonigle et al. 1996). Cytoplasmic subcellular localization of FBP11, a Petunia SEPALATA-like protein, has been shown by using FRET-spectral imaging microscopy. Nuclear localization of FBP11 was observed when FBP11 interacted with FBP2, FBP5 or FBP9 (Immink et al. 2002). AGL15 (AGAMOUS-like15) protein is also localized in the cytoplasm of cells of the female germ unit before fertilization (Perry et al. 1996), and moves into the nucleus after the first few cell divisions in the embryo (Perry et al. 1999). It is not understood how MADS protein complex localization is regulated, although complex formation itself might affect localiza-
tion. The AGL24 protein is localized exclusively in the nucleus only in meristematic tissue where MRLK is expressed. One possible explanation is that MRLK activity promotes the translocation of AGL24 from the cytoplasm to the nucleus by protein phosphorylation. However, it is not clear whether nuclear localization is achieved directly by AGL24 alone or indirectly through MADS protein complex formation containing AGL24. Use of phosphorylation site-disrupted AGL24 and other genetic manipulations will provide insight as to the regulation of AGL24 localization by MRLK kinase.

If AGL24 is a direct target protein of MRLK, the MRLK-mediated signal transduction pathway may be relatively simple. Although the MAPK cascade was expected to be involved in the CLV1 pathway due to the association of Rop in CLV1 complexes, supporting experimental data were rather limited. In animals, a signal transduction pathway mediated by a serine/threonine kinase, TGF-β (transforming growth factor-β) receptor family has been well characterized to directly regulate SMAD transcription factors (Attisano and Wrana 2002). TGF-β receptor complexes are activated by binding of TGF-β and then phosphorylate SMADs causing them to accumulate in the nucleus (Kretzschmar et al. 1999, Attisano and Wrana 2002). Like the TGF-β signaling pathway, activated MRLK may affect localization of AGL24 proteins by phosphorylation. Alternatively, phosphorylation may affect various properties such as transactivation activities, DNA binding affinities, and protein–protein interactions. Recent studies on several MADS-box genes, such as SEPELLATA genes, indicated that MADS-box proteins function in multimers (Honma and Goto 2001). AGL24 was shown to interact with AP1 and CAL by the yeast two-hybrid system (Pelaz et al. 2001, M. Takemura et al. unpublished observations). Thus, AGL24 may form heterodimers or heteromultimers with other MADS-box proteins, and MRLK may influence their interactions. We do not know how MRLK regulates AGL24 and what the fundamental role of its signaling cascade is in plants. Recent analysis of AGL24 has shown the role of this gene in flowering (Yu et al. 2002, Soc1 primer M3 (TaKaRa, Kyoto, Japan). The amplified fragment was inserted into the yeast vector, pGBK7 (Matchmaker two-hybrid system; Clontech Laboratory Inc., Palo Alto, U.S.A.), resulting in fusion with the DNA binding domain of GAL4 (pGBT9-MRLK-KD). A cDNA library was prepared from mRNA of Arabidopsis inflorescence tissues. Synthesized cDNAs using ZAP-cDNA synthesis kit were introduced into corresponding sites of the HybriZAP vector (Stratagene, La Jolla, U.S.A.). The phagemid, pAD-GAL4 vector was excised from the HybriZAP vector. The yeast strain HF7c (MATa, ura3-52, his3-200, lys2-801, ade2-101, trpl-101, leu2-3, 112, GAL41-HIS3, URA3::(GAL4 17mers) /CYC1-lacZ) was used. Yeast transformation, HIS3 reporter assays and membrane filter assays for β-galactosidase activities were carried out according to the manufacturer’s instructions (Matchmaker two-hybrid system; Clontech Laboratory Inc.).

### Measurement of β-galactosidase activity in yeast

For measurement of β-galactosidase activities, yeast transformants were grown in the appropriate selecting media to an OD$_{600}$ of 0.5–1.0. Colorimetric β-galactosidase activity was determined using o-nitrophenyl-β-D-galactoside as substrate in a method described by Miller (Miller 1972). Incubation was stopped in 90 min by adding 1 M Na$_2$CO$_3$ as described in protocols. The Miller Unit (MU) was calculated by $1,000 \times$ OD$_{420}$/A$_{600}$ x time in min × volume in milliliters.

### Isolation of full-length cDNAs and sequence analysis

To isolate full-length cDNAs for AGL24, At2g04630 and At3g14180, a ZIPLOX (Invitrogen Corp., Carlsbad, U.S.A.) cDNA library generated from Arabidopsis inflorescence tissues was screened. For screening, cDNA fragments of genes in pAD-GAL4 vectors were used as probes. cDNA fragments inserted in λ phage vectors were excised and formed into phagemids (pZL1). Sequences of all cDNA clones were determined by using Dye Terminator Thermosequenase (Amersham Pharmacia, Buckinghamshire, UK). For deduced amino acid sequences of the three genes, database searches were performed with BLASTX. For estimation of α-helix regions in AGL24, secondary protein structure was predicted by the Chau and Fasman method and an algorithm described by Kneller et al. (1990).

### Protein expression and in vitro binding assay

The MRLK-KD was amplified by PCR using primers, MRLKK1 and M13 primer M3, and introduced into the pGEX4T-1 (Amersham Pharmacia). Protein purification was carried out according to the manufacturer’s protocol. The entire coding sequences of AGL24, At2g04630 and At3g14180 were amplified by PCR and inserted into pET21a vector (Novagen, Madison, U.S.A.). The following primers were used for amplification: 5'-CCGGAATTCATGGCGAGAGAAAGATAAGGAGA-3' and 5'-TACGGGCGCGTCTCCAAAGATGGAAAGC-CAA-3' for AGL24; 5'-CTGAATTCTGCGACGGATGATGTAGGAAGGTACGG-3' and 5'-TACGGGCGCGCATTCCACCCGACTATTACTTGT-3' for At2g04630; 5'-ACGTCGACTGCGTATCCCAATGGAAAGTGC-3' and...
and purified by a Ni-
His-tagged AGL24 and At3g14180 proteins were expressed in PBS buffer for 30 min at room temperature. The beads were washed with 10 bed volumes of PBS buffer 5x and then were washed with 10 bed volumes of PBST buffer [PB [S + 0.5% Tween-20] 8x. Beads were loaded onto a 10% SDS-PAGE. Gels were dried and subjected to autoradiography.

In vitro phosphorylation assay
GST-MRLK-KD and GST proteins expressed in Escherichia coli were purified using glutathione Sepharose 4B (Amersham Pharmacia). His-tagged AGL24 and At3g14180 proteins were expressed in E. coli and purified by a Ni²⁺ column (Novagen). For the kinase assay, 2 µg of GST-MRLK-KD and 1 µg of GST, AGL24, and At3g14180 proteins were mixed in a 10 µl reaction mixture (50 mM Tris-HCl (pH 7.5), 1 µM DTT, 10 mM MnCl₂, 20 µM ATP and 10 µCi [γ-³²P]ATP). The phosphorylation reaction was done by incubation for 1 h at room temperature. Proteins were separated on 10% SDS-PAGE for AGL24 and GST control or 12.5% SDS-PAGE for At3g14180, and then the gel was subjected to autoradiography.

Expression analysis of AGL24 and MRLK by RT-PCR
Total RNAs from various tissues of Arabidopsis plants at 4, 6, 14, 20, 35, and 50 d after sowing (DAS) were extracted. First strand cDNAs were synthesized by 1 µg of total RNAs with the First-Strand Synthesis Kit (Amersham Pharmacia), using primers specific for AGL24, MRLK and UBQ5 (AGL24s1, 5'-TTCCCAAGATGGAA-GCCCAA-3'; MRLKB, 5'-ACCGCAATGACCAACCAATGAGG-3'; and UB12, 5'-CTACAAACAGATCAACGTCA-3', respectively). The PCR reaction was performed using 1 µl of the 7.5 µl first strand DNA solution with primers: AGL24-1, 5'-ATGGCCGAGAAGAGATGAA-GA-3'; AGL24s1, MRLKB and MRLKK2, 5'-CTCCGAATGATCTC-TTGTGGTC-3'; and UB12, 5'-GGGCTGCTAAGAAGAGAAGA-3', and UB12 for AGL24, MRLK and UBQ5, respectively. The PCR conditions were one denaturation step at 95°C for 1 min, followed by cycles of 95°C at 30 s, 50°C at 30 s, and 68°C at 1 min. AGL24 and MRLK were amplified for 35 cycles. UBQ5 fragments were amplified for 25 cycles.

In situ hybridization
In situ hybridization was performed as previously described by Lincoln et al. (1994) and by Aida et al. (1999). A partial CDNA fragment containing both K and C domains of AGL24 was labeled with DIG by RNA labeling mix (Roche Diagnostics, Mannheim, Germany). Samples were hybridized at 45°C overnight and washed at 45°C. Sections were observed using a Zeiss Axiopt microscope attached with a digital camera for image acquisition.

Construction of truncated AGL24 plasmids
Fragments containing a series of AGL24 domains were amplified from AGL24 cDNA. In all cases, amplified PCR fragments were digested with EcoRI and Sall and fused to GAL4-AD of pGAD424 (Matchmaker two-hybrid system, Clontech Laboratory Inc.). For constructs, AGL24-MIK (MFE, CRS), AGL24-IC (IFE, CRS), AGL24-KC (KFE, CRS), AGL24-MIK (MFE, KRS), AGL24-M (MFE, IRS), AGL24-IK (IFE, KRS), AGL24-M (MFE, MRS), AGL24-I (IFE, IRS), AGL24-K (KFE, IRS), AGL24-C (CFE, CRS) and AGL24-KCA7 (KFE, IRS), the following primers were used:
MFE: 5'-CCGAATTCATGGGCGAGAGAGATAAGGA-3'
MRS: 5'-ATGTCGACGAGAAGAGGTCCCGGG-3'
IFE: 5'-GGCAATTCTTCCACACGAAATGAGAGAC-3'
IRS: 5'-TTGTCCGCTACTCCGGAGATGAGTAG-3'
KFE: 5'-GGCAAATCCATGTCACTTGCAFAGGGG-3'
KRS: 5'-ATGTCGACTCCTCCGGATCCGATAAGA-3'
CFE: 5'-GGGAAATTCTCAGGAAATGGTGGGATGGA-3'
CRS: 5'-TAGTCGACCTTCCCAAGATGGAAGCCCAAG-3'
KIFE: 5'-GGCAATTCCATTGGAACATTTAGAAAAGAG-3'

Subcellular localization analysis with the GFP reporter
AGL24 coding sequences were amplified by PCR with the primers AGL24M1 (5'-CTTCAATGGCAGAAGAGAAGA TAGG-3') and AGL24R1 (5'-TACTCGAGCCCAAGATGGAAGCCCAAG-3') that contain Ncol and Xhol sites, respectively. The digested fragment was inserted into the plant GFP expression vector, pTH2Xa (Kohchi et al. 2001) to make a translational fusion with a GFP. The fragment containing DNA for the fusion protein and the 35S promoter was inserted into the pHII12 vector (Jefferson et al. 1987), replacing theUidA gene to make a construct for stable transformation. The construct was then transformed into Arabidopsis via Agrobacterium (C588r') by vacuum infiltration (Bechtold and Pelletier 1998). Transformants were selected in MS plates containing kanamycin at a final concentration of 30 µg ml⁻¹. So that roots and hypocotyl could be observed, transgenic plants were grown on MS plates without sucrose for 4 d and stained with propidium iodide (10 µg ml⁻¹). Shoot apex was embedded in 5% agar and sliced by a microscissor into 100 µm thick sections. GFP fluorescence was visualized using a confocal laser scanning microscope (LSM 510, Zeiss). The FITC channel was used for GFP, and the rhodamine channel was used for propidium iodide and autofluorescence.

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