MARIS plays important roles in Arabidopsis pollen tube and root hair growth

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Abstract  In flowering plants, male gametes are delivered to female gametophytes for double fertilization through pollen tubes. Therefore, pollen tube growth is crucial for double fertilization. Despite its importance to sexual reproduction, genetic mechanisms of pollen tube growth remain poorly understood. In this study, we characterized the receptor-like cytoplasmic protein kinase (RLCK) gene, MARIS (MRI) that plays critical roles in pollen tube growth. MRI is preferentially expressed in pollen grains, pollen tubes and roots. Mutation in MRI by a Ds insertion led to a burst of pollen tubes after pollen germination. Pollen-rescue assay by pollen and pollen tube-specific expression of MRI in the mri-4 mutant showed that loss of MRI function also severely affected root hair elongation. MRI protein interacted with the protein kinase OXIDATIVE SIGNAL INDUCIBLE1 (OXI1) in the in vitro and in vivo assays, which functions in plant defence and root hair development, and was phosphorylated by OXI1 in vitro. Our results suggest that MRI plays important roles in pollen tube growth and may function in root hair elongation through interaction with OXI1.

Keywords: Arabidopsis; pollen tube; protein kinase; receptor-like cytoplasmic protein kinases; root hair


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INTRODUCTION

In flowering plants, male gametes (sperm cells) are delivered into female gametophyte (embryo sac) for double fertilization through pollen tube. Pollen tube is generated by vegetative cell of pollen grain after landing on stigma during pollination. Then the pollen tube invades into the stigma, elongates in the transmitting tract and finally enters the embryo sac where it releases the two sperm cells for double fertilization. Therefore, growth of pollen tube is essential for double fertilization in flowering plants (McCormick 1993, 2004; Krichevsky et al. 2007).

Pollen tube growth is a polarized-elongation event (Zerzour et al. 2009; Qu et al. 2013). Many genes are involved in the process, including the the protein kinase genes (Takeuchi and Higashiyama 2011). So far, several protein kinases have been identified as playing important roles in pollen tube growth. For example, the Arabidopsis receptor-like protein kinase AtPRK2 participates in the ROP1 GTPase-based signaling in pollen tube growth. Mutation in AtPRK2 leads to a significant reduction in pollen germination and drastic defect in pollen tube growth (Chang et al. 2013). ANXU1 (ANX1) and ANX2, which belong to the Catharanthus roseus RLK1-like (CrRLK1L) protein kinase subfamily, are required to maintain cell wall integrity of pollen tubes. The ANX1 ANX2 double mutation results in burst of pollen tube after pollen germination (Boisson-Dernier et al. 2009). In tomato, the pollen-specific receptor-like protein kinases LePRK1 and LePRK2 are localized in the plasma membrane and involved in pollen-pistil interactions (Salem et al. 2012). These results indicate that the receptor-like protein kinases (RLKs) are involved in many processes of pollen tube growth.

In general, the RLKs contain a signal peptide, an extracellular domain, a transmembrane domain and a cytoplasmic kinase domain (Wu and Zhou 2013). In Arabidopsis, there are more than 100 protein kinases which do not contain extracellular domain and are identified as receptor-like cytoplasmic protein kinases (RLCKs) (Shiu and Bleecker 2001a, 2001b, 2003; Wang et al. 2007). These RLCKs are classified into 12 subgroups. The members from the RLCK VIII subgroup share high identities in amino acid sequence with Solanum lycopersicum Pto-interacting 1 (SIP1) (Zhou et al. 1995; Shiu and Bleecker 2001a, 2001b, 2003; Anthony et al. 2006). Therefore, the 11 members from RLCK VIII subgroup share high identities in amino acid sequence with Solanum lycopersicum Pto-interacting 1 (SIP1) (Zhou et al. 1995; Shiu and Bleecker 2001a, 2001b, 2003; Anthony et al. 2006). In this group, PTI1-1, PTI1-2, PTI1-3 and PTI1-4 have been characterized (Anthony et al. 2006; Forzani et al. 2011). The four PTI1 proteins physically interact with the AGC (named after cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C) protein kinase OXIDATIVE SIGNAL INDUCIBLE1 (OXI1) and are phosphorylated by OXI1 in response to phosphatidic acid (PAs), H2O2, fliC22 and xylanase (Anthony et al. 2006; Forzani et al. 2011). Genetic study has shown that OXI1 is involved in plant defence and root hair development (Rentel et al. 2004). These results suggest that the PTI-like proteins participate in diverse biological processes and play important roles in signal transduction networks in responses to a wide spectrum of stimuli.
Recently the protein kinase MARIS (MRI) from PTI group was reported to play important roles in pollen tube growth and root hair elongation (Boisson-Dernier et al. 2015). Here we report our independent study of MRI that functions in pollen tubes and root hairs by characterizing another mri mutant allele, mri-4 which was generated by the transposon Dissociation (Ds)-insertion in MRI (At2g41970). MRI is highly expressed in mature pollen grains, pollen tubes and root tissues. The MRI protein labeled with the green fluorescent protein (GFP) was distributed in the cells with the strongest signals in the plasma membrane and cell wall regions at tips of pollen tubes. Mutation in MRI led to burst of pollen tubes at the early growth stage in in vitro culture. Pollen-rescue experiment showed that growth of the mutant root hairs also was severely affected. The yeast two-hybrid (Y2H), luciferase complementation imaging (LUC) and co-immunoprecipitation (Co-IP) assays showed that MRI interacted with OXI1 in vitro and in vivo. Kinase activity assays showed that MRI had kinase activity and was phosphorylated by OXI1 in vitro. Taken together, our results suggest that MRI plays important roles in pollen tube growth and may function in root hair elongation through interaction with OXI1.

RESULTS
Isolation and genetic analysis of mri-4 mutant
The mri-4 mutant was identified in a screening for male gametophyte-defective mutants from a collection of gene-trap and enhancer-trap Dissociation (Ds) insertion lines in Arabidopsis ecotype Landsberg erecta (Ler) (Sundaresan et al. 1995). The Ds insertion conferred a selective marker of kanamycin resistance and expression of a β-glucuronidase reporter gene (GUS). When the heterozygous mri-4 (mri-4/+ ) plants were self-pollinated, the resulting progeny exhibited a segregation ratio of approximately 1 kanamycin-resistant (KanR) (1,232) to 1 kanamycin-sensitive (KanS) (1,250) (Table 1), indicating that mri-4 was defective in gametophytic function. The Ds-carried GUS reporter gene was expressed in pollen grains and pollen tubes, distinguishing the mri-4 mutant pollen grains and pollen tubes from those of wild-type (Figure 1). To investigate whether the mri-4 mutation affected male or female gametophytic function, reciprocal crosses were performed between mri-4/+ and wild-type plants. When mri-4/+ plants were used as females to cross with wild-type plants, approximately 50% of the progeny seedlings (n=2,303) were resistant to kanamycin (Table 1). When mri-4/+ plants were used as males to cross with wild-type plants, all the resulting seedlings (n=1,130) were sensitive to kanamycin (Table 1). These results indicated that mri-4 mutation completely disrupted genetic transmission of the male mutant gametophytes, but did not affect female gametophytic function.

To investigate how the mri-4 mutation affected male gametophytic function, mri-4 mutant was introgressed into the quartet1 (qrt1) background (Preuss et al. 1994; Copenhaver et al. 2000). In the homozygous qrt1 mutant, the tetrads pollen grains remain attached together after maturation, but their functions are unaffected (Preuss et al. 1994; Copenhaver et al. 2000). Therefore, a quartet from the mri-4+/+;qrt1/qrt1 plant has two mri-4;qrt1 pollen grains (mutant) and two +;qrt1 pollen grains (representing wild-type). Morphology, viability and nuclear division of the mri-4 mutant pollen grains were examined by scanning electron microscope (SEM) (Figure S1A), Alexander staining (Alexander 1969) (Figure S1B) and 4’,6-diamidino-2-phenylindole (DAPI) staining (Figure S1C). No differences in the four pollen grains of the quartets from mri-4+/+;qrt1/qrt1 plants were observed, compared to the pollen grains from qrt1/qrt1 plants (Figure S1D–F), indicating that mri-4 mutation did not affect pollen formation.

Pollen germination and pollen tube growth in mri-4 mutant were then examined. Under the in vitro condition, 81.4% (1,782/2,189) of wild-type pollen grains germinated normally (Figure 2A) and produced normal pollen tubes (Figure 2B). In contrast, many pollen grains from mri-4/+ plants did not germinate normally or had burst after germination (Figure 2C). Only 39.4% (888/2,254) of the pollen grains from mri-4/+ plants germinated normally and produced normal pollen tubes (Figure 2A, C). 47.7% (1,075/2,254) of the pollen grains from mri-4/+ plants had burst after germination (Figure 2C), compared to 6.9% ruptured pollen tubes found among the wild-type pollen grains (Table 2). The pollen grains from complementated mri-4/+ plants germinated normally and produced normal pollen tubes like those from wild-type plants (Figure 2D). In qrt1 quartet assay, most of the qrt1 pollen grains from qrt1/qrt1 plants (representing wild-type) germinated normally and produced normal pollen tubes (Figure 2E), whereas the GUS-labelled mri-4+/+;qrt1 mutant pollen tubes in the quartets from the mri-4+/+;qrt1/qrt1 plants had burst after germination (Figure 2F, C). Furthermore, when a limited number of the quartets from mri-4+/+;qrt1/qrt1 plants were pollinated to wild-type pistils, half of the pollen tubes were abnormal at 48 h after pollination (Figure 2H). These results suggest that the mri-4 mutation leads to burst of pollen tubes in vitro and abnormal pollen tube growth in vivo.

| Table 1. mri-4 mutant is defective in male gametophyte |  |
|---|---|---|---|---|---|---|
| Crosses (female × male) | KanR seedlings (%) | KanS seedlings (%) | KanR/KanS | TEF | TEM |
| mri-4/+ selfed | 1,232 | 1,250 | 0.99 | NA | NA |
| mri-4/+ × WT | 1,179 | 1,124 | 1.05 | 100% | NA |
| WT × mri-4/+ | 0 | 1,130 | 0.00 | NA | 0 |

KanR, kanamycin-resistant; KanS, kanamycin-sensitive; TE, genetic transmission efficiency = (KanR/KanS) × 100%; TEF, female TE; TEM, male TE; NA, not applicable; Selfed, selfed pollination; WT, wild-type.
MRI is required for tip growth

Figure 1. The Ds-insertion conferred GUS-expression in mri-4 pollen
(A) A wild-type flower. (B) Wild-type pollen grains with pollen tubes. (C) A mri-4/+ flower showing GUS expression (blue stains) in the anthers. (D) The pollen grains from the mri-4/+ flower, showing GUS stains and burst of pollen tubes in the mri-4 pollen grains as indicated by black arrows. Pg, pollen grains; Pt, pollen tubes; WT, wild-type. Bars = 1 mm in (A, C), 20 μm in (B, D).

Molecular characterization of mri-4 mutant
Isolation of the Ds-inserted gene by thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (Liu et al. 1995) showed that the Ds element was inserted in the second intron of At2g41970, 148 bp downstream of start coden ATG (Figure S2A). The Ds insertion site was further confirmed by PCR using gene-specific and Ds border-specific primers (Figure S2B). Sequencing the resulting DNA fragments showed that the Ds insertion created a 7 bp repeat sequence (TTTCCCAC) adjacent to both ends of the Ds element (Figure S2A). To confirm that the phenotype of mri-4 mutant was caused by the mutation in MRI, a 4.3 kb full-length genomic DNA fragment of At2g41970, including the 2.1 kb promoter region upstream from start coden ATG, entire coding region and 3’ terminal region, was cloned into Ti-derived vector pCAMBIA1300 (CAMBIA, Queensland, Australia, http://www.cambia.org) and introduced into mri-4/+ plants. The resulting transgenic lines were selected using hygromycin and kanamycin double selection in T1 generation. 43 independent transformant lines were obtained. 24 out of the 43 lines were selected for further genetic analysis. The progeny seedlings from all the self-pollinated transgenic plants exhibited a segregation ratio of approximately 2 KanR:1 KanS (Table S1), indicating that defect in male gametophytic function was restored by introduction of wild-type MRI gene in the transgenic mri-4/+ mutant plants. The pollen grains from the independent complemented lines germinated normally and produced normal pollen tubes under the in vitro culture condition. Moreover, the germination rate of the pollen grains from the transgenic mutant plants was increased to 80%, similar to 81.4% of wild-type pollen (Figure 2A, D; Table 2). These results indicate that defect of pollen tube growth in mri-4 results from the MRI gene mutation.

MRI is expressed highly in pollen grains, pollen tubes and roots
Quantitative RT-PCR (qRT-PCR) assays showed that MRI was expressed highly in pollen grains, pollen tubes and root tissues (Figure 3A). To further investigate the expression pattern of MRI, a 2.1 kb promoter fragment of MRI was fused to GUS reporter gene in Ti-derived vector pCAMBIA1300 and introduced into wild-type plants. Totally 29 independent transgenic lines exhibited GUS activities. In T2 transgenic plants, GUS stains were observed strongly in mature pollen grains (Figure 3B), pollen tubes (Figure 3C), roots (Figure 3D), root hairs (Figure 3E) and anthers (Figure 3F). The GUS activity was detected weaker in cotyledons (Figure 3D), but not in hypocotyls (Figure 3D) and leaves (Figure 3G).

MRI-GFP was distributed in the cell with the strongest signal in plasma membrane of growing pollen tubes and root hairs
To determine the subcellular localization of MRI protein, the pPPME1:MRI-GFP and pLAT52:MRI-GFP constructs were generated for MRI expression under the control of pollen/pollen tube-specific promoters PPME1 and LAT52 (Muschietti et al. 1994; Tian et al. 2006), respectively. Both constructs were subcloned into vector pCAMBIA1300 and introduced into mri-4/+ plants. Genetic analyses showed that both pPPME1: MRI-GFP and pLAT52:MRI-GFP constructs could complement mri-4 phenotypes (Tables S2, S3), indicating that MRI-GFP protein function normally in pollen grains and pollen tubes. The GFP signals were observed in cytoplasm of the pollen grains (Figure 4A, B) and elongating pollen tubes (Figure 4C–E) from pLAT52:MRI-GFP transgenic mri-4 plants. The strongest signals were detected in the tip areas of growing pollen tubes (Figure 4F–H), which were overlapped with the signals of membrane-specific dye FM4-64 (Figure 4C, D). Similar results
Figure 2. The mri-4 mutation affected pollen tube growth

(A) Comparison of in vitro germination rates of pollen grains from wild-type (Ler), mri-4/+ and complemented mri-4 plants (Comp). (B–D) The in vitro germination of pollen grains from wild-type (B), mri-4/+ (C) and complemented mri-4 (D) plants. (E–F) The in vitro germination of pollen grains from qrt1/qrt1 (E) and mri-4/++;qrt1/qrt1 (F) plants. (G) Quantitative analysis of pollen tube growth in the quartets from the mri-4/++;qrt1/qrt1 plants. (H) The in vivo growth of the pollen tubes in a quartet from mri-4/++;qrt1/qrt1 plant. The black arrow indicates the pollen grains that did not germinate. The white arrows indicate the ruptured or abnormal pollen tubes. Bars = 30 μm in (B–F) and (H).
Table 2. *In vitro* germination of pollen grains from wild-type, mri-4/+ and complemented mri-4 mutant (mri-4/mri-4;gMRI/gMRI) plants

<table>
<thead>
<tr>
<th>The plant genotypes</th>
<th>Rates of pollen grains (%)</th>
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<tr>
<td></td>
<td>With normal pollen tubes</td>
<td>With burst pollen tubes</td>
<td>Failed to germinate</td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>81.4 ± 2.8</td>
<td>7.7 ± 2.1</td>
<td>10.9 ± 2.2</td>
<td></td>
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<tr>
<td>mri-4/+</td>
<td>39.4 ± 4.1</td>
<td>47.7 ± 2.7</td>
<td>12.9 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>mri-4/mri-4;gMRI/gMRI</td>
<td>80.1 ± 3.5</td>
<td>8.5 ± 4.8</td>
<td>11.4 ± 2.7</td>
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All results represent the average of three biological replicates with the standard deviation. For each independent replicate, at least 300 pollen grains were examined. gMRI, the cloned genomic DNA fragment of MRI gene.

Figure 3. Expression patterns of MRI

(A) Expression of MRI in different tissues, as revealed by qRT-PCR. ACT2 expression levels were used as the internal controls for RNA quantitative normalization. (B–G) The pMRI:GUS assays, showing the GUS activities in the transgenic mature pollen grains (B), pollen tubes (C), seedling (D), root (E) and inflorescence (F), but not in leaf (G). An, anthers; Cot, cotyledon; Hc, hypocotyl; Pg, pollen grain; Pt, pollen tube; Rh, root hair. Bars = 25 μm in (B) and 100 μm in (C), 1 mm in (D–F) and 0.5 mm in (G).
were obtained from root hair assays. MRI-GFP signals were also detected in the cytoplasm and plasma membrane at the tips of growing root hairs (Figure 4I, J) when it was expressed in root hairs under control of the root hair-specific promoter AtEXPA7 (Lin et al. 2011). Fluorescence intensity measurement (arbitrary units) across pollen grains, shank regions of pollen tubes and root hairs (Figure 4A, C–F) were further performed to investigate whether the signals were correlated to plasma

Figure 4. The MRI-GFP protein was distributed in the cells with higher intensity signals in the plasma membrane and cell wall regions at the tips of pollen tubes and root hairs

(A) The fluorescent images of a transgenic mature pollen grain. (B) The fluorescent images of a germinating transgenic pollen grain with emerging pollen tube. (C) The fluorescent images of a growing transgenic pollen tube at the early stage. (D) The fluorescent images of a growing transgenic pollen tube at the later stage. (E) The fluorescent images of a root hair. (F–J) The fluorescence intensity distribution in the crossed regions (red lines) of (A–E), respectively. The highest GFP fluorescence intensities (measured by arbitrary units, AU) were detected in the plasma membrane and cell wall regions at the tips of pollen tube (D, I) and root hair (E, J). Bars = 5 μm.
membrane. The results showed that the highest fluorescence intensities were correlated to the regions of plasma membranes and cell walls (Figure 4G–K). To further investigate whether the signals were in plasma membrane or cell wall, a plasmolysis assay was performed by transient expression of MRI-GFP fusion protein in onion epidermal cells under control of 35S promoter (p35S: MRI-GFP). The stronger GFP signals were detected in the most outer region related to plasma membrane. Taken together, all of these results indicate that MRI-GFP is likely localized in both cytoplasm and plasma membrane in growing pollen tubes and root hairs.

**Pollen-rescue assay revealed that mri-4 mutation also affected root hair growth**

The expression pattern analysis indicates that MRI is expressed in roots and root hairs (Figure 3). Therefore, we further investigated the roles of MRI in root and root hair development. Due to complete loss of male gametophytic function in mri-4, homozygous mri-4 plant was not naturally available. Therefore, pollen-rescue was used to generate homozygous mri-4 plants for studying the impact of mri-4 on root and root hair development, using the promoter of the pollen and pollen tube-specific gene AtPPME1, which encodes pectin methyltransferase 1 and was identified as being expressed specifically in pollen and pollen tubes (Tian et al. 2006). The expression cassette pPPME1: MRI-GFP was generated in pCAMBIA1300 and introduced into mri-4/+ plants. 23 independent pPPME1: MRI-GFP transgenic plants were obtained. The kanamycin segregation analysis of the T2 transgenic plants showed that the function of the mri-4 male gametophytes had been fully restored (Figure 2C; Table S2). The pollen-rescued mri-4 homozygous plants (PRmri) were selected from the progeny of self-pollinated transgenic mri-4/+ plants. qRT-PCR analysis showed that expression levels of MRI in root tissues of PRmri plants were significantly reduced, compared to that in wild-type root tissues (Figure 5A). Root hairs of the transgenic mri-4 homozygous plants were obviously shorter than wild-type root hairs (Figure 5B–E). Morphological analyses showed that the root hairs of the PRmri plants appeared severely abnormal (Figure 5C–D), compared to those of wild-type plants (Figure 5E). These results suggest that loss of MRI function also affects root hair growth.

**MRI is a member of Arabidopsis PTI1-like protein kinase family**

MRI is encoded by At2g41970 and identified as a putative protein kinase of 365 amino acids with an estimated pl 8.3 and a molecular weight of 39 kDa. The basic local alignment search tool-protein (BLASTP) analysis using the full length amino acid (aa) sequence of MRI showed that MRI shared 63% amino acid sequence identity with the tomato SIPT1 (Zhou et al. 1995) and high amino acid sequence similarities with PTI1-like kinases from other higher plant species (Dissanayake et al. 2004; Tian et al. 2004; Herrmann et al. 2006) (Figure 5A). It has been reported previously that the PTI1 kinase family in Arabidopsis has 10 members (Anthony et al. 2006; Forzani et al. 2011). In this study, we found one more PTI1-like kinase, namely At1g48220, which shared 67% amino acid sequence identity with the tomato SIPT1 and 43% amino acid sequence identity with MRI, respectively. Considering that PTI1 to PTI8-7 have been named previously (Anthony et al. 2006; Forzani et al. 2011), we named At2g41970 protein as PTI-5, and the other six members as PTI-6 to PTI-11, respectively (Figure S5). Recently, however, At2g41970 protein was published as MARIS (MRI) (Boisson-Dernier et al. 2015). Therefore, we renamed the PTI-5 as MRI. The MRI has 53% amino acid sequence identity with PTI-1, 52% with PTI-2, 55% with PTI-3, 53% with PTI-4, 47% with PTI-6, 54% with PTI-7, 43% with PTI-8, 52% with PTI-9, 50% with PTI-10 and 50% with PTI-11, respectively. The results indicate that MRI belongs to Arabidopsis PTI1 family that has 11 members (Figure S4).

RT-PCR assays showed that MRI, PTI-3 (At3g539350) and PTI-7 (At2g43230) were highly expressed in mature pollen grains and root tissues (Figure S5A). Furthermore, PTI-2, PTI-3, PTI-4 and PTI-7 share high amino acid sequence identities with MRI. To test whether they had the same biochemical function as MRI, we performed complementation of mri-4 mutant by expression of these genes in mri-4/+ mutant plants under control of the pollen-specific LAT52 promoter. The results showed that these genes could not complement the phenotypes of mri-4/+ mutant (Tables S4–S7), implying that the roles of MRI in growth of pollen tube may not be replaced by its homologous proteins tested.

**MRI interacts with OXI1**

The OXI1 and its closest homolog AGC2-2 in AGC protein kinase family have been identified as positive regulators that interact with PTI-1, PTI-3 and PTI-4 in Arabidopsis defence process (Anthony et al. 2006; Forzani et al. 2011). MRI shares 67% and 66% amino acid sequence identities with PTI-2 and PTI-4, respectively (Figure S3). To investigate whether MRI could interact with OXI1 and AGC2-2, yeast two-hybrid assays (Y2H) were performed. The yeast cells co-transformed with construct pairs pGBK7-TIT1-5/pGADT7-OXI1 and pGBK7-TIT1-5/pGADT7-AGC2-2 could grow on the medium-stringency selective media (DOD/Trp-Leu) and high-stringency media (QDO/Trp-Leu-His-Ade), respectively. These results indicated that MRI interacted with both OXI1 and AGC2-2 in the yeast, respectively (Figure 6A).

The interaction between MRI and OXI1 were further examined in tobacco leaves by luciferase complementation imaging (LCI) assay. In the assay, the constructs MRI-NLuc and Cluc-OXI1 were generated to test the interaction, while the construct combinations of MRI-NLuc/Cluc-OXI1 and Cluc-NLuc and Cluc-oxi1 were used as negative controls. The results showed that MRI also interacted with OXI1 in tobacco leaves (Figure 6B). The co-immunoprecipitation (Co-IP) assay was further applied to examine the interaction of MRI with OXI1. In the assay, the transgenic Arabidopsis plants expressing Myc-tagged MRI and Flag-tagged OXI1 driven by G35S promoter (pSuper: MRI-Myc and pSuper: Flag-OXI1) were generated. MRI-Myc and Flag-OXI1 fusion proteins were immunoprecipitated from plant extracts using anti-Myc IgG and anti-Flag IgG antibodies, respectively. The MRI-Myc and Flag-OXI1 were detected in the immunocomplex from the transgenic Arabidopsis. In contrast, no signal with anti-Flag IgG was detected in the negative control lane containing only MRI-Myc (Figure 6C). In summary, all the results indicate that MRI interacts with OXI1 in yeast, plant cell and in vitro assays.
MRI was phosphorylated by OXI1 in the in vitro assay

Previous studies showed that OXI1 phosphorylated PTI1-2 and PTI1-4 in vitro (Anthony et al. 2006; Forzani et al. 2011). To examine whether MRI could be phosphorylated by OXI1 in vitro, the purified His-tagged MRI and OXI1 recombinant proteins were incubated with [$\gamma^{32}$P]-ATP. As shown in Figure 7A lane 2, the OXI1 band exhibited a strong auto-phosphorylation signal. When the OXI1 was incubated with the generic kinase substrate MBP, MBP also exhibited a strong phosphorylation signal (Figure 7A, lane 3), indicating that OXI1 has phosphorylation activity. MRI did not have auto-phosphorylation activity (Figure 7A, lane 4), but when it was incubated with the generic kinase substrate MBP, MBP also exhibited phosphorylation signal (Figure 7A, lane 6).

Figure 5. The PRmri plant had shorter root hairs compared to wild-type plant

(A) The quantitative RT-PCR assay showed that expression of MRI in the PRmri roots was significantly reduced, compared to that in wild-type. (B) Comparison of root hair lengths between PRmri and wild-type plants. The root hairs in the differentiation zones were measured (mean ± SE, n > 500 from at least 30 individual roots). Data are representative of three independent experimental replicates. (C–E) The roots from PRmri-2 (C), PRmri-3 (D) and wild-type (E) plants. The arrows indicate the shorter root hairs. Bars = 0.5 mm.
Furthermore, the auto-radiographic signal in MBP was significantly reduced when the kinase-inactive mutant protein MRI\textsuperscript{K100E} was used in the assays (Figure 7A, lane 7). Moreover, when OXI1 and MRI were incubated together, the MRI band exhibited a strong phosphorylation signal (Figure 7B, lane 3), indicating MRI was phosphorylated by OXI1. Taken together, these results suggest that MRI is a functional protein kinase and may be the substrate of OXI1.

**DISCUSSION**

**MRI is crucial to pollen tube growth**

We report here functional characterization of MRI, which is involved in the pollen tube growth. MRI encodes a putative protein kinase that shares high similarity with other PTI family proteins (Anthony et al. 2006; Forzani et al. 2011; Figure S3). The genetic data suggested that mri-4 was a male gametophyte-defective mutant. Microscopic observation showed that mri-4 did not affect pollen formation, indicating that MRI does not involve in pollen development. In the in vitro and in vivo growth assays, mri-4 pollen tubes had burst and grew abnormally, suggesting its crucial roles in pollen tube growth. MRI is localized in cytoplasm of pollen tubes and partially co-localized with membrane-specific dye in plasma membrane at tips of pollen tubes, which is consistent with the data reported by Boisson-Dernier et al. (2015). Moreover, the MRI-GFP signals increases dramatically during pollen tube growth, particularly at the tip of pollen tube. The dynamic changes suggest that MRI protein may be involved in the growth and development of pollen tubes.
biosynthesis of new plasma membrane and secretion of cell wall materials.

So far, several mutants have been identified to exhibit pollen tube burst phenotype, for example vanguard 1 (vgd1) (Jiang et al. 2005). VGD1 encodes a pectin methylesterase (PME) that involves in cell wall modification. Loss of VGD1 function caused burst of pollen tubes in vitro and retarded growth of pollen tubes in vivo, indicating that alternation of cell wall integrity causes pollen tube burst in vitro and affects pollen tube growth in vivo. The mri-4 exhibited a phenotype similar to
that of vgd1. Furthermore, studies have showed that the PMEs are transported to the tips of pollen tubes during tube elongation (Tian et al. 2006). This raises a question whether MRI is also related to cellular transportation or cell wall biosynthesis. So far, we did not have any evidence to address this question. Nevertheless, more studies are required to elucidate the mechanism of MRI in pollen tube growth.

As mentioned above, the anxi anx2 double mutation in receptor-like protein kinases ANXi and ANX2 causes pollen tube burst in the transmitting tract before reaching to embryo sacs, indicating that ANXi and ANX2 also function in maintaining of pollen tube integrity (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). Boisson-Dernier et al. (2015) recently reported that the point mutation R240C by substitution of the conserved arginine (R240) with cysteine in MRI can rescue the anxi anx2 pollen tube burst phenotype, indicating that MRI is likely related to ANXs. Furthermore, the mutation point is next to the conserved threonine (T239) in the kinase activation loop of At2g41970, namely MRI (Boisson-Dernier et al. 2015). Overexpression of the mutant protein MRI<sup>R240C</sup> in wild-type plants inhibited pollen germination, compared to overexpression of wild type MRI, suggesting that MRI<sup>R240C</sup> is a more active form of the MRI kinase. MRI has been proposed to function downstream of ANXs (Boisson-Dernier et al. 2015). Therefore, MRI may function in pollen tube growth through ANX signaling pathway.

**MRI is important for root hair elongation**

Expression of MRI in root and root hairs (Figure 3) implies its potential functions in roots. Our pollen-rescue assay showed that the pollen-rescued mri-4 plant (PRmri) was defective in root hair growth, demonstrating that MRI also plays important roles in root hairs. MRI also is expressed in the root tissues other than root hairs. However, we did not observe any defect in other root tissues except root hairs. Therefore, it probably is not required for the development of the other root tissues except root hairs.

Previous studies showed that mutants in a set of genes exhibited abnormal root hair phenotypes, similar to that in mri-4. For example, function-loss mutations in transcription factor gene maMYB, root hair-specific expansin gene ATEXPA7 and receptor-like protein kinase gene FERONIA (FER) all led to significantly shorter root hairs, respectively (Duan et al. 2010; Lin et al. 2011; Slabbaugh et al. 2011). Recently, MRI has been demonstrated as being a downstream component of FER signaling in root hairs. Expression of MRI in fer-4 mutant partially rescued the root hair-defective phenotype of fer-4 (Boisson-Dernier et al. 2015). Taken together, the results suggest that MRI is related to FER signaling in root hairs.

**MRI plays roles in root hair elongation possibly through interaction with OXI1**

OXI1 is a protein kinase from the AGC VIII subgroup. Mutations in OXI1 is involved in pollen tube growth. AGC2-2 is the closest homologue of OXI1. The AGC2-2 is ubiquitously expressed in mature pollen grains and other tissues (Figure S5B). Y2H assay showed that MRI also interacted with AGC2-2 in yeast (Figure 6). However, we did not have any agg2-2 mri or oxil mri double mutant available to address whether OXI1 or AGC2-2 actually function in pollen tube growth. Nevertheless, more studies are required to address the mechanisms of MRI underlying pollen tube growth as well as root hair development.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

The wild-type and mutant Arabidopsis thaliana plants used in this study were in Landsberg erecta (Ler) background. Mutant mri<sup>-4/+</sup> was screened from an Arabidopsis mutant pool of Ds insertion lines as described by Sundaresan et al. (1995). The seeds of mri<sup>-4</sup>/+; wild-type and all transgenic plants were surface-sterilized with 10% bleach for 10 min, then washed five times with sterile water, and plated on the Murashige and Skoog (MS) media with or without 50 mg/L kanamycin or 20 mg/L hygromycin. The plates were placed in 4°C for 2 d, and then transferred into growth chambers under conditions of 16 h light/8 h dark cycles at 22°C. The 7-d-old seedlings on MS media were transplanted into soil for further growth under the abovementioned conditions. Tobacco (Nicotiana benthamiana) plants used in this work were grown in soil under the cycles of 16 h light/8 h dark at 28°C.

**Phenotypic analyses**

The genetic analyses were performed as previously described (Jia et al. 2009; Cui et al. 2015). To examine pollen viability and nuclear division, the mature pollen grains were harvested and dipped in Alexander’s solution (Alexander 1969) and 4',6-diamidino-2-phenylindole (DAPI) buffer for 20 min, respectively. Then the pollen grains were spread on microscope slides and observed under a fluorescence microscope (Leica, DM2500, http://www.leica.com). For pollen in vitro germination assays, mature pollen grains were collected and spread on the pollen germination medium containing 10 mM CaCl₂, 10 mM Ca(NO₃)₂, 10 mM MgSO₄, 10 mM H₃BO₃, 1.8% sucrose and 0.1% agarose. After incubation in the darkness for 6 h at 25°C, pollen germination was examined as previously described (Jia et al. 2009; Cui et al. 2015).

**Molecular cloning of MRI and complementation experiments**

Isolation of the flanking sequences adjacent to Ds element by TAIL-PCR was carried out as previously described (Liu et al. 1995; Jiang et al. 2005). The genotypes of the mri<sup>-4</sup>/+ plants were confirmed by PCR using the primer pairs listed in Table S8. For complementation experiments, a 4.3 kb full-length genomic DNA fragment of MRI was amplified using the gene-specific primers (Table S8). The resulting fragment was cloned into pCAMBIA1300 vector. The full-length cDNA of MRI was amplified and cloned into the pCAMBIA1300 vector driven by a pollen-specific promoter PPME1 (Tian et al. 2006; Liu et al. 2007).
The constructs were then introduced into the heterozygous mri-4 plants (mri-4/+) using the Agrobacterium-mediated infiltration method (Bechtold and Pelletier 1998). The transformants were selected on MS media containing both 20 mg/L hygromycin and 50 mg/L kanamycin.

Quantitative RT-PCR analyses
Total RNAs were extracted from different Arabidopsis tissues using TRIzol kit (Invitrogen, Catalog R1051, California, USA, http://www.invitrogen.com). The first-strand cDNA synthesis was carried out in a 20 μL reaction mixture containing 1 μg of DNase-treated total RNAs using the superscript III reverse transcriptase (Promega, Wisconsin, USA, http://www.promega.com) and random primers (Promega) according to instructions from the manufacturer. Two nanograms of cDNA products were used in each RT-PCR reaction. Quantitative RT-PCR reactions were performed using an ABI PRISM 7500 Real-time PCR system (Applied Biosystems, California, USA, http://www.appliedbiosystems.com) with SYBR Green PCR Master mix (Applied Biosystems, PN 4309155) and gene specific primers (Table S8) following instructions of the supplier’s protocol.

GUS staining
The promoter activity of MRI was monitored by GUS staining as previously described (Sundaresan et al. 1995; Jiang et al. 2005). The tissues were immersed in GUS staining solution (1 mg/mL X-Glucuronic acid, 1 mM potassium ferricyanide, 10 mM EDTA, 0.1% Triton X-100, and 100 mg/mL chloramphenicol in 50 mM sodium phosphate buffer, pH 7.0) for 2–3 h at 37 °C in the dark. Then the stained samples were washed with water and clarified in ethanol/lactic acid solution, and then observed under a Leica microscope (Leica DM2500, http://www.leica.com).

Root hair observation
Lengths of root hairs in 5-d-old seedlings grown on MS medium were measured under a microscope. The data were analyzed using the ImageJ software (http://rsb.info.nih.gov/ij). To ensure the data from different samples are comparable, all the root hairs from the same root region (1.7–2.0 mm from the root tip) were measured. Three plants were randomly selected for measurement from each genotype and three independent experimental replicates were performed.

Subcellular localization of MRI protein
The MRI coding sequence was cloned into the pCAMBIA1300 vector to generate MRI-GFP fusion protein expression cassette under control of the pollen-specific promoter LAT52 (Muschietti et al. 1994) or root hair-specific promoter AtEXPAT7 (Lin et al. 2011). The resulting constructs pLAT52-MRI-GFP and pAtEXPAT7-MRI-GFP were then introduced into mri-4/+/ and wild-type plants. The germinated pollen grains and roots were mounted in FM4-64 solution or distilled water under glass coverslips. Confocal laser scanning microscopy (CLSM) data were collected using a Zeiss LSM510 META microscope (Carl Zeiss, http://www.zeiss.com). The GFP signals at 505−530 nm were collected using the excitation light at 488 nm. The FM4-64 signals were excited at 543 nm and collected at 585–615 nm. The images were captured with the LSM510 image acquisition software (Carl Zeiss). The transient expression assay in onion epidermal cells was performed as described previously (Cui et al. 2015).

Yeast two-hybrid assay
The yeast two-hybrid assay was performed using Gal-4 system vectors (Clontech, California, USA, http://www.clontech.com). The full-length coding sequences of Arabidopsis MRI, OXI1, AGC2-2 and PTI1-3 were amplified by PCR using the primers listed in Table S9, respectively. The MRI and PTI1-3 CDNA fragments were cloned into the pGBK7T vector. The OXI1 and AGC2-2 cDNA fragments were cloned into the pGADT7 vector. The resulting constructs were co-transformed into yeast strain AH109 and examined following the instructions in the Yeast Protocols Handbook from the suppliers (Clontech, CA, USA, http://www.clontech.com/).

Firefly luciferase complementation imaging assay
The coding sequence of MRI was fused to upstream of N-Luc in the pCAMBIA-NLuc vector, and OXI1 was fused to downstream of C-Luc in the pCAMBIA-CLuc vector. The resulting constructs were transformed into Agrobacterium strain GV3101, and then infiltrated into Nicotiana benthamiana leaves. After 2 to 4 d, 1 mM luciferin was sprayed on the leaves, and the tobacco plants were then placed in the dark for 5 min. A low-light cooled charge-coupled device camera (ikon-L936, Andor Tech, Delaware, USA, http://www.andor.com) was used to capture the LUC signals. The camera was cooled to −90 °C and used to measure the relative LUC activity as described by Hua et al. (2012).

Co-immunoprecipitation assays
The coding sequence of MRI was fused to the N-terminal of Myc, and the coding sequence of OXI1 was fused to the C-terminal of Flag in the pCAMBIA1300 vector, respectively. The resulting constructs were transformed into Arabidopsis Ler plants. The transgenic plants from both MRI-Myc and Flag-OXI1 were selected in MS media containing 20 mg/L hygromycin. Then total proteins from the transgenic plants were extracted in the extraction buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 1 mM PMSF and 1× protease inhibitor cocktail (Roche, Basel, Swiss, http://www.roche.com). Then the extraction mixtures were centrifuged at 13,000 g for 35 min at 4 °C. Anti-Myc agarose (20 mL) or anti-Flag agarose (20 mL) (Sigma-Aldrich, Missouri, USA, http://www.sigma-aldrich.com) was incubated with the protein extract for 3 h at 4 °C. After washing with 100 mL of PBS buffer for five times, the immunoprecipitating products were examined by immunoblot analysis with anti-Myc or anti-Flag antibodies.

Recombinant fusion protein production
The coding sequences of MRI and OXI1 were amplified and cloned into vector pET30a(+) to express recombinant proteins fused with His-tag (GE Healthcare, Buckinghamshire, UK, http://www.gehealthcare.com). To generate the mutation form of MRI and OXI1 proteins, site-directed mutagenesis (see Table S8 for primer information) was performed as previously described (Wang et al. 2011), and the mutations were verified by sequencing. All the resulting constructs were transformed into E. coli strain BL21(DE3). The recombinant proteins were induced and purified with Ni sepharose 6 fast-flow (GE
Protein kinase assays
The kinase activity assays were performed as described previously (Wang et al. 2011). One microgram of fusion proteins and 5 μg MBP were incubated in kinase reaction buffer containing 50 mM Tris-Cl (pH 7.5), 1 mM DTT, 10 mM MgCl₂, 10 mM MnCl₂, 50 μM ATP, and 1 μCi of [γ-32P] ATP in a final volume of 20 μL. The reaction mixtures were incubated for 30 min at 30°C, then stopped by adding equal volume of 2 x SDS sample buffer, and then heated at 95°C for 5 min. The samples were then fractionated in a 12% (w/v) SDS-PAGE and autoradiographed to reveal the phosphorylation signals.

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AUTHORS CONTRIBUTIONS

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-3040.2013.02484.suppinfo

Figure S1. The mri-4 mutation did not affect pollen development
Figure S2. Molecular characterization of mri-4 mutant
Figure S3. Assay for subcellular localization of the MRI protein in onion epidermal cells
Figure S4. MRI belongs to Arabidopsis PTI1-like protein kinase family
Figure S5. Comparison of MRI expression patterns with those of the Arabidopsis PTI1 genes and OXI1 by RT-PCR arrays
Table S1. Segregation of the seedlings from the self-pollinated complemented mri-4/+ plants
Table S2. The segregation of the progeny seedlings from self-pollinated pollen-rescued (pPPME1:MRI-GFP transgenic) mri-4/+ plants
Table S3. The segregation of the progeny seedlings from self-pollinated pLAT52:MRI-GFP transgenic mri-4/+ mutant plants
Table S4. The segregation of the progeny seedlings from self-pollinated pLAT52:PTI1-2-GFP transgenic mri-4/+ mutant plants
Table S5. The segregation of the progeny seedlings from self-pollinated pLAT52:PTI1-3-GFP transgenic mri-4/+ mutant plants
Table S6. The segregation of the progeny seedlings from self-pollinated pLAT52:PTI1-4-GFP transgenic mri-4/+ mutant plants
Table S7. The segregation of the progeny seedlings from self-pollinated pLAT52:PTI1-7-GFP transgenic mri-4/+ mutant plants
Table S8. The sequences of the primers used in this study
Table S9. The sequences of the primers used in the assays for interaction of MRI with OXI1