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Spatial and temporal AtRMR1 expression patterns in *A. thaliana*

Sequence analysis revealed that the *A. thaliana* genome harbors six RMR homologues, named AtRMR1 to AtRMR6 (Fig. S1), that display 29–38% amino acid sequence homology to one another. RMR homologues that are encoded by JR700 and JR702, which are referred to here as AtRMR1 and AtRMR2,

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AtRMR5   --MNYSWITIMSLLVICKLASA-KVVLIGKNTILSFDDVEATFTPIVRNSGECGILYVAE 57
AtRMR6   ------------------------------MFS--------APVVRRSGEYGLLYAAE 20
AtRMR4   --MIRSSIVILSLLLISHLVSA-KVLLIGNSTSLSFDDVEATFTPMIKRSDQGGVLYVAE 57
AtRMR2   MN--RALVLLLYVCTVSCLASS-KVILMRNNITLSFDDIEANFAPSVKGTGEIGVVYVAE 57
AtRMR3   MNLVVLLILTLLLFIVSYVVDAGQVILVDSNITRSFVDMEADFSPSVT-TVETGVVYVAE 59
AtRMR1   MRLVVSSCLLVAAPFLSSLLRVSLATVVLNSISASFADLPAKFDGSVTKNGICGALYVAD 60
                       .     .      ..   *  .  :     :  .   * :*.*:

AtRMR5   PLEACSDITN--MAEKRSKYRSSYVLIVLGGCSFEEKVRKAQKAGYKAAIVYNDGYDELL 115
AtRMR6   PLDACSYLTN--MAEKGSKFRPSYVLIVRGGCSFEEKIRNAQEAGYKAAIVYNDRYEELL 78
AtRMR4   PLDACSDLVNTVNVKNGTTVSPPYVLIIRGGCSFEDKIRNAQKAGYKAAIVYD--YED-- 113
AtRMR2   PLDACQNLMN--KPEQSSNETSPFVLIVRGGCSFEEKVRKAQRAGFKAAIIYDNEDRGTL 115
AtRMR3   PLNACRNLRN--KPEQSPYGTSPLVLIIRGGCSFEYKVRNAQRSGFKAAIVYDNVDRNFL 117
AtRMR1   PLDGCSPLLHAAASNWTQHRTTKFALIIRGECSFEDKLLNAQNSGFQAVIVYDNIDNEDL 120
         **:.*  : ::   :      .  .**: * **** *: :**.:*::*.*:*:.

AtRMR5   VPMAGNSSGVDIHGLLVTRASGEVLKGYADQDEMKLWLIP-GFGISSWSIMG-ITFISLL 173
AtRMR6   VRR--NSSGVYIHGVLVTRTSGEVLKEYTSRAEMELLLIP-GFGISSWSIMA-ITFVSLL 134
AtRMR4   ------------FGFLVS-TTGEVLKEYAGRTDFEVWLMP-SFETSAWSIMA-ISFISLL 158
AtRMR2   IAMAGNSGGIRIHAVFVTKETGEVLKEYAGFPDTKVWLIP-SFENSAWSIMA-VSFISLL 173
AtRMR3   SAMGGDSDGIKIQAVFVMKRAGEMLKKYAGSEEMEVMLVPPNTEDSVWSLYASIALILSL 177
AtRMR1   IVMKVNPQDITVDAVFVSNVAGEILRKYARGRDGECCLNP-PDRGSAWTVLA-ISFFSLL 178
              .. .    ..:*   :**:*: *:   : :  * *     * *:: . :::.  *

AtRMR5   AMSAILATCFVVRRHQIRQSVRDLPHGGQGLSCMPRDLLQSMPTEVYSGVLEESSTSVTC 233
AtRMR6   VISAVLASYFSVRRHRIRQHVRDLHHGGQGHSRMPKDLLQSMPTEVYTGVLEEGSTSVTC 194
AtRMR4   AMSAVLATCFFVRRHRVRR-RRILALNGNDFHRMPKSMIIRMPTTIFNGICDEATTSILC 217
AtRMR2   AMSAVLATCFFVRRHRIRR-RTSRSSRVREFHGMSRRLVKAMPSLIFSSFHEDNTTAFTC 232
AtRMR3   AIFCVMVTCVFFYRYCSTI-RNSTS----QFNGMCRRTVKAMPSVTFTCAKIDNTTGFSC 232
AtRMR1   LIVTFLLIAFFAPRHWTQWRGRHTR-----TIRLDAKLVHTLPCFTFTDSAHHKAG-ETC 232
          :  .:   .   *:                  :    :  :*   :.    . :    *

AtRMR5   AICIDDYCVGEKLRILPCKHKYHAVCIDSWLGRCRSFCPVCKQNPRTGNDVPPASETTPL 293
AtRMR6   AICIDDYRVGEILRILPCKHKYHAVCIDSWLGRCRSFCPVCKQNPRTGNDVPPASETTPL 254
AtRMR4   CICLENYEKGDKLRILPCHHKFHVACVDLWLGQRKSFCPVCKRDARSISTDKPPSEHTPF 277
AtRMR2   AICLEDYTVGDKLRLLPCCHKFHAACVDSWLTSWRTFCPVCKRDARTSTGEPPASESTPL 292
AtRMR3   AICLEDYIVGDKLRVLPCSHKFHVACVDSWLISWRTFCPVCKRDARTTADEPLATESTPF 292
AtRMR1   AICLEDYRFGESLRLLPCQHAFHLMCIDSWLTKWGTSCPVCKHDIRTETMSSEVHKRESP 292
         .**:::*  *: **:*** * :*  *:* **    : *****:: *:        :  .

AtRMR5   ISPSP------------NSITSLQS------------FYDLPIVVRVYL----------- 318
AtRMR6   ISPGP------------NSITSLQS------------FYDLPIVVRVYL----------- 279
AtRMR4   LSRTPSMTPTSSFLLSSSSTTPLQS------------SHELPISIRVDPSLPSTSMQPHT 325
AtRMR2   LSSAASS----------FTSSSLHSSVRSSALLIGPSLGSLPTSISFSPAYASSSYIRQS 342
AtRMR3   LSSSIAT----------SSLVCIDS----------PPLG---SSVSFSPAHVSSSFIHQF 329
AtRMR1   RTDTSTS----------------------------------------RFAFAQSSQSR---- 310
          :   :                                        .  :  : :

AtRMR5   ------------------------------------------------------------
AtRMR6   ------------------------------------------------------------
AtRMR4   VPMYLSHSRSHTSFQNGSNRFSRPIPVSRSSADLRNAVSQRSYNSPHQVSLPRFLHSRYT 385
AtRMR2   FQSSSNRRSPPISVSRSSVDLRQQAASPSPSPSQRSYISHMASPQSLGYPTISPFNTRYM 402
AtRMR3   VRSS------PMNGSRISENLRRQAS-PLQSSSQRSHLS-MKSSHSLGYSTMSPLNAMGM 381
AtRMR1   ------------------------------------------------------------


AtRMR5   ---------------------------------------------
AtRMR6   ---------------------------------------------
AtRMR4   HILGPGNASRSQVVGLLTSQREHSLHQNDSRRSFIHFASASSLPGW 431
AtRMR2   SPYRPSPSNASPAMAGSSNYPLNPLRYSESAGTFSPYASANSLPDC 448
AtRMR3   SPYRPYPSNASPGLFSSTNHLLS----NYTANTFSHFASAHSLPD- 422
AtRMR1   ---------------------------------------------
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Figure S1. **Sequence alignment of AtRMR homologues.** AtRMR homologues were aligned by using Clustal W1.62. Asterisks indicate conserved amino residues among the six RMR homologues. Numbers on the right indicate amino acid positions of the proteins. AtRMR1, At5g66160 (JR700); AtRMR2, At1g71980 (JR702); AtRMR3, At1g22670; AtRMR4, At1g09560; AtRMR5, At1g35630; AtRMR6, At1g35625.

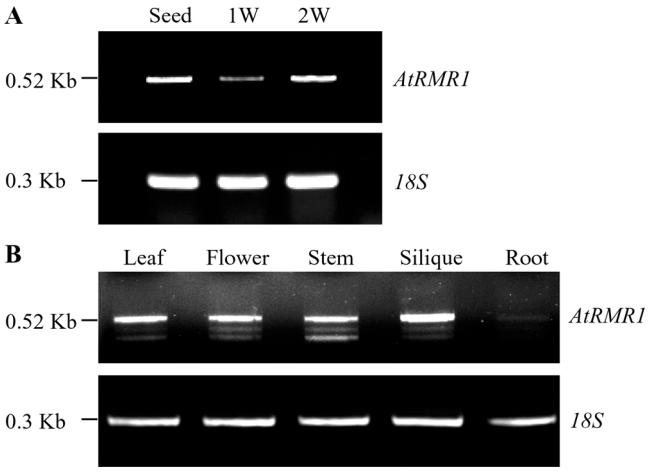

Figure S2. **Tissue-specific and temporal expression patterns of *AtRMR1* in *A. thaliana*.** Total RNA was extracted from plants at different growth stages (A) or from different tissues (B) and subjected to semiquantitative RT-PCR for *AtRMR1*. PCR products from 18S rRNA served as an internal standard. The nucleotide sequence of the PCR products (0.52 kb) was confirmed by sequencing. 1W, 1 wk; 2W, 2 wk.

respectively, were previously shown to be expressed in root tip cells of *A. thaliana* (Jiang et al., 2000). To understand the biological role of these AtRMRs in intracellular trafficking, we decided to specifically study AtRMR1, which is a polypeptide of 32 kD. First, we examined its temporal and tissue-specific expression patterns by semiquantitative RT-PCR analysis using *AtRMR1*-specific primers. Total RNA was prepared from imbibed seeds, 1- and 2-wk-old *A. thaliana* plants, and from leaves, flowers, stems, siliques, and root tissues. The 18S ribosomal RNA level served as a control. The *AtRMR1* transcript was detected at similar levels at different growth stages (Fig. S2 A). In addition, *AtRMR1* was expressed at similar levels in all of the different tissues except for in the roots, in which it was expressed at significantly lower levels (Fig. S2 B). Thus, *AtRMR1* is expressed in most *A. thaliana* cells regardless of the growth stage of the plant.

Isolation of genomic DNA

1 g of leaf tissues that were harvested from 3-wk-old *A. thaliana* (Columbia) and tobacco (*Nicotiana tabaccum*) plants were ground thoroughly in liquid N_2. Lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM EDTA, 0.5% [wt/vol] SDS, and 10 mM β-mercaptoethanol) was then added. After 1 h, the sample was centrifuged, and the supernatant was extracted by phenol/chloroform. Genomic DNA was precipitated with ethanol.

RT-PCR analysis

Total RNA was extracted from imbibed seeds, cotyledons from 1-wk-old plants, and leaf tissues from 2-wk-old plants by using the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA with Superscript II-RNase H reverse transcriptase (Invitrogen), and PCR was performed with the 5′-ATGAGACTCGTCGTCTCA-3′ and 5′-CGCGTACTTTCTCAAAAT-3′ primers for the LU of AtRMR1. The 5′-ATGATAACTCGACGGATCGC-3′ and 5′-CCTCCAATGGATCCTCGTTA-3′ primers for 18S rRNA were used for 30 and 16 cycles, respectively, at 94, 48, and 72°C for 30 s.

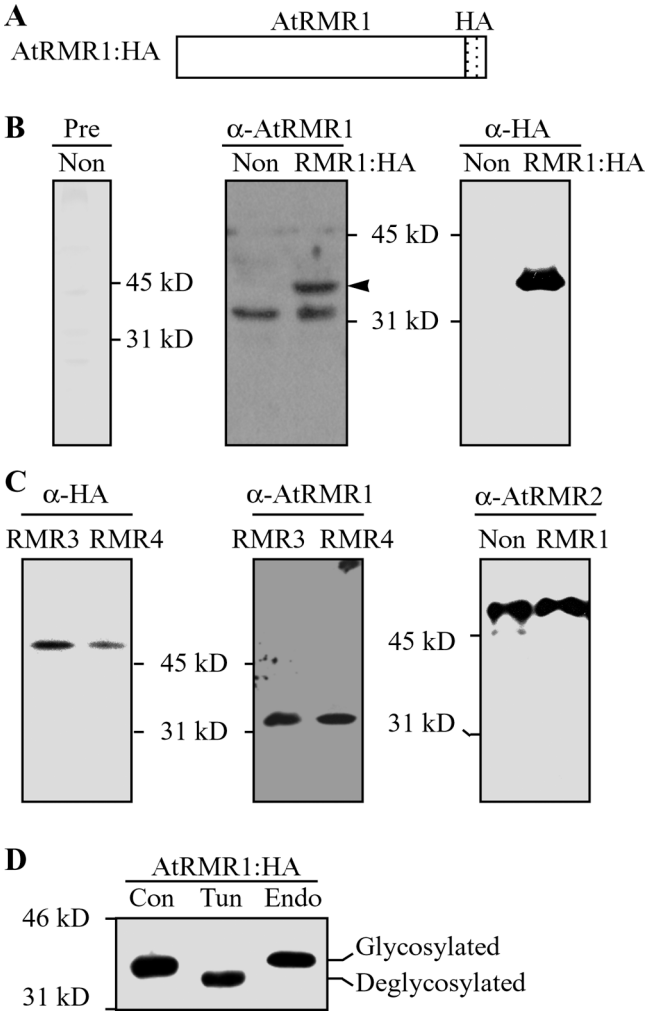

Figure S3. **Western blot analysis of endogenous and transiently expressed AtRMR1.** (A) Schematic depiction of the construct used. AtRMR1 was epitope-tagged with HA at their COOH terminus. (B) Western blot analysis. Protein extracts were obtained from untransformed (Non) and *AtRMR1-HA*–transformed (RMR1-HA) protoplasts and subjected to Western blot analysis using anti-AtRMR1 (α-AtRMR1) and anti-HA (α-HA) antibodies. As a control, preimmune serum (Pre) was used to stain the Western blot. Arrowhead indicates transiently expressed AtRMR1-HA. (C) Specificity of anti-AtRMR1 antibody. Protein extracts were prepared from untransformed protoplasts (Non) and protoplasts transformed with *AtRMR1-HA* (RMR1), *AtRMR3-HA* (RMR3), or *AtRMR4-HA* (RMR4) and were used for Western blot analysis using anti-AtRMR1 (α-AtRMR1) or anti-RMR2 (α-AtRMR2) antibodies. (D) AtRMR1-HA glycosylation pattern. Protoplasts were transformed with *AtRMR1-HA* and incubated in the presence (Tun) and absence (Con) of tunicamycin. Protein extracts that were obtained from untreated protoplasts were also digested with endoH (Endo). These protein extracts were subsequently analyzed by Western blot analysis using anti-HA antibody.

Characterization of anti-RMR1 antibody

First, we examined the specificity of the anti-AtRMR1 antibody using protein extracts that were obtained from wild-type plants and protoplasts transformed with *AtRMR1-HA* (Fig. S3 A; Jin et al., 2001). The antibody specifically recognized a protein band of 32 kD in wild-type protein extracts and two bands of 32 and 33 kD from protein extracts that were obtained from *AtRMR1-HA*–transformed protoplasts (Fig. S3 B). The 33-kD band was also specifically detected with the anti-HA antibody. In contrast, the preimmune serum did not detect any protein. These results indicate that the anti-AtRMR1 antibody specifically recognizes AtRMR1. As a control, we used anti-AtRMR2 antibody raised against the COOH-terminal peptide of AtRMR2 (Jiang et al., 2000). The anti-AtRMR2 antibody specifically detected a 48-kD band corresponding to that of AtRMR2 (Fig. S3 C). To further test the specificity of anti-AtRMR1 antibody, we expressed AtRMR3 and AtRMR4 tagged with HA in protoplasts and examined their reactivity with this antibody. Although the anti-HA antibody specifically detected bands of 46 and 47 kD, which corresponded to AtRMR3 and AtRMR4, respectively, the anti-AtRMR1 antibody did not recognize these bands (Fig. S3 C), indicating that the anti-AtRMR1 antibody is specific for AtRMR1. Next, we examined whether AtRMR1 is modified by glycosyla-

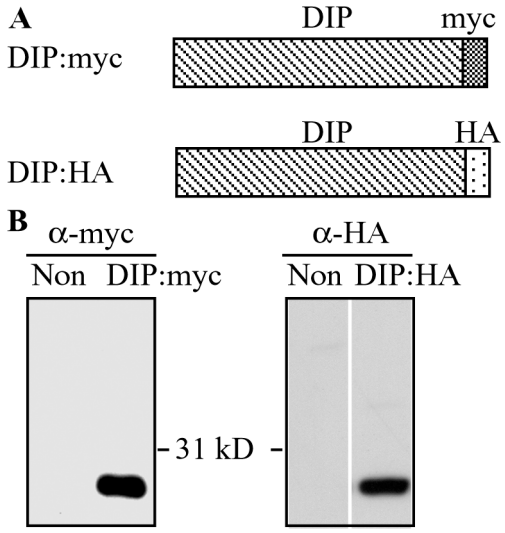

Figure S4. **Transient expression of epitope-tagged DIP in *A. thaliana* protoplasts.** (A) Schematic depiction of the constructs used. DIP was epitope-tagged with HA or myc at the COOH terminus. (B) Expression of myc- and HA-tagged DIP in protoplasts. Protein extracts were prepared from protoplasts that were transformed with *DIP-myc* or *DIP-HA* and were used for Western blot analysis using anti-myc (α-myc) or anti-HA (α-HA) antibodies, respectively. Non, untransformed protoplasts.

tion. Protein extracts were prepared from protoplasts expressing AtRMR1-HA in the presence and absence of tunicamycin, which is an inhibitor of NH_2 glycosylation (Leavitt et al., 1977). AtRMR1-HA that was prepared from tunicamycin-treated protoplasts migrated faster than that prepared from untreated protoplasts (Fig. S3 D). Thus, AtRMR1-HA is glycosylated in *A. thaliana* leaf protoplasts. Then, we examined the endoH sensitivity of the NH_2 glycan moiety on AtRMR1-HA (Kornfeld and Kornfeld 1985; Crofts et al., 1999). EndoH treatment did not change the mobility of AtRMR1-HA (Fig. S3 D), which suggests that the NH_2 glycan moiety of AtRMR1-HA is the Golgi-modified complex type.

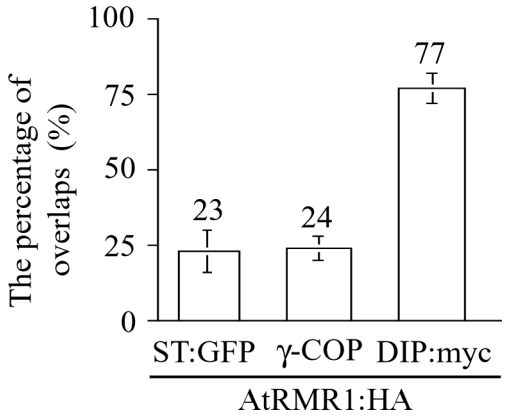

Figure S5. **Quantification of the overlap of AtRMR1-HA with ST-GFP, γ-COP, or DIP-myc.** The number of AtRMR1-positive punctate stains that colocalized with ST-GFP, γ-COP, or DIP-myc–positive punctate stains were counted to estimate the degree of overlap. More than 200 punctate stains were counted for each comparison. Three independent experiments were performed to obtain means and SEM (error bars).

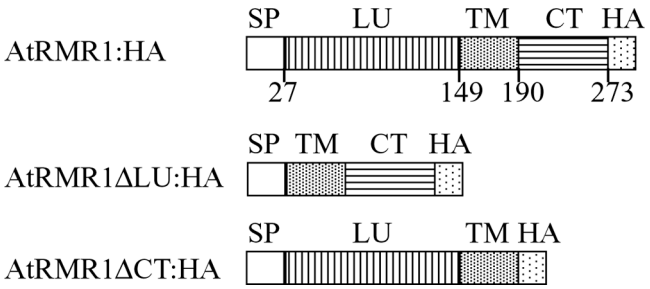

Figure S6. **Schematic depiction of the constructs used.** The LU and COOH-terminal ring H2 domains of AtRMR1 were deleted in AtRMR1ΔLU-HA and AtRMR1ΔCT-HA, respectively. Numbers indicate amino acid positions of AtRMR1. SP, leader sequence; LU, lumenal domain; TM, transmembrane domain; CT, ring H2 finger domain.

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