Ubiquitin-Related Modifiers of *Arabidopsis thaliana* Influence Root Development

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Abstract

Ubiquitins are small peptides that allow for posttranslational modification of proteins. Ubiquitin-related modifier (URM) proteins belong to the class of ubiquitin-like proteins. A primary function of URM proteins has been shown to be the sulfur transfer reaction leading to thiolation of tRNAs, a process that is important for accurate and effective protein translation. Recent analyses revealed that the Arabidopsis genome codes for two URM proteins, URM11 and URM12, which both are active in the tRNA thiolation process. Here, we show that URM11 and URM12 have overlapping expression patterns and are required for tRNA thiolation. The characterization of *urm11* and *urm12* mutants reveals that the lack of tRNA thiolation induces changes in general root architecture by influencing the rate of lateral root formation. In addition, they synergistically influence root hair cell growth. During the sulfur transfer reaction, URM proteins of different organisms interact with a thiouridylase, a protein-protein interaction that also takes place in Arabidopsis, since URM11 and URM12 interact with the Arabidopsis thiouridylase ROL5. Hence, the sulfur transfer reaction is conserved between distantly related species such as yeast, humans, and plants, and in Arabidopsis has an impact on root development.

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Introduction

Ubiquitins (Ub) are small peptides that allow posttranslational modification of proteins. Ubiquitylation is the reversible attachment of Ub to proteins involving activation, conjugation, and ligation of Ub via corresponding E1, E2, and E3 ligase activities, respectively [1]. While polyubiquitylation targets proteins to degradation via the proteasome, single ubiquitylation has non-proteolytic effects on cellular processes such as transcription, chromatin modifications, or vesicle dynamics. In addition to ubiquitin, a number of ubiquitin-like modifiers are present in most eukaryotes that are also able to tag proteins, usually in a transient manner [2–4].

In addition to Ub, ubiquitin-related modifiers (URMs) were identified that are not highly homologous to ubiquitin in respect to the amino acid sequence but share a \(\beta\)-grasp motif as typical structural feature of this type of protein. The primary identified function of URM and URM-related peptides in many different organisms such as archaea, yeast, and eukaryotes is as sulfur carriers in tRNA thiolation [5–10]. This process involves activation of URM by an E1-like protein such as Uba4p of yeast which adenylates URMs and transfers sulfur to the terminal glycine resulting in a thio-carboxylate. With the activity of thiouridylases such as Nsc2p and Ncs6p in yeast, the thiol group is then transferred onto uridine residues of tRNAs, a modification which is thought to increase translation efficiency [11]. The function of URMs in thiolation of tRNAs is reminiscent of sulfur transfer reactions in prokaryotes in the synthesis of molybdopterin and thiamine. The MoaD/ThiS proteins involved in this process are not homologous in sequence to URMs, yet also show the \(\beta\)-grasp motif [12]. Hence, URM-type proteins appear to have an activity different from other ubiquitin-related proteins and, because of their similarity to prokaryotic sulfur transfer systems, are considered to be evolutionary intermediates between prokaryotic sulfur transfer and eukaryotic ubiquitin-like protein conjugation systems [13]. In addition to the established role of URM proteins in tRNA thiolation, there is increasing evidence for a second role of URMs in urmylation, a protein modification similar to ubiquitylation in which URMs are conjugated to lysine of target proteins [14, 15].

Recently, two Arabidopsis genes, *URM11* and *URM12*, were identified encoding proteins that are involved in tRNA thiolation [16]. *URM11* and *URM12* show homology to URM proteins of other organisms and share the \(\beta\)-grasp motif and the terminal di-glycine motif typical for these proteins. In addition to *URM11* and *URM12*, the Arabidopsis homologs of the yeast E1-ligase Uba4p and thiouridylase Ncs6p were identified as CNX5/SIR1 and ROL5, respectively. Both these Arabidopsis proteins have been shown to be involved in the tRNA thiolation process. *cnx5/sir1* mutants show a severe growth defect, whereas the *rol5* mutant is mainly affected in root growth and shows changes in cell wall architecture. The enhanced severity of the *cnx5/sir1* mutant phenotype compared to *rol5* is likely caused by a general defect in sulfur transfer reactions in this mutant that also affects molybdopterin biosynthesis [16–19].
This work presents a more detailed characterization of URM11 and URM12 of Arabidopsis. Our data support the finding that URM11 and URM12 are involved in tRNA thiolation. The protein interactions of the URM proteins are conserved in plants, as they interact with the thiouridylase ROL5. Even though URM11 is expressed to a higher level than URM12, there is a significant synergetic interaction between the two proteins. Finally, the analysis of mutants including a urm11 urm12 double mutant shows that the lack of tRNA thiolation has an effect on the general root architecture but also on root cell development.

Results

Arabidopsis Possesses Two Proteins with High Sequence and Functional Similarity to Yeast Urm1p

Proteins with significant homology to ubiquitin-related modifier (URM) proteins have been identified in a number of organisms. The genome of Arabidopsis harbors two URM genes, At2g45695 and At3g61113, respectively which were termed URM11 and URM12 [16]. URM11 and URM12 are homologous to the yeast and human URM proteins, particularly in the C-terminal half including the terminal di-glycine motif essential for URM protein function. This suggests that the C-termius is less tolerant to variations in amino acid sequence. The two URM proteins of Arabidopsis share high homology to each other with an identity of 97% and a similarity of 91% (Figure 1; [16]).

The involvement of URM11 and URM12 in the sulfur carrier protein important for the thiolation of eukaryotic cytoplasmic transfer RNAs (tRNAs) was indicated by the successful complementation of the yeast ∆urm1 mutant defective in tRNA thiolation [8] with URM11 and URM12 [16]. To assess whether URM proteins fused to reporter proteins can be used to assess localization of URM proteins, complementation efficiency of the yeast ∆urm1 mutant by URM and GFP-URM proteins was compared. The ∆urm1 mutant transformed with cDNAs for URM11 or GFP-URM11 and GFP-URM12 constructs under the control of a constitutively active yeast promoter was analyzed for the presence of thiolated tRNAs. The binding of N-acryloylaminophenyl mercuric chloride (APM) to 2-thiouridine residues leads to the presence of thiolated tRNAs. The binding of N-acryloylamino control of a constitutively active yeast promoter was analyzed for URm11 and URM12 are active with an additional GFP reporter reaction in yeast. Furthermore, this experiment shows that Arabidopsis URM proteins are functional in the sulfur transfer process important for the thiolation of eukaryotic cytoplasmic transfer RNAs (tRNAs) was indicated by the successful complementation of the yeast ∆urm1 mutant defective in tRNA thiolation [8] with URM11 and URM12 [16]. To assess whether URM proteins fused to reporter proteins can be used to assess localization of URM proteins, complementation efficiency of the yeast ∆urm1 mutant by URM and GFP-URM proteins was compared. The ∆urm1 mutant transformed with cDNAs for URM11 or GFP-URM11 and GFP-URM12 constructs under the control of a constitutively active yeast promoter was analyzed for the presence of thiolated tRNAs. The binding of N-acryloylaminophenyl mercuric chloride (APM) to 2-thiouridine residues leads to the presence of thiolated tRNAs. The binding of N-acryloylamino

Figure 1. Homology between URM proteins of Arabidopsis and yeast. Alignment of the Arabidopsis URM11, URM12, and the yeast Urm1p. Identical positions are indicated in black, colons indicate conservative amino acid substitutions, and periods indicate similar amino acids.

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Arabidopsis URM Proteins Interact with ROL5

The protein network leading to tRNA thiolation has been investigated in detail in yeast. Within this network, Urm1p interacts with the thiouridylase Ncs6p [7], [8]. To get an insight into the degree of conservation of this network in Arabidopsis, URM11 and URM12 were tested for interaction with ROL5, the Arabidopsis thiouridylase and functional homolog of Ncs6p [19]. To this end, a yeast-two-hybrid experiment was performed using ROL5 as the bait protein and URM11 or URM12 as the prey proteins. For both URM proteins, an interaction with ROL5 was observed as cells grew on selective medium and resulted in GUS activity. Control experiments with the ROL5-containing bait vector and an empty prey vector or the empty bait vector with the URM11- or URM12-containing prey vectors revealed no autoactivation of any of the proteins (Figure 2C). The interaction of URM11 and URM12 with ROL5 provides further evidence for the conservation of the process of sulfur transfer leading to tRNA modification across a wide range of species.
with the empty second plasmid did not result in yeast growth and galactosidase activity. Transformation with only one of two constructs HA-URM11 and HA-URM12 interaction of URM11 and URM12 with ROL5, resulting in yeast growth. 

Bulk tRNA was extracted from wild-type (WT) or urm11-1 urm12-2 double mutants. A representative result of total RNA extracted from homozygous mutants revealed that the urm12-1 line still produced URM12 mRNA (data not shown). By contrast, both urm11-1 and urm12-2 lack detectable levels of gene expression of URM11 and URM12, respectively (Figure 4B).

To test whether the absence of URM expression has an effect on tRNA thiolation, tRNAs were isolated from wild-type and urm11-1 urm12-2 double mutant plants. Previously, a strong but not complete reduction in tRNA thiolation has been shown for the urm11-1 mutant [16]. As a control, tRNA of the rol5-1 mutant was isolated which was previously shown to lack thiolated tRNAs [19]. A shifted tRNA band, i.e. thiolated tRNAs, were observed only in the wild type but neither in rol5-1 nor in the urm11-1 urm12-2 double mutant (Figure 4C), indicating that this tRNA modification is impaired in the absence of URM11 and URM12.

To investigate the importance of URM11 and URM12 for plant development, T-DNA insertion lines of these loci were identified. For URM11, one insertion line (urm1-1) was used which contains a T-DNA insertion in the first intron. For URM12, two insertion lines were used: urm12-1 harbors the insertion 250 bp upstream of the start codon and urm12-2 in the first intron (Figure 4A, allele nomenclature according to [16]). RT-PCR experiments on total RNA extracted from homozygous mutants revealed that the urm12-1 line still produced URM12 mRNA (data not shown). By contrast, both urm11-1 and urm12-2 lack detectable levels of gene expression of URM11 and URM12, respectively (Figure 4B).

Mutations in the URM Genes Affect tRNA Modification and Root Development

To determine the significance of URM11 and URM12 for plant development, T-DNA insertion lines of these loci were identified. For URM11, one insertion line (urm11-1) was used which contains a T-DNA insertion in the first intron. For URM12, two insertion lines were used: urm12-1 harbors the insertion 250 bp upstream of the start codon and urm12-2 in the first intron (Figure 4A, allele nomenclature according to [16]). RT-PCR experiments on total RNA extracted from homozygous mutants revealed that the urm12-1 line still produced URM12 mRNA (data not shown). By contrast, both urm11-1 and urm12-2 lack detectable levels of gene expression of URM11 and URM12, respectively (Figure 4B).

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To investigate the importance of URM11 and URM12 for plant development, the urm11-1 urm12-2 double mutant was analyzed, since URM11 and URM12 appear to have a very similar if not identical activity and thus are likely to be functionally redundant. A defect in lateral root development was observed in the double mutant. After growth for ten days, double mutant seedlings had developed a lower density of lateral roots compared to the wild type (Figure 5A). No obvious defect or retardation in shoot development was observed in the double mutant. To test whether the lateral root phenotype is indeed caused by the urm11-1 and urm12-2 mutations, the double mutant line was complemented with a 35S:HA-URM11 or 35S:HA-URM12 construct. Complementation with either of the two constructs resulted in wild type-like lateral root formation (Figure 5A), confirming that the absence of URM11 and URM12 induces the reduction in lateral root formation and that both HA-URM proteins are functional.

The locus coding for the URM11 and URM12-interacting protein ROL5 was previously identified as a suppressor of the root hair formation mutant lxl [19]. Since ROL5, URM11, and URM12 are involved in the same process, we explored whether mutations in the URM genes have the same effect on lxl. As shown in Figure 5B, root hairs are regularly formed in wild-type

**URM11 and URM12 are Ubiquitously Expressed**

According to microarray data of the Genevestigator platform [21], URM11 and URM12 are expressed at all developmental stages of Arabidopsis. To investigate URM11 and URM12 expression patterns in more detail, the promoter sequences of URM11 and URM12 were fused to the GUS gene and transformed into Arabidopsis. Several independent transgenic lines were then screened in the T₂ generation for GUS activity at seedling and adult stage and representative examples are shown in Figure 3. At the seedling stage, the URM11 promoter induced homogenous GUS expression while URM12 promoter-induced GUS expression was mainly detectable in the vasculature. In adult plants, GUS activity was found in most tissues, with URM11:GUS resulting in a stronger GUS staining than URM12:GUS, which is in agreement with microarray data that found URM11 to be expressed at a higher level [21]. Again, staining was particularly strong in the vascular tissue. This shows that expression of URM11 and URM12 is largely overlapping.

**Figure 2. Properties and activities of URM11 and URM12.**

(A) Bulk tRNA was extracted from wild-type (WT), urm1 and urm1 mutant yeast strains complemented with URM11 or URM12. Thiolated tRNAs (arrow) show slower migration than non-thiolated tRNAs (bottom of gel) in an acrylamide gel containing APM. A band of unknown nature (arrowhead) occasionally occurred. URM11 and the GFP-URM fusion proteins of Arabidopsis are functional in yeast, resulting in tRNA thiolation in the otherwise thiolation-defective lxm1 mutant. A representative result of several repetitions is shown. (B) Western blotting of total protein extracts and purified nuclei of urm11-1 urm12-2 double mutants expressing HA-URM11 or non-transgenic double mutants. Immunolabelling was done with an anti-HA (upper lane) and an anti-histone H3 (lower lane) antibody. The experiment was performed twice with comparable outcome. (C) A representative result of the yeast-two-hybrid experiment (performed three times independently) revealed the interaction of URM11 and URM12 with ROL5, resulting in yeast growth on selective medium and blue staining of the cells due to β-galactosidase activity. Transformation with only one of two constructs with the empty second plasmid did not result in yeast growth and β-galactosidase activity.

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seedlings, whereas they are malformed or absent in the lrx1 mutant. This defect is indeed suppressed in the lrx1 urm11-1 urm12-2 triple mutant, which showed wild-type-like root hair development. By contrast, the lrx1 urm11-1 and lrx1 urm12-2 double mutants showed largely an lrx1 phenotype (Figure 5B). Hence, the urm11-1 and urm12-2 mutations synergistically interact, which provides further evidence for URM11 and URM12 having similar functions during root development. The urm11-1 urm12-2 double mutant, however, did not reveal aberrant root hair morphology compared to the wild type (data not shown). Finally, the lrx1 urm11-1 urm12-2 triple mutant was complemented with 35S:HA-URM11 and 35S:HA-URM12 constructs. Transgenic lines expressing either of the two URM genes developed an lrx1-like root hair phenotype (Figure 5C), confirming that the mutations in the two URM loci account for suppression of lrx1.

Discussion

Ubiquitin-related modifier proteins (URMs) are found in phylogenetically distantly related species. They can have a low level of identity or similarity, as found for Arabidopsis URM11 and URM12 versus the yeast Urm1p or human Urm1 [16], but are conserved in the β-grasp, a characteristic structure consisting of a core with a pocket of four β-strands and diagonally arranged α-helices, and the C-terminal di-glycine motif [1], [3], [4]. Despite the limited conservation in the primary sequence, Arabidopsis URM genes are able to complement the tRNA thiolation defect of the yeast Aum1l mutant ([16]; this work). Our data also show that GFP-URM fusion proteins are functional, which allows a GFP-based analysis of protein accumulation in future studies. In the sulfur transfer reaction, URM proteins interact with a thiouridylase. This interaction is also conserved in Arabidopsis, since URM11 and URM12 interact with the Arabidopsis thiouridylase ROL5, a protein that is essential for the sulfur transfer reaction, as the rol5 mutant is also defective in tRNA thiolation [19]. In addition to ROL5, URM11 and URM12 have been shown to interact with the E1 ligase SIR1/CNX5 which is important for activation of the URM proteins and essential for tRNA thiolation [16]. Hence, to this point, interactions within the sulfur transfer reaction are well conserved in a diverse range of organisms.

Mutations in URM11 and URM12 Affect Root Development

The thiolation of the uridine in the wobble position of tRNAs conferred by the protein network involving URM proteins is assumed to increase codon-anticodon accuracy, while blocking this tRNA modification is expected to have a negative impact on translation efficiency [11]. However, absence of tRNA thiolation does not have a deleterious impact on the organism. Both the yeast Aum1l mutant [5], [22] and the Arabidopsis urm11-1 urm12-2 double mutant are missing detectable levels of thiolated tRNAs but are viable. However, in both organisms, the growth process is affected. The Arabidopsis urm11-1 urm12-2 double mutant develops a modified root architecture with a lower lateral root density compared to the wild type. Lateral root formation is under the control of auxin and cytokinin, but is also strongly influenced by nutrient availability [23–25]. This is similar to yeast where the mutant Aum1l is impaired in pseudohyphal growth, a developmental response to nutrient limitation [22]. Thus, URM proteins of yeast and Arabidopsis, and possibly URM proteins in general, affect processes that are modified by environmental conditions.

In addition to lateral root formation, mutations in URM11 and URM12 also affect root hair development. Even though the urm11-1 urm12-2 double mutant does not show impaired root hair growth, it does suppress the root hair formation mutant lrx1. LRX1 is an extracellular protein that is involved in root hair cell wall formation [26–26]. The thiouridylase-defective rol5 mutant was initially isolated as a suppressor of lrx1 [19], which is the reason why a suppression of lrx1 was tested in the lrx1 urm11-1 urm12-2 triple mutant. The comparable effect of rol5 and the urm11-1 urm12-2 double mutant suggests that interfering with tRNA modification is causing the suppression of lrx1. Since lrx1 is a cell wall formation mutant, suppression is likely induced by changes in cell wall structures. Indeed, the rol5 mutant was shown to induce modifications in cell wall structures [19]. A possible mechanism by which changes in tRNA thiolation can affect root architecture and cell wall formation is via the TOR (Target Of Rapamycin) signaling network. The TOR network is a growth controller in eukaryotic cells that senses growth factors and nutrient availability and modulates cellular processes such as translation, ribosome biogenesis, mitochondrial activity, or cytoskeletal dynamics [29]. Alterations in tRNA thiolation modify
translation efficiency and have been shown to modulate TOR signaling [11], [22]. In addition, tRNAs are involved in nutritional stress responses via modulating TOR activity [30].

In Arabidopsis, inhibiting the TOR network by rapamycin, a macrocyclic lactone specifically inhibiting the TOR kinase [31], leads to fewer lateral roots, modification of cell wall structures, and suppression of the lrx1 root hair mutant phenotype [19], [32], [33]. Thus, the phenotypes caused by interfering with TOR signaling by rapamycin treatment are comparable to those observed in the rol5 and urm11-1 urm12-2 mutant lines, supporting the hypothesis that alterations in tRNA thiolation have an impact on TOR signaling.

Figure 4. urm11 urm12 double mutant fails to thiolate tRNAs. (A) Schematic structure of URM11 and URM12. Black boxes represent exons and white boxes introns. T-DNA insertions are highlighted by black arrows and are located in the first intron for urm11-1 and urm12-2, which were further analyzed. (B) RT-PCR on total RNA of entire seedlings revealed absence of URM11 and URM12 mRNA in the corresponding mutants. The ACTIN2 gene was amplified as a control for comparable RNA extraction efficiency. PCR on genomic DNA reveal larger products due to introns. (C) The urm11-1 urm12-2 double mutant is impaired in tRNA thiolation. In the presence of APM, thiolated tRNAs show slower migration in an acrylamide gel, non-thiolated tRNAs migrate faster (bottom of the gel). In contrast to the wild type, rol5-1 and urm11-1 urm12-2 mutants lack thiolated tRNAs (arrow). Bands of unknown nature (arrowhead) occasionally occurred. Representative examples of several independent experiments are shown. Col: wild-type Columbia.

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Figure 5. Effects of urm11-1 urm12-2 on root development. (A) Lateral root density is reduced in the urm11-1 urm12-2 double mutant compared to the wild type. Complementation with a 3SS:HA-URM11 or 3SS:HA-URM12 construct restores lateral root formation. Error bars represent the standard error, the asterisk indicates the only value significantly different from the others (two-sided t-test; p = 0.01; n ≥ 25). (B) In contrast to the wild type (Col), lrx1 mutants frequently have collapsed root hairs. While urm11-1 or urm12-2 have no effect on lrx1, an lrx1 urm11-1 urm12-2 triple mutant shows suppression of lrx1 and develops wild type-like root hairs. (C) Suppression of lrx1 by urm11-1 urm12-2 is complemented with either 3SS:HA-URM11 or 3SS:HA-URM12, resulting in the lrx1 root hair phenotype. Col: wild-type Columbia. Bar = 0.5 mm.

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The data presented here support the view that the tRNA thiolation process is conserved across distantly related species. This assumption is corroborated by the analysis of URM protein function in Arabidopsis (16), and this work) and the interaction of URM11 and URM12 with ROL5, which is equivalent to the interaction of the yeast Urm1p with Nse6p (8). A protein-protein interaction requires an overlapping localization of the proteins in the cell. This study revealed that the Arabidopsis URM11 localizes to the cytoplasm and nucleus. Considering the functional similarity of URM11 and URM12, e.g. both being able to complement the yeast Δurm1 mutan, it is quite probable that URM12 shows a localization pattern that is comparable to URM11. Since the HA-URM11 construct is under the control of the strong 35S promoter, a possible ectopic detection of HA-URM11 protein in the nucleus cannot be excluded. Yet, in a high-throughput analysis of yeast protein localization, Urm1p was also localized to the cytoplasm and the nucleus. Even though this localization was later changed to cytoplasmic (manual curation of the localization data), there seems no direct experimental evidence for this restriction (www.yeastgenome.org). The URM11 and URM12 interaction partner ROL5, however, appears to predominantly colocalize with mitochondria (19). Based on the function of ROL5 in the modification of cytoplasmic tRNAs, ROL5 must, at least transiently, be present in the cytoplasm. Dual localization of proteins in different compartments and organelles is not unusual (34), suggesting that ROL5 is a mobile protein that translocates between mitochondria and the cytoplasm. The biological significance of ROL5 in mitochondria and the URM proteins in the nucleus remains to be elucidated.

Initially, URM-like proteins were expected to serve in a protein conjugation process, comparable to ubiquitylation by ubiquitin [4]. Indeed, target proteins of Urm1p were identified in yeast and human cells where urmylation seems to be induced during oxidative stress [14], [15], [35]. The HA-tagged URM11 and URM12 proteins are functional and will serve as tools in future oxidative stress [14], [15], [35]. The HA-tagged URM11 and URM12, e.g. both being able to complement the yeast Δurm1 mutan, it is quite probable that URM12 shows a localization pattern that is comparable to URM11. Since the HA-URM11 construct is under the control of the strong 35S promoter, a possible ectopic detection of HA-URM11 protein in the nucleus cannot be excluded. Yet, in a high-throughput analysis of yeast protein localization, Urm1p was also localized to the cytoplasm and the nucleus. Even though this localization was later changed to cytoplasmic (manual curation of the localization data), there seems no direct experimental evidence for this restriction (www.yeastgenome.org). The URM11 and URM12 interaction partner ROL5, however, appears to predominantly colocalize with mitochondria [19]. Based on the function of ROL5 in the modification of cytoplasmic tRNAs, ROL5 must, at least transiently, be present in the cytoplasm. Dual localization of proteins in different compartments and organelles is not unusual (34), suggesting that ROL5 is a mobile protein that translocates between mitochondria and the cytoplasm. The biological significance of ROL5 in mitochondria and the URM proteins in the nucleus remains to be elucidated.

Materials and Methods

DNA Constructs

For complementation of the yeast Δurm1 mutant, cDNA clones of the Arabidopsis URM11 and URM12 were amplified using the primer pairs URM11_for (GAATTCATGCAATACCTCTTGGAATTCCGGG)/URM11_rev (TAATCCACCATGCAAAATGGTAAGAAAT) and URM12_for (GAATTCATGCAATACCTCTTGGAATTCCGGG)/URM12_rev (TCATCCACCATGCAAAATGGTAAGAAAT) and digested with the same enzymes. For cloning into pGEM-T, the fragment was cloned into BanHI and 3′-NotI cassette of pGEM-T easy for sequencing. The clones of URM11 and URM12 were digested with XbaI and BanHI and cloned into pGEM-DH (Dysystems) cut with the same enzymes. The clones of URM11 and URM12 were digested with XbaI and BanHI and cloned into pGEM-DH (Dysystems) cut with the same enzymes.

For expressing HA-tagged versions of URM11 and URM12, genmic clones were amplified using the primer pairs

URM11_HA_gen_for
TCTCGAGATGTAACCCATACGAGATTCGCA-GATTCGACATGCAATTACCTCTTGGAATTCCGGG/GTAACCCATACGAGATTCGCA-GATTCGACATGCAATTACCTCTTGGAATTCCGGG/URM11_HA_gen_rev (TCTAGAGAAGAACACTTAAAATGGAAAACTCTAAATATGCAATTACCTCTTGGAATTCCGGG)/URM12_HA_gen_for (TCTCGAGATGTAACCCATACGAGATTCGCA-GATTCGACATGCAATTACCTCTTGGAATTCCGGG)/(GAATTCATGCAATACCTCTTGGAATTCCGGG)/URM12_HA_gen_rev (TCTAGAGAAGAACACTTAAAATGGAAAACTCTAAATATGCAATTACCTCTTGGAATTCCGGG).

For sequencing, the fragments were ligated into pGEM-T easy. Correct clones were digested with XbaI and XbaI and cloned into the expression cassette of pART7 (37), containing a 35S CaMV promoter and GUS terminator, cut with the same enzymes. For plant transformation, the 35S-HAURM-OCS cassettes were cut out with XbaI and cloned into the plant transformation vector pBART (38) which is identical to pART27 (37) but contains a gene for resistance to kanamycin.

The N-terminal genomic GFP fusion constructs for transient expression in plants were produced by XbaI digestion of pART7-HA-URM11 and insertion of an XbaI-GFP cassette. The GFP gene was amplified from the vector pMDC33 (39) using the primer pair

GFP_XbaI_for (TCTCGAGATGTAACCCATACGAGATTCGCA-GATTCGACATGCAATTACCTCTTGGAATTCCGGG)/GFP_3′_rev (CTTCAGAATGGAACATGTCAACAGCGGAGAACTTAAAATGGAAAACTCTAAATATGCAATTACCTCTTGGAATTCCGGG)

and into pENTR (Invitrogen). A correct clone was then used for cloning of the URM11 promoter into the gateway vector MDC164 containing the GUS gene [39].

For obtaining the URM12 promoter-GUS construct, a 1.8 kb of promoter sequence 5′ upstream of the ATG start codon was PCR-amplified using the primers URM11Prom_F (TCTCGAGATGTAACCCATACGAGATTCGCA-GATTCGACATGCAATTACCTCTTGGAATTCCGGG) and URM11Prom_R (TCTAGAGAAGAACACTTAAAATGGAAAACTCTAAATATGCAATTACCTCTTGGAATTCCGGG) and cloning into pGPTV-Bar (40) digested with the same enzymes.

Plant Material and Growth Conditions

All plant lines used are Arabidopsis thaliana, accession Columbia. The bx1 allele is described in (41). The urm11-1 and urm12-2 allele are the Salk lines 024513 and 070672.90. Respectively. The urm12-1 allele not further used in this study is the line ET5108 and is of the accession Landsberg erecta.

Seeds were sown in vitro and in soil as described [9]. In brief, seeds were surface sterilized, washed and grown in a vertical orientation with a 16-h-light/8-h-dark cycle at 22°C on plates containing half-strength MS medium. For further growth and propagation, seeds were transferred to soil and grown with a 16-h-light/8-h-dark cycle at 22°C.

Selection of transgenic plants produced by the standard floral dip method was done on 20 µg/mL basta (for pBART and pGPTV-bar vectors) or 20 µg/mL hygromycin (pMDC164).

Yeast Strains and Growth Conditions

Yeast strains used in this study were obtained from EUROSCARF, Frankfurt, Germany. The wild-type strain is BY4741 with the relevant genotype MATa; his³Δ1; leu²Δ0; met15Δ0; and propagation, seedlings were transferred to soil and grown with a 16-h-light/8-h-dark cycle at 22°C. The bx1 allele is described in (41). The urm11-1 and urm12-2 allele are the Salk lines 024513 and 070672.90. Respectively. The urm12-1 allele not further used in this study is the line ET5108 and is of the accession Landsberg erecta. Seeds were sown in vitro and in soil as described [9]. In brief, seeds were surface sterilized, washed and grown in a vertical orientation with a 16-h-light/8-h-dark cycle at 22°C on plates containing half-strength MS medium. For further growth and propagation, seeds were transferred to soil and grown with a 16-h-light/8-h-dark cycle at 22°C. Selection of transgenic plants produced by the standard floral dip method was done on 20 µg/mL basta (for pBART and pGPTV-bar vectors) or 20 µg/mL hygromycin (pMDC164).
ura3Δ0, and the Aum1 strain has the relevant genotype BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YIL006w::kanMX4. Yeast strains were grown at 30°C for 2 d on SD plates supplemented with His, Leu, Ade for strains complemented with pFL61 constructs and His, Leu, Ade and Ura for growth of the wild type.

Transient Gene Expression in Onion Epidermal Cells

For transient gene expression, onion epidermal cells were transformed by particle bombardment as described [42]. Bombarded tissue was incubated for 1 d at room temperature and the fluorescence pattern was microscopically analyzed.

Isolation of Nuclear Proteins and Western Blotting

Nuclei were isolated from 1 g fresh weight Arabidopsis 2-week old seedlings grown under sterile conditions following the protocol of [43].

For western blotting, proteins were prepared by mixing nuclear extracts or total seedsling material with standard SDS-PAGE loading buffer prior to heat denaturation. SDS-PAGE, blotting onto nitrocellulose, and immunodetection by ECL technology was performed as described by Ringli [44]. Immunodetection was performed using a rat anti-HA antibody (Roche, # 11867423001) and a rabbit anti-histone H3 antibody (Abcam, Ab # 1791), followed by horseradish-coupled goat anti-rabbit and anti-rat antibodies (Santa Cruz Biotechnology, # sc-2004 and sc-2006, respectively), all of which were used in 1:3000 dilutions.

Mcroscopy

Epidermal GFP fluorescence was analyzed using a Zeiss Imager Z1 microscope equipped with an Axiocam HRC. GFP fluorescence of yeast cells was analyzed with a Leica DM6000 equipped with a Leica BFC 350FX. Phenotypic observations and GUS expression analysis were done with a Leica LZ M125 stereomicroscope. Data points of lateral root development represent ≥25 seedlings. The experiment was done several times. For the root hair phenotype, over 30 seedlings of each line were analyzed.

RNA Extraction and Analysis

The seedlings were grinded in liquid nitrogen and the material was extracted two times with 8 ml acidic phenol (Sigma), 0.8 ml chloroform and once with 4 ml acidic phenol, 0.4 ml chloroform. Yeast strains were grown at 30°C in 50 ml liquid SD media supplemented with His, Leu, Ade for strains complemented with pFL61 constructs and His, Leu, Ade and Ura for growth of the wild type or Aum1 mutant. The tRNA was extracted 2 times with 4 ml acidic phenol, 0.4 ml chloroform.

After extraction of the plant or yeast material, tRNA was purified with AX100 columns from MACHEYER NAGEL following manufacturer’s instructions. For analysis, the purified tRNA was separated on an acrylamide gel supplemented with N-acryloylaminophenyl mercuric chloride (APM) by the method adapted from [11].

Accession Numbers

The Arabidopsis genes discussed in this study have the following accession numbers: URM11: At2g45695; URM12: At3g61113; LRX1: At1g12040; ROL5: At2g44270.

Supporting Information

Figure S1 Arabidopsis GFP-URM11 localizes to the cytoplasm and the nucleus. Transient transformation of 35S::GFP-URM11 and 35S::GFP into onion cells results in a comparable pattern of cytoplasmic and nuclear fluorescence. For each construct, at least 15 transformed cells were analyzed. Bar = 100 μm.

(TIF)

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Author Contributions

Conceived and designed the experiments: FJ MP R-ML CR. Performed the experiments: FJ MP R-ML SE CR. Contributed reagents/materials/analysis tools: FJ MP R-ML SE CR. Wrote the paper: FJ CR.

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