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Conserved and unique features of the homeologous maize Aux/IAA proteins ROOTLESS WITH UNDETECTABLE MERISTEM 1 and RUM1-like 1

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Abstract

The maize (Zea mays L.) Aux/IAA protein RUM1 (ROOTLESS WITH UNDETECTABLE MERISTEM 1) is a key regulator of lateral and seminal root formation. An ancient maize genome duplication resulted in the emergence of its homeolog rum1-like1 (rul1), which displays 92% amino acid sequence identity with RUM1. Both, RUL1 and RUM1 exhibit the canonical four domain structure of Aux/IAA proteins. Moreover, both are localized to the nucleus, are instable and have similar short half-lives of ~23 min. Moreover, RUL1 and RUM1 can be stabilized by specific mutations in the five amino acid degron sequence of domain II. In addition, proteins encoded by both genes interact in vivo with auxin response factors (ARFs) such as ZmARF25 and ZmARF34 in protoplasts. Although it was demonstrated that RUL1 and RUM1 can homo and heterodimerize in vivo, rul1 expression is independent of rum1. Moreover, on average rul1 expression is ~84-fold higher than rum1 in the 12 tested tissues and developmental stages, although the relative expression levels in different root tissues are very similar. While RUM1 and RUL1 display conserved biochemical properties, yeast-two-hybrid in combination with BiFC experiments identified a RUM1-associated protein 1 (RAP1) that specifically interacts with RUM1 but not with RUL1. This suggests that RUM1 and RUL1 are at least in part interwoven into different molecular networks.

Key words: Aux/IAA, maize, protein interaction, root, RAP1, RUM1, RUL1.

Introduction

Maize (Zea mays L.) plays an important role as fodder, human food and a source of bioethanol. The maize root system is formed by embryonic primary and seminal roots and post-embryonic shoot-borne crown and brace roots, which are instrumental for water and nutrient uptake and for anchorage of plants in soil (Hochholdinger and Tuberosa, 2009). All these root-types form post-embryonic lateral roots, which are initiated from pericycle and endodermis cells and make up the major backbone of the plant (Hochholdinger et al., 2004).
The phytohormone auxin is a key regulator of almost all development processes including root formation (Hochholdinger and Zimmermann, 2008; Peret et al., 2009; Jansen et al., 2012). Application of exogenous auxin or auxin transport inhibitors to roots of Arabidopsis thaliana and maize suggest that polar auxin transport is required for lateral root initiation (Reed et al., 1998; Casimiro et al., 2001; Jansen et al., 2012). The semi-dominant maize mutant rum1 (rootless with undetectable meristem I) is affected in the initiation of embryonic seminal and postembryonic lateral roots of the primary root (Woll et al., 2005). The mutant rum1 showed an 83% reduction of polar auxin transport and delayed gravitropic response in the primary root (Woll et al., 2005). The rum1 gene encodes an Aux/IAA (auxin/indole-3-acetic acid) protein which is a key regulator of auxin signal transduction (von Behrens et al., 2011). Canonical Aux/IAA proteins have four functional domains. Domain I is responsible for transcriptional repression (Tiwari et al., 2004). In Arabidopsis, domain I is also predicted to be a protein-protein interaction domain. For instance, domain I of BD/IAA12 is responsible for interaction with TOPLESS which is required as a co-repressor for IAA12 (Szemenyei et al., 2008). Domain II of Aux/IAA proteins is related to their instability of Aux/IAA proteins (Worley et al., 2000; Dreher et al., 2006). The degron motif GWPPV of domain II binds to the E3 ubiquitin ligase complex SCF (Tan et al., 2007) leading to Aux/IAA protein ubiquitination and subsequent proteasomal degradation (Gray et al., 2001). The maize rum1 mutant and several Arabidopsis Aux/IAA gain-of-function mutants were identified due to mutations in the core region of domain II (Benjamins and Scheres, 2008; von Behrens et al., 2011) resulting in increased stability of Aux/IAA proteins (Worley et al., 2000; Dreher et al., 2006; von Behrens et al., 2011). Aux/IAA proteins can form homo- or heterodimers by their dimerization domains III and IV with Aux/IAA or ARF (auxin response factor) proteins (Kim et al., 1997; Woodward and Bartel, 2005; Ludwig et al., 2014). The Aux/IAA-ARF complex binds to AuxREs (auxin responsive elements) repressing the transcription of early/primary auxin-responsive genes such as Aux/IAAs, SAURs and GH3s at low intracellular auxin concentrations (Woodward and Bartel, 2005). These genes often contain a conserved 5’ TGTCTC 3’ motif in their upstream regulatory sequence (Ulmasov et al. 1997, 1999; Lau et al., 2011). However, at high cellular auxin levels, ARF proteins are released from the Aux/IAA-ARF complex to promote transcription of auxin-responsive genes, whereas Aux/IAA proteins are degraded by the proteasome (Abel, 2007; von Behrens et al., 2011).

The genome of a maize progenitor was subjected to a whole genome duplication 5–12 million years ago. In the course of evolution in many instances one copy of the duplicated genes was lost, a process called partial fractionation (Schnable and Freeling, 2011). In modern maize, ~50% of all syntenic genes are pairs of homeologs while the remaining 50% of genes are single copy genes (Schnable and Freeling, 2011). Thus far only a small number of homeologous maize gene pairs have been characterized in more detail. Among those, dwarf plant8 (d8) and dwarf plant9 (d9) encode DELLA proteins, which are negative regulators of gibberellin signaling (Lawit et al., 2010). Moreover, colored aleurone1 (cl) and purple plant1 (pl1) encode MYB transcription factors (Cone et al., 1993), while colored 1 (cl) and colored plant (bl) encode bHLH transcriptional regulators (Chandler et al., 1989), which all control the biosynthesis of anthocyanin. Finally, discordial (dcd1) and its close relative alternative discordial (add1) encode a putative B’’ regulatory subunit of the PP2A phosphatase complex that is involved in preprophase band formation during cytokinesis (Wright et al., 2009). In the present study, we characterized the unique and conserved features of the homeologous maize genes rum1 and rul1, which encode Aux/IAA proteins.

Materials and methods

Plant material and growth conditions

Seeds of the maize inbred line B73, the mutant rum1-R (rum1-Reference) and its wild-type siblings were sterilized with 6% hypochlorite for 5 min under vacuum at 500 mPa and then rinsed five times with distilled water. Subsequently, seeds were germinated in paper rolls in a plant growth chamber in 16 h light at 28°C and 8 h dark at 21°C as previously described (Ludwig et al., 2013). Five-day-old seedlings of the maize inbred line B73 were treated with the auxin analog 1-Naphthaleneacetic Acid (1-NAA, working solution 5 μM) for 3 h. Primary roots were harvested after 0, 1, 2 and 3 h of 1-NAA exposure, then immediately frozen in liquid nitrogen and stored at ~80 °C until RNA isolation (Zhang et al., 2015).

qRT-PCR expression analyses

For qRT-PCR, total RNA was isolated from distinct maize samples with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). All RNA samples were treated with RNase-free DNaseI (Fermentas, St. Leon-Roth, Germany) and were subsequently tested for contamination with genomic DNA by PCR as previously described (Zhang et al., 2015). cDNA was synthesized from 500ng total RNA using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, USA). qPCR was performed as previously described (Zhang et al., 2014). Each genotype or treatment was assayed in four biological replicates by qRT-PCR. Each biological replicate was subjected to three qRT-PCR reactions (technical replications). An internal control gene (Genbank AC: 486909G09.x1; primers: 486909G09.x1-5’; 486909G09.x1-3’) was used in the qPCR as previously described for maize primary roots (Hoecker et al., 2006). The oligonucleotide primers rum1-fw and rum1-rv, rul1-fw and rul1-rv, rap1-fw and rap1-rv were used for rum1, rul1, and rap1 gene expression studies, respectively (Supplementary Table S1 available at JXB online). Differential gene expression was determined by Student’s t-test (*, P<0.05; **, P<0.01; ***, P<0.001; n=4). Correlation of expression values was calculated based on Student’s t-distribution (degree of freedom, n-2; tails, 2).

Subcellular localization

To construct the vector pucHA-GFP for transient transformations, pUC-SPYCE was double digested by the restriction enzymes SmaI and SacI, then ligated with the HA-GFP fragment replacing the SPYCE fragment (Lab AC: 765). The HA-GFP open reading frame was amplified by PCR from vector pCF203-GFP using the oligonucleotide primers HA-GFP-SmaI-fw and HA-GFP-SacI-rv (Supplementary Table S1). For the rul1-GFP fusion construct, the open reading frame of rul1 which was previously cloned into the pENTR/D-TOPO vector (Invitrogen, Darmstadt, Germany) was amplified using the oligonucleotide primers rul1-KpnI-fw and rul1-BamHI-rv (Supplementary Table S1) introducing 5’ KpnI and 3’ BamHI restriction sites and deleting the stop codon of the rul1 cDNA. Subsequently, this PCR

Table S1
product was introduced into the \textit{KpnI} and \textit{BamHI} restriction sites of the pucHA-GFP vector yielding a construct containing a constitutive cauliflower mosaic virus (CaMV) 35S promoter at the 5' end of the coding sequence of \textit{rul1} and a 3' in-frame GFP sequence followed by the nopaline synthase (NOS) terminator (Lab AC: 818). Site-directed mutagenesis was used to change the proline (P) amino acid residues at positions 121 and 122 of RUL1 to lysine (L) according to the manufacturer's instructions (Stratagene). The oligonucleotide primers \textit{rul1-P121L-fw}, \textit{rul1-P121L-rv} and \textit{rul1-P122L-fw}, \textit{rul1-P122L-rv} were used for site-directed mutagenesis of \textit{rul1-P121L} and \textit{rul1-P122L} (Lab ACs: 820 and 821), respectively (Supplementary Table S1). All of the nucleotide sequence insertions were confirmed by sequencing.

Subcellular localization experiments were performed by transiently transforming the plasmids 35S::\textit{GFP}, 35S::\textit{rul1-GFP}, 35S::\textit{rul1-P121L-GFP} and 35S::\textit{rul1-P122L-GFP} into Arabidopsis Col-0 protoplasts. Cell cultures were incubated at 26°C in MSCol medium overnight in dark (Liu et al., 2003). Protoplasts were generated as described in Negrutiu et al. (1987). Transformation was performed according to a PEG protocol (Merkle et al., 1996). After incubation overnight, the transformed protoplasts were examined with a HCX PL APO 63×/1.2W CORR water immersion objective (Leica Microsystems) of a TCS SP2 AOBS confocal microscope (Leica Microsystems). GFP was excited with an argon laser at 488 nm and the emitted fluorescence was detected with an argon-krypton laser at 599 nm. Image processing was performed with Leica Confocal Software (Leica Microsystems).

Table 1. Stability of RUL1, \textit{rul1-P121L} and \textit{rul1-P122L}.

| Stability | RUL1 | \textit{rul1-P121L} and \textit{rul1-P122L} |
|-----------|------|-----------------------------------------------|
| GFP localization | Col-0 | Arabidopsis Col-0 |
| Fluorescence intensity | ++ | ++ |
| Fluorescence signal intensity | ++ | ++ |

Results

BiFC and flow cytometric analysis

Generation of fusion proteins of full length \textit{rum1} and the Aux/IAA domains of ZmARF25 and ZmARF34 with YFP (Walter et al., 2004) or YFPN (Li et al., 2010) has been previously described (von Behrens et al., 2011). Moreover, subdomains of \textit{rum1} were amplified with the oligonucleotide primers \textit{DI-rum1-BamHI-fw} and \textit{DI-rum1-KpnI-av} (domain I), \textit{DII-rum1-BamHI-fw} and \textit{DII-rum1-KpnI-rv} (domain II) and \textit{DIII-IV-rum1-BamHI-fw} and \textit{DIII-IV-rum1-KpnI-rv} (domain III–IV) (Supplementary Table S1) using Takara Taq Polymerase (Lonza, Basel, Switzerland) from the pENTR-TOPO-\textit{rul1} vector (Lab AC: 473), which was constructed as previously described (von Behrens et al., 2011). Subsequently, \textit{BamHI} and \textit{KpnI} fragments of domain I, domain II and domain III–IV of \textit{rum1} were introduced into pUC-SPYCE and the modified pUC-SPYNE-152, respectively (Lab ACs: 767 and 778, 780 and 770). Similarly, full length \textit{rul1} was amplified with the oligonucleotide primers \textit{rum1-BamHI-fw} and \textit{rul1-KpnI-rv} (Supplementary Table S1) from the pENTR-TOPO-\textit{rul1} (Lab AC: 475) vector, which was generated as described above. Subsequently, \textit{BamHI} and \textit{KpnI} fragments were introduced into pUC-SPYCE and the modified pUC-SPYNE-152 (Lab ACs: 529 and 530). Furthermore, the oligonucleotide primers \textit{rum1-R-Xbal-fw} and \textit{rum1-R-Smal-rv} (Supplementary Table S1) were used to amplify the full length sequence of mutated \textit{rum1-R} cDNA using pENTR-TOPO-\textit{rum1-R} as a template (Lab AC: 474) (von Behrens et al., 2011). This PCR product was introduced into the restriction sites \textit{Xbal} and \textit{SmaI} of pUC-SPYCE and the modified vector pUC-SPYNE-152 (Lab ACs: 710 and 711). The inserts were confirmed by sequencing and transformed into Arabidopsis Col-0 protoplasts for BiFC analyses.

Flow cytometry was performed as previously described (von Behrens et al., 2011). Briefly, 300 µl of the transfected protoplasts per experiment were filtered through a 40 µm sieve and fluorescence signal intensity was analysed with a Modular Flow (Beckman Coulter, Brea, CA, USA) cyometer. YFP fluorescence was excited with a 488 nm (50 mW) argon laser and its principal emission was captured in FL1 (530/40) and plotted against autofluorescence in FL2 (580/30). After gating out cellular debris detected in the FSC/ SSC plot, BiFC expressing cells were identified as those whose fluorescence signal intensity was increased in the FL1 channel compared to the negative controls (NUO-YFPCE and NUO-YFPNE-152, Lab ACs: 705 and 706) as previously described (von Behrens et al., 2011). Data acquisition and analysis was performed with MoFlo Summit 4.3 software.

Protein expression of each fusion protein described above was analysed in Arabidopsis protoplasts. Western blot analyses were performed as previously described (Saleem et al., 2010) using a primary anti-HA antibody (Roche, Germany) and a secondary anti-mouse IgG antibody (Sigma Aldrich, Germany). The secondary antibody was detected with NBT/BCIP (Roche, Germany) as previously described (Saleem et al., 2010).

Results

RUL1 is the homeolog of the Aux/IAA protein RUM1 and localizes to the nucleus

Based on their microsynteny it has been demonstrated that \textit{rum1}, which is located on chromosome 3, and \textit{rul1} \textit{rul1-likel}; GRMZM2G163848), which maps to chromosome 8, are homeologs (von Behrens et al., 2011). A maize progenitor
underwent a whole genome duplication in ancient times. Therefore, many genomic regions of modern maize can be attributed to either of two subgenomes 1 and 2. While the rum1 gene belongs to subgenome 1 its homolog rul1 was attributed to subgenome 2. RUL1 is predicted to contain the canonical four domain structure of Aux/IAA proteins and a bipartite nuclear localization signal (NLS; Fig. 1A).

To determine the subcellular localization of RUL1, a RUL1-GFP fusion protein was transiently expressed in Arabidopsis Col-0 protoplasts. This experiment localized RUL1-GFP to the nucleus (Fig. 1B). Similarly, the fusion proteins rul1-P121L-GFP and rul1-P122L-GFP containing point mutations in the degron sequence of RUL1 were also localized to the nucleus. By contrast, the GFP control protein displayed a constitutive localization in the nucleus and the cytoplasm.

RUL1 is unstable

Aux/IAA proteins are unstable proteins that are rapidly degraded at increased cellular auxin levels. Therefore, the stability of the RUL1 wild-type protein was compared with two mutated isoforms of the protein, rul1-P121L and rul1-P122L, in which the proline (P) at amino acid positions 121 and 122 was changed into lysine (L), respectively. Relative GFP-fluorescence intensities of RUL1-GFP, rul1-P121L-GFP and rul1-P122L-GFP fusion proteins were measured between 0 and 120 min with a flow cytometer (Fig. 1C). As a control, relative GFP-fluorescence intensities of constitutively expressed GFP were determined (Fig. 1C). This experiment demonstrated the instability of RUL1 with an average half-life of ~23 min, compared to the stable GFP protein (Fig. 1C). As predicted, the P-to-L amino acid exchange within the degron sequence of RUL1 at position 122 stabilized the protein.

**Fig. 1.** Characteristics of rul1. (A) Alignment of the amino acid sequences encoded by the homologous genes rul1 and rum1 revealed ~92% identity. The domain structures and the nuclear localization signal (NLS) are indicated. (B) Subcellular localization of RUL1, rul1-P121L-GFP and rul1-P122L-GFP and a GFP control. (C) Protein stability assay of RUL1-GFP, rul1-P121L-GFP and rul1-P122L-GFP was confirmed at time points 0, 30, 120 min by Western blot analyses using an anti-GFP antibody.
the protein significantly. While the relative GFP fluorescence of wild-type RUL1 was reduced to ~10% within 120 min, GFP fluorescence of rul1-P122L was still at ~85% which is close to the GFP control. By contrast, the P-to-L amino acid exchange within the degron sequence of RUL1 at position 121 did not stabilize the protein. The rul1-P121L protein displayed a similar half-life of ~28 min as the wild-type RUL1 protein and after 120 min only ~20% of the GFP fluorescence was remaining (Fig. 1C). Western blot experiments with total protein extracts from protoplasts overexpressing RUL1-GFP, rul1-P121L-GFP and rul1-P122L-GFP fusion proteins confirmed these results by showing half-lives similar in value to those observed in flow cytometry (Fig. 1C).

Overall higher expression in rul1 compared to rum1

To compare the temporal and spatial expression patterns of *rum1* and *rul1*, 12 distinct tissues and developmental stages of the maize inbred line B73 were analysed by qRT-PCR (Fig. 2A). In all comparisons, *rul1* was significantly higher expressed than *rum1*. On average, *rul1* displayed a ~84-fold higher expression than *rum1*. Both, *rum1* and *rul1* displayed the highest expression in the cortex and stele of 3-day-old primary roots (Fig. 2A). Among all tested tissues, *rum1* and *rul1* displayed the lowest expression in the elongation zone of 3-day-old primary roots. Furthermore, both genes were expressed at higher levels in seminal and crown roots than in all other tested stages of primary roots. Despite the significant differences in overall expression, across all tissues *rum1* and *rul1* expression was significantly correlated (Fig. 2B).

For all analysed tissues a Pearson correlation coefficient of R=0.8 (P<0.01) was calculated. Pairwise t-tests comparing expression of the two genes in all tested tissues are summarized in Supplementary Table S2. Thus far two types of auxin response promoter elements (AuxRE), 5′ TGTCCTC 3′ and 5′ TGTC 3′, have been described. Promoter analysis of 1 kb upstream of the ATG start codon of *rul1* revealed six 5′ TGTCCTC 3′ (once) and 5′ TGTC 3′ (six times) (Fig. 4A). Auxin induction of *rap1* in 5-day-old wild-type primary roots was demonstrated after a 3 h treatment with 5 µM 1-NAA (Fig. 4B).

Subsequently, BiFC experiments were performed to analyse the interaction of RAP1 (RUM1 ASSOCIATED PROTEIN 1) with RUM1, RUL1 and rum1-R, a mutated form of RUM1 lacking 24 amino acids in domain II. These experiments confirmed the interaction of RUM1 and rum1-R with RAP1 in Arabidopsis protoplasts. Remarkably, interaction of the mutant protein rum1-R with RAP1 in Arabidopsis protoplasts was significantly stronger than RUM1-RAP1 interaction (Fig. 4C), possibly due to the increased stability of the mutated proteins. In contrast, no interaction was observed between RUL1 and RAP1 compared to the corresponding negative controls in BiFC analyses (Fig. 4C). Western blot analyses were performed with Anti-HA (haemagglutinin) antibodies against the YFPC and anti-c-Myc antibodies against the YFPN tag, which demonstrated the expression of the corresponding proteins in Arabidopsis protoplasts (Supplementary Fig. S1).

RUL1 interacts with ZmARF25 and ZmARF34, RUM1 and itself

Aux/IAA proteins are characterized by their capability of interacting with ARF proteins. Interactions of RUL1 with ZmARF25 and ZmARF34, which have been previously demonstrated to interact with RUM1 (von Behrens et al., 2011), were surveyed in BiFC (Bimolecular Fluorescence Complementation) experiments. This *in vivo* technique is based on the detection of a YFP (Yellow Fluorescent Protein) signal which is emitted when N (YFPN) and C (YFPC) terminal YFP parts come in close proximity by the interactions of fusion proteins coupled to these YFP subunits.

Quantification of the YFP fluorescence by flow cytometry demonstrated significant interactions of RUL1 with ZmARF25 and ZmARF34 in Arabidopsis protoplasts compared to the control experiments (Fig. 3A). Moreover, significant homo-interaction was observed for RUM1-RUM1 and RUL1-RUL1. Furthermore, hetero-interaction of RUM1-RUL1 in both orientations in comparison to the corresponding negative controls was demonstrated (Fig. 3B). Each experiment was performed in three biological replicates. The fusion proteins NUO-YFPC and NUO-YFPN, which were used as a negative control (von Behrens et al., 2011), did not display any interaction with RUL1, ARF25, and ARF34. Expression of fusion proteins in BiFC assays according to Fig. 3A, B were confirmed by Western blot experiments (Supplementary Fig. S1).

RAP1 specifically interacts with RUM1 but not with RUL1

To identify novel interaction partners for RUM1 and RUL1, a yeast two-hybrid assay was performed using RUM1 as bait and a cDNA expression library generated from mRNA of 2.5-day-old maize primary roots as prey (methods). This experiment revealed known interaction partners of RUM1 and RUL1 such as RUM1 and ARF25 (Supplementary Table S3). To identify interaction partners of RUM1 and RUL1 involved in auxin signal transduction, 1 kb promoter sequences upstream of the ATG start codon of yeast two-hybrid candidate genes were screened for auxin response elements (AuxREs). The promoter of a gene designated *rap1* contained seven AuxREs of the type 5′ TGTCCTC 3′ (once) and 5′ TGTC 3′ (six times) (Fig. 4A). Auxin induction of *rap1* in 5-day-old wild-type primary roots was demonstrated after a 3 h treatment with 5 µM 1-NAA (Fig. 4B).
experimental fusion-proteins in Arabidopsis protoplasts were confirmed by Western blot assays (Supplementary Fig. S1).

The RAP1 family in maize

Homology searches using the maize RAP1 protein sequence as query revealed six additional proteins of this family ZmRAP1-like1 (ZmRAL1) to ZmRAP1-like6 (ZmRAL6). Four of seven maize genes were assigned to maize subgenome 1, while the remaining three members were not assigned to a subgenome. All four maize genes assigned to subgenome 1 (ZmRAL1, ZmRAL3, ZmRAL5, ZmRAL6) have an ortholog in rice and sorghum (Supplementary Table S4).

Discussion

RUM1 and RUL1 display characteristics of canonical Aux/IAA proteins

About 5–12 million years ago a maize progenitor had undergone a whole genome duplication which led to the emergence
The Aux/IAA homeologs RUM1 and RUL1 of two subgenomes (Schnable et al., 2011). During evolution, subgenome 2 experienced more gene loss than subgenome 1. Moreover, mutant phenotypes identified in forward genetic screens are often the result of a mutation in genes of maize subgenome 1 (Schnable and Freeling, 2011). This observation is explained by the hypothesis that subgenome 1 genes have predominantly retained the ancestral function while subgenome 2 genes potentially adopted new, or less essential functions (Schnable and Freeling, 2011). In line with this, several genes controlling maize root development such as *rtcs* (Taramino et al., 2007), *rth3* (Hochholdinger et al., 2008) and *rum1* (von Behrens et al., 2011) belong to subgenome 1. Their homeologs in maize subgenome 2 are designated *rtcl* (Taramino et al., 2007), *rtl3* (Hochholdinger et al., 2008) and *rul1* (von Behrens et al., 2011).

The proteins encoded by *rum1* and *rul1* display the canonical four domain architecture defining Aux/IAA proteins (Liscum and Reed, 2002). Aux/IAA proteins are involved in the transcriptional regulation of auxin responsive genes (Quint and Gray, 2006) and are therefore localized in the nucleus. In the present study, nuclear localization of RUL1 was demonstrated by RUL1-GFP localization in Arabidopsis protoplasts (Fig. 1B). This localization pattern was also observed for RUM1 (von Behrens et al., 2011) and several other Aux/IAA proteins (Ludwig et al., 2014).

Aux/IAA proteins are unstable with short half-lives of between six and 80 minutes due to their interaction with the SCF<sup>TIR</sup> complex via domain II and subsequent proteasomal degradation (Abel et al., 1994; Gray et al., 2001; Ouellet et al., 2001). The instability of Aux/IAA proteins is conferred by interaction with the SCF<sup>TIR1</sup> complex at the conserved degron sequence GWPPV (Dreher et al., 2006). Point mutations in this short amino acid stretch are often sufficient to prohibit the interaction and thus enhance the stability of Aux/IAA proteins (Tian et al., 2003). In the present study, wild-type RUL1-GFP displayed a half-life of ~23 min (Fig. 1C) which was similar to RUM1 (~22 min; von Behrens et al., 2011). Remarkably, while the point mutation that led to a P-to-L exchange in position 122 was sufficient to stabilize the rul1-P122L protein, the same amino exchange at position 121 did not stabilize the protein (Fig. 1C) suggesting that SCF<sup>TIR1</sup> interaction and subsequent proteasomal degradation is still possible in this mutated protein to a considerable degree. In Arabidopsis several mutants with a developmental phenotype have been identified with P-to-L exchanges that correspond to position 121 (Rogg et al., 2001; Tatematsu et al., 2004) and 122 (Rouse et al., 1998; Knox et al., 2003; Tatematsu et al., 2004) in the degron sequence. These aberrant phenotypes are likely conditioned by stabilized mutated Aux/IAA proteins. In maize, as suggested by rul1-P121L-GFP.
 Aux/IAA proteins repress the transcription of early auxin-responsive genes by their interaction with ARF proteins (Woodward and Bartel, 2005). In the present study, we demonstrated interaction of RUL1 with ZmARF25 and ZmARF34 (Fig. 3A) as previously demonstrated for RUM1 (von Behrens et al., 2011). It was suggested that this interaction blocks lateral root formation in non-precursor pericycle cells (von Behrens et al., 2011). In Arabidopsis, multiple models of Aux/IAA-ARF-dependent auxin response signalling involved in lateral root development were proposed. First, the IAA28-ARF-dependent model...
mediates the specification of lateral root founder cell identity (De Rybel et al., 2010). Second, the SLR/IAA14-ARF7-ARF19 module controls the division of early founder cells of lateral roots, and subsequently the BDL/IAA12-ARF5 module regulates lateral root initiation and organogenesis (De Smet et al., 2010). Hence, RUM1 and RUL1 might also be involved in different pathways involved in lateral root formation.

In addition to the canonical features of RUL1, it was demonstrated in the present survey that RUM1 and RUL1 can form homo- and heterodimers in vivo (Fig. 3B). It was previously demonstrated that the Arabidopsis proteins IAA1, IAA2 and the pea protein IAA4 can form homodimers in vitro (Kim et al., 1997). Nevertheless, the function of Aux/IAA interactions still remains elusive and it has been suggested that it might allow interaction with downstream genes without the formation of Aux/IAA-ARF complexes (Paciorek and Friml, 2006). Similarly, direct binding of RUM1 to the promoter of lrp1 (lateral root primordia 1) has also been suggested (Zhang et al., 2015).

**In general rul1 displays higher expression than rum1**

It was observed that on average genes of maize subgenome 1 were expressed at higher levels than their homoeologous genes in subgenome 2 (Schnable et al., 2011). In contrast to this trend, rull (subgenome 2) displayed on average a ~84-fold higher expression than rum1 (subgenome 1) in the 12 tissues surveyed in the present study (Fig. 2A). Despite highly correlated expression patterns (Fig. 2B), the significantly differential expression intensities of rull and rum1 might suggest distinct functions of these two homoeologous proteins in root development.

Promoter analysis of the sequence 1 kb upstream of the ATG start codon revealed 13 putative AuxREs in rum1 and six AuxRE in rull. Despite the different number and position of AuxRE elements it was demonstrated that rull is auxin inducible as previously demonstrated for rum1 (von Behrens et al., 2011). Moreover, by comparing rull expression in wild-type and rum1-R mutant primary roots it was demonstrated that the expression of rull was not regulated by rum1. This result supports the notion that these genes might act in different molecular pathways and might therefore have different functions in root development.

For the recessive loss-of-function mutants rts and rth3, different functions compared to their homoeologous genes were demonstrated since the homoeologs were not able to complement the mutant phenotypes (Taramino et al., 2007; Hochholdinger et al., 2008). However for the semi-dominant mutation rum1-R the situation is different. The rum1-R mutant phenotype is conferred by a stabilization of the rum1-R/ARF complex, which inhibits the expression of downstream gene expression (von Behrens et al., 2011). Hence, RUL1 cannot complement the mutant phenotype because downstream gene expression is already blocked by the gene product of the gain-of-function allele rum1-R which may not allow redundancy in this process.

**RAP1 specifically interacts with RUM1 but not with RUL1**

AtSPR1 is a plant-specific small protein. Homology searches revealed similarity of AtSPR1 with a nitrilase-associated protein (GenBank AC: Z96936) in Arabidopsis (Nakajima et al., 2004). Yeast two-hybrid experiments demonstrated interaction of RUM1 with a novel protein which is a homolog of AtSPR1 and which we designated RAP1. Homology searches (ensembl.gramene.org) identified a total of seven homoeologous maize genes rap1 and rap1-like1 (ral1) to rap1 (Supplementary Table S4). Four of seven rap1-like gene family members (57%) were assigned to maize subgenome 1 (Supplementary Table S4). This tendency was also observed for the lrpl-like gene family where five of nine (56%) assigned to subgenome 1. The remaining three (43%) rap1-like genes likely emerged by single copy duplications after the last whole genome duplication because they did not map to any of the subgenomes. Similarly, seven Aux/IAA genes were not associated with a subgenome, suggesting that they emerged after the ancient genome duplication of maize (Ludwig et al., 2013).

Quantification of the interaction of RUM1, rum1-R and RUL1 with RAP1 (Fig. 4C) demonstrated interaction of RUM1 with RAP1, and an even stronger interaction of rum1-R with RAP1 in vivo. This is most likely a consequence of the observation that rum1-R is more stable than RUM1 (von Behrens et al., 2011). The homeologous maize proteins RUM1 and RUL1 share 92% identity on the protein level (von Behrens et al., 2011). However, no interaction was detected between RUL1 and RAP1. A domain interaction analysis revealed that the interaction of domain I of RUM1 with RAP1 is significantly stronger than the interaction of domains III–IV, while no interaction was detected with domain II in BiFC experiments (Fig. 4D). It has been demonstrated that domain I of Aux/IAA is also responsible for protein-protein heterodimerization. For example, domain I of BDL/IAA12 was sufficient to interact with TOPESS which is a co-repressor regulating embryogenesis in Arabidopsis (Szemenyei et al., 2008).

In summary, we demonstrated that both RUM1 and RUL1 display all characteristics of functional Aux/IAA proteins. Distinct functions of the two proteins are suggested by a different promoter architecture and overall differences in gene expression levels. Moreover, it was demonstrated that rull is not regulated by RUM1 suggesting their activity in independent pathways. Finally, RUM1 specific interaction with RAP1 suggests that these homoeologous genes, despite their role as Aux/IAA proteins, have at least in part diverse interaction partners and might thus be functioning in distinct molecular networks.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Expression of fusion proteins in Arabidopsis Col-0 protoplasts detected by Western blot experiments.

Table S1. Sequences of oligonucleotide primers used in this study.
Table S2. Pairwise comparison of rum1 (upper table) and rul1 (lower table) expression in different tissues and at different developmental stages according to Fig. 2A.

Table S3. RUM1 interaction partners identified via yeast two-hybrid experiments.

Table S4. Characteristics of members of the maize raph-like gene family in maize.

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