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RESEARCH ARTICLE

Conserved *LBL1-ta-siRNA* and miR165/166-*RLD1/2* modules regulate root development in maize

Vibhav Gautam[1,2], Archita Singh[1], Sandeep Yadav[1], Sharmila Singh[1], Pramod Kumar[1], Shabari Sarkar Das[3] and Ananda K. Sarkar[1,*]

ABSTRACT

Root system architecture and anatomy of monocotyledonous maize is significantly different from dicotyledonous model *Arabidopsis*. The molecular role of non-coding RNA (ncRNA) is poorly understood in maize root development. Here, we address the role of LEAFBLADELESS1 (LBL1), a component of maize trans-acting short-interfering RNA (ta-siRNA), in maize root development. We report that root growth, anatomical patterning, and the number of lateral roots (LRs), monocot-specific crown roots (CRs) and seminal roots (SRs) are significantly affected in *lbl1-rgd1* mutant, which is defective in production of ta-siRNA, including tasiR-ARF that targets *AUXIN RESPONSE FACTOR3* (*ARF3*) in maize. Altered accumulation and distribution of auxin, due to differential expression of auxin biosynthesis and transporter genes, created an imbalance in auxin signalling. Altered expression of *microRNA165/166* (*miR165/166*) and its targets, *ROLLED1* and *ROLLED2* (*RLD1/2*), contributed to the changes in *lbl1-rgd1* root growth and vascular patterning, as was evident by the altered root phenotype of *Rld1-O* semi-dominant mutant. Thus, *LBL1*/ta-siRNA module regulates root development, possibly by affecting auxin distribution and signalling, in crosstalk with *miR165/166-RLD1/2* module. We further show that ZmLBL1 and its *Arabidopsis* homologue AtSGS3 proteins are functionally conserved.

KEY WORDS: Small RNA, Ta-siRNA, *LBL1*, Root development, Vascular patterning, Maize

INTRODUCTION

Angiospermic plant groups, such as dicotyledons and monocotyledons, display significant variation in their root system architecture (RSA), which comprises primary root (PR) and root branches (Rich and Watt, 2013; Yruela, 2015). Dicots develop a tap root system, which consists of a PR and lateral roots (LRs), whereas monocots have a fibrous root system that, in early growth phase, consists of embryonic PR and seminal root (SR), post-embryonic crown root (CR) and LRs (Rich and Watt, 2013; Yruela, 2015). However, in monocot maize, shoot-borne CRs dominate during the later growth phase, which are absent in eudicot *Arabidopsis thaliana (Arabidopsis)*. Roots of monocot and dicot plants also differ anatomically in terms of quiescent centre (QC) cell number, cortical cells/cell files, root initials and vascular pattern (Hochholdinger and Zimmermann, 2008; Orman-Ligeza et al., 2013). Maize root possesses polyarch type of stelar organization consisting of several xylem poles alternating with the same number of phloem pole, which differs from that of *Arabidopsis* (Hochholdinger et al., 2018; Orman-Ligeza et al., 2013).

Despite having architectural and anatomical differences, recent studies have shown the role of some common genetic factors in regulation of shoot-borne CRs and root-borne LRs in monocots and dicots (Hochholdinger et al., 2018; Orman-Ligeza et al., 2013). Monocot specific root types, such as SRs and CRs, involve some level of distinct genetic regulation when compared with the eudicot *Arabidopsis*. Maize *ROOTLESS CONCERNING CROWN AND SEMINAL LATERAL ROOTS* (*RTCS*) and its rice homologue *CROWN ROOTLESS1* (*CRL1*) mutants fails to develop CRs, though PRs and LRs remain unaffected (Inukai et al., 2005). On the other hand, the *lateral rootless1* (*lrt1*) maize mutant lacks LR formation, while CRs remain normal (Hochholdinger and Feix, 1998). Although some LR regulatory genes are conserved between *Arabidopsis* and rice, about one quarter of *CRL1*-regulated genes are rice specific, indicating distinct regulation of CRs, SRs and LRs in monocot (Coudert et al., 2010; Zhao et al., 2009). This suggests that root type-specific regulation of developmental programme exists in maize and rice (Hochholdinger et al., 2018; Orman-Ligeza et al., 2013).

Besides protein-coding genes and phytohormones, several ncRNAs have been shown to regulate plant development by negatively regulating their target genes (Ambros et al., 2003; Chen, 2009; Petricka et al., 2012; Singh et al., 2018; Ubeda-Tomas et al., 2012). Molecular regulation of root growth and LR development is relatively well characterized in the eudicot model *Arabidopsis* compared with monocot plants (like rice and maize). microRNAs (miRNAs) and small interfering RNAs (siRNAs) constitute two major classes of endogenous small RNAs in plants. Ta-siRNAs are produced from *TRANS ACTING siRNA* (*TAS*) loci through the activity of specific miRNAs in both monocot and dicot (Nogueira et al., 2009; Yoshikawa et al., 2005). Maize LEAFBLADELESS1 (LBL1) is a homologue of *Arabidopsis* SUPPRESSOR OF GENE SILENCING3 (SGS3), which is hypothesized to be involved in the stabilization of the miRNA-cleaved transcripts generated from the *TAS* loci. Mutation in *LBL1*, as observed in the strong allele *lbl1-rgd1*, leads to abaxialized radial leaves and the retarded plant fails to enter reproductive state, which is partially different and much more severe than observed in the *Arabidopsis sgs3-11* mutant (Peragine et al., 2004; Timmermans et al., 1998). A relatively weaker allele *lbl1-ref* also shows a leaf phenotype (Nogueira et al., 2007). This suggests the function of *LBL1* in maize leaf development is slightly different from that of *SGS3*. Besides the *TAS3*-derived ta-siRNAs pathway, miR166 also contributes to the establishment of adaxial/abaxial leaf polarity by restricting the spatial expression domain of *CLASS III HOMEODOMAIN-LEUCINE*

[1]National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India. [2]Centre of Experimental Medicine and Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India. [3]Department of Botany and Forestry, Vidyasagar University, Midnapore, WB 721104, India.

*Author for correspondence (aksarkar@nipgr.ac.in)

A.S., 0000-0003-0116-6267; S.Y., 0000-0001-8858-4271; P.K., 0000-0001-9976-1368; S.S.D., 0000-0001-7952-7531; A.K.S., 0000-0001-7169-4298

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ZIPPER (*HD-ZIP III*) genes that specify adaxial fate (Husbands et al., 2009). The opposing activities of *TAS3*-derived tasiR-ARFs, which target *AUXIN RESPONSE FACTOR3* (*ARF3*), and miR165/166 (*Zm*-miR165/166) specify the polarity of developing maize leaves (Nogueira et al., 2006). Expression of miR165/166 in the abaxial domain restricts the expression of *ROLLED1* and *ROLLED2* (*RLD1/2*), a member of *HD-ZIP III* gene family in maize, to the adaxial side of the leaf (Juarez et al., 2004a). A semi-dominant *Rld1-Original* (*Rld1-O*) mutation in a miR166 complementary site leads to the inability of miR166 to recognize and cleave the *RLD1* transcript, resulting in the increased accumulation of its transcripts and adaxialized leaf fate (Juarez et al., 2004a). miR165/166-*HD-ZIP III* are known to have crosstalk with different phytohormones and their signalling (Dello Ioio et al., 2012; Singh et al., 2017). The phytohormone auxin is a major regulator of root growth and branching, both in monocot and dicot plants (Balzan et al., 2014; Benkova and Hejatko, 2009; Coudert et al., 2010). Auxin-mediated regulation of these processes is achieved through differential accumulation of auxin in various root cells and tissue types, which is attained by the spatiotemporal activity of several auxin biosynthesis genes and transporters (Orman-Ligeza et al., 2013). However, possible hormonal crosstalk with the maize ta-siRNA-*ARF* and miR165/166-*RLD* module in root development remains to be investigated.

Considering the known morphological, anatomical and genetic/molecular difference in root development between dicot and monocot models, we have anticipated distinct roles for small RNAs and their crosstalk in maize root development. In this study, we have uncovered distinct roles for the ta-siRNA pathway in maize root growth, branching and vascular patterning. We have addressed the crosstalk of ta-siRNA, miR165/166 and auxin signalling in maize root development. Additionally, we demonstrate some level of evolutionarily functional conservation of *LBL1* and *SGS3* between *Arabidopsis thaliana* and *Zea mays*.

RESULTS

Mutation in *LBL1* affects root development, vascular patterning, cortical cell number and cell division

To understand the possible molecular role of *LBL1* in maize root development, we compared the phenotype of mutant *lbl1-rgd1* roots at 7 days after germination (dag; 7-day-old) with that of wild type. We observed that *lbl1-rgd1* has longer PR and a reduced number of lateral branches (SR, CR and LR) when compared with that of wild type (Fig. 1A). The primary root is about 72.61% longer in *lbl1-rgd1* when compared with wild type (Fig. 1B). To determine whether increased PR length in *lbl1-rgd1* was associated with a change in the root meristem size, we analysed longitudinal sections (LSs) of the PR. Meristem size was determined by calculating the distance from QC to the approximate first elongated cortical cell (Fig. S1A,B). The root meristem of *lbl1-rgd1* was 34.09% longer in size than that of the wild type (Fig. S1B). We performed bromodeoxyuridine/5-bromo-2′-deoxyuridine (BrdU) staining, which marks nuclei of actively dividing cells of the root tip, to investigate the cell division activity in *lbl1-rgd1* (Fig. S1C). We observed more actively dividing nuclei in the *lbl1-rgd1* root meristem region, indicating increased cell division when compared with wild type (Fig. S1C). We further studied the expression levels of cell cycle regulators *ZmCycB1*, *ZmCycD2*, *ZmCycD3* and *ZmCycD4*, and found that these genes were upregulated in *lbl1-rgd1* PR tip (0-1 cm) when compared with wild type (Fig. S2A) (Hu et al., 2010). These results suggest that increased cell division activity contributes to the increase in root meristem size, and thus longer PR, in *lbl1-rgd1*.

Next, we analysed LRs and monocot-specific SRs and CRs in *lbl1-rgd1*. We observed that *lbl1-rgd1* seedlings have 50% fewer CRs and 41.67% fewer SRs compared with wild type (Fig. 1C,D). The density of emerged LRs was also reduced in *lbl1-rgd1* when compared with wild type by 67.31% (Fig. 1E). Root network 'bushiness' and area were reduced in *lbl1-rgd1*, which could be due to the lower number of SRs, CRs and LRs (Fig. S4). This experiment was performed on a 14-day-old plant; the parameters were checked using the GiA root online tool (Galkovskyi et al., 2012). As the number of emerged LRs was lower in the *lbl1-rgd1* seedling, we checked the expression of the cell wall remodelling-related genes, such as *ZmLAX1*, *ZmLAX2* and *Zmα-EXP* (homologues of *AtEXP7* and *AtEXP18*), which have been hypothesized to be involved in LR development or emergence (Zhang et al., 2014) in *Arabidopsis*. We found that the expression levels of *ZmLAX1*, *ZmLAX2* and *Zmα-EXP* were downregulated in the LR-forming region (1-2 cm) of the *lbl1-rgd1* root (Fig. S2B). We further studied the expression levels of cell cycle regulators, *ZmCycB1*, *ZmCycD2*, *ZmCycD3* and *ZmCycD4*, and found that these genes were downregulated in the *lbl1-rgd1* LR region (1-2 cm) when compared with wild type (Fig. S2C). As *lbl1-rgd1* showed altered root growth and branching, we compared the cellular anatomy between different regions of *lbl1-rgd1* and wild-type root. To examine the root anatomy, we analysed the serial transverse sections (TSs) along the regions of the 7-day-old PRs that included 0-1 cm tip region (Fig. 1F) and 1-2 cm above tip region (Fig. S1D). The root tip (0-1 cm) of *lbl1-rgd1* showed about 41.94% more metaxylem cells (Fig. 1H), and about 34.02% more cortical cell layers when compared with wild type (Fig. 1G). The average number of large metaxylem cells was 6.2 in wild type and 8.8 in *lbl1-rgd1* in the 0-1 cm region of root tip (Fig. 1H). The average number of cortical cell layers was 6.3 in wild type and 8.4 in in 0-1 cm of *lbl1-rgd1* root tip (Fig. 1G). As the density of emerged LRs was also reduced in *lbl1-rgd1* when compared with wild type, we next asked whether LR emergence is affected in *lbl1-rgd1*. To study delay in LR emergence, first we analysed the root tip using a confocal microscope. We observed that in the 1-2 cm region of 7-day-old seedling roots, there were one emerged and two embedded (non-emerged) lateral root primordia (LRP) in *lbl1-rgd1*, whereas there were four emerged LRP in wild type (Fig. 1I). Comparative study of LR emergence between wild type and *lbl1-rgd1* is also represented graphically (Fig. S5D). To further study the changes at a histological level, we made longitudinal sections of the 1-2 cm region of root and observed that LRP were emerged in wild type but not in *lbl1-rgd1* (Fig. 1J). As the number of emerged nodal roots (CR and SR) was also reduced in *lbl1-rgd1*, we checked whether their emergence was affected. In transverse sections of the nodal region of 7-day-old seedlings, we observed three embedded (non-emerged) CRs in *lbl1-rgd1*, whereas in wild type we found two emerged and one non-emerged CR (as indicated by dark-red staining in Fig. S5). This suggests that the reduction in nodal roots in *lbl1-rgd1* is due to delayed emergence. Increased cell numbers in most of the tissue layers indicate that the LBL1-mediated ta-siRNA pathway contributes to cell division in the vascular and cortical regions of the root.

A decrease in tasiR-ARFs production leads to upregulation of *ZmARF2/3* family target genes in *lbl1-rgd1*

As LBL1 is involved in ta-siRNA biogenesis, we investigated whether the production of *TAS3*-derived tasiR-ARFs was affected in *lbl1-rgd1* root. We analysed the accumulation of tasiR-ARF using *in situ* hybridization in the PR tip and LR formation region of wild-

Development (2021) 148, dev190033. doi:10.1242/dev.190033

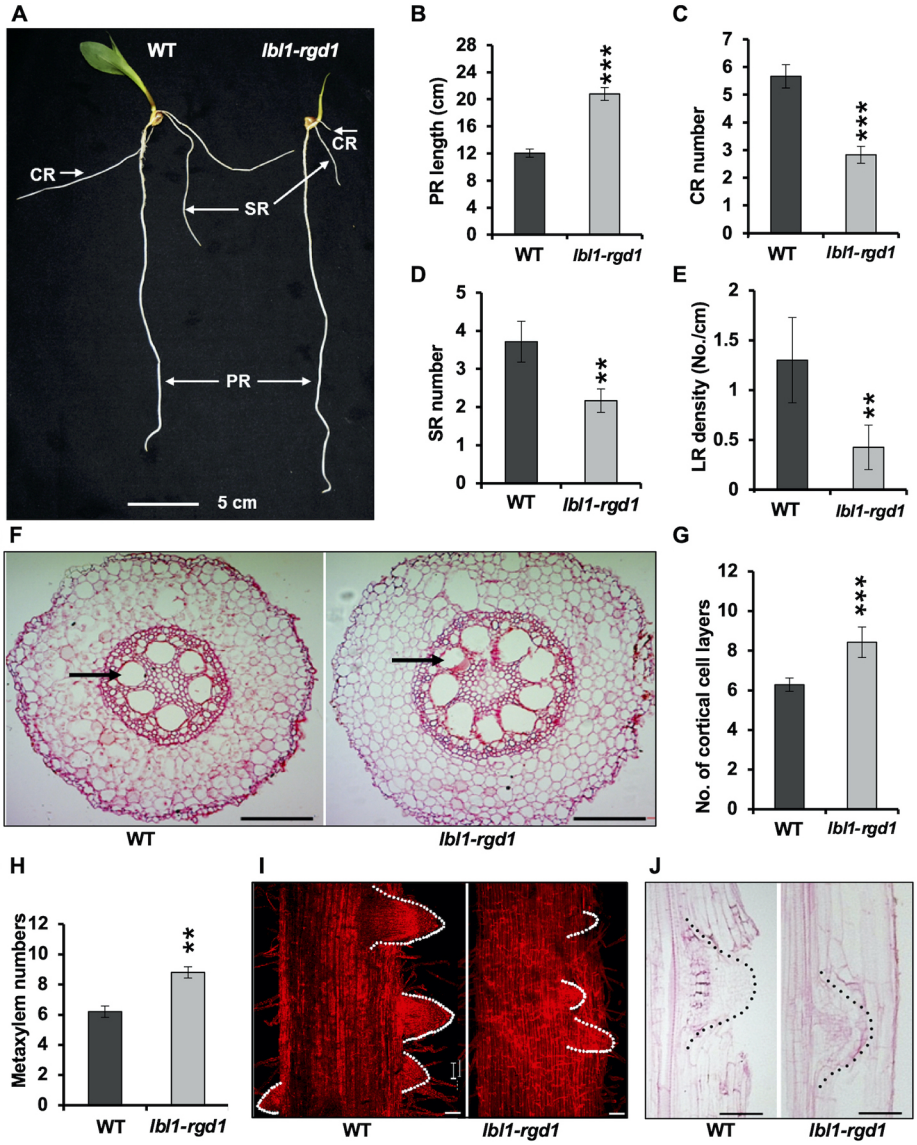

Fig. 1. *lbl1-rgd1* show perturbed root growth with altered vascular patterning.
(A) Representative image of 7-day-old *lbl1-rgd1* and wild-type seedling. Scale bar: 5 cm. (B) Primary root (PR) length. (C) Crown root (CR) number. (D) Seminal root (SR) number. (E) Lateral root (LR) density. *n*=20. (F) Transverse sections of the 0-1 cm region of 7-day-old wild-type and *lbl1-rgd1* root (black arrows indicate metaxylem cells). Scale bars: 0.1 mm. (G) Quantification of the number of cortical cell layers in the wild-type and *lbl1-rgd1* PR tip (0-1 cm). (H) Quantification of the metaxylem number in the wild-type and *lbl1-rgd1* PR tip (0-1 cm). (I) Confocal image of 7-day-old *lbl1-rgd1* and wild-type root (1-2 cm). *n*=10. Scale bar: 100 µm. (J) Longitudinal section of 7-day-old wild-type and *lbl1-rgd1* root (1-2 cm). Scale bars: 0.1 mm. Dotted lines in I,J indicate developing LRPs. Error bars represent the standard error of the results from three independent experiments. One-way ANOVA was used to calculate significant statistical differences (***P<0.001, **P<0.01).

type and *lbl1-rgd1* root (Fig. 2A,B). Mature tasiR-ARFs were highly expressed in wild-type root meristem and developing LRP (Fig. 2A,B). However, in *lbl1-rgd1*, the accumulation of mature tasiR-ARFs was drastically reduced in both PR and LRP (Fig. 2A, B). This result suggests that the *LBL1*-mediated tasiR-ARF pathway contributes to maize root development.

As the accumulation of tasiR-ARFs was reduced in *lbl1-rgd1* root, we have analysed the expression levels of *ZmARF2* (predicted target and closest homologue of *AtARF2*; also referred to as *ZmARF10* by Matthes et al., 2019) and *ZmARF3a-e* (*ZmARF3a*, *ZmARF3b*, *ZmARF3c*, *ZmARF3d* and *ZmARF3e*) genes by qRT-PCR; gene IDs are provided in Table S2 (Dotto et al., 2014). In comparison with wild type, the expression levels of *ZmARF2*, *ZmARF3a*, *ZmARF3b*, *ZmARF3c*, *ZmARF3d* and *ZmARF3e* were increased in *lbl1-rgd1* root (Fig. 2C). Among *ZmARF3a-e*, *ZmARF3b* showed maximum upregulation (4.2-fold) in *lbl1-rgd1* root (Fig. 2C). We further analysed the spatial expression pattern of *ZmARF3b* in PR and developing LRP in wild-type and *lbl1-rgd1* root using *in situ* hybridization. The expression of *ZmARF3b* was significantly stronger in the PR and LRP of *lbl1-rgd1* than in wild type, indicating the potential contribution of *ZmARF3b* to maize root growth and development (Fig. 2D,E).

Auxin accumulation, biosynthesis and transport are affected in *lbl1-rgd1* root

As the distribution of auxin plays an important role in root growth and branching (Balzan et al., 2014; Benkova and Hejatko, 2009; Coudert et al., 2010; Orman-Ligeza et al., 2013), we asked whether auxin homeostasis was affected in *lbl1-rgd1* root, which has altered RSA. To address this, we quantified endogenous auxin levels along different regions of the wild-type and *lbl1-rgd1* roots. Maize root was divided into four regions – (a) (0-1 cm), (b) (1-2 cm), (c) (2-5 cm) and (d) (above 5 cm region) – starting from the root tip (Fig. 3A). We found reduced endogenous auxin levels in the 0-1 cm PR tip region of *lbl1-rgd1* when compared with wild type (Fig. 3B,C). In the regions (b) (1-2 cm), (c) (2-5 cm) and (d) (above 5 cm), endogenous auxin level was higher in *lbl1-rgd1* than in wild type (Fig. 3B). We performed the auxin immunolocalization to further confirm the change in endogenous auxin abundance in the root tip region. We found reduced auxin accumulation in the *lbl1-rgd1* root tip, more specifically in columella, the QC area, cortical cell layers and the vascular region, in comparison with wild type (Fig. 3C). As the endogenous auxin level was reduced in the *lbl1-rgd1* PR tip, we analysed the expression of genes involved in auxin biosynthesis and transport. The expression of *ZmYUCCA1* (*ZmYUC1*), *ZmYUC2* and

Development (2021) 148, dev190033. doi:10.1242/dev.190033

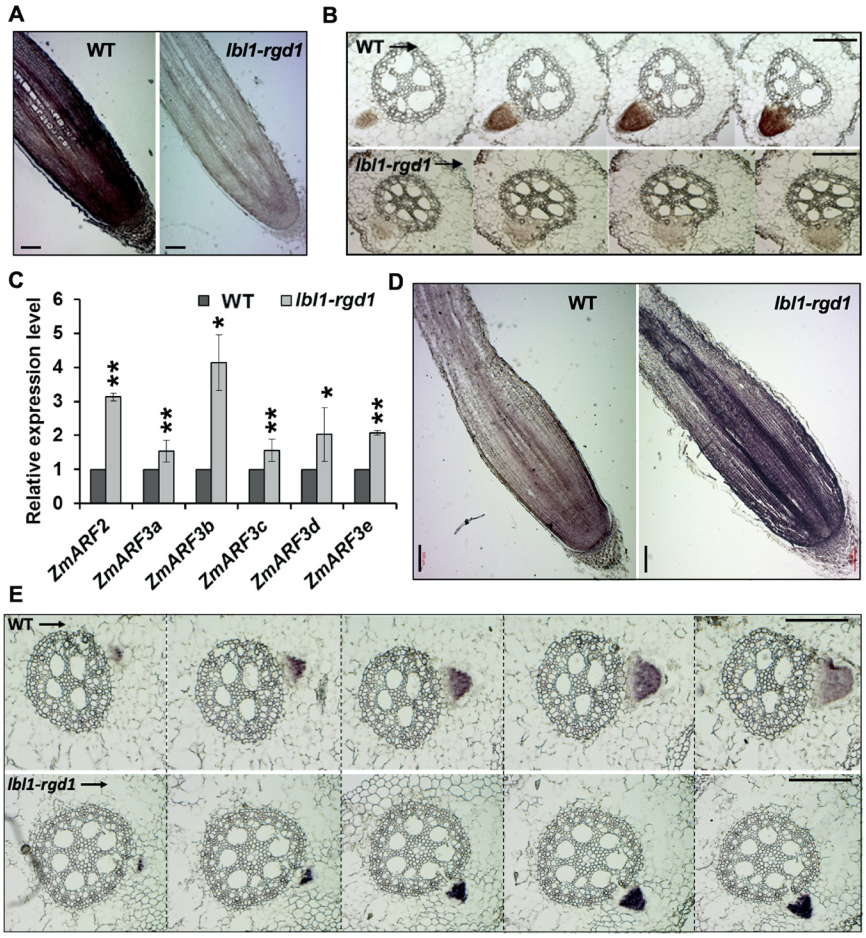

Fig. 2. Tissue-specific localization and expression analysis of ta-siRNA and their targets (tasiR-ARFs). (A) *In situ* localization of tasiR-ARF in wild-type and *lbl1-rgd1* 0-1 cm PR. (B) *In situ* localization of tasiR-ARF in successive sections of developing LR in the 1-2 cm region of wild type and *lbl1-rgd1* root. (C) An elevated level of the target *ZmARF* gene expression was observed in the *lbl1-rgd1* root. (D) *In situ* localization of *ZmARF3b* in *lbl1-rgd1* 0-1 cm PR tip. (E) *In situ* localization of *ZmARF3b* in successive sections of developing LR in the 1-2 cm region of wild-type and *lbl1-rgd1* root. Dark-brown staining indicates the expression. *n*=10. Error bars represent standard error of the results from the three independent experiments. One-way ANOVA was used to calculate significant statistical differences (***P*<0.01, **P*<0.05). Scale bars: 100 in A,B,D,E.

ZmYUC3 were downregulated in the *lbl1-rgd1* PR tip region (Fig. S3A). We analysed the transcript levels of auxin efflux carriers (transporter), *ZmPINFORMED1* (*ZmPIN1a*, *ZmPIN1b*, *ZmPIN1c* and *ZmPIN1d*), *ZmPIN2* and *ZmPIN7* in *lbl1-rgd1* and wild-type root tip. Expression analysis showed that *ZmPIN1a*, *ZmPIN1d* and *ZmPIN7* were significantly upregulated in *lbl1-rgd1* root (Fig. S3B). Furthermore, we have performed *Zm*PIN1 protein immunolocalization to substantiate the changes in auxin transport in

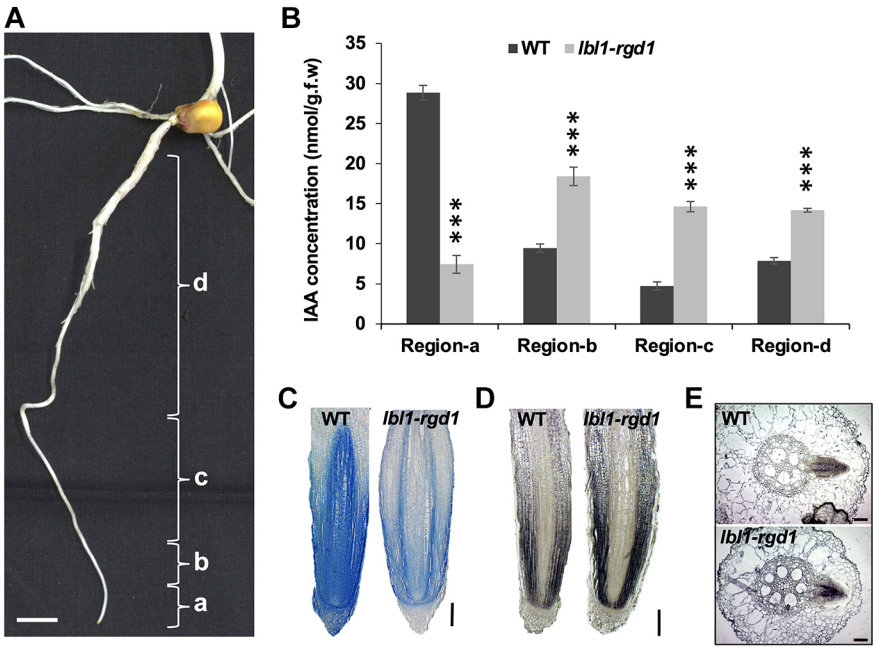

Fig. 3. *lbl1-rgd1* root tissues show altered auxin accumulation and transport. (A) Different regions (a, b, c and d) marked in the maize seedling, used for HPLC. (B) Quantification of the auxin in different regions (a, b, c and d) of the 7-day-old wild-type and *lbl-rgd1* root tissue. (C) Auxin immunolocalization in 0-1 cm wild-type and *lbl1-rgd1* PR tip. (D) ZmPIN1 immunolocalization in the 0-1 cm PR tip of the wild type and *lbl1-rgd1*. (E) ZmPIN1 immunolocalization in the 1-2 cm LR primordia of the wild-type and *lbl1-rgd1* roots. *n*=10. Error bars represent standard error of the results from the three independent experiments. One-way ANOVA was used to calculate significant statistical differences (****P*<0.001). Scale bars: 1 cm in A; 100 µm in C-E.

Development (2021) 148, dev190033. doi:10.1242/dev.190033

the PR tip. We found that the accumulation of PIN1 protein increased in *lbl1-rgd1* PR tip, with more abundance in the cortical cells (Fig. 3D). In LR primordia, the expression domain of ZmPIN1 protein expanded in *lbl1-rgd1*, in comparison with wild type (Fig. 3E).

Mutation in *LBL1* alters the expression of miR165/166 and targets *RLD1* and *RLD2* in maize root

As LBL1 regulates miR165/166 expression in maize shoot (Chitwood et al., 2007; Juarez et al., 2004a), and the miR165/166-*HD-ZIP III* module regulates root growth and vascular patterning in *Arabidopsis* (Carlsbecker et al., 2010; Miyashima et al., 2011; Singh et al., 2014; Turchi et al., 2015), we asked whether this regulatory module is conserved in maize root development. To address this, we studied the expression of miR165/166, *RLD1* and *RLD2* in *lbl1-rgd1* root. We observed that miR165/166 was upregulated, and *RLD1* and *RLD2* were downregulated in *lbl1-rgd1* root compared with wild type (Fig. 4A). Furthermore, we confirmed increased miR165/166 accumulation in *lbl1-rgd1* root through *in situ* hybridization using a LOCKED NUCLEIC ACID (LNA) probe (Fig. 4B). To investigate whether the altered expression of miR165/166, *RLD1/2* (*HD-ZIP III*) might have contributed to the root phenotype of *lbl1-rgd1* in maize, we analysed the root growth and anatomy of the gain-of-function mutant *Rld1-O*, the transcripts of which are resistant to miR165/166-mediated cleavage. We observed that the primary root of *Rld1-O* was shorter than in wild type (Fig. 4C,D) with no significant difference in the CR and SR number, although occasional alteration was observed (Fig. 4C,E,F). Interestingly, there were six cortical cell layers in *Rld1-O* compared with eight in wild-type root (Fig. 4G,I). Moreover, *Rld1-O* showed reduced average number of metaxylems and cortical cells, in comparison with wild type (Fig. 4G,I). We also observed that the root diameter (0.68 mm) and stele diameter (0.42 mm) were much narrower in *Rld1-O* mutant than in wild-type root (0.84 mm) and stele (0.54 mm) (Fig. 4H,I). Our results suggest that the miR165/166-*RLD1/2* module regulates root growth and vascular or anatomical patterning in maize and this module acts downstream of the LBL1/ta-siRNA pathway.

Maize LBL1 can rescue the leaf and root phenotype of *sgs3* in *Arabidopsis*

As both maize LBL1 and its *Arabidopsis* homologue SGS3 proteins show 65% of amino acid similarity (Nogueira et al., 2007), we asked whether they are functionally conserved. First, we carefully analysed the phenotypic similarities of *lbl1-rgd1* and *sgs3*, in terms of leaf and root development. Consistent with a previous report, we observed that *sgs3-11* leaves were elongated and downwardly curled (Fig. S3C) (Peragine et al., 2004). However, strong allele *lbl1-rgd1* showed a severe phenotype with radialized and abaxialized leaves in maize (Timmermans et al., 1998). Two independent alleles, *sgs3-11* and *sgs3-13*, which were genetically cleaned through backcrossing, showed longer PR and reduced length of LRs in *Arabidopsis* (Fig. 5A,B). The number of LRs was increased in *sgs3* root (Fig. 5A). To determine whether both LBL1 and SGS3 are functionally conserved, we developed a complementation construct by expressing *ZmLBL1* under the *Arabidopsis SGS3* promoter (*pAtSGS3:ZmLBL1*) and transformed that into homozygous *sgs3-11* plants, thus making *sgs3-11/-* (*pAtSGS3:ZmLBL1*). We found that the leaf curling phenotype of *sgs3-11* was restored in the *sgs3-11/-* (*pAtSGS3:ZmLBL1*) line in *Arabidopsis* (Fig. S3C). As *lbl1-rgd1* produced a longer PR, similar to *sgs3*, we investigated whether LBL1 could rescue the root growth phenotype of *sgs3-11*. Interestingly, we observed that the complementation line could restore the root length

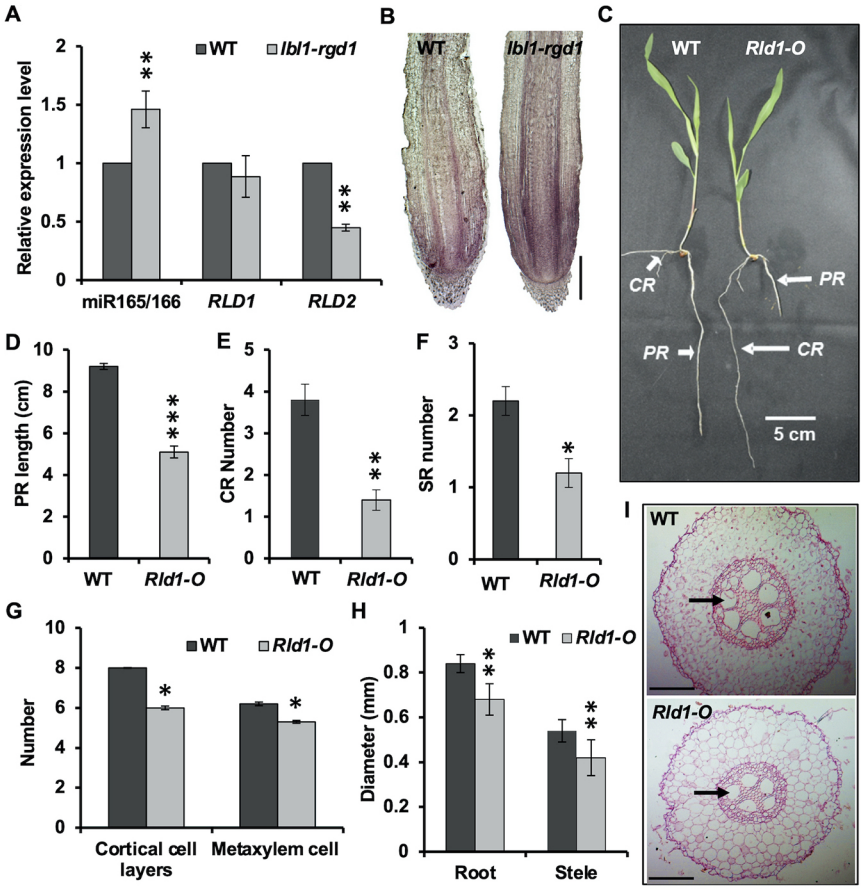

Fig. 4. miR165/166 and target *RLD1/2* regulates root growth in maize. (A) The expression level of the target *RLD1* and *RLD2* was reduced with increased expression of the miR165/166 in *lbl1-rgd1*. (B) *In situ* localization of the miR165/166 in wild-type and *lbl1-rgd1* 0-1 cm PR tip. (C) Root phenotype of 7-day-old *Rld1-O* and wild-type seedlings. PR length was reduced in the *Rld1-O* mutant. (D) Quantification of PR length in wild type and *Rld1-O*. (E) Quantification of CR number. (F) Quantification of SR number. (G) Quantification of cortical cell layers and metaxylem number in wild-type and *Rld1-O* root tip. (H) Quantification of the average root diameter and stele diameter in wild-type and *Rld1-O* PR tip (0-1 cm). (I) Transverse section of PR tip region of wild-type and *Rld1-O* mutant (0-1 cm). *Rld1-O* mutants show reduced number of metaxylem cells. n=10. Error bars represent standard error of the results from the three independent experiments. One-way ANOVA was used to calculate significant statistical differences (***P<0.001, **P<0.01, *P<0.05). Scale bars: 170 µm in B; 100 µm in I.

Development (2021) 148, dev190033. doi:10.1242/dev.190033

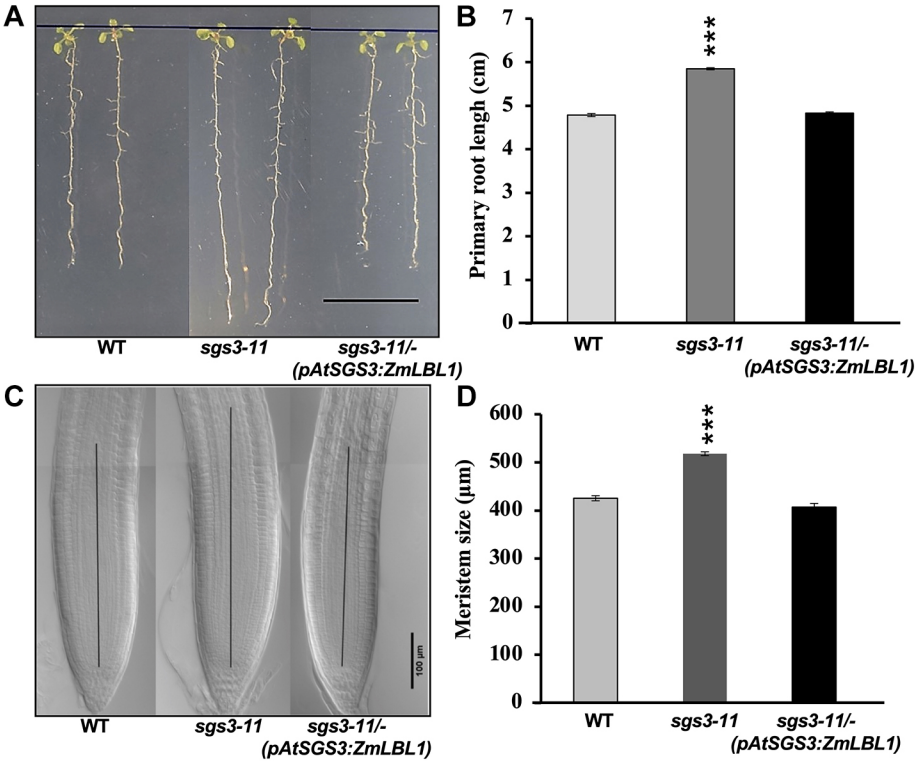

Fig. 5. AtSGS3 and ZmLBL1 proteins are functionally conserved. (A) Growth assay of 7-day-old wild-type, *sgs3-11*, *sgs3-11/-* (*pAtSGS3:ZmLBL1*) lines, *n*=50. Scale bar: 2 cm. (B) Quantification of PR length using Image J. (C,D) Meristem size determination of 5-day-old wild-type, *sgs3-11* and *sgs3-11/-* (*pAtSGS3:ZmLBL1*) lines. Scale bar: 100 µm. *n*=25. Error bars represent standard error of the results from the two independent experiments. One-way ANOVA was used to calculate significant statistical differences (***P<0.001).

to wild-type levels (Fig. 5A,B). Additionally, we observed that the root meristem size of *sgs3-11* was longer than in wild-type plants, whereas the root meristem size of *sgs3-11/-* (*pAtSGS3:ZmLBL1*) was restored to wild-type levels (Fig. 5C,D). Furthermore, we checked the expression level of the tasiR-ARF target genes *AtARF2*, *AtARF3* and *AtARF4* in wild type, *sgs3-11* and the complementation line. In *sgs3-11*, *AtARF2*, *AtARF3* and *AtARF4* were upregulated due to absence of tasiR-ARFs production, whereas in the complementation line, *AtARF2*, *AtARF3* and *AtARF4* expression levels were restored to those in wild type (Fig. S3D). Thus, our results showed that LBL1 is able to rescue the leaf and root developmental defects of *sgs3-11*, possibly by restoring functional tasiR-ARF production in *sgs3-11/-* (*pAtSGS3:ZmLBL1*) plants. This indicates functional conservation of the LBL1- and SGS3-mediated ta-siRNA pathways between dicots and monocots.

DISCUSSION

The developmental pattern of different root types (PR, SR, CR and LR) is regulated by a partially independent genetic pathways. Molecular genetic evidences indicates that the developmental programmes of different root types are at least partially distinct between monocot and dicot plants (Orman-Ligeza et al., 2013). Our study underscores the role of small RNAs (ta-siRNA and a miRNA) in maize root development and their functional conservation with *Arabidopsis*. We show that maize LBL1, which is involved in ta-siRNA biogenesis, regulates root growth and branching possibly by modulating phytohormone auxin and miR165/166-*RLD1/2* module.

LBL1-mediated ta-siRNA function is required for proper root growth, anatomical patterning and branching in maize

LBL1 has been previously shown to regulate maize leaf polarity through ta-siR-ARF (Juarez et al., 2004b; Timmermans et al., 1998). In this study, we showed that the *lbl1-rgd1* mutant produced longer PR and showed reduced number of SRs, CRs and LRs (Fig. 1A-E). The number of metaxylem and cortical cell layers was also reduced in *lbl1-rgd1* root (Fig. 1F). This suggests that LBL1 regulates root growth, branching and anatomy in maize. BrdU staining and meristem cell count results indicate increased cell division in the *lbl1-rgd1* root, which might result in enhanced root growth (Fig. S1C).

In maize, *LBL1* is required for the biogenesis of *TAS3* locus-derived tasiR-ARFs production, which targets transcripts of *ZmARF3* family genes in shoot apex (Dotto et al., 2014; Nogueira et al., 2007). The leaf polarity defects observed in *lbl1-rgd1* are caused by reduced tasiR-ARF accumulation and thus increased activity of *ZmARF3* genes in vegetative apices (Dotto et al., 2014). Recently, five *ZmARF3* genes (*ZmARF3a-e*) were shown to be the targets of tasiR-ARF, and the expression of all *ZmARF3* genes, except *ZmARF3b*, was upregulated in *lbl1-rgd1* vegetative apices, suggesting that *ZmARF3b* may not contribute to the leaf polarity defects in *lbl1-rgd1* (Dotto et al., 2014). We showed that tasiR-ARF accumulation was reduced in root meristem and developing LRP of *lbl1-rgd1*, which correlate to the upregulated expression of *ZmARF2* (a predicted target) and all five *ZmARF3* (*a-e*) genes in *lbl1-rgd1* root (Fig. 2C). Thus, impaired tasiR-ARF production and availability in *lbl1-rgd1* leads to increased abundance of *ZmARF3b* and other *ZmARF2/3* genes that contribute to the altered root phenotype. The upregulated expression of *ZmARF2*, a predicted target (not validated in maize through RNA ligase-mediated-rapid amplification of cDNA ends, RLM-RACE), indicates its potential contribution to root development. In contrast to this observation in maize, the mutation in *TAS3* ta-siRNA pathway genes or their targets affects LR development, but not PR growth, in *Arabidopsis* (Marin et al., 2010; Yoon et al., 2010). Thus, our results suggest that LBL1-mediated balanced expression of tasiR-ARF and their target *ZmARF3s* contributes to proper root growth, anatomy and branching in maize. However, we cannot ignore the possible contribution of other ta-siRNAs and/or other phased siRNAs (phasiRNAs) to LBL1-mediated regulation of root development, which need to be explored further.

Interestingly in *lbl1-rgd1* mutants, the misexpression of tasiR-ARF and target Zm*ARF3* is known to cause adaxial-abaxial polarity defect in leaf (Juarez et al., 2004a); however, this phenotype is not

Development (2021) 148, dev190033. doi:10.1242/dev.190033

obvious in root development (current study). Moreover, some overlap in expression of tasiR-ARF and specific target *ZmARF* genes indicates their distinct regulatory interaction in root. It is also likely that, not only the spatial distribution, but also the maintenance of a balanced dose of ta-siRNA target is important for proper root development in maize.

The LBL1-mediated ta-siRNA pathway regulates root development by altering the auxin homeostasis

The phytohormone auxin, which is biosynthesized in young apical regions and transported to different parts of the plant by various auxin influx (AUX/LAX) and efflux (PINs) carriers, plays a crucial role in root growth and branching (Orman-Ligeza et al., 2013; Overvoorde et al., 2010; Peret et al., 2009). The tissue-specific biosynthesis and transport leads to differential accumulation of auxin along various root tissues, which is required for proper growth of PR and LR formation, and emergence in *Arabidopsis* and rice (Coudert et al., 2010; Hochholdinger and Tuberosa, 2009; Orman-Ligeza et al., 2013; Peret et al., 2009). *OsIAA11/13* and *OsCRL1* are required for auxin-dependent formation of LR and CR, respectively, in rice (Inukai et al., 2005; Kitomi et al., 2012; Zhu et al., 2012). Our results showed that accumulation or distribution of endogenous auxin in various regions along the root was affected in *lbl1-rgd1* (Fig. 3B,C), which could be caused by misregulation of biosynthesis and transporter genes. In *lbl1-rgd1*, reduced expression of the auxin biosynthesis genes *ZmYUC2* and *ZmYUC3* (Fig. S3A) altered expression of *PIN* family genes (Fig. S3B), and PIN1 protein (Fig. 3D) could have contributed to the change in auxin homeostasis observed in different regions of the root (Fig. S3A and B; Fig. 3D). As *lbl-rgd1* has a leaf defect (Timmermans et al., 1998), we cannot rule out the partial contribution of a possible change in shoot-derived auxin and metabolite flow to root phenotype, which is an aspect for future study. However, *LBL1* appears to make an important contribution to the local biosynthesis, transport and tissue-specific distribution of auxin along the zones of the root, which is known to play a pivotal role in root growth and branching (Ditengou et al., 2008; Peret et al., 2009; Singh et al., 2020). The reduction in auxin accumulation in PR ('a' region) of *lbl1-rgd1* might contribute to the change in root growth, whereas altered distribution of auxin in the branch formation regions of root ('c' and 'd' regions) might be responsible for reduced emergence and growth of LRs or CRs (Fig. 3B,C). It has been shown that auxin distribution and maxima formation, and even the subcellular distribution, play crucial roles in root growth and branching (Ditengou et al., 2008). It will be interesting to address in future studies whether subcellular auxin distribution is affected in the LR-forming cells and vascular initials of *lbl1-rgd1* root.

Cytokinins (CKs) regulate root growth and branching (LR and CR) by antagonistically affecting auxin transport and biosynthesis (Benkova and Hejatko, 2009; Laplaze et al., 2007; Peret et al., 2009; Rani Debi et al., 2005). *OsCRL5* is induced by auxin and regulates CR initiation through repression of CK signalling in rice (Kitomi et al., 2011; Zhao et al., 2009). Auxin and overexpression of *OsYUC1* induces the expression of *WUSCHEL-RELATED HOMEOBOX11* (*OsWOX11*), which further interacts with ETHYLENE RESPONSE FACTOR3 (ERF3) and regulates CR initiation and elongation by modulating CK signalling in rice. This indicates the importance of auxin-CK crosstalk in root development (Zhao et al., 2015). We showed that the expression of maize homologue of *Arabidopsis ISOPENTENYL TRANSFERASE* genes (*AtIPTs*), which are involved in CK-biosynthesis, was altered in PR and branching regions of the *lbl-rgd1* root (Fig. S3E,F). This altered CK biosynthesis might be regulating LR formation and emergence by antagonistically affecting auxin biosynthesis, transport and signalling (Fig. S3A,B; Fig. 2C). This indicates that an imbalance of auxin-CK homeostasis along the root could contribute to the altered root growth and branching phenotype observed in *lbl-rgd1*. It is possible that the LBL1-mediated ta-siRNA pathway helps in maintaining auxin homeostasis and auxin-CK balance, which is required for proper root growth and branching in maize through regulation of downstream pathway genes.

In *Arabidopsis*, *LAX3* expression in the cells overlying LRP promotes auxin influx into these cells to induce cell-wall remodelling genes and facilitate lateral root emergence (Balzan et al., 2014; Peret et al., 2013; Swarup et al., 2008). *ZmLAX1* and *ZmLAX2* were downregulated in the root branching mutant *rootless with undetectable meristems1* (*rum1*) in maize (Zhang et al., 2014). In both dicot and monocots, *EXPANSIN* (*EXP*) plays a role in root development (Cho and Cosgrove, 2002; Marowa et al., 2016). Reduced expression levels of *ZmLAX1*, *ZmLAX2* and *Zmα-EXP* (Fig. S2B) may possibly contribute to delayed emergence of LRs or CRs in *lbl1-rgd1* in maize, although this needs to be substantiated with further genetic studies.

The *LBL1*-mediated tasiR-ARF-*ZmARF2/3* module affects the miR165/166-*RLD1/2* module to regulate maize root development

The opposing activities of *TAS3*-derived tasiR-ARF and miR165/166 regulate leaf polarity in maize by negatively regulating target *ARF2/3* and *RLD1*/*RLD2*, respectively (Chitwood and Timmermans, 2007; Juarez et al., 2004a,b). LBL1 regulates miR165/166 expression in maize shoot (Juarez et al., 2004a; Nogueira et al., 2009, 2007), and the miR165/166-*HD-ZIP III* module regulates root growth and vascular patterning in *Arabidopsis* (Carlsbecker et al., 2010; Dello Ioio et al., 2012; Miyashima et al., 2011; Singh et al., 2014; Turchi et al., 2015). We show that the *LBL1* mediated ta-siRNA pathway regulates root growth and branching, and that the expression of miR165/166 was upregulated in a broader domain, whereas *RLD1* and *RLD2* were downregulated in *lbl1-rgd1* root (Fig. 4A). Interestingly, *Rld1-O* plants, which produce *RLD1* transcripts that are insensitive to miR165/166-mediated cleavage, developed a smaller and narrower primary root with a narrower stele and reduced ground tissues than in wild type, which is in contrast to the longer PR and broader stele and ground tissue of *lbl1-rgd1* (Fig. 4C,D,G-I). The variation in reduction of expression could be due to very weak expression of *RLD1* in root meristem, in comparison with *RLD2*. Thus, it is possible that *RLD2* contributes more to the root phenotype of *lbl1-rgd1* than to *RLD1*. Like *REVOLUTA* (*AtREV*) (a homologue of *RLD1/2* in *Arabidopsis*), *RLD1/2* may regulate root development by modulating the expression of downstream target genes as reported in *Arabidopsis* (Brandt et al., 2012). It would be interesting to further study the possible distinct and/or redundant functional contribution of *RLD1* and *RLD2* to maize root development.

In *Arabidopsis*, endodermis-derived miR165/166-mediated repression of *HD-ZIP III* (*PHB*) in the stele in a dose-dependent manner is required for the specification of xylem, pericycle and ground tissue (Carlsbecker et al., 2010; Miyashima et al., 2011; Ursache et al., 2014). High levels of *PHB* promote metaxylem specification (numbers) and increase in ground tissue in *Arabidopsis* root (Carlsbecker et al., 2010; Miyashima et al., 2011). However, unlike *Arabidopsis*, *Rld1-O* (with high *RLD* transcript) root showed less metaxylem and ground tissue (cortical cell layers) (Fig. 4G,I). Together, these results suggest that miR165/166 and *HD-ZIP III* (*PHB* and *RLD1/2*) play similar roles in primary root growth; however, they have distinct functions in vascular and ground tissue patterning in monocot (maize) and eudicot (*Arabidopsis*) plants.

Development (2021) 148, dev190033. doi:10.1242/dev.190033

In *Arabidopsis,* auxin modulates the expression of miR165/166 and targets, *HD-ZIP III* genes, and regulates root growth (Singh et al., 2017, 2014), whereas root-based auxin biosynthesis and polar auxin transport regulate vascular patterning (Ursache et al., 2014). In *lbl1-rgd1* root, altered auxin biosynthesis and transport leading to altered auxin homeostasis along the root might cause upregulation of miR165/166 and downregulation of *RLD1/2* (Fig. S3A,B; Fig. 3B-D; Fig. 4A,B). It is possible that auxin-modulated expression of miR165/166 and *RLD1/2* is mediated by the tasiR-ARF target *ZmARF2/3* genes, which were upregulated in *lbl1-rgd1* root (Fig. 2C). In *Arabidopsis*, HD-ZIP III proteins regulate auxin biosynthesis and transport in a feedback loop (Baima et al., 1995, 2001; Brandt et al., 2012; Huang et al., 2014; Turchi et al., 2015). We cannot rule out the possibility that *Zm*ARFs and *RLD1/2* mediate feedback regulation of auxin transport and/or biosynthesis in maize, which would require further studies. Moreover, altered expression of *IPT* genes in *lbl1-rgd1* root indicates that LBL1-mediated balanced activity of auxin and CK might be required for proper cell division and differentiation, and thus root growth (Fig. S3E,F). We have conceived a hypothetical model demonstrating the role of potential molecular players involved in LBL1-mediated maize root growth and branching (Fig. 6). Taken together, our results suggest that an orchestrated crosstalk between LBL1-mediated ta-siRNA and miR165/166-*RLD1/2* modules is mediated by auxin and is required for root growth and vascular patterning in maize.

LBL1 shows functional conservation with SGS3

AtSGS3 and ZmLBL1 have a 65% amino acid similarity, and each of them has been implicated in leaf development involving the tasiR-ARF-ARF2/3 module in eudicot *Arabidopsis* and monocot maize, respectively (Nogueira et al., 2007; Peragine et al., 2004; Timmermans et al., 1998). In *sgs3*, leaves are downwardly curled with no polarity defect; however, *lbl1-rgd1* leaves become radial and abaxialized (Peragine et al., 2004), which suggests an additional role for LBL1 in moncot leaf development (Dotto et al., 2014; Peragine et al., 2004; Timmermans et al., 1998). We show that *ZmLBL1* can rescue the leaf defect of *sgs3-11* in *Arabidopsis* (Fig. S3C), which suggests the partially conserved function of AtSGS3 and ZmLBL1 in leaf development.

Despite morphological and anatomical differences, monocot and dicot root developmental programmes use some similar and distinct factors, which might be attributed to their molecular evolution (Hochholdinger et al., 2018; Orman-Ligeza et al., 2013). The loss of function of *SGS3* resulted in increased PR length and LR density, whereas the loss of function of *LBL1* led to the increase in PR length, number of cortical and metaxylem cells, and reduction in the number of CR, SR and LRs (Figs 1 and 5). We show that *ZmLBL1* can rescue the root defect of *sgs3-11* in *Arabidopsis*, which suggests the conserved function of AtSGS3 and ZmLBL1 in root development.

Moreover, we show that the expression of tasiR-ARF targets (*ARF2/3/4*) is restored to wild-type levels in the *sgs3-11/–* (*pAtSGS3:ZmLBL1*) complementation line, which further confirms the functional conservation of *ARF2/3/4* at the molecular level (Fig. S3D). Thus, our results suggest that the LBL1- and SGS3-mediated ta-siRNA pathways have conserved functions in root and leaf development in monocot and dicot. However, the phenotypic differences of *lbl1* and *sgs3-11* indicate their distinct function, at some level, that they might have acquired in the course of evolution through sub-functionalization. It is possible that crosstalk of ta-siRNA-ARFs and miR165/166-*RLD1/2* modules and phytohormone signalling contribute to this molecular variation.

MATERIALS AND METHODS

Plant materials and growth conditions

Generation of *lbl1-rgd1* mutant population in maize (*Zea mays*) *B73* inbred line and molecular nature of the mutation have been described previously

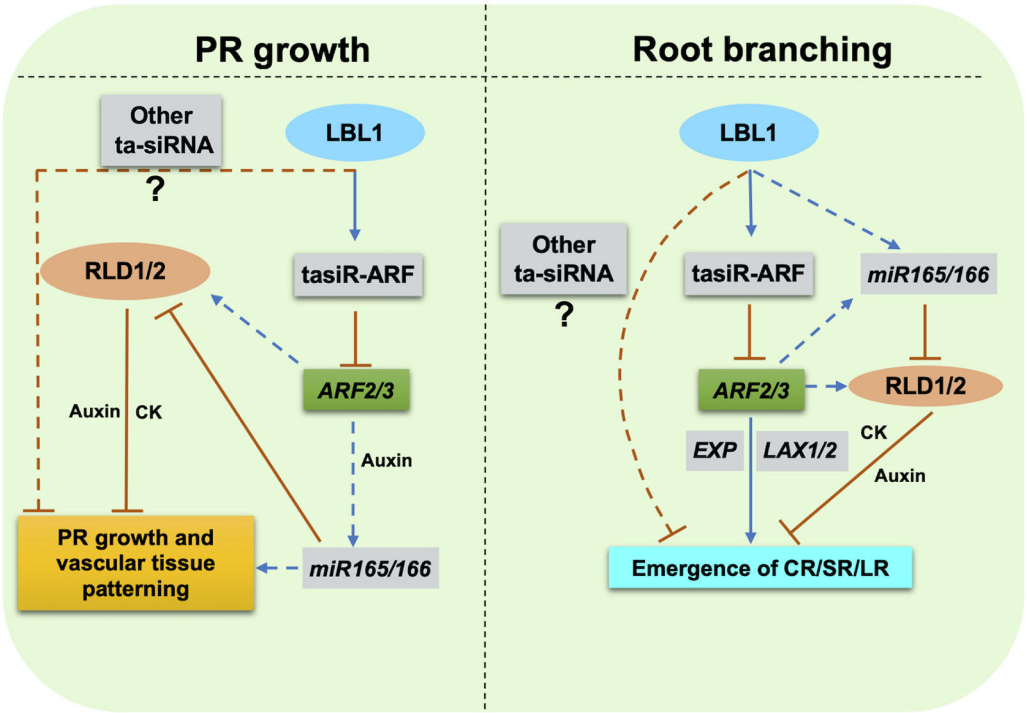

Fig. 6. A putative model of *LBL1*-mediated root development in maize. *LBL1* regulates the activity of downstream target and non-target *ARF* genes. *miR165/166* regulates the activity of the target RLD1/2 to regulate root growth and vascular patterning in maize. The solid arrows indicate positive regulation; dotted blue lines indicate the probable positive or negative regulation; dotted orange lines indicate possible regulation of root development through other ta-siRNAs.

Development (2021) 148, dev190033. doi:10.1242/dev.190033

(Nogueira et al., 2007; Timmermans et al., 1998). A heterozygous population in the *B73* background, segregating wild type, *lbl1-rgd1/−* and *lbl1-rgd1/+*, was used for phenotypical analysis. As homozygous plants were sterile, the mutant was maintained in heterozygous condition. *Rld1-O* is a semi-dominant mutant in maize *T43* inbred line and has been described previously (Juarez et al., 2004a; Timmermans et al., 1998). A heterozygous population in *T43* background, segregating wild type and *Rld-O* were used for phenotypical analysis of root (Juarez et al., 2004a; Nelson et al., 2002; Timmermans et al., 1998). *Arabidopsis thaliana* (ecotype Col-0), and *sgs3-11* and *sgs3-13* mutants have been described previously (Peragine et al., 2004) and were procured from the Arabidopsis Biological Research Center (ABRC), USA. We have cleaned both the mutants by backcrossing to wild type (Col-0) three times.

Maize seeds were germinated in a composite soil (agropeat: vermiculite= 3:1) and were grown in a green house chamber at 28±2°C with ∼70±5% relative humidity and under a photoperiod of 16 h/8 h (day/night) (with ∼300 µmoles/m^2/s light). Age of the seedling was determined as days after germination (dag), which we have used throughout the text as day-old (e.g. 7 dag has been referred to as 7-day-old). *Arabidopsis* seeds were grown on half-strength Murashige and Skoog medium containing 0.8% agar and 1% sugar plates (Murashige and Skoog, 1962) for root growth assays, or on soil, as described previously (Singh et al., 2012). The above experiments were repeated at least thrice with replicates to ensure the reproducibility of observed phenotype.

Confocal microscopy

For visualizing maize LR root, imaging was carried out using a SP5 confocal microscope (Leica); 0.1 mg/ml of PI was used for staining the root tissue for 30 min followed by vacuum infiltration. The selected dye has excitation/emission maxima of 535/617 nm. Meristem size was calculated by measuring the distance between the QC and TZ (transition zone) where cells start elongating. The number of cortical cells within the meristem (as above) was used to determine the meristem (cortical) cell number.

Immunolocalization in plants

Auxin immunolocalization in maize root tissue was carried out by fixing the plant root samples in 4% paraformaldehyde in 100 mM Na_3PO_4 (pH 7.2), vacuum infiltrated and rotated overnight at 4°C. Tissue was dehydrated in graded series of ethanol for half to 1 h each, diluted in tert-butanol. Sample embedding was performed at 65°C by changing the paraffin wax twice in 1 day for a period of 3 days. Samples were sectioned using a rotary microtome (8-12 µM) and were placed on the glass slides. PIN1 antibody used for immunolocalization was procured from Dr Klaus Palme (Pasternak et al., 2015). A detailed protocol of the auxin immunolocalization has been described previously (Forestan and Varotto, 2013).

Real-time quantitative PCR

For quantitative real time PCR (qRT-PCR), gene-specific primers were designed using IDT software and were custom synthesized by Sigma Aldrich. cDNA synthesis and qRT-PCR have been described previously (Singh et al., 2020). *ZmUBQ6* or *18s* rRNA were used as an endogenous control. Dotto et al. have assigned *ZmARF3a* (*GRMZM2G030710*), *ZmARF3b* (*GRMZM2G441325*), *ZmARF3c* (*GRMZM2G056120*), *ZmARF3d* (*GRMZM2G437460*) and *ZmARF3e* (*GRMZM5G874163*) based on their homology to *AtARF3*. In the current study, we have assigned *GRMZM2G338259* as *ZmARF2* as it is the closest homologue of *AtARF2* (Dotto et al., 2014). For the *in silico* predicted target (*ZmARF2*) and previously validated targets (*ZmARF3a-e*) of tasiR-ARF, primers were designed in the region flanking the tasiR-ARF target sites. At least two biological and three technical replicates were used. Relative expression of genes were calculated using the $\Delta\Delta C_t$ method, as described previously (Pfaffl, 2001; Singh et al., 2017, 2012). All primers are listed in Table S1.

Auxin quantification using high-performance liquid chromatography (HPLC)

Root tissues of 7-day-old maize (wild type and *lbl1-rgd1*) seedlings were harvested from different regions (a, b, c and d) (Fig. 3A), and extraction was carried out with 100% methanol (2.5 ml/gram fresh weight). Plant extracts and standard substances were resolved in the reverse phase C-18 column (Apollo C–18, Altech) with a HPLC system (Simadzu). A solvent gradient programme was optimized for indole-3-acetic acid (IAA) and indole-3-propionic acid (IPA) separation in the presence of 0.3% acetic acid. The elution profile was traced with a UV detector (Nakurte et al., 2012). For comparative auxin measurement, equivalent amounts of tissues were taken from four similar regions of wild-type and *lbl1-rgd1* root, and extraction was carried out. Following HPLC, auxin normalization and measurement were carried out accordingly as described previously (Kim et al., 2006; Singh et al., 2020).

Tissue fixation for histology

Different regions (along the root) of 7-day-old maize seedling (*lbl1-rgd1* mutant and wild type) were dissected, fixed in fixative, embedded in paraplast (Sigma) and sectioned using a rotary microtome (Leica RM2265). Tissue fixation and processing have been described previously (Gautam et al., 2016). Embedding and block preparation was performed at 60°C. Successive transverse sections were made in each region of root. Longitudinal sections were made for root tip, including meristem. Sections were stained with 0.5% safranin stain (Sigma Aldrich), mounted on a glass slide and visualized under a bright-field Nikon 80i or Zeiss AxioImager2 microscope. Cell size, area and diameter were calculated using ImageJ (http://imagej.nih.gov/ij/) (Collins, 2007).

In situ hybridization

In situ hybridization was carried out for visualizing the tissue specific expression of both small RNAs and target transcripts. Small RNA localization was carried out using the previously published methods with modifications (Gautam et al., 2019; Javelle and Timmermans, 2012; Singh et al., 2014). Localization of the mRNA transcripts was carried out using a previously described method with modifications (Javelle and Timmermans, 2012; Sarkar et al., 2007; Singh et al., 2014). The elaborate method of tissue fixation/processing and *in situ* hybridization is provided in the supplementary Materials and Methods. LNA probes for miR165/166 (Eurogentec) were as described (Singh et al., 2017), and LNA probes for tasiR-ARF were used for *in situ* localization. Riboprobes of *ZmARF3* were prepared and labelled with digoxigenin (DIG) through *in vitro* transcription using T7 RNA polymerase (Roche), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA: ***P<0.001, **P<0.01, *P<0.05.

BrdU staining

For carrying out the BrdU staining reaction, we used the BrdU Staining Kit (Invitrogen, 93-3943). Longitudinal sections of 7-day-old maize root tips were mounted on glass slides, and incubated in BrdU Labeling Reagent (Thermo Fisher Scientific, 000103) for 24 h at room temperature. BrdU was incorporated into proliferating cells (S phase) and was marked by the staining of nuclei in blue. We have followed the entire protocol for the BrdU staining according to the manufacturer's instructions (http://tools.thermofisher.com/content/sfs/manuals/933944_Rev1009.pdf).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: V.G., A.K.S.; Validation: V.G., A.K.S.; Methodology: V.G., A.S., S.S., S.S.D.; Formal analysis: V.G., A.K.S.; Investigation: V.G., A.K.S.; Data curation: V.G.; Writing - original draft: V.G., A.K.S.; Writing - review & editing: V.G., A.S., S.Y., S.S., S.S.D., A.K.S.; Visualization: V.G., A.S., S.Y., P.K.; Supervision: A.K.S.; Project administration: A.K.S.; Funding acquisition: A.K.S.

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Supplementary information

Supplementary information available online at
https://dev.biologists.org/lookup/doi/10.1242/dev.190033.supplemental

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