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MERISTEM ACTIVITYLESS (*MAL*) is involved in root development through maintenance of meristem size in rice

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Abstract

Key message **Rice *MERISTEM ACTIVITYLESS (MAL)*, a RING-H2 finger domain (RFD)-containing gene, regulates meristem cell viability after the initiation of root primordia mediated by cytokinin signaling.**

Abstract Genes in the RING-H2 finger domain (RFD) family play various roles during plant development and in biotic/abiotic stress responses. Rice gene *MERISTEM ACTIVITYLESS* (*MAL*), being contained in the RING-H2 finger domain (RFD), is characterized by a transmembrane domain at the N-terminal and a C3H2C3 zinc finger domain at the C-terminal. To elucidate the physiological and molecular functions of *MAL*, we generated *MAL* knockdown transgenic plants by RNA interference. *MAL* RNA-interfered (*MRi*) transgenic plants exhibited a phenotype with shorter crown root length and lower crown root number, accompanied by a lower cell division rate. The low division rate was observed in the root meristem exactly where *MAL* was expressed. Furthermore, transcriptome data revealed that cell wall macromolecule metabolism-related genes and redox-related genes were enriched in *MAL* RNAi lines. Most of these differentially expressed genes (DEGs) were induced by exogenous cytokinin. Hence, we conclude that *MAL*, as a novel regulatory factor, plays a major role in maintaining cell viability in the meristem after the initiation of root primordial formation, mediated by cytokinin signaling and reactive oxygen species (ROS).

Keywords Rice · *MAL* · Root meristem activity · Cytokinin signaling · ROS

Introduction

Understanding the mechanisms behind plant root development is important for regulating root growth in order to improve crop performance. The root system of rice is composed of three root types that are formed during consecutive developmental stages. Among these types, crown roots (CRs) [also named shoot-borne roots or adventitious roots (ARs)], which are specific to cereals, initiate from the ground meristem located in stem nodes of coleoptile sections during the postembryonic development process (Marcon et al. 2013) and constitute the main part of the fibrous root system (Zhao et al. 2009). The elongation of crown roots primarily expands the volume of soil explored. It is now widely accepted that many aspects of crown root development, including the temporal sequences of crown root formation, the growth rate and angle, influence the ability of the root system to exploit different spatial and temporal soil niches and are key to optimizing the capture of soil resources. The formation of crown roots leads to an increased number of functional root meristems, which has a profound impact on

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root architecture. Therefore, it is vital to characterize the key genes which function in crown root meristem development. Over the past decades, significant progress has been made in understanding rice crown root morphogenesis. For instance, developmental stages such as initiation, emergence, and elongation have been clearly distinguished (Itoh et al. 2005; Coudert et al. 2010; Kitomi et al. 2011b), and several key genes involved in the control of crown root development have been identified and characterized (Inukai et al. 2005; Liu et al. 2005, 2009; Kitomi et al. 2011a; Zhao et al. 2009; Zhou et al. 2017). However, the molecular mechanisms of crown root development remain elusive.

As is known, the formation of proper root morphology is mainly dependent on the maintenance of the root apical meristem (RAM), which is controlled by phytohormones, transcription factors, as well as Ca^{2+} signaling (Lee et al. 2013; Leitao et al. 2019). It has been reported that cytokinin and auxin are the main hormones involved in RAM maintenance in *Arabidopsis* (Dello Ioio et al. 2007; Dello Ioio et al. 2008; Moubayidin et al. 2009; Di Mambro et al. 2017). In general, cytokinin functions to stimulate cell differentiation by suppressing auxin signaling and transport, whereas auxin plays a role in promoting cell division by inhibiting cytokinin signaling (Lee et al. 2013). Previous studies have also revealed that auxin and cytokinin signaling mutually interact with each other in the root stem cell niche in *Arabidopsis*. For example, PIN-FORMED (PIN) and the AUXIN RESISTANT 1 (AUX1)/LIKE AUX1 (LAX) family transporters form an auxin gradient along the RAM and an auxin maximum at the quiescent center (QC) (Blilou et al. 2005; Grieneisen et al. 2007; Ugartechea-Chirino et al. 2010). Spatial profiling of *PLETHORA* (*PLTs*) expression in RAM can be determined from the auxin distribution, which is vital for stem cell niche specification and its daughter cell proliferation (Scheres and Krizek 2018). Meanwhile, AUXIN RESPONSE FACTOR 5 (ARF5) and ARF7 regulate the expression of *PLT1* and *PLT2* during the development of the embryonic root (Aida et al. 2004). 26S proteasome (26SP) containing AAA-ATPase 5a (RPT5a) has been reported to be involved in adjusting the activity of TIR1/AFB-dependent auxin signaling to an appropriate level required for maintenance of RAM size, especially under high-boron conditions in *Arabidopsis* (Sakamoto et al. 2019). *CROWN ROOTLESS 5* (*CRL5*), which is activated by AUXIN RESPONSE FACTOR (ARF1), promotes crown root development by repressing type A cytokinin-response factor, *RESPONSE REGULATOR 1* (*RR1*) (Kitomi et al. 2011a). OsNAC2, a NAC transcription factor, mediates the crosstalk between auxin and cytokinin signaling pathways to regulate the initiation and elongation of rice roots (Mao et al. 2020). *WUSCHEL-Related Homeobox 11* (*WOX11*) is also induced by auxin and cytokinin, and interacts with ETHYLENE-RESPONSIVE FACTOR 3 (ERF3) protein, which might enhance WOX11-mediated repression of *RR2* or inhibit its function on *RR2* activation during crown root elongation (Zhao et al. 2009, 2015). Root meristem size in rice is also found to be modulated by an AP2 transcription factor SHOEBOX mediated by GA biosynthesis, and SHOEBOX regulates the elongation and proliferation of meristem cells in a developmental stage-specific manner (Li et al. 2015). Intriguingly, nuclear Ca^{2+} signaling in the nucleus of *Arabidopsis* root cells is correlated with meristem development and auxin homeostasis to sustain primary root (PR) growth (Leitao et al. 2019). The miR156/SPL module also plays a role in *Arabidopsis* PR growth by altering root meristem activity, mainly via regulating cytokinin responses (Barrera-Rojas et al. 2019). Taken together, these findings suggest the important role of root apical meristem (RAM) formation and maintenance in controlling root system architecture. However, the regulatory mechanisms underlying the control of RAM activity remain largely unknown.

The ARABIDOPSIS TOXICOS EN LEVADURA (ATL) family is one of the plant-specific RING-H2-type ubiquitin ligases (Aguilar-Hernandez et al. 2011). ATL54 plays an essential role in secondary cell wall formation in *Arabidopsis* (Noda et al. 2013). Meanwhile, as a member of RING-H2, EL5 with RING-type E3 activity is involved in maintenance of root meristem viability via the effect of cytokinin on nitrogen in rice (Koiwai et al. 2007; Takai et al. 2002; Mochizuki et al. 2014). Further analysis suggests that EL5 membrane localization is necessary for its physiological function in rice root development (Koiwai et al. 2007). In this study, we identified another member of RING-H2, viz. *MAL* (*MERISTEM ACTIVITYLESS*), and found that it plays a role in CR growth by altering root meristem activity, mainly via modulating cytokinin responses, in rice. *MAL* exhibits a high transcription level in crown root meristem. Cytological analysis showed that *MAL* is involved in meristematic cell division activity. RNA-interfered plants exhibited root growth with shorter root length and fewer crown roots. *MAL* showed increased sensitivity to exogenous cytokinin. Transcriptome profiling of root tips revealed many cytokinin-related genes with an altered expression level in *MAL* RNAi plants. Taken together, these results suggest that a *MAL*-mediated cytokinin-triggered pathway has an effect on root meristem size in rice.

Materials and methods

Plant materials and growth conditions

Rice cultivar (*Oryza sativa* ssp. *Japonica/Geng*) Zhonghua11 (ZH11) was used in this study. Seeds were surface-sterilized and germinated on MS medium [0.8% agar and 3% (w/v) sucrose] at 28 °C (in light) and 24 °C (in dark)

with a 14/10 h light/dark cycle. Full-length cDNA used in the following experiment was amplified from total cDNA of ZH11 using primers *MAL*-F and *MAL*-R (Table S3).

Vector construction and rice transformation

To construct the RNAi vector, a 304-bp cDNA fragment (Fig. S1A) of *MAL* was amplified from the *MAL* cDNA using primers *MAL*-RNAi F and *MAL*-RNAi R (Table S3) and inserted into the *Kpn* I and *Bam*Hi I sites (for forward insert) and the *Sac*I and *Spe* I sites (for the reverse insert) of the pDS1301 vector (Zhao et al. 2009).

Agrobacterium tumefaciens (strain *EHA105*)-mediated transformation of ZH11 was conducted according to the method of Zhao et al. (2009).

RNA isolation and RT-qPCR

Total RNA of rice root tips was isolated using TRIzol (Invitrogen, 15596026) according to the manufacturer's instructions. For reverse transcription, 4 µg total RNA was digested by 1 µg DNase I. Oligo dT was conjugated with poly A tail by treatment at 65 °C for 10 min and cooled down on ice for 2 min. MLV (reverse transcriptase, Invitrogen, 1906298) with RRI (RNase inhibitor, Takara, 2313A) and DTT (dithiothreitol, Invitrogen, 1906300) were added for reverse transcription at 37 °C for 1.5 h. Products were diluted by adding 140 µl ddH_2O. RT-qPCR analysis was performed in a 96-well plate with an ABI StepOnePlus real-time PCR system (Applied Biosystems). The following thermal profile was used for all reactions: 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The rice *ACTIN1* gene was used as internal control. The relative expression level of each gene in transgenic plants was compared with the wild type, after normalization with *ACTIN1* transcript and averaged from three biological replicates. Bars represent the standard deviation (SD). The sequences of the primers used are listed in Table S3.

In situ hybridization

In situ hybridization and immunological detection were conducted as described by Zhao et al (2009). The *MAL* fragment was amplified from the *MAL* cDNA using the primer set *MAL*-RNAi F and *MAL*-RNAi R (Table S3) and inserted into pGEM-T (Promega, A3600), followed by transcription with either T7 (Roche, 10881767001) or SP6 (Roche, 10810274001) transcriptase by using the Digoxigenin RNA labeling kit (Roche, 11277073910).

Exogenous hormone treatment

Plants were transferred to media with or without 10^{-6} M NAA, 10^{-5} M 6-BA for the indicated hours after germination on agar medium. For kinetics assay, total RNA of root tips (3–5 mm) was extracted and analyzed by RT-qPCR as mentioned before (Zhao et al. 2015; Jiang et al. 2017).

5-Ethynyl-2′-deoxyuridine (EdU) staining

EdU staining was performed as described by Li et al. (2015). Briefly, root tips of 5 days after germination (DAG) rice seedling was labeled in 50 µM EdU (RiboBio, C10310) solution for 2 h. Root tips were fixed by 4% paraformaldehyde, then embedded in 5% agarose. These samples were sectioned for 100 µm and incubated with Apollo. Images were captured with a Leica TCS SP2 confocal laser scanning microscope.

RNA-Seq analysis

Total RNA was extracted from root tips of 5 DAG *MRi* and wild type (ZH11) plants using TRIzol reagent (Invitrogen, 15596026). The collected root tips were just the sections where *MAL* is expressed. RNA libraries were collected according to the protocol provided by Illumina. Briefly, 4 mg total RNA was used for mRNA purification and cDNA synthesis. After first- and second-strand cDNA was synthesized, indexing adapters (TruSeq ChIP Sample Preparation kit, Illumina) were added at 5′ and 3′. DNA fragments were enriched by PCR with 10 cycles. The purified DNA libraries were sequenced with the Illumina HiSeq-3000 system. High-throughput sequencing resulted in the generation of ~ 40 million raw reads for each sample. Two independent biological repeats were analyzed.

For data analysis, after removing low-quality tags with Trimmomatic (v0.32) (Bolger et al. 2014), clean tags were aligned to the rice genome (RGAP, v7.0) by TopHat (v2.1.1). Cufflinks (v2.2.1) software suite was used to assemble transcripts and find differentially expressed genes (DEGs) (fold change > 2, $P < 0.05$) (Trapnell et al. 2012).

To search for cytokinin-inducible genes, published data (GSE39429) were used (https://ricexpro.dna.affrc.go.jp/) (Sato et al. 2013). The cytokinin induction ability of all genomic genes was calculated. Fold change was defined as treatment value (experimental group versus mock group) divided by pretreatment value. Genes with fold changes greater than or equal to 2 times were defined as cytokinin-inducible genes. The website https://ricearray.org/ was used for gene ontology (GO) enrichment analysis.

Results

MAL is expressed in the apical meristem zone of crown roots

To study the molecular mechanism of *MAL* during the crown root formation process, we first analyzed *MAL* expression patterns in different rice tissues/organs. Total RNA was isolated from leaf (Le), stem (St), crown root tips (about 5 mm) (Rt), panicle (Pa), seeds (3 days after pollination, DAP) (Se), and callus (Ca). RT-qPCR results revealed that *MAL* was ubiquitously expressed in all examined tissues/organs and exhibited a high transcript level in root tips but a relatively low level in 3-DAP seeds (Fig. 1A). These results indicate that *MAL* might play a role in controlling root growth. To provide insight into the spatiotemporal variation of *MAL* during crown root formation in more detail, we further explored the expression profile of *MAL* at rice crown root initiation, emergence, and elongation stages by in situ hybridization. The results showed that the transcript of *MAL* was mainly detected in crown root tips, especially in the meristem region [Fig. 1B(a)] as well as during crown root emergence (Fig. 1B(c)). Together, these results indicate a role for *MAL* in establishing and maintaining the RAM in rice.

MAL-downregulated transgenic plants displayed abnormal crown root development

To further reveal the functions of *MAL* during rice crown root development, a 304-bp segment downstream zinc finger domain specific to *MAL* was inserted in inverted repeats fashion to build a construct by RNA interference (RNAi) (Fig. S1A). The construct was transformed into a *Japonica* (*Geng dao*) variety 'Zhonghua11' (ZH11). More than 10 independent transgenic lines were produced, and the relative transcript levels of *MAL* in the root tips of transgenic plants were analyzed using specific primer pairs. Most transgenic plants showed decreased expression of the endogenous gene *MAL*, suggesting the effect of RNAi (Fig. S1B). Two of theRNAi transgenic lines (*MRi-2*, *MRi-3*) were selected for subsequent analysis. Seven days after germination, *MRi* seedlings developed fewer crown roots compared with wild type (Fig. 2a, b). In addition, the primary root length and the plant height were also decreased (Fig. 2a, b; Table 1). To further examine whether *MAL* modulated the initiation of crown root primordia, cross sections of the coleptilar nodal region of 3- and 5-DAG *MRi* and ZH11 seedlings were stained with toluidine blue, respectively. Histological observation revealed that emergence of crown roots in *MRi* transgenic lines was retarded compared with wild type (Fig. 2c). Furthermore, 1-month-old *MRi* seedlings also displayed reduced plant height and many fewer crown roots (Fig. S1C). These results indicate that *MAL* might be essential for crown root initiation and emergence.

MAL participates in regulating meristem size and cell division activity

Continuous root growth and development are sustained by RAM, a reservoir of undifferentiated cells that gives rise to mature root architecture. To determine whether *MAL* regulates root meristem size, longitudinal sections of root tip of wild-type and *MRi* transgenic lines were stained with toluidine blue. The root meristem size of *MRi* plants was shorter than that of

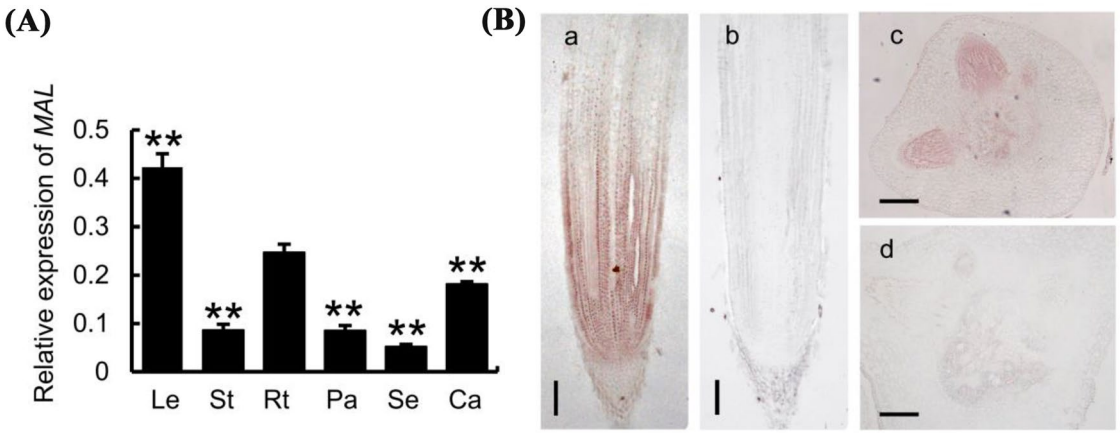

Fig. 1 Expression patterns of *MAL*. **A** Detection of *MAL* transcript by RT-qPCR in leaf (Le), stem (St), crown root tip (Rt), panicle (Pa), seed (Se), and callus (Ca). *ACTIN1* gene was used as internal control. Bars show mean ± SD of three technical replicates. **B** In situ hybridization detection of *MAL* transcripts in crown root tip (a, b) and crown root primordia (c, d) with an antisense or sense probe. Bar: 50 μm in (a, b) and 250 μm in (c, d)

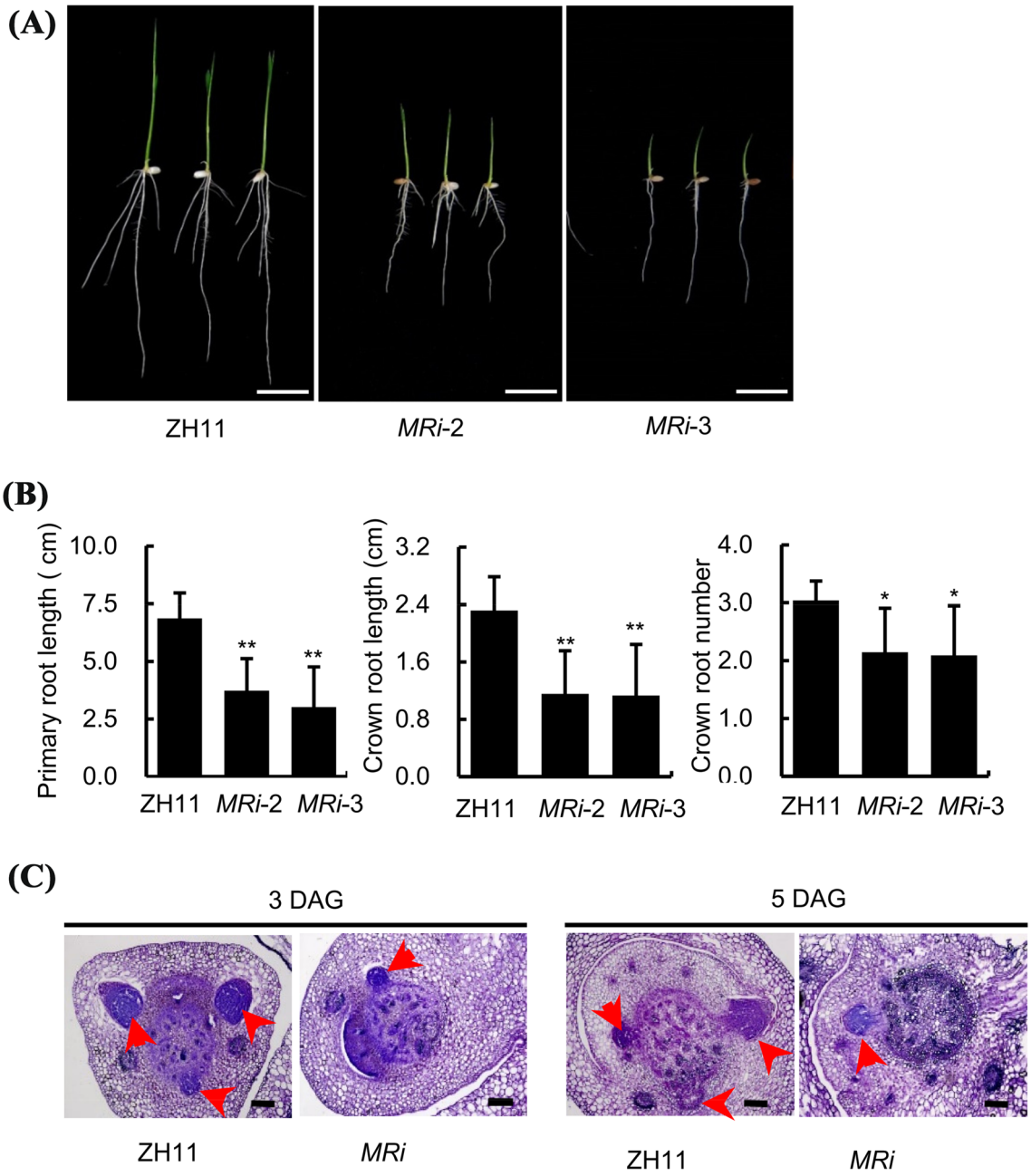

Fig. 2 Transgenic plants with downregulation of *MAL* (*MRi*) displayed severe crown root development effects. **a** Root phenotypes of *MAL* RNAi (*MRi*) and ZH11 plants at 7 DAG (days after germination). ZH11 represents wild type, *MRi*-2 and *MRi*-3 represents two independent transgenic lines. Bar = 3 cm. **b** Statistical analysis of primary root length, crown root length, and crown root number for each genotype. Data represent the mean ± SD of 30 independent biological samples. **c** Crown root primordia numbers in *MAL* RNAi and wild-type (ZH11) seedlings at 3 DAG (left) and 5 DAG (right) as indicated. Arrowheads indicate emerging crown root primordia. Bar = 100 μm. Significance determined by *t*-test: Asterisks indicate significant difference between wild type and *MRi* lines at $*P < 0.05$ and $**P < 0.01$ level

wild type (*MRi*-2, 379.67 ± 34.62**; *MRi*-3, 355.00 ± 58.41**; wild type, 496.88 ± 62.53; data obtained from three lines of transgenic genotype with $n > 15$ for each line) (Fig. 3a, b). Root meristem size is correlated with cell number and cell longitudinal length in the meristem region, so we examined both of them in root-tip longitudinal sections of transgenic and wild-type plants. The results showed that the root meristem of *MRi* plants contained fewer cells (*MRi*-2, 56.33 ± 6.56**; *MRi*-3, 52.50 ± 8.19**) than wild type (81.00 ± 2.55) (Fig. 3b), while no clear difference in cell longitudinal length in root meristem

Table 1 Statistical data of 7-DAG seedlings from *MAL* RNAi lines and ZH11, respectively

Trait	ZH11	*MRi*-2	*MRi*-3
Plant height (cm)	5.2 ± 0.8	3.1 ± 0.7**	2.9 ± 0.8**
Length of primary root (cm)	6.9 ± 1.1	3.7 ± 1.4**	3.0 ± 1.7**
Length of crown root (cm)	2.3 ± 0.5	1.2 ± 0.6**	1.1 ± 0.7**
Number of crown roots	3.0 ± 0.3	2.1 ± 0.7*	2.1 ± 0.8*

Data collected from wild-type (ZH11) and *MAL* RNAi transgenic positive lines (*MRi*-2, *MRi*-3) in generation T3. Data represent mean ± SD for each line

*$P < 0.05$

**$P < 0.01$, *t*-test, two sided ($N = 20$)

was observed between *MRi* and wild-type plants (Fig. 3b). These data suggest that *MAL* knockdown impaired cell proliferation of root meristem in rice. Consistently, EdU staining indicated that *MRi* noticeably decreased cell proliferation in root meristem (Fig. 3c, d). All of these data suggest that *MAL* is involved in promoting cell division of root meristem in rice.

MAL is involved in response to exogenous cytokinin and in cytokinin-related pathway

Cytokinin and auxin have central, but opposite, regulatory functions in root development. To ascertain whether *MAL* expression is responsive to exogenous cytokinin and auxin treatments, analysis of the promoter sequence of *MAL* gene was performed by using the PLACE program (https://www.dna.affrc.go.jp/PLACE/?action=newplace). The results showed that *MAL* promoter included 36 cytokinin response element NGATT (Ross et al. 2004; Sakai et al. 2000), 4 auxin response element TGTCTC (Ulmasov et al. 1999) or GAGAC (Maruyama-Nakashita et al. 2005), and 4 ABA response element MACGYGB (Kaplan et al. 2006) (Fig. S2A). Furthermore, we examined the expression levels of *MAL* in 7-DAG seedling root tips treated with 10^{-5} M 6-benzylaminopurine (6-BA) and 10^{-6} M 1-naphthaleneacetic acid (NAA) during different time courses by RT-qPCR, respectively. These results showed that, apart from NAA treatment, the transcript of *MAL* was greatly induced by 6-BA 0.5 h post treatment, compared with control (Fig. 4a; Fig. S2B). Additionally, in situ hybridization results also revealed that the transcript level of *MAL* in cytokinin-treated crown roots was higher than in control [Fig. 4B(a, b)]. Collectively, these results indicate that *MAL* might be involved in cytokinin-mediated crown root development.

Knockdown of *MAL* affects expression of cytokinin-related genes

To reveal the physiological function of *MAL*, the transcript changes in crown root tips of two different *MRi* transgenic lines and wild type (ZH11) were investigated. The sequence data displayed high reproducibility (coefficient of association, $R > 0.99$) (Table S1). Above 97% of the reads were of high quality, of which about 95% aligned well to the rice genome sequence. Above 80% of the aligned reads were unique (Table S1). The uniquely aligned reads were used to calculate the relative abundance of transcripts. A total of 1179 differentially expressed genes (DEGs) (405 upregulated, 774 downregulated) exhibited greater than twofold change ($P < 0.05$) in *MRi* plants versus wild type (Fig. 5a). Most of the DEGs fell into the posttranslation, proteolysis, metabolism, stress, and cell components categories (Fig. 5b), while about 35% of the DEGs had no annotated function. More interestingly, we found that a noticeable number of the down- (103) and upregulated (17) genes could be inducible during cytokinin treatment (https://ricexpro.dna.affrc.go.jp/; Fig. 5c), including cytokinin dehydrogenase precursor (*LOC_Os01g56810*) and *cis*-zeatin *O*-glucosyltransferase (*LOC_Os04g47720*) (Table S2). We selected 12 DEGs to verify the accuracy of the RNA-Seq results and found a high correlation coefficient between the two datasets (Fig. 6a). To further determine whether these genes involved in cytokinin metabolism and signaling were regulated by *MAL*, we examined the relative expression levels of not only these DEGs, but also other cytokinin responsive regulators (RRs) and cytokinin oxidases/dehydrogenases in wild type (ZH11) and *MRi* root tips. We found that all these genes were highly activated in *MRi* transgenic plants compared with wild type (Fig. 6b, Fig. S3). This result suggests that *MAL* regulates rice root development by mediating cytokinin homeostasis/signaling.

Discussion

Root growth is mainly dependent on cell proliferation in the RAM zone (Takatsuka and Umeda 2014; Dello Ioio et al. 2012). The root cell proliferation speed and root meristem size are determined by the cell division rate in the root meristem zone. In rice, crown root growth is modulated by many factors, such as phytohormones (Lin and Sauter 2019; Sun et al. 2015; Li et al. 2015), transcription factors (Inukai et al. 2005; Liu et al. 2005; Zhao et al. 2009; Li et al. 2015), microRNAs (miRNAs) (Cho and Paszkowski 2017; Bian et al. 2012), and the interplay between them. In this study, we identified *MAL* as a novel regulatory gene involved in the development of rice crown roots by controlling meristem size. Our results suggest that *MAL* has a high expression level in root meristem (Fig. 1). Knockdown of *MAL* significantly decreased the number and length of crown roots (Fig. 2, Fig. S1). Histological analysis further showed that *MRi* plants displayed retardation of crown root primordia initiation and impairment of

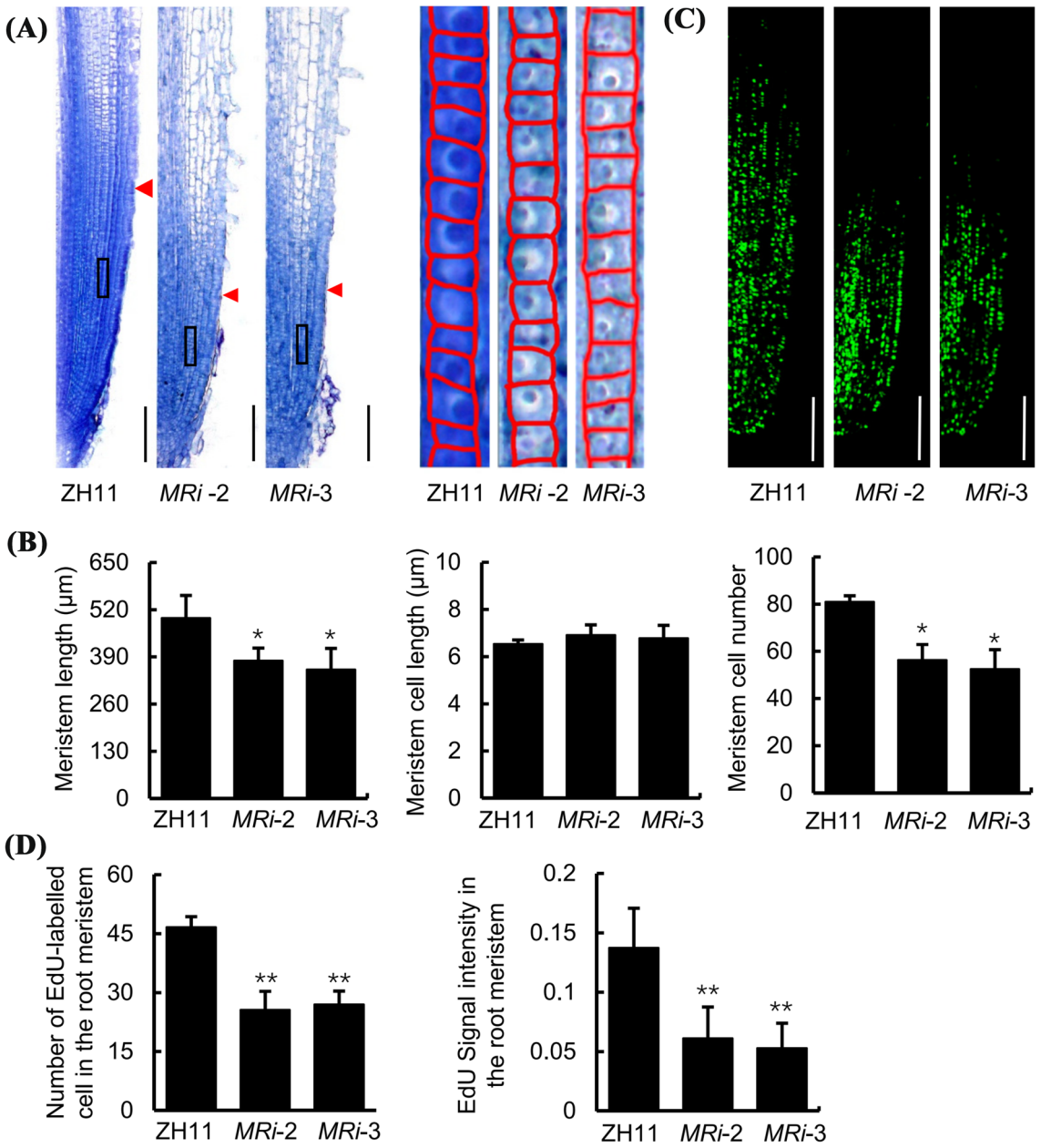

Fig. 3 *MRi* plant roots show decreased root meristem size and activity of meristem. **a** Median longitudinal sections through crown root tips of 5-DAG ZH11 and *MAL* RNAi (*MRi*) seedlings. Red arrowheads indicate the proximal end of the root meristem. Black box marks region selected for enlargement in meristem region. **b** Quantification analysis done at the fourth cortical layer in the whole meristem region. **c** Median longitudinal view of EdU staining in root meristem of ZH11 (left) and *MRi* (right) seedlings. **d** Quantification analysis of EdU-stained cell number and signal intensity (using ImageJ software) at the fourth cortical layer in the whole meristem region. Bar = 150 μm in (a, b). Error bars represent SD ($n = 10$). *$P < 0.05$, **$P < 0.01$, *t*-test

cell mitosis activity in RAM (Fig. 2b). These results suggest that *MAL* preferentially acts in root development after initiation of root primordia by maintaining the viability of meristematic cells. The expression profile and histological phenotype of *MAL* were similar to those of *WOX11* (Zhou et al. 2017), and the transcript level of *MAL* was also reduced in *wox11* (Fig. S3A). However, no WOX11 binding site was found in the *MAL* promoter and gene body. These results demonstrate that the expression of *MAL* might be regulated indirectly by WOX11. In addition, expression of *WOX11* was mainly detected in emerging crown root primordia and later growth stage (Zhao et al. 2009), whereas *MAL* was expressed during the initiation of crown root primordia [Fig. 1B(c)]. These results

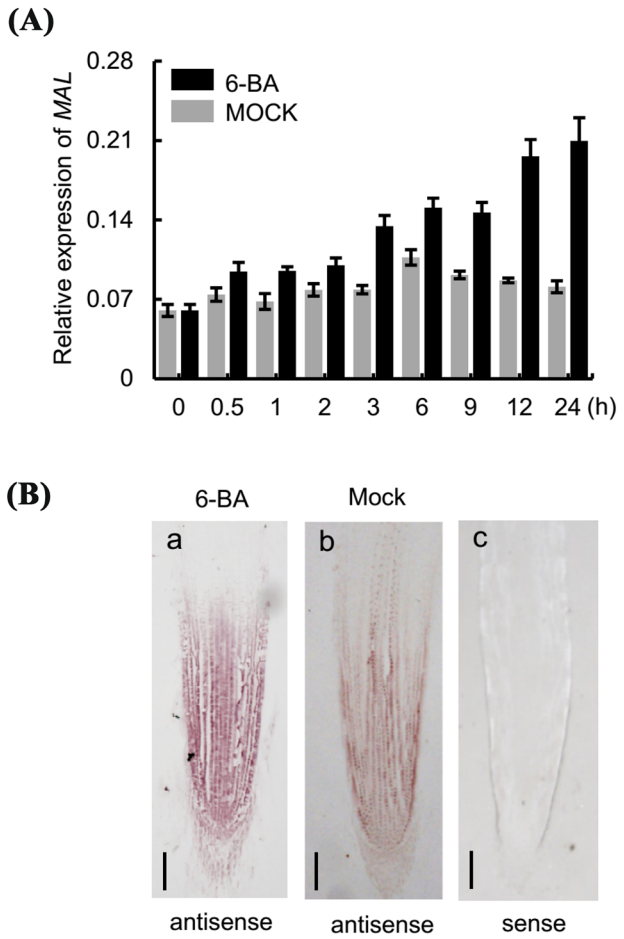

Fig. 4 *MAL* is responsive to exogenous cytokinin treatment. **A** Kinetics of induction of *MAL* in response to 6-BA. The transcript level of *MAL* in roots of 7-DAG light-grown wild-type seedlings treated with 6-BA (black) or water (white) for the indicated durations. PCR signals normalized with those of *ACTIN1* transcripts. Bars are mean ± SD of three technical replicates. **B** In situ hybridization detection of *MAL* transcripts in crown root tip treated with 6-BA (a) and mock (b) with antisense (a and b) or sense probe (c). Bar = 250 μm

suggest that *MAL* and *WOX11* might be involved in early development stages of crown roots. To investigate whether *MAL* and *WOX11* regulated crown root development by a similar pathway, the expression levels of downregulated genes in *wox11* (Jiang et al. 2017) were also detected in *MRi* plants (Fig. S3B). However, these gene transcripts were not significantly different in *MRi* lines compared with wild type, indicating that *MAL* controlled crown root development via a different pathway than *WOX11*. It has been reported that *CRL5* regulates crown root development through activation of *OsRR1* (Kitomi et al. 2011a). OsRR1 and OsRR2 regulate *OsCKX4* by directly binding to its promoter (Gao et al. 2014). Interestingly, in *MRi* crown root tips, the expression levels of *CRL5*, *OsRR1/2*, and *OsCKX4* were clearly decreased (Fig. S3A; Fig. S6B). These results demonstrate that *MAL* might act upstream of *CRL5* and be involved in crown root development through cytokinin signaling.

Cytokinin plays a crucial role in determining root meristem size by controlling the switch from cell proliferation to cell differentiation (Takatsuka and Umeda 2014), and inhibiting root elongation by reducing the number of dividing cells and the size of root meristem (Hwang et al. 2012; Moubayidin et al. 2009; Schaller et al. 2015; Vanstraelen and Benkova 2012; Werner et al. 2003). In *Arabidopsis*, dysregulation of cytokinin-related genes could affect root meristem activity, resulting in abnormal root growth and development (Miyawaki et al. 2006; Sakamoto et al. 2006; Hirose et al. 2007; Cheng et al. 2010), and an increase in the level of endogenous cytokinin further leads to inhibition of primary root growth (Sakamoto et al. 2006). Disruption of eight of the ten type-A ARR genes affects root development by altering the size of the apical meristem (Zhang et al. 2011). Mutation of a maize type-A RR gene, *ABPHL1* (*ABPH1*), induced an increased root meristem size (Giulini et al. 2004). In rice, overexpression of *OsRR3*, *OsRR5*, and *OsRR6* affects crown root development (Hirose et al. 2007; Cheng et al. 2010). In this study, exogenous cytokinin greatly induced *MAL* expression in crown root tips (Fig. 4a, b). In the root tips of *MAL* RNAi transgenic plants, the expression levels of many genes encoding cytokinin synthesis, cytokinin response regulators (RRs), and oxidase/dehydrogenase were obviously changed (Fig. S3C). These results suggest that *MAL* plays a partial role in crown root development by mediating cytokinin signals, which is necessary for induction of cell division and elongation as well as maintenance of meristem identity. Similarly, *EL5*, another ATL member, was reported to maintain the viability of root meristem through the effect of cytokinin on nitrogen in rice (Mochizuki et al. 2014). However, in our study, the transcript level of *EL5* was not affected in *MAL* RNAi plants roots (Fig. S3A). These results indicate that *MAL* and *EL5* might play different roles in rice crown root development mediated by cytokinin signaling. The molecular mechanism by which *MAL* affects root meristem cell activity through cytokinin-triggered pathway remains to be further elucidated.

Our transcriptome data show that the DEGs in *MAL* RNAi plants were enriched in cell wall macromolecule metabolism and oxidation–reduction (Fig. S4), and many of them were cytokinin-induced genes, such as *LOC_Os01g54620*, *LOC_Os01g61160*, and *LOC_Os01g61940* (Figs. 5c, 6a; Table S2). It was recently reported that changes in cell size were essential for the initial steps of cell differentiation, which were related to cytokinin activation of expansion (Pacifici et al. 2018), thus triggering the differentiation through the mechanical control of cell walls. The cell wall loosening consequently induced cell elongation, driving cell differentiation in the

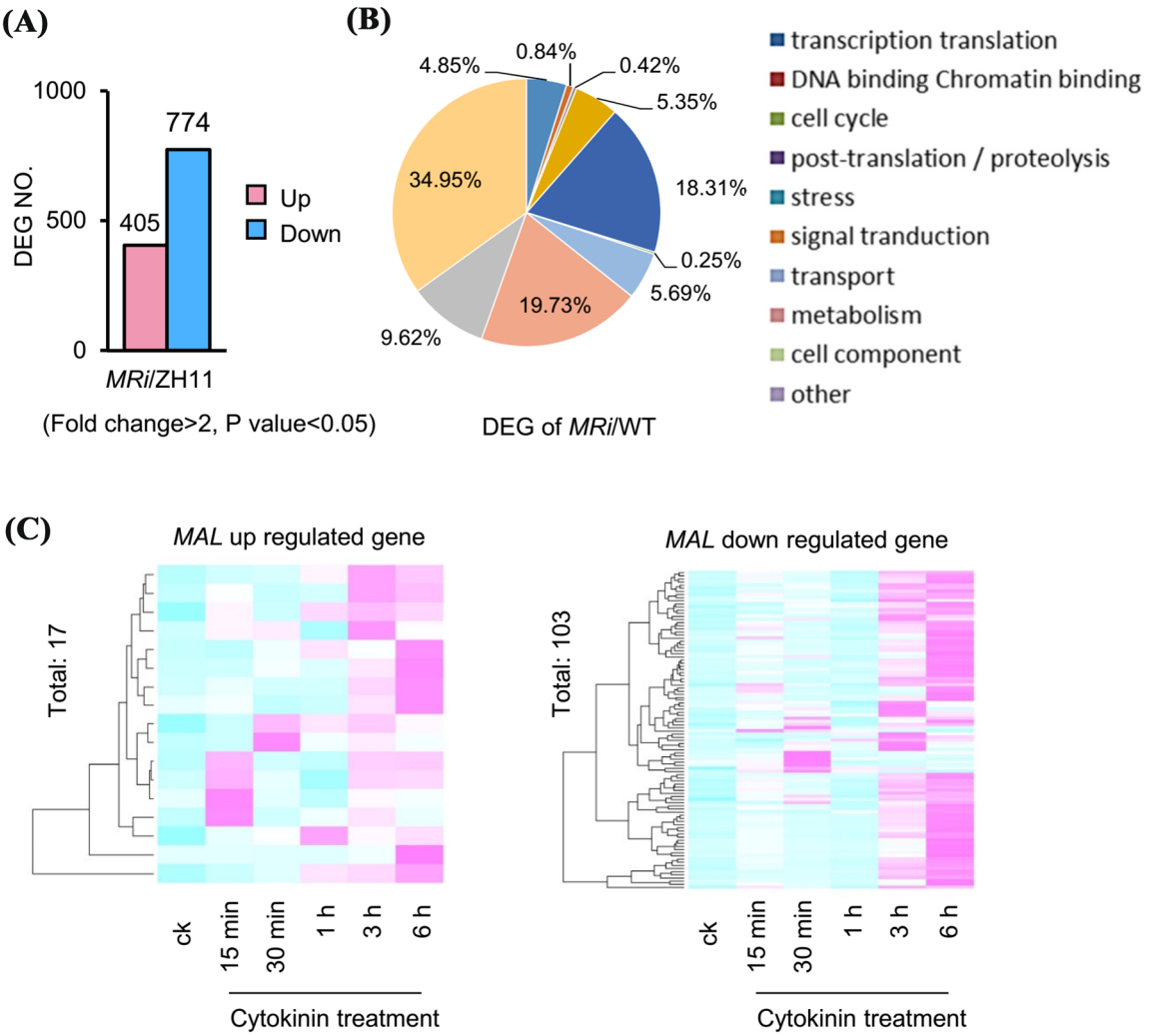

Fig. 5 Knockdown of *MAL* affects the transcripts of a large number of cytokinin-regulated genes. **a** Differentially expressed gene numbers of *MRi* compared with wild type. **b** Functional categorization of 1,179 genes with altered expression in *MRi* crown root by gene ontology analysis. **c** Relative transcript level of identified differentially expressed genes (17/103 up/down) that are responsive to cytokinin

root meristem. We found that some DEGs related to cell wall formation were also responsive to exogenous cytokinin (Fig. 5c). These results indicate that MAL might be involved in cell wall metabolism by mediating cytokinin-regulated cell differentiation and growth. Higher H_2O_2 level in *vtc-1* mutant RAM led to increased QC cell number and periclinal division (Kka et al. 2018). This result reveals that H_2O_2 level and cell division exhibit a certain correlation during RAM size maintenance. Accumulating evidence demonstrates that the cell redox state has an impact on its proliferation/differentiation program; For instance, in *Arabidopsis* embryonic roots, cells in the oxidized state were accelerated during divisions of the G1 phase, whereas cells in the reduced state were slowed down (de Simone et al. 2017). These results suggest that regulating cell redox is a key sign of cell activity in root apical meristem. Meanwhile, overexpression of *IPT* increased cytokinin synthesis, which alleviated inhibition of root growth in response to drought through ROS scavenging system activation in *Agrostis stolonifera* (Xu et al. 2016). These results demonstrate the interplay between cytokinin and oxidative state to maintain better root growth. In *MRi* plants, oxidation reduction-related genes were enriched (Fig. S4), and importantly, expression levels of these DEGs were also induced by cytokinin (Fig. 5c). These results demonstrate that *MAL* might play a role in the crosstalk between ROS and cytokinin (Fig. 7). At present, although there is limited information about the relationship between MAL, ROS, and cytokinin in regulation of root meristem size responsible for crown root growth, our results provide a theoretical basis for further research into the interaction.

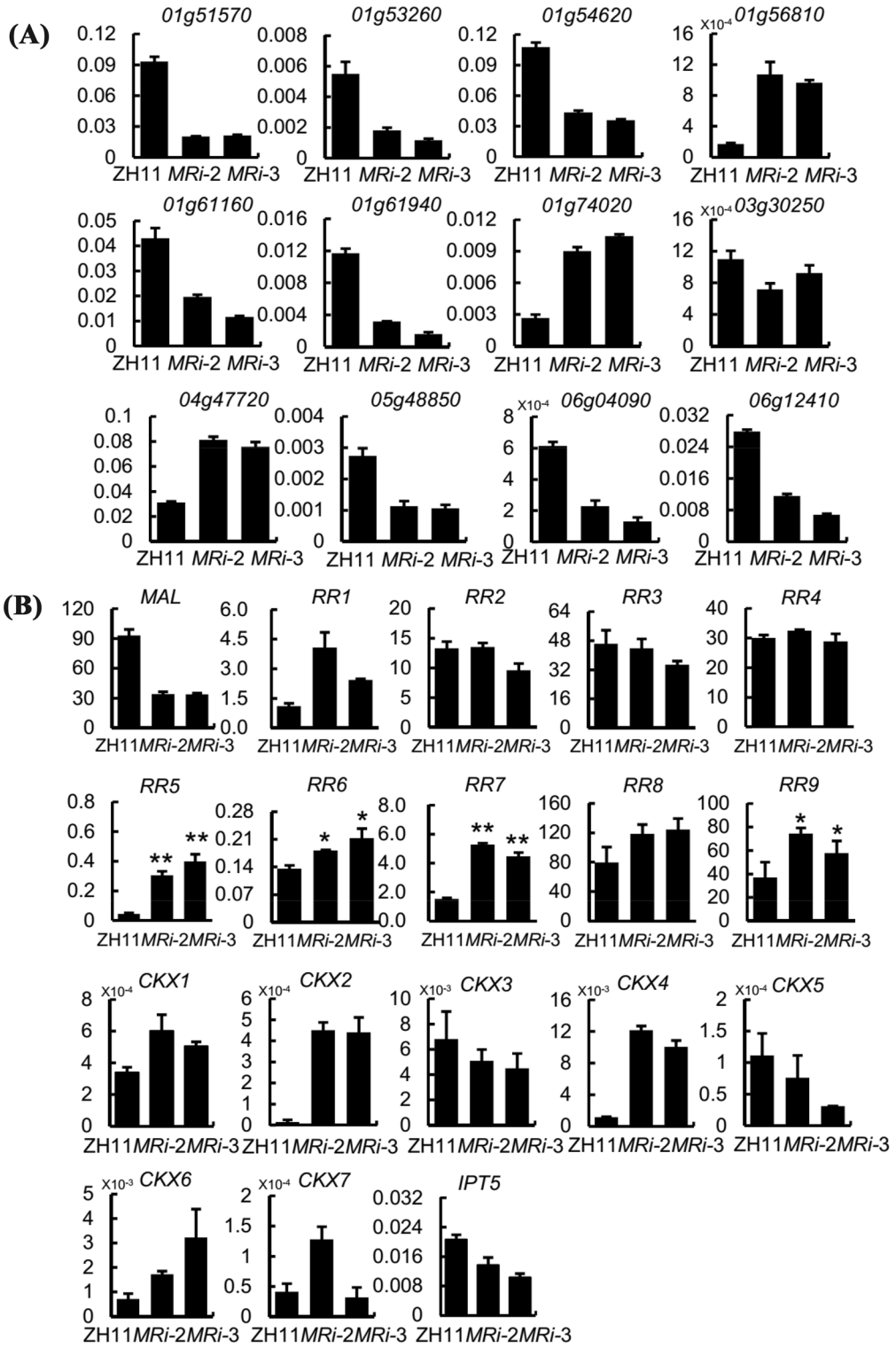

Fig. 6 Determination of relative transcript levels of putative target genes in the root tips of two *MAL* RNAi lines (*MRi*-2 and *MRi*-3) compared with wild type (ZH11). Values are relative to *ACTIN1* transcript. Bars indicate mean ± SD of three technical replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test

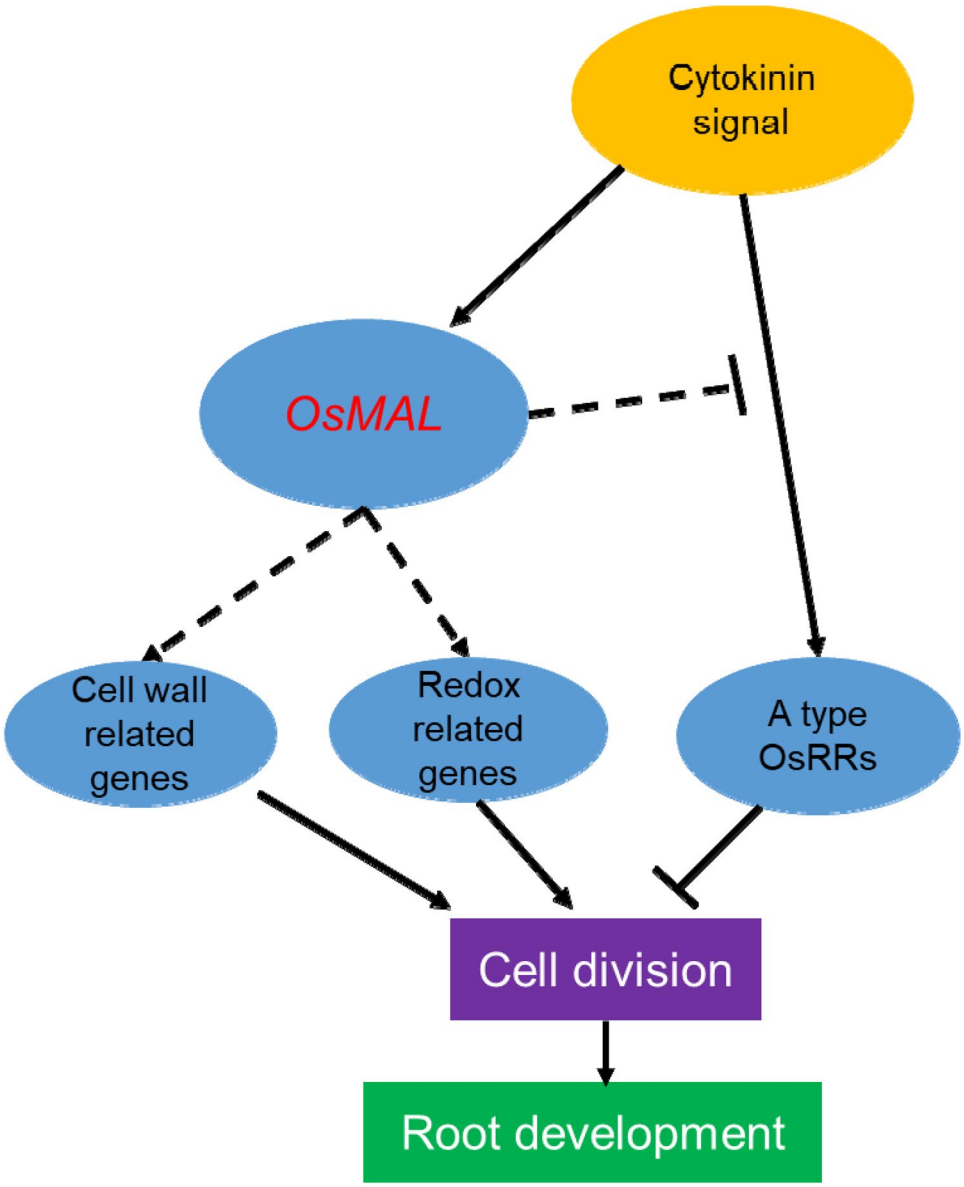

Fig. 7 Hypothetical gene regulatory network regulated by *OsMAL* in crosstalk between ROS and cytokinin

Accession numbers

Sequence data from this article can be found on the Rice Genome Annotation Project website (https://rice.plantbiology.msu.edu/) under the following accession numbers: *MAL*, LOC_Os06g19680; *EL5*, LOC_Os02g57490; *CKX1*, LOC_Os05g31040; *CKX2*, LOC_Os01g10110; *CKX3*, LOC_Os01g09260; *CKX4*, LOC_Os01g71310; *CKX5*, LOC_Os06g37500; *CKX6*, LOC_Os06g37500; *CKX7*, LOC_Os02g12770; *CKX8*, LOC_Os01g56810; *CKX9*, LOC_Os08g35860; *RR1*, LOC_Os04g36070; *RR2*, LOC_Os02g35180; *RR3*, LOC_Os02g58350; *RR4*, LOC_Os01g72330; *RR5*, LOC_Os04g44280; *RR6*, LOC_Os04g57720; *RR7*, LOC_Os07g26720; *RR8*, LOC_Os08g28900. The RNA-Seq described in this paper have been deposited in the National Center for Biotechnology Information databases (GSE141854).

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Author contributions W.J. and Y.Z. designed the experiment. W.J. S.Z. H.H., and H.S. performed the experiments. Q.Z. managed the fields. W.J., S.Z., and Y.Z. analyzed the data. J.W. and Y.Z. wrote the article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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