Expression of MYB transcription factor gene *ZmMYB59* affects seed germination in *Nicotiana tabacum* and *Oryza sativa*

Kaihui Zhai

Zhejiang A and F University

Guangwu Zhao (✉ gwuzhao@126.com)

Zhejiang A and F University <https://orcid.org/0000-0001-5646-9922>

Hongye Jiang

Zhejiang A and F University

Caixia Sun

Zhejiang A and F University

Jingyu Ren

Zhejiang A and F University

Research article

Keywords: MYB transcription factor, ZmMYB59, negative regulation, seed germination

Posted Date: March 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-19878/v1>

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Abstract

Background

MYB transcription factors are involved in many biological processes, including metabolism, development and responses to biotic and abiotic stresses. In our previous work, a new MYB transcription factor gene, ZmMYB59 was induced by deep sowing and down-regulated during maize seed germination via Real-Time PCR. However, there are few reports on seed germination regulated by MYB proteins and the functions of ZmMYB59 remain unknown.

Results

In this study, to examine its functions, *Agrobacterium* -mediated transformation was exploited to generate ZmMYB59 transgenic tobacco and rice. In T₂ generation transgenic tobacco, germination rate, germination index, vigor index and hypocotyl length were significantly decreased by 25.0~50.9%, 34.5~54.4%, 57.5~88.3% and 21.9~31.2% compared to wild-type (WT) lines. In T₂ generation transgenic rice, germination rate, germination index, vigor index and mesocotyl length were notably reduced by 39.1~53.8%, 51.4~71.4%, 52.5~74.0% and 28.3~41.5%, respectively. On this basis, relative physiological indicators were determined. The activities of catalase, peroxidase, superoxide dismutase, ascorbate peroxidase and proline content of transgenic lines were significantly lower than those of WT, suggesting that ZmMYB59 reduced their antioxidant capacity. As well, ZmMYB59 expression extremely inhibited the synthesis of gibberellin A₁ (GA₁) and cytokinin (CTK), and promoted the synthesis of abscisic acid (ABA) concurrently, which implied that seed germination was repressed by ZmMYB59 in hormone levels. Furthermore, cell length and cell number of hypocotyl/mesocotyl in transgenic plants were notably decreased.

Conclusions

Taken together, it proposed that ZmMYB59 plays a negative regulation during seed germination in tobacco and rice, which also contributes to illuminate the molecular mechanisms regulated by MYB transcription factors.

Background

Seed germination is a remarkably pivotal stage in the biological clock of plants [1], involving the fast absorbing water by the dry seeds until all of the seed organs are hydrated thoroughly; a dynamic equilibrium in which the water potential of the seed remains unchanged; and a phase of rapid combination water for radical elongation [2–4]. For most plants, the germination stages are the most delicate to biotic and abiotic stresses. On that account, the investigation into seed germination is recommended to assess and breed the ecological adaptation of the crops. Recently, some researches have documented that MYB transcription factors have noteworthy influences on this developing phases [5–8].

MYB, the largest transcription factor family in plants, is widely distributed in both monocotyledons and dicotyledons [9, 10]. MYB proteins are characterized by a highly conserved DNA-binding domain: the MYB domain about 52 amino acids, the C-terminal region alters strikingly from one MYB protein to another, thereby allowing MYB superfamily to perform a considerable assortments of structures and functions [6, 11]. Depending on the number of conserved motifs, the superfamily is divided into four classes: R1-MYB, R2R3-MYB, R1R2R3-MYB and 4R-MYB [12]. The functions of MYB proteins have been probed in plentiful plant species such as Arabidopsis, maize, rice, petunia, snapdragon, grapevine, poplar and apple [9], involving the regulation of cell differentiation, plant development, organ morphogenesis, phytohormone response, stress tolerance, secondary metabolism [13, 14].

In view of the regulation of seed germination by MYB transcription factors, AtMyb7 mutants produced a lessened lateral root length, playing a key role in establishment of homeostasis during seed germination in Arabidopsis [6]. RSM1, an Arabidopsis MYB protein could modulate seed germination in response to ABA and salinity [7]. LcMYB2 involved the potential to increase root growth to enhance drought tolerance during sheepgrass seed germination [8]. However, there are relatively few reports on seed germination regulated by MYB proteins and the explicit mechanisms remain unidentified.

In our previous study, a new MYB transcription factor gene named ZmMYB59, was cloned from the B73 inbred line. Affymetrix GeneChip was employed to analyze the gene expression patterns of mesocotyl tissue during maize seed germination at 20 cm sowing depth, indicating that MYB genes were significantly expressed [15]. Thereafter, Real-Time PCR showed the expression of ZmMYB59 in maize mesocotyl was fairly down-regulated during seed germination [16]. In this study, ZmMYB59 transgenic tobacco and rice were produced by genetic transformation. Afterwards, germination experiment, antioxidant capacity, cellular morphology and phytohormone content were measured. The objective of this study was to further investigate the functions of ZmMYB59 during seed germination in exogenous expressed tobacco and rice, which will also contribute to elucidate the regulatory mechanisms by MYB transcription factors affecting seed germination.

Results

Verification of the integration of the ZmMYB59 gene into the tobacco and rice genomes

The expression vector pCAMBIA3301-Bar-ZmMYB59 construct was transferred into immature embryos to gain transgenic tobacco and rice. The regeneration of somatic embryos and their conversion into plants was attempted to each transgenic line. The different stages of plant regeneration of stable transgenic lines were described in Fig. 1. T_0 generation transgenic plants were continually self-pollinated until T_2 generation. Then, to validate the presence of the ZmMYB59 gene, T_2 generation transgenic tobacco and rice were tested by PCR amplification, wild-type was as negative control, ZmMYB59 gene was as positive control. PCR products were tested by agarose gel electrophoresis. In tobacco, the result of electrophoresis showed that 18 of 22 transgenic lines were consistent with the positive control (Additional file 1: Figure S1). In rice, the results suggested that 16 of 19 lines were successfully transformed (Additional file 1:

Figure S2). During all electrophoretic bands, three over-expression lines were selected to be employed in this study (OE1, OE2, OE3 in tobacco, OE2, OE4, OE6 in rice), which was showed in Fig. 2a, 2b.

Effect of ZmMYB59 expression on seed germination

To reveal the effect of ZmMYB59 on seed germination, germination experiment was performed in both wild-type and three independent homozygous transgenic lines (Table 2). In T₂ generation transgenic tobacco, germination rate, germination index, vigor index and hypocotyl length were significantly decreased by 25.0 ~ 50.9%, 34.5 ~ 54.4%, 57.5 ~ 88.3% and 21.9 ~ 31.2% compared to WT lines (Table 2, Fig. 3a). In T₂ generation transgenic rice, germination rate, germination index, vigor index and mesocotyl length were significantly reduced by 39.1 ~ 53.8%, 51.4 ~ 71.4%, 52.5 ~ 74.0% and 28.3 ~ 41.5%, respectively (Table 2, Fig. 3b). The above results suggested that ZmMYB59 played a negative regulatory role in the process of seed germination. Meanwhile, it can be seen that the most affected lines by ZmMYB59 were OE2 of tobacco, OE2 of rice (Table 2). Therefore, the above two corresponding transgenic lines were screened out for follow-up experiments.

Table 1 The nucleotide sequences of the primer pairs used to identify the PCR products of expressed *ZmMYB59* gene

primer	sequence5'- 3'	T _m (°C)	Product size (bp)
<i>ZmMYB59-F</i>	ATTGAGCTCCATGCTCGGTG	60	580
<i>ZmMYB59-R</i>	TAGCTGAGTGGCCTGACCAA	60	

Table 2
Measurement of phenotypic indexes of wild-type and ZmMYB59 transgenic plants

Species	Lines	Germination rate (%)	Germination index	Vigor index	Hypocotyl/Mesocotyl length (cm)
Tobacco	WT	91.7 ± 8.1 a	2.61 ± 0.30 a	105.94 ± 23.20 a	0.32 ± 0.02 a
	OE1	51.0 ± 6.0 bc	1.37 ± 0.18 b	26.08 ± 5.38 bc	0.23 ± 0.02 b
	OE2	45.0 ± 5.3 c	1.19 ± 0.11 b	12.42 ± 2.40 c	0.22 ± 0.01 b
	OE3	68.8 ± 7.5 b	1.71 ± 0.44 b	45.05 ± 8.61 b	0.25 ± 0.03 b
Rice	WT	58.0 ± 3.4 a	1.40 ± 0.03 a	40.04 ± 6.10 a	0.53 ± 0.07 a
	OE2	26.8 ± 4.2 b	0.40 ± 0.01 c	10.43 ± 3.20 b	0.31 ± 0.01 b
	OE4	35.3 ± 8.6 b	0.68 ± 0.08 b	19.01 ± 8.25 b	0.38 ± 0.10 b
	OE6	28.7 ± 6.3 b	0.49 ± 0.04 c	13.85 ± 5.37 b	0.33 ± 0.08 b
Note: WT and OE represent wild-type and ZmMYB59 transgenic plants, respectively. Means with standard deviations that do not followed by the same lower case letter between OE and WT lines significantly differ by ANOVA analysis at 5% level of significance.					

Effect of ZmMYB59 expression on antioxidant capacity

To investigate whether ZmMYB59 expression decreases antioxidant capacity, the contents of malondialdehyde (MDA), proline content and the activities of CAT, POD, SOD and APX were measured (Table 3). Compared to WT lines, the MDA content of OE2 tobacco and OE2 rice was enhanced by 21.4% and 8.0%, respectively. However, there were no significant differences between transgenic and WT lines. Compared to WT lines, the proline content of OE2 tobacco and OE2 rice was significantly reduced by 60.5%, 10.9%, respectively. Moreover, in T₂ generation transgenic tobacco of OE2, the activities of CAT, POD, SOD and APX were significantly decreased by 46.2%, 25.3%, 18.9%, 29.0%, respectively. In T₂ generation transgenic rice of OE2, the above enzymatic activities were decreased by 12.8%, 19.4%, 43.5%, 59.8%, respectively. These result proposed that the ZmMYB59 significantly decreased antioxidant capacity of transgenic tobacco and rice, which was generally consistent with the results of germination experiment.

Table 3
Measurement of antioxidant capacity of wild-type and ZmMYB59 transgenic plants

Species	Lines	MDA ($\mu\text{mol/g}$)	Proline ($\mu\text{g/g}$)	CAT (U/g·min)	POD (U/g·min)	SOD (U/g)	APX (U/g·min)
Tobacco	WT	22.8 $\pm$ 2.0 a	97.1 $\pm$ 14.0 a	318.0 $\pm$ 39.0 a	125.6 $\pm$ 8.0 a	88.2 $\pm$ 10.0 a	50.4 $\pm$ 9.0 a
	OE2	27.7 $\pm$ 3.0 a	38.3 $\pm$ 10.0 b	171.1 $\pm$ 9.0 b	93.8 $\pm$ 6.0 b	71.5 $\pm$ 3.0 b	35.8 $\pm$ 4.0 b
Rice	WT	116.9 $\pm$ 7.2 a	18.3 $\pm$ 0.4 a	10.9 $\pm$ 0.2 a	48.9 $\pm$ 3.6 a	53.3 $\pm$ 1.4 a	10.4 $\pm$ 0.3 a
	OE2	126.3 $\pm$ 1.6 a	16.3 $\pm$ 0.3 b	9.5 $\pm$ 0.1 b	39.4 $\pm$ 2.2 a	30.1 $\pm$ 2.5 b	4.2 $\pm$ 0.1 b
Note: WT and OE represent wild-type and ZmMYB59 transgenic plants, respectively. MDA, CAT, POD, SOD, APX represent malondialdehyde, catalase, peroxidase, superoxide dismutase, ascorbate peroxidase, respectively. Means with standard deviations that do not followed by the same lower case letter between OE and WT lines significantly differ by Student's t test at 5% level of significance.							

Effect of ZmMYB59 expression on cellular morphology

Considering that ZmMYB59 reduced hypocotyl/mesocotyl length, cellular morphology of hypocotyl/mesocotyl in tobacco and rice was observed in this experiment to determine whether and how MYB affects cell proliferation and elongation. This is indeed the case (Fig. 4, Table 4). In T₂ generation transgenic tobacco of OE2, cell number and cell length of hypocotyl were significantly decreased by 22.2% and 42.7% compared to WT lines (Fig. 4a, 4b). In T₂ generation transgenic rice of OE2, cell number and cell length of mesocotyl were significantly decreased by 23.8% and 17.5% (Fig. 4c, 4d). The above results suggested the decrease of hypocotyl/mesocotyl length caused by ZmMYB59 was attributed to the inhibition of cell growth including cell number and cell length.

Table 4
Measurement of cell length and cell number of wild-type and ZmMYB59 transgenic plants

Species	Lines	Cell number	Cell length (μm)
Tobacco	WT	18.0 $\pm$ 0.1 a	103.8 $\pm$ 14.0 a
	OE2	14.0 $\pm$ 0.1 b	59.5 $\pm$ 5.0 b
Rice	WT	22.0 $\pm$ 0.6 a	242.1 $\pm$ 7.1 a
	OE2	15.8 $\pm$ 1.0 b	199.6 $\pm$ 30.7 b
Note: WT and OE represent wild-type and ZmMYB59 transgenic plants respectively. Means with standard deviations that do not followed by the same lower case letter between OE and WT lines significantly differ by Student's t test at 5% level of significance.			

Effect of ZmMYB59 expression on content of phytohormone

To investigate the influence of ZmMYB59 expression on endogenous phytohormones during seed germination, the contents of ABA, IAA, GA₁, GA₃, GA₄, CTK in hypocotyl/mesocotyl were determined. In T₂ generation transgenic tobacco of OE2, compared to WT lines, the contents of endogenous GA₁, GA₃, GA₄, IAA, CTK were reduced by 39.2%, 29.4%, 15.4%, 7.5% and 44.8%, whereas the content of ABA was increased by 43.9% (Table 5). In T₂ generation transgenic rice of OE2, the contents of endogenous GA₁, GA₃, GA₄, IAA and CTK were decreased by 47.6%, 22.2%, 24.3%, 10.0% and 37.8%, whereas the content of ABA was increased by 26.9% (Table 5). Among them, the changes of endogenous GA₁, CTK and ABA reached significant level while there were no significant changes in those of endogenous GA₃, GA₄ and IAA. These results indicated that the expression of ZmMYB59 might have contributed to the promotion of endogenous GA₁ and CTK synthesis and the inhibition of endogenous ABA synthesis.

Table 5
Measurement of phytohormone contents in wild-type and ZmMYB59 transgenic lines

Species	Lines	GA ₁ (ng/g)	GA ₃ (ng/g)	GA ₄ (ng/g)	CTK (ng/g)	IAA (ng/g)	ABA (ng/g)
Tobacco	WT	0.166 ± 0.022 a	0.187 ± 0.035 a	0.123 ± 0.016 a	18.544 ± 2.152 a	1.657 ± 0.528 a	4.832 ± 0.486 b
	OE2	0.101 ± 0.013 b	0.131 ± 0.028 a	0.104 ± 0.010 a	10.234 ± 0.989 b	1.532 ± 0.472 a	6.951 ± 0.349 a
Rice	WT	0.187 ± 0.029 a	0.202 ± 0.030 a	0.169 ± 0.046 a	45.142 ± 5.317 a	1.914.±0.355 a	7.235 ± 0.561 b
	OE2	0.098 ± 0.021 b	0.157 ± 0.042 a	0.128 ± 0.057 a	28.085 ± 3.391 b	1.722 ± 0.863 a	9.179 ± 0.380 a

Note: WT and OE represent wild-type and ZmMYB59 transgenic lines, respectively. GA, CTK, IAA, ABA represent gibberellin, cytokinin, indole-3-acetic acid, abscisic acid, respectively. Means with standard deviations that do not followed by the same lower case letter between OE and WT lines significantly differ by Student's t test at 5% level of significance.

Discussion

Seed germination is directly related to field establishment and crop yield. There are some documents that some transcription factors normally defined as proteins are capable of mobilizing or suppressing plant growth [17]. Nonetheless, the contributions of MYB transcription factors during seed germination have not yet been functionally characterized. In our previous study, to elucidate the molecular mechanisms of maize seed germination at 20 cm sowing depth, gene expression of mesocotyl was analyzed by Affymetrix GeneChip [15]. The results demonstrated that there was the highest expression level of MYB genes, accounting for 8.15% of total number of transcription factor family [15].

However, its functions of MYB protein on seed germination have not been investigated thoroughly. The limited research for MYB transcription factors in plant primarily concentrated on *Arabidopsis thaliana*. MYB family was positively involved in plant-specific processes including physiological and biochemical processes and various biotic and abiotic stresses [18, 19], such as AtMYB15, AtMYB30, AtMYB60 and AtMYB96 [20]. On the other hand, AtMYB59, was reported to negatively regulate cell cycle progression of root tips, and inhibited root growth by extending the metaphase of mitotic cells [21]. Recently, AtMYB59 was found to be involved in plant growth and stress responses by acting as a negative regulator of Ca^{2+} signaling and homeostasis [10]. These reports suggested that MYB59 transcription factor might play important roles in plant growth, however, its functions on seed germination remained unclear.

The sequence of ZmMYB59 gene was first issued in 2009. Through BLASTp of NCBI, comparing the amino acid sequence of ZmMYB59 protein [GenBank: ACG37097.1] with AtMYB59 protein [GenBank: NP_200786.1], sequence identity between them attained 53.65%, suggesting that ZmMYB59 is indeed the homologous gene of AtMYB59. We speculated that ZmMYB59 might also play negative regulation and made some research to prove our conjectures. The expression of ZmMYB59 in maize mesocotyl was remarkably down-regulated after incubation for 6 ~ 8 d [16]. It was well known that seed germination was attributable primarily to the elongation of the mesocotyl and the first internode [22, 23]. In like manner, AtMYB30 was highly expressed in brassinosteroid pathway to manipulate hypocotyl cell prolongation during *Arabidopsis thaliana* seed germination [24]. Consequently, mesocotyl/hypocotyl length was an essential index to inflect germination. In this study, germination experiments revealed that hypocotyl/mesocotyl length of transgenic tobacco and rice was significantly lower than wild-type lines. To further detect the effect of ZmMYB59 expression on cell growth, cell morphology of hypocotyl/mesocotyl was observed. Our results suggested that the reduction of hypocotyl/mesocotyl length caused by ZmMYB59 was due to reducing both cell length and cell number.

Seed germination involved high metabolic activity and generation of reactive oxygen species (ROS) [25]. ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\text{OH}\cdot$), and singlet oxygen ($^1\text{O}_2$), was generated in both stressed and unstressed cells [26]. Abiotic stress could cause the accumulation of ROS, which might initiate destructive oxidative processes, such as lipid peroxidation (inflected by MDA content), chlorophyll and protein oxidation [27]. Besides, increased production of ROS might lead to cellular damage resulting in seed deterioration [28]. To minimize the negative effects of ROS, aerobic organisms had evolved both non-enzymatic and enzymatic antioxidant defence [29]. CAT, POD, SOD, APX, as major antioxidant enzymes of plants provided cells with highly efficient machinery to scavenge the ROS. SOD catalyzes the dismutation of O_2^- , CAT, POD and APX mainly scavenge H_2O_2 [30]. Meanwhile, proline, under extreme adversity, will protect plant protein from osmotic stress [31]. In this study, compared to the wild-type lines, MDA content was increased and proline content and the activities of CAT, POD, SOD, APX were decreased in ZmMYB59 transgenic tobacco and rice. These results implied that ZmMYB59 expression could inhibit seed germination by reducing antioxidant capacity.

The phytohormones GA, CTK, ABA were reported to play antagonistic roles in the control of seed germination [30]. GA released dormancy and stimulates seed germination by enhancing the proteasome-mediated destruction of RGL2 (RGA-LIKE2), a key DELLA factor repressing germination [32, 33]. ABA biosynthesis was associated with the maintenance of seed dormancy, leaf senescence and inhibited germination [34, 35]. Cytokinin (CTK) regulated diverse processes from embryonic development to adult plant growth [36]. It can be safely concluded that some MYB transcription factors played important roles in phytohormone regulation. For example, AtMYB60 and AtMYB96 could synergistically control stomatal aperture, drought and disease resistance by ABA signal pathway [9]. GAMYB expression in the first internode was substantially increased by GA₃ application in a wheat variety, named Hong Mang Mai [37]. AtMYB7 negatively regulated ABA-induced inhibition of seed germination by blocking the expression of a bZIP transcription factor ABI5 [6]. Overexpression of OsMYBR1 conferred improved drought tolerance and decreased ABA sensitivity in rice [38]. CLAU was a MYB transcription factor that modulated leaf morphogenesis by constraining the morphogenetic potential, in part due to attenuation of CTK signaling [39].

Gibberellin (GA) was essential intermediate in the stimulation of seed germination, including GA₁, GA₃, GA₄ [40, 41]. In our previous study, discovering that the expression level of ZmMYB59 in maize mesocotyl was inhibited when seed soaked with 10⁻⁵ M GA₃, and in plumules and roots were strongly increased by ABA [13]. Furthermore, after GA₃ soaking, endogenous GA₃, GA₄ and ABA were controlled at a relatively low level, but endogenous GA₁ was controlled at a relatively high level in seed embryos of Masson pine [41]. The results showed that exogenous GA₃ improves seed germination through lowering the ABA level and stimulating GA₁ biosynthesis, which indicated that endogenous GA₁ might play more important roles during seed germination rather than endogenous GA₃, GA₄ [41]. In this study, our results showed that ZmMYB59 expression decreased the total levels of endogenous GA₁, GA₃, GA₄, IAA, CTK and increased the level of endogenous ABA, but only endogenous GA₁, CTK and ABA had significant changes. Therefore, it could be concluded that the inhibitory effect of ZmMYB59 was attributed to endogenous GA₁, but not GA₃ and GA₄. Taken together, the results suggested that endogenous GA₁ could play more important roles during seed germination in ZmMYB59 transgenic tobacco and rice, which was generally consistent with the research results of embryos of Masson pine according to the study of Zhao et al. (2014) [41]

In summary, the molecular mechanisms regulated by ZmMYB59 gene during seed germination of tobacco and rice can be elucidated in Fig. 5. Expressing ZmMYB59 inhibited the synthesis of endogenous GA₁ and CTK, and promoted the synthesis of endogenous ABA. This effect reduced the antioxidant capacity, which directly affected cell growth. All above effects together inhibited hypocotyl/mesocotyl elongation, which suggested that ZmMYB59 gene was a negative regulatory factor during seed germination in tobacco and rice. In our future work, genetic transformation of ZmMYB59 gene in maize will be performed to further validate its functions. Gene knockout is advised as an effective strategy to breeding new maize varieties, which improve seed germination.

Conclusions

The results reported here demonstrated that ZmMYB59 heterogenous expression in tobacco and rice had a negative effect on seed germination by inhibiting the synthesis of GA, CTK and IAA and promoting the synthesis of ABA, then reducing the contents of proline and the activities of CAT, POD, SOD, APX, as a result, repressing cell proliferation and elongation. The result also confirms that endogenous GA₁ may play more important roles during seed germination rather than GA₃, GA₄. Our findings suggest that ZmMYB59 plays a negatively regulatory role in tobacco and rice, which will contribute to elucidate the mechanisms of seed germination regulated by MYB transcription factors and also provides a key gene affecting seed germination.

Methods

Plant materials

T₂ generation transgenic seeds of tobacco (*Nicotiana tabacum* L.), rice (*Oryza sativa* L. ssp. Japonica) were provided by Hangzhou Biogle Co., LTD by Agrobacterium-mediated transformation. The embryogenic callus from wild-type plants was inoculated with agrobacterium tumefaciens strain EHA105 and expression vector plasmid pCAMBIA3301-Bar-ZmMYB59 to generate ZmMYB59 transgenic tobacco, rice. In each crop, three independent ZmMYB59 transgenic lines were used in this study.

RNA isolation and PCR amplification

Total RNA was extracted using All-in-one DNA/RNA Mini-preps kit (B618203, Sangon Biotech, shanghai, China) with a genomic DNA purification according to the manufacturer's instructions. RNA concentration and purity were measured with a NanoDrop ONE (Thermo Fisher Scientific, Madison, USA), and RNA integrity was estimated by 1% agarose gel electrophoresis. cDNA (20 µL) was synthesized from 1000 ng of total RNA using the One-step gDNA Removal and cDNA Synthesis Kit using PrimerScript™ RT reagent Kit with gDNA Eraser (RR047, Takara, Beijing, China). Primers were designed by Primer Premier 5.0 software and showed in Table 1. The amplification reaction system was as follows (20 µL): Master mix 10 µL, ddH₂O 7 µL, 10 µmolL⁻¹ up-stream and down-stream primers 1 µL, cDNA 1 µL. PCR reaction procedure: 95 °C for 5 min; followed by 30 s at 95 °C, 30 s at 55 °C; 40 s at 72 °C for 35 cycles; finally maintained at 72 °C for 10 min.

Table 1
The nucleotide sequences of the primer pairs used to identify the PCR products of expressed ZmMYB59 gene

primer	sequence(5'-3')	T _m (°C)	Product size (bp)
ZmMYB59-F	ATTGAGCTCCATGCTCGGTG	60	580
ZmMYB59-R	TAGCTGAGTGGCCTGACCAA	60	

Germination experiment

Germination experiment was conducted on three replicates. Twenty seeds were randomly selected for each replicate. Wild-type and ZmMYB59 transgenic seeds were evenly flooded in the germination boxes. In tobacco, culture conditions were set up in 16 h at 20 °C dark and 8 h at 30 °C light. In rice, culture conditions were set up in 8 h at 25 °C dark and 16 h at 30 °C light. Considering the fact that sowing covered with soil was generally required in crop production, seeds of tobacco and rice were sowed at the depth of 1 cm and 4 cm, respectively. After incubation for 2 d, the number of germinated seeds was recorded each day until 14 d. Germination rate, germination index, vigor index were determined according to following formulas:

Germination rate = n_1 within 14 days / $n_2 \times 100\%$. Here n_1 is the number of germinated seeds; n_2 is the number of tested seeds.

Germination index = $\sum Gt / Dt$.

Vigor index = $\sum (Gt / Dt) \times S$. Here Gt is corresponding number of seeds germinated in the t day; Dt is time corresponding to Gt in days; S is the average length of 10 seedlings.

Determination of antioxidant capacity

Malondialdehyde (MDA) content was measured by using 2-thiobarbituric acid [27]. Fresh leaves 0.5 g were ground with 5 ml 0.6% TBA in 10% trichloroacetic acid. The mixture was heated at 100 °C for 15 min and then cooled in ice bath. Finally, read the absorbance at 450, 532 and 600 nm. computational formula was as follows: $\mu\text{mol MDA g}^{-1}$ fresh weight (FW) = $6.45 (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \text{OD}_{450}$. The proline content was assayed from freeze dried leave material, using a 3% sulfosalicylic and ninhydrin extraction buffers [42]. Dry leaves 0.04 g was homogenized with 3% sulfosalicylic acid and centrifuged at 3000 g/min for 10 min. 200 μl supernatant was mixed with 400 μl reagent mixture and heated in sealed test tubes at 100 °C for 1 h. After cooling down, 4 ml toluene was added to each sample. Proline content was measured by spectrophotometer at 520 nm.

Until the seedlings grew out of 4 ~ 5 cm, the activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX) were measured by employing 0.5 g samples in 5 mL extraction buffer containing 0.05 M phosphate buffer [30, 43]. CAT was determined spectrophotometrically based on the decrease in absorbance of H_2O_2 at 240 nm. POD was measured as the absorbance at 470 nm. SOD was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). APX was assayed from the decrease in absorbance at 290 nm as ascorbate was oxidized [44].

2.4 Observation of cell morphology

Hypocotyl/mesocotyl of wild-type and transgenic seedlings was cut longitudinally and the cell sections were made to determine the changes of cell length and cell number. Firstly, the middle position of

hypocotyl/mesocotyl was cut and fixed with 25% glutaraldehyde stationary solution initially. Next five visual fields were randomly selected and cell length of hypocotyl/mesocotyl was measured by calibrated eyepiece, and cell number was counted by pictures.

2.5 Determination of phytohormone content

The concentration of the endogenous phytohormones involving gibberellin A1 (GA₁), gibberellin A3 (GA₃), gibberellins A4 (GA₄), cytokinin (CTK), abscisic acid (ABA), indole-3-acetic acid (IAA) during seed germination was determined by LC-MS/MS system [45, 46]. Each sample was transferred into a 2 mL LC/MS glass vial for LC-MS/MS analysis. MS system: ion spray voltage – 4500 V, temperature 550 °C, Ion source Gas 1:50/Gas 2:50. Chromatographic system: HSS T3 liquid chromatography column (100 ≤ 2.1 mm, 1.8 μm), mobile phase A (0.1% formic acid-aqueous solution), mobile phase B (0.1% formic acid-acetonitrile). Multiple reaction monitoring detection method was used for the quantification of all analytes. Each sample was extracted three times.

2.6 Statistical analysis

Analysis of variance (ANOVA) was carried out with SPSS 19.0 (IBM SPSS Statistics, Chicago, USA). Duncan's multiple range test or Student's t test was employed to determine if there were significant differences between the determination indicators of transgenic and wild-type lines at $p < 0.05$.

Abbreviations

WT: Wild-type; OE: Over-expression; GA: Gibberellin; CTK: Cytokinin; ABA: Absciscic acid; PCR: Polymerase chain reaction; GA1: gibberellin A1; GA3: gibberellin A3; GA4: gibberellin A4; CAT: Catalase; POD: Peroxidase; SOD: Superoxide dismutase; APX: Ascorbate peroxidase; MDA: Malondialdehyde; ROS: Reactive oxygen species; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; ANOVA: Analysis of variance

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the necessary data generated or analyzed during this study have been included in this article.

Competing interests

All the authors declared that they had no conflict of interest.

Funding

This research was supported by Zhejiang Natural Science Foundation (LY18C130001, LY13C130011), National Natural Science Foundation of China (31371712), Zhejiang Key Project for New Variety Breeding of Agriculture (Grain) (2016C02050-9-5), The National Key Research and Development Program of China (2018YFD0100900), Special Fund for Agro-scientific Research in the Public Interest of China (201303002), The Key Research and Development Program of Zhejiang (2019C02013). The funder bodies have no role in the design of the study and collection, analysis, and interpretation of data and writing the manuscript.

Authors' contributions

G.Z. designed, supervised the study and analyzed the data. K.Z. wrote the manuscript and analyzed the data. H.J. performed the experiments of rice. C.S. and J.R. performed the experiments of tobacco. All authors read and approved the manuscript.

Acknowledgements

We acknowledge all the members of the research team for their assistance in the field and laboratory work. Meanwhile, we thank Biogle Genome Editor Centre for assistance with genetic transformation and seed propagation.

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Figures

Figure 1

Description of regeneration from callus of ZmMYB59 transgenic plants. (a)~(f) Tobacco transformation: (a) EHA105 infected leaves, (b) Differentiation of callus, (c) Formation of resistant seedlings, (d) Rooting culture, (e) Transgenic seedlings, (f) Transplantation. (g)~(l) Rice transformation: (g) Screening seeds, (h) Co-culture with *Agrobacterium tumefaciens* containing target gene vector at EHA105, (i) Subculture of callus, (j) Screening resistant seedlings, (k) Differentiation of callus, (l) Rooting culture.

Figure 2

Electrophoretic results to confirm the presence of the ZmMYB59 gene in T2 generation transgenic tobacco and rice. (a) Detection of PCR in T2 generation transgenic tobacco. M: DL10000 DNA Marker, 1: positive control, 2: negative control, OE1~OE3: ZmMYB59 transgenic tobacco lines. (b) Detection of PCR in T2 generation transgenic rice. M: DL2000 DNA Marker, 1: negative control, 2: positive control, OE1~OE6: ZmMYB59 transgenic rice lines.

(a)

(b)

Figure 3

Phenotypic observation of wild-type and ZmMYB59 transgenic seedlings. (a) T2 generation transgenic tobacco with wild-type tobacco. (b) T2 generation transgenic rice with wild-type rice.

Figure 4

Microstructure of mesocotyl cells of wild-type and ZmMYB59 transgenic plants. (a) Wild-type tobacco. (b) T2 generation transgenic tobacco. (c) Wild-type rice. (d) T2 generation transgenic rice. The image is a 4 × fluorescence microscope, and the arrow refers to the longitudinal boundary position of the cell.

Figure 5

The regulatory mechanisms of seed germination regulated by ZmMYB59 in tobacco and rice. GA1, CTK, ABA represent gibberellin A1, cytokinin, abscisic acid, respectively. CAT, POD, SOD, APX represent catalase, peroxidase, superoxide dismutase, ascorbate peroxidase, respectively.

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