Rice MYB transcription factor OsMYB1R1 negatively regulates drought resistance

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Research Article

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Rice MYB transcription factor *OsMYB1R1* negatively regulates drought resistance

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Abstract

MYB transcription factors have been demonstrated to play an important role in plant growth, development and abiotic stresses. This study isolated a rice MYB gene, *OsMYB1R1*(Os04g0583900), and functionally characterized its role in tolerance to drought stress by generating transgenic rice plants with overexpressing (OE) and RNA interference (RNAi) *OsMYB1R1*. Expression of *OsMYB1R1* was down-regulated by drought stress. The tissue-specific expression analysis indicated that *OsMYB1R1* was expressed at high level in panicle, but relatively low in the other parts of rice. No difference in germination rate among *OsMYB1R1*-OE, RNAi and wild-type (WT) seeds under mannitol treatments. No differences in phenotypes, physiological indicators and agronomic traits among WT, OE and RNAi plants were observed under normal grown conditions. Under drought stress, the RNAi plants were more tolerant to drought stress and higher survival rate after re-watering than WT plants, whereas, the overexpressing plants have found just the opposite. The *OsMYB1R1*-OE plants exhibited increased relative electrical conductivity (REC), increased malondialdehyde (MDA) content, and decreased proline content compared with the wild type, whereas lower REC and MDA content and higher proline content were found in the RNAi plants. These results suggest that *OsMYB1R1* functions as a negative regulator in response to drought stresses, and may be used as a candidate gene for molecular breeding of drought-tolerant crop varieties.

Keywords Rice. MYB. *OsMYB1R1*. Drought stress. Mannitol tolerance

Introduction

As one of the most important food crops, rice (*Oryza sativa*) is consumed by more than 75% of the population in Asia (Fitzgerald et al. 2009). Drought is one of the major stress factors that can seriously affect plant growth. However, rice is more sensitive to drought stress than other cereals (Huang et al. 2014). According to the statistical data from 1995 to 2007, the loss of rice production caused by drought in North China accounted for 0.13 % - 0.43 % of the total rice yield in China (Lin et al. 2013). Therefore, improving the rice resistance to drought stress has been one of the main objectives of agricultural production.

Transcription factors are key players in the regulatory networks underlying plant responses to abiotic stresses (Golldack et al. 2014). The MYB transcription factor family which is one of the richest transcription factor families in rice have been studied for their involvement in the regulation of abiotic stress response, as recently reviewed (Li et al. 2015). For example, *OsMYB6* and *OsMYB48-1* overexpressing plants were more tolerant to drought and salt stress in rice (Tang et al. 2019; Xiong et al. 2014). Under normal growth conditions, there was no difference between *OsMYB2*-overexpressing and WT plants, but *OsMYB2*-overexpressing plants showed increased tolerance to salt, cold and dehydration stresses and more sensitive to abscisic acid than WT plants (Yang et al. 2012). In rice, *OsMYB91* played a coordinating role in rice plant growth and salt tolerance, but over-expressing *OsMYB91* showed reduced plant growth and accumulation of endogenous ABA under control conditions (Zhu et al. 2015). *OsMYB3R-2* transgenic rice enhanced the tolerance to chilling stress by mediating the alteration of cell cycle and ectopic expression of stress genes (Ma et al. 2009), and overexpression of *OsMYB3R-2* could improve the tolerance of transgenic *Arabidopsis thaliana* to freezing, drought and salt stress (Dai et al. 2007). The rice high-affinity potassium transporter1;1 which regulated by an MYB-type transcription factor was involved in salt tolerance (Wang et al. 2015). The ectopic overexpression of the rice *OsMYB4* gene increased cold and drought tolerance (Vannini et al. 2004; 2006; Mattana et al. 2005; Baldoni et al. 2013; Park et al. 2010; Pasquali et al. 2008). El-Kereamy et al. (2012) concluded that the rice R2R3-MYB transcription factor *OsMYB55* was involved in the tolerance to high temperature and modulates amino acid metabolism. In addition, a novel *MYBS3*-dependent pathway has also been demonstrated to confer cold tolerance in rice (Su et al. 2010). While little is known about the function of rice transcription factor *OsMYB1R1* (Os04g0583900) in drought stress response.

This study isolated the *OsMYB1R1* gene from rice and tested the expression pattern of *OsMYB1R1* in different tissues. The role of the *OsMYB1R1* gene in tolerance to drought stress was characterized by generating transgenic rice plants with overexpressing and RNA interference *OsMYB1R1* in rice. The

effects of *OsMYB1R1* on rice seeds germination and agronomic traits of rice were studied. The work not only provides valuable information for exploring the role of the *OsMYB1R1* genes in response to abiotic stress in rice, but also provides a candidate gene in molecular breeding to increase crop drought stress resistance.

Material and Methods

Generation of transgenic rice plants

The full-length cDNA clones of *OsMYB1R1* (AK101209.1) was obtained from the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). To create the overexpression construct of *OsMYB1R1*, the complete ORFs of *OsMYB1R1* was PCR amplified with the primers 5'-CGGGATCCTCGATTCAAGTCTGGAGATGG-3' (BamHI site underlined) and 5'-GCTCTAGAG CAAGCATCTATAGTTGCAGTGAT-3' (Xba1 site underlined). The full-length cDNA plasmids were used as templates. Then the PCR products were cloned in pCAMBIA1301-Multi (modified from pCAMBIA1301) under the control of the CaMV35S promoter respectively. To make double-strand RNAi construct, the antisense fragment *OsMYB1R1*-A was PCR amplified with XhoI-KpnI (indcated by underline) linker primers 5'-ATGGATCCGATATCTACCCGCTTCGCGTCG-3' and 5'-GGGAA TTCATTGTTTGCCTCCCTGCTGC-3', the sense fragment *OsMYB1R1*-S was PCR amplified with PstI-XbaI (indcated by underline) linker primers 5'-CGGGATCCTGCAAACTGTGGC AGACATG-3' (BamHI sites underlined) and 5'-GCCCTCGAGCATTTCATCTTCGTTTCTGTTTCC-3' (Xho1 sites underlined), using the cDNA clone AK101209.1 as the template. The schematic diagram of the recombinant plasmid *OsMYB1R1*-OE and *OsMYB1R1*-RNAi were as Fig.1a and b. Both of the constructs were transformed into rice (*Oryza sativa ssp. japanica var.* Nipponbare) by the *Agrobacterium*-mediated transformation method (Toki et al. 2006).

Drought and mannitol stress treatments

To test the transcription level of *OsMYB1R1* in different tissues, we collected tissue samples of roots, stems, leaves, sheaths and spikes from Nipponbare rice plants which grown in the field or climate chamber as described previously (Zou et al. 2009). To check the expression level of *OsMYB1R1* gene under drought stress, Nipponbare rice seeds were germinated on 1/2 MS agar for one week and then transplanted into a grown chamber (14-h-light/10-h-dark) at 28 ℃ and 75 % relative humidity for three weeks. The seedlings at four-leaf stage were treated with 20 % PEG6000 for 0 (used as control), 0.5, 1,

2, 4, 8 and 24 h separately.

For estimating the osmotic tolerance of transgenic seed germination, T2 generation seeds of *OsMYB1R1* transgenic lines and the WT were placed on 1/2 MS medium containing 0, 150 and 200 mmol/L mannitol respectively. The germination rate of was scored after 7 days, the shoot heights and root length were measured at 10 days after being transferred. To detect the osmotic stress tolerance of transgenic seedling, the one-week-old WT and the *OsMYB1R1* transgenic seedlings were planted on 1/2 MS agar plates containing 0, 200 and 250mmol/L mannitol respectively. After 7 days, the root length and shoot height were calculated.

To test drought tolerances of transgenic rice seedling, T2 generation seeds of *OsMYB1R1* transgenic lines were germinated in 1/2 MS medium containing 50 mg/L hygromycin; the WT was placed in normal 1/2 MS medium. Drought tolerance of rice seedlings were evaluated under two drought-stressed conditions. For seedlings vitro drought stress, the two-week-old rice seedlings were pulled out and washed the medium. After drained of water, we moved them into a growth chamber at 28 ℃and 75 % relative humidity for 10 h. Then it were transplanted into 1/2 MS medium for culture and re-watered for additional 3 days. The survival rate of transgenic seedling and the WT seedling were scored. For seedling natural drought stress, the one-week-old rice seedlings were grown in plastic pots filled with sandy soil. After two weeks growing in the growth chamber, the seedlings were un-watered for drought treatment. When the WT rice seedlings exhibited the lethal effects of dehydration, watering was resumed. The REC, MDA and proline contents of rice seedlings were measured at the beginning and at the end of natural drought treatment.

Drought testing at the reproductive stage was conducted in plastic pots. After one week germination on 1/2 MS medium, the seedlings were transplanted into field soil under the same growth conditions. Then we moved them into plastic pots when they reached booting stage with flag leaf just pulled out. After two days, drought stress was initiated at the booting stage by discontinuing watering of plants. When soil water contents lower than 20 %, the plants were recovered with irrigation. The rate of water loss (RWL), REC, MDA and proline contents in flag leaf of rice at booting stage were measured at the beginning and at the end of drought treatment.

All above experiments were performed three replications. The phenotype of *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT plants under different treatments was observed and photographed.

RNA isolation, RT-PCR and semi-quantitative PCR

Total RNA isolation, reversed transcription into cDNA, real-time quantitative PCR and

semi-quantitative PCR analysis were performed according to Zou et al. (2009). The rice *Actin1* gene (Os03g0718100) was used as the endogenous control. The primes were: for *OsMYB1R1*, 5'-ATTTGGCAAGAGGGTTATGGTG-3' and 5'-ACACTCGGGTCCAAGGTTGA-3'; for *Actin1*: 5'-CTTCAACACCCCTGCTATG-3' and 5'-TCCATCAGGAAGCTCGTAG-3'. The PCR thermal cycle used was 94 ℃ for 5min and 28 cycles (*OsMYB1R1*-OE) or 35 cycles (*OsMYB1R1*-RNAi) of 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s (*OsMYB1R1*-OE) or 10 min (*OsMYB1R1*-RNAi).

Measurements of RWL, REC, MDA and proline content

The leaf RWL was performed according to the method of Xiang et al. (2013). The leaf REC, MDA and proline content were measured at beginning and at the end of drought stress as the method described by Yu et al. (2006), Kuk et al. (2003) and Bates et al. (1973) respectively.

Statistical analysis of agronomic characters

Twenty mature *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT plants were randomly selected to analyze the tiller number, plant height, flag leaf length, flag leaf width, first stem node length, second stem node length, spike length, 1000 grain weight and seed setting rate.

Statistical analysis

All data were analyzed by analysis of variance with Student's *t* test using SPSS 19.0 software (IBM Corporation, Chicago, IL, USA), values of $P<0.05$ were considered statistically differences.

Results

Expression patterns of *OsMYB1R1* in rice

Semi-quantitive RT-PCR analysis was used to examined spatial and temporal expression level of *OsMYB1R1*. As presented in Fig.1, the tissue-specific expression analysis indicated that *OsMYB1R1* expression was high in panicle, but relatively low levels in the other parts of rice (Fig. 1a). The expression level of *OsMYB1R1* gene at the time points of 0, 0.5, 1, 2, 4, 8 and 24 h after drought stress were shown in Fig. 1b. The semi-quantitative PCR analysis of *OsMYB1R1* gene expression demonstrated that an decrease in the *OsMYB1R1* transcript was observed after 2 h of exposure to drought stress.

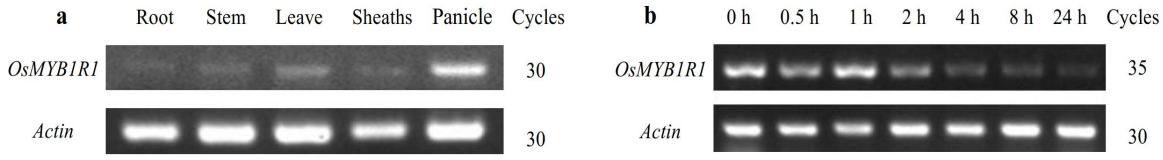


Fig. 1 Expression analysis of the *OsMYB1R1* gene in rice. (a) Spatial expression pattern of *OsMYB1R1* in rice; (b) Temporal expression pattern of *OsMYB1R1* under drought stress

Vectors construction and RT-PCR Assays

To test the effect of *OsMYB1R1* on drought stress tolerance, *OsMYB1R1*-OE (Fig. 2a) and RNAi (Fig. 2b) vectors were constructed and were transformed into Nipponbare separately. The expression level of *OsMYB1R1* in transgenic plants was analyzed by RT-PCR (Fig. 2c, d). Two independent *OsMYB1R1*-OE (OE4 and OE5) lines and four *OsMYB1R1*-RNAi lines (Ri1, Ri2, Ri5 and Ri8) were proved and chosen for drought stress tolerance test.

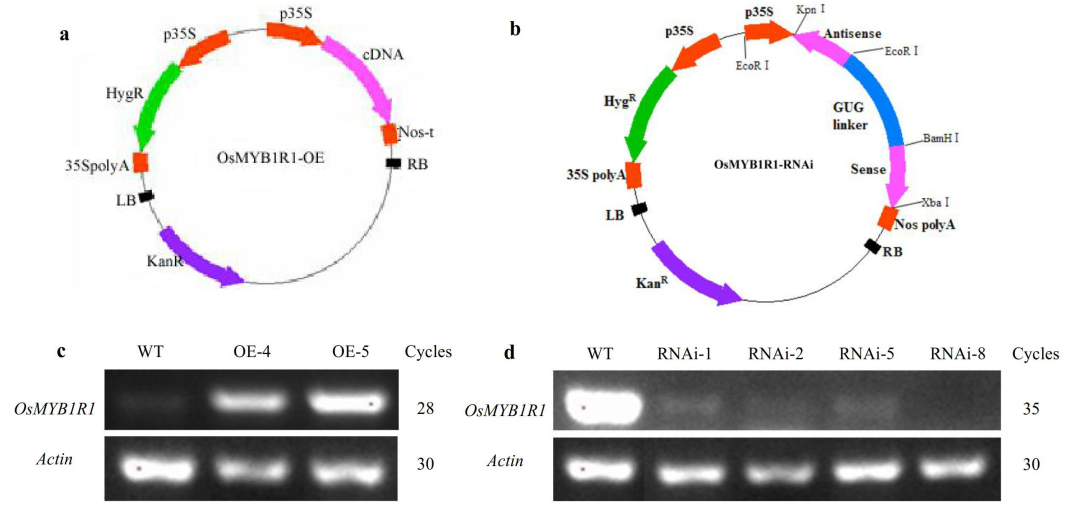


Fig. 2 Expression analysis of *OsMYB1R1* in transgenic rice lines and WT under normal condition. (a,b) The schematic diagram of the recombinant plasmid *OsMYB1R1*-OE and *OsMYB1R1*-RNAi. c Expression level of *OsMYB1R1* in *OsMYB1R1*-OE lines and WT under normal condition. d Expression level of *OsMYB1R1* in *OsMYB1R1*-RNAi lines and WT under normal condition. The rice *Actin1* gene was used as the control for constitutive expression.

Osmotic stress on seed germination and the postgermination stage rice seedlings

To test the effect of *OsMYB1R1* in transgenic rice lines on seed germination under osmotic stress, we counted the germination rate, shoot height and root length of *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT seeds under different concentrations of mannitol stress (Fig. 3). The results showed that the germination rate and shoot height of *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT seeds decreased with the increase of mannitol

concentration, while the root length of the three rice genotypes increased with the the increase of mannitol concentration. However, there were no significant difference among the three genotypes under the same mannitol concentration. These results indicated that overexpression or RNA interference of *OsMYB1R1* does not affect seed germination under normal condition and mannitol stress, suggesting that the effect of *OsMYB1R1* gene on drought tolerance might not reflected in the stage of seed germination.

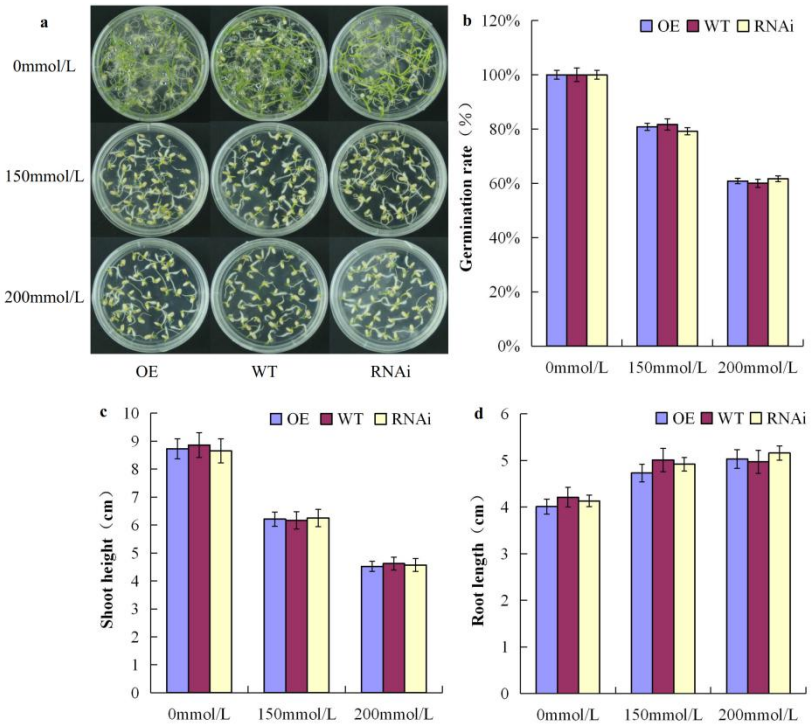
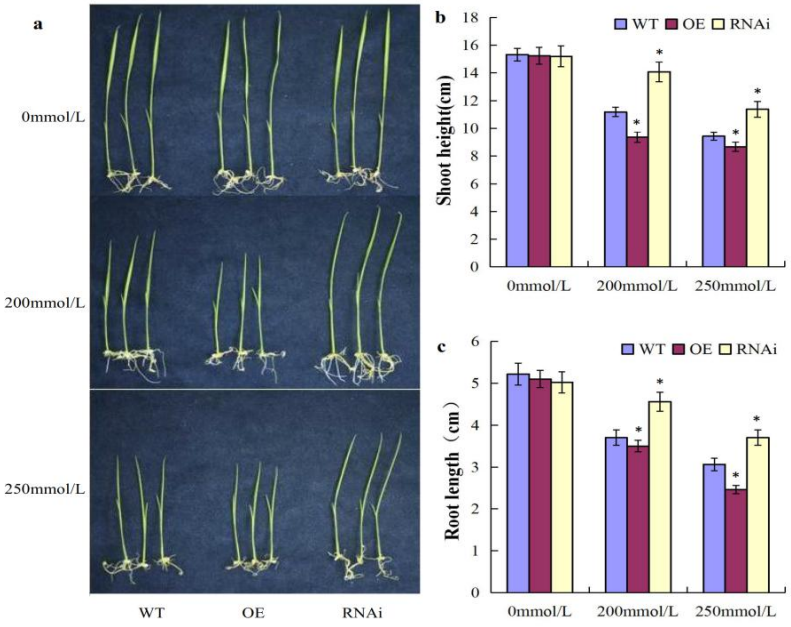


Fig.3 Seedling growth and germination under different concentrations of mannitol treatments. (a,b) The growth condition and seed germination rate in one-week-old *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT plants under mannitol treatments. (c,d) Plant height and root length of the three rice genotypes under mannitol treatments. Plant height and root length were measured after the seedlings growing for 10 days.

Osmotic stress was further performed at the postgermination stage rice seedling (Fig. 4). The results showed that there were no significant differences in shoot height and root length among the three rice genotypes before mannitol stress tolerance. After the treatment, the shoot height and root length of *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT seedlings decreased with the increase of mannitol concentration. Under the same mannitol concentration, the shoot height and root length of *OsMYB1R1*-OE seedlings were significantly lower than those of the WT, but the *OsMYB1R1*-RNAi plants were significantly higher than those of the WT. It means that the increase of *OsMYB1R1* expression enhanced the sensitivity of rice seedlings to drought stress, while the decrease of *OsMYB1R1* expression reduced the sensitivity of plants to drought stress.



183 Fig. 4 Osmotic stress on the postgermination stage rice seedling.(a) The growth of one week seedlings
184 in culture medium containing different concentrations of mannitol; (b,c) Statistical results of shoot
185 height and root length of two-week-old *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT plants under
186 mannitol treatments. (*P<0.05)

187 **OsMYB1R1 negatively regulates drought tolerance in rice**

188 Drought tolerance of rice seedlings were evaluated under two drought-stressed conditions. For
189 seedlings vitro drought stress, two-week-old WT and transgenic plants were withheld water for 10 h
190 and then re-watered for additional 3 days (Fig. 5). After withholding water, the two-week-old
191 *OsMYB1R1*-OE plants was discovered to exhibit significant dehydration and wilting, while the leaves
192 of WT seedlings were curled but not completely. However, most of the *OsMYB1R1*-RNAi plants was
193 still stretched. There were remarkable difference after re-watering. The majority of *OsMYB1R1*-OE
194 plants leaves showed further withered and the survival rate of seedlings was only 23 %. While most of
195 the WT plant were recovered and the survival rate of seedlings were 70 %. However, the
196 *OsMYB1R1*-RNAi plants were almost restored normal growth and the survival rate of seedlings were
197 90 %. The same result was obtained from the natural drought stress treatment three-week-old plant
198 seedlings (Fig. 6a-c). Next, whether the physiological indicators changed within the period after the
199 drought was investigated (Fig. 6d-f). The contents of REC, MDA and proline showed that there were
200 no significant differences between the transgenic lines and WT plants before drought stress. After the
201 treatment, the MDA and REC content of *OsMYB1R1*-OE seedlings were significantly higher than those
202 of the WT and *OsMYB1R1*-RNAi (Fig. 6c, f). While the lowest proline accumulation was observed in

the *OsMYB1R1*-OE plants (Fig. 6e). The REC and MDA content were lower in the *OsMYB1R1*-RNAi seedlings than in the WT. While there were no significant differences between the *OsMYB1R1*-RNAi and WT plants in proline accumulation.

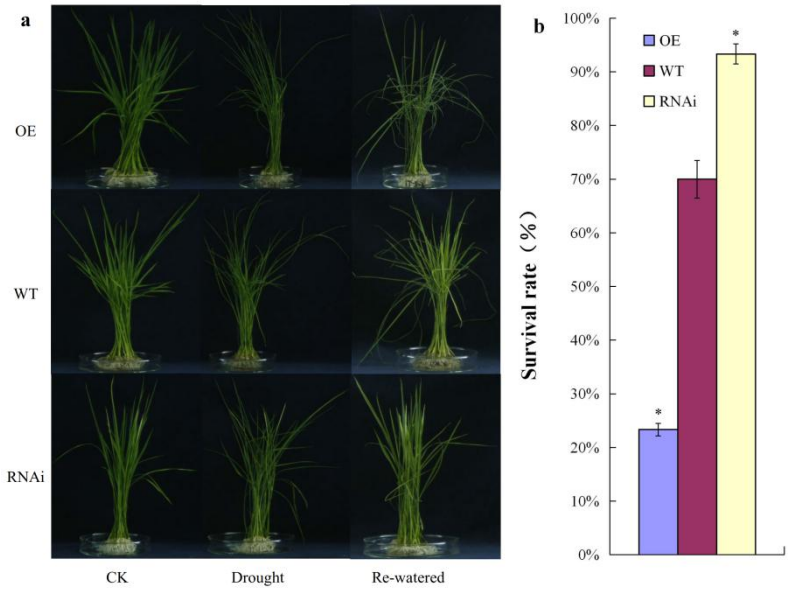


Fig. 5 Phenotypic and the survival rate of WT and *OsMYB1R1* transgenic plants under vitro drought treatments. a Two-week-old plants before drought treatment (left), after 10 h drought treatment (middle) and after re-watering for 3 days (right); b Survival rate of the *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT plants recovered for 3 days after drought stress. (*P<0.05).

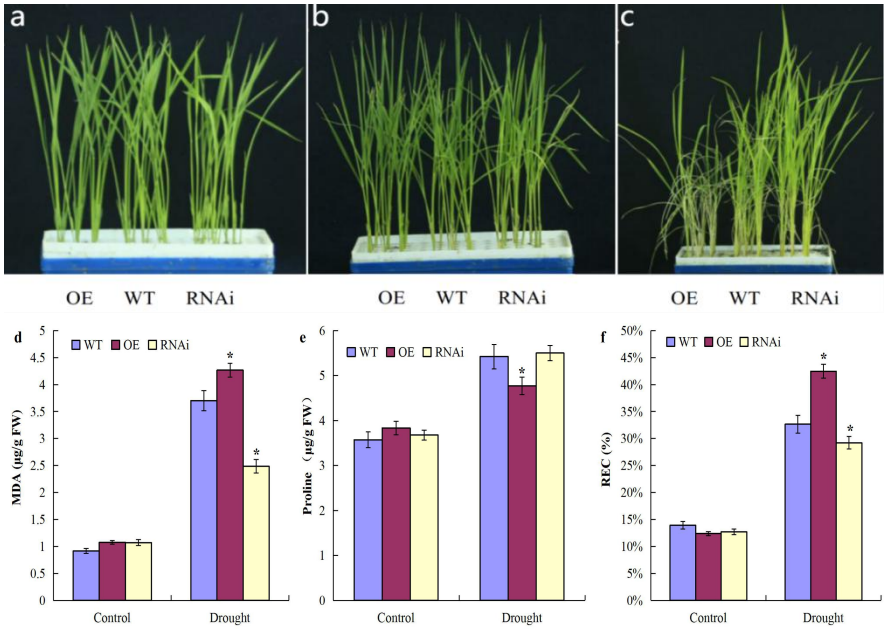


Fig. 6 Phenotypic and the biochemical index changes of WT and *OsMYB1R1* transgenic plants under natural drought treatments. (a) Seedlings before treatment. (b) Seedlings were un-watered 8 days for drought treatment. (c) Seedlings were re-watered for 5 days after the treatment.(d-f) Assay of MDA, proline and REC content. (*P<0.05)

Booting stage is one of the most critical periods in rice growth cycle. Drought stress was also performed at this stage by withheld water for 5 days and then re-watered for additional 7 days (Fig.7). The results showed that all leaves of WT and transgenic plants became completely rolled after drought treated for 5 days (Fig.7 b,e). After re-recovering with irrigation for 7 days, there were remarkable difference among the three genotypes. Compared with WT, the majority of *OsMYB1R1*-OE plant leaves turned yellow and withered (Fig.7c), while he *OsMYB1R1*-RNAi plants were almost restored normal growth (Fig. 7f). The detached leaves among the *OsMYB1R1-OE, OsMYB1R1-RNAi* and WT plants were exposed to air to compare the RWL in leaves (Fig.8a). The results showed that the leaves from *OsMYB1R1*-RNAi had the lowest RWL, while the leaves from *OsMYB1R1*-OE plants had higher RWL than that of the WT and RNAi. It means that the difference of drought tolerance of the three genotypes had a certain relationship with leaf water loss rate.

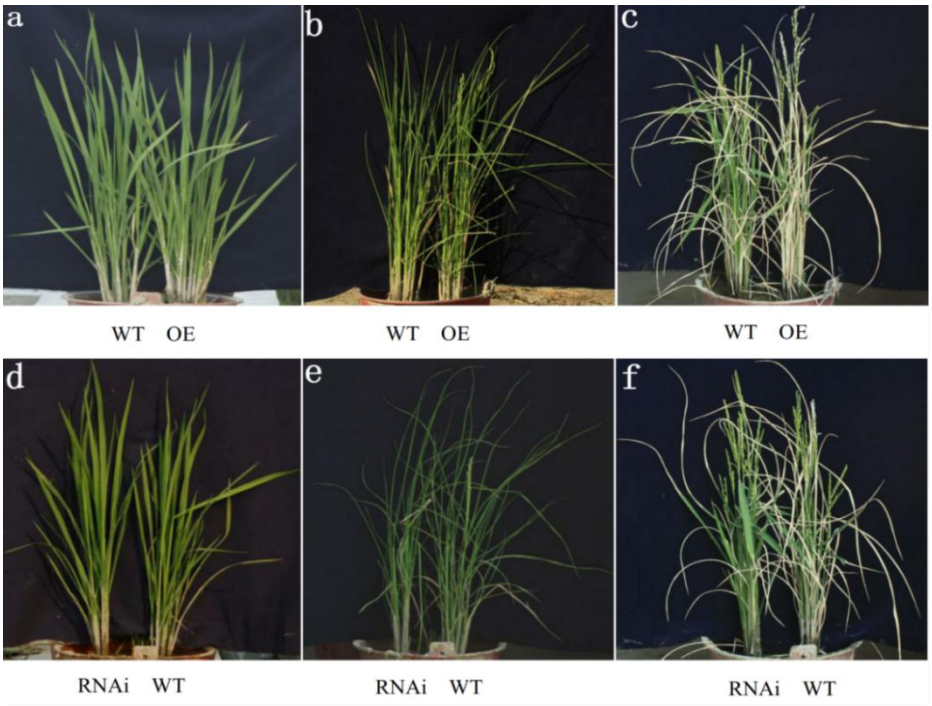


Fig. 7 Phenotype of the *OsMYB1R1* over-expression and RNAi transgenic plants in response to drought treatment at booting stage. (a, d) Rice plants before treatment. (b, e) Rice plants were un-watered 5 days for drought treatment. (c, f) Rice plants were re-watered for 7 days after the treatment.

The REC, MDA content, and proline content as physiological indicators often used to reflect various abiotic stresses, it were also very sensitive physiological indexes. The REC, MDA content, and proline content revealed that there were no significant differences between the transgenic lines and WT plants before drought stress. After the treatment, the MDA and REC content of *OsMYB1R1*-RNAi plants were significantly lower than those of the WT and *OsMYB1R1*-OE. While there were no

significant differences between the *OsMYB1R1*-RNAi and WT plants in proline accumulation. The lowest proline accumulation and highest REC content were observed in the *OsMYB1R1*-OE plants. While there were no significant differences between the *OsMYB1R1*-OE and WT plants in MDA content (Fig.8b-d).

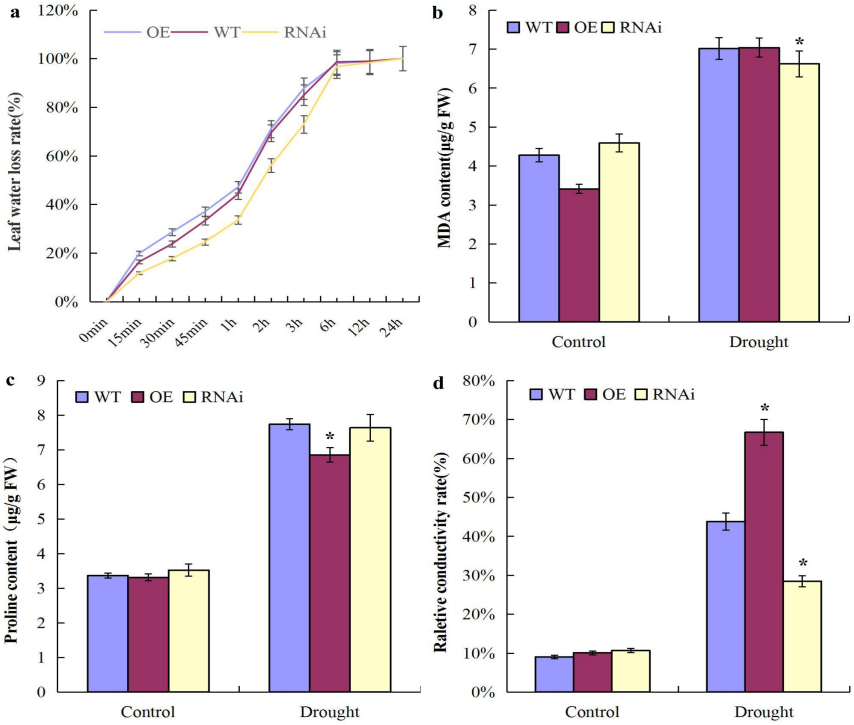


Fig. 8 RWL, MDA content, proline content and relative conductivity of *OsMYB1R1*-OE and RNAi rice booting stage after drought treatment.

Effect of OsMYB1R1 expression on growth and development of rice

The statistical data on the main agronomic traits of *OsMYB1R1*-OE, *OsMYB1R1*-RNAi and WT rice plants were shown in Fig. 9. The results showed that there were no significant differences in tiller number, plant height, flag leaf length, flag leaf width, first stem node length, second stem node length, panicle length, 1000 grain weight and seed setting rate among the three genotypes. Our data suggest that over-expression and RNA interference *OsMYB1R1* in rice, it does not affect the development and yield of transgenic rice.

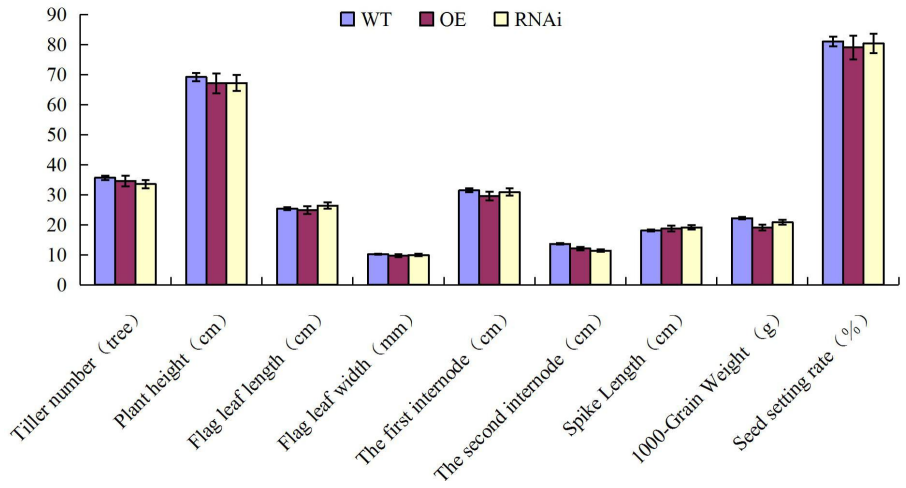


Fig. 9 *OsMYB1R1* over-expression and RNAi plant agronomic traits

Discussion

MYB transcription factors contain a large number of family, with 185 members in rice (Dubos et al., 2010). In this study, we isolated a rice MYB gene, *OsMYB1R1*, and functionally characterized its role in tolerance to drought stress by generating transgenic rice plants with overexpressing and RNA interference *OsMYB1R1*. Based on the performance of transgenic plants under drought stress and agronomic traits, we conclude that *OsMYB1R1* is a drought stress response gene and that, when overexpressed and RNA interference in rice, it does not affect the rice seed germination, development and yield of transgenic rice, but negatively regulates drought resistance of rice.

Expression of *OsMYB1R1* was detected in all the tissues tested, with the maximum level in panicle, but relatively low levels in the other parts of rice. The previous study revealed that *AtCIR1*, as a closely related homologous gene of *OsMYB1R1*, was identified to be related to plant photoperiod (Zhang et al. 2007). Therefore, the high expression of *OsMYB1R1* in the photoperiod sensitive young panicles of rice might be due to its role in photoperiod.

Previous research showed that *OsMYB1R1* (Os04g0583900) was strongly induced in rice cultivar 996 under heat stress, and the expression level of *OsMYB1R1* was early elevated and then gradually reduced to lower increased level at the time point of 2 h after heat stress (Zhang et al. 2012). In our study, *OsMYB1R1* (Os04g0583900) was greatly down-regulated by drought stress, and the expression level of *OsMYB1R1* began to decrease at the time point of 2 h after drought stress. It shows that that abiotic stress could induce the expression of *OsMYB1R1* rapidly. *OsMYB1R1* might be of great importance for stress tolerance.

Under drought stress, the *OsMYB1R1*-RNAi plants showed increased tolerance to drought stress and higher survival rate after re-watering than WT plants, whereas the overexpressing plants were more

sensitive to drought stress and lower survival rate after re-watering than WT plants. Previous work demonstrated that drought-tolerant plants have a more perfect defense mechanism to maintain low levels of MDA (Xie et al. 2008). The abundance of REC and MDA can be used as indicators of cell membrane damage, and lower REC and MDA indicates that less membrane damage occurred (Bajji et al. 2002; Marnett et al. 1999). By contrast, proline enrichment as effective indicator of plant stress tolerance is a general response to various abiotic stresses (Akram et al. 2007). In the present study, compared with the wild type plants, the *OsMYB1R1*-overexpressing plants exhibited increased REC and MDA content and decreased proline content, while RNAi plants showed lower REC and MDA content and higher proline content. And the above results were obtained in the drought treatment experiments at seedling and booting stage, which further confirmed the negative regulatory role of *OsMYB1R1* gene in plant response to drought stress. The results of vitro drought experiment and RWL measurement indicated that *OsMYB1R1* gene might increase water loss in plants by opening stomata, thus reducing the drought tolerance of plants.

No difference in germination rate among *OsMYB1R1*-overexpressing, WT and RNAi seeds under mannitol treatments. No differences in phenotypes, physiological indicators and agronomic traits among WT, overexpressing, and RNAi plants were observed when grown under normal conditions. This results indicate that *OsMYB1R1* is a drought stress response gene and that, when overexpressed and RNA interference in rice, it does not affect the rice seed germination, development and yield of transgenic rice, but only negatively regulates drought resistance of rice. These results enhance our understanding of the role of rice *MYB* transcription factor in the regulation of abiotic stress response, and provide a candidate gene for molecular breeding of drought-tolerant rice varieties.

Acknowledgements

Compliance with ethical standards

Conflict of interest All authors have approved the manuscript and declare no conflict of interests.

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