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Rice MYC2 (OsMYC2) modulates light-dependent seedling phenotype, disease defence but not ABA signalling

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Arabidopsis MYC2 (AtMYC2) is a bHLH class transcription factor that mediates light-dependent seedling development, disease defence, JA and ABA signalling. *AtMYC2* gene modulates hypocotyl elongation and expression of *chlorophyll A/B binding protein 1* (*CAB1*) and *rubisco small subunit protein1* (*RBCS1*) under blue light. The *atmyc2* mutants are resistant against virulent bacterial pathogens. MYC2 orthologues from several crop plants have been characterized. The rice gene *Os10g42430* has been referred earlier as OsMYC2 and has been shown to promote expression of JA-inducible genes. However, the role of OsMYC2 in seedling development under ABA, dark or light of specific wavelengths was not known. It was also not known whether OsMYC2 complements AtMYC2 function in *Arabidopsis*. We show here that expression of *OsMYC2* in the *atmyc2* mutant of *Arabidopsis* complements the blue-light-mediated defects in hypocotyl elongation and expression of *CAB1* and *RBCS1*. We generated multiple transgenic rice lines for over-expression and RNAi-mediated suppression of *OsMYC2*. In agreement with AtMYC2 function, *OsMYC2* over-expression and RNAi lines showed enhanced and suppressed seedling growth compared to WT plants respectively under blue light, and showed little effect under white light or dark. In agreement with the negative regulatory role of AtMYC2 in disease defence, the RNAi lines showed enhanced resistance against bacterial pathogen *Xanthomonas oryzae* pv *oryzae*. However, in contrast to AtMYC2 function, *OsMYC2* influences seedling development under red light and show no effect in ABA-mediated seed germination. Thus, the results suggest evolutionarily conserved as well as the distinct role of OsMYC2 in comparison with AtMYC2.

Keywords. ABA; AtMYC2; Light; OsMYC2; Rice; Seedling development

1. Introduction

AtMYC2 is a basic helix-loop-helix (bHLH) class transcription factor that modulates diverse processes in *Arabidopsis*, including light signalling, stress responses, growth and development (Abe *et al.* 2003; Yadav *et al.* 2005; Dombrecht *et al.* 2007; Gangappa and Chattopadhyay 2010; Maurya *et al.* 2015). MYC2 acts as point of crosstalk in light signalling, ABA signalling, SA signalling and JA/Et signalling (Abe *et al.* 2003; Anderson *et al.* 2004; Boter *et al.* 2004; Lorenzo *et al.* 2004).

Light controls the early seedling development in all plants. When seedlings are grown under dark they exhibit skotomorphogensis or etiolated phenotype, which includes elongated hypocotyl and folded cotyledons. In contrast, seedlings exhibit photomorphogensis or de-etiolated phenotype with reduced hypocotyl elongation and unfolding of cotyledon under light (Ang *et al.* 1998). MYC2 binds with Z box and G box elements present in promoters of light-regulated gene and acts as blue-light-specific repressor of photomorphogenesis (Yadav *et al.* 2005). The *atmyc2* mutant plants show reduced hypocotyl length compared to WT plants under blue light, but has no effect seedling growth under red, far-red or dark condition (Yadav *et al.* 2005). In addition to light, ABA also regulates seed germination and seedling growth. AtMYC2 acts as positive regulator of ABA signalling; *atmyc2* mutants are relatively less sensitive towards exogenous application of ABA during seed germination (Abe *et al.* 1997; Lorenzo *et al.* 2004; Yadav *et al.* 2005).

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AtMYC2 homologs/orthologs have been characterized in many plant species including *Lycopersicon esculentum*, *Nicotiana tabacum, Nicotiana benthamiana* and *Catharanthus roseus* (Boter *et al.* 2004; Shoji and Hashimoto, 2011; Zhang *et al.* 2011, 2012). CrMYC2, an MYC2 orthologue of *C. roseus,* binds to G-Box-like elements and activates the expression of JA responsive genes (Zhang *et al.* 2011). Two orthologues of MYC2 in banana (*Musa acuminate*), MaMYC2a and MaMYC2b, induce JA signalling and provide cold stress tolerance (Zhao *et al.* 2013). Recently, a rice homologue OsMYC2 has been shown to modulate JA-mediated gene expression, spikelet development and resistance against bacterial pathogen and senescence (Uji *et al.* 2016, 2017; Ogawa *et al.* 2017a, b). However, it was not known whether OsMYC2 functionally complements *Arabidopsis* MYC2 gene. Moreover, influence of OsMYC2 in skotomorphogenesis or wavelength-specific photomorphogenesis is not known.

In this study, we identified the putative homologues of AtMYC2 from rice, OsMYC2, which show 47% amino acid sequence identity with AtMYC2. We have shown that OsMYC2-1 efficiently complements blue light signalling in *Arabidopsis* with respect to seedling phenotype. Similar to AtMYC2, OsMYC2-1 acts as a negative regulator of blue-light-mediated photomorphogenesis in rice. OsMYC2-1 also complements AtMYC2 in terms of disease defence in *Arabidopsis*. However, ABA signalling is not affected in both OsMYC2-1 Oex and RNAi lines.

2. Materials and methods

2.1 *Materials*

AtMYC2 over-expression and mutants were described by us previously (Yadav *et al.* 2005). Transgenic rice plants in Japonica variety TP309 was generated in this study. Untransformed TP309 was used a WT control along with transgenic plants. Pathogens were available in the laboratory. Vector pTCK303 was received from Kang Chong (Wang *et al.* 2004). Plant growth materials were procured from local market. Oligonucleotide primers were synthesized and listed in the supplementary table 1.

2.2 *Bioinformatic analysis*

To identify rice orthologue of AtMYC2, we made homology based search from rice genome. Amino acid sequence of AtMYC2 protein was retrieved from The *Arabidopsis* Information Resource website (TAIR; *www.Arabidopsis.org*). This was then used as a query sequence to search for the rice homologues of AtMYC2 by using tBLASTn tool of NCBI. Scanning for functional domains was done by using 'prosite scan' through *http://prosite.expasy.org/*.

2.3 *Generation of transgenic Arabidopsis plants*

Maize ubiquitin promoter of pTCK303 vector (Wang *et al.* 2004) was replaced by cauliflower mosaic virus 35S (CaMV35S) promoter for efficient expression of the transgene in *Arabidopsis*. For complementation study, OsMYC2 full length CDS was cloned under 35SCaMV within *KpnI* and *SpeI* sites. *Arabidopsis* transgenic plants were raised by using C58 strain of *Agrobacterium tumefaciens* as described previously (Bhattacharjee *et al.* 2017; Roy and Nandi 2017). The seeds were screened on MS media plates supplemented by 25 mg/L hygromycin. WT and transgenic *Arabidopsis* plants were grown as described earlier (Swain *et al.* 2015; Banday and Nandi, 2017), in a growth chamber at 22 °C temperature at 60% humidity and 12–12 h light (80 μM/m^2/s) - dark cycle.

2.4 *Vector construction and generation of transgenic rice plants*

To generate OsMYC2 RNAi lines in rice, we used a RNAi vector pTCK303 (Wang *et al.* 2004; Bhattacharjee *et al.* 2015). The vector contains two specific multiple cloning sites (MCSs) which are separated by a rice intron of 478 bp. The OsMYC2 RNAi target of 397 bp was cloned under the maize ubiquitin promoter in opposite orientations on both the sides of the intron at *BamHI-Kpn*I and *Spe*I-*Sac*I sites. Embryogenic calli of TP309 were used for transformation by using EHA105 strain of *Agrobacterium* as described earlier (Singh *et al.* 2016a; Singh *et al.* 2016b). Two rounds of selections were done for 21 days each on NB_6 selection media supplemented with 50 g/L hygromycin. After regeneration, rooting was done in liquid ½ MS media containing 50 mg/L hygromycin. The plantlets were then transferred to soil and kept in culture room for about a week under plastic dome before the final shifting to glass house.

2.5 *RNA isolation, RT-PCR and small RNA northern blot hybridization*

Gene expression analysis in *Arabidopsis* plants were carried out by quantitative reverse-transcription PCR (qRT-PCR). RNA extraction, cDNA preparation and expression analysis were carried out as described earlier (Giri *et al.* 2014; Banday and Nandi 2017). We used *ACTIN2* (At3g18780) for normalization. For expression analysis in rice, total RNA was isolated from 200 mg of leaves of WT and transgenic rice leaves using trizol reagent. RT-PCR was carried out as described previously (Singh *et al.* 2013). For northern blot, approximately 10 μg of total RNA was separated in a denaturing agarose gel (Swain *et al.* 2011) and probed with radio-labelled *OsMYC2* cDNA as probe. For detecting

siRNA, low-molecular-weight RNA was extracted from 700 mg rice leaves (Carra *et al.* 2007). For each line, 20 µg of small RNA was loaded on to 10% polyacrylamide gel and transferred to nylon membrane by vacuum blotting. The nylon membrane was then probed with the radio-labelled cDNA of OsMYC2.

2.6 *Seedling phenotype study*

Surface sterilized *Arabidopsis* or rice seeds were placed on MS medium and incubated in dark at 4 °C for 3 days before placing under white light (100 µmol m^{-2} s^{-1}), or blue light (30 µmol m^{-2} s^{-1}) or red light (30 µmol m^{-2} s^{-1}), or dark at 23 °C. Six days post-germination, hypocotyl length for *Arabidopsis* and coleoptile length for rice was recorded. Seedling photographs were taken at the same distance by using a digital camera. For studying the effect of ABA, surface sterilized rice seeds were placed on only MS or MS supplemented with ABA and germinated under white light as described above.

2.7 *Pathogen culture and infection of plants*

Two months old rice plants were clip inoculated with 8×10^8 CFU/mL bacterial culture of *Xanthomonas oryzae* pv *oryzae* (*Xoö*) resuspended in water with 0.01% silwet (Singh *et al.* 2013). Plants were covered with a clear plastic for 2 days. After 10 days of inoculation, a photograph of the inoculated leaves were taken.

3. Results and discussion

3.1 *Identification of OsMYC2 as functional orthologue of AtMYC2*

Initially, an OsJAZ interacting bHLH transcription factor OsbHLH148, which confers tolerance against abiotic stress was thought to be the functional homolog of AtMYC2 in rice, because of its inducibility upon MeJA, ABA treatment, low temperature, dehydration, high salinity and wounding (Seo *et al.* 2011; Kazan and Manners 2013). However, recently the *Os10g42430* gene has been referred as OsMYC2, which binds to some of the JAZs in a JAZ-interacting domain (JID)-dependent manner and modulates JA-dependent phenotypes (Uji *et al.* 2016). However, comparative homology between AtMYC2 and putative OsMYC2 has not been shown.

Basic local alignment search tool (BLAST) identified nine hits from rice genome (supplementary figure 1). Among these, only OsMYC2 (Os10g42430 -MSU/TIGR gene ID, or Os10g0575000 as RAP-DB) showed comparable overall

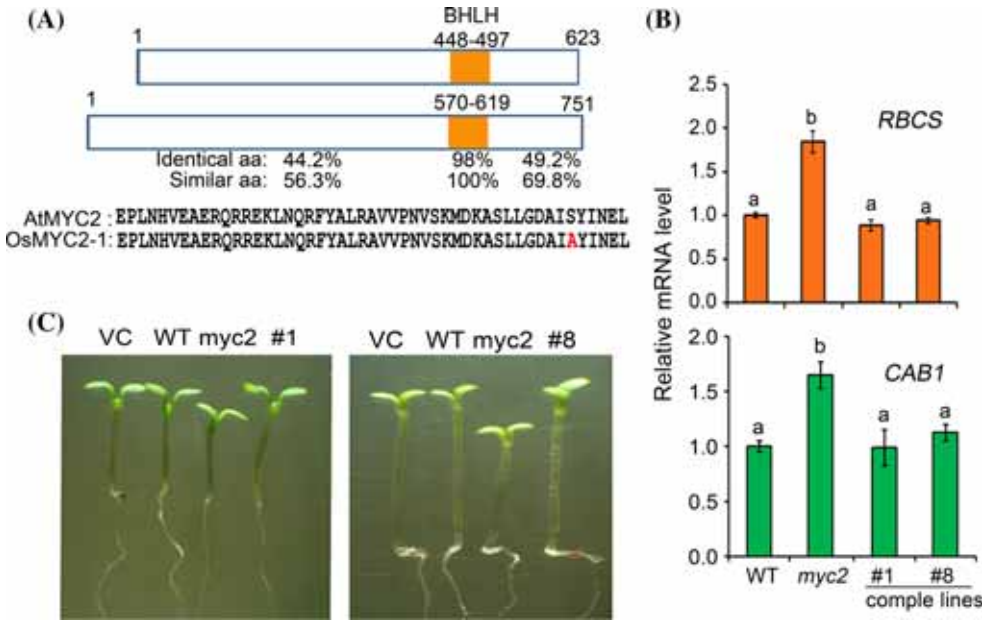

Figure 1. Structural similarity between OsMYC2 and AtMYC2 protein and functional complementation. (**A**) Schematic diagram represents amino acid (aa) sequence similarity of AtMYC2 and OsMYC2. Boxes indicate bHLH domain. The amino acid sequence of bHLH domain of OsMYC2 is near identical except for a serine to alanine (red colour font). (**B**) Relative mRNA level of RBCS and CAB1 gene in WT(Col0), *myc2* and two independent complementation lines (#1 and #8) under blue light. Each bar represents mean ± SD (n = 3). Different letters above the bars indicate significant difference ($P \leq 0.05$) in mean as obtained by one-way ANOVA. (**C**) Six-day-old blue light grown seedlings phenotype of VC (Vector control), WT(Col-0), *myc2* and complementation lines (#1 and #8).

length with AtMYC2 (figure 1A) and also showed the highest level of sequence similarity. OsMYC2 and AtMYC2 share 47% identical and 60% similar amino acid sequences throughout the length (supplementary figure 2). *In silico* scanning for functional domain identified only bHLH domain in both the proteins. Interestingly, bHLH domain of these two proteins contains all but one identical amino acid sequences (figure 1A). Though the remaining parts of these two proteins do not include any particular functional motif, share a high level of homology. The N-terminals to bHLH domain share 44.2% similar and 56.3% identical amino acids, whereas C-terminals share 49.2% identical and 69.8% similar amino acids.

To investigate whether *OsMYC2* is a functional orthologue of *AtMYC2*, we generated *OsMYC2* expressing plants in the *atmyc2* background (supplementary figure 3). AtMYC2 is a negative regulator of *CAB* and *RBCS* transcription and blue-light-mediated photomorphogenic growth of *Arabidopsis* seedlings (Yadav *et al.* 2005). As expected, *atmyc2* plants showed more expression of *RBCS* and *CAB* genes than WT plants under blue light, which was reverted to normal WT-level in *OsMYC2* expressing *atmyc2* plants (figure 1B). Seedlings of *atmyc2* mutants show reduced hypocotyl elongation compared to WT plants under blue light (figure 1C; Yadav *et al.* 2005). Transgenic lines with constitutive expression of OsMYC2 in *atmyc2* mutant showed full complementation of hypocotyl elongation phenotype (figure 1D). The results altogether demonstrate that OsMYC2 is a functional orthologue of AtMYC2.

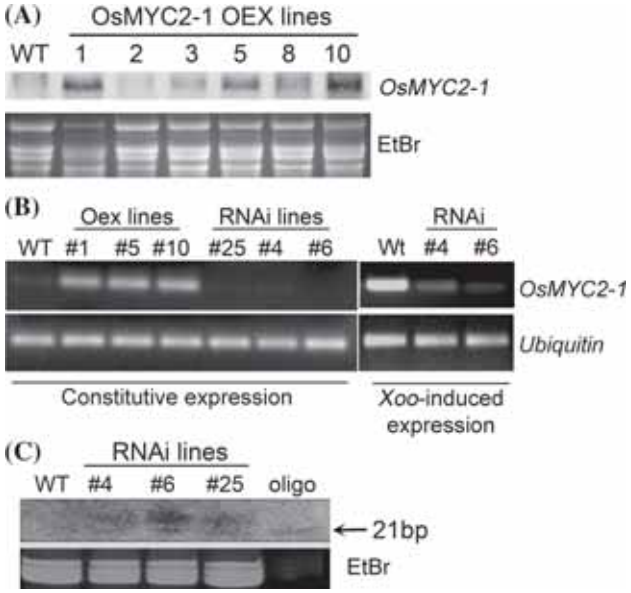

Figure 2. Conformation of rice transgenic plants. (**A**) Detection OsMYC2 transcript in untransformed wild type (WT) and in over-expression plants by northern blot hybridization with radio-labelled OsMYC2 sequence probe. Ethidium bromide (EtBR)-stained gel indicates the level of RNA loaded in the gel. Numbers above the lane represents the independent transgenic lines. (**B**) RT-PCR analysis to check the induction of *OsMYC2* transcipt after *Xoo* inoculation. WT-TP309, #1, 5 and 10 are three independent OsMYC2 Oex lines and #25, 4 and 6 are three independent OsMYC2 RNAi lines. RT-PCR with ubiquitin-specific primer indicate relative level of cDNA used for the experiment. (**C**) Northern blot for siRNA detection in three independent (#4, #6 and #25) OsMYC2 RNAi lines. EtBR-stained gel photo indicates relative level of total RNA used.

3.2 *Generation of transgenic over-expression and RNAi lines of OsMYC2 in rice*

To investigate the role of OsMYC2 in rice, we generated over-expression and the siRNA lines of *OsMYC2* by tissue culture technique. Over-expression plants were created by expressing OsMYC2 coding sequences under constitutive rice ubiquitin promoter (supplementary figure 4A). The presence of the transgene in the regenerated plants was confirmed by PCR with *hpt* specific primers (supplementary figure 4B). Further, the transgenic plants were confirmed by GUS reporter expression (supplementary figure 4C). Levels of *OsMYC2* transcript accumulation in the transgenic plants were determined by northern blot analysis (figure 2A). Out of five independent transgenic lines tested, four showed the much higher level of *OsMYC2* transcript than untransformed plants (figure 2A). To generate siRNA lines, we selected a stretch of 397 bp that is specific for *OsMYC2* gene as siRNA target (supplementary figure 5), and cloned in the rice RNAi vector pTCK303 (supplementary figure 6A; Wang *et al.* 2004). Transgenic plants were confirmed by GUS reporter expression (supplementary figure 6B), and by PCR with *hpt*-specific primers (supplementary figure 6C). To determine the efficacy of RNAi-mediated suppression, we monitored *OsMYC2* transcript accumulation by reverse-transcription PCR (RT-PCR). As controls, we used untransformed WT plant and overexpresser lines. As expected, we observed a higher level of *OsMYC2* mRNA in overexpresser lines than WT plants but significantly lower levels in RNAi lines (figure 2B). We also noted that basal level expression of *OsMYC2* in rice was quite low (figure 2A and B). To visualize the effect of RNAi-mediated suppression, we inoculated WT and RNAi plants with *Xanthomonas oryzae* Pv. *Oryzae* (*Xoo*), a virulent rice bacterial pathogen, with an assumption that it would enhance *OsMYC2* expression. Indeed, we observed enhanced *OsMYC2* expression in *Xoo*-inoculated WT plants. Compared to the WT plants, *Xoo*-inoculated RNAi plants showed significantly reduced of *OsMYC2* transcript accumulation. Further, to confirm siRNA production, we performed northern blot hybridization using siRNA target as the probe. As a control, we loaded one 21-mer oligonucleotide that was used as the primer for generating siRNA target (supplementary table 1). As

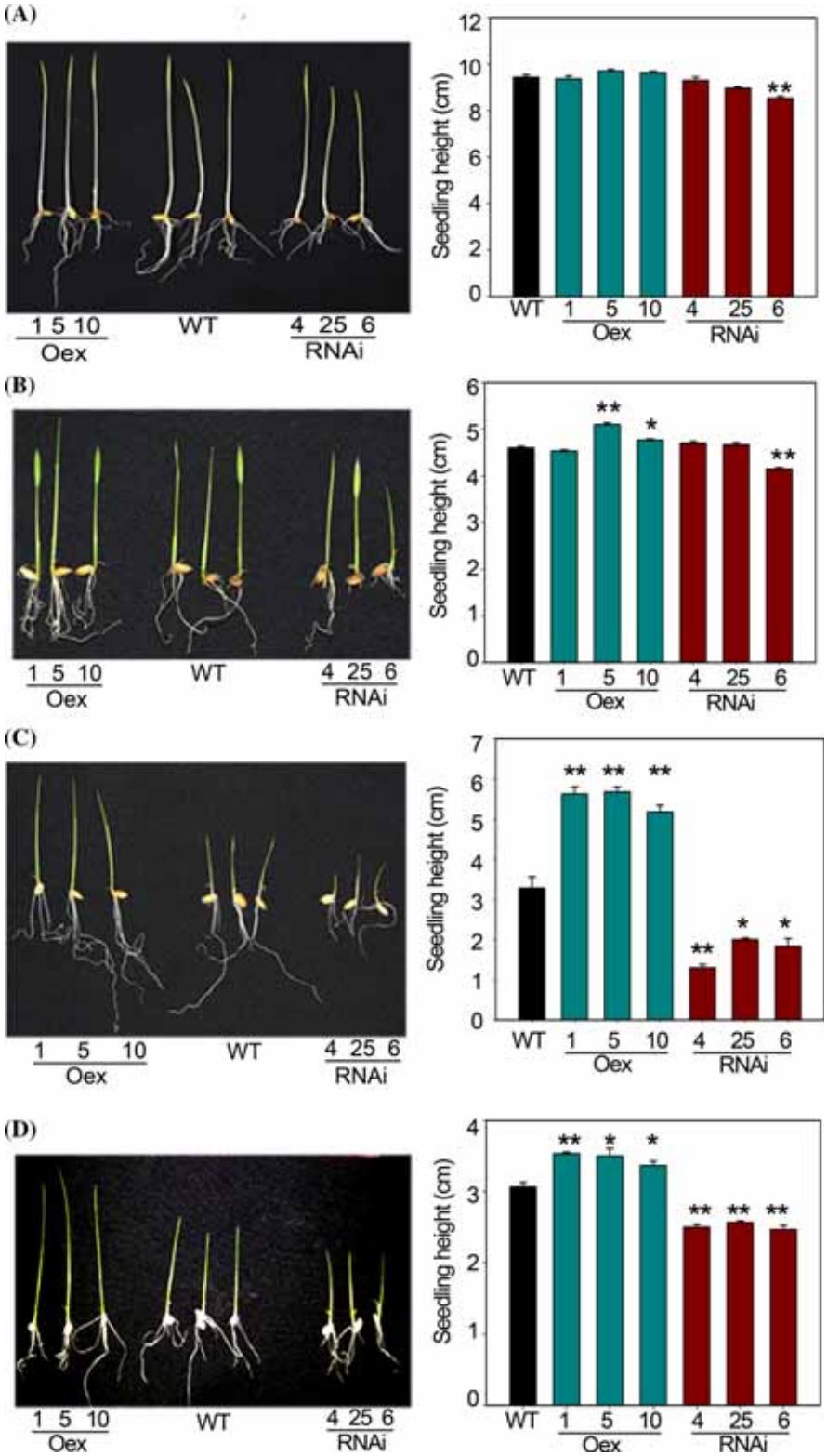

Figure 3. Rice seedling phenotypes at under different light or dark condition: (**A**) dark grown, (**B**) white light, (**C**) blue light, (**D**) red light. Photographs were taken at 6 days post-germination. Graphs in the right indicate mean ± SD (n = 6) of seedling height. Significant difference in mean with WT as obtained by Student's *t* test are indicated by *($P \leq 0.05$) or **($P \leq 0.001$).

expected, we observed siRNAs in RNAi lines but not in WT plants (figure 2C). The results described above confirmed the successful generation of *OsMYC2* over-expression and RNAi lines.

3.3 *OsMYC2 negatively regulates photomorphogenesis with most prominent effect under blue light*

The AtMYC2 function is required for WT-level hypocotyl elongation, specifically when plants are grown under blue light (Yadav *et al.* 2005). The *atmyc2* mutant shows retarded hypocotyl elongation compared to WT plants under blue-light but show no difference in dark or red light. To study the role OsMYC2 in coleoptile elongation, we germinated WT, *OsMYC2* over-expression and RNAi lines under different light or dark conditions. Similar to *atmyc2* mutant plants, over-expression and RNAi lines of rice did not show much difference with WT in coleoptile length under dark (figure 3A). There was only a modest reduction (∼10%) in coleoptile length in one RNAi line compared to the WT plants, and no difference was observed in overexpresser lines. Influence of OsMYC2 in seedling development under white light was also marginal (figure 3B). However, the influence of OsMYC2 in seedling growth was obvious under blue light. We observed significant reduced and enhanced seedling growth in RNAi and overexpresser lines respectively compared to WT plants (figure 3C). Since suppression of seedling height is a photomorphogenic phenotype, the results suggested that OsMYC2 negatively regulates photomorphogenesis under blue light. This phenotype was in full agreement with AtMYC2 function in *Arabidopsis*. However, in contrast to AtMYC2 function, we also observed the negative regulatory role of OsMYC2 in photomorphogenesis under red light, though the extent at which transgenic lines differ from the WT under red light was significantly lower compared to blue light condition (figure 3D).

3.4 *OsMYC2 acts as negative regulators of disease defence in rice*

AtMYC2 negatively regulates defence against bacterial pathogens in *Arabidopsis* (Laurie-Berry *et al.* 2006). To investigate whether OsMYC2 complements AtMYC2 function, we monitored bacterial (*Pseudomonas syringae* pv *maculicola* ES4326; *Psm*) growth in *atmyc2* mutant, complemented lines and WT *Arabidopsis* plants. As expected, *atmyc2* mutant plants supported lower *Psm* growth than WT plants (figure 4A). The complemented lines showed bacterial numbers that were comparable to the WT plants, suggesting a perfect functional complementation of *atmyc2* by OsMYC2 regarding disease defence. To further examine the function of OsMYC2 in disease defence in rice, we inoculated WT and two independent RNAi plants with *Xanthomonas oryzae* pv *oryzae* (Xoö). After 10 days of inoculation we observed visibly much reduced disease symptom in RNAi lines compared to WT plants (figure 4B). The results suggest OsMYC2 is a negative regulator of defence against bacterial pathogen Xoö.

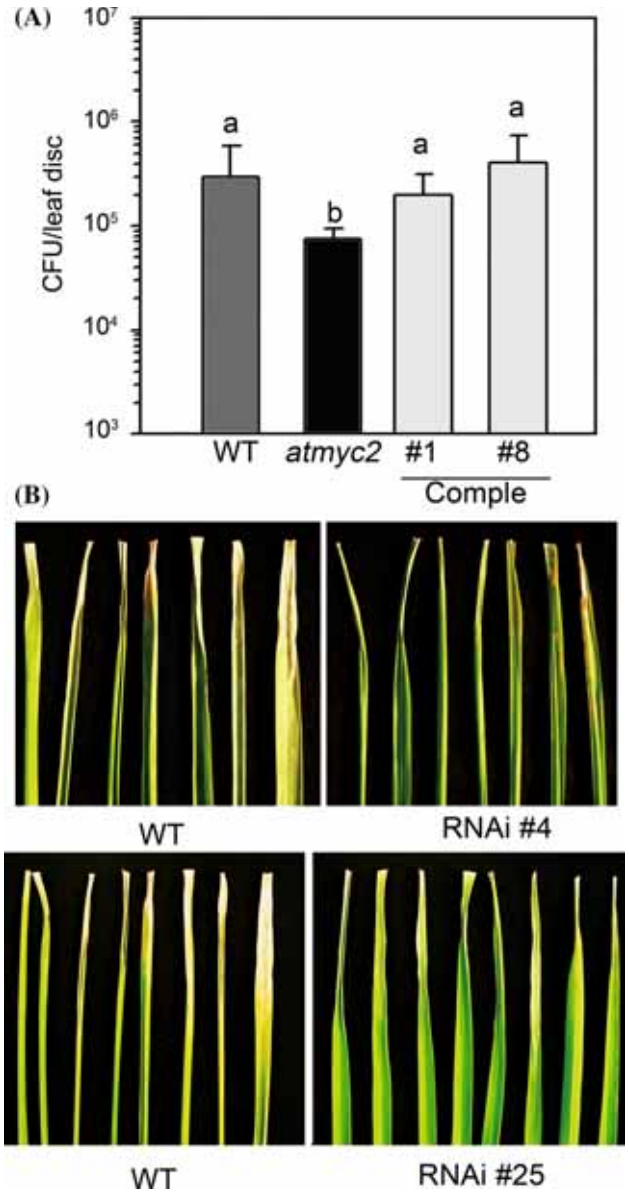

Figure 4. (**A**) Bacterial count in WT(Col-0), *atmyc2* and OsMYC2 complementation lines (#1 and #8) after 3 days of *Psm* inoculation. Each bar represents mean ± SD of 4 samples each carrying 4 discs of 5 mm diameter. Different letters above the bars indicate significant difference ($P \leq 0.05$) in mean as obtained by one-way ANOVA. (**B**) Disease symptoms after 10 days of *Xanthomonas oryzae* inoculation in WT(TP309) and two independent (#4 and 25) *OsMYC2RNAi* lines.

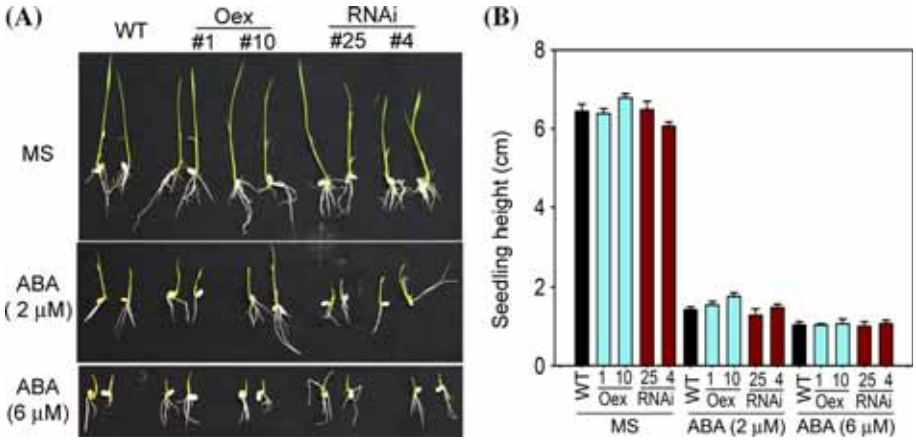

Figure 5. (**A**) Photograph showing Seedling germination efficiency of WT (TP309), *OsMYC2* Oex and OsMYC2 RNAi lines. Seeds of respective genotype were grown for 6 days in MS with or without ABA. (**B**) Quantification of seedling height at 6 days post-germination.

3.5 *OsMYC2 does not play a significant role in ABA-mediated inhibition of seedling germination and growth*

ABA inhibits seed germination and seedling growth. The *atmyc2* mutant lines show reduced sensitivity to ABA treatment compared to WT plants. The rate of germination of *atmyc2* plants was better than WT plants when germinated in the presence of ABA (Abe *et al.* 2003; Yadav *et al.* 2005). In contrast, over-expression of AtMYC2 makes seeds and seedlings hypersensitive to exogenous ABA application (Abe *et al.* 2003; Lorenzo *et al.* 2004). Thus, we investigated the probable role of OsMYC2 by germinating transgenic rice seeds in the presence of ABA. WT, overexpresser and RNAi line plants were germinated in MS plate supplemented with 2 or 6 µM concentration of ABA. As a control, we used only MS plate. We did not observe any difference in seed germination or seedling growth among WT, overexpresser or RNAi lines (figure 5A). We measured height of the seedlings at 6 days post-germination, and found no significant difference among the lines (figure 5B). The results suggest that OsMYC2 does not influence ABA-mediated seed germination or seedling growth.

4. Conclusion

Our results demonstrate that OsMYC2 is a functional orthologue of AtMYC2. The defects of *atmyc2* in terms of seedling development and expression of light-dependent genes are rectified when *OsMYC2* is expressed in *atmyc2* mutant background. Transgenic rice plants with over-expression and under-expression of OsMYC2 shown *Arabidopsis*-like seedling phenotype under dark, blue and red light. However, unlike *AtMYC2*, the *OsMYC2* gene does not influence ABA sensitivity regarding seed germination and seedling growth.

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