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The *NtMYC2-NtABI5* module regulates abscisic acid mediated seed germination in tobacco

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Abstract

In tobacco, the basic helix-loop-helix (bHLH) transcription factor MYC2 contributes significantly to the biosynthesis and transport of nicotine, and also participates in the regulation of pollen development. However, their biological functions in seed germination remain largely unknown. Here, we examined the regulatory roles of *NtMYC2a* and *NtMYC2b* in abscisic acid (ABA) mediated seed germination. The expression of both *NtMYC2a* and *NtMYC2b* was repressed by ABA at both mRNA and protein levels. Simultaneous knock out of *NtMYC2a* and *NtMYC2b* reduced ABA signaling to promote seed germination, while overexpression of *NtMYC2a* or *NtMYC2b* resulted in the germinated seeds being more ABA-sensitive. Gene expression analysis showed that the expression of both *NtABI3* and *NtABI5* was reduced in *Ntmyc2a/2b* double mutants, but enhanced in transgenic plants overexpressing *NtMYC2a* or *NtMYC2b*. Both electrophoretic mobility shift assays (EMSA) and transient dual-luciferase reporter assays, revealed that both NtMYC2a and NtMYC2b could bind to the promoter of *NtABI5* to activate its expression. Furthermore, NtMYC2a and NtMYC2b could interact with each other to form a heterodimer and function synergistically to activate the expression of *NtABI5*. Thus, these results provide evidence that the NtMYC2-NtABI5 module may function as a novel component in ABA signaling to positively modulate ABA-mediated seed germination in tobacco.

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Introduction

Flowering plants have evolved the capacity to sense favorable seasons to accurately initiate the seed dormancy-to-germination transition during their life cycles, leading to their successful adaptation and high suitability to an ever-changing environment[1,2]. Seeds usually become dormant during maturation in their mother plants. After the completion of seed development, the seeds perceive multiple exogenous cues, such as light, temperature, soil nitrate levels, and smoke, which provide information about soil status and time of year, facilitating them to determine whether to terminate seed dormancy and initiate germination[3–6]. Numerous studies have demonstrated that the phytohormones, mainly abscisic acid (ABA) and gibberellin (GA), function as key integrators between endogenous developmental cues and environmental conditions, to modulate gene transcription during seed dormancy-to-germination transition. Specifically, the dormancy and germination status of seeds are primarily determined by the dynamic balance between ABA and GA levels or sensitivity[7–9].

As the primary phytohormone in controlling seed dormancy and germination, ABA is perceived by the well-known receptors PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR (RCAR), which leads to the formation of PYR/RCAR–PROTEIN PHOSPHATASE 2C (PP2C) complex, and to their subsequent repression[10,11]. The inhibition of PP2Cs releases SNF1-related kinases 2 (SnRK2s) which phosphorylate downstream transcription factors (TFs), thereby initiating the ABA-responsive signaling cascade[12–16]. As a master transcription factor in ABA signaling, ABI5 (ABSCISIC ACID INSENSITIVE5) was revealed to directly bind and activate the promoters of downstream ABA-responsive genes like *EM1* (*EARLY METHIONINE-LABELED 1*) and *EM6*, ultimately leading to the inhibition of seed germination[17]. Dissecting novel ABA signaling-associated components, especially critical transcription modulators, may contribute to the explanation of the molecular basis of ABA signaling or its crosstalk with other signaling pathways during seed germination.

The bHLH transcription factors are regulatory proteins that constitute a superfamily in plants. In tobacco, the bHLH transcription superfamily comprises over 190 members that can be divided into 23 subfamilies based on their protein sequence similarity[18]. Although much progress in bHLH transcription factors' functional research has been obtained in the past decades, especially in the model plant *Arabidopsis thaliana*, the functional elucidation of most *bHLH* genes in tobacco represent a major challenge. A body of genetic and molecular evidence has revealed that bHLH transcription factors perform crucial functions in plant development as well as in responses to both biotic and abiotic stresses[19,20]. Among them, the bHLH transcription factor MYC2 was revealed to function as the master regulator of the JA signaling cascade and participates in the regulation of various plant physiological processes, including JA-induced leaf senescence, root growth, and both biotic and abiotic stresses[21,22]. Furthermore, MYC2 can also participate in ethylene, salicylic acid, GA, and ABA signaling to mediate various plant developmental processes and defense responses[23]. In *Nicotiana* species, MYC2 transcription factors, including NtMYC2a/b/c from tobacco and their homolog NbbHLH2 from *N. benthamiana*, function as key regulators to control nicotine biosynthesis possibly through directly binding to the target G-box sequences present in the promoters of

nicotine biosynthetic pathway genes, such as *NtPMT1a/2*, *NbPMT*, *NtQPT2*, *NtA622*, and *NtMATE1*[24–27]. One recent study also demonstrated that NtMYC2a participates in pollen development through modulating carbohydrate metabolism in tobacco[28]. Interestingly, their possible roles in seed germination in tobacco and the mechanisms involved still remain unclear.

Here, this study employed diverse molecular approaches to investigate the role of *NtMYC2a* and *NtMYC2b* in the regulation of ABA-mediated seed germination. We found that both mutation and overexpression of *NtMYC2a* and *NtMYC2b* can affect ABA-mediated seed germination. Furthermore, NtMYC2a and NtMYC2b can interact with each other to form a heterodimer and synergistically activate *NtABI5* expression, leading to the transmission of ABA signaling during seed germination. Our results thus provide evidence that both *NtMYC2a* and *NtMYC2b* function as positive regulators to modulate ABA responses during seed germination in tobacco.

Materials and methods

Materials

The seeds used in this study were all derived from the genetic background *Nicotiana tabacum* (cv. Coker176). Both the *NtMYC2a/2b* mutants and overexpression lines were kindly gifted by Yulong Gao (Yunnan Academy of Tobacco Agricultural Sciences). Apart from ABA (Sigma Co. Ltd.), all other chemicals were sourced from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China).

Treatment of ABA

ABA working solution (100 μM) was prepared by diluting the 100 mM ABA stock solution, and six-week-old seedlings were used in this study. Samples were collected at 0, 1, 2, 4, 8, 12, and 24 h after treatment with H_2O or ABA. For each time point, three biological replicates were set, with each replicate consisting of pooled tissues from at least three individual seedlings. All samples were immediately frozen in liquid nitrogen after collection, and stored at −80 °C until RNA extraction.

RT-qPCR analysis

Total RNA was isolated with Trizol reagent according to the manufacturer's protocol. Total RNA (1 μg) pre-treated with DNase1 was reverse-transcribed using Superscript II. Then, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed on a Roche Light Cycler 480 real-time PCR machine. All gene-specific primers used for RT-qPCR are listed in Supplementary Table S1.

Western blotting

Seeds of *35S:MYC-NtMYC2a/2b* transgenic plants were harvested after treatment with ABA at the indicated time, and total proteins were extracted using RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 0.1% Nonidet P-40, 150 mM NaCl, 1% SDS, 0.5% sodium deoxycholate, and a protease inhibitor cocktail [CWBIO]). The anti-MYC (Abmart) and anti-ACTIN (Abiocode) were used to detect MYC-NtMYC2a/2b and Actin, respectively.

Measurement of germination or greening rates

Seeds of WT and *NtMYC2a/2b* mutants or overexpression lines were harvested at the same time and dried for 2 weeks. Seeds were stratified for 3 d at 4 °C and then germinated at 24 °C under a 16 h light/8 h dark photoperiod. Seeds were plated on 1/2 MS medium supplemented with different ABA concentrations (0, 0.5, or 1 μM) to assess ABA sensitivity of seeds. Germination was defined by the appearance of visible radicles, while seedling greening was determined based on the emergence of green cotyledons.

EMSA

The full-length CDSs of *NtMYC2a/2b* were inserted into the expression vector *pET-28a* to generate *pET-28a-NtMYC2a/2b*. His-NtMYC2a/2b recombinant proteins were purified using the His PurNi-NTA resin. The biotin-labeled WT or mutant *NtABI5* promoter fragments were used as probes, and the biotin-unlabeled WT fragments were used as competitors. Electrophoresis mobility shift assays were conducted using a Chemiluminescent EMSA Kit following the manufacturer's protocol.

Molecular interaction simulation

The amino acid sequences of the two proteins were submitted to AlphaFold3 to predict the structure of the protein complex. The visualization of the interacting structures was achieved in PyMOL by uploading the protein structure. The image of molecular interaction was exported from PyMOL as png file.

Pull-down assay

The full-length cDNA of *NtMYC2a* was fused to *pGEX-4T-1*, and the full-length cDNA of *NtMYC2b* was cloned into *pET-28a*. Then these two plasmids were transformed into *Escherichia coli* BL21 cells, and the recombinant proteins were induced with isopropyl-beta-thio-galactopyranoside. Crude NtMYC2a-GST and GST recombinant protein extracts were obtained and then immobilized to glutathione affinity resin. NtMYC2b-His protein was co-incubated with the immobilized NtMYC2a-GST and GST fusion proteins for 4 h at 4 °C. Co-incubated proteins were eluted with glutathione elution buffer, and the eluted proteins were then subjected to western blot analysis. The membranes were probed respectively with anti-His and anti-GST antibodies to determine protein interaction. The primers used for pull-down are listed in Supplementary Table S2.

Split luciferase complementation assay

To confirm the interactions between NtMYC2a and NtMYC2b, their full-length CDS were fused to *pCAMBIA1300-cLUC* or *pCAMBIA1300-nLUC* to generate *cLUC-MYC2b* and *nLUC-MYC2a* respectively. All plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105, and then infiltration of *N. benthamiana* leaves was performed as described in Zhang et al[29]. Infiltrated plants were kept under standard growth conditions for 48 to 72 h to allow optimal protein expression and potential interaction. Luciferase imaging was performed using an *in vivo* imaging system. The leaves were sprayed uniformly with a 1 mM D-luciferin substrate solution (in 0.1% Triton X-100), and placed in darkness for 5 min to minimize background luminescence. Bioluminescence signals were then captured using a low-light, cooled CCD camera imaging apparatus (Tanon-5200, China) with an exposure time optimized to avoid signal saturation. The split luciferase complementation assays were carried out with at least three independent biological replicates, with similar results. The primers used for this study are listed in Supplementary Table S2.

Transient expression assay

The native promoters of *NtABI5* were amplified and cloned into the *pGreenII 0800-LUC* vector as a reporter. Full-length CDSs

of *NtMYC2a*, *NtMYC2b*, and *GFP* were amplified and cloned into the *pGreenII 62-SK* vector as effectors. *Agrobacterium tumefaciens* EHA105 (pSoup) harboring the above constructs was infiltrated into five-week-old *N. benthamiana* leaves using a needleless syringe for transactivation analyses. The low-light cooled CCD imaging apparatus was used to capture the LUC image and count luminescence intensity. The transient expression assays were carried out with at least three independent biological replicates with similar results. The primers used for this study are listed in Supplementary Table S2.

Results

Expression pattern of*NtMYC2a* and *NtMYC2b* upon ABA treatment

Previous studies have demonstrated that NtMYC2s function as critical regulators in the synthesis and transport of nicotine and also participate in the regulation of carbohydrate metabolism during pollen development[22,27,28]. However, their distinctive roles in various physiological processes and stress responses in tobacco are still poorly characterized. This study performs experiments to determine their possible roles in ABA-mediated seed germination. To determine their roles in ABA signaling, we first conduct RT-qPCR analysis to explore their expression upon ABA treatment. As shown in Fig. 1a, b, the expression of both *NtMYC2a* and *NtMYC2b* was inhibited upon ABA treatment compared with the control treatment. To further reveal the expression patterns of *NtMYC2a* and *NtMYC2b*, the stabilities of both NtMYC2a and NtMYC2b proteins during ABA treatment was determined using *35S:MYC-NtMYC2a/2b* transgenic plants. The western blotting analysis showed that both NtMYC2a and NtMYC2b protein accumulations were decreased upon ABA treatment (Fig. 1c, d). These results indicated that *NtMYC2a* and *NtMYC2b* may participate in the regulation of ABA signaling.

Knock out of *NtMYC2a* and *NtMYC2b* renders the seeds more insensitive to ABA

Based on the findings that both *NtMYC2a* and *NtMYC2b* are down-regulated by ABA, we speculated that they may be involved in the regulation of ABA associated responses, such as ABA-mediated seed germination. Then, the phenotypes of the wild type, *Ntmyc2a* and *Ntmyc2b* single and double mutants were compared, in response to ABA during seed germination[27] (Supplementary Figs. S1, S2). No notable phenotypic differences were observed among the WT, *Ntmyc2a* and *Ntmyc2b* single mutants, and the double mutants on half-strength MS medium (Fig. 2a–c). However, both the *Ntmyc2a* and *Ntmyc2b* single mutant seeds exhibited a slight but consistent reduction in ABA sensitivity during seed germination and early seedling growth compared to the WT, especially at a concentration of 0.5 μM ABA. Furthermore, the *Ntmyc2a/2b* double mutant seeds were more insensitive to ABA compared with that of WT, and their single mutants, suggesting a redundant function of *NtMYC2a* and *NtMYC2b* during ABA-mediated seed germination (Fig.2a–c). Thus, our results implied that NtMYC2a/2b may act as positive regulators in ABA mediated seed germination and early seedling growth.

Overexpression of *NtMYC2a* or *NtMYC2b* renders the seeds more sensitive to ABA

To further characterize the role of *NtMYC2a* and *NtMYC2b* in ABA-mediated seed germination, we also obtained transgenic tobacco plants constitutively expressing *NtMYC2a* or *NtMYC2b* under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. In contrast to the *Ntmyc2a/2b* mutants, seeds from *NtMYC2a/2b* overexpression lines exhibited significantly reduced germination and cotyledon greening relative to the WT (Fig. 3a–c). Thus, constitutive overexpression of *NtMYC2a/2b* led to enhanced sensitivity to ABA during seed germination and post-germinative growth, and further confirm that NtMYC2a and NtMYC2b function as positive regulators in ABA-mediated seed germination.

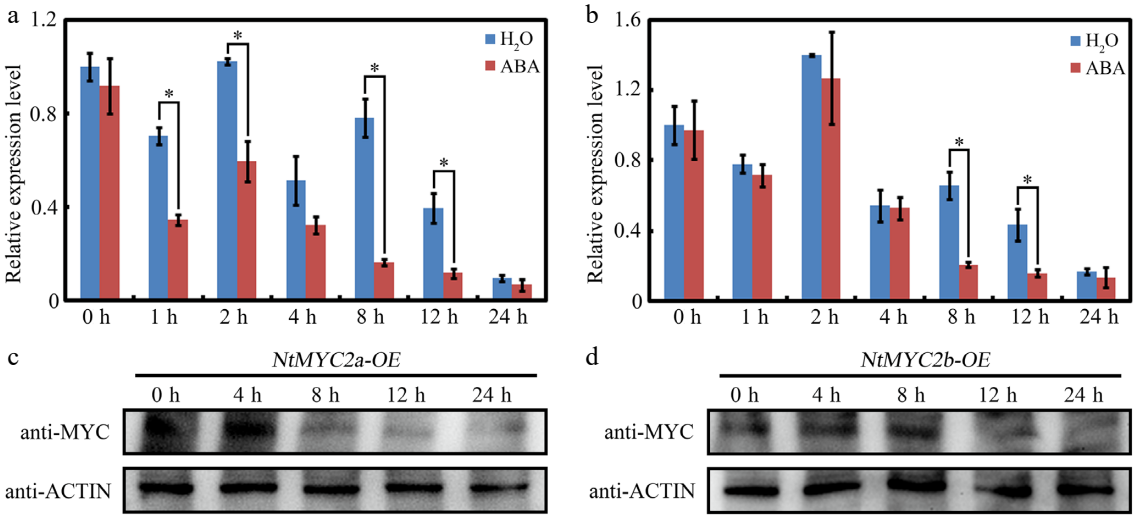

Fig. 1 Expression of *NtMYC2a* and *NtMYC2b* upon ABA treatment. (a) and (b) RT-qPCR analysis of transcript levels of *NtMYC2a* and *NtMYC2b* after ABA treatment. Samples were collected after ABA treatment for the indicated time. All data are presented as the mean ± SD from three independent biological replicates. Asterisks indicate Student's *t*-test significant differences as compared to controls, * $p < 0.05$. (c) and (d) Western blot analysis of NtMYC2a/2b protein accumulation in transgenic *35S:MYC-NtMYC2a/2b* plants after ABA treatment for the indicated time. An anti-MYC antibody was used to detect MYC-NtMYC2a/2b. Actin was used as an internal control.

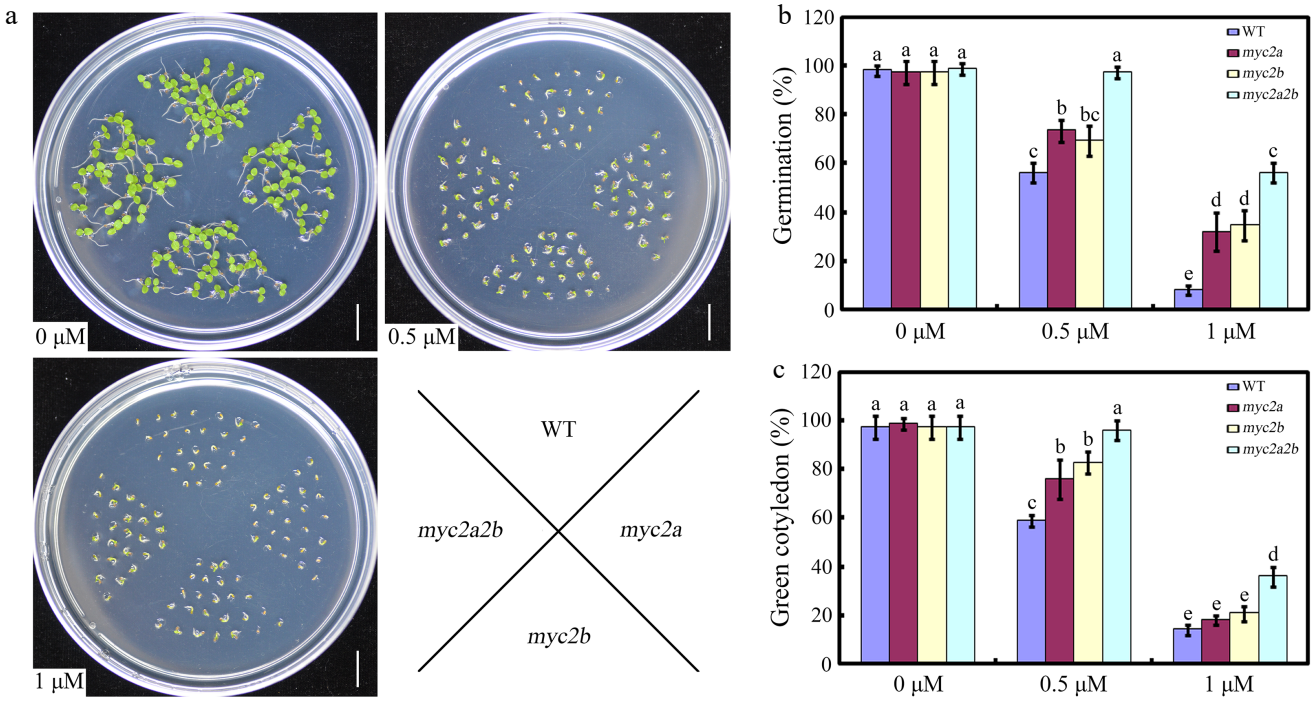

Fig. 2 The effect of *NtMYC2a* and *NtMYC2b* mutation on seed germination. (a) Seedlings of the indicated genotypes observed 7 d after germination on medium supplemented with 0, 0.5 or 1 μM ABA. (b) Statistic analysis of germination rate in the indicated genotypes. The seeds were scored 5 d after stratification on medium containing different concentrations of ABA. Germination is defined by radical emergence. (c) Statistic analysis of the indicated genotypes for cotyledon greening. Cotyledon greening was scored 7 d after stratification on medium containing different concentrations of ABA. All data are presented as the mean ± SD from three independent biological replicates. These data were analyzed by a two-way ANOVA using Tukey's HSD test. Bars with different letters are significantly different from each other ($p < 0.05$).

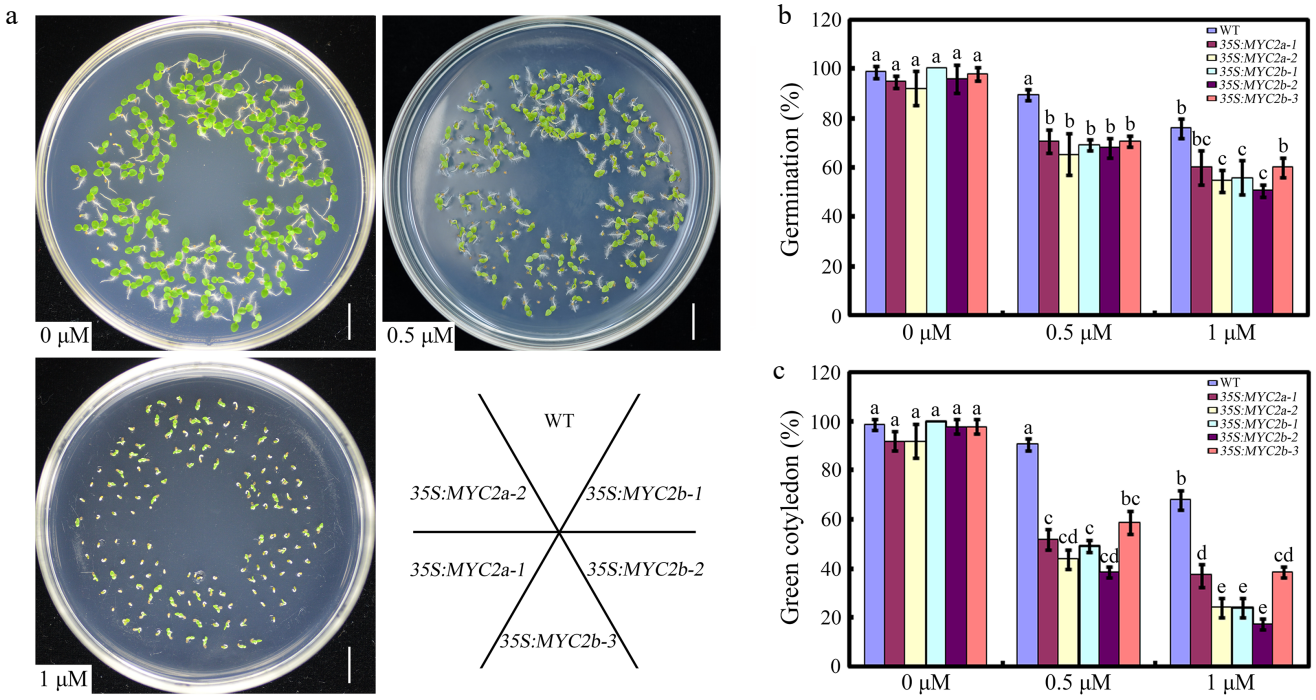

Fig. 3 The effect of *NtMYC2a* or *NtMYC2b* overexpression on seed germination. (a) Seedlings of the indicated genotypes observed 10 d after germination on medium supplemented with 0, 0.5 or 1 μM ABA. (b) Statistic analysis of germination rate in the indicated genotypes. The seeds were scored 8 d after stratification on medium containing different concentrations of ABA. Germination is defined by radical emergence. (c) Statistic analysis of the indicated genotypes for cotyledon greening. Cotyledon greening was scored 10 d after stratification on medium containing different concentrations of ABA. Error bars (mean ± SD) are derived from three biological replicates. Data were analyzed by a two-way ANOVA using Tukey's HSD test. Bars with different letters are significantly different from each other ($p < 0.05$).

NtMYC2a and *NtMYC2b* affect the expression of *NtABI3* and *NtABI5*

Seeds of the *Ntmyc2a/2b* mutant or constitutive overexpression of *NtMYC2a/2b* showed contrasting phenotypes with respect to ABA mediated seed germination. This may indicate that MYC2a/2b may participate in this process by affecting the expression of germination associated genes. To test this possibility, we analyzed the expression of several ABA-related genes in *NtMYC2a/2b* mutant seeds or seeds of constitutive overexpression of *NtMYC2a/2b* by RT-qPCR. The results demonstrated that the expression levels of ABA signaling genes, including *NtABI3* and *NtABI5*, decreased in *Ntmyc2a/2b* to different degrees compared with the WT, while enhanced in seeds that constitutively expressing *NtMYC2a* or *NtMYC2b* (Fig. 4). These results demonstrated that *NtMYC2a/2b* may participate in ABA mediated seed germination by affecting the expression of ABA signal transduction-related genes.

NtMYC2a and NtMYC2b bind the promoter of *NtABI5* to activate its expression

MYC2, as a bHLH transcription factor, was revealed to perform its biological functions through directly binding to the G-box element. Interestingly, one G-box element was found in the promoter of *NtABI5*, implying that NtMYC2a and NtMYC2b may bind to the G-box element to regulate *NtABI5* expression. EMSA was performed to test whether NtMYC2a and NtMYC2b can directly bind to the G-box element. Incubation of NtMYC2a/2b proteins and the labeled probes resulted in a band shift, whereas inclusion of the unlabeled competitor DNA reduced the electrophoretic mobility shift in a dosage-dependent manner. Meanwhile, mutation of the G-box element in the probe abolished the band shift (Fig. 5a), confirming that NtMYC2a and NtMYC2b bind directly and specifically to the G-box element in the *NtABI5* promoter.

Based on the above results that NtMYC2a/2b binds directly to the promoter of *NtABI5*, the next steps examined whether NtMYC2a/2b could directly modulate *NtABI5* expression through transient expression analysis in *N. benthamiana* leaves using constructs in which the *NtABI5* promoter sequence was fused to *LUCIFERASE* (*LUC*) as a reporter. LUC signals were significantly enhanced when NtMYC2a or NtMYC2b was co-expressed with the construct (Fig. 5b–d), further implying that both NtMYC2a and NtMYC2b can act as transcriptional activators to directly enhance *NtABI5* expression during seed germination.

NtMYC2a interacts with NtMYC2b

Because NtMYC2a and NtMYC2b function redundantly during ABA mediated seed germination, and both of them can directly activate *NtABI5* expression during this process, we speculate that they may interact with each other to synergistically modulate *NtABI5* expression. In order to determine our prediction, we first employed AlphaFold and PyMOL to analyse the potential interaction between NtMYC2a and NtMYC2b (Fig. 6a). Then, the *in vitro* pull-down assay was performed to detect their interaction. The pull-down assay demonstrated that GST-NtMYC2a can pull down NtMYC2b-His, while the GST control did not (Fig. 6b). Interaction of NtMYC2a and NtMYC2b was further corroborated by split luciferase complementation (split-LUC) assays. *NtMYC2a* and *NtMYC2b* were fused to the N- and C-terminal halves of the firefly luciferase, respectively (*nLUC-NtMYC2a* and *cLUC-NtMYC2b*). When fused nLUC-NtMYC2a was co-infiltrated with cLUC-NtMYC2b into *N. benthamiana* leaves, strong luminescence was observed (Fig. 6b). However, no luminescence was detected in all negative controls. Taken together, this data demonstrates a physical interaction between NtMYC2a and NtMYC2b, suggesting their possible synergistic action as a heterodimer in diverse stress responses.

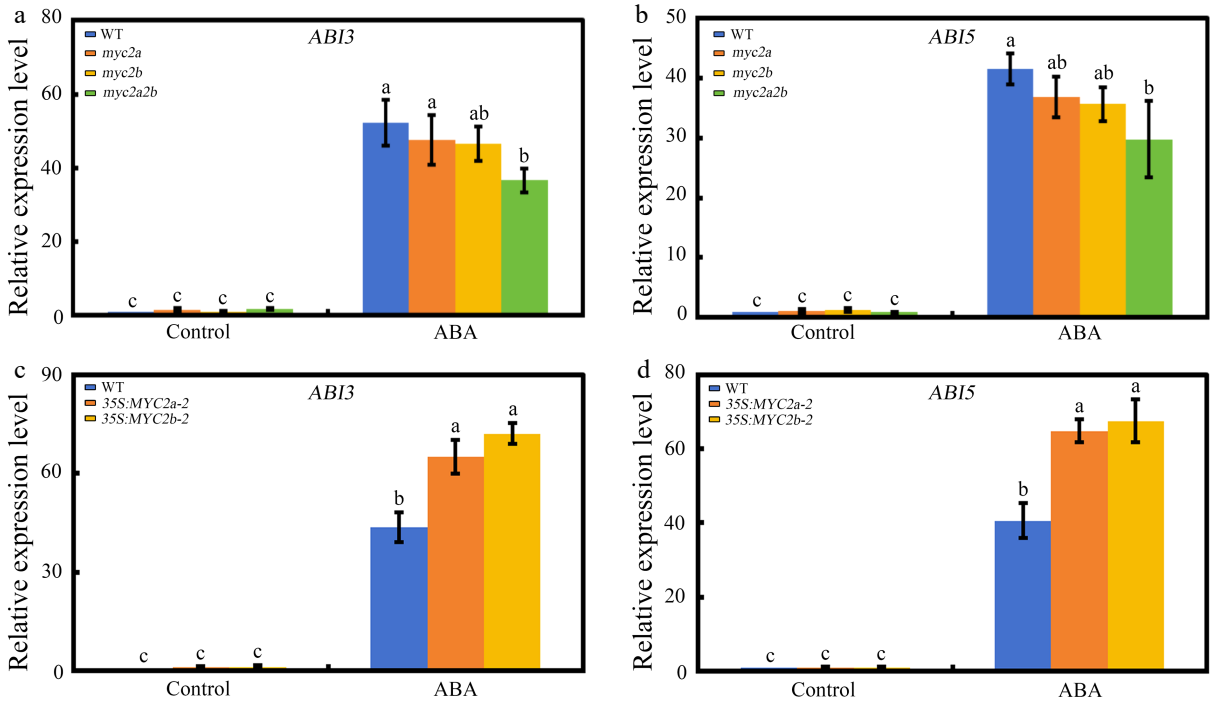

Fig. 4 The effect of *NtMYC2a/2b* mutation or overexpression on *NtABI3* and *NtABI5* expression. (a) and (b) Expression levels of *NtABI3* and *NtABI5* in *Ntmyc2a* and *Ntmyc2b* single mutant, *Ntmyc2a2b* double mutant and wild-type tobacco seeds grown on medium containing 1 μM ABA or not for 3 d. (c) and (d) Expression levels of *NtABI3* and *NtABI5* in *NtMYC2a* or *NtMYC2b* overexpression line and wild-type tobacco seeds grown on medium containing 1 μM ABA or not for 3 d. The data is shown as mean ± SD, error bars represent SD from three independent biological replicates, which were analyzed by a two-way ANOVA using Tukey's HSD test. Bars with different letters are significantly different from each other ($p < 0.05$).

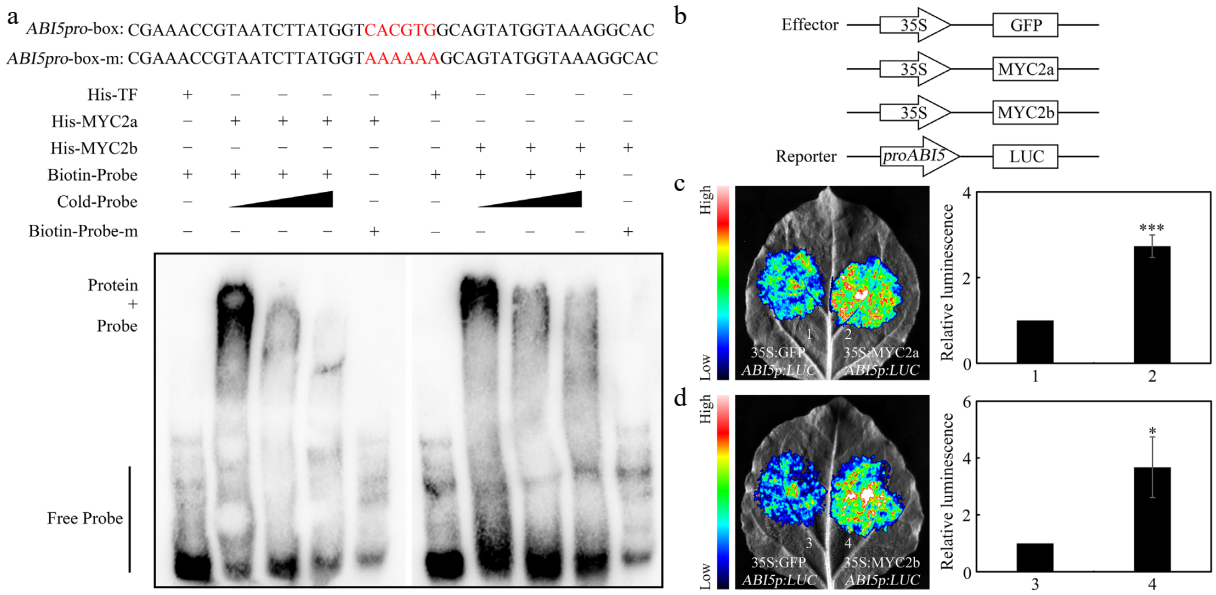

Fig. 5 NtMYC2a/2b bind the promoter of *NtABI5* to activate its expression. (a) EMSA results revealed that NtMYC2a/2b can bind to the promoter of *NtABI5*. The probes were synthesized according to the G-box element within the *NtABI5* promoter. The red sequence signifies G-box sequence and the mutated base in the G-box element. His, His-NtMYC2a/2b, biotin-probe, biotin-probe-m and cold probes were present (+), or absent (–) in each reaction. (b) Schematic of the *NtABI5p:LUC* reporter and *NtMYC2a/2b* and *GFP* effectors. (c) and (d) Transient transcriptional activity assays. NtMYC2a/2b could activate *NtABI5* expression in *N. benthamiana* leaves. Both representative leaf images and quantification of corresponding relative luminescence intensities were presented. Error bars represent ± SD. Asterisks indicate student's *t*-test significant differences as compared to controls, * $p < 0.05$, *** $p < 0.001$.

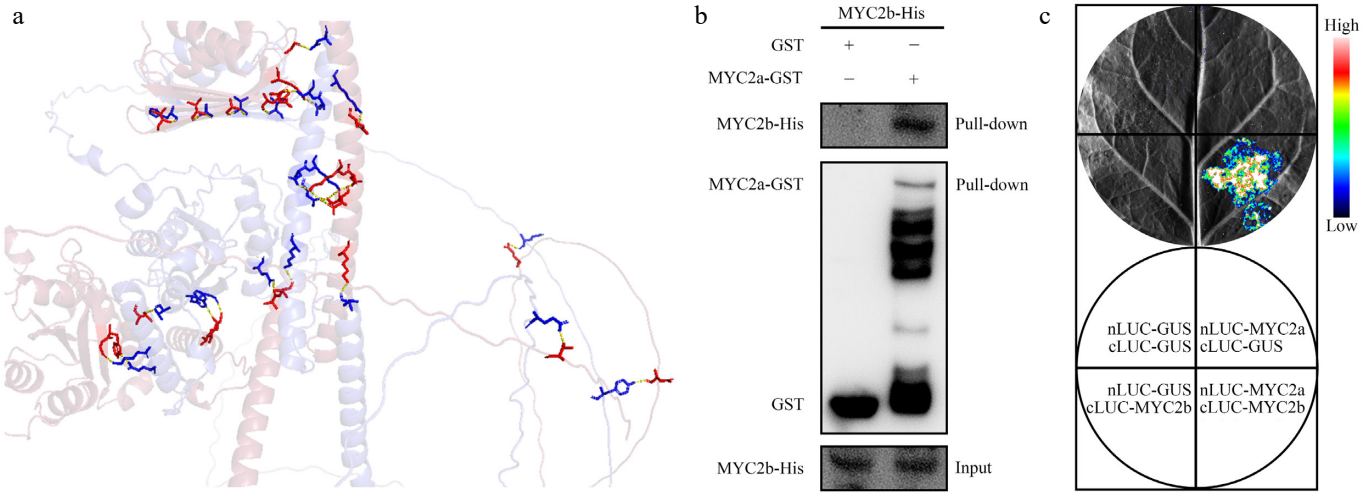

Fig. 6 Interaction between NtMYC2a and NtMYC2b. (a) The predicted cartoon diagram showing the interaction between NtMYC2a and NtMYC2b, as analysed via AlphaFold and PyMOL. The hydrogen bonds were shown as yellow dashed lines, the protein complex structures were shown as colored sticks, blue represents NtMYC2a, red represents NtMYC2b. (b) Pull-down assay confirming the interaction between NtMYC2a-GST and NtMYC2b-His. GST alone served as a negative control. (c) Split-luciferase complementation (split-LUC) assay confirming NtMYC2a-NtMYC2b interaction in *N. benthamiana*. A representative image of leaf taken 48 h after infiltration is shown, with luminescence intensity indicated by the pseudocolor bar.

NtMYC2a and NtMYC2b synergistically activate *NtABI5* expression

Since NtMYC2a and NtMYC2b function redundantly in ABA-mediated seed germination, and they can interact with each other to form a heterodimer, it can be speculated that they may function synergistically to activate *NtABI5* expression. To assess this possibility, a LUC reporter assay was performed to examine the effects of NtMYC2a on the transcriptional activity of NtMYC2b in *N. benthamiana* leaves. As shown in Fig. 7, co-expression of NtMYC2a with NtMYC2b increased LUC expression driven by the *NtABI5* promoter, compared with the expression of NtMYC2a or NtMYC2b alone. These results suggest that NtMYC2a and NtMYC2b may function synergistically to promote *NtABI5* expression during ABA-mediated seed germination.

Discussion

Seeds have evolved sophisticated gene-regulated networks to perceive favorable environmental conditions, such as moisture, temperature, and light quality, to determine timing of germination[3,5,6]. These cues then modulate the accumulation and perception of GA and ABA, leading to the promotion and repression of seed germination respectively[1]. Numerous studies have demonstrated that GA

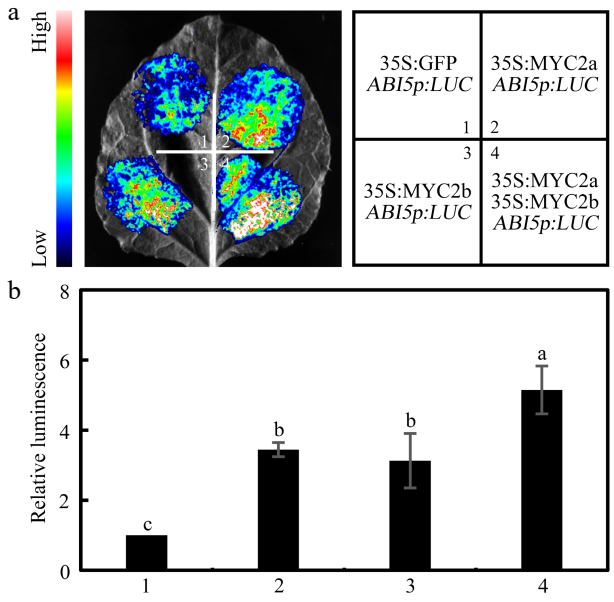

Fig. 7 NtMYC2a and NtMYC2b synergistically activate *NtABI5* expression. Transient expression analysis in *N. benthamiana* leaves to show that NtMYC2a and NtMYC2b can synergistically activate *NtABI5* expression. Both the (a) representative leaf image, and the (b) corresponding relative luminescence intensity, were presented. Three biological replicates data were analyzed by a one-way ANOVA using Tukey's HSD test. Bars with different letters are significantly different from each other ($p < 0.05$).

and ABA control seed germination in an antagonistic manner[30,31]. ABA, perceived by the PYR/RCAR–PP2C complex and transduced by ABA response transcription factors, accumulates during seed development and facilitates the seeds to enter a physiologically dormant state, which represents an adaptive strategy under unfavorable conditions. Identifying new components which function in ABA-mediated seed germination will help reveal the molecular basis of ABA signaling. In this study, we demonstrated that NtMYC2a/2b may function as critical components of ABA signaling during ABA-mediated seed germination in tobacco.

Numerous studies have demonstrated that MYC2 plays a critical role in diverse respects of plant growth and development, stress tolerance, and secondary metabolisms[23]. Despite its diverse roles, MYC2 mainly performs its biological functions by directly binding to the cis-acting G-box element (CACGTG), or G-box-like motifs (AACGTG or CATGTG) present in its target promoters[32–35]. In *Tanacetum cinerariifolium*, TcMYC2 binds to the E/G-box elements in the promoters of *TcCHS*, *TcAOC*, and *TcGLIP* and acts as a positive regulator of these pyrethrin biosynthesis genes upon methyl jasmonate (MeJA) treatment[36]. In *Arabidopsis*, AtMYC2 directly targets the jasmonate-induced *LIPOXYGENASE2* to modulate jasmonate-induced anthocyanin accumulation and root growth inhibition[37]. Here, we also revealed that NtMYC2a/2b can directly target *NtABI5* through the G-box in its promoter, and subsequently regulate ABA-mediated seed germination (Fig. 5). Based on the contrasting expression pattern of *NtMYC2a/2b* and *NtABI5* in *NtMYC2a/2b* mutants and overexpression plants, together with the enhanced LUC intensity in transient expression assays, we concluded that NtMYC2a/2b act as positive regulators of *NtABI5*. Thus, our results implied that NtMYC2a and NtMYC2b may act upstream of *NtABI5* to positively mediate the ABA-mediated germination process.

Protein-protein interaction represents an important mechanism for the functional performance of proteins. Upon receipt of various signals, plants usually amplify them through ordered interactions between proteins, thereby triggering various physiological responses[38]. Numerous studies have demonstrated that MYC2 can interact with various types of proteins which function as key components of various signaling pathways to mediate various plant developmental processes, and both biotic and abiotic stresses[23]. For example, NtMYC2a and NtMYC2b can interact with NtJAZ1 and modulate multiple jasmonate-inducible genes in the nicotine biosynthetic pathway[26]. *Arabidopsis* AtMYC2 physically associates with AtPHR1 to synergistically enhance Pi deficiency-activated jasmonate signaling[37]. Thus, it will be interesting to further determine whether certain proteins can interact and form complexes with MYC2 to modulate its DNA binding ability, or transcriptional activation, or repression ability. In this study, NtMYC2a and NtMYC2b was found to interact with each other to form a functional heterodimer and synergistically activate *NtABI5* expression, suggesting that they may function together during ABA-mediated seed germination. Identification of their interacting partners can contribute to the elucidation of NtMYC2-mediated ABA responses during seed germination.

In this study, an interesting finding is that NtMYC2a and NtMYC2b can interact with each other to form a hetreodimer and synergistically activate *NtABI5* expression during ABA-mediated seed germination. This finding may be contradictory to the inhibited expression patterns of *NtMYC2a* and *NtMYC2b* upon ABA treatment. Furthermore, ABA can also attenuate the interaction between NtMYC2a and NtMYC2b (Supplementary Fig. S3). These findings suggested that seeds may actively reduce the RNA and protein levels of NtMYC2a and NtMYC2b and also their interaction strength during seed germination, which may contribute to the avoidance of over accumulation of *NtABI5* transcripts under high ABA levels. It may represent a feedback regulation of ABA signaling on the *NtABI5* transcripts through the regulation of *NtMYC2a* and *NtMYC2b* expression and interaction. However, this hypothesis needs to be further determined in the future.

Seeds are irreplaceable genetic resources, and their rapid and synchronous germination and subsequent seedling establishment are the prerequisites of successful agricultural production and sustainable agriculture[39]. This study has revealed the molecular mechanisms underlying the regulation of ABA-mediated seed germination by NtMYC2a/2b in tobacco. Our results demonstrated that NtMYC2a/2b may act as critical components of the seed germination regulatory network and participates in the ABA-mediated seed germination process in tobacco. It will be interesting to further investigate how NtMYC2's transcription and translation is regulated during seed germination processes. These results are consistent with the role of AtMYC2 in ABA-mediated seed germination[40], implying that the function of MYC2 in different plant species may be conserved. Considering the role of NtMYC2a/2b in ABA-mediated seed germination, it will also be interesting to determine their possible involvement in plant abiotic stresses in the future. The molecular mechanisms revealed in this study contribute to the understanding of the regulatory mechanism of MYC2 in plants.

Author contributions

The authors confirm their contributions to the paper as follows: designed the research: Chen L, Yao H; performed experiments: Jing Y, Wang R, Zhao Z, Shi L, Wang B, Yang D, Pan W; analyzed data: Jing Y, Wang R, Chen L; wrote the paper: Chen L. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The original contributions presented in the study are included in the article or supplementary material, further inquiries can be directed to the corresponding author/s.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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