MYB3 plays an important role in lignin and anthocyanin biosynthesis under salt stress condition in Arabidopsis

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Abstract

Key message  Nuclear-localized Arabidopsis MYB3 functions as a transcriptional repressor for regulation of lignin and anthocyanin biosynthesis under high salt conditions.

Abstract  Salinity stress is a major factor which reduces plant growth and crop yield worldwide. To improve growth of crops in high salinity environments, plant responses to salinity stress must be tightly controlled. Here, to further understand the regulation of plant responses under high salinity conditions, the function of the MYB3 transcription factor was studied as a repressor to control accumulation of lignin and anthocyanin under salt stress conditions. Nuclear-localized MYB3 forms a homodimer. It is ubiquitously expressed, especially in vascular tissues, with expression highly induced by NaCl in tissues such as roots, leaves, stems, and flowers. \textit{myb3} mutant plants exhibited longer root growth in high NaCl conditions than wild-type plants. However, several NaCl responsive genes were not significantly altered in \textit{myb3} compared to wild-type.

Interestingly, high accumulation of lignin and anthocyanin occurred in \textit{myb3} under NaCl treatment, as well as increased expression of genes involved in lignin and anthocyanin biosynthesis, such as phenylalanine ammonia lyase 1 (\textit{PAL1}), cinnamate 4-hydroxylase (\textit{C4H}), catechol-O-methyltransferase (\textit{COMT}), 4-coumaric acid-CoA ligase (\textit{4CL3}), dihydroflavonol reductase (\textit{DFR}), and leucoanthocyanidin dioxygenase (\textit{LDOX}). According to yeast two-hybrid screenings, various transcription factors, including anthocyanin regulators Transparent Testa 8 (TT8) and Enhancer of Glabra 3 (EGL3), were isolated as MYB3 interacting proteins. MYB3 was characterized as a transcriptional repressor, with its repressor domain located in the C-terminus. Overall, these results suggest that nuclear-localized MYB3 functions as a transcriptional repressor to control lignin and anthocyanin accumulation under salinity stress conditions.

Keywords  MYB3 · Lignin · Anthocyanin · Transcriptional repressor · Phenylpropanoid biosynthesis

Introduction

Regulation of gene expression at the transcriptional level controls many crucial biological processes in eukaryotes (Spitz and Furlong 2012). An estimated 7\% of the plant genome encodes for transcription factors (TFs); these can

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be divided into six groups according to typical DNA-binding motifs (AP2/ERF, MYB, BZIP, WRKY, MYC and NAC) (Century et al. 2008; Hwang et al. 2014). The MYB proteins belong to a large family of transcription factors in eukaryotic organisms (Katayama et al. 2012). Depending on the numbers of adjacent imperfect repeats, MYB proteins in plants are classified into five subfamilies: R1-, R2-, R2R3-, R1R2R3-MYB (3R-MYB), and 4R-MYB, with the last group containing four R1/R2-like repeat motif structures with a length of approximately 50–55 amino acids (Ambawat et al. 2013). Each MYB domain forms three α-helices, with the second and third helices building a helix–turn–helix structure that intercalates in the major DNA groove when the MYB protein binds to DNA (Dubos et al. 2010). The R2R3-MYB proteins, which have two adjacent repeat motifs in the N-terminal (R2 and R3) and a highly variable C-terminal domain, are unique to higher plants and are the predominant family of MYB proteins in plants (Dubos et al. 2010; Ambawat et al. 2013). R2R3-MYB transcription factors play important roles in regulating plant development and growth, biotic and abiotic stress responses, and other physiological mechanisms in plants. Arabidopsis MYB72 plays an important role in roots during the early signaling steps of induced systemic resistance mediated by beneficial fungi and bacteria (Segarra et al. 2009; Van der Ent et al. 2008). R2R3-type MYB60 and MYB96 proteins work through the ABA signaling cascade to regulate stomatal movement (Cominelli et al. 2005). MYB96-mediated ABA signals enhance plant pathogen resistance by elevating salicylic acid biosynthesis (Seo and Park 2010).

Phenylpropanoid is a natural product derived from the amino acid l-phenylalanine through deamidation by a l-phenylalanine ammonia degrading enzyme (PAL) (Biala and Jasiński 2018). The phenylpropanoid pathway serves as a source of abundant metabolites required for the biosynthesis of lignin and anthocyanin in plants and serves as a starting point for the regulation of excessive lignin and anthocyanin biosynthesis in plant tissue, and anthocyanin is strongly accumulated in plants through this pathway (Gonzalez et al. 2008). Furthermore, proanthocyanidins (PAs) biosynthesis is dependent on the transcriptional activity of R2R3-type MYB123/TT2, MYB5, R/B-like bHLH (GL3 (GLABRA 3)/bHLH001, EGL3/bHLH002, and TT8/bHLH042) transcription factors, which form ternary complexes (MBW; MYB–bHLH–WD Repeat) with TTG1, a WD repeat-containing protein in the seed coat of Arabidopsis (Xu et al. 2014). Recently, MYB5 was proposed to be partially redundant with MYB123/TT2 in regulating the heat stress response in Arabidopsis (Jacob et al., 2021).

We previously reported that the MYB3 transcription factor interacts with the C-terminal of Arabidopsis CPL1 (C-terminal domain phosphatase-like 1) phosphatase under NaCl- and ABA-treated conditions (Bang et al. 2008). Here, MYB3 is further characterized as an important growth regulator under high salinity conditions. MYB3 is highly expressed by NaCl and forms a homodimer in the nucleus. NLS is located in the N-terminal of MYB3. According to yeast two-hybrid screening, several transcription factors involved in phenylpropanoid and flavonoid biosynthesis strongly interact with MYB3. Lignin and anthocyanin are significantly increased in myb3 compared to those of wild-type plants under NaCl treatment. All together, these results suggest that nuclear-localized MYB3 interacts with other transcription factors to function as a transcriptional repressor for the regulation of excessive lignin and anthocyanin accumulation under salinity stress conditions.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Col-0, myb3 (Gabi-Kat 815G05) and 2 kb MYB3pro::GUS transgenic plants were used in this study. Seeds were sterilized by soaking in 1% bleach solution for 10 min followed by washing five times with sterilized water. Surface-sterilized seeds were sown on 1/2 Murashige and Skoog (MS) agar medium including 1% (w/v) sucrose and 0.5% (w/v) phytagel as well as 0.05% (w/v) MES. pH was adjusted to approximately 5.7 using KOH. Seeds and plates were kept for 3 days at 4 °C in the dark, and then subsequently transferred to a growth chamber with 16 h of light illumination per day and 70% humidity at 22 °C.
Isolation of protoplasts and analysis of subcellular localization

Full-length, N- or C-terminal MYB3 CDS for the GFP-fused constructs were prepared with a PCR reaction and fused to the 5′ end of GFP in the pENSOTG GFP vector for transient expression. To isolate Arabidopsis protoplasts, the 4-week-old plants were incubated with 1 M mannitol for 30 min. 30 mL of 0.22 μm filter-sterilized enzyme solution was added, which contained 10 mM MES-KOH (pH 5.7), 0.4 M mannitol, 1 mM CaCl₂, 1% cellulase (Onozuka R-10), 0.25% macerozyme (R-10), 1% BSA (Goldbio) and 0.035% β-mercaptoethanol (Kim et al. 2016). The plates were then covered with parafilm and aluminum foil for dark conditions. After gentle agitation at 21 °C for 8–10 h, the enzyme solution containing protoplasts was gently filtered through 100 μm nylon mesh into a 50 mL tube. The protoplasts were gently covered with 10 mL W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM CaCl₂, and 5 mM KCl) without disturbing the 21% sugar content gradient, followed by centrifugation for 8 min at 100 × g. Approximately 10 mL of intact protoplasts floating on the sucrose solution added to 20 mL of W5 solution were carefully transferred to a new 50 mL tube. An aliquot of 15 mL W5 solution was added followed by centrifugation for 5 min at 60 × g. Subsequently, the protoplasts were washed with 15 mL of W5 solution and centrifuged again for 5 min at 60 × g. Pelleted protoplasts were resuspended in 5 mL MaMg solution containing 4 mM MES pH 5.7, 0.4 M mannitol and 15 mM MgCl₂. Plasmids encoding the proteins to be expressed were transfected into protoplasts using the plasmid-PEG-calcium transfection method. Briefly, 20 μg of each construct (1 μg/μL) was added to 300 μL of W5 solution and mixed gently. 320 μL of PEG solution was added and gently mixed with DNA-protoplasts by tapping. This mixture was incubated at 21 °C for 30 min. The transfection mixture was diluted and washed with 1 mL W5 solution 5 times at 5 min intervals, then centrifugated at 50 × g for 4 min at room temperature. The protoplasts were resuspended with 2 mL W5 solution and incubated for 24 h at least under dark conditions for further experiments. Transformed protoplasts were observed 2 days after transformation with Olympus AX-70 fluorescence microscope, and the images were captured with a cooled charge-coupled device camera (Olympus, Tokyo, Japan).

Transient expression assay

A pUC19-derived plasmid containing the GUS reporter gene under the control of a Gal4 and D1-3 minimal promoter was used as the reporter plasmid (Hwang et al. 2014). For the effector plasmids, full-length MYB3 CDS was inserted into a plant expression vector (pHBT95) containing the Gal4-BD domain. Effector and reporter plasmids were co-introduced into Arabidopsis protoplasts; GUS activity was analyzed as described previously (Hwang et al. 2014). Protoplasts were isolated from 3-week-old plants as described previously (Kim et al. 2016). In each transformation, 2 × 10⁶ protoplasts were transformed using PEG with 20 μg of plasmid DNA carrying a reporter plasmid plus empty effector vector or the reporter plasmid plus various effector plasmids. The transformed protoplasts were incubated in the dark at 21 °C for 16 h. A construct carrying the CaMV35S promoter fused to the luciferase (LUC) gene was used as an internal control in each transformation. To correct the variations in transformation efficiency, data normalization was performed by dividing the GUS activity of the cell lysate by the LUC activity.

RT- and quantitative RT-PCR (qRT-PCR) analysis

For RT- and quantitative RT-PCR (qRT-PCR) analysis, 2-week-old seedling plants were incubated in sterile one-half-strength liquid MS at room temperature overnight. Plants were immediately harvested after treatment with various abiotic stresses and phytohormones by flash-freezing in liquid nitrogen. For hormone treatment, 100 μM of each hormone [gibberellin (GA) (Sigma, USA), salicylic acid (SA) (Sigma, USA), 1-naphthaleneacetic acid (NAA) (Sigma, St Louis, MO, USA), 1-aminocyclopropane-1-carboxylic acid (ACC; Ethylene) (Sigma, USA) for ethylene] was added to a Murashige and Skoog liquid medium (Duchefa, Haarlem, Netherlands) in which 2-week-old plants were growing. Various salts were applied by adding 100 mM NaCl, 300 mM KCl, 300 mM LiCl and 200 mM Mannitol for 3 h under continuous light. Harvested samples were ground in a mortar and pestle with liquid nitrogen until a superfine powder. Total RNA was extracted using Trizol reagent (Invitrogen, 15596018) according to the manufacturer’s instructions. The RNA concentration was estimated using a NanoDrop spectrophotometer (ND2000) and RNA was subsequently treated with Turbo DNA-free DNase (Invitrogen, AM2238). Five micrograms of total RNAs were used for first-strand cDNA synthesis using reverse transcriptase (Promega, M-MLV Reverse Transcriptase, PRM1705). The real-time PCR was performed using the SYBR™ Green PCR Master mix (Applied Biosystems, Power SYBR® Green PCR Master Mix, 4368702) following the manufacturer’s instructions. RNA levels were normalized against the expression of the Actin (Actin2). The primer sequences used in RT- or qRT-PCR analysis are listed in Supplementary Table S1.

Histochemical GUS staining

Histochemical staining of GUS activity was performed as previously described (Kim et al. 2016). Briefly, whole seedlings or various tissues were immersed in histochemical
staining solution containing 50 mM KH₂PO₄ buffer (pH 7.2), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide and 0.2% Triton X-100 containing 1 mM X-GlcA (Duchefa, Netherlands). After incubation in a vacuum for 10 min, the seedlings were incubated at 37 °C for 6–12 h depending on staining status. Chlorophyll was cleared from the plant tissues by immersing them in 70% ethanol then washing with 70% EtOH repeatedly until tissue was clear. Stained tissues were observed and digital images were obtained using an Olympus Stereo-master microscope (Olympus SZX12 stereo scope).

**Yeast two-hybrid assay**

To isolate MYB3 interacting proteins, a yeast two-hybrid assay was performed according to yeast protocols Handbook (Clontech, USA). The cDNA fragment encoding MYB3 was amplified by PCR using the Arabidopsis cDNA library as a template and then ligated into the pBUTE plasmid, resulting in pBUTE-MYB3 as a bait. Subsequently, the pBUTE-MYB3 construct was transformed with combinations of pACT constructs or cDNA library (activation domain; AD) into the yeast strain PJ69-4a using the PEG method and was screened via auxotrophic selection on Leu/Ura/His drop-out media containing 5 mM of 3-amin o-1,2,4-triazole (3-AT) eliminating the leaky expression of the HIS3 reporter gene. To validate positive interactions, the transformed PJ69-4a yeast colonies containing each construct were analyzed by auxotrophic selection and confirmed by β-galactosidase assay.

**Quantification of lignin contents**

The thioglycolic acid (TGA) assay was carried out according to the previously reported protocol in order to determine lignin contents (Brinkmann et al. 2002). Plants were flash-frozen immediately harvested in liquid nitrogen. Harvested leaves were ground in a mortar and pestle with liquid nitrogen until a superfine powder. Approximately 50 mg of powder was extracted with 70% ethanol until undetectable at 280 nm absorbance. The residual substance was then extracted with 100% DMSO for 12 h at room temperature. This extraction step was repeated two more times until the samples were starch-free. The cell wall residues were washed six times with 70% ethanol to remove residual DMSO solution and finally washed two more times with 100% acetone. The cell wall residues were collected by centrifugation at 16,000 × g for 10 min and were air-dried. After 1 mL 100% methanol was added to the cell wall residues, the tube was capped, mixed to resuspend the pellet and incubated at 80 °C for 2 h. Insoluble materials were collected by centrifugation at 10,000 × g for 5 min. The pellet was resuspended in 1 mL distilled water by vortexing. The pellet was then mixed with 750 μL distilled water, 250 μL concentrated HCl and 100 μL thioglycolic acid. The tube was capped, mixed and incubated at 80 °C for 3 h. The insoluble materials were collected by centrifugation at 16,000 × g for 15 min and the supernatant was discarded. The pellet was resuspended in 1 mL distilled water and recollected by centrifugation at 16,000 × g for 15 min. The resulting pellet was resuspended in 1 mL 1 M NaOH. The capped tubes were gently agitated for 12 h at room temperature. Insoluble materials were collected by centrifugation at 10,000 × g for 5 min and the collected precipitates were removed. The supernatant was transferred to a fresh 2 mL tube and mixed with 200 μL concentrated HCl. The tubes were capped, shaken vigorously for 5 s, and incubated for 4 h at 4 °C. The resulting precipitate was collected by centrifugation at 10,000 × g for 5 min. The pellet was dissolved in 1 mL NaOH. After a 50-fold dilution with 1 M NaOH, the absorbance of the samples at 280 nm was recorded.

**Quantification of anthocyanin contents**

Anthocyanins were extracted from rosette leaves of 2-week-old plants treated NaCl for 5 days by immersing in 99% methanol plus 1% HCl at 4 °C for 16 h. Approximately 100 mg of powder was used for quantification of total anthocyanin contents. The relative level of anthocyanin was calculated from the absorbance at 530 and 637 nm, as described previously (Mancinelli 1984). All experiments were independently performed three times (biological repeats) per treatment.

**Statistical analysis**

All experiments were performed in triplicate. Relative transcript levels were normalized to ACT2. In above graphs, individual anthocyanin content or anthocyanin biosynthesis genes were quantified using ImageJ and Excel, and analyzed using the GraphPad Prism 8 software. Data shown as mean ± SEM of three (or more, as indicated) replicates, p value indicates significance, *p* < 0.05, **p* < 0.01, ***p* < 0.001, ****p* < 0.0001 and was determined by one-sided ANOVA with unpaired or two-tailed Student’s t test.

**Accession numbers**

Sequences of the genes in this paper may be found in the GenBank/EMBL database library under the following accession numbers: At1g22640 (MYB3), At4g34990 (MYB32), At1g35515 (MYB8), At3g18780 (ACT2), At5g52310 (RD29a), At5g25610 (RD22), At5g15960 (KN1), At5g15970 (KN2), At2g42540 (COR15), At2g37040 (PAL1), At3g53260 (PAL2), At2g30490 (C4H), At5g54160 (COMT), At1g65060 (4CL3), At4g26220

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(CCoAOMT), At5g13930 (CHS), At3g51240 (F3H), At3g51240 (F3′H), At4g22880 (LDOX), At5g42800 (DFR), At5g24520 (TTG1), At4g09820 (TT8), At1g63650 (EGL3), At3g27810 (MYB21), At5g46590 (ANAC096), At3g10580 (MYB-LIKE DNA-BINDING DOMAIN protein).

**Results**

**MYB3 forms a homodimer and the N-terminus of MYB3 is crucial for nuclear localization**

In previous reports, MYB3 is shown to serve as a transcriptional regulator and is localized to the nucleus (Bang et al. 2008; Zhou et al. 2017). In this study, we predicted the nuclear localization sequence (NLS) in the N-terminus of MYB3 using the SUBA (https://suba.live/) and NLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) programs (Supplementary Fig. S1). To demonstrate the NLS of MYB3, GFP-fused full-MYB3, N-terminal MYB3 (1–184 a.a) and C-terminal MYB3 (185–257 a.a) were transformed into Arabidopsis protoplasts. The GFP signal was observed 48 h after transformation. As expected, N-terminus deleted MYB3 did not mainly localize to the nucleus but instead remained in the cytoplasm (Fig. 1a). This indicates that the NLS sequence is located in the N-terminal of MYB3. In previous reports, the homo- or heterodimerization of transcription factors is essential for their function as a transcriptional regulator (Dubos et al. 2010). To determine MYB3 homodimerization, yeast two hybrid screening was performed. With this method, it was determined that MYB3 forms a homodimer. MYB32 and MYB8 were used as negative controls (Fig. 1b). These results clearly indicate that NLS is located in the N-terminus of MYB3, which forms a homodimer in the nucleus.

**MYB3 is expressed in vascular tissues and is highly induced by salt and various hormones**

To understand tissue-specific **MYB3** expression patterns, GUS staining and RT-PCR were performed. To confirm this, 2 kb of **MYB3** promoter fragments fused with the β-glucuronidase (GUS) reporter gene was prepared in order to examine the expression of **MYB3**. Interestingly, **MYB3** is highly expressed in the flowers but weakly expressed in various tissues such as roots, leaves and stems under normal growth conditions (Fig. 2a). This was confirmed by GUS staining in various tissues, especially in vascular compartments (Fig. 2b). It has previously been shown that the **MYB3** is highly induced by ABA and NaCl treatments (Fig. 2c, d). In addition, **MYB3** is not only induced by ABA, but also highly expressed by various phytohormones such as a GA, SA, NAA and ethylene (Fig. 2e). Taken together, these results indicate that nuclear-localized homodimer MYB3 is highly expressed under salt conditions and when treated with several plant growth regulators and stress-responsive phytohormones.

**MYB3 is involved in NaCl response**

To understand functional characterization of MYB3, T-DNA inserted **myb3** was obtained from the Gabi-Kat collection (https://www.gabi-kat.de/) (Fig. 3a). To confirm the **myb3** mutant, genotyping and RT-PCR were
performed. As shown in Fig. 3b, T-DNA was inserted homozygously in the 3rd exon of the MYB3 chromosome (Fig. 3a, b) This was confirmed by RT-PCR analysis. As a result, MYB3 was completely undetectable in this mutant, so myb3 is characterized as a knock-out mutant (Fig. 3c).

To determine the biological role of myb3 in high salt conditions, wild-type and myb3 mutant seeds were germinated at the same time and after 3 days, transferred to a 100 mM NaCl containing MS medium and then vertically grown for 5 more days to measure primary root growth. As shown in the results, primary root length was significantly increased in the myb3 mutant plants compared to those of wild-type plants under high NaCl treatment (Fig. 3d, e).

To identify the target genes of the MYB3 transcriptional regulator under NaCl stress conditions, transcriptional levels of various salt responsive genes, such as a RD29a, RD22, KIN1, KIN2 and COR15 were examined by RT-PCR analysis. RD29a and RD22 expression was slightly higher in the myb3 background under normal growth conditions; however, most of the examined stress-responsive genes did not show significantly different levels when compared to the WT and myb3 plants under NaCl treatments (Fig. 3f).

These results suggest that MYB3 may not function via an initial direct activation or repression of salt stress-responsive such genes as a transcriptional regulatory pathway.

**myb3 displays increased lignin content under NaCl treatments**

The above results show that MYB3 effects plant growth under NaCl treatment, but that it does not significantly regulate the expression of various salt responsive genes. Therefore, how could the myb3 mutant show longer root growth in the NaCl-treated environment? Interestingly, MYB3 was widely detected in the vascular tissue in various organs including roots, leaves, and flowers, and its expression was highly induced by NaCl. It has been reported that such gene expression patterns are importantly involved in the stress response through the biosynthesis of lignin or anthocyanin under various stresses, especially in response to high NaCl conditions (Eryılmaz 2006; Mahmood et al. 2016; Chen et al. 2020). This evidence suggests that MYB3 functions through either the synthesis of lignin or anthocyanin in vascular tissues in response to high salt stress. First, lignin contents were examined with a TGA assay in WT and myb3 plants to investigate the relationship between MYB3 and lignin biosynthesis under NaCl treatments. The total lignin contents significantly increased in myb3 up to twofold greater than the WT plants (Fig. 4a). These results indicate that MYB3 plays an important role as a negative regulator for lignin biosynthesis in Arabidopsis. Thus, transcripts of
genes related to lignin biosynthesis from both WT and myb3 plants were analyzed by RT-PCR. Transcript levels of PAL2, C4H, COMT and 4CL3 from myb3 plants were higher than those of WT plants under NaCl conditions (Fig. 4b). However, a significant difference in the transcript levels was not observed in CCoAOMT, CHS, F3H and F3’H compared to WT and myb3 under NaCl treatment (Fig. 4c). This indicates that MYB3 plays an important role as a negative regulator in lignin biosynthesis under NaCl stress conditions.

High accumulation of anthocyanins in myb3 under high NaCl condition

When plants are exposed to abiotic stresses, they may change anthocyanin content, owing to differential expression of genes related with anthocyanin biosynthesis. Recently, MYB3 was reported to be involved in the synthesis of anthocyanins under normal growth conditions (Zhou et al. 2017). In order to analyze anthocyanin contents under NaCl stress conditions, anthocyanin contents were quantified in WT and myb3. Under normal conditions, anthocyanins were slightly higher in myb3 plants as previously reported. Remarkably, anthocyanin content in myb3 was significantly higher than those in wild-type at 5 days under high salt conditions (Fig. 5a). To analyze the effect of MYB3 on the biosynthesis of anthocyanins, the transcripts of genes related with flavonoid biosynthesis, including LDOX and DFR, were examined under normal and NaCl conditions. Transcripts of LDOX and DFR were much higher increased under NaCl treatment in myb3 compared to wild-type (Fig. 5b, c). However, no significant differences were observed in the transcript levels of TTG1 between the WT and myb3 plants.
under normal growth or high salt conditions (Fig. 5d). These results indicate that MYB3 plays an important role as a negative regulator in both \textit{LDOX} and \textit{DFR} gene expression to control excessive anthocyanin accumulation in NaCl stress conditions.

\textbf{MYB3 forms hetero-oligomer with other transcription factors and functions as a transcriptional repressor}

Transcription factors either act as activators or repressors in gene expression, and either increase or decrease transcription through sequence-specific DNA-binding and protein–protein interactions. In previous work (Ambawat et al. 2013), it was described that MYB4 contains an LNL[E/D]L motif in the C-terminus which belongs to subgroup 4. This motif acts as a negative regulator of Cinnamate-4-hydroxylase (C4H) in the core phenylpropanoid pathway and serves an important function in its repression activity (Wang et al. 2020). Collectively, it can be proposed that MYB3 carrying the LNL[E/D]L motif plays a role as a transcriptional repressor. To determine whether MYB3, a R2R3-type MYB transcription factor, acts as an activator or a repressor, a transactivation assay was performed. Gal4-BD-MYB3 showed repression of GUS reporter gene expression when compared to the BD-Gal4 control treatment (Fig. 6a, b). To address whether the repressor domain is located in the C-terminus of MYB3, a deletion of MYB3 was constructed. As expected, transcriptional repression was abolished in the C-terminal deletion of MYB3 (Fig. 6c). To identify MYB3-interacting proteins, yeast two hybrid screening was performed using the Arabidopsis \textit{CD4-10} cDNA prey library. Five individual clones, TT8 (At4g09820), EGL3 (At1g63650), MYB21 (At3g27810),...
ANAC096 (At5g46590) and putative MYB transcription factor (At3g10580), were isolated as putative MYB3 interacting proteins and confirmed with an X-gal test (Fig. 6d, Table 1). These results indicate that MYB3 is a transcriptional repressor that forms a hetero-oligomer with other transcription factors in the nucleus.

**Discussion**

**MYB3 functions to inhibit excessive biosynthesis of lignin and anthocyanin due to high salinity conditions**

One common plant response to abiotic stressors like high salinity is the rapid synthesis of lignin and anthocyanin (Eryılmaz 2006). However, excessive synthesis of lignin and anthocyanin adversely affects plant growth (Chen et al. 2020; Naing and Kim 2021). Therefore, the synthesis of lignin and anthocyanin in such an unfavorable environment must be controlled very precisely. Recently, there has been a strong interest in research to elucidate the function of this negative transcriptional regulator. Recent studies have showed that several R2R3-type MYB transcription factors are important for the control of phenylpropanoid biosynthesis.
biosynthesis genes in plants (Cone et al. 1986; Preston et al. 2004; Dubos et al. 2010). For example, MYB4 and MYB32, which belong to subgroup 4 along with MYB3, regulate phenylpropanoid biosynthesis genes (Jin et al. 2000; Preston et al. 2004). It has been reported that AtMYB4 is a negative regulator of C4H, which is a gene that is involved in the upstream biosynthesis of phenylpropanoid, and that the LNL[E/D]L motif was crucial for MYB4 function (Jin et al. 2000). In our studies, lignin contents dramatically increased in the myb3 mutant background under high salinity conditions (Fig. 4a). Furthermore, the expression of lignin biosynthesis genes such as PAL2, C4H, COMT and 4CL3, were highly induced in the myb3 mutant under NaCl conditions (Fig. 4b). However, CCoAOMT, CHS, F3H and F3’H were not significantly altered in myb3 (Fig. 4c). This absence of induction of gene involved in later stages of lignin biosynthesis indicates that MYB3 is selectively involved in the regulation of early stages of lignin biosynthesis. In addition, anthocyanin accumulated strongly in myb3 (Fig. 5a). LDOX and DFR expression in the myb3 mutant was highly induced under NaCl treatment (Fig. 5b, c). LDOX encodes leucoanthocyanidin dioxygenase, which is involved in proanthocyanin biosynthesis. As the leucoanthocyanidin dioxygenase (LDOX) reaction produces cyanidin, the substrate for BAN, this places LDOX before BAN in the pathway (Abrahams et al. 2003). DFR encodes dihydroflavonol reductase, which catalyzes the conversion of dihydroquercetin to leucocyanidin in the biosynthesis of anthocyanins (Nesi et al. 2000). However, TTG1 was not significantly altered in the myb3 mutant under NaCl treatment. In previous reports and our studies, it is strongly suggested that MYB3 selectively functions to inhibit excessive biosynthesis of lignin and anthocyanin under high salinity conditions.

The C-terminus of MYB3 is important for its function as a repressor, and the N-terminus is essential for its nuclear localization

Members of MYB subgroup 4 share the presence of a conserved C2 repressor core LNL[E/D]L motif associated with the ethylene response factor-associated amphiphilic repression (EAR) motif (Kranz et al. 1998; Jin et al. 2000). Two Antirrhinum majus MYB factors, AmMYB308 and AmMYB330, have been reported as transcriptional repressors of lignification and phenolic acid metabolism. These MYB transcription repressors can suppress the gene expression of CAD (cinnamyl alcohol dehydrogenase), 4CL (4-coumarate: CoA ligase) and C4H (cinnamate-4-hydroxylase) in transgenic tobacco plants (Tamagnone et al. 1998). MYB4 regulates sinapate esters involved in UV protection through downregulation of C4H (cinnamate-4-hydroxylase) in Arabidopsis (Jin et al. 2000; Wang et al. 2020). The C-terminal motif, LNL[E/D]L, of AtMYB4, found to be conserved in the analysis presented here, is required for repression of transcription at target promoters (Wang et al. 2020). AtMYB3, which contains a LNL[E/D]L motif in the C-terminal region similar to MYB4 in subgroup 4, was confirmed as a transcriptional repressor by transient expression assay (Fig. 6a–c).

In previous reports, MYB3 was identified as a CPL1 phosphatase interacting partner and GFP-tagged MYB3 was shown to localize in the nucleus. To detect the NLS signal of MYB3 in more detail, we investigated the subcellular localization of MYB3 in protoplast cells using a construct with truncated R2R3 MYB domain regions in the N-terminus or C-terminus to function as a repressor. In recent reports, the N-terminal region of poplar MYB transcription factor 3 (PtrMYB3) contained a short sequence of nuclear localization signal (NLS). Here, the nuclear localization signal (NLS) in the N-terminal region of MYB3 was predicted using the NLS mapper program (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). As expected, NLS is located in the N-terminus of MYB3 (Fig. 1a). Therefore, we determined the MYB3 protein to be a nuclear-localized transcription repressor operated by a modular system in which the functions of N-terminus and C-terminus are distinguished.

**MYB functions by interacting with other TFs in the nucleus**

In previous reports, MYB and bHLH transcription factors have been shown to interact and form transcriptional complexes that regulate anthocyanin biosynthesis in plants (Holton and Cornish 1995; Schwinn et al. 2006). These complexes can be bound to the promoter of flavonoid genes directly. In previous studies, maize ZmC1MYB and ZmBbHLH bind to the promoter of DFR, a Flavonoid structural gene (Goff et al. 1992); petunia PhAN2 and PhJAF13 transcriptional regulators directly bind to the promoters of spinach DFR and ANS (Shimada et al. 2007). The WD-repeat protein and homeobox protein are also involved in anthocyanin biosynthesis; the petunia WD-repeat protein AN11 acts upstream of DFR, a Flavonoid structural gene (Goff et al. 1992); maize ZmC1MYB and ZmBbHLH bind to the promoter of DFR, a Flavonoid structural gene (Goff et al. 1992); petunia PhAN2 and PhJAF13 transcriptional regulators directly bind to the promoters of spinach DFR and ANS (Shimada et al. 2007). The WD-repeat protein and homeobox protein are also involved in anthocyanin biosynthesis; the petunia WD-repeat protein AN11 acts upstream of AN2 (de Vetten et al. 1997). In Arabidopsis, the WD-repeat protein TTG1 regulates the expression of DFR gene, while the homeobox protein ANL2 can control anthocyanin accumulation in the subepidermal layer of leaf tissues (Shirley et al. 1995; Sompornpailin et al. 2002). In our studies, MYB3 directly interacts with TT8 and EGL3, which function in the late steps of the flavonoid biosynthesis pathway. Interestingly, the expression of DFR and LDOX anthocyanin biosynthesis genes are increased in myb3 under high salinity conditions, which results in high anthocyanin accumulation levels. Therefore, it will be an important goal in future research to reveal that MYB3 directly represses...
Anthocyanin biosynthesis genes
Lignin biosynthesis genes

Fig. 7 Hypothetical model of MYB3. Arabidopsis nuclear-localized MYB3 forms a homodimer, and NLS is located in its N-terminus. Various analyses showed high expression of MYB3 transcripts and longer root growth in high NaCl conditions. Interestingly, high accumulation of lignin and anthocyanin was displayed in myb3, and expression of genes related to lignin and anthocyanin biosynthesis was higher in myb3 than that of the wild-type under NaCl treatment. According to protoplast analysis, MYB3 was characterized as a transcriptional repressor, and its C-terminus LNL[E/D]L motif was shown to be essential for repression. Overall, these results suggest that the nuclear-localized MYB3 transcriptional repressor interacts with TT8, EGL3, MYB and NAC transcription factors to regulate anthocyanin and lignin biosynthesis under high salt conditions (Fig. 7).

the promoters of DFR and LDOX to control the excessive anthocyanin accumulations under NaCl stress conditions in plants.

In summary, we demonstrated that Arabidopsis nuclear-localized MYB3 forms homodimers, and that NLS is located in its N-terminus. GUS and RT-PCR analysis shows high expression of MYB3 transcripts under NaCl treatment, as well as longer root growth in high salinity conditions. MYB3 may not function via an initial activation or repression of salt stress-responsive genes as a transcriptional regulator. Interestingly, high accumulation of lignin and anthocyanin content was displayed in myb3, and expression of genes related to lignin and anthocyanin biosynthesis, such as PAL1, C4H, COMT, 4CL3, DFR and LDOX, was much higher in myb3 than that of the wild-type under NaCl treatment. According to protoplast analysis, MYB3 was characterized as a transcriptional repressor and its C-terminus LNL[E/D]L motif was shown to be essential for repression. Overall, these results suggest that nuclear-localized MYB3 functions as a transcriptional repressor for the precise control of lignin and anthocyanin accumulation under salinity stress conditions (Fig. 7).

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Author contribution statement DK, SJJ and SK designed and mainly performed the experiments. The manuscript was written primarily by D. Kim and revised by SY, HSK and SK supervised the study and edited the manuscript. All authors discussed the results and commented on the manuscript.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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