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StMYB66, a R2R3-myb Transcription Factor, Regulates Plant Growth and Anthocyanin Accumulation in Potato (*Solanum tuberosum* L.)

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Abstract. **Potatoes are a vital noncereal staple crop that plays a critical role in global food security. Identifying key genes involved in potato growth and development is essential for improving yield and sustainability. In this study, we characterized *StMYB66,* a potato gene homologous to *AtMYB88* and *AtMYB124,* encoding an R2R3 MYB transcription factor. The full-length open reading frame of *StMYB66* is 1413 bp, encoding 470 amino acids. Transgenic lines overexpressing *StMYB66* were developments using polymerase chain reaction identification and selection medium cultivation. The highest expression of *StMYB66* was observed in sprouting tubers, with significantly increased expression following *Phytophthora infestans* infection and low nitrogen treatment. Elevated expression levels were also observed in red tubers compared with yellow ones. Overexpression of *StMYB66* resulted in shorter plant height, increased root biomass, and purple stem coloration, suggesting its role in anthocyanin accumulation. Comparative transcriptomic analysis between the receipt line (Atlantic) and the overexpression line (OE-T01) revealed that *StMYB66* primarily regulates genes involved in cell wall remodeling, influencing cellulose pectin, and lignin biosynthesis, thereby modulating potato growth. These findings demonstrate that *StMYB66* is a key regulator of potato growth, anthocyanin biosynthesis, and stress response, making it a promising candidate for potato breeding programs.**

Potato (*Solanum tuberosum* L.) is a significant noncereal crop, with annual growth and yield figures profoundly influencing global food security (Tang et al. 2022). Identifying genes that govern potato growth and mediate responses to abiotic and biotic stressors is imperative, as such genes can be used to improve existing potato varieties, enhance their resilience, and achieve higher yields. Transcription factors play a pivotal role in regulating gene expression, driving various developmental processes and environmental stress responses. Among these, the MYB transcription factor (TF) family is one of the largest in potatoes, comprising ~158 genes (Sun et al. 2019). These genes are divided into two subfamilies based on amino acid sequences and gene structures associated with the MYB domain, referred to as R2R3-MYB and R1R2R3-MYB. The R2R3-MYB family is further categorized into 20 subgroups (Sun et al. 2019; Yuan et al. 2021), all sharing a MYB domain that forms a helix-turn-helix motif (i.e., R1, R2, and R3) and that is characterized by a distinctive set of imperfectly repeated amino acids (Heine et al. 2004; Rosinski and Atchley 1998).

The *Arabidopsis* R2R3-MYB TF *FOUR LIPS* (*FLP*, *AtMYB124*) and its paralog *AtMYB88* regulate terminal divisions during stomatal development (Lai et al. 2005; Yang and Sack 1995), female reproductive processes (Makkena et al. 2012), various stress responses (Xie et al. 2010), and gravity stimulation in primary and lateral roots (Wang et al. 2015). It interacts with E2Fs transcription factors and functions in conjunction with RETINOBLASTOMA-RELATED (RBR) protein to negatively regulate cell cycle progression (Boudolf et al. 2004; Lee et al. 2013). Moreover, *AtMYB88* and *AtMYB124* are recognized as pivotal for their critical roles in regulating plant growth and conferring abiotic stress tolerance. Given these roles, there is significant value in identifying homologous genes in potato and elucidating their potential functions.

The MYB family is known to play an important role in potato growth, development, and abiotic stress response (Chacón-Cerdas et al. 2020; Li et al. 2019; Lin et al. 2021; Liu et al. 2020; Sun et al. 2019). Several potato MYB genes are known to regulate anthocyanin synthesis and participate in abiotic stress responses. For example, *StAN1* plays a key role in regulating anthocyanin levels, which in turn enhances the expression of anthocyanin biosynthetic pathway genes and causes the accumulation of anthocyanins. Recent studies of variants showing differences in the C terminus of *StAN1* have emphasized the importance of this region for the gene's function in activating

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anthocyanin accumulation. Moreover, two other R2R3 MYB TFs, *StMYBA1*, which is highly homologous to the protein encoded by *StAN1*, and *StMYB113*, are also known to activate anthocyanin accumulation in tobacco leaves (Liu et al. 2016). In addition, light signals may induce *StMYBA1* to positively regulate anthocyanin biosynthesis in potato (Liu et al. 2017). Another study found that low-temperature treatments induced the expression of *StMYB113* and *StMYB308*, both of which promoted anthocyanin accumulation (Chen et al. 2024). Two other R2R3-MYB TFs, *StMYB12A* and *StMYB12C*, both show high levels of expression in flowers but low levels in tubers. Recent work using overexpression lines reported that OE lines accumulated flavonols and other phenylpropanoids, indicating that these genes are involved in flavonol and other phenylpropanoid accumulation (Chen et al. 2022). Finally, we also note that the MYB gene *MYB-RELATED PROTEIN Hv1* (*MYB-Hv1*) is also involved in the regulation of flavonoid biosynthesis.

Despite limited research, StMYBs may also play diverse roles in potato growth and adaptive responses to environmental conditions. For instance, recent studies have shown that StMYBs are involved in potato tuber development; upon treatment of tubers with gibberellic acid and abscisic acid, the expression of several StMYBs was significantly upregulated (Zaki and Radwan 2022). Another study reported that in seven potato lines, StMYB expression increased in response to drought stress, suggesting a potential role in drought tolerance (Lin et al. 2021). Additional investigations have explored the involvement of StMYBs in growth regulation and responses to environmental stress. However, there is limited research focusing on characterization of specific MYB transcription factors in potato, and there are even fewer studies performing functional analyses using transgenic lines.

In this study, we employed bioinformatic methods to investigate *StMYB66*, a member of the R2R3 MYB TF family in potato, homologous to *AtMYB88* and *AtMYB124*. We first analyzed the basic characteristics of *StMYB66*, including protein sequences, physicochemical properties, homologous genes in model plants, and motifs in its promoter region. Subsequently, we examined its expression patterns across diverse tissues and under varying conditions. Finally, to elucidate its function, we generated transgenic lines overexpressing *StMYB66*, conducted phenotypic surveys, and performed comparative transcriptomic analyses to uncover its role in potato growth and regulatory mechanism. Overall, this study aimed to clarify the role of *StMYB66* in potato growth and assess its potential for future genetic engineering of new potato varieties.

Materials and Methods

Potato material and growth conditions. The tetraploid potato variety Atlantic, known for its attractive appearance and broad adaptability, is also well suited for transgenic applications. Atlantic obtained from the International Potato Center-China Asia–Pacific Center was used in all experiments, including transgenesis. All plants, including transgenic lines, were grown in plant incubators under the following conditions: a light period of 16 h at approximately 16,000 lx, followed by 8 h of darkness. The temperature during the light phase was maintained at 21 and 20 °C during the dark phase.

Cloning of the full-length *StMYB66* gene and construction of the pBWAHS-StMYB66 vector. The full-length coding sequence of *StMYB66* was amplified from the leaf cDNA of the Atlantic potato variety using NEB Q5 high-fidelity DNA polymerase in a 50-μL polymerase chain reaction (PCR). The reaction mixtures comprised 2 μL of cDNA template, 5 μL of 5× Q5 buffer, 1 μL of 2.5 mM dNTP, 1.5 μL each of forward (ATGATTGATTGGTTGGTTATT) and reverse (GGCACTC CTCCAAAGCCTATGA) primers, and 0.25 μL of Q5 high-fidelity DNA polymerase. The PCR program was as follows: initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 5 s, annealing at 55 °C for 10 s, and extension over 35 cycles of 72 °C for 30 s. This was followed by a final extension at 72 °C for 10 min. PCR products were separated via 1% agarose gel electrophoresis and purified using an Monarch PCR and DNA cleanup kit (New England Biolabs, Ipswich, MA, USA). Adapter primers were added to both ends of *StMYB66* using the same PCR procedure. The *StMYB66* fragment and the pBWA(V)HS vector, linearized with BsaI, were assembled using the NEBbuilder assembly kit (E2621; New England Biolabs) following the manufacturer's instructions. The resulting pBWAHS-StMYB66 construct was confirmed via Sanger sequencing (Tsingke Technology, Beijing, China).

Bioinformatic analysis of StMYB66. The isoelectric point and molecular weight of the StMYB66 protein were predicted using the ExPASy Protparam toolset (https://web.expasy.org/protparam/). Protein domains were analyzed using the SMART tool (http://smart.embl-heidelberg.de/), and structural modeling of each domain was done using SWISS-Level structure MODEL (https://swissmodel.expasy.org/). Homologous protein sequences were identified through BLAST-p searches of the National Center for Biotechnology Information (NCBI) database. Conserved motifs among R2R3 MYB TFs were detected using the MEME Suite (http://meme-suite.org/). Protein sequences for alignment were downloaded from the NCBI, including StMYB66 and two *Arabidopsis thaliana* proteins, two *Malus domestica* proteins, and one *Fragaria vesca* protein. Multiple sequence alignment were conducted using DNAMAN, version 5.2.

Phylogenetic analysis of StMYB66. StMYB66 protein sequences were aligned with those from *A. thaliana*, *M. domestica*, *Arachis hypogaea*, *F. vesca*, *Solanum lycopersicum*, *Glycine max*, *Saccharum spontaneum*, and *Cichorium intybus* using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were constructed using the neighbor-joining algorithm in MEGA with bootstrap values based on 1000 replicates to assess tree topology and consistency (Liu et al. 2023).

Analysis of motifs present in *StMYB66* promoters. The *StMYB66* promoter region was identified by aligning cDNA sequences to the potato reference genome (DMs 1–3 516 R44) using BLAST-N (NCBI). Promoter sequences, extended upstream by 2 kb, were analyzed for motifs using JASPAR CORE tool (https://jaspar.elixir.no) with an 80% relative profile score threshold.

Generation of StMYB66 overexpression lines. Frozen (−80 °C) *Agrobacterium tumefaciens* harboring pBWA(V)HS-StMYB66 plant expression binary vector (V) was thawed and cultured in YEP lipid medium at 28 °C with shaking at 150 rpm. The cells were centrifuged, resuspended in infection medium (4.43 g/L MS basal medium with vitamins, 20 g/L sucrose, pH

5.5, 100 mg/L acetosyringone), and adjusted to an optical density at 550 nm (OD_{550}) wavelength of ~0.5. Potato explants were immersed in the bacterial suspension for 20 min with gentle shaking and transferred to COA medium (4.43 g/L MS basal medium with vitamins, 20 g/L sucrose, 80 mg/L acetosyringone, 1 mg/L zeatin, 2 mg/L 1-Naphthaleneacetic acid (NAA), 8 g/L agar, pH 5.8). After 2 d of cocultivation in the dark at 21 °C, the explants were transferred to selection medium containing 50 mg/L kanamycin. Regenerated shoots (≥5 cm) were transferred to MS30 medium for rooting.

Reverse transcription–quantitative PCR analysis of *StMYB66* expression in transgenic lines. Total RNA was extracted from transgenic and wild-type potato leaves using the Yisheng MolPure plant RNA kit. cDNA was synthesized from 2 μg of RNA using NovoScript Plus All-in-one SuperMix. Relative *StMYB66* expression levels were quantified via reverse transcription–quantitative PCR using EF1α as the reference gene. The comparative Ct method ($2-\Delta\Delta Ct$) was employed to calculate fold changes. PCRs (20 μL) consisted of 10 μL of SYBR quantitative PCR Master Mix, 0.4 μL of up/downstream primers (10 μM), 1 μL of RNA, and 7.8 μL of RNase-free water. The reactions were run in triplicate. The primers sequence are shown in Supplemental Table 1.

Phenotypic analysis of transgenic lines. Phenotypic traits of the Atlantic cultivar and two overexpression lines (T01 and T16) were assessed after in vitro subculturing in MS30 medium under controlled growth conditions (21 °C/20 °C, 16 h of light/8 h of dark. After 4 weeks, shoot and root lengths were measured with a ruler, and weight was recorded using a balance. Stem color was evaluated after 2 months of subculturing.

Leaf samples collection, RNA sequence and gene functional enrichment analysis. The healthy leaves on the top of the canopy were selected; three leaves per plant of eight plants for Atlantic and OE-T01, respectively, were selected for transcriptome research. There are four replicate samples per line. Leaf samples were stored in RNA*later* (Thermo Fisher Scientific Inc., Waltham, MA, USA) immediately after collection. Leaf total RNA was extracted by TRIzol solution following the manufacturer's protocol.

RNA sequencing was completed by Majorbio biotechnology company (Shanghai, China) with the Illumina sequencing platform. Paired-end sequencing with each read length 150 bp was used. Clean reads (filtered out reads with an adapter, N ratio greater than 10%, all A-base, mass value $Q \leq 30$, and bases accounting more than 50% of the entire read) and sequence data quality were obtained and maintained by the fastp tool. In the GenBank DNA sequence database of NCBI, the ribosome database was generated by obtaining the sequence with rRNA keywords in the annotation by searching for the genus name of potato. These clean data were aligned to the potato reference genome (DM8.1) by HISAT2 (Yang et al. 2023), in which transcript was reconstructed by Stringtie, calculated expression levels of all genes in each sample. DESeq2 software (version 1.20.0), ran with R (version 3.6.0) in Rstudio was used to analyze the different expression genes. Normalized read counts, calculated *P* value according to model, obtained the false discovery rate value by multiple-hypotheses testing corrections. The gene expression levels were calculated using fragments per kilobase of exon per million fragments mapped. Genes with a P value $<$ 0.05 and $|\log2\ FC| > 1$ were screened as differentially expressed genes (DEGs). DEGs were mapped to the Gene Ontology (GO) database (version 3.8.2, http://www.geneontology.org/). The list of genes in each term and the specific GO functions with the number of genes were obtained.

Statistical analyses. One-way analysis of variance was used to determine significant differences between genotypes while Student's t tests ($^*P \leq 0.05$) were used for pairwise comparisons. Statistical analyses were performed using SPSS (version 17.0) software.

Results

Phylogenic identification of AtMYB88 and AtMYB124 homologs in potato. To identify potato homologs, we first aligned StMYB66 with five other proteins, including AtMYB88 and AtMYB124 from *A. thaliana*, MdMYB88 and MdMYB124 from *M. domestica* Borkh, and FvMYB82 from *F. vesca*. We found that these proteins are highly conserved in their predicted R2 and R3 MYB DNA-binding domains (Fig. 1A). Notably, conserved amino acids were identified near the C-terminal region, marked in dark blue, confirming their association with the R2 and R3 MYB DNA-binding domains. Additionally, residues conserved between StMYB66 and AtMYB88, AtMYB124, MdMYB88, and MdMYB124 proteins, shown in pink, were greater in number compared with those conserved with FvMYB82. This difference was especially pronounced near the N-terminal region, underscoring variation across the proteins. These findings indicate that StMYB66 shares close conservation with AtMYB88, AtMYB124, MdMYB88, and MdMYB124.

To explore evolutionary relationships, we performed a phylogenetic analysis based on the MYB TF domain sequences. A phylogenetic tree was constructed using 12 full-length MYB TF protein sequences (Fig. 2), including two from *A. thaliana* (AtMYB88 and AtMYB124), three from *M. domestica* Borkh (MdMYB88, MdMYB23, and MdMYB124), and one each from *A. hypogaea* L. (AhMYB30), *F. vesca* (FvMYB82), *S. lycopersicum* L. (SiMYB15), *G. max* L. (GmMYBJ1), *S. spontaneum* (SsMYB18), *C. intybus* (CiMYB5), and StMYB66. The phylogenic and domain analyses suggest that StMYB66 is closely related to AtMYB88 and/or AtMYB124 of *A. thaliana*, supporting the hypothesis that StMYB66 is homologous to these genes. In *M. domestica* Borkh, the closest homologs to *StMYB66* were *MdMYB88* and *MdMYB124*, but not *MdMYB23*. Similarly, in *S. lycopersicum* L., homologous genes included *SiMYB15* and *MdMYB23*. Other homologous genes, such as *GmMYBJ1, SsMYB18,* and *CiMYB5* formed a separate cluster (Fig. 1B). These results collectively demonstrate that StMYB66 exhibits strong conservation with two homologs from *A. thaliana* (AtMYB88 and AtMYB124) and two from *M. domestica* (MdMYB88 and MdMYB124) while diverging significantly from homologs in other plant species such as *G. max* L., *S. spontaneum*, and *C. intybus*.

Cloning and bioinformatic analyses of *StMYB66*. The full-length *StMYB66* gene (*Soltu.DM.04G004510*) was successfully cloned from *S. tuberosum* var. Atlantic. The gene spans 1413 bp and encoded 470 amino acids (Fig. 2A). The theoretical molecular weight of the *StMYB66* protein is 53.3 kDa, with an isoelectric point of 6.71 mA (Fig. 2B). Analysis of the amino acid composition revealed that Ser (9.4%), Leu (8.9%), Lys (7.4%), and Thr (7.2%) were the predominant residues. The total number of negatively charged residues (Asp + Glu) was 59,

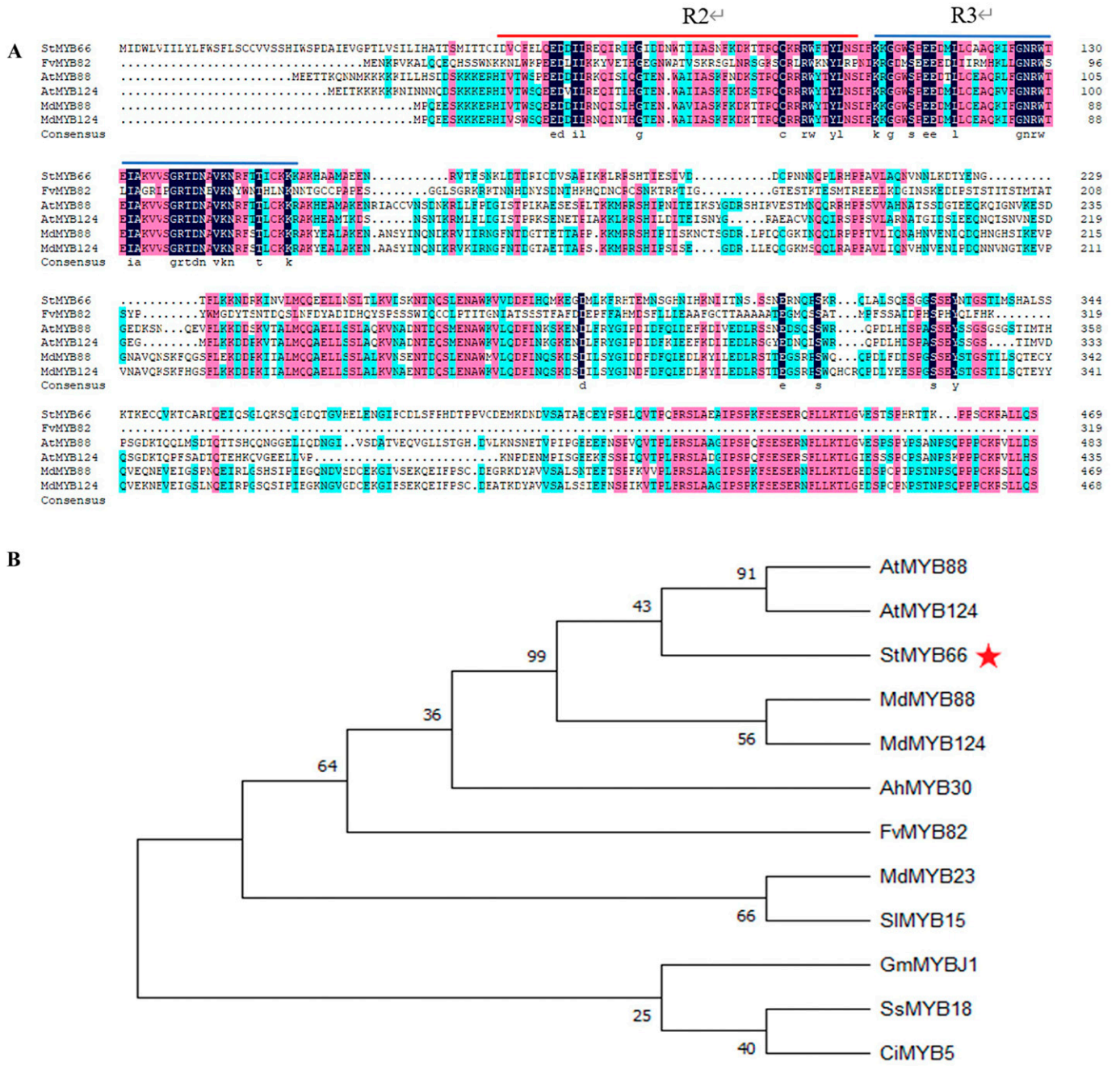
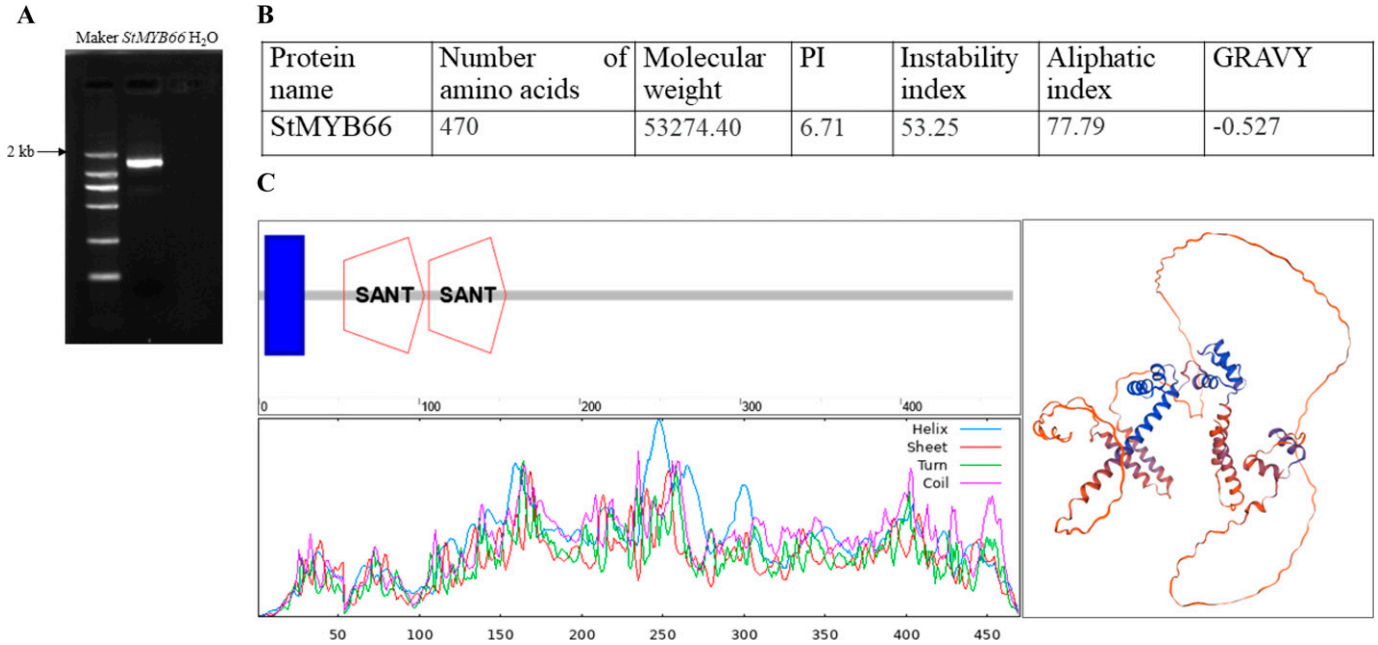


Fig. 1. Homologous genes of *StMYB66* in model plants. (**A**) Alignment of MYB domains from homologous genes. *StMYB88*, *AtMYB88*, and *AtMYB124* from *A. thaliana*, MdMYB88 and MdMYB124 from *M. domestica*, and FvMYB82 from *F. vesca* were aligned. R2 and R3 MYB DNA-binding domains are marked by pink and light blue lines, respectively. Conserved residues are shaded based on their conservation level: dark blue (all proteins), pink (five proteins), and light blue (four proteins). (**B**) Phylogenic tree of *StMYB66* and its homologous genes. The tree was constructed using MYB transcription factor domains from 12 proteins, including those from *A. thaliana* (*AtMYB88* and *AtMYB124*), *M. domestica* (*MdMYB88*, *MdMYB23*, and *MdMYB124*), *A. hypogaea* (*AhMYB30*), *F. vesca* (*FvMYB82*), *S. lycopersicum* (*SlMYB15*), *G. max* (*GmMYBJ1*), *S. spontaneum* (*SsMYB18*), and *C. intybus* (*CiMYB5*). *StMYB66* (highlighted with a star) clusters closely with *AtMYB88* and *AtMYB124* in *A. thaliana* and *MdMYB88* and *MdMYB124* in *M. domestica*.

while the positively charged residues (Arg + Lys) totaled 57. The aliphatic index of StMYB66 was calculated to be 77.79, and its instability index was 53.25, suggesting that the protein is moderately unstable. The Kyte–Doolittle hydropathy plot and overall average hydropathicity value of −0.527 indicates that StMYB66 is predominantly hydrophilic (Fig. 2B).

Using the SWISS-MODEL tool, we determined that the StMYB66 protein contains two conserved SANT DNA-binding domains, located at amino acid positions 53–103 and 106–154, respectively (Fig. 2C). The secondary structure, predicting using SOPMA, revealed that the composition of StMYB66 protein as follows: 42.34% α-helices, 4.89% β-turns, 11.49% extended strands, and 41.28% random coils (Fig. 2C). The tertiary structure prediction, also performed with SWISS-MODEL, identified a helix-turn-helix region, aligning with the predicted secondary structure features of StMYB66 (Fig. 2C). These



Protein name	Number of amino acids	Molecular weight	PI	Instability index	Aliphatic index	GRAVY
StMYB66	470	53274.40	6.71	53.25	77.79	-0.527

Fig. 2. Cloning and bioinformatic analysis of *StMYB66*. (**A**) *StMYB66*, with a full-length gene of 1413 bp encoding a 470-amino acid protein, has a theoretical molecular weight of 53.3 kDa and an isoelectric point (PI) of 6.71. (**B**) Functional domains and protein structure. StMYB66 contains two SANT conserved DNA-binding domains. Its secondary structure comprises 42.34% α-helices, 4.89% β-turns, 11.49% extended strands, and 41.28% random coils, with a helix-turn-helix tertiary structure.

findings provide comprehensive structural insights into the StMYB66 protein and highlight its potential role as a transcription factor with DNA-binding capabilities.

MADS-box factor binding motifs predominate in the *StMYB66* promoter. Analysis of the *StMYB66* promoter revealed the presence of three distinct types of MADS (MCM1, AGAMOUS, DEFICIENS, Serum Response Factor)-box factor binding motifs: MA0001.1, MA0001.2, and MA0001.3. These motifs correspond to the MIKC, MIKC, and type II families, respectively, and are characterized by unique sequence features. The occurrences of these motifs within the *StMYB66* promoter were 28, 17, and 27 sites, respectively (Table 1). This indicates that MADS-box motifs are the most abundant regulatory elements within the promoter region. The core sequence of the MADS-box motif, ccaaaattag, is rich in AT bases, which are known to be preferential bind site for MADS-box transcription factors (Fujisawa et al. 2011). The high AT content may facilitate specific interactions with these TFs. MADS-box transcription factors are crucial regulators of developmental processes, particularly in flower and fruit development. It is plausible that these factors bind to the identified motifs to modulate the expression of *StMYB66* in a tissue-specific or developmental stage–specific manner. This suggests that the regulation of *StMYB66* may be closely tied to key growth and reproductive processes in *S. tuberosum*.

Expression patterns of *StMYB66*. Gene expression data for *StMYB66* were obtained from the Spud DB database (http://spuddb.uga.edu/) and analyzed comprehensively. The results revealed that *StMYB66* expression was highest in sprouting tubers and in whole plant grown in vitro. During tuber development, from young to mature stages, *StMYB66* expression declined. Similar expression levels were observed across various tissues, including stamens, flowers, leaves, stems, stolons, petioles, and roots. When plants were subjected to water stress treatments, *StMYB66* expression increased in leaves (Fig. 3A). Following infection with *Phytophthora infestans*, *StMYB66* expression in resistant potato lines exhibited a delayed but significant increase. No expression was observed at early time points, but by 2 d postinfection, expression reached a level of 0.099. In susceptible lines, *StMYB66* expression increased more dramatically, showing a 4-fold rise following infection (Fig. 3B). Low nitrogen treatments significantly enhanced *StMYB66* expression across three potato varieties, demonstrating a strong response to nitrogen supplementation (Fig. 3C). However, long-term drought stress did not significantly alter *StMYB66* expression in potato varieties (Fig. 3D). These findings suggest that *StMYB66* expression is responsive to environment stresses, with specific patterns dependent on the type of stress. The gene appears to play a dynamic role in responding to water stress, pathogen infection, and nitrogen availability while remaining unaffected by prolonged drought conditions.

***StMYB66* is involved in the growth regulation of potato shoots and roots.** To investigate the role of *StMYB66* in potato development, the vector pBWA(V)HS-StMYB66 was constructed, incorporating *StMYB66* downstream of a 35S promoter (Fig. 4A). Successful insertion of the target gene was confirmed through Sanger sequencing. After selection on kanamycin sulfate, genetically transformed potato seedlings were screen by PCR, identifying 11 transgenic lines as positive based on the amplification of the target gene (Fig. 4B). Further confirmation of these lines was achieved by subculturing in a selection medium supplemented with 100 mg/L kanamycin. Positive transgenic lines demonstrated robust root systems after 2 weeks, while negative lines exhibited a dwarf phenotype with significantly fewer roots (Fig. 4C). Quantitative real-time PCR was used to assess *StMYB66*

Table 1. Motif information in the promoter of *StMYB66*.

Matrix ID	Name	Family	Class	Sites	Sequence logo
MA0001.1	AGL3	MIKC	MADS box factors	28	
MA0001.2	AGL3	MIKC	MADS box factors	17	
MA0001.3	AGL3	Type II	MADS box factors	27	
MA0002.1	RUNX1	Runt-related factors	Runt domain factors	8	
MA0002.2	Runx1	Runt-related factors	Runt domain factors	7	
MA0002.3	Runx1	Runt-related factors	Runt domain factors	11	
MA0003.1	TFAP2A	AP-2	Basic helix-span-helix factors (bHSH)	3	
MA0003.3	TFAP2A	AP-2	Basic helix-span-helix factors (bHSH)	4	

expression levels in the transgenic lines, using EF1α as an internal reference. Results indicated considerable variability among the lines. Notably, line T01 exhibited the highest *StMYB66* expression, ∼1000-fold higher than the wild-type control (Atlantic). Line T16 also displayed high pression level, with an increase of about 400-fold. Other lines (e.g., T02, T04, T05, T08, T13, T17, T18, T20, and T25) exhibited relatively lower expression levels comparable to the control Atlantic line (Fig. 5A).

Phenotypic analysis revealed that *StMYB66* overexpression significantly affected plant growth. Lines OE-T01 and OE-T16 produced shorter plants with reduced height and increased root weight compared with the control after 4 weeks of growth (Fig. 5A). Further quantitative measurements showed that overexpression lines had significantly shorter plant height and heavier root fresh weight, while shoot fresh weight and root length remained similar to those of the control (Fig. 5B). These findings indicate that *StMYB66* plays a role in regulating potato shoot and root growth. Its overexpression leads to reduced shoot height and enhanced root biomass, suggesting its involvement in developmental and physiology pathways crucial for root and shoot development in potato plants.

***StMYB66* is involved in the regulation of anthocyanin synthesis.** Previous research has demonstrated a strong correlation between *StMYB66* expression and anthocyamin accumulation. For instance, *StMYB66* expressed levels were significantly higher in fresh red tubers compared with yellow tubers during all developmental stages, from tuber initiation (S1) to maturation (S3) (Fig. 6A) (Liu et al. 2023). Similarly, another study reported that *StMYB66* expression was ∼2.5-fold higher in purple flesh than in white flesh ones (Fig. 6B) (Riveros-Loaiza et al. 2022). These observations suggest that *StMYB66* may play a role in anthocyanin homeostasis. In this study, we observed that overexpression line OE-T01 exhibited a noticeable phenotypic change: the stems appeared purple after 2 months of subculturing, whereas the stems of the control remained green (Fig. 6C). This phenotypic variation suggests that *StMYB66* influences anthocyanin biosynthesis in stems, potentially through pathways similar to those operating in tubers. These results collectively indicate that *StMYB66* may regulate anthocyanin synthesis, particularly in tissues such as tubers and stems, and its activity likely contributes to pigmentation differences observed in potato cultivars.

Transcriptome analysis between Atlantic and overexpression line OE-T01. To uncover the dynamic regulatory pathways influenced by overexpression of *StMYB66* in potato growth, we conducted RNA-sequencing analysis of leaves from Atlantic and OE-T01 line. The RNA-sequencing data were processed and aligned to the potato reference genome (DM8.1) (Yang et al. 2023) with match rates exceeding 94% on average (Q30; Supplemental Table 2). The mapped reads were used for subsequent analyses, with principal component analysis clearly separating the two genotypes into distinct cluster (Supplemental Fig. 1). This result indicates that the differential gene expression patterns observed were induced by *StMYB66* overexpression. The genes with their expression level in different samples were shown in Supplemental Table 3. A comparison between Atlantic and OE-T01 identified a total of 1564 DEGs, with 664 genes especially expressed in OE-T01 and 900 Atlantic (Fig. 7A). Among these, 281 genes were upregulated and 287 were downregulated in OE-T01 (Fig. 7B, Supplemental Table 4).

GO enrichment analysis revealed that most DEGs were associated with molecular function processes, such as oxidoreductase activity (GO:0016705), xyloglucan:xyloglucosyl transferase activity (GO:0016762), catalytic activity (GO:0003824), vitamin binding (GO:0019842), organic acid binding (GO:0043177), carboxylic acid binding (GO:0031406), glycosyltransferase activity (GO:0016757), xyloglucan metabolic process (GO:0010411), L-ascorbic acid binding (GO:0031418), glucosyltransferase activity (GO:0046527), and cell wall–related processes (Fig. 7C, Supplemental Table 5).

The gene *DM8C01G44330* encoded a UDP-glycosyltransferase family with elevated expression in OE-T01 (Fig. 7D). *DM8C02G16010* and *DM8C02G33650*, members of glycosyltransferase family 2, play central role in polysaccharide synthesis, a key component of plant cell wall. Notably, the homolog of *DM8C02G16010* in *Arabidopsis* (*AT4G18780*) is involved in cell wall development by influencing cellulose crystallization (Glass et al. 2015). Similarly, *DM8C02G20070*, encoding pectinesterase, modified the cell wall via pectin demethylesterification. Its homolog, *AT1G11890*, regulated cell morphogenesis in *Arabidopsis* (Guan et al. 2021). *DM8C03G02430* encoded a class III peroxidase, a cell wall–localized protein that modulates wall loosening and stiffening by producing reactive oxygen species and oxidizing aromatic compounds linked to polysaccharides.

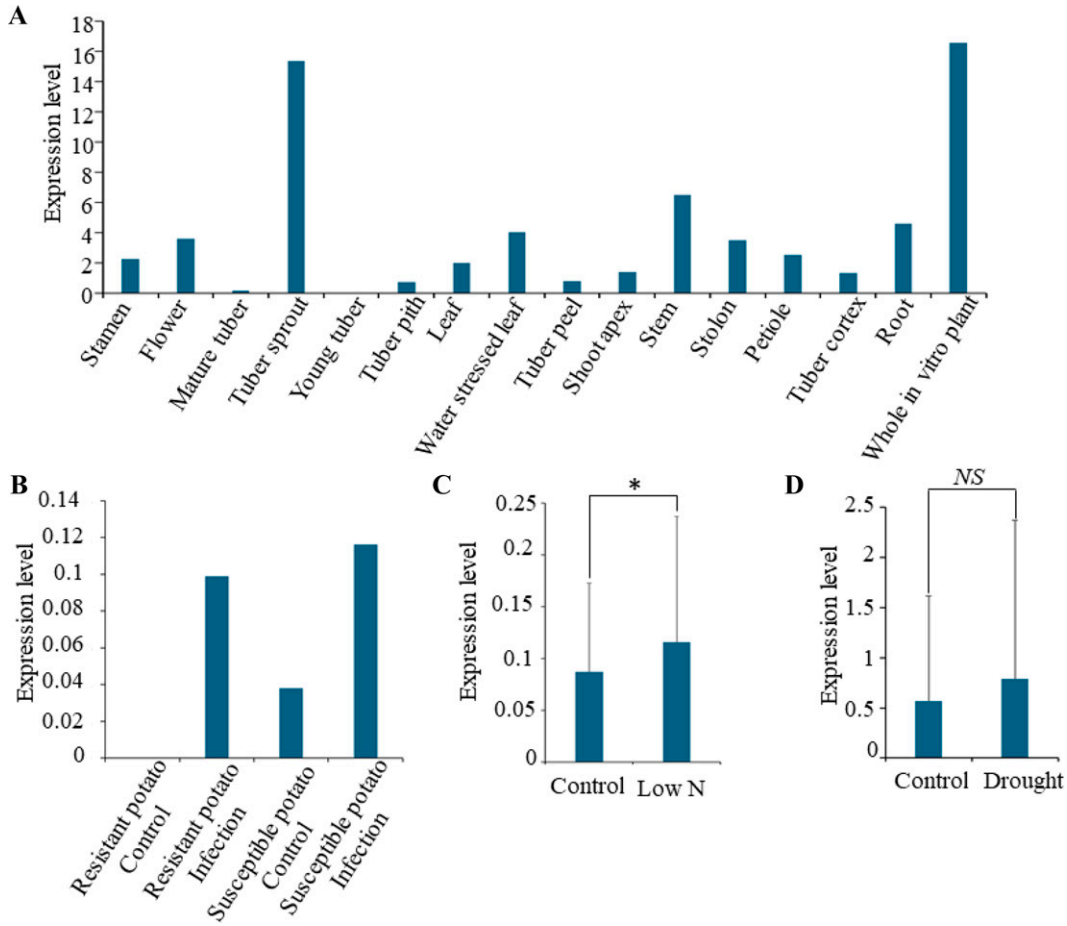


Fig. 3. Expression pattern of *StMYB66*. (**A**) Tissue-specific expression of *StMYB66* in various potato organs. (**B**) *StMYB66* expression significantly increased following *P. infestans* infection in both resistant and susceptible potato lines. (**C**) Nitrogen treatment significantly enhanced *StMYB66* expression. (**D**) *StMYB66* expression remained unchanged under drought conditions. An asterisk (*, $P < 0.05$) denotes significant differences determined by Student's *t* tests. NS indicates no significance.

Additionally, *DM8C03G15570*, *DM8C03G15580*, *DM8C03G15590*, *DM8C07G18690*, and *DM8C02G27260* encoded xyloglucan:xyloglucosyl transferase, which exhibited significantly lower expression in OE-T01 compared with Atlantic (Fig. 7D). These enzymes, categorized under glycoside hydrolase family 16, are crucial for cell wall remodeling and expansion (Stratilová et al. 2020). For instance, the *Arabidopsis* homolog of *DM8C03G15580* (*AT4G25810*) is involved in lateral root emergence via the auxin pathway. Furthermore, *DM8C03G02920* encoded expansin A13, which may mediate pH-dependent cell wall loosening (Supplemental Table 6).

The plant hormone ethylene also plays a role in the *StMYB66* growth regulation pathway. Notably, *DM8C01G31600*, encoding 1-aminocyclopropane-1-carboxylate synthase, a key enzyme in ethylene biosynthesis, was differentially expressed between Atlantic and OE-T01. Nitrogen metabolism is another pathway influenced by *StMYB66*. The DEG *DM8C01G14390* encoded glutamine synthetase, a key enzyme for ammonium assimilation and metabolism. It catalyzed the ATP-dependent addition of ammonium (NH^{4+}) to glutamate to form glutamine, a central component of nitrogen flow in plants. The *Arabidopsis* homolog *AT3G53180* regulates root morphogenesis through nitrogen assimilation pathway (Doskočilová et al. 2011). This suggests that the nitrogen-dependent expression of *MYB66* may influence *DM8C01G14390* expression, contributing to growth regulation (Fig. 3C). Micronutrients also appear to be involved in *StMYB66*-mediated growth regulation. Gene such as *DM8C01G02260*, *DM8C02G15120*, *DM8C02G15140*, *DM8C01G02200*, and *DM8C01G07730* encoded iron ascorbate–dependent oxidoreductase. Homologous genes in *Arabidopsis* regulated iron reduction, which is crucial for primary root growth under phosphate-deficient conditions (Clúa et al. 2024).

Discussion

Potato growth and development are influenced by environmental factors, including various biotic and abiotic stresses such as fungi, drought, and nutrition limitation. Among these, late blight disease caused by *P. infestans* has a severe negative effect on potato yield, as do water and nutrient deficiencies (Arora et al. 2014; Haverkort and Struik 2015). Understanding the function of genes involved in environmental response and growth regulation is crucial for enhancing potato yield, resistance, and adaptability. Such knowledge can support genetic improvement programs to promote sustainable agriculture (Ma et al. 2022; Pixley et al. 2022).

In this study, we identified *StMYB66,* a potato R2R3 MYB transcription factor homologous to *AtMYB88* and *AtMYB124*.

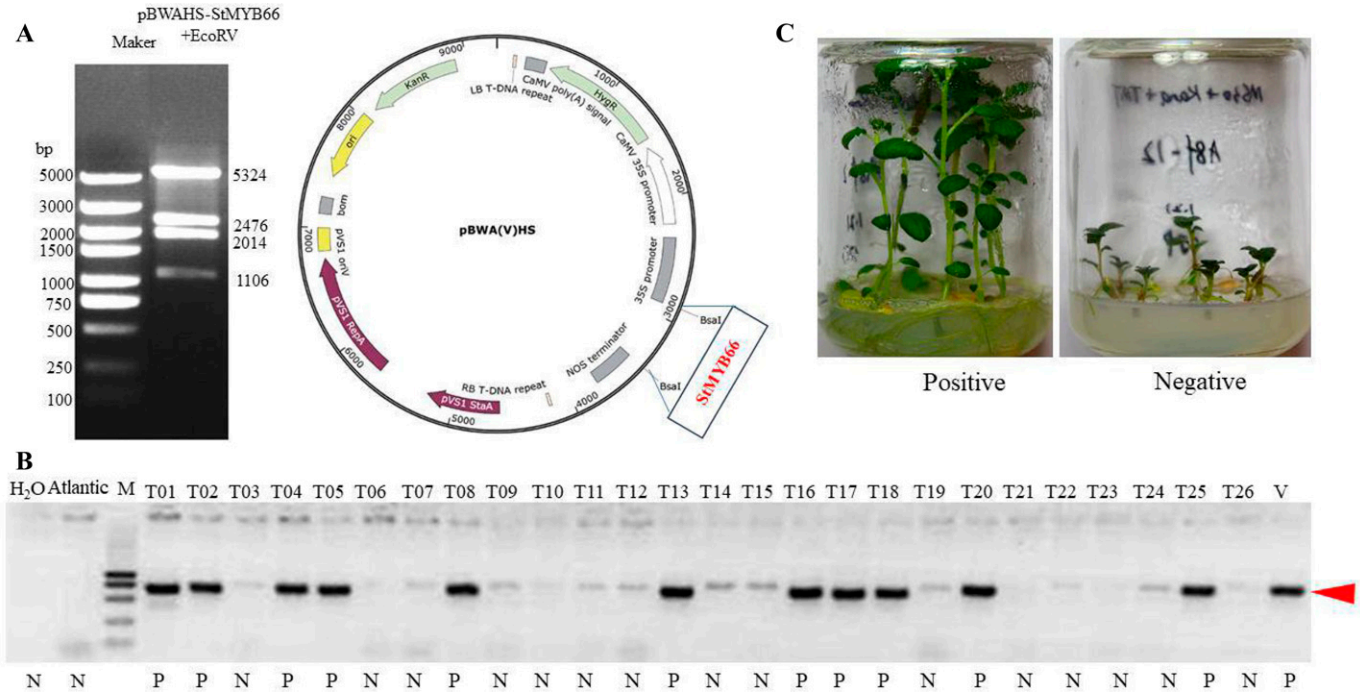


Fig. 4. Identification of *StMYB66* overexpression lines. (**A**) Construction and validation of the *StMYB66* overexpression vector, pBWA(**V**)HS-StMYB66, confirmed by EcoRV digestion, yielding fragments of 1106, 2014, 2476, and 5324 bp. The vector map highlights *StMYB66* downstream of the 35S promoter. (**B**) Polymerase chain reaction screening of transgenic seedlings. Nontransformed plants served as negative controls (N), water as a bank, and a positive control (P) validated transformation. (**C**) Transgenic seedlings grown on selective media.

Bioinformatic analyses revealed the protein's physicochemical properties and identified homologous genes in other species. For example, *MdMYB88* and *MdMYB124*, homologs in *M. domestica*, regulate cold and drought tolerance through pathways involving *COLD SHOCK DOMAIN PROTEIN 3* (*MdCSP3*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*MdCCA1*) (Geng et al. 2018; Sedeek et al. 2019; Xie et al. 2018). In our study, overexpression of *StMYB66* in potato seemed to confer drought tolerance, characterized by reduced shoot length and increased root weight (Fig. 5B). These traits align with drought-adaptive strategies that prioritize root growth to enhance water and nutrient uptake (Shoaib et al. 2022).

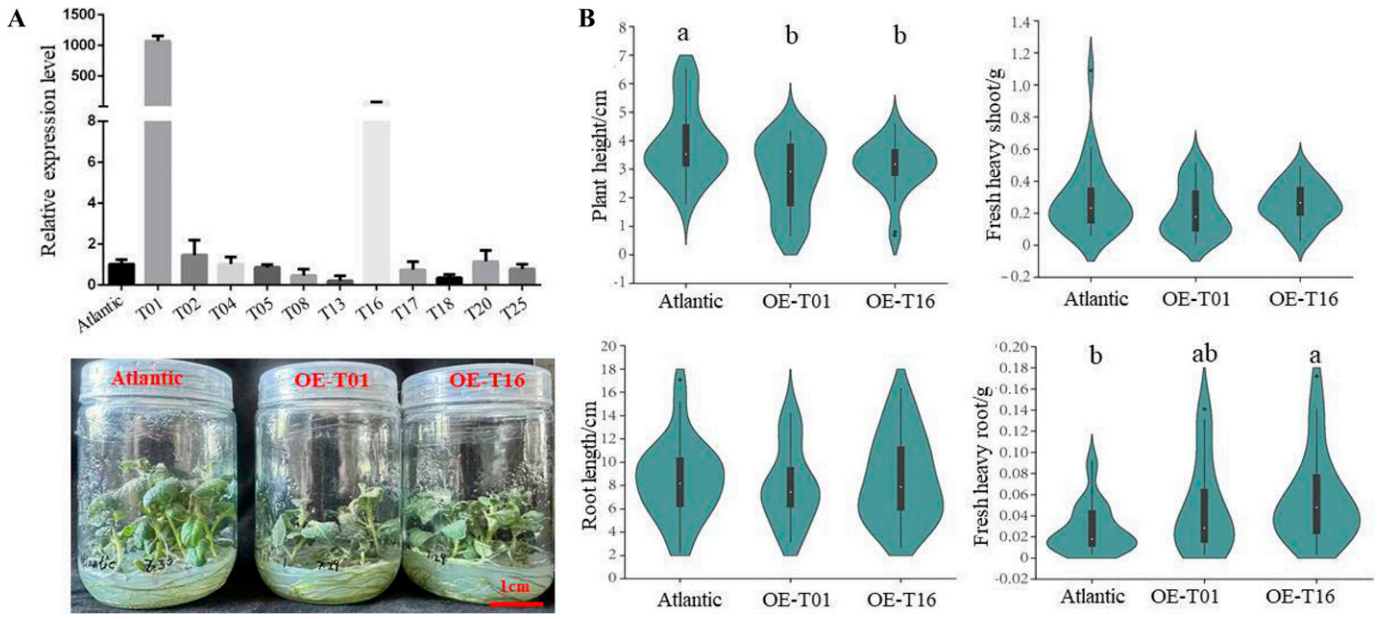


Fig. 5. Expression analysis and phenotypic characterization of *StMYB66* overexpression lines. (**A**) Expression levels of *StMYB66* in OE-T01 and OE-16 lines, showing ~1000-fold and 400-fold increases, respectively, compared with the control. The scale bar indicates 1 cm. (**B**) Phenotypic effects of overexpression. OE-T01 and OE-T16 exhibited reduced shoot height and increased root biomass compared with non-transgenic Atlantic. Different letters indicate significant differences (Tukey's test, $P < 0.05$).

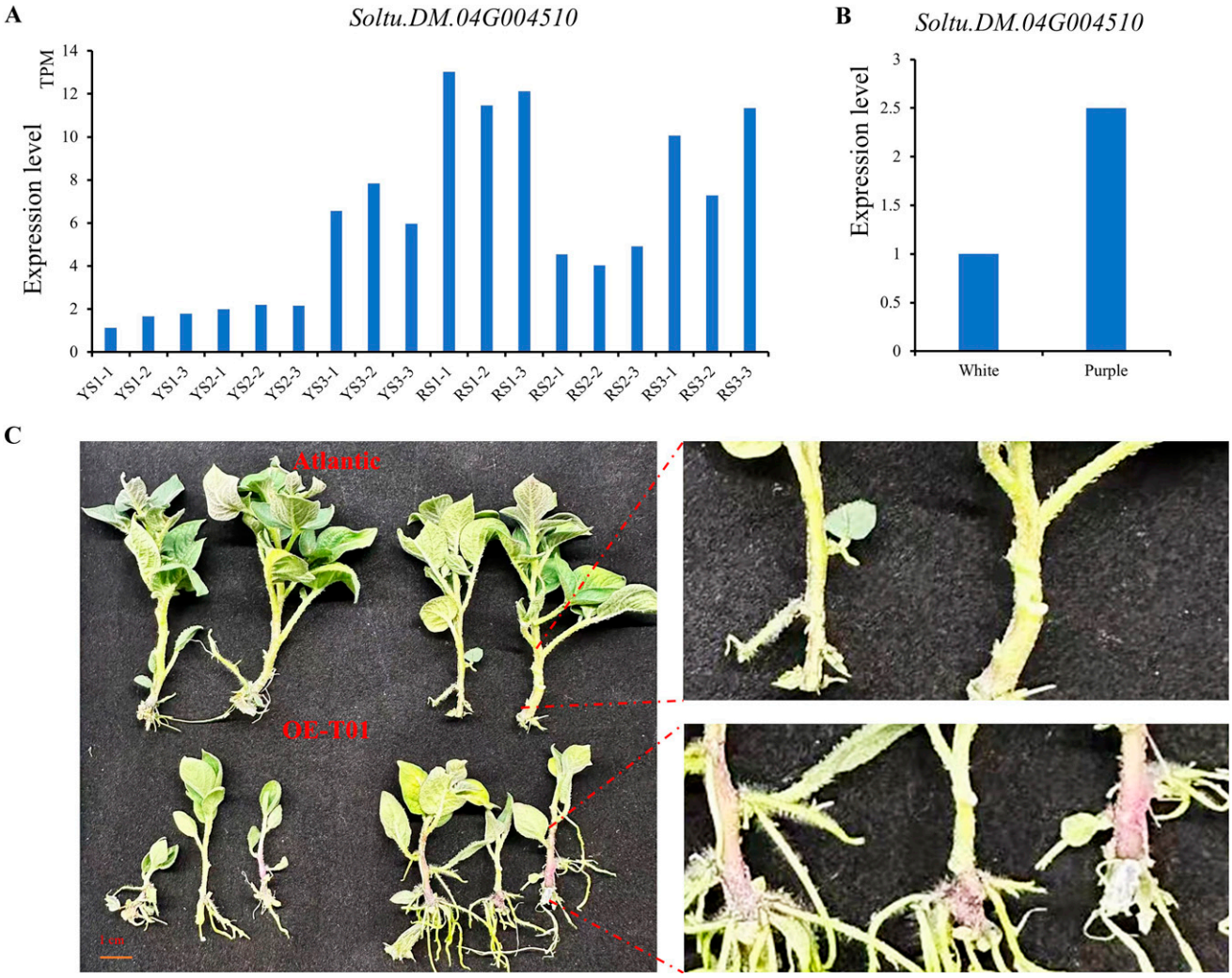


Fig. 6. Role of *StMYB66* in potato anthocyanin synthesis. (**A**) *StMYB66* expression was higher in red-fleshed tubers during tuberization (S1), bulking (S2), and maturing (S3) stages compared with yellow-fleshed tubers. (**B**) *StMYB66* expression was elevated in purple-fleshed tubers relative to light-fleshed ones. (**C**) OE-T01 plants displayed red stems, while Atlantic plants had green stems. The scale bar indicates 1 cm.

Promoter analysis revealed MADS-box motifs in the *StMYB66* regulatory region, suggesting that MADS-box TFs may control *StMYB66* expression. MADS-box TFs are known to regulate diverse developmental processes, including root growth flowering and fruit development (Becker and Theissen 2003). Increased *StMYB66* expression following nitrogen treatment suggests its role in regulating nitrogen response pathway. However, the lack of response to prolonged drought stress indicates a possible involvement in short-term stress responses (Cominelli et al. 2010).

In addition to stress tolerance, *StMYB66* appears to influence anthocyanin biosynthesis. Overexpression lines exhibited purple stems, in contrast to green stems in control plants. Previous studies reported that higher *StMYB66* expression in purple- and red-fleshed tubers compared with yellow-fleshed tubers during tuber development (Liu et al. 2023; Riveros-Loaiza et al. 2022). This suggests that *StMYB66* regulates anthocyanin accumulation in both tubers and stems, enhancing pigmentation.

Plant cells are critical for plant root and shoot growth, morphogenesis, and stress response mechanisms (Cosgrove 2024). These plant cell walls are primarily composed of polysaccharides grouped into three major classes: cellulose, pectins, and hemicelluloses (Voiniciuc et al. 2018). Lignin interacted directly or indirectly with cellulose, xylan, and other polysaccharides, contributing to the structural integrity of the cell wall. In *Arabidopsis*, numerous MYB transcription factors play vital roles in regulating plant development and metabolism by influencing cell wall formation. For instance, *AtMYB46* and *AtMYB83* coordinate transcription factors involved in secondary wall synthesis, while *AtMYB20*, *AtMYB42*, *AtMYB43*, and *AtMYB85* regulate lignin biosynthesis (Geng et al. 2020). Additionally, *AtMYB58*, *AtMYB61*, and *AtMYB63* act as lignin-specific transcription factors that directly regulate genes in the monolignol biosynthesis pathway (Geng et al. 2020; Newman et al. 2004; Zhou et al. 2009).

In this study, comparative transcriptome analysis revealed that *StMYB66* regulates shoot and root growth by affecting the biosynthesis of key cell wall components, including cellulose, xylan, and lignin. Specifically, *StMYB66* may regulate lignin biosynthesis through genes such as *DM8C01G44330*, *DM8C02G16010*, and *DM8C02G33650*, which encode glycosyltransferase. These glycosyltransferases function similarly to UGT72B1 in *A. thaliana*, which is essential for normal cell wall lignification (Lin et al. 2016).

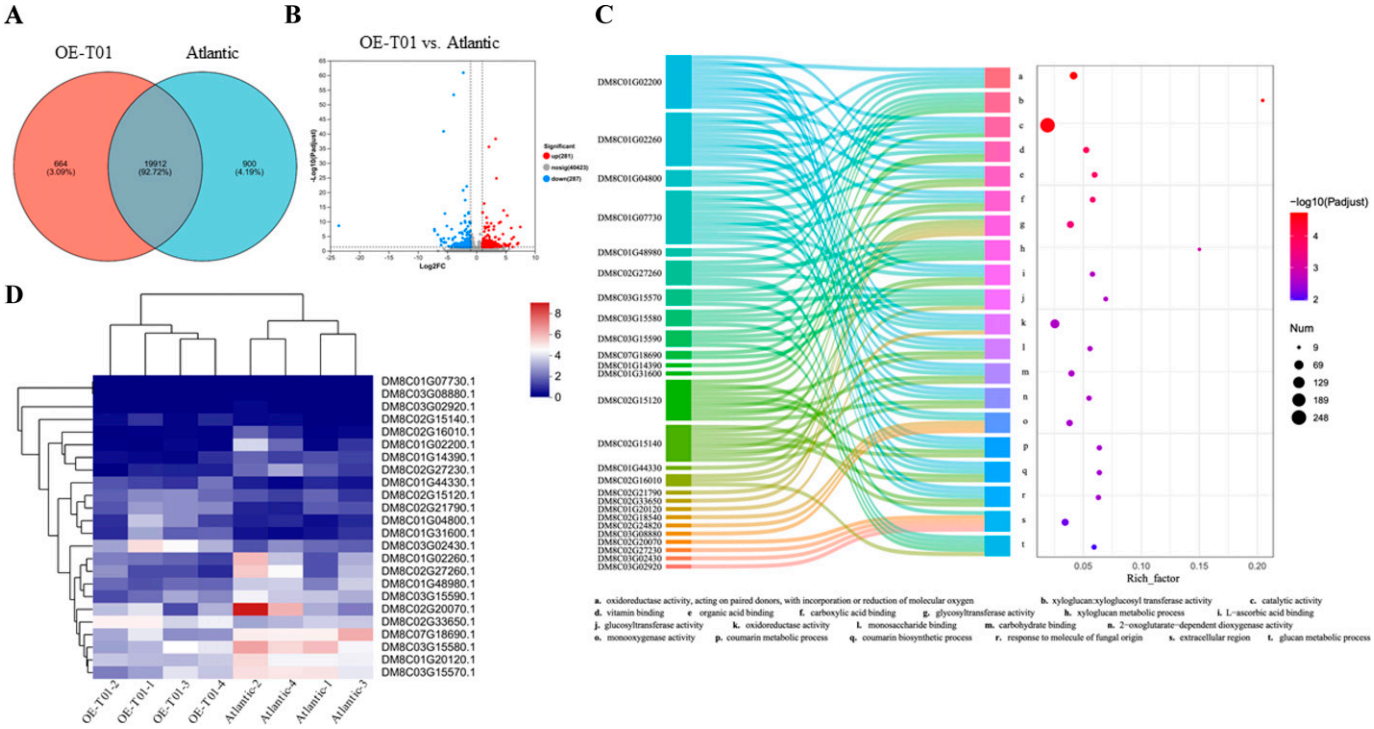


Fig. 7. Transcriptome analysis between Atlantic and OE-T01 (**A**) Venn diagram of expressed genes in Atlantic and OE-T01. (**B**) Volcano plot of differentially expressed genes (DEGs). (**C**) Gene ontology (GO) analysis of DEGs. (**D**) Heat map of the high frequency DEG expression level between Atlantic and OE-T01.

StMYB66 also regulates cellulose and pectin biosynthesis by modulating the expression level of the glycosyltransferase gene (*DM8C02G16010*) and the pectinesterase gene (*DM8C02G2007*).

Although six MYB transcription factors were differentially expressed between Atlantic and OE-T01 lines, none were associated with the main GO terms. Their homologous genes in *Arabidopsis* are involved in processes such as gibberellic acid biosynthesis, jasmonate regulation pathway, and cold stress response (Supplemental Tables 4 and 6). These findings suggest that *StMYB66* directly influences the expression of key genes regulating lignin, cellulose, and xylan biosynthesis, thereby affecting cell wall formation and, consequently, regulated potato shoot and root growth. Additionally, *StMYB66* influences other growth and stress response pathways, including ethylene biosynthesis (*DM8C01G31600*), epidermal patterning factors (*EPF*, *DM8C02G27230*), and other mechanisms (Supplemental Table 6). These findings highlight the central role of *StMYB66* in regulating potato growth and environmental responses.

Conclusions

This study identifies *StMYB66*, a potato R2R3 MYB transcription factor, as a critical regulator of potato growth and stress responses. By integrating bioinformatics analyses, transcriptome profiling, and phenotypic evaluation of *StMYB66* overexpression lines, we demonstrate its involvement in regulating root and shoot growth through cell wall biosynthesis pathways. Specifically, *StMYB66* influences the expression of genes involved in the biosynthesis of key cell wall components, including lignin, cellulose, and xylan, which are vital for structural integrity and growth modulation. Additionally, *StMYB66* responds to nitrogen treatment and *P. infestans* infection and may play a role in anthocyanin accumulation, linking it to both metabolic regulation and environmental adaptability. These findings establish *StMYB66* as a pivotal regulator of potato development and stress adaptation, with potential applications in breeding programs aimed at improving yield, stress resilience, and nutrient utilization. Further exploration of its regulatory mechanisms will enhance our understanding of its role in plant biology and its potential for genetic enhancement in sustainable agriculture.

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