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Mutation of MYB36 affects isoflavonoid metabolism, growth, and stress responses in *Lotus japonicus*

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Abstract

Isoflavonoids are mostly produced by legumes although little is known about why and how legumes are able to regulate the biosynthesis of these particular compounds. Understanding the role of potential regulatory genes of the isoflavonoid biosynthetic pathway constitutes an important topic of research. The LORE1 mutation of the gene encoding the transcription factor MYB36 allowed the identification of this gene as a regulator of isoflavonoid biosynthesis in *Lotus japonicus* plants. The levels of several isoflavonoid compounds were considerably lower in two lines of *Ljmyb36* mutant plants compared to the WT. In addition, we found that *Ljmyb36* mutant plants were significantly smaller and showed a substantial decrease in the chlorophyll levels under normal growth conditions. The analysis of plants subjected to different types of abiotic stress conditions further revealed that mutant plants presented a higher sensitivity than WT plants, indicating that the MYB36 transcription factor is also involved in the stress response in *L. japonicus* plants.

1 | INTRODUCTION

It is well established that flavonoids and isoflavonoids are important secondary metabolites which have roles in plant growth and development, and in the defense against biotic and abiotic stress acting as phytoalexins (García-Calderón et al., 2020; Liu et al., 2015). Therefore, the study of the biosynthesis of flavonoids and isoflavonoids is a very important topic of research which, in addition, may have important consequences for human nutrition. For example, soybean isoflavonoids (isoflavones) have several beneficial effects on human health including the prevention of different types of cancers and cardiovascular diseases (Chu et al., 2017).

(Iso)flavonoid compounds are of special interest because of their role in the nodulation process in legumes. They are crucial for the determination of the host range, since only a specific combination of (iso)flavonoids exudated by the legume root is able to attract the symbiont and activate *NOD* genes transcription in a compatible rhizobial strain (García-Calderón et al., 2020; Liu & Murray, 2016). It has been described that isoflavonoids are also involved in the systemic autoregulation of nodule number (Dong & Song, 2020). Different (iso)flavonoids can be used by the host in order to exert an inhibitory effect on incompatible rhizobial strains (Dong & Song, 2020). Although the main role for flavonoids or isoflavonoids in legume nodulation is to induce the Nod factor production by rhizobia, they also regulate other rhizobial responses that are important for symbiosis, including alterations in growth and motility, P and Fe acquisition and others (Liu & Murray, 2016 and references therein).

Interestingly, isoflavonoids are mostly produced by legume species. However, little is known about why this group of plants produces these particular compounds and how they regulate them. The response is also quite species-specific, for example, while several legumes accumulate flavonols in response to abiotic stress, different

works from our group indicate that the model legume *Lotus japonicus* uses a peculiar strategy accumulating the isoflavonoid vestitol in response to different kinds of abiotic stresses (García-Calderón et al., 2015; Kaducová et al., 2019). A recent co-expression network was built in our laboratory to establish the possible interconnections between expression patterns of genes of the isoflavonoid biosynthetic pathway and other *MYB* and *MYB*-related genes, in order to search for putative regulatory genes of isoflavonoid biosynthesis (García-Calderón et al., 2020). By using this new gene co-expression network, six different MYB transcription factors (TFs) were identified. In the present paper mutants affected in one of these genes have been characterized phenotypically under different stress conditions.

2 | MATERIALS AND METHODS

2.1 | Plant material, growth conditions and experimental designs

The model legume *L. japonicus* (Regel) K. Larsen ecotype Gifu (B-129-S9) was used in all experiments. Seeds were originally obtained from Jens Stougaard (Aarhus University, Denmark) and self-propagated at the University of Seville. Homozygous mutant plants affected in the *MYB36* gene were identified and selected from the progeny of different lines containing the LORE1 transposon inserts. WT and mutant seeds were scarified and surface sterilized and germinated in 1% (w/v) agar in Petri dishes. Phenotype characterization was determined with five seedlings planted in each pot using vermiculite as a solid support and grown in a growth chamber under 16/8 h day/ night, 20/18°C with a photosynthetic photon flux density of 200 µmol m^{-2} s^{-1} and a constant humidity of 70%. Non-nodulated plants were irrigated with 'Hornum' nutrient solution containing 5 mM NH_4NO_3 and 3 mM KNO_3. Nodulated plants were inoculated with the compatible symbiont *Mesorhizobium loti* and watered with nitrogen-free 'Hornum' medium supplemented with 3 mM KCl (Handberg & Stougaard, 1992). *Mesorhizobium loti* TONO JA76 (Kawaguchi et al., 2002) was grown in liquid YM medium (Vincent, 1970) at 28°C to an optical density of 1.0 at 600 nm, and then collected by centrifugation for 30 min at 2408 *g* and resuspended in 0.75% (w/v) NaCl. Once sown in the pots, the plants were inoculated by the addition of 200 µL of this bacterial suspension. 15 plants per genotype were analyzed to determinate the growth patterns under non-symbiotic and symbiotic conditions.

For the stress treatments, WT and mutant plants were irrigated with a 'Hornum' nutrient solution and grown under the above conditions, with some exceptions according to the different experimental designs described below.

UV-B radiation was applied together with the continuous visible-light illumination on plants that had been previously grown as described above for 30 days, in pots filled with vermiculite. Three biological replicates grown in the same pot, consisting of five plants each, were used for each genotype. Leaf tissue samples collected at the beginning of the experiment just before the UV treatment (t = 0 h) were used as controls. Samples were exposed to UV-B treatment for 16 h using a UV-B G8T5E fluorescence lamp (Sayko Denki, 310 nm). The distance between the leaves and the lamp was approximately 23 ± 3 cm. The spectral output of the UV tubes at the top of the plant canopy was determined with a spectral radiometer (Sola-Scope 2000 from Solatell, resolution 0.5 nm) and weighted using the biological spectral weighting function (Flint & Caldwell, 2003); the bioactive UV-B dose was 0.68 mW cm^{-2}. Tissues for metabolite analysis were dried at 70°C for 24 h. All leaves from five control or treated plants were collected and pooled for the different measurements carried out (Kaducová et al., 2019). All leaf samples used in this work were harvested 4 h after the beginning of the light period.

For salt stress treatments, a single seedling was sown per pot four days after imbibition, and salt stress treatment was applied four days after sowing. In total, 180 plants per genotype were analyzed. The salt content in the nutrient solution was gradually increased by 50 mM for salinity acclimation in steps of four days till reaching lethal salt conditions (300 mM NaCl). Mortality rate was determined when the whole plant or all leaves were wilted or dead as described by Sánchez et al. (2011) with minor modifications.

The experiment to determinate the irradiance sensitivity was performed by growing control plants at the standard photosynthetic flux density described above. Treated plants were provided with a higher light intensity corresponding to 300 µmol m^{-2} s^{-1}. All plants were grown in these conditions for 56 days. 30 plants were examined for each genotype.

The sensitivity to high temperature was analyzed by increasing the temperature of the growth chamber to 25°C and 30°C during the day photoperiod while maintaining 18°C during the night. Control plants were grown under 20/18°C day/night as mentioned above. All plants were grown in these conditions for 56 days. 15 plants were examined for each genotype.

2.2 | Identification of *Ljmyb36* LORE1 mutants

DNA extraction and cDNA synthesis methods were carried out as described by Pérez-Delgado et al. (2016). *Ljmyb36* genotypes were detected through genotyping by PCR. Forward primers were used with LORE1-specific primer P2 to detect loci with LORE1 insertions. Reverse primers together with forward primers yielded a PCR product only when the locus they flank does not contain an insertion (Urbański et al., 2012). Knockout lines were detected through forward and reverse specific primers. Real-time qPCR of cDNA samples from WT and mutant plants was performed as described by Kaducová et al. (2019). The housekeeping gene ubiquitin carrier protein 10 from *L. japonicus* (*LjUBC10*) was used as cDNA loading control gene. All the primers used are listed in Table S2.

2.3 | Determination of chlorophyll content

0.05 g of fresh leaves were homogenized with 1 mL of 100% pure acetone. The extracts were centrifuged at room temperature for

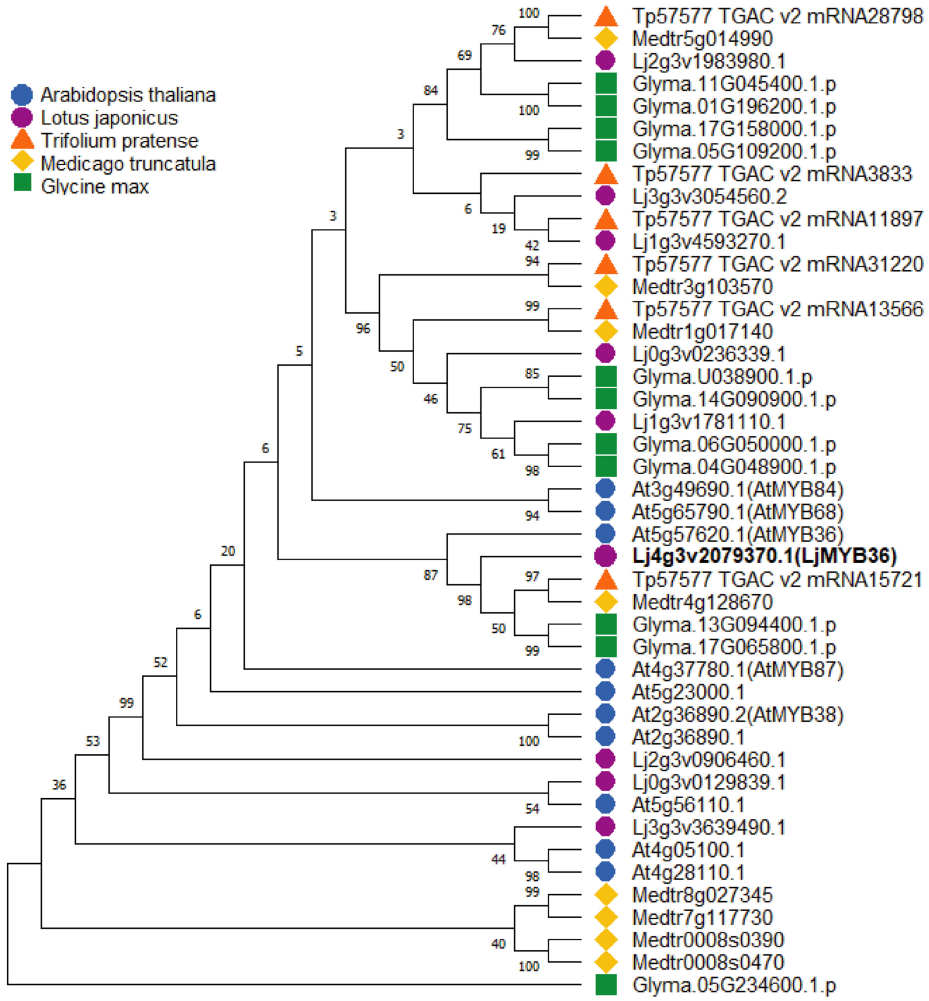

FIGURE 1 Phylogenetic analysis of the *LjMYB36* gene. (A) Phylogenetic tree of complete amino acid sequence from the sequence Lj4g3v2079370.1 from *Lotus japonicus* and other R2R3-MYB proteins from different plant species (*Arabidopsis thaliana*, *Glycine max*, *Medicago truncatula and Trifolium pratense*).

10 min at 19 510 *g*. The absorbance was measured at 661.6 and 644.8 nm, and chlorophyll *a* and *b* contents were calculated according to Lichtenthaler and Buschmann (2001).

2.4 | Metabolite Profiling Analysis

For HPLC analysis, the leaves were dried for 24 h at 60°C and stored in the dark in desiccator at room temperature until used. Previous studies showed that the incubation of leaf samples at this temperature did not significantly change the main flavonoid profiles (García-Calderón et al., 2015). 50 mg of dry tissue were extracted with 1.5 mL of 80% (v/v) methanol for analysis of flavonoid glycosides, phenylpropanoids and isoflavonoids. The extracts were centrifuged at 760 *g* for 5 min and filtered using a 0.2 µm nylon membrane. Samples were analyzed by gradient reversed phase HPLC using an *Agilent 1260 Infinity Quaternaly LC* system with *1260 Infinity DAD* detector and *Kromasil* C_{18} 250 x 4.6 mm x 5 µm column (Nouryon). The solvent was delivered to the column at a 0.7 cm^3 min flow rate. A gradient system with two mobile phases was used: 5% (v/v) acetonitrile with 3% (v/v) trifluoroacetic acid (A) and 80% (v/v) acetonitrile (B). The gradient program utilized for determination of flavonoids, phenypropanoids, and isoflavonoids was as follows: 0 min A/B (90:10); 5 min A/B (86:14); 30 min A/B (76:24); 35 min A/B (60:40); 50 min A/B (0:100); 55 min A/B (0:100); 60 min A/B (90:10). The gradient program utilized for determination of isoflavonoids was as follows: 0 min A/B (90:10); 5 min A/B (60:40); 24 min A/B (0:100); 30 min (0:100); 35 min (90:10). Compounds were detected at 220 and 350 nm.

Peak identities were identified based on their retention time and UV–VIS spectra measurements carried out during the analysis by comparison of commercially available standards of *p*-coumaric acid, *p*-ferulic acid, isoferulic acid, cinnamic acid, isoliquiritigenin, biochanin A, kaempferol-3-O-glucoside (Sigma Aldrich), quercetin-3-O-rhamnoside (Roth), vestitol (Biorbit), medicarpin (Plantech, UK) or using the standards previously isolated from *L. japonicus* leaves and identified by NMR methods (García-Calderón et al., 2015). The identities of some peaks were also verified according to Kaducová et al. (2019).

For quantification, kaempferol-3-O-glucoside was used as a standard for MS identified kaempferol glycosides, quercetin-3-O-rhamnoside was used for MS identified quercetin glycosides, p-coumaric acid was used as a standard for coumaric acid isomers and vestitol was used as the reference for its derivative and sativan. All flavonols and isoliquiritigenin were evaluated at $\lambda = 350$ nm, phenylpropanoids at $\lambda = 280$ nm, vestitol, vestitol derivatives, sativan and medicarpin at $\lambda = 220$ nm and expressed as µmol g^{-1} dry weight.

2.5 | Phylogenetic analyses

A phylogenetic tree was constructed using the complete amino acid sequence from sequence Lj4g3v2079370.1 from *L. japonicus* together with those of R2R3-MYB proteins from different plant species. In this case, three other commonly used legume species (*Glycine max*, *Medicago truncatula* and *Trifolium pratense*) were analyzed, together with *Arabidopsis thaliana* as reference. The BLAST algorithm was used to select the 10 sequences most closely related to each of the plant species analyzed. These sequences were identified by searching the genome in specific databases. SoyBase (https://www.soybase.org/) for *G. max*, TAIR (https://www.arabidopsis.org/) for *A. thaliana*, Lotus Base (https://lotus.au.dk/) for *L. japonicus*, and PlantTFDB (http://planttfdb.gao-lab.org/) for the rest of the species. The amino acid sequences were aligned using the MUSCLE multiple alignment method in MEGA11 ver. 11.0.13 (Tamura et al., 2021). Phylogenetic relationships between sequences and tree reconstruction were inferred by using the Maximum Likelihood method and the w/freq. model (Jones et al., 1992). The percentage of trees in which the associated taxa are clustered is shown beside the branches. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories [+ G, parameter = 1.1158]). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 8.61% sites). This analysis involved 45 amino acid sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option).

TABLE 1 Percent amino acid (aa) and DNA sequence identity of Lj4g3v2079370.1 with the most closely related MYBs from *A. thaliana*

ID	Gene name	% Identity (aa)	% Identity (DNA)
At5g57620.1	AtMYB36	58.57	63.21
At4g37780.1	AtMYB87	52.65	53.53
At3g49690.1	AtMYB84	50.80	55.40
At5g65790.1	AtMYB68	47.02	54.50
At2g36890.1	AtMYB38	45.56	52.37
At2g36890.2	AtMYB38	45.56	52.37
At5g23000.1	AtMYB37	44.94	49.47
At5g56110.1	AtMYB103	40.00	45.67
At4g05100.1	AtMYB74	39.83	43.63
At4g28110.1	AtMYB41	39.07	43.51

2.6 | Statistical data analysis

Significant differences between control and mutants under the different growth conditions analyzed were determined for each genotype by ANOVA ($p < 0.05$) with a post-hoc Turkey HSD test.

3 | RESULTS

3.1 | Isolation of LORE1 mutants affected in the *L. japonicus* MYB36 gene

Previous results established a high degree of interconnections between different transcription factor genes from *L. japonicus* and

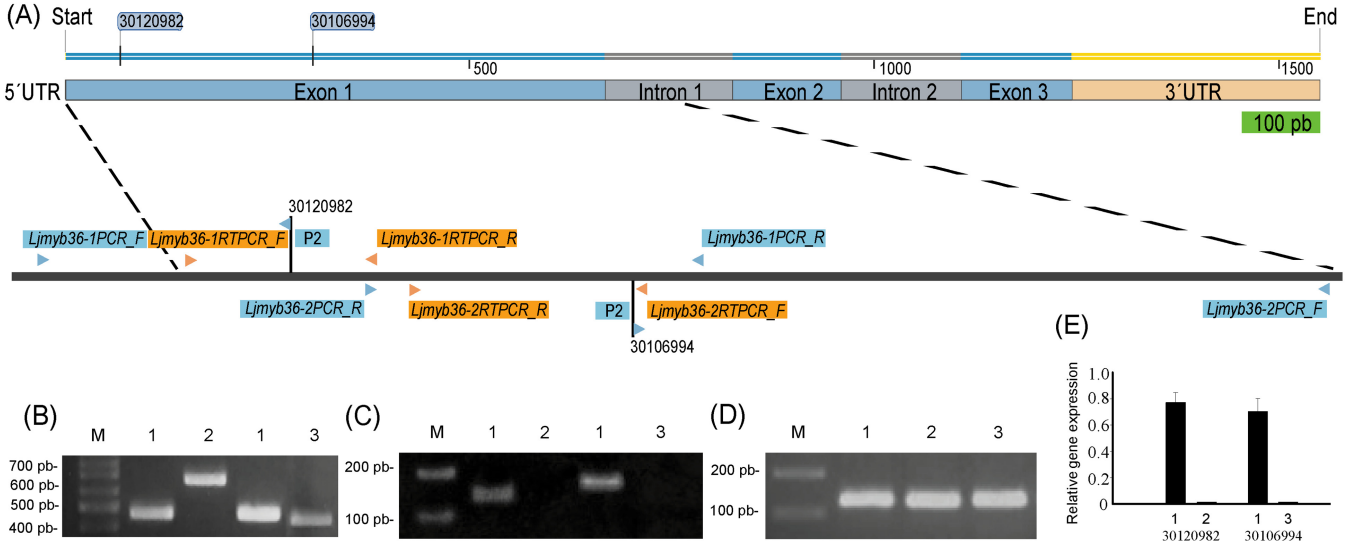

FIGURE 2 Molecular analysis of *Ljmyb36* mutants. (A) Mapping of LORE1 insertions in *Ljmyb36-1* and *Ljmyb36-2* mutants, (B) genotyping of DNA products by PCR to detect genotypes of *Ljmyb36*, (C) PCR of cDNA to confirm *Ljmyb36* gene knockout, (D) PCR carried out to confirm the correct extraction of cDNA using *LjUBC10*, a housekeeping gene, and (E) real-time qPCR of cDNA samples from WT and mutant plants. M ladder, 1: WT, 2: *Ljmyb36-1*, 3: *Ljmyb36-2*.

several genes of the isoflavonoid biosynthetic pathway from this plant, based on a co-expression analysis (García-Calderón et al., 2020). One of the sequences identified in this co-expression network (Lj4g3v2079370.1) was initially assigned to the *LjMYB84* gene based on database descriptions. In the present paper the Figure 1 shows a detailed phylogenetic analysis carried out comparing the complete amino acid sequence from this TF to other homologous amino acid sequences from *Arabidopsis thaliana* and different legume species. This analysis showed that Lj4g3v2079370.1 was grouped in a clade which included the corresponding gene from the other legume species analyzed, most closely related, together with one particular gene from Arabidopsis (Figure 1A). The results obtained indicate that among all Arabidopsis sequences analyzed, the sequence Lj4g3v2079370 from *L. japonicus* shows the highest degree of sequence identity of around 58–63% to the At5G57620.1 sequence, corresponding to the *AtMYB36* gene (Table 1).

Homozygous mutant lines affected in the different TF genes from *L. japonicus* determined in the co-expression network were identified by genotyping the self-fertilization progeny of the LORE1 mutant seed resource available for these particular genes in our laboratory (Małolepszy et al., 2016). A preliminary analysis of these mutant plants subjected to stress conditions showed that the mutant plants affected in the *MYB36* gene presented a higher sensitivity to stress than the other mutant plants tested. For this reason, MYB36 mutant plants were selected to study the role of this gene in *L. japonicus* plants under different growth conditions. Figure 2A indicates the position of the two different mutations corresponding to the two mutant lines studied in the present work. For the rest of the paper, mutant line 30120982 will be called *Ljmyb36-1* while mutant line 30106994 will be *Ljmyb36-2*, respectively, according to standard nomenclature given for this plant (Stougaard et al., 1999). PCR analysis further confirmed that both mutant lines show the presence of LORE1 insertion where expected (Figure 2B) and lack of the corresponding *MYB36* transcripts (Figure 2C) while having normal levels of the *LjUBC10* gene, used as a control by cDNA extraction and loading in the gels (Figure 2D). No expression of *LjMYB36* gene in mutant plants was confirmed by real-time qPCR of cDNAs (Figure 2E). Therefore, these mutant lines were considered complete knockout mutants for further studies.

3.2 | *Ljmyb36* mutants are affected in isoflavonoid biosynthesis

Previous studies have reported that UV-B irradiation increases the levels of isoflavonoids in *L. japonicus* (García-Calderón et al., 2015; Kaducová et al., 2019). Therefore, *Ljmyb36-1* and *Ljmyb36-2* mutants were subjected to UV-B treatments and the determination of the levels of different flavonoids and isoflavonoids was carried out. At 16 h of UV-B treatment, important differences in the levels of some compounds were detected, particularly in the levels

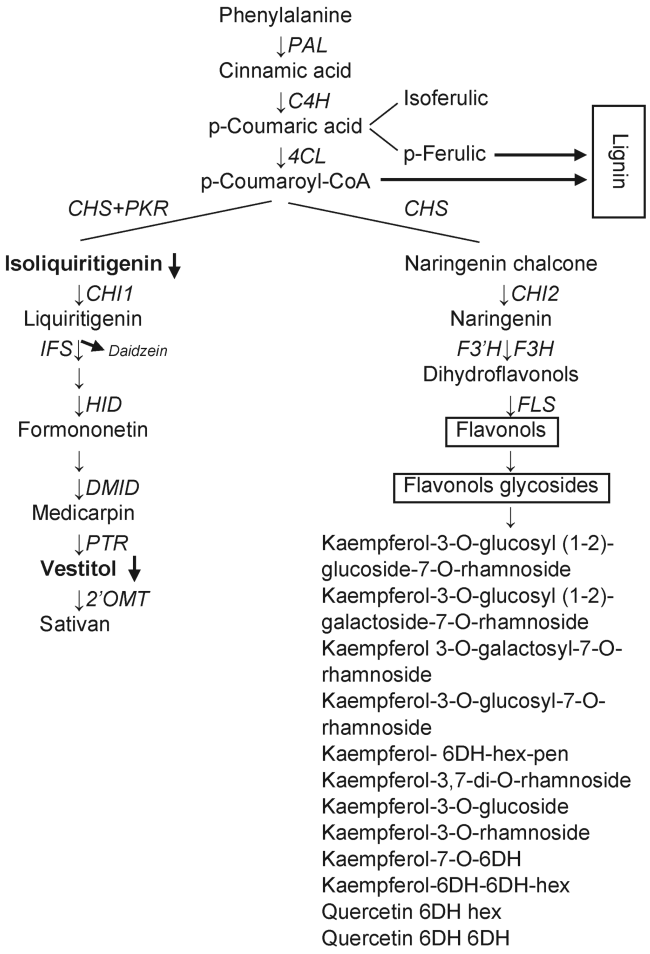

FIGURE 3 Scheme of major changes observed in flavonoids / isoflavonoids biosynthetic pathways in *Ljmyb36* mutants. Samples were analyzed after 16 h of UV-B irradiation treatment. In bold are the compounds with significant increase or decrease. These changes are highlighted with arrows. Abbreviations: hex, hexose; pen, pentose; 6DH, 6-deoxyhexose.

of isoflavonoids isoliquiritigenin and vestitol, which were substantially reduced by more than 2-fold in both mutant lines, compared to the WT (Figure 3). A full list of the levels of metabolites can be found in Supplemental Table 1, where all significant differences of mutants with regard to the WT are shown, as well as the differences obtained before and after UV-B treatment which increased specifically the levels of these isoflavonoids. These results indicate that the mutation of the transcription factor MYB36 from *L. japonicus* results in an alteration of isoflavonoid levels, thus suggesting that this TF may be involved in the regulation of the biosynthesis of these compounds. However, the measurements carried out before UV-B treatment indicates that the levels of a coumaric acid derivative and several kaempferol glycosides were also significantly diminished in mutant plants compared to WT. In addition, increased levels of sativan and a quercetin glycoside were obtained in *Ljmyb36* mutant plants in the absence of UV-B irradiation.

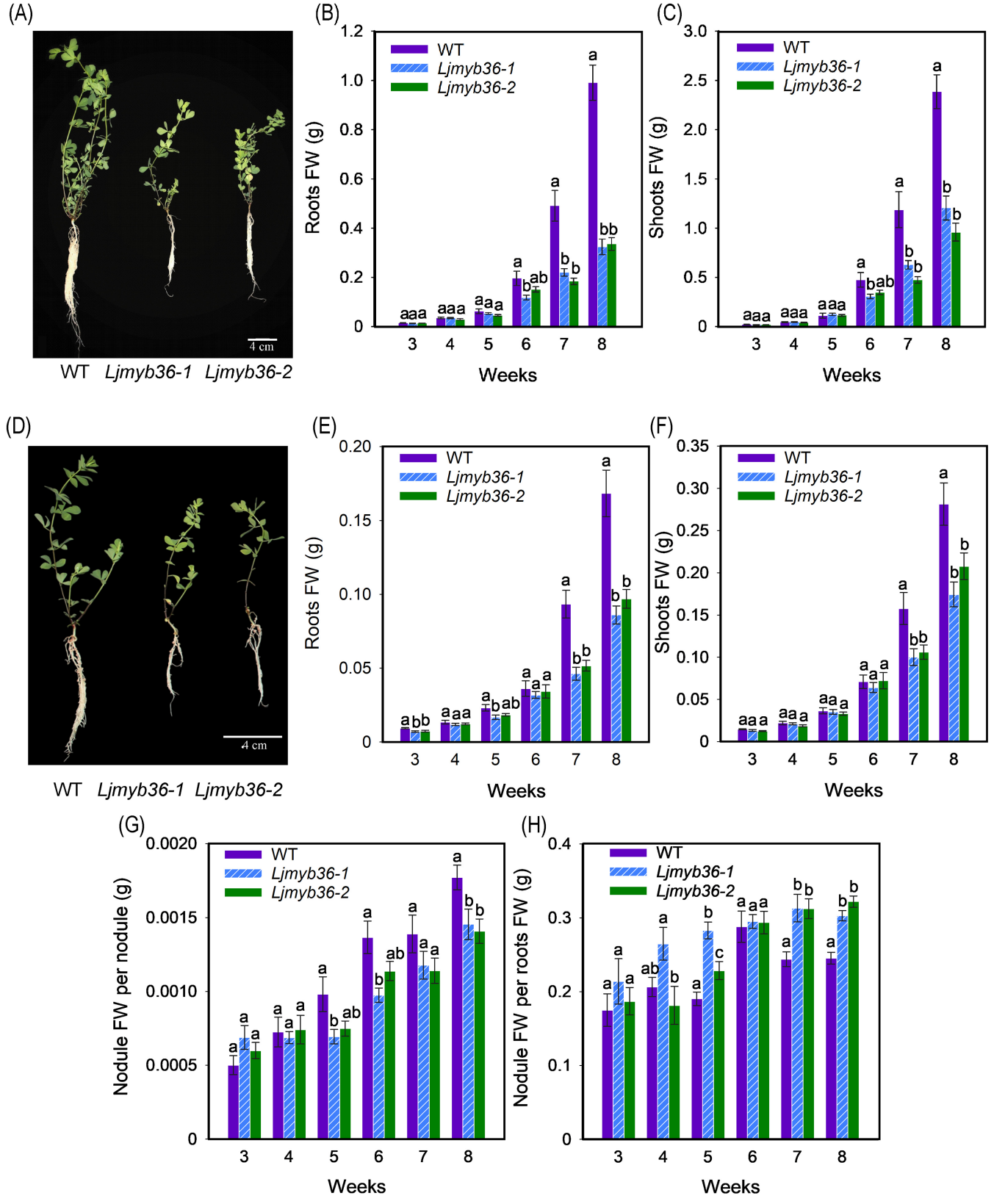

FIGURE 4 Growth patterns under non-symbiotic conditions of WT and *Ljmyb36* mutant plants. (A) Phenotype of WT and *Ljmyb36* mutants after eight weeks of growth, (B) shoots and (C) roots fresh weight at different times of growth. Growth patterns under symbiotic conditions of WT and *Ljmyb36* mutant plants. (D) Phenotype of WT and *Ljmyb36* mutants after eight weeks of growth, (E) roots and (F) shoots fresh weight at different times of growth, (G) fresh weight of nodules per nodule, (H) fresh weight of total plant nodules per fresh weight of roots. Values are the means ± SE of 15 plants per genotype. Letters indicate significant differences ($P < 0.05$) between treatments according to ANOVA with a post-hoc Tukey HSD test.

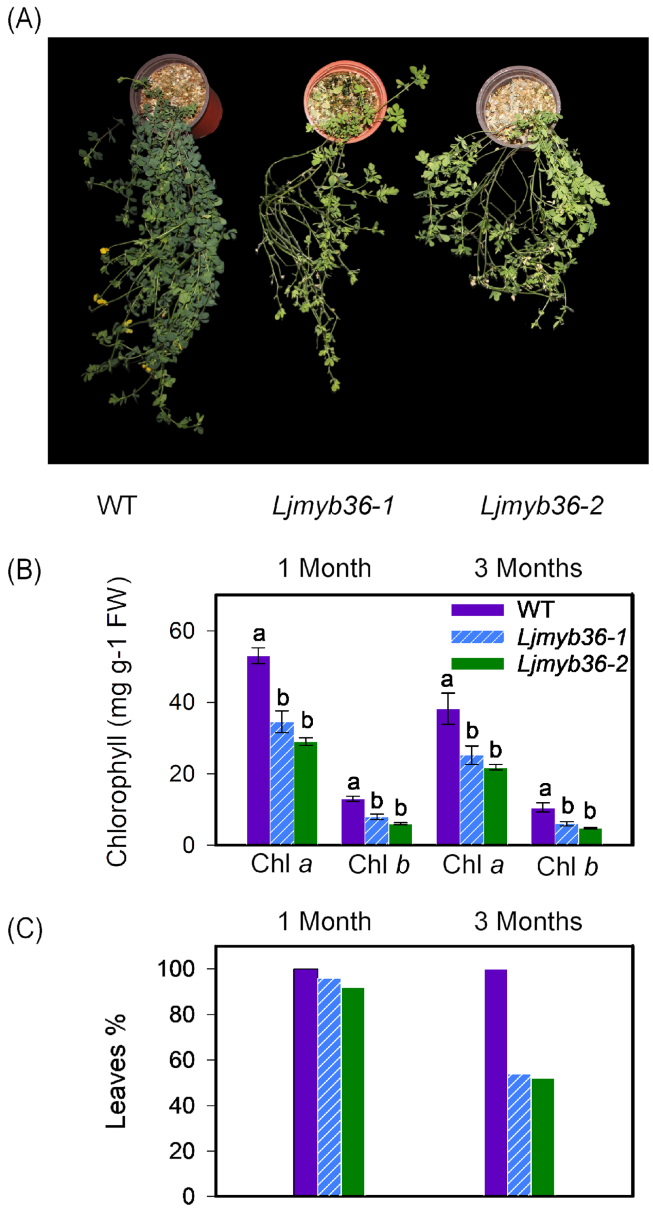

FIGURE 5 Growth differences between WT and *Ljmyb36* mutant mature plants. (A) Phenotype of WT and *Ljmyb36* mutants after three months of growth, (B) chlorophyll *a* and *b* content in *Lotus japonicus* leaves at one month (left) or three months (right) of growth, (C) percent of leaves present in mutant plants at one or three months of growth with respect to WT plants.

3.3 | Effects in growth of *Ljmyb36* mutants

The differences in growth observed for WT and *Ljmyb36* mutant plants grown either under non-symbiotic or symbiotic conditions are shown in Figure 4. A much lower size of the plants was detected under nitrate-grown conditions (Figure 4A) affecting both the growth of roots (Figure 4B) and shoots (Figure 4C) which were significantly smaller in both *Ljmyb36-1* and *Ljmyb36-2* mutants compared to WT plants after five weeks of growth. A similar result was detected for plants grown in the absence of nitrate under dinitrogen fixing conditions as a result of symbiosis with *Mesorhizobium loti* (Figure 4D). In this case differences in growth were mostly observed at seven and eight weeks of growth (Figure 4E and F). These results indicate that the mutation of the *LjMYB36* gene causes an important effect in the growth of the plants, independently of these plants being nodulated or non-nodulated. Interestingly, the fresh weight of the nodules produced by the plants was also substantially reduced, particularly at seven and eight weeks of growth (Figure 4G). However, the fresh weight of nodules referred to root biomass was significantly increased (Figure 4H) due to the lower growth of the roots.

Analysis of *Ljmyb36* mutants was also carried out at a later stage of growth (three months). Figure 5 illustrates that at this time of growth the mutant plants showed not only a reduction in growth but also an increase in the proportion of yellow leaves (Figure 5A). In fact, a substantial decrease in the levels of chlorophyll *a* and *b* was detected in these plants (Figure 5B). Interestingly, Figure 5B also shows that even at one month of growth, a lower content of chlorophyll could be detected in *Ljmyb36* mutants in comparison with the WT, although this change in chlorophyll is not properly noticeable by eye. In addition, Figure 5C shows that the number of leaves was reduced by 50% in older plants (three months grown), thus indicating that *Ljmyb36* mutations result not only in retardation of growth, as mentioned above, but also in an early senescence phenotype.

3.4 | *Ljmyb36* mutants show higher sensitivity to salt, light and thermal stress

The response of *Ljmyb36* mutants to a gradual increase in NaCl, carried out as previously reported for this plant species (Sánchez et al., 2011) is shown in Figure 6A. The results obtained indicate that a much higher proportion of dead plants were found at 200–300 mM NaCl for both lines of *Ljmyb36* mutants, compared to the WT. In fact, more than 50% of mutant plants were dead at the beginning of the 300 mM NaCl treatment, while no significant death was observed for the WT plants.

Response of *Ljmyb36* mutants to an increase in light irradiance was also analyzed. A much higher proportion of dead plants were obtained when mutant plants were provided with 300 μmol m^{-2} s^{-1} light intensity for 56 days (Figure 6B). In fact, 60–80% of the mutant plants died as a consequence of this treatment, while only 10% of WT plants were affected. In contrast, only a minor proportion of dead plants could be detected under control standard illumination conditions.

Growth of WT and *Ljmyb36* mutants was comparatively examined at 20°C, 25°C and 30°C (Table 2). The results obtained indicated an important reduction of shoot and root growth in the mutant plants compared to the WT when grown at 20°C (standard growth conditions). However, when temperature was increased to 25°C and 30°C, a much lower growth rate was obtained for the mutants compared to the WT, depending on the temperature.

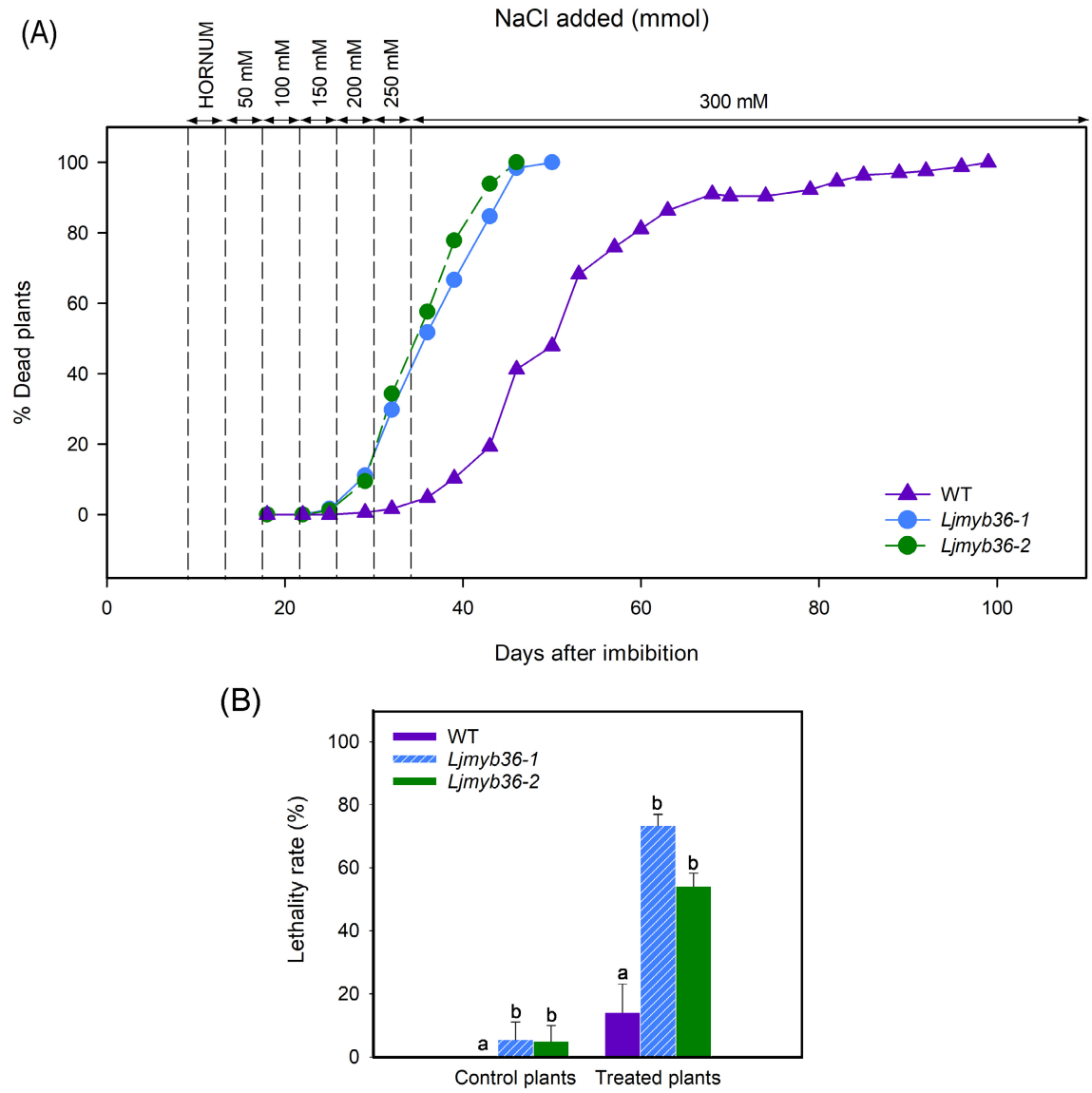

FIGURE 6 Response of the mutant lines to abiotic stress. (A) Lethality of WT and *Ljmyb36* mutant plants under NaCl stress treatment. The stepwise increase in total NaCl added to each pot is shown at the top. Values are the means ± SE of 180 plants per genotype. (B) Increase in the proportion of dead plants after 56 days of growth under high irradiance versus control conditions. Values are the means ± SE of 30 plants per genotype and treatment. Letters indicate significant differences ($P < 0.05$) between treatments according to ANOVA with a post-hoc Tukey HSD test.

TABLE 2 Changes in fresh weight of shoots and roots between WT and *Ljmyb36* mutants in response to temperature increase. Values are the means of 15 plants per genotype and treatment

	FW Shoots			FW Roots		
	20°C	25°C	30°C	20°C	25°C	30°C
WT	100	100	100	100	100	100
Ljmyb36-1	55	43	22	40	24	11
Ljmyb36-2	49	37	35	42	18	13

4 | DISCUSSION

The results shown in this paper indicate that in the model legume *L. japonicus*, two different knockout mutations of the *MYB36* gene lead to significant alterations in the isoflavonoid biosynthetic pathway. These results are in accordance with the previous identification of this gene within a co-expression network of isoflavonoid genes together with other potential regulatory genes from this plant. In this co-expression network, it was found that the *LjMYB36* gene is highly connected to the *LjHID4* (2-hydroxyisoflavanone dehydratase) and *LjI2′H2* (isoflavone-2′-hydroxylase) genes, both involved in the isoflavonoid pathway (García-Calderón et al., 2020). The fact that isoflavonoid levels are altered in the mutant plants suggests that the MYB36 TF is involved in the regulation of isoflavonoid biosynthesis. In addition, the results obtained clearly support a major role of isoflavonoids in growth and abiotic stress protection in this plant, since mutants show an important retardation in growth as well as early senescence

symptoms, and a higher sensitivity to saline, high irradiance and temperature stresses. Previous works have indicated that stress conditions increase the levels of isoflavonoids in *L. japonicus* WT plants (García-Calderón et al., 2015; Kaducová et al., 2019).

The observed alterations in the levels of different flavonoid compounds and precursors in *Ljmyb36* mutants (Supplemental Table 1) indicate that LjMYB36 is not only involved in isoflavonoid biosynthesis control, but also in the levels of several flavonols and other steps of the phenylpropanoid metabolism, thus revealing a crucial importance of this TF. In plants, flavonoids carry out many different functions like regulating cell growth, attracting pollinator insects and protecting against biotic and abiotic stresses (Agati et al., 2012; Liu et al., 2021; Winkel-Shirley, 2001). Previous works have described that flavonoids may effectively control key steps of cell growth and differentiation, thus acting by regulating the development of the whole plant and individual organs (Agati et al., 2012). Quercetin modulates root growth by limiting cell proliferation and enhancing the cell elongation phase. It is assumed that the meristematic region of the roots faces high limitations of cell proliferation under quercetin treatment (Silva-Navas et al., 2016; Singh et al., 2021). The hormone auxin plays an important role in defining root meristem size through formation of the PLETHORA (PLT) gradient that regulates cell proliferation. Treatment of plant roots with quercetin reduced this gradient along with the meristem size, so quercetin content regulates meristem size through regulation of the auxin-PLT gradient (Silva-Navas et al., 2016). Therefore, the lower growth rate and those growth defects observed in *Ljmyb36* mutant plants could be related with the deficiency in the MYB36 TF.

However, the results obtained indicate no clear connection between the deficiency of the *MYB36* gene and the nodule numbers in *L. japonicus*, since mutants are still able to nodulate in a similar pattern than the WT. Nevertheless, further studies of the infection process such as the quantification of the number of root hair infection threads and others are necessary to elucidate the involvement of this TF in the symbiosis process in *L. japonicus* plants. Recent work has been able to show that different phenolic acids, particularly ferulic acid, but not (iso)flavonoids, are a novel type of *NOD* gene inducers in *L. japonicus-Mesorhizobium* symbiosis (Shimamura et al., 2022). Since the levels of ferulic and other phenolic acids were found not to be altered in *Ljmyb36* mutants, all these results could explain why the nodule number of the mutant plants may not be significantly affected in these mutants.

Different TFs have been identified to regulate the phenylpropanoid pathway in higher plants, such as MYB, bZIP, WRKY, MADS box and WD40. Plant MYB proteins can be classified into four subfamilies according to the DNA-binding domains: the R1-MYB related group (one single domain), the R2R3-type group (two domains), the R1R2R3-MYB group (three domains) and a minor subfamily of MYB genes carrying four domains (Campos et al., 2016). A high number of genes encoding R2R3-MYB TFs have been identified in plants (Paz-Ares et al., 1987; Pucker et al., 2020; Stracke et al., 2001; Zheng et al., 2017). These TFs are the most abundant type and constitute one of the largest transcription factor families being involved in the regulation many biological processes like cell shape and identity, developmental control, secondary metabolite biosynthesis, hormone signal transduction and response to abiotic stresses and pathogen defense (Zheng et al., 2017). In legumes, less than 1% of these have been characterized genetically, although TFs likely played seminal roles in legume evolution and clearly now play crucial roles in plant development and differentiation (Udvardi et al., 2007). For this reason, it is of paramount importance to elucidate the role of every single TF in legume plants like in this study. In *L. japonicus* the involvement of different subgroups of R2R3MYB TF that act coordinately to induce the flux to isoflavonoids and/or reduce the flux of metabolites through competing branches of phenylpropanoid metabolism has been shown (Shelton et al., 2012). However, MYB36 TF has not been analyzed so far in this plant.

Different roles of the *MYB36* gene have been described in several works. Liberman et al. (2015) showed that the MYB36 TF from Arabidopsis plants activated genes involved in Casparian strip formation and represses genes involved in proliferation, suggesting that MYB36 is a critical regulator of developmental timing in the root endodermis by promoting the transition from proliferation to differentiation. Other authors demonstrated that MYB36 directly and positively regulates the expression of the Casparian strip genes CASP1 (Casparian strip domain proteins), PER64 (Peroxidase 64) and ESB1 (Enhanced Suberin 1) in Arabidopsis (Hosmani et al., 2013; Kamiya et al., 2015). ESB1 was suggested to mediate bimolecular coupling during the lignin biosynthesis (Hosmani et al., 2013). This sparks an intricate developmental program, in which the transcription factor MYB36 plays a role in the synthesis and deposition of lignin between endodermal cells (Li et al., 2018; Kamiya et al., 2015). Likewise, another study identified *PpMYB36* as a regulator involved in lignin accumulation and russet coloration in *Pyrus pyrifolia* (Ma et al., 2021). Furthermore, MYB36 plays a critical role in restricting cell division at larger stages of lateral root primordia development by regulating a subset of PER genes, and perhaps other genes that modulate ROS balance and cell proliferation potential (Fernández-Marcos et al., 2016). In addition, through a new network with connected genes related to anthocyanin synthesis, a high co-expression between *VvMYB36* with *bHLH041* and *WRKY75* was found, regulating four PAL genes and one 4CL gene (Zhong et al., 2022). However, effects in (iso)flavonoid metabolism, growth and stress responses of MYB36 mutants have not been described.

The results shown in the present paper indicate that LjMYB36 is involved in controlling phenylpropanoid metabolism and, particularly, (iso)flavonoid biosynthetic pathways. We establish a major role of MYB36 in the biosynthesis of phenolic compounds and growth of *L. japonicus* plants. Consequently, MYB36 is a new interesting TF identified that can be added to the complex picture of TFs involved in the cooperative and homeostatic regulation of the biosynthesis of flavonoid and other phenolic compounds such as lignin, which constitute a very interesting topic of research (Shi et al., 2022; Zhang et al., 2021). In addition, it has been also reported that the MYB36 TF could be a key TF that may drive floral integrators for flowering time regulation in orchids (Ahmad et al., 2022). All these studies emphasize the importance of the MYB36 TF in plants.

Many reports have described that MYB TFs are involved in response to abiotic stress in different plants (Ambawat et al., 2013; Jung et al., 2008; Zhang et al., 2011). In several works, different MYB TFs were up-regulated by stress conditions and different approaches have demonstrated the role of MYB genes in response to abiotic stress (An et al., 2018; Du et al., 2022; Wang et al., 2021; Yang et al., 2012; Zhou et al., 2022). Nevertheless, there are no works describing a possible role of the *MYB36* gene in response to stress conditions in plants. In this paper we describe the high sensitivity of two lines of mutant plants deficient in the *MYB36* gene with regard to different stress conditions and the alterations obtained in the isoflavonoid levels, suggesting a key role of this TF controlling the response to abiotic stress in *L. japonicus* plants. Measurements of several enzymatic and non-enzymatic antioxidant systems such as catalase, ascorbate peroxidase, total peroxidases activities, as well as proline accumulation, have been performed (data not shown), indicating that *Ljmyb36* mutant plants have no lower levels of these parameters compared to WT plants under either control or stress conditions that may explain the high susceptibility of these plants to abiotic stress. In addition, chlorophyll content is definitely lower in *Ljmyb36* mutant plants from *L. japonicus* compared to the WT (Figure 5) in accordance with chlorophyll fluorescence measurements (not shown). Other works have reported chlorophyll-deficient soybean plants with a concomitant reduction in isoflavone levels (Mikuriya et al., 2017; Shiroshita et al., 2021). Also interestingly, a recent work in cucumber has reported that *Csmyb36* mutants have also lower chlorophyll content concluding that CsMYB36 may regulate the yellow-green coloration in cucumber by interacting and/or changing the expression levels of a pigment protein gene and other genes (Hao et al., 2018).

Although the MYB TF family is very large, the results obtained in this work suggest that there is no other TF that is able to compensate for the function of the *LjMYB36* gene when it is missing. No other MYB TF is affected in *Ljmyb36* mutant plants, as demonstrated by genotyping analysis. Finding that MYB36 is a regulator of isoflavonoid biosynthesis, in agreement with previous co-expression networks developed, it constitutes an important advance for the future knowledge of why legumes produce isoflavonoid compounds, particularly in response to stress. All these results can be very important for future projects aimed to attempt to increase growth and stress protection in legumes.

AUTHOR CONTRIBUTIONS

M.D.M-R carried out most of the experimental work and made the Figures. P.P-B and K.T. performed the quantitative analysis of flavonoids and isoflavonoids in plant samples. M.G-C, M.B. and A.J.M supervised the experimental work. All authors contributed to experimental design, discussion of results and the writing of the manuscript.

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DATA AVAILABILITY STATEMENT

All data supporting the findings of this study can be found within the manuscript and its supporting information.

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