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Contribution of the regulatory miR156-SPL9 module to the drought stress response in pigmented potato (*Solanum tuberosum* L.)

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ABSTRACT

Potato (*Solanum tuberosum* L.) is nowadays an important component of diversified cropping systems due to its adaptability, yielding capacity, and nutrition contribution. Breeding programs aiming at raising potato's nutritional value have mainly focused on the accumulation in potato tubers of health-promoting phytochemicals such as anthocyanins. In different plant species, increased amounts of anthocyanins in vegetative tissues have been associated with enhanced tolerance to abiotic and biotic stresses that challenge agrifood systems in the current context of global climate change. In the present study, we aimed at gaining insight into the effect of anthocyanin accumulation on the potato plants response to drought stress using three different potato genotypes with differential canopy and tuber pigmentation: the purple fleshed commercial variety Bleuet; the red fleshed breeding clone DAR170; and the non-pigmented commercial variety Monalisa. The varieties Bleuet and DAR170 exhibiting higher anthocyanin content in vegetative tissues than the Monalisa variety showed a remarkable inhibition of stem growth development under drought stress treatment suggestive of an anthocyanin-mediated physiological shift from growth to resilience as a mechanism of stress tolerance. The results of the expression analysis of stu-miR156a and its target *StSPL9* gene in the potato plants with different anthocyanin content, as well as their change in response to drought stress support the participation of the conserved miR156-SPL9 regulatory module in coordinating potato plants development and plant responses to drought stress, involving precise fine-tuning of anthocyanin biosynthesis.

1. Introduction

The cultivated potato (*Solanum tuberosum* L.) is produced in approximately 140 countries for a total reported estimated harvested area of over 18 million of hectares and an estimated production of over 376 million tons (IBM Corp., 2019; https://www.fao.org/faostat/en/#data/QCL). It is the third most important staple food after rice and wheat for human nutrition (Bethke et al., 2017), and breeding efforts have aimed at obtaining cultivars with diverse characteristics, coupling good productivity with the best adaptation to different environments and response to biotic and abiotic stresses. Tetraploid varieties ($2n = 4x = 48$) with high productivity constitute the core of modern potato breeding, despite the complex tetrasomic inheritance (Bradshaw, 2007).

The climate change scenario mainly comprising increasing temperatures as consequence of global warming associated with long periods of drought impacts crops performance, yield and consequently, food security. Potato is especially susceptible to climate change-induced stresses, either abiotic, like heat and drought, and biotic (e.g. differences in pest distribution and/or host interactions, for a review see Lal et al., 2022) with temperature being a key factor for potato production. These stresses are known to impair tuberization process and tubers growth, thus affecting potato yield. Indeed, potato cultivation is hampered by water shortage (Demirel, 2023), and requires narrow optimal temperatures for both vegetative growth and tuber bulking. Therefore, it is easy to foresee the consequences of climate changes occurring in temperate areas on its productivity being yield losses linked to the stress severity, its duration and potato developmental stage (Nasir and Toth, 2022). Hijmans and coworkers (Hijmans, 2003) using LINTUL-potato model predicted that climate changes related abiotic and biotic stresses could

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lead to over 30% yield loss worldwide by 2050. With other potato crop simulation models, yield losses can vary according to the potato varieties, region of origin and type of climate data available (for a review, see Adekanmbi et al., 2024).

Potato's nutritional value has long been neglected, and it has been recently acknowledged that this crop is not only a valuable source of starch, vitamin C and fibres, but also of proteins, potassium, unsaturated fatty acids, and well characterized phytochemicals polyphenols and carotenoids with antioxidant properties (Zaheer and Akhtar, 2016). In recent years, breeding programs aimed at raising potato's nutritional value have been mainly focused on the accumulation in the tubers of carotenoids and anthocyanins (Brown et al., 2003). Since carotenoids and anthocyanins exhibit antioxidant activity, their enhanced levels in the tubers have been considered a desirable trait.The secondary metabolites levels and composition in the potato tubers are genetically determined; however, developmental, tissue specific and environmental cues also act as key players (Bassolino et al., 2022).

Anthocyanins conferring colourful phenotypes to flowers and fruits are widely distributed in land plants and comprise one of the best studied pathways including multiple sub-classes derived from common precursors and/or enzymes (Bassolino et al., 2022; Holton and Cornish, 1995). The biosynthesis of anthocyanins (e.g., delphinidin and pelargonidin derived pigments) and its regulation in potato tubers and vegetative organs has been elucidated (Liu et al., 2023; Strygina et al., 2019). In potato, both early and late biosynthetic enzymes encoding genes leading to colourless dihydroflavonols and coloured anthocyanins, respectively, were identified (André et al., 2009; De Jong et al., 2003; Stushnoff et al., 2010). In addition, among the transcription factors (TFs) belonging to MYB and bHLH sub-families that play regulatory roles in the conserved mechanism governing anthocyanins synthesis in dicots (Bassolino et al., 2022), the MYB gene *S. tuberosum ANTHOCYANIN 1* (*StAN1*) (also annotated as *StAN2*) is recognised as a master regulator of anthocyanins accumulation both in tubers and in vegetative tissues (D'Amelia et al., 2014; De Jong et al., 2003).

Small RNAs also regulate the accumulation of flavonoid metabolites by targeting transcription factors and rate-limiting enzymes that act in the flavonoid biosynthesis pathway, both at transcriptional and post-transcriptional level (Deng and Lu, 2017; Yang et al., 2022). Early reports demonstrated the regulatory role of miR156 and miR828 on the phenylpropanoid pathway in *Arabidopsis* (Gou et al., 2011; Luo et al., 2012), and in tomato with respect to miR858 (Jia et al., 2015). In potato tubers, strong association between miR828 and the purple colour of tuber tissues suggested the involvement of this miRNA in phenylpropanoid accumulation by targeting R2R3 MYBs (Bonar et al., 2018). Recent analysis based on small RNA and degradome sequencing identified differentially expressed miRNAs and their target genes involved in anthocyanin synthesis during different developmental stages in purple potato, also demonstrating that stu-miR156 was involved in anthocyanin biosynthesis by targeting StSPL9 (Squamosa Promoter Binding Protein-Like 9) (Wu et al., 2022). Further, Qi et al. (2023) performed a comparative transcriptome analysis of four potato varieties exhibiting different tuber colour and anthocyanin content in tuber tissues. They identified six genetic isoforms of stu-miR156 that may influence potato tuber anthocyanin content via regulating StSPL6 and StSPL12 TFs levels and found that miR858 was expressed differently in each variety, suggesting that anthocyanin synthesis regulation might be genotype specific.

The SPL plant transcription factor family is characterized by a highly conserved SBP-box domain and plays an important role in plant growth, development, and stress tolerance (Jerome Jeyakumar et al., 2020). Previous studies have shown that SPL9 under the negative control of miR156 represses anthocyanin biosynthesis through interfering with the formation of the MYB-bHLH-WD40 complex by directly interacting with R2R3 MYBs (Cappellini et al., 2021). It has been proposed that in plants undergoing salt and drought stress, in which miR156 was induced and SPL9 was repressed, the anthocyanin biosynthesis pathway was activated (Cui et al., 2014).

Anthocyanins, as for other secondary metabolites, can be generally conceived as plant response molecules to environmental challenges. Indeed, drought-induced anthocyanin synthesis is mainly related to their role as osmoregulators and radical oxygen species (ROS) scavenging ability and protection against photo-oxidative stress (Landi et al., 2015), although the physiological function of this response is still an unsolved debate. Several studies suggested a positive correlation between anthocyanin accumulation and drought stress tolerance (reviewed in Naing and Kim, 2021) and higher anthocyanin amounts are associated to higher tolerance to drought (Naing et al., 2017, 2018).

In the present work, aimed at gaining insight into the anthocyanin mediated tolerance to drought stress in three potato genotypes of *S. tuberosum* characterized by different anthocyanin content, we analysed the stress response of stu-miRNA156a and its target *StSPL9* gene under drought conditions.

2. Material and methods

2.1. Plants

Three tetraploid *S. tuberosum* genotypes exhibiting different tuber flesh colour and canopy pigmentation were used in the experiments: clone DAR170 belonging to CREA-Research Centre for Cereal and Industrial Crops (CREA-CI) Bologna breeding programme (red fleshed tuber, pigmented canopy) and the commercial varieties Bleuet (purple fleshed tuber, pigmented canopy) and Monalisa (yellow fleshed tuber, non-pigmented canopy). Plants by Ø 35–40 mm virus-free and no cut tuber-seed were grown in plastic round pots filled with potting substrate for horticulture under greenhouse conditions at day and night temperatures of 22 ± 4 °C and 20 ± 4 °C, respectively, with 16/8 h (light/dark) and 60% relative humidity. No chemical treatments on the potato plants were applied during the experiment, pests management and diseases.

2.2. Anthocyanin extraction

Anthocyanin extraction was performed as described by Jia et al. (2015). Fresh stems of potato plants of the different varieties were weighed and homogenized in 3 mL of 1% acidic methanol (1% HCl v/v). The mixture was incubated overnight at 4 °C under constant agitation. To remove chlorophylls, 3 mL of chloroform and 2 mL of double-distilled water were added. Following centrifugation (14000×*g* for 15 min), the upper aqueous phase was collected, and spectrophotometric measurements were taken at 530 nm (A_{530}) and 657 nm (A_{657}) with a Genesys 10S UV–Vis spectrophotometer (ThermoScientific, MA, USA). The total amount of anthocyanins was calculated as (A_{530}-A_{657})/g fresh weight. Mean values were calculated from three independent values.

2.3. Drought stress

In drought stress experiments, watering was automatically controlled using Ridder MultiMa irrigation regulation system (Ridder). Four 15-day old potato plants of each of the three different genotypes were deprived of water for 18 days and four additional plants of each genotype were kept irrigated throughout as control. For each plant in every group, the plant height and number of stems was recorded and determinations of net stomatal conductance (transpiration rate, mol H_2O m^{-2} s^{-1}) and photosynthetic CO_2 assimilation rate (μmol CO_2 m^{-2} s^{-1}) were performed using a portable photosynthesis system LICOR 6400 (LI-COR, Lincoln, NE, USA). In addition, leaf tissue from 3rd and 4th leaves was collected from each plant in every group, and frozen in liquid Nitrogen.

2.4. Total RNA isolation and cDNA synthesis

Total RNA was isolated from potato plant leaves using the Plant/

Fungi Total RNA Purification kit (Norgen Biotek, Ontario, Canada). TURBO DNA-free kit (Ambion, Cat. No. AM1907) was used to remove contaminating genomic DNA, and RNA quality was evaluated by 1% agarose gel electrophoresis and quantified spectrophotometrically (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA).

Total RNA was retrotranscribed to complementary DNA (cDNA), using the PrimeScript™ kit (Takara, Kusatsu, Japan) following the manufacturer instructions.

2.5. miRNA amplification

For miRNA amplification, 1 g of total RNA was polyadenylated using 1 μL of 10x poly(A) polymerase buffer, 1 mM of ATP, and 1 unit of poly(A) polymerase (New England Biolabs, Ipswich, MA, USA), and incubated at 37 °C for 15 min and then at 65 °C for 20 min. Polyadenylated RNA was reverse transcribed to complementary DNA (cDNA) using the Universal RT-primer (Integrated DNA Technologies, Coralville, IA, USA) described in Balcells et al. (2011) (5′-CAGGTCCAGTTTTTTTTTTTTTTTVN-3′, where V is A, C, and G, and N is A, C, G, and T). Reverse transcription reaction was performed using PrimeScript™ RT reagent Kit (Takara, Kusatsu, Japan) according to manufacturer protocol.

2.6. RT-qPCR analysis

The StepOnePlus Real-Time PCR system (Applied Biosystems, MA, USA) was used to analyse gene expression, following the steps recommended by the manufacturer. The sequences of the primers used (Condalab) are shown in Table S1. The cycling parameters were as follows: 95 °C for 30 s to activate the polymerase, 40 cycles of denaturation at 95 °C for 5 s and extension and hybridization at 60 °C for 30 s. The analysis was performed using the mean value of three technical replicates for each of the four biological replicates. The relative expression of each gene was calculated using as reference gene the ribosomal protein *StRPS18* gene (GenBank: 3950409).

To analyse the expression of miRNAs, the same procedure described above for the genes was followed. The reference gene used to calculate relative miRNA expression was the *StU6* snRNA gene of *S. tuberosum* (GenBank: X51447.1).

LinRegPCR software (Ruijter et al., 2009) was employed for the analysis of RT-qPCR data.

2.7. Statistical analysis

Four biological replicates were used in all experiments. The data were analysed using IBM SPSS Statistics (version 26.0). One-way analysis of variance (ANOVA) test was used to compare differences among groups, followed by Duncan's multiple range test for post hoc comparisons. Normality and homoscedasticity of data was assessed using a Shapiro-Wilk's and a Levene's test, respectively. If the assumption of normality was not met, the Kruskal–Wallis nonparametric test for independent samples with Bonferroni correction was performed. When homoscedasticity was not fulfilled, Welch test for independent samples with unequal variances followed by Dunnet's T test was used. Comparisons between two groups were carried out using Student's t-test. In all cases, $p < 0.05$ values were considered statistically significant.

2.8. Bioinformatic analysis

The bioinformatics software MATCH 1.0 (http://gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) was used to identify transcription binding sites in the *Solanum tuberosum SPL9* gene sequence (PGSC_DMv3_219_ch10:43832152.43837763) using default parameters.

3. Results and discussion

3.1. Drought stress response of potato genotypes with different anthocyanin content

Anthocyanin accumulation in the vegetative parts of potato plants and their response to drought stress was investigated among three tetraploid genotypes: 1) the red fleshed breeding clone DAR170 (pigmented canopy), 2) the commercial purple fleshed variety Bleuet (pigmented canopy), and 3) the commercial light-yellow fleshed variety Monalisa (non-pigmented canopy) (Fig. 1A). Accordingly with the presence of colour in the tuber flesh, the amount of anthocyanin in stems was higher in the Bleuet and DAR170 than in the Monalisa plants, although no statistically significant differences in the anthocyanin content between the two pigmented genotypes were found (Fig. 1B).

The three potato plants subjected to 18 days of water deprivation showed a slight loss of leaf turgor compared to well-watered control plants. Therefore, this time point was selected to compare the genotype-specific potato plant response to drought. The stem height of Monalisa subjected to water scarcity was only slightly decreased compared to control plants (Fig. 2A and B). In contrast, the two anthocyanin enriched potato genotypes exhibited a significantly reduced growth under drought stress treatment (Fig. 2A and B). The number of stems was reduced under water deprivation in all three potato genotypes, but this difference was only statistically significant in the Bleuet and DAR170 genotypes (Fig. 2C).

Both transpiration rate and CO_2 assimilation rate were reduced as a consequence of drought stress in all three genotypes (Fig. 2D and E) indicating a common avoidance strategy to cope with water deficit based on closing stomata to limit transpiration, which in turn, by restricting the CO_2 supply, leads to a lower photosynthetic output thus, decreasing the canopy growth (Nasir and Toth, 2022). No statistically significant differences were observed among transpiration rates of the three potato genotypes in well-watered or water-deprived plants. Despite that a reduction of leaf transpiration rate upon drought stress is a consistent observation in the literature, in some studies differences in transpiration rates among well-watered potato genotypes were reported whereas in others no differences among the genotypes chosen were found (Gervais et al., 2021; Salehi-Soghadi et al., 2023) suggesting that differences in transpiration rate are genotype-dependent. Regarding CO_2 assimilation, agreeing with the differences detected in the average stem height of the two anthocyanins enriched genotypes, a significant higher CO_2 assimilation rate was also observed in Bleuet and DAR170 compared to non-pigmented Monalisa genotype, under well-watered and water-deprived conditions (Fig. 2B–E).

In transgenic tobacco plants overexpressing the potato *StAN1* gene, increased anthocyanin content in plant leaves correlated with higher CO_2 assimilation rates and lower dry biomass reduction compared to wild-type plants when subjected to drought stress, suggesting higher plant resilience under water scarcity (Cirillo et al., 2021). Interestingly, Tattini et al. (2017) reported higher photosynthetic performance in cyanic than in acyanic basil leaves exposed to excess light due to blue light attenuation by epidermal anthocyanins evoking shade avoidance response (SAR). SAR is an adaptive response of plants growing within dense vegetation triggered by changes in light quality and quantity caused by the proximity of other plants (Sessa et al., 2018). Stem elongation, reduced branching, hyponastic leaf orientation, early flowering and accelerated senescence, are collectively termed the shade avoidance syndrome (SAS) (Liu et al., 2021). Xie et al. (2017) described that SAS in *Arabidopsis* is being enhanced by phytochrome-interacting factors direct suppression of *MIR156* expression, which promote the increased expression of miR156-targeted Squamosa Promoter Binding Protein-Like (SPL) family of genes involved in plant development.

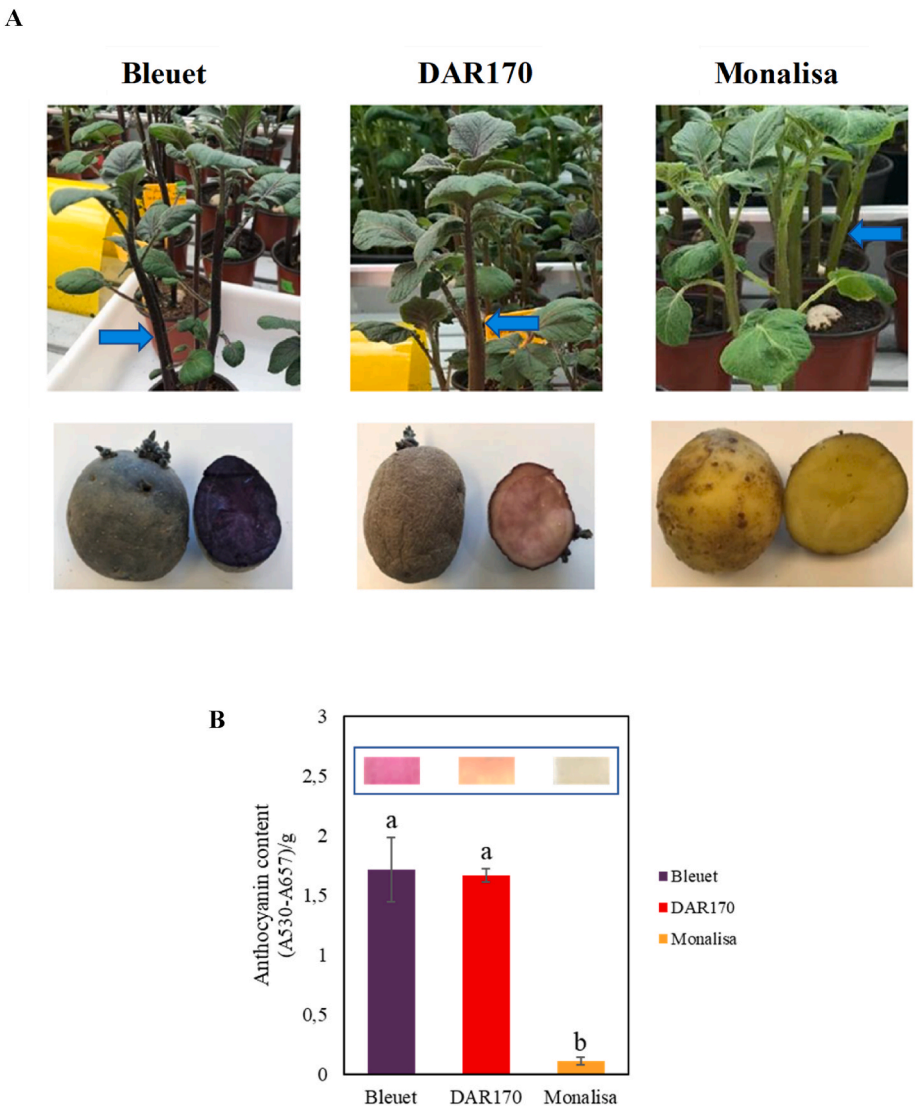

Fig. 1. (A) Images of potato tubers and vegetative parts of Bleuet, DAR170 and Monalisa genotypes. Blue arrows point to the plants stems exhibiting distinct coloration. (B) Anthocyanin content in the stems of each potato plant genotype. The upward inset shows the upper aqueous phase of the anthocyanin extraction of the corresponding samples. The data shown are the means of at least three independent replicates ± standard deviation (SD). Means with the same lowercase letter are not significantly different (one-way ANOVA test for independent samples followed by Duncan multiple range test, $p < 0.05$).

3.2. Expression analysis of stu-miR156a and its target gene StSPL9 in potato plant varieties with different anthocyanin content undergoing drought stress

The conserved miR156/SPLs regulatory module among plants controls vegetative phase change, flowering, branching/tillering and leaf initiation (Wang and Wang, 2015) and participates in stress responses and biosynthesis of secondary metabolites, including anthocyanins (Jerome Jeyakumar et al., 2020). *Arabidopsis* plants undergoing salt or drought stresses, displayed induced expression of miR156 genes (*MIR156A* and *MIR156C*), which seemed to maintain plants in the juvenile stage for longer time, by downregulation of SPLs genes expression and enhanced anthocyanin accumulation (Cui et al., 2014). Hence, miR156-SPLs coordinate development and abiotic stress response by affecting anthocyanin synthesis (Zheng et al., 2019). To gain insight into the role of the miR156-SPLs regulatory module in the potato plants response to water deficit, in the present work, we analysed stu-miR156a and *StSPL9* gene expression by RT-qPCR in leaves of the Bleuet, DAR170 and Monalisa genotypes (Fig. 3).

In control plants, higher amounts of stu-miR156a in Bleuet and DAR170 compared to the Monalisa variety inversely correlated with the StSPL9 transcript levels (lower in Bleuet and DAR170 and higher in Monalisa). This indicates involvement of potato miR156a-SPL9 regulatory module in anthocyanin biosynthesis in potato vegetative tissues, as previously reported in purple potato tubers (Wu et al., 2022). Very recently, Luo et al. (2024) demonstrated that in potato transgenic plants overexpressing Stu-miR156, *StSPL9* expression levels were downregulated, and confirmed that the miR156/SPLs module is involved in the regulation of potato plant height and root growth. Moreover, as described in *Arabidopsis*, induction of stu-miR156a expression and downregulation of *StSPL9* gene expression was observed in all three genotypes under drought stress. However, despite substantial difference in the drought-induced stu-miR156a levels among the three genotypes, *StSPL9* showed similar extent of downregulation (Fig. 3). As reported in *Arabidopsis* (He et al., 2018), it is likely that there is a threshold-dependent repression of *SPL* gene expression by miR156 explaining a non-linear response in stu-miR156a-mediated StSPL9 levels under drought stress. Delaying the transition from the juvenile vegetative to reproductive-competent adult vegetative phases through miR156-mediated SPLs function might be an important strategy for

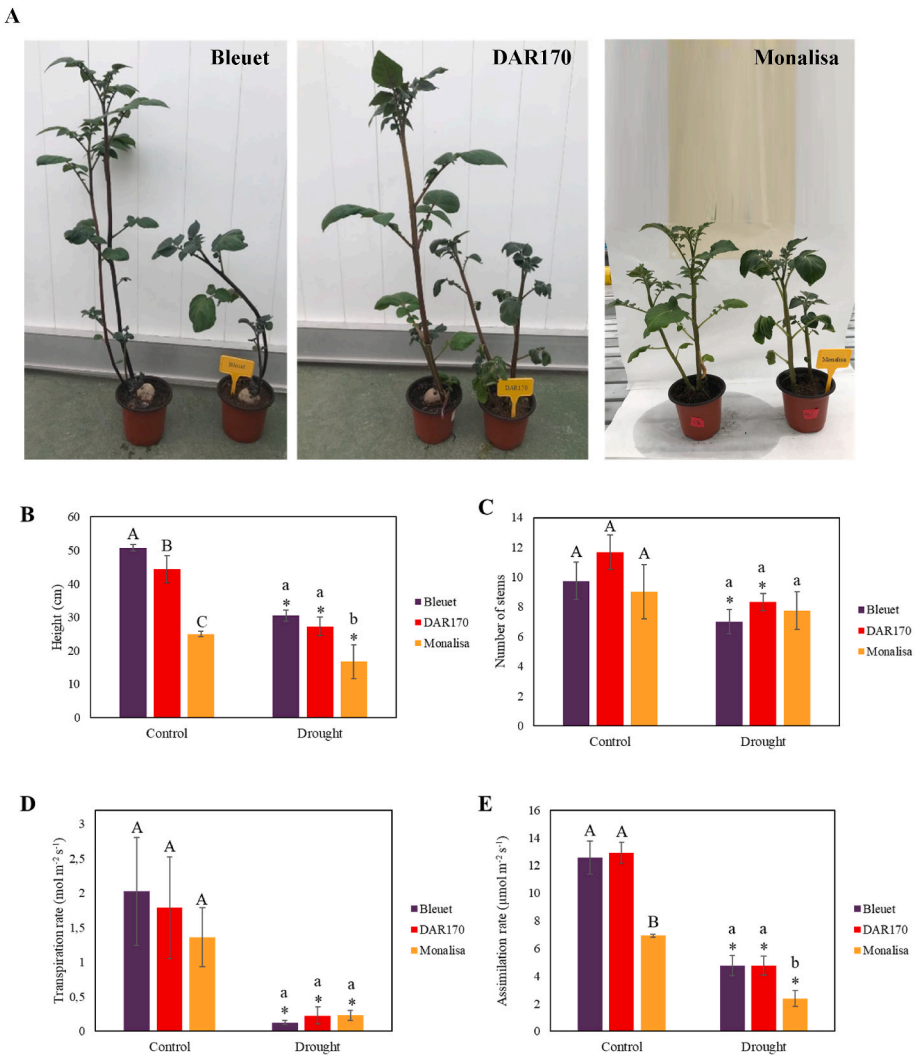

Fig. 2. (A) Bleuet, DAR170 and Monalisa potato plants in control condition (left) and drought stress (right). Bar charts represent the height (B), number of stems (C), transpiration rate (D) and CO_2 assimilation rate (E) of each genotype in both conditions (control and drought). The data shown are the means of at least three independent replicates ± standard error (SD). For each condition (control and drought), differences among the three genotypes were analysed by one-way ANOVA test for independent samples followed by Duncan multiple range test, $p < 0.05$ or Kruskal-Wallis with Bonferroni correction for the height. Means with the same letter (capital letters for control condition and lowercase letters for drought condition) are not significantly different. For each of the three genotypes, differences between conditions (control and drought) were analysed by Student's *t*-test. Asterisks indicate significant differences obtained between conditions ($p < 0.05$).

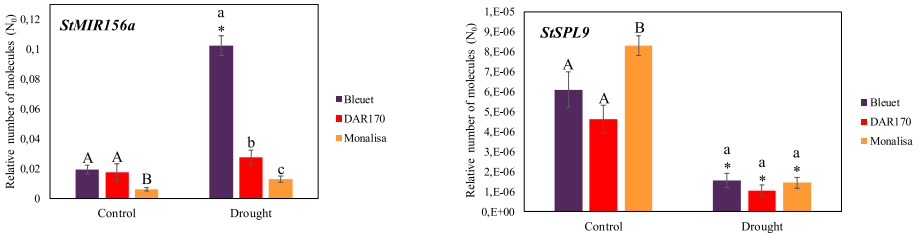

Fig. 3. RT-qPCR analyses of stu-miR156a and its *StSPL9* target gene in leaves of potato plants of the three genotypes in control and drought stress conditions. The data shown are the means of at least three independent replicates ± standard deviation (SD). For each condition (control and drought), differences among the three genotypes were analysed by one-way ANOVA test for independent samples followed by Duncan multiple range test, $p < 0.05$ (stu-miR156a) and Welch test for independent samples with unequal variances followed by Dunnet's T test, $p < 0.05$ (*StSPL9*). Means with the same letter (capital letters for control condition and lowercase letters for drought condition) are not significantly different. For each of the three genotypes, differences between conditions (control and drought) were analysed by Student's *t*-test. Asterisks indicate significant differences obtained between conditions ($p < 0.05$).

potato plants to cope with drought stress and to relocate important stress tolerance resources. Under adverse conditions, the role of miR156 might be more complex than simply switching on or off the expression of SPLs since it is also central to abiotic stress memory in plants (Charng et al., 2023).

Alternative mechanisms of *SPL9* repression (other than via miR156 control) might also participate in the *StSPL9* downregulation observed among potato genotypes undergoing drought stress. Upregulation of

SLP9 expression is also influenced by MYB33 TF as reported in *Arabidopsis* (Guo et al., 2017) as well as in potato where it has been shown to be involved in the response to water shortage too (Wyrzykowska et al., 2022). Using Match 1.0, a tool for the identification of TF binding sites, a putative binding sequence of a c-Myb TF was predicted in exon 1 (position 92–109) in the potato *StSPL9* gene (Fig. 4A). Interestingly, the expression analysis of StMYB33 by RT-qPCR (Fig. 4B), showed a significant downregulation of *StMYB33* under water deficit conditions in all three genotypes, which might also account for the *StSPL9* gene repression (Fig. 3). However, further research is needed to validate this hypothesis and confirm whether under drought stress, StMYB33 might be acting as a modifier of vegetative phase change in potato plants.

3.3. Regulation of anthocyanin biosynthesis in potato plants with different anthocyanin content undergoing drought stress

Mitigation of drought stress by anthocyanin accumulation has been reported in several plant species where its accumulation correlated with anthocyanin genes upregulation (Kaur et al., 2023). In *Solanaceae* plants, dihydroflavonol reductase (DFR) enzyme reduces dihydroflavonols to their respective colourless leucoanthocyanidins, which are then converted into coloured anthocyanidins (e.g., cyanidin, pelargonidin, and delphinidin) by anthocyanidin reductase (ANS) enzyme. Both enzymes encoding genes are regulated by a MYB-bHLH-WD40 transcriptional activation complex which includes the R2R3-MYB transcription factor StAN1 in potato (Bassolino et al., 2022). *StAN1* gene is highly expressed in anthocyanin-pigmented potato tubers and displayed a positive correlation with the transcript levels of anthocyanin synthesis structural genes (Liu et al., 2021). To gain more insights, the expression of the two structural genes *StDFR* and *StANS*, as well the St*AN1* regulatory gene was analysed by RT-qPCR, in leaves of the Bleuet, DAR170 and Monalisa (Fig. 5).

Results demonstrated genotype-specific regulation of the anthocyanin biosynthetic pathway under water shortage, with the DAR170 and Monalisa displaying significant downregulation of *StDFR*, *StANS* and *StAN1* genes under drought stress, unlike the Bleuet variety (Fig. 5). Downregulation of genes associated with phenylpropanoid and carotenoid biosynthesis has also been reported in potato tubers in response to drought (Da Ros et al., 2020), but it is known that the impact depends upon the severity of drought, stage of plant development, and susceptibility of the genotype to drought stress (Nasir and Toth, 2022). Intriguingly, the repression of *StAN1* gene that we have detected in drought-stressed potato plants (Fig. 5) might overrule the putative positive effect on anthocyanin biosynthesis due to downregulation of *StSLP9* in water-deficient plants (Fig. 3). As the sharp downregulation of *StSLP9* gene might be crucial for delay in vegetative phase change transition in response to drought stress (He et al., 2018; Poethig and Fouracre, 2024), it seems likely that regulatory counteractions (e.g., *StAN1* gene repression) might be activated to prevent the detrimental effects associated with excessive anthocyanin accumulation. In line with this, Steyn et al. (2002) argued that accumulation and maintenance of anthocyanins carries an energy cost, may reduce light capture and ultimately carbon assimilation. Therefore, they put forward that plant acclimatation to new conditions may entail the replacement of anthocyanins by physical, photosynthetic or metabolic adjustments to re-establish homeostasis between the plant and the environment, pointing to a role of anthocyanin accumulation during developmental or environmental changes as a transitory strategy.

The miR156 clade is extremely well conserved among flowering plants and studies in many different plant species have shown that miR156 levels change in response to abiotic stress (Jerome Jeyakumar et al., 2020). The induction of miRNA156 expression, specifically during the recovery period after heat stress, was found crucial for conveying regulation of plant thermotolerance to recurring stress through targeting

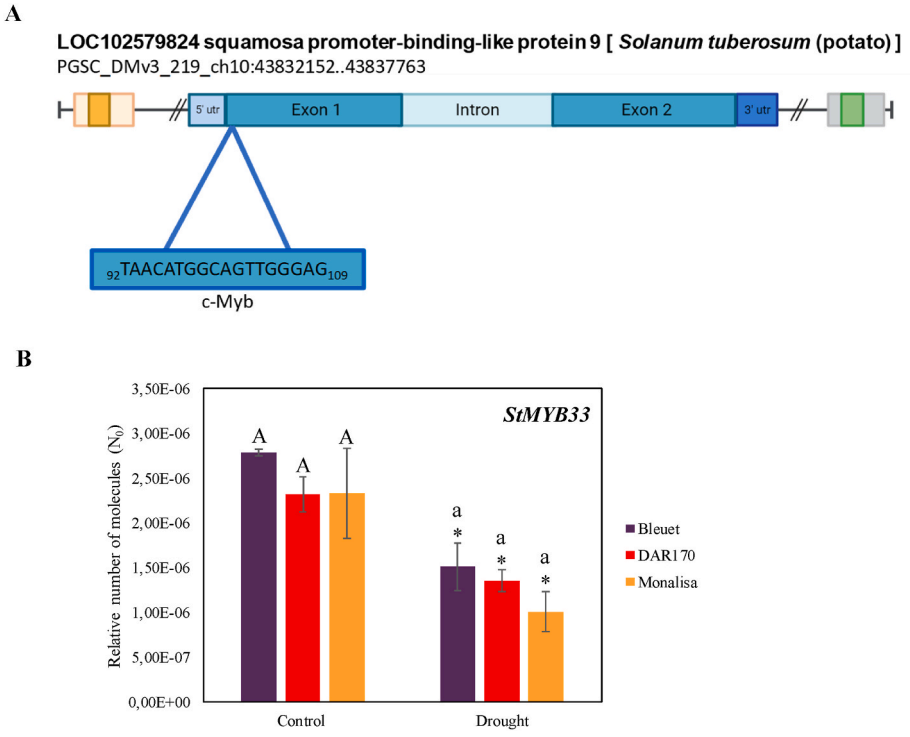

Fig. 4. (A) Putative binding sequence of a c-Myb TF, located in exon 1, position 92 in the *StSPL9* gene of *Solanum tuberosum* (analysis performed using Match 1.0). (B) RT-qPCR analysis of *StMYB33* gene expression in leaves of control and drought-stress treated plants of the three potato genotypes. The data shown are the means of at least three independent replicates ± standard deviation (SD). For each condition (control and drought), differences among the three genotypes were analysed by one-way ANOVA test for independent samples followed by Duncan multiple range test, $p < 0.05$. Means with the same letter (capital letters for control condition and lowercase letters for drought condition) are not significantly different. For each of the three genotypes, differences between conditions (control and drought) were analysed by Student's *t*-test. Asterisks indicate significant differences obtained between conditions ($p < 0.05$).

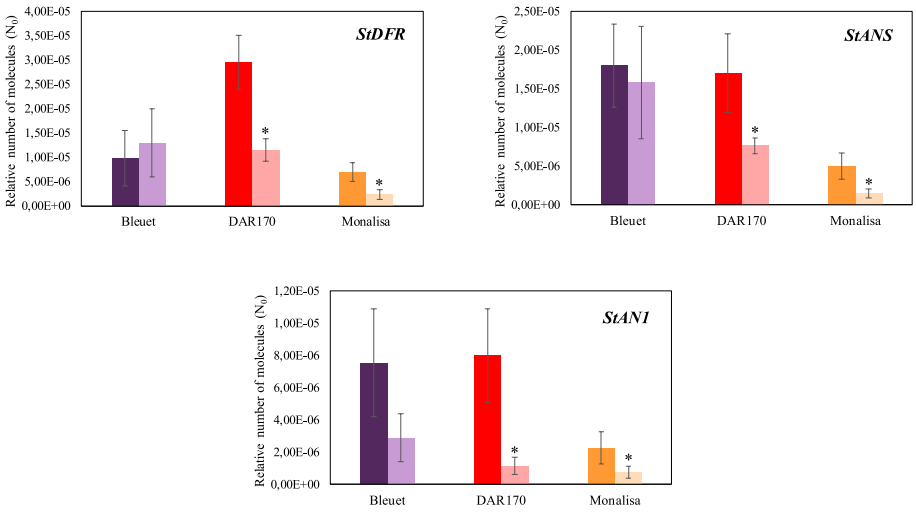

Fig. 5. RT-qPCR analysis of *StDFR*, *StANS* and *StAN1* gene expression in leaves of potato plants of the three genotypes in control (dark coloured) and drought stress (light coloured) conditions. The data shown are the means of at least three independent replicates ± standard deviation (SD). Asterisks indicate significant differences obtained by Student's *t*-test between control and drought-stressed plants of each genotype ($p < 0.05$).

the SPL family of transcription factors (Brown et al., 1993; Charng et al., 2023). To address concerns regarding potato crop resilience in the context of climate change, future work would be needed to identify the role of miR156/SPL9 module and other miR156-SPL targets in the plant recovery after drought stress and understand how plant stress memory mechanisms interlocked by miR156 integrate stress response and development.

4. Conclusions

Collectively, the results obtained in the present study support the contribution of the miR156-SPL9 regulatory module in coordinating potato plants development and plant responses to drought stress, which involve precise fine-tuning of anthocyanin biosynthesis. As a potential benefit of naturally enriched anthocyanin potato plants varieties in crop settings, we highlight their higher photosynthetic rate under conditions of complete lack of irrigation that mimic severe drought situations in the current context of climate change.

Author contributions

Sara Pescador-Dionisio: Investigation; Writing -review & editing. Aida Robles-Fort: Investigation; Writing -review & editing. Bruno Parisi: Resources; Writing -review & editing. Inmaculada García-Robles: Formal analysis; Writing -review & editing. Laura Bassolino: Formal analysis; Writing -review & editing. Giuseppe Mandolino: Formal analysis; Writing -review & editing. M. Dolores Real: Conception; Formal analysis; Writing -original draft. Carolina Rausell: Conception; Formal analysis; Writing -original draft.

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Declaration of competing 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109195.

Data availability

Data will be made available on request.

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