Functional analysis of MYB alleles from *Solanum chilense* and *Solanum lycopersicum* in controlling anthocyanin levels in heterologous tobacco plants

Patharajan Subban | Shanmugam Prakash | Amir Bootbool Mann | Yaarit Kutsher | Dalia Evenor | Ilan Levin | Moshe Reuveni

Agricultural Research Organization, Volcani Center, Plant Science Institute, Rishon LeZion, Israel

Correspondence
Moshe Reuveni, Plant Science Institute, Agricultural Research Organization, Volcani Center, 68 Hamakabim Road, P.O. Box 15159, Rishon LeZion 7528809, Israel. Email: vhmoshe@agri.gov.il

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Abstract
Flavonoids are natural pigments occurring in plants and are present in fruits, leaves, stems, roots, and flowers. Tobacco plants transformed with an MYB regulatory gene from either *Solanum chilense* (Sc) or *S. lycopersicum* (Sl) demonstrate that ScANT1 induces a higher level of anthocyanin accumulation in comparison to SlANT1 and that this gene is sufficient to promote increased anthocyanin levels. We compared the aptitude of ScANT1 protein to induce anthocyanin accumulation to that of SlANT1 protein in tobacco plants. We also tested the effect of amino acid substitutions in ScANT1 and SlANT1. We examined these synthetic alleles' effect following the over-expression of additional anthocyanin synthesis regulators, such as the tomato bHLH (SJAF13) protein. Our results show that the amino acid changes that differentiate ScANT1 from SlANT1 are the main contributors to the advantage that ScANT1 has over SlANT1 in anthocyanin accumulation per transcript unit. We further demonstrated that altering the amino acid composition of SlANT1 could increase anthocyanin accumulation, while reciprocally modifying ScANT1 lowers the anthocyanin level. These results confirm the increased anthocyanin level in tobacco is attributed to the amino acid differences between ScANT1 and SlANT1. We also show that the co-expression of SJAF13 with SlANT1 in tobacco plants represses the anthocyanin production.

1 | INTRODUCTION

Flavonoids are polyphenolic compounds that occur naturally in most plants and are present in fruits, leaves, stems, roots, and flowers. There are about 8000 different flavonoids that, based on their core structure, can be categorized into five major groups: chalcones, flavones, dihydroflavonols, flavonols, and anthocyanins. Flavonoids are involved in many aspects of plant growth and survival, including pathogen resistance, recruitment of pollinators via floral pigmentation, UV light protection, pollen germination, modulation of auxin transport, and seed coat development (Bovy et al., 2002; Buer & Muday, 2004; Dixon & Paiva, 1991; Dooner et al., 1991; Jones et al., 2003; Koes et al., 2005).

Evidence suggests that flavonoids are potent health-promoting components in the human diet ascribed to their antioxidant activity and their ability, in vitro, to induce human protective enzymes (Bovy et al., 2002; Koes et al., 2005). Also, flavonoid compounds such as anthocyanins provide attractive colors to leaves, stems, roots,
flavonoids are secondary plant metabolites synthesized from phenylalanine and Acetyl-CoA. All the biosynthetic enzymes in this pathway are known. CHALCONE SYNTHASE (CHS) is the initial enzyme in this pathway that converts \( p \)-coumaroyl-CoA with malonyl-CoA to create naringenin chalcone. Naringenin chalcone is then subjected to rapid isomerization by CHALCONE ISOMERASE (CHI) to naringenin. From naringenin, the pathway splits to different flavanones and dihydroflavonols through the addition of hydroxyl groups to the A and B rings of the naringenin skeleton by the enzymes FLAVANONE-3-HYDROXYLASE (F3H) and FLAVONOID 3’-HYDROXYLASE (F3’H), respectively.

Further modifications of these core compounds are achieved by introducing double bonds and loss of oxygen from the B ring, resulting in the basic aglycone structures of the flavonoids and anthocyanins. These aglycones are further modified by the additions of various glycosides such as glucose, rhamnose, and mannose, rendering these hydrophobic molecules water-soluble and creating a large variety of flavonoids found in the plant kingdom (Forkmann & Heller, 1999; Holton & Cornish, 1995). Based on studies using Arabidopsis and petunia hybrid, three proteins that form an MYB-bHLH-WD40 complex (MBW complex) regulate the expression of the genes encoding the enzymes of the flavonoid biosynthetic pathway. bHLH (basic Helix-Loop-Helix; also known as MYC) and MYB are transcription factors known to control the transcription of flavonoid biosynthetic genes. The WD40 protein acts as a scaffold to connect these transcription factors with their targets, presumably the flavonoid gene promoters (Ramsay & Glover, 2005). The MYB and bHLH components were shown to bind to the WD40 protein under environmental conditions such as cold and intense light, which induces the synthesis of flavonoid compounds, including anthocyanins (Baudry et al., 2004; Broun, 2005; Martin et al., 2001; Vom Endt et al., 2002). Several negative regulatory proteins are from the R2R3-MYB or R3-MYB protein family; they disrupt the MBW complex’s activity and directly affect the transcription of anthocyanin accumulation (Colanero et al., 2018; Zhu et al., 2009). An inhibitory R3-MYB encoding gene was identified in tomato as the source of the ATV mutation (Colanero et al., 2018). Thus, the MYB gene family has an activation or repression or and competition function between the different MYB genes that regulate anthocyanin production.

The R2R3-MYB component of the MBW complex seems to determine the set of target genes that the MBW complex will activate in the anthocyanin biosynthetic pathway. The MYB gene family is classified into several subgroups with different functions in plant-specific processes, such as development, signal transduction, resistance to pathogens, and metabolism (including anthocyanin synthesis) (Dubos et al., 2010). The group members represented by genes similar to the petunia AN2 gene are classified as Sub-Group 6 (SG6) and they all share a short amino acid signature for anthocyanin regulation (Albert et al., 2011; Dubos et al., 2010).

Tomato (Solanum lycopersicum) is an important food crop worldwide (Bovy et al., 2002; Willits et al., 2005). Tomato fruits accumulate small amounts of flavonoids, mainly in their peel (Minaggio et al., 2003). Efforts were invested in increasing and/or diversifying flavonoid content in the tomato fruit by traditional breeding and transgenic approaches with encouraging results (Bovy et al., 2002; Schreiber et al., 2011; Verhoeven et al., 2002).

In tomato fruits, three MYB genes have been thus far implicated in controlling either an increase or a decrease of flavonoid and anthocyanin levels, SIMYB12 (Adato et al., 2009; Ballester et al., 2010), SIAN1 (Boches, 2009; Mathews et al., 2003; Sapir et al., 2008), and the tightly linked homolog SIAN2 (Sestari et al., 2014). SIAN1, coding for a MYB-type transcription factor, was first identified by activation-tagging in tomato as a regulator of anthocyanin biosynthesis (Mathews et al., 2003). SIAN1 was identified as the gene responsible to the ANTHOCYANIN FRUIT (AFT) phenotype that originating from S. chilense (Sapir et al., 2008; Schreiber et al., 2011). SIAN1 is categorized as a SG6-MYB type that regulates a small family of genes contributing to the color of different plant parts (Albert et al., 2011). The AFT trait by itself or synergistically combined with the high pigment (hp) mutations \( hp-2, hp-3, hp-2^{h}, hp-1, \) and \( hp-1^{m} \) that control carotenoid biosynthesis, can increase flavonoid content, in particular anthocyanins, in tomato fruit peels (Sapir et al., 2008; Sestari et al., 2014).

AFT (ANTHOXYANIN FRUIT) was originally identified by Georgiev in 1972 (Giorgiev, 1972) and was later shown to segregate as a single dominant gene (Jones et al., 2003). Sapir et al. (2008) analyzed AFT at the molecular and metabolomics level and showed that AFT mutant lines are characterized by significantly higher flavonols and anthocyanins in tomato peels. It was also demonstrated that AN1 displays nucleotide polymorphisms between the AFT genotype and its cultivated counterparts and that it is genetically associated with the AFT trait. The nucleotide polymorphisms observed in AFT result in eight amino acid substitutions in the predicted protein sequence (Sapir et al., 2008). Sequencing followed by alignment of the predicted amino acid sequence of AN1 homologs from Solanaceous species, such as pepper, petunia, and several wild tomatoes differing in their anthocyanin accumulation patterns in fruits or flowers, showed that proline\(^{187}\) might be associated with the non-accumulation of anthocyanin, while substituting proline\(^{187}\) into any other amino acid may result in anthocyanin accumulation (Figure S1). Thus, we hypothesized that AN1 controls anthocyanin accumulation leading to the AFT genotype and that the amino acid substitution may be significant for anthocyanin accumulation.

An earlier study (Schreiber et al., 2011) aimed at dissecting the AFT (Anthocyanin Fruit) locus genetically and validating that the ScANT1 coding region’s mutations are sufficient to cause the AFT phenotype. Tomato plants were transformed with either ScANT1 (ANT1 from S. chilense) or SIAN1 (ANT1 from S. lycopersicum) to test if there is an effect on the sequence changes in ScANT1 in comparison to SIAN1. The results obtained show that ScANT1 induces a higher level of anthocyanin accumulation in comparison to SIAN1 in tomato (Schreiber et al., 2011) and is sufficient to induce increased anthocyanin production without an additional bHLH gene.

This study’s objectives were to compare the capability of ScANT1 and SIAN1 to induce anthocyanin accumulation. The earlier study did...
not show the effect of amino acid substations, the impact of R3-MYB, or the effect of SIJIF13. In this study, we specifically wanted to remove the tomato side effect caused by overexpression of ScANT1 (like reduced fertility and reduced growth) and concentrate on a heterologous system with fewer side effects that show just the impact of the substitution of the amino acids on anthocyanin synthesis. Tobacco has been used in numerous studies to show that both regulatory and structural genes from heterologous species can affect anthocyanin production (Aharoni et al., 2001; Geekiyanage et al., 2007; Sui et al., 2018; Zhang et al., 2009; Zhu et al., 2009).

Our results show that the amino acid changes that differentiate ScANT1 from SIAN1 are associated with increased anthocyanin accumulation per transcript unit in tobacco. We further show that altering a single amino acid in SIAN1 can increase anthocyanin accumulation, while modifying the same amino acid in ScANT1 lowers the anthocyanin content. This indicates that the increased anthocyanin level is due to the amino acid differences between ScANT1 and SIAN1. We also show that adding the tomato bHLH (SIJAF13) to tobacco plants overexpressing the tomato SIAN1 does not further enhance anthocyanin accumulation but acts as an inhibitory protein.

2 | MATERIALS AND METHODS

2.1 | Plasmid construction, plant transformation, and mutation insertion

ScANT1- or SIAN1-containing plasmids used are described in Schreiber et al., 2011 and introduced to tobacco Nicotiana tabacum L. cv. SR1 leaf segments (Shaya et al., 2012). ScANT1 or SIAN1 were sent to GeneScript Company USA for site-specific mutagenesis and verified by sequencing. FaMYB1-containing plasmid was a gift from Asaph Aharoni from the Weizmann Institute Israel. SIJIF13 was cloned into pK7wg2D using a Gateway system and verified by sequencing.

2.2 | Tissue sampling for metabolomics and transcriptional characterization

Sepal tissue was sampled for both RNA and pigment extraction in paired observations, allowing coupling between the transcriptional and the metabolomics analyzes. For the transcriptional profiling and anthocyanin content analyzes, both flower corolla and sepal tissues were used in at least five biological replicates for each transgenic line. Tobacco sepals (from five plants pooled three replicate sepals or corolla petals) were chosen to be the tissue for analyzing anthocyanin content as the background is very low (Figure 1A) and, unlike in corolla tissue, the background anthocyanin production is not affected by temperature. We used corolla tissue to compare to sepal tissue since most studies use corolla tissue to study anthocyanin accumulation. Figure 1A shows that anthocyanins accumulated in all aerial parts of the plant.

2.3 | Transgenic characterization of plants and quantitation of mRNA

All PCR primers used throughout this study were purchased from Hy Labs Ltd. Real-Time PCR (qRT-PCR) analyzes were done as described by Sapir et al. (2008).

Genomic DNA was extracted from young leaves of individual plants, according to Shaya et al., 2012. Validation of the transgenic lines was with primers complementary to the CaMV 35S promoter and the ANT1 gene. The primers’ sequence for the genes tested in this study is in Figure S2.
The PCR reactions were performed in a T-GRADIENT thermal cycler (Biometra, Analytik Jena).

Total RNA was extracted from seeds and flowers of tobacco using the TRIzol reagent system (Invitrogen Corp). Genomic DNA contaminants were digested with Turbo DNA-free DNase (Ambion Inc.). The remaining RNA was then used as the template for cDNA synthesis using the Masterscript cDNA synthesis kit with random hexamer primers (KAPA Biosystems).

The qRT-PCR analysis was performed using the KAPA SYBER FAST Master Mix (KAPA Bio systems); DNA sequences complementary to 18S RIBOSOMAL RNA were used as a reference.

Three technical replicates were performed for every biological repeat with three biological repeats. The qRT-PCR analyzes were done using the Rotor-Gene Q detection system analyzed with the Rotor-Gene 6000 software (Qiagen Corbett Life Science). The relative abundance of the examined gene transcripts was calculated by the formula, $2^{-\Delta CT_{examined\,gene}-\Delta CT_{reference\,gene}}$, where CT represents the fractional cycle number at which the fluorescence crosses a fixed threshold.

2.4 | Extraction and quantification of anthocyanins

All chemicals and solvents were purchased from Sigma (St Louis). Samples were ground in liquid nitrogen, and pigments were extracted as shown before (Schreiber et al., 2011) in the dark with 2 ml of cold methanol:water:acetic acid (11:5:1). Three replicates were taken for every biological sample. Anthocyanins were determined by measuring the OD at 530 nm by spectrophotometer in a UV-2401 PC (Shimadzu Co.) (Schreiber et al., 2011).

2.5 | Protein extraction and analysis

Total leaf proteins were extracted as previously shown (Reuveni et al., 1990) by adding 10 volumes of ice-cold acetone to ground leaf tissue, overnight incubation at −20°C, and precipitation in a cooled centrifuge at 12,000g for 10 min. The protein pellets were dissolved in 0.1 M NaOH, and protein concentration was determined by Bradford (BioRad). Ten microgram of protein (Laemmli, 1970) was loaded onto SDS-PAGE (about 1.5 h at a constant 100 V), transferred to nitrocellulose membranes (Whatman), and blotted overnight at 4°C with specific antibodies. Primary anti HA (Sigma) diluted 1:2000, and detection was by alkaline phosphatase assays secondary antibody (Santa Cruz) diluted 1:10,000 (Reuveni et al., 1990).

2.6 | Protein structural modeling

Amino acid sequences were analyzed using computerized modeling by the I-Tasser program from the University of Michigan (https://zhanglab.ccmb.med.umich.edu/; Yang et al., 2015). Peptide analysis for the presence of peptidase cutting sites was based on peptide cut-ter (http://www.expasy.ch/tools/peptidecutter/).

2.7 | Statistical analyzes

Analysis of variance (ANOVA) was performed with the SAS/JMP software (SAS Institute Inc). Differences among means were calculated based on the Tukey–Kramer honestly significant difference (HSD) test for three or more treatments (Azari et al., 2010) and Student t-test for two treatments (Schreiber et al., 2011).

3 | RESULTS

3.1 | ScANT1 increases anthocyanin content more significantly than SIAN1 in tobacco plants

Both AN1 alleles significantly increased anthocyanin accumulation in all aerial parts compared to wild-type tobacco plants (Figure 1A). The increase in anthocyanin production could stem from two possible mechanisms. In the first one, there is more ScANT1 allele than the SIAN1 allele, inducing more anthocyanin. In the other mechanism, both alleles' expression is the same but one is more efficient in activating anthocyanin production than the other.

T0 plants expressing the ScANT1 MYB allele accumulated significantly more anthocyanin than plants transformed with its SIAN1 counterpart [Figure 1B; the average level was 41 ± 4 for ScANT1 expressing plants 4 ± 1 for SIAN1 expressing plants, $P(F) = 2 \times 10^{-5}$]. When the two AN1 alleles' transcript level was plotted against the anthocyanin level of T0 plant (Figure 1C), the ScANT1 allele causes a higher accumulation of anthocyanin in tobacco plants than SIAN1 allele (Figure 1C). We found no statistically significant differences between the average mRNA transcript levels of tomato AN1 alleles between lines transformed with ScANT1 to those transformed with SIAN1 [SIAN1 transcript level in transformed tobacco was 0.0281 ± 0.0059 and ScANT1 transcript level was 0.0185 ± 0.0060, $P(F) = 0.26$]. These insignificant differences remained unchanged for three generations (Figure 2A). When anthocyanin levels were normalized to the AN1 mRNA transcript levels of the two alleles, it was clear the ScANT1 produces a greater increase in the accumulation of anthocyanins than SIAN1 at equal levels of ScANT1 and SIAN1 transcripts (Figure 2B).

MYBs are transcription factors known to control the transcription of the flavonoid biosynthetic genes together with WD40 and bHLH proteins and act as a complex transcription factor and their targets are the flavonoid gene promoters (Ramsay & Glover, 2005). SIAN1 and ScANT1 are members of the group represented by the petunia AN2 gene and are classified as Sub-Group 6 (SG6) (Albert et al., 2011; Dubos et al., 2010). Sub-Group 6 MYBs control the late flavonoid biosynthetic genes leading to anthocyanin biosynthesis (Albert et al., 2011, Dubos et al., 2010). Transcript levels of several structural tobacco genes of anthocyanin biosynthesis significantly changed following the over-expression of ScANT1 and SIAN1. The late biosynthetic genes, DFR and ANS, increased about threefold in plants expressing ScANT1 compared to plants expressing SIAN1 (Figure 2C). Transcript levels of the early genes CHS, CHI, and PAL did not differ significantly between the two
groups of plants, each overexpressing one of the two tomato alleles (shown just CHS in 2C).

3.2 Mutations in ScANT1 causing alteration in anthocyanin accumulation

The ScANT1 protein has eight amino acids modified compared to the SIAN1T1 protein (Figure 3A). Comparison of ANT1 protein in several tomato and Solanaceae species shows that the amino acid at position 187 is either Q187 or E187 in anthocyanin-accumulating plants, while P187 is present in non-accumulators (Sapir et al., 2008; Figure S1).
Another interesting change between ScANT1 and SIAN1 is in the SG6 consensus sequence: amino acid P144 in ScANT1 changes to R144 in SIAN1, and thus seems to have the potential to affect anthocyanin accumulation (Figure 3A). However, the amino acid number 144 in the SG6 consensus sequence in pepper (Capsicum annuum), petunia (P. hybrida), and S. pimpinellifolium is different compared to ScANT1 and SIAN1 without correlation to anthocyanin accumulation (Figure S1), making amino acid 144 a lesser target for site-directed mutagenesis as means to affect anthocyanin accumulation.

Three single amino acid alterations were made in ScANT1 and SIAN1. In ScANT1, Q187 was substituted with P, T126 with I, and Q252 with P. To test the importance of each of these amino acid substitutions in affecting anthocyanin accumulation, the reverse modifications were made in SIAN1, P187 was substituted with Q, I126 with T, and P252 with Q, thus creating six permutations of amino acids to test.

The T126-to-I126 and Q252-to-P252 substitutions in ScANT1 caused a significant increase in anthocyanin accumulation [Figure 3B; P (F) = 0.01]. The Q187-to-P187 substitution in ScANT1 caused a minor decrease in anthocyanin accumulation. The P187-to-Q187 substitution in SIAN1 caused a marked increase [183%, P(F) = 3.2 x 10−4] in anthocyanin accumulation as predicted (Figure 3C). The I126-to-T126 and P252-to-Q252 modifications in SIAN1 caused a significant decrease in anthocyanin accumulation [Figure 3C; P(F) = 7.6 x 10−5]. The control amount of anthocyanins was 10 times higher in ScANT1 over-expressing plants than in the SIAN1 over-expressing plants (Figure 3).

The P187-to-Q187 amino acid substitution in SIAN1 enhanced anthocyanin accumulation significantly by almost two-fold without affecting the ANT1 protein (Figure 3D). The amount of ScANT1 protein in plants carrying the reverse Q187-to-P187 amino acid substitution was not affected as well (Figure 3D). However, in ScANT1-expressing plants, only a small but significant decrease in anthocyanin accumulation was observed. Moreover, the use of a HA tag to track protein levels indicated that the addition of a HA tag (a short amino acids sequence for antibody binding) did not affect protein level except for the substitution P252-to-Q252 in SIAN1-expressing plants where a large reduction in the amount of the ANT1 protein was observed. A possible explanation for the decrease in the tagged protein in substitution P252-to-Q252 is that in SIAN1 overexpressing plants the HA tag is removed and thus influencing protein stability (Figure 3D). Analysis of the peptide sequence around amino acid 252 showed that P252-to-Q252 substitution resulted in an additional protease cleavage site, marked with a red circle in Figure 3A. The reciprocal Q252-to-P252 substitution in plants expressing ScANT1 enhanced anthocyanin accumulation by 28% without affecting protein stability.

The T126-to-I126 substitution in ScANT1 dramatically enhanced the already boosted by 1.5-fold the anthocyanin accumulation in plants expressing ScANT1, while the reciprocal I126-to-T126 substitution in SIAN1 in plants decreased anthocyanin accumulation by 50%. No peptidase cleavage-site was created after changing position 126 in the ANT1 alleles (Figure 3B).

3.3 ScANT1 MYB interacts differently with SIJAF13 and FaMYB than SIAN1

We crossed tobacco plants over-expressing FaMYB1 [an inhibitory MYB from Stawberry (Fragaria × ananassa) (Aharoni et al., 2001)] with plants over-expressing either ScANT1 or SIAN1 together with tomato bHLH (SIMYC1 or SIJAF13). The crosses were carried out to determine whether the tomato ANT1 protein alleles differ in the effects of MYB proteins inhibiting anthocyanin accumulation in tobacco (Aharoni et al., 2001; Matsui et al., 2008). Over-expression of tomato bHLH protein (SIJAF13) alone did not affect anthocyanin accumulation in either sepal or corolla tissue compared to SR1 (Nicotiana tabacum L. cv. SR1) control tobacco plants (Figure 4). Combining SIJAF13 with either ScANT1 or SIAN1 reduced anthocyanin accumulation by 32 and 56%, respectively [P(F) = 5.2 x 10−12]. FaMYB1 significantly reduced anthocyanin accumulation [P(F) = 2.05 x 10−13] in ScANT1-expressing tobacco by about 80%, while lowering anthocyanin accumulation in SIAN1-expressing tobacco plants by only 40%, similarly to FaMYB1 reduction of anthocyanins accumulation in non-transgenic corolla tissue [Figure 4A; [P(F) = 1.96 x 10−5]]. Co-expressing the SIAN1 and SIJAF13 or ScANT1 and SIJAF13 anthocyanin regulatory genes with the inhibitory FaMYB1 in the same tobacco plant abolished the FaMYB inhibitory effect, bringing anthocyanin accumulation to similar levels obtained by expressing ANT1 alleles alone (Figure 4).

Co-expression of FaMYB1 with the various single mutations displayed in Figure 3 showed that the inhibitory FaMYB1 protein decreased anthocyanin accumulation compared to their non-FaMYB1 counterpart. The substitution of amino acid Q187 to P187 in SIAN1 and T126-to-I126 in ScANT1 was less sensitive to the presence of FaMYB1 protein compared to the original sequence or the other modifications (Figure 5). On the other hand, the reciprocal modification in SIAN1 amino acid 126 and iScANT1 amino acid 187 showed similar inhibition by FaMYB1 protein compared to SIAN1 with no changes (Figure 5). The substitutions we made at amino acid 126 and 187 showed that these amino acids are important to R3-MYB induced inhibition. The anthocyanin inhibition in the presence of FaMYB1 was reduced from 60 to 40% in SIAN1 (P187-to-Q187; Figure 5B), and from 80% to 60% in ScANT1 T126-to-I126 (Figure 5A). Both results correlate well with the observations that the amino acid 126 of ScANT1 and 187 in SIAN1 are important to the function of ANT1 as a regulator of anthocyanin accumulation (Figures 3 and 5).

3.4 ScANT1 MYB has a different postulated 3D structure than SIAN1

The amino acid sequence changes might lead to a structural alteration that makes the ScANT1 protein different from the SIAN1 protein. Using the I-Tasser program (https://zhanglab.ccmb.med.umich.edu/), we modeled the two tomato ANT1 alleles (Figure 6). The models in Figure 6 show that when we change amino acid at position 187 (red...
arrow) from Glutamine (Q) to Proline (P) in ScANT1, the full 3D modeled structure of the non-R2-R3 domain (Figure 6C) changes to look like SIAN1 (Figure 6B). When we change amino acid at position 187 (red arrow) from Proline (P) to Glutamine (Q) in SIAN1 (Figure 6D), the complete 3D modeled structure changes to look like ScANT1 (Figure 6A). Altering amino acid 252 (black arrow) between ScANT1 protein (Q) to SIAN1 protein (P) does not cause a change in the modeled 3D structure. The reverse change does not affect the model either. However, the original mutation in amino acid 126 (blue arrow) in ScANT1 compared to SIAN1 turns the protein relative to the DNA helix (yellow line) by 180° (Figure 6A compared to 6B). No change occurred in ScANT1 in the non-R2-R3 portion of the protein, as seen by modeling the substitution in position 126. However, the model shows that the non-R2-R3 part in SIAN1, where amino acid 126 was substituted from Ile to Thr, seems more like ScANT1 than SIAN1 (Figure S3).

4 | DISCUSSION

We designed this study to analyze the difference in function between two alleles of the tomato ANT1 gene: ScANT1 from S. chilense and SIAN1 from its cultivated S. lycopersicum counterpart. ANT1 was previously mapped to the tomato chromosome 10 as a candidate gene controlling anthocyanin and flavonols accumulation in fruit peels of the AFT genotype (Sapir et al., 2008). It was shown to increase anthocyanin and flavonols accumulation when over-expressed in tomato (Figure 6D), the complete 3D modeled structure changes to look like ScANT1 (Figure 6A). Altering amino acid 252 (black arrow) between ScANT1 protein (Q) to SIAN1 protein (P) does not cause a change in the modeled 3D structure. The reverse change does not affect the model either. However, the original mutation in amino acid 126 (blue arrow) in ScANT1 compared to SIAN1 turns the protein relative to the DNA helix (yellow line) by 180° (Figure 6A compared to 6B). No change occurred in ScANT1 in the non-R2-R3 portion of the protein, as seen by modeling the substitution in position 126. However, the model shows that the non-R2-R3 part in SIAN1, where amino acid 126 was substituted from Ile to Thr, seems more like ScANT1 than SIAN1 (Figure S3).
(Schreiber et al., 2011). There are eight amino acid changes in the protein sequence between ScANT1 and SlANT1 without expression level change. Thus we assume that these amino acid modifications make ScANT1 more efficient than SlANT1 in anthocyanin accumulation in tomato (Schreiber et al., 2011) and tobacco. Here, we show that the two ANT1 constitutive over-expressions led to increased accumulation of anthocyanin pigments in tobacco plants' vegetative and reproductive tissues, with a much stronger induction of anthocyanin accumulation in plants over-expressing ScANT1 than plants over-expressing SlANT1. While this was shown for tomato (Schreiber et al., 2011), we use single amino acid substitution and other anthocyanin synthesis regulatory proteins to deduce the mechanism by which ScANT1 is more efficient than SlANT1.

The two ANT1 alleles over-expressed in tobacco plants increased anthocyanin accumulation at similar mRNA transcript levels. ScANT1 overexpressing at the same mRNA transcript levels produces more anthocyanins of than SlANT1 overexpressing plants. This observation is showing that this enhanced ability to produce more anthocyanins is built in the S. chilense ANT1 protein with its eight mutations.

The difference between the two ANT1 alleles was not due to transcript levels, but the difference in anthocyanin accumulation capability between ScANT1 protein and SlANT1 protein is within the protein-coding sequence. There was no difference in anthocyanin composition between tobacco plants overexpressing SlANT1 or ScANT1 alleles. Both activated the same late structural genes in anthocyanins' biosynthesis, implying that the tomato ANT1 gene controls the late biosynthesis genes similarly to NtAn2 (Liu et al., 2018; Zhu et al., 2015). A direct correlation between the expression levels of late biosynthesis genes and anthocyanin content is observed in many plants (Liu et al., 2018), suggesting that variations in late biosynthesis genes expression determine the quantitative increase in anthocyanin content.

We hypothesized that a possible explanation for the observed differences in the ability to induce anthocyanin synthesis between the two tomato alleles rests in the sequence of the non-R2-R3 part of the gene. ScANT1 protein has eight amino acid changes compared to SlANT1. Thus, the modified ScANT1 protein may interact in a different/better manner with other components of the bHLH-MYB-WD40 complex to activate the transcription and perhaps translation of the late structural enzymes in anthocyanins biosynthesis, leading to increased synthesis and accumulation of more anthocyanins. The amino acid sequence changes might lead to a structural difference between ScANT1 and SlANT1 proteins, thus making one more efficient in activating the late biosynthetic pathway. Using the i-Tasser
program (https://zhanglab.ccmb.med.umich.edu/; Yang et al., 2015), we modeled the two tomato ANT1 proteins (Figures 6 and S2). The models in Figures 6 and S2 show that ScANT1 and SIAN1 proteins have different structures and that altering amino acid 187 from Proline (P) to Glutamine (Q) in SIAN1 (red arrow in Figure 6) changes the 3D modeled structure of SIAN1 to a structure closer to ScANT1 (Figure 6A compared to 6D) and increase anthocyanins accumulation (Figure 3C). The reciprocal change in amino acid 187 in ScANT1 from Glutamine (Q) to Proline (P) (Figure 6C) alters the structure of the non-R2-R3 domain to be similar to SIAN1 (Figure 6B) and reduces anthocyanins accumulation (Figure 3B). The amino acid at position 126, located closer to the DNA binding R2-R3 domain (blue arrow in Figure 6), changes the protein orientation compared to the DNA, affecting this domain and binding to DNA (yellow structure in Figure S2). The computed model predicts that ScANT1 binds to DNA with 13 amino acids in the R2-R3 domain, while SIAN1 binds with 11 amino acids. Changing amino acid 126 in ScANT1 from Thr to Ile reduces the number of amino acids predicted to bind the DNA coil to only 10. In contrast, modifying amino acid 126 in SIAN1 from Ile to Thr increases the number of predicted amino acids that attach to the DNA coil to 13 and changes the predicted non-R2-R3 3D structure to SIAN1-like (Figure S2). The results suggest that single modifications in the regulatory proteins that control anthocyanin biosynthesis can yield various plants coloration variants.

The MYB family contains some negative regulatory proteins that are R2R3-MYB type or R3-MYB type proteins. These proteins disrupt the MBW complex' activity and affect anthocyanin accumulation directly (Colanero et al., 2018; Zhu et al., 2009). The tomato ATROVIOLACIUM (ATV) mutation (that increases anthocyanins) was identified as a malfunctioning R3-MYB encoding gene (Colanero et al., 2018; Sestari et al., 2014), indicating that repressor R3-MYB functions in tomato. The strawberry repressor R3-MYB (FaMYB1) reduces anthocyanin production in over-expressing ScANT1 and SIAN1 plants as expected (Figure 4). The tomato bHLH (SUJAF13) protein also diminishes anthocyanin accumulation in plants overexpressing ScANT1 and SIAN1 (Figure 4). It seems that the tomato bHLH protein has a lesser effect on ScANT1. At the same time, the tomato bHLH protein reduced anthocyanin accumulation in ScANT1 plants compared to SIAN1 plants. The FaMYB1 is having the opposite impact and inhibits anthocyanin accumulation in ScANT1 plants more strongly than in SIAN1 plants (Figure 4). A homolog of the strawberry inhibitory MYB gene was identified in tomato as the ATROVIOLACIUM (ATV) gene, when ATV is crossed with ScANT1 plants it does not increase anthocyanin accumulation compared to SIAN1 plants (Sestari et al., 2014). The atv mutant is defective in the inhibitory R3-MYB that inhibits anthocyanin production in its natural state, and when mutated, its inhibitory effect is diminished and thus increases anthocyanin production. We analyzed the numbers published on the effect of ATV in Micro-Tom plants in the absence of SIAN1 (WT) or presence of ScANT1 (AFT) and observed that ATV does not increase anthocyanin production equally in ScANT1 or SIAN1 background (in SIAN1 100% increase and ScANT1 50% increase in anthocyanin production) (Sestari et al., 2014). The difference reported by Sestari et al. (2014) may indicate that ScANT1 protein has increased affinity to R3-MYB proteins, and even mutated ATV can still affect ScANT1. Colanero et al. (2018) postulated that the inhibitory R3-MYB binds to the MBW complex and thus inhibits its activity. The usual model for anthocyanin regulation is that the MBW complex dimerizes and binds to DNA and activates the anthocyanin synthesis genes (Colanero et al., 2018). We propose that there might be a different way the R3-MYB inhibits the MBW complex activity. The R3-MYB binds to the MBW complex with either the R2-R3 MYB or the bHLH together, thus the three proteins compete for the WD-repeat site of binding. This is supported by the cancelation of bHLH and R3-MYB inhibitory effect when co-expressed. The simple explanation is that the FaR3-MYB and SibHLH proteins bind together and do not impede the ability of the R2-R3 MYB to activate tobacco anthocyanin biosynthesis genes. When the R3-MYB or bHLH are separate, they inhibit the MBW complex's binding by binding to the R2-R3 MYB and preventing the initial dimerization of R2-R3 MYB-bHLH of tobacco. The above simple explanation shaded light on the mode of action of R3-MYB inhibitory proteins.

In conclusion, our results show that ScANT1 binding properties were modulated due to one or more amino acid changes compared to SIAN1. Thus, the altered protein is more efficient in inducing anthocyanins biosynthesis. We also show the inhibitory effect of the SIAF13 protein as a repressor, and hence regulator, of anthocyanin production.

Anthocyanin accumulation in plants can be affected by directed single amino acid substitution, as we show by changing amino acid 126 or 187 to increase or decrease anthocyanins synthesis.

**AUTHOR CONTRIBUTIONS**

Moshe Reuveni conceptualized and designed the experiments, Patharajan Subban, Shammugam Prakash, and Amir B. Mann performed experiments and analyzed the data. Yaarit Kutsher and Dalia Evenor produced the transgenic plants and crosses. Moshe Reuveni and Ilan Levin wrote initial draft of manuscript and edited the paper. All the authors have read, edited, and approved the final manuscript.

**DATA AVAILABILITY STATEMENT**

Data sharing is not applicable to this article as all generated data is already included in this article or in the supplementary material.

**ORCID**

Moshe Reuveni https://orcid.org/0000-0002-8762-4179

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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