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ORIGINAL RESEARCH

Targeted mutagenesis of *StISAC* stabilizes the production of anthocyanins in potato cell culture

Vincenzo D'Amelia[1] | Annalisa Staiti[2] | Fabio D'Orso[3] | Maria Maisto[4] | Vincenzo Piccolo[4] | Riccardo Aversano[2] | Domenico Carputo[2]

[1]Institute of Biosciences and Bioresources (IBBR), National Research Council of Italy, Portici, Italy

[2]Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy

[3]Research Centre for Genomics and Bioinformatics (CREA-GB), Council for Agricultural Research and Economics, Rome, Italy

[4]Department of Pharmacy, University of Naples Federico II, Naples, Italy

Correspondence
Domenico Carputo, Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055 Portici, Italy.
Email: carputo@unina.it

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Abstract

To increase the production of decorated anthocyanins in potato cell cultures, we knocked out a novel potato gene, named *Inducer Silencing of Anthocyanins in Cell culture* (*StISAC*), using CRISPR-Cas9 editing. Our results provided evidence that mutant cell lines doubled the accumulation level of anthocyanins biosynthesized. Moreover, the production of these important pigments was stabilized over time. Our study overcame important challenges in the efficient biotechnological production of these valuable pigments and reported the function of a novel anthocyanin biosynthesis repressor gene.

KEYWORDS
calli, CRISPR-Cas9, R3-MYB, *Solanum tuberosum*

1 | INTRODUCTION

Anthocyanins are flavonoid pigments mostly responsible for the red, blue, and purple colors of plant tissues. They are used as colored additives and bioactive ingredients in processed foods (Neri-Numa et al., 2020). Among plant species, the cultivated potato *Solanum tuberosum* offers a wide and unique range of anthocyanins that can be extracted from pigmented tubers (Oertel et al., 2017). According to a *market data forecast* (www.marketdataforecast.com) report, the market size of anthocyanins was estimated at 318 million USD in 2020 and is expected to increase at a compound annual rate of 4.6%. Since direct extraction from plant matrices does not guarantee a sustainable and efficient production on demand, innovative methodologies and technologies can be used. Among them, plant cell cultures represent a very versatile and powerful system for metabolite production. The main drawback is that cell cultures are quite unstable in producing anthocyanins over time. This has a strong negative impact on their production in bioreactors (Appelhagen et al., 2018; D'Amelia et al., 2020). CRISPR-Cas9-based gene editing is the emerging technique that can promote the accumulation of anthocyanins in plants

(Yan et al., 2020). In the study presented here, we exploited CRISPR-Cas9 system to generate an edited potato cell culture line that stably accumulates a high level of complex anthocyanins. We undertook a two-stage study to accomplish our goal. In the first stage, we pointed out *StISAC* as a novel promising target gene encoding for a negative R3-MYB regulator of anthocyanin biosynthesis. Then, we knocked out *StISAC* by employing CRISPR-Cas9-induced mutagenesis. Edited cells showed not only an increased amount of anthocyanins but also a stable production of these pigments during successive subcultures.

2 | RESULTS AND DISCUSSION

2.1 | Re-annotation of *StMYBATV in StISAC*

In D'Amelia et al. (2020), we isolated a R3-MYB transcription factor we named StMYBATV due to its phylogenetic relatedness to the tomato anthocyanin repressor SlMYBATV (MF197509). Based on the potato genome annotation (v. 6.1., DM 1-3 516 R44), we found that *StMYBATV* localized on chromosome 12 (*StMYBATV-chr12*; *Soltu. DM.12G023200*), whereas *SlMYBATV* is placed on tomato chromosome 7. These data indicated that *StMYBATV* is not the direct ortholog of *SlMYBATV*, given the conserved genomic synteny between the two Solanaceae (Peters et al., 2012). Instead, *StMYBATV-chr12* is the direct ortholog of *SlMYBATV-like* (MF197514), another tomato R3-MYB encoding gene. It is localized on chromosome 12 (Cao et al., 2017), but it has not been functionally characterized yet. The phylogenetic analysis of the encoded proteins (Figure 1a) indicated that the tomato SlMYBATV (with its different alleles, SlMYBATV-X1, SlMYBATV-X2, SlMYBATV-X3; Cao et al., 2017) grouped (cluster IIa) with a potato protein encoded by a gene placed on chr07 (StMYBATV-chr07, Soltu.DM.07G017910), while StMYBATV-chr12 (StMYBATV in D'Amelia et al., 2020) and SlMYBATV-like grouped apart (cluster IIb). A signature motif Asp-Ser-Thr-Arg-Val-Val within the R3 domain contradistinguished the two classes of proteins (Ia and IIb). Indeed, it was present only in proteins of cluster IIa (Figure 1b). Differences in exon-intron structure suggest possible distinct evolutionary histories among these MYBs (Figure 1c), where the protein-encoding genes of cluster IIa have four exons rather than the three found in the cluster IIb encoding-proteins. Based on the functional analyses described below, we re-named StMYBATV-chr12 as *ISAC* (*Inducer Silencing of Anthocyanins in Cell culture*).

2.2 | *StISAC* editing increases and stabilizes anthocyanin accumulation in potato cells

StISAC has been proposed to act against anthocyanin accumulation similarly to SlMYBATV by re-recruiting anthocyanin bHLH co-partners. This process might not allow the formation of the MYB-bHLH-WD40 (MBW) anthocyanin promoter complex (D'Amelia et al., 2020). Therefore, to knock out *StISAC*, we edited its sequence in cells of the potato variety "Blue Star" through CRISPR-Cas9. This system has been efficiently used to mutate alleles of the tetraploid potato for several desirable traits such as biotic stress tolerance and starch quality (reviewed in Tiwari et al., 2022). However, it has never been used to improve anthocyanin content in potato cells. In total, more than 20 kanamycin-resistant callus lines were obtained and subcultured for more than 12 months along with the *wild type (Wt)*. The target region for the putative mutations was amplified and sequenced on the gDNA extracted from five randomly selected calli. Allele discrimination was carried out by CRISPR-ID® software analysis (Dehairs et al., 2016). As represented in Figure 1d, the end of the first exon was the target region for two SgRNA guides, and five type mutations were characterized in this region. Overall, deletions of nucleotides between PAMs of Sg1 and Sg2 were produced. *Mut_1*, *mut_2*, and *mut_3* led to a frameshift mutation and a premature stop codon, which was predicted to truncate the encoded protein after about the first 20 amino acids. *Mut_4* and *mut_5* were predicted to produce sense mutations (i.e., D11N, F14S, I15S, or I15T) and to delete five amino acids. High resolution melting-based genotyping (HRM) revealed the heterogenic composition of the five selected calli as regards the *StISAC* allelic composition. Most alleles showed heteroallelic mutations (Figure S1). They were gathered together in main peaks in Figure S1B.

All cultures derived from edited calli were fully dark purple, in contrast to a "mosaic" (white and colored cells and pale purple) culture generated from *Wt* potato explants (Figure 1e). The edited cells (*Stisac*) produced about twice as many anthocyanins as Wt cells produced and 14-fold more anthocyanins than tubers of "Blue Star" (Figure 1f,g). Compared with other sources of anthocyanins, the total amount of pigment accumulated by the *Stisac*-line was similar to that produced by blackberries (Figure 1g). However, the rich potato metabolite biodiversity may cover all of the six main aglycon anthocyanidins, adding further value to the potato-edited cell platforms. Moreover, potato accumulates several acylated anthocyanin forms (Oertel et al., 2017). This is felt to be important since acylation provides increased stability during technological processes (Zhao et al., 2017). Anthocyanin profiling of *Stisac*-edited lines highlighted a different increase of anthocyanin fractions favoring *p*-coumarylated forms. Malvidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside increased more than other fractions and about 150% more than *Wt* cells (Figure 1h). We hypothesize that the lack of functionality of the R3-MYB repressor had positively impacted the main flux leading to the biosynthesis of flavonoid precursors (including *p*-coumaroyl-CoA) and affected the side routes less (e.g., feruoyl-CoA).

Wt lines contained subpopulations of cells that accumulated no (or very small amounts of) anthocyanins. In contrast, edited cell culture, grown at the same conditions, showed more stable and homogeneously colored cell populations (Figure 1e,i). Associated with the increased total amount of anthocyanins, anthocyanoplasts were observed within vacuoles of *Stisac* cell lines (Figure 1i). In addition, cells from edited cultures showed cylindrical forms with a mean length of 120 μm, whereas *wt* cells were round-shaped (diameter about 50 μm; Figure 1j). This difference might be due to the augmented volume of the vacuolar lumen of *Stisac*-cells determined by an improved anthocyanin vacuolar uptake. We cannot exclude that *StISAC* can directly affect cell shape. Indeed,

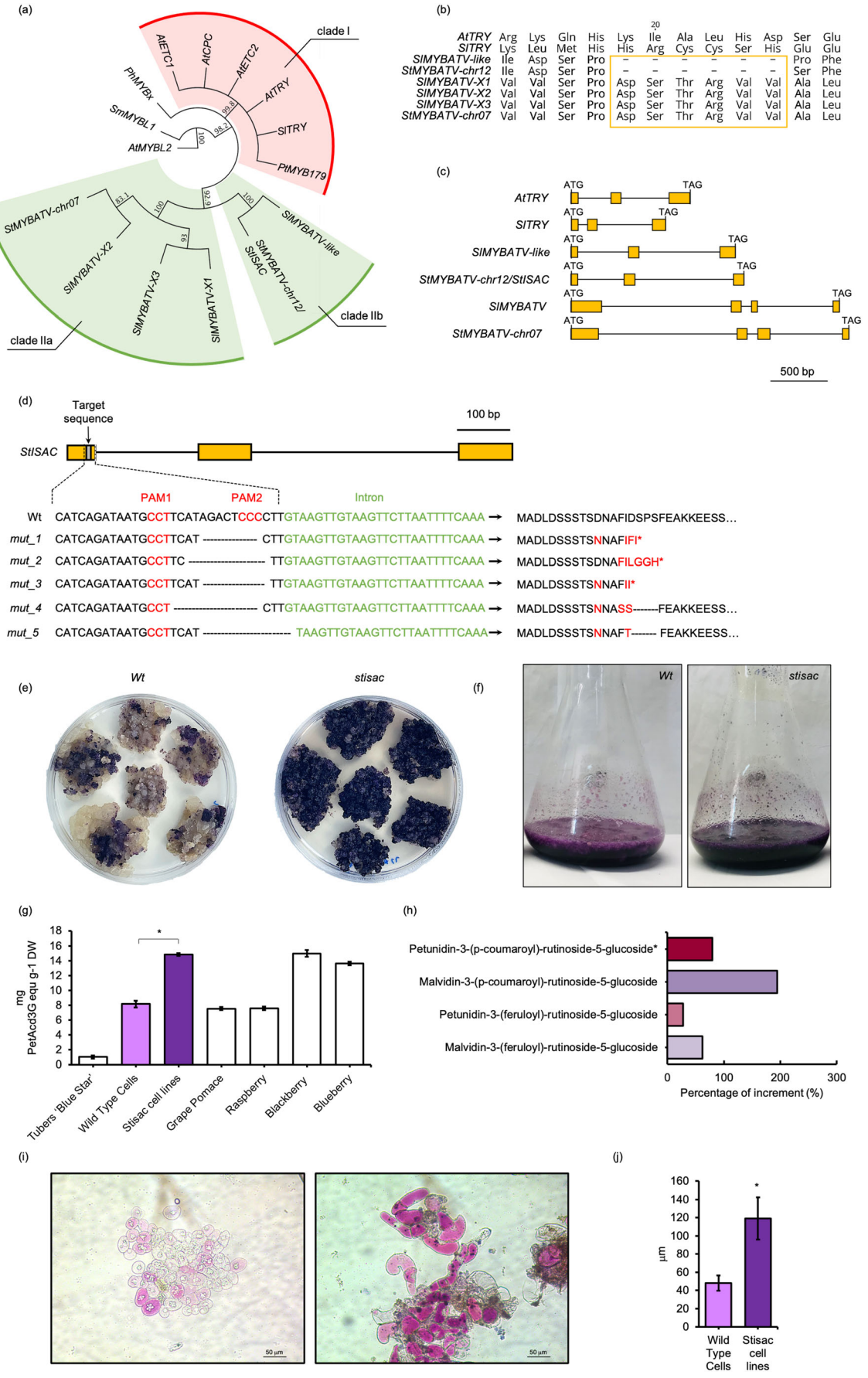

FIGURE 1 Legend on next page.

FIGURE 1 CRISPR/Cas9-mediated editing to stabilize anthocyanin production in potato cells. (a) Phylogenetic tree of CPC-like R3-MYBs. Values lower than 7% of bootstrap are not shown. (b) Particular of the alignment of R3-MYBs of clade I and II of the phylogenetic tree in (a). Yellow box underlines an amino acid motif which is held by components of clade IIa, but which lacks in clade IIb. (c) Genomic structures components of phylogenetic tree in (a). (d) A schematic map of the SgRNA targeted sites on *StISAC* and sequencing results of the *Stisac* cell lines (*mut*) obtained after transformation. The predicted mutation at amino acid level was also reported. In (c) and (d), introns (not drawn to scale) are shown as lines and exons are shown as yellow boxes. (d, f) Phenotype of a representative *Stisac* cell line (*Stisac*) at about 12 months after the transformation, respectively, as calli and liquid suspension culture. (g) Total anthocyanin content of *Wt* and *Stisac* edited cell lines in comparison with other source of anthocyanins. (h) Relative percentage of anthocyanins in three representative *Stisac* cell lines compared with Wt, [1]petunidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside is present as two different stereoisomers. (i, j) Microscopic images of the suspension cultures and average cell length. * $P < 0.05$ according to Student's *t* test.

members of clade II are closely related to CPC-type R3-MYBs (Figure 1a, LaFountain & Yuan, 2021), which are known to be involved in cell determination (Ma & Constabel, 2019). Future experimental works are needed to shed light on the potential involvement of Clade IIb members in cell shape determination.

3 | CONCLUSIONS

To date, gene editing has never been used in potato or in cell cultures to change anthocyanin content. In this work, we functionalized a new potato anthocyanin repressor named *StISAC* and provided a method (based on CRISPR-Cas9) for stabilizing anthocyanin production in cell culture. Potato cell culture is advantageous for anthocyanin production because colored potatoes display all the six most common anthocyanidins with different acylation level. The method presented here is transferable to other potato varieties and related Solanaceae species.

4 | MATERIALS AND METHODS

4.1 | Generation of potato calli and suspension cell culture

Potato calli were obtained from leaf explants of the potato variety "Blue Star" (2n = 4x = 48) as described in D'Amelia et al. (2020). Briefly, callus formation was induced in young leaves and internodes for approximately 15–20 days on MS agar plates supplemented with 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma, St. Louis, USA) and 30 g/L sucrose; pH 5.8. Calli were cultured at 24°C in the dark and were subcultured every 2 weeks to a fresh medium. Cell suspension cultures were initiated by culturing approximately 1 g callus in 250-Erlenmeyer's flasks. Cell suspension cultures were maintained under continuous agitation at 110 rpm on a shaker (Infors AG, CH-4103, Switzerland) and incubated under same culture conditions as described for the induction of callus culture.

4.2 | Vector construction

The final CRISPR construct was developed by using the Golden Gate cloning method (Engler et al., 2008). Two sgRNAs (Sg1 and Sg2) were designed to target the first exon of *StISAC* at sites adjacent to CCT-PAM1 and CCC-PAM2. These chosen sequences (5′-TCATA-GACTCCCCTTGTAAG-PAM1-3′ and 5′-CTTGTAAGTTCTTAATTTTC–PAM2-3′) were assembled by PCR to a sgRNA backbone contained in pICSL70001 plasmid, which has been used as an amplification template. The two different amplicons were assembled, respectively, in vectors pICH47732 and pICH47742 along with pU6 promoter contained in pICSL90001. The two obtained vectors containing the expression cassettes pU6:sgRNA were used in a Golden Gate digestion-ligation reaction to make the final vector in pICSL002203 backbone containing the Cas9 driven by the 2 × 35S CaMV and Kanamycin selection cassette according to the Golden Gate methodology. All the assembly steps to obtain a complete transcription unit was obtained by means Golden Gate digestion-ligation reactions (http://synbio.tsl.ac.uk/). The final vector was expressed in potato cells via *Agrobacterium tumefaciens* strain LBA4404-pAL4404 by electroporation.

4.3 | Potato transformation

A. tumefaciens grew in YEP broth supplemented with 100 mg L^{-1} of streptomycin, rifampicin, and kanamycin at 28°C. An overnight culture was centrifuged at 7000 rpm for 10 min. The pellet was resuspended in callus culture liquid medium with the addition of 100 μM acetosyringone (Sigma, St. Louis, USA) and diluted to $OD_{600} = 0.7$. Potato explants were infected by immerging in *A. tumefaciens* inoculum for 20 min. Then, explants were blotted on a sterile filter paper and placed on a solidified callus induction medium, followed by 2-day co-cultivation at 26°C in the dark. Subsequently, for Agrobacterium elimination, explants were rinsed with sterile water containing 400 mg L^{-1} cefotaxime and were transferred to a fresh callus culture medium supplemented 100 mg L^{-1} kanamycin for transformed callus selection. Single, small calli developed after 3 weeks of selection were picked up and transferred periodically to the same selection medium until whole callus tissues were obtained.

4.4 | High-resolution melting analysis, amplicon sequencing and CRISPR mediated indels detection

Genomic DNA was extracted from five calli using the DNeasy Plant Mini Kit (Qiagen, www.qiagen.com). A nucleotide region flanking the

target region of sgRNAs was amplified by PCR and sequenced (Eurofins, www.eurofins.com) using Mix2Seq kit. To identify indels directly from Sanger sequencing traces without sub-cloning, we used the web tool CRISPR-ID. Reads were manually aligned to the reference sequence using the Geneious software to detect induced mutations.

HRM analysis was conducted with Rotor-Gene™ 6000® (Corbett, Life Science). Reaction was performed in of 20 μl containing 10 ng of gDNA, 0.4 μM of each primer, 0.8 μM dNTPs, Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific), 1× PCR buffer, and 1× EvaGreen Dye (Biotium). PCR products (149 bp) were melted from 65 to 90°C with a melting rate of 0.1°C s^{-1}. All the HRM data collected were analyzed by the dedicated HRM software (Rotor-Gene 6000 Series Software 1.7).

4.5 | Spectrophotometric quantification

Five independent pieces of edited and wild-type calli were used. Five hundred milligrams of calli were dispersed in ethanol/water blend (70:30 v/v, 25 ml). Then, an ultrasonic extraction was carried out for 30 min at the power of 50 W. After centrifugation (15 min, 5000 rpm), the extractive solution (supernatant) was evaporated under reduced pressure at 40°C to remove ethanol and then freeze-dried, producing a powder stored under vacuum until use. The pH differential method was employed to quantify the total content of monomeric anthocyanins from cell culture.[3] Anthocyanin profiles of purple potato extracts were obtained using high-performance liquid chromatography coupled to mass spectrometry with a heated electrospray ionization interface (HPLC-HESI-MS). LTQ XL mass spectrometer (Thermo Fisher Scientific) equipment, coupled with HPLC DIONEX UltiMate 3000 (Thermo Fisher Scientific) instrument, were used for the analysis. The samples' analytes were separated using a C18 reversed-phase column (250 × 4.6 mm, 5 μm, Phenomenex). The separation conditions were as follows: Column temperature was set at 30°C, inject volume was 5 μl, and the solvent flow rate was set at 1 ml/min. The mobile phase consisted of A (formic acid/water at the ratio of 0.3/99.7 v/v) and B (formic acid/acetonitrile/water at the ratio of 0.3/30/69.7 v/v/v). Elution gradient was as follows: 0 min: 20% (B), 3 min: 20% (B), 10 min: 30% (B), 40 min: 90% (B), 45 min: 90% (B), 45.1 min: 30% (B), 53 min: 30% (B). The source was a heated electrospray interface (HESI), operated in positive ionization mode using the following parameters: sheath gas flow rate: 35 ml/min; auxiliary gas flow rate: 10 ml/min; capillary temperature: 320°C; source heater temperature: 150°C; source voltage 3.5 kV; source current 100 μA; capillary voltage: 32 V; tube lens: 80 V. Data acquisition was performed using X-Calibur software and HPLC system was controlled by Chromeleon software.

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CONFLICT OF INTEREST

VDA, AS, RA, and DC declare to be co-authors of a patent governing the information disclosed here (Italian patent application n. 102021000000785, filled on 18 Jan 2021). The other authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

VDA conceived the idea; VDA, AS, FD, MM, and VP performed experiments; VDA, DC, and RA wrote the manuscript.

ORCID

Vincenzo D'Amelia https://orcid.org/0000-0002-8548-6825
Fabio D'Orso https://orcid.org/0000-0002-4906-0026
Vincenzo Piccolo https://orcid.org/0000-0003-4480-5022
Riccardo Aversano https://orcid.org/0000-0002-4041-3702

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SUPPORTING INFORMATION

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

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