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Functional characterization of tomato SP3C gene in a heterologous system, Arabidopsis thaliana

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Dissertation submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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ABSTRACT

SANTIBÁÑEZ, Patricio Andrés Segundo Delgado, M.Sc., Universidade Federal de Viçosa, February, 2025. **Functional characterization of tomato SP3C gene in a heterologous system, Arabidopsis thaliana**. Adviser: Agustin Zsogon. Co-advisers: Karla Gasparini dos Santos and Adriano Nunes Nesi.

A heterologous system is a biological tool in which a gene of interest is introduced into a different genetic background for various purposes. By introducing a gene into a well-characterized organism, such as *Arabidopsis thaliana*, we can gain deeper insight into its functional roles. The aim of this study was to better understand the pleiotropic role of the antiflorigenic *SELF-PRUNING 3C* (*SP3C*) gene from tomato (*Solanum lycopersicum* L.), using the extensively characterized flowering regulatory network of Arabidopsis as a model. We generated three independent homozygous Arabidopsis lines overexpressing *SP3C* (*SP3C*#8, #9, and #11). Under short-day (SD) conditions, the *SP3C* overexpression lines showed a significant delay in flowering, larger rosette areas, and increased biomass, particularly in roots and shoots, compared to the Col-0 control. In contrast, under long-day (LD) conditions, no differences were observed between transgenic and control plants, suggesting that the anti-florigenic function of *SP3C* is photoperiod-dependent. In vitro root growth assays revealed longer primary roots and increased formation of secondary roots in the transgenic lines, consistent with phenotypes previously observed in tomato plants. Seed germination assays under NaCl treatment (30, 75, and 150 mM) demonstrated enhanced salinity tolerance in the transgenic lines, particularly at 75 mM NaCl. Drought experiments conducted under severe dehydration (25% relative water content) revealed significant changes in sugar metabolism in the *SP3C* overexpression lines, specifically an increase in starch content in the rosettes. This effect was strongly dependent on drought conditions, as no metabolic differences were observed under control conditions. Interestingly, this shift in carbon allocation was accompanied by a marked increase in proline accumulation, likely compensating for the reduced availability of sucrose as an osmoprotectant. Further drought experiments are needed to better understand the interactions between *SP3C*, photoperiod, water availability, and carbon allocation.

Keywords: heterologous expression; *SP3C* gene; *Arabidopsis thaliana*; flowering regulation; water stress.

RESUMO

SANTIBÁÑEZ, Patricio Andrés Segundo Delgado, M.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Caracterização funcional do gene SP3C do tomateiro em um sistema heterólogo, Arabidopsis thaliana**. Orientador: Agustin Zsogon. Coorientadores: Karla Gasparini dos Santos e Adriano Nunes Nesi.

Um sistema heterólogo é uma ferramenta biológica na qual um gene de interesse é introduzido em um contexto genético diferente para diversos propósitos. Ao introduzir um gene em um organismo bem caracterizado, como *Arabidopsis thaliana*, podemos obter uma compreensão mais profunda das suas funções. O objetivo deste estudo foi entender melhor o papel pleiotrópico do gene antiflorígeno *SELF-PRUNING 3C* (*SP3C*) de tomate (*Solanum lycopersicum* L.), utilizando a rede regulatória da floração de Arabidopsis como modelo. Foram geradas três linhagens independentes e homozigóticas de Arabidopsis superexpressando *SP3C* (*SP3C*#8, #9 e #11). Sob dias curtos (SD), as linhagens transgênicas apresentaram atraso significativo na floração, maiores áreas de roseta e aumento de biomassa, especialmente em raízes e caules, em comparação ao controle Col-0. Em contraste, sob dias longos (LD), não foram observadas diferenças entre plantas transgênicas e controle, sugerindo que a função antiflorígena de *SP3C* é dependente do fotoperíodo. Ensaios de crescimento radicular in vitro revelaram raízes primárias mais longas e maior formação de raízes secundárias nas linhagens, consistentes com fenótipos já descritos em tomate. Ensaios de germinação sob NaCl (30, 75 e 150 mM) demonstraram maior tolerância à salinidade, especialmente em 75 mM. Experimentos de seca sob desidratação severa (25% de conteúdo relativo de água) revelaram mudanças significativas no metabolismo de açúcares, com aumento do teor de amido nas rosetas. Esse efeito foi dependente da seca, pois não houve diferenças metabólicas em condições controle. Curiosamente, essa alteração na alocação de carbono foi acompanhada por acúmulo de prolina, provavelmente compensando a menor disponibilidade de sacarose como osmoprotetor. Mais experimentos são necessários para compreender melhor as interações entre *SP3C*, fotoperíodo, disponibilidade hídrica e alocação de carbono.

Palavras-chave: expressão heteróloga; gene *SP3C*; *Arabidopsis thaliana*; regulação da floração; estresse hídrico.

LIST OF ABBREVIATIONS

***ABA* –** Abscisic acid

***AP1* –** *APETALA 1*

***ATC* –** *ARABIDOPSIS THALIANA CENTRORADIALIS*

***BFT* –** *BROTHER OF FT AND TFL1*

***CEN* –** *CENTRORADIALIS*

***CO* –** *CONSTANS*

COL1 – CO-like protein 1

GA – Gibberellins

GOI – Gene of interest

FD – *FLOWERING LOCUS D*

***FM* –** Floral meristems

FT – *FLOWERING LOCUS T*

IM – Inflorescence meristem

LD – Long day

***LFY* –** *LEAFY*

***MFT* –** *MOTHER OF FT AND TFL1*

ND – Neutral day

PEBP – Phosphatidylethanolamine-binding proteins

RWC – Relative water content

SAM – Shoot apical meristem

SD – Short day

SFT – TWIN SISTER OF FT

***SOC1* –** *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*

SP – *SELF PRUNING*

SP3C – *SELF PRUNING 3C*

***SFT* –** *SINGLE-FLOWER TRUSS*

TFL1 – *TERMINAL FLOWER 1*

TF – Transcription factor

CONTENTS

1. INTRODUCTION

A heterologous system is a modern and versatile biotechnological tool suitable for diverse applications in different fields, including pharmaceutical industry and biology research. It involves the cloning and the expression of a gene of interest (GOI) from a donor species into a different genome context, a host species, a process commonly known as transgenesis **(**Kramer, 2015**)**. The choice of the host species depends on the finality of the heterologous system and often implies a well-characterized model organism. The technique is widely used in the pharmaceutical industry, where the objective is commonly to produce a large amount of specific genetic product by introducing a GOI coding sequence into microorganisms, like bacteria or yeast, leveraging their reproductive characteristics (Alberti *et al*., 2016). However, bacteria or yeast may not provide comprehensive insights into the specific functionality of a GOI within plant systems. In such cases, *Arabidopsis thaliana* (thale cress) is the preferred model organism for heterologous expression studies due to its well-characterized genome and favorable traits for plant research **(**Zhang *et al*., 2006**)**.

Arabidopsis thaliana is a small eudicotyledonous plant native to Western Eurasia that belongs to the Brassicaceae family **(**Diaz, 2019**)**. Its unique traits have made it the primary model for plant biology research, integrating genetic studies with molecular biology tools **(**Koornneef; Meinke, 2010**)**. Arabidopsis was the first flowering plant to have its genome fully sequenced in the year 2000 (The Arabidopsis Genome Initiative, 2000). Its compact genome (~135 Mbp), distributed across five chromosomes (n=5), facilitates genomics research (Krämer, 2015). Arabidopsis is also ideal for laboratory studies due to its small size, suitability for cultivation in limited spaces like Petri dishes, and a short life cycle of approximately eight weeks, optimizing research timelines (Ferjani *et al*., 2023). Each Arabidopsis plant produces a large number (~10000) of seeds and the plants' self-compatibility and high self-fertilization rates support efficient genetic screening for specific traits or mutations (Charlesworth; Vekemans, 2005). Furthermore, the simple and well-characterized anatomy of Arabidopsis, with few tissue layers, facilitates detailed studies on anatomy and plant development (Krämer, 2015).

Recently, various genes cloned from different species have been successfully expressed into Arabidopsis, contributing to a deeper understanding of their functionality across plant species. These genes commonly include abiotic stress tolerance genes sourced from crops like rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*) (Ding *et al.*, 2024; Gu *et al.*,

2024; Zhong *et al.*, 2024). Additionally, heterologous expression in Arabidopsis is particularly useful when transformation techniques are complicated or unavailable in the original model organism (Yu *et al*., 2009). On the other hand, genes governing plant growth and development, even playing a pivotal role in determining crop yield and harvest efficiency (Scheres; van der Putten, 2017), are less frequently studied using this approach, probably due to the complexity of interpreting the results. An interesting example of this is found in Vrebalov *et al*., 2009. Working with the tomato fruit ripening gene *AGAMOUS-LIKE* (*AGL1*) through heterologous expression, the authors demonstrated that it is functionally distinct from the native homologous Arabidopsis *SHATTERPROOF/2* (*SHP1/SHP2*) at the molecular level. Suggesting that the transcription factors (TFs), *AGL1* and *SHP1/SHP2*, have undergone evolutionary changes, reflecting the divergence between different fruit types, such as the fleshy berry of tomato and the siliqua of Arabidopsis. In this study, we aimed to use Arabidopsis as a genetic background to conduct a heterologous expression of *SELF PRUNING 3C* (*SP3C*) gene that is involved in tomato plant development, as will be discussed further, influencing flowering responses.

Flowering is a result of a transition in the fate and identity of the shoot apical meristem (SAM). The timing of this transition is tightly controlled in flowering plants because it is critical to ensure its reproductive success (Jiang *et al*., 2022). Flowering involves a set of concepts and molecular mechanisms that must be considered here due to the difference between Arabidopsis and tomato. Both species are considered facultative flowering plants, so in addition to autonomous regulation the flowering response is sensitive to environmental cues. However, these environmental cues have different quantitative effects in the flowering response of the two species (Samach; Lotan, 2007). Long day (LD) treatments tend to accelerate flowering of Arabidopsis plants and short day (SD) treatments tend to delay it. Therefore, Arabidopsis is considered sensitive to photoperiod and classified as a LD plant. On the other hand, wild tomato species commonly flower under SD conditions but domesticated tomato species lost the sensitivity to photoperiod, consequently other environmental cues passed to play a more significant role to define the flowering response in tomato plants. For that reason tomato cultivated species are commonly classified as neutral day (ND) plants (Higuchi *et al*., 2018).

Molecular mechanisms that regulate flowering responses in the LD plant Arabidopsis have been extensively studied. The research is now focussed in understanding the molecular

mechanisms of flowering response in agro-economical important crops like tomato (Baranov *et al*., 2024). Autonomous regulation of flowering in Arabidopsis is defined by the age of the plant including the interaction between miRNA156 and miRNA172 (Wang, 2014). Phytohormones like exogenous gibberellins (GA) and sugar disponibility can also influence flowering responses in Arabidopsis (Hisamatsu; King, 2008). However, environmental cues such as temperature and light signals have a pivotal role in the flowering of this facultative species. Light signals include light intensity and the day length, which is commonly known as photoperiod.

In Arabidopsis, the photoperiod flowering pathway is under the control of *CONSTANS* (*CO*) gene expression that occurs primarily in the companion cells of the phloem (Reyes *et al.*, 2024). The expression of *CO* reaches a peak approximately 12 h after the beginning of the photoperiod in a circadian dependent-manner. The darkness promotes the ubiquitination and the degradation of CO protein and constant light promotes its stability (Takagi *et al*., 2023). Under LD conditions circadian increased expression of *CO* gene overlaps with constant light, improving CO protein stability and increasing its abundance. CO protein is a TF that upregulates *FLOWERING LOCUS T* (*FT*) gene, this protein is translocated by the phloem to the SAM where binds to the bZIP *FLOWERING LOCUS D* (*FD*) protein (Wang *et al.*, 2022). In the SAM, FT-FD complex promotes expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) gene that triggers the transition of the vegetative SAM into a inflorescence meristem (IM). The FT-FD complex and *SOC1* upregulates *LEAFY* (*LFY*) and *APETALA 1* (*AP1*) genes that activate floral homeotic genes and generate floral meristems (FM) (Ho; Weigel, 2014). The coincidence between the high expression level of *CO* gene and constant light does not occur in SD conditions and for this reason flowering is delayed in Arabidopsis, by a mechanism explained below (Imaizumi; Kay, 2006).

In recent years, CO-like protein *Sl*COL1 was identified in tomato and showed a similar circadian behavior and interaction with light signals, however, acting as a repressor of homologue of *FT* in tomato, called *SINGLE-FLOWER TRUSS* (*SFT*) (Cui *et al*., 2022). Therefore, under LD treatments the constant light increases *Sl*COL1 abundance that repress SFT and dalay the flowering of the plants, probably like in the previously SD rice flowering model (Yang *et al*., 2020). This mechanism appears to have lost predominance in the process of domestication of tomato plants in a manner that is still under research.

There are genes that repress the flowering, like *TERMINAL FLOWER 1* (*TFL1*) gene in Arabidopsis and *SP* genes in tomato plants. These genes are necessary to ensure a correct vegetative development of the plants under different environmental conditions improving reproductive success (Matsoukas, 2015). Antiflorgens interact with the molecular mechanisms described above in manners that were intensively studied in Arabidopsis but poorly understood in tomato species. As they influence the architecture of the plants, it is necessary to consider beforehand some issues regarding the different growth habits of the two species. In monopodial plants like Arabidopsis, the SAM remains indeterminate until the death of the plant. During the vegetative phase, it generates leaves, while in the reproductive phase, it transitions to produce the inflorescence and flowers. In sympodial plants, such as wild tomato species, the SAM is determinate. Upon entering the reproductive phase, it produces a single inflorescence, and the plant growth is sustained by lateral meristems (Reinhardt; Kuhlemeier, 2002).

In Arabidopsis TFL1 protein is expressed in the SAM and through competition with FT binds to FD and maintains a vegetative state of the meristem influencing the length and the architecture of the inflorescence in *Arabidopsis* (Wickland; Hanzawa, 2015). In tomato *SFT* is upregulated in old leaves and *SP* in young leaves, therefore the age of the plant plays an important role in determining flowering. Non-functional allele (*sp*) produces a determinate growth habit that is characterized by early cessation of inflorescence production and, therefore, shorter, bushy plants with simultaneous fruit ripening (Pnueli *et al*., 2001). In contrast to its orthologue *TFL1*, that influences inflorescence architecture in Arabidopsis, *SP* gene influences the overall plant architecture, probably in function of the sympodial growth habit of tomato, as opposed to the monopodial habit of Arabidopsis (Pnueli *et al.*, 1998). The discovery of *sp* marked an important milestone in the tomato green revolution, increasing its yield and contributing to large-scale field production (Rajendran *et al*., 2021; Kang *et al*., 2022).

In addition to *CENTRORADIALIS* (*CEN*) gene from *Antirrhinum majus*, *TFL1* from Arabidopsis and *SP* from tomato, are commonly classified as part of the *CETS* gene family (Prewitt *et al*., 2018). These genes encode phosphatidylethanolamine-binding proteins (PEBPs), a kind of globular protein highly conserved across organisms, including animals, with multiple functions in angiosperms (Jin *et al.*, 2021). There are three phylogenetic clades of PEBP in Arabidopsis, *FT*-like clade, that include the genes that promote the flowering, *FT*

and *TWIN SISTER OF FT* (*SFT*) genes, *TFL1*-like clade that repress flowering, including *TFL1*, *BROTHER OF FT AND TFL1* (*BFT*) and *A. THALIANA CENTRORADIALIS* (*ATC*) genes and *MOTHER OF FT AND TFL1* (*MFT*)-like clade that is principally related to the germination process (Bellinazzo *et al*., 2024). Even with the highly conserved structure between PEBPs, small changes in its amino acid sequence have important implications for its functionality. FT is structurally highly similar to TFL1 protein, even with opposite roles as florigen and anti florigen respectively. Both have putative anion-binding pockets in Y85 for FT and in H89 for TFL1, a putative 14-3-3 binding domain and a segment B loop (Wang *et al*, 2017). The segment B is structurally highly divergent between FT and TFL1 and has a significant influence on the function of the protein. In fact, some works have turned TFL1 protein into an inductor of flowering by changing only one amino acid in segment B loop (Taoka *et al*., 2013).

BLAST analysis revealed twelve *SP* paralogues in sequenced genomes of tomato (Cao *et al*., 2016). Based on amino acid sequence alignment; these genes were classified in three different clades by their maximum likelihood with the *CETS* founding members from Arabidopsis (Moreira *et al.*, 2022). *FT*-like genes, *SP5G*, *SP6A*, *SP11B.1*, *SP11B.2* and *SP3D, TFL1*-like genes, *SP3C*, *SP1C.2*, *SP1C.1*, *SP9D* and *SP* and MFT-like genes *SP2G* and *SP3I*. With the exception of the antiflorigen *SP*, which was the first discovered, and *SP5G* that is associated with the loss of sensitivity to photoperiod on domesticated tomato species the role of the other *SP* genes is poorly understood (McGarry; Ayre, 2012). Recently, *SP3C* paralogue has been characterized by our research group and revealed pleiotropic roles in tomato plants. Sequence analysis defined *SP3C* gene in the *TFL1*-like clade as anti-florigen (Cao *et al*., 2016). Additionally, MYB-binding motifs, commonly associated with abiotic stress tolerance, were found on the promoter sequence of *SP3C* gene (Zhao *et al.*, 2018). We measured the expression levels of different *SP* genes in tomato plants subjected to drought treatments and the results showed a significant increase in the expression of *SP3C* among the other *SP* genes. Further characterization by overexpression and CRISPR/Cas9 approaches revealed that when overexpressed, *SP3C* causes delayed flowering and germination, along with an increase in the number of lateral roots contributing to drought tolerance in tomato plants (Moreira *et al*., 2022).

2. OBJECTIVE

In this study, we aimed to use *Arabidopsis thaliana* as a genetic background, taking advantage of its well-established characteristics, to gain new insights about the pleiotropic functions of *SP3C* gene on processes such as flowering regulation, seed germination, root development, and abiotic stress tolerance. Additionally, these new perspectives aim to expand our knowledge of how *SP3C* interacts with the intricate flowering signaling networks and influences the transition between vegetative and reproductive growth under abiotic stress.

3. MATERIALS AND METHODS

3.1. Plant Material

The transformation was performed in the genetic background *A. thaliana* Columbia-0 (Col-0) using the *Agrobacterium tumefaciens*-mediated floral dip method, as described by Clough and Bent (1998). *SP3C* coding sequence was previously purified from *Solanum lycopersicum* cv. Micro-Tom leaves and cloned into pCR8/GW/TOPO® (Invitrogen), as described in Moreira *et al.*, (2022). The cloned sequence was combined into an overexpression vector (pK7WG2) with kanamycin resistance by Gateway® technology (Karimi *et al.,* 2002). The correct assembly of the construct was confirmed in the DNA of T_0 plants by PCR amplification of the *nptII* (Neomycin phosphotransferase) gene using specific primers (F: 5'-GAG GCT ATT CGG CTA TGA CTG G-3' and R: 5'-ATC GGG AGC GGC GAT ACC GTA-3') (Supplementary figure S01.A). The presence of the *35S::SP3C* insert was further verified through a second PCR, where the forward primer targeted the CaMV 35S sequence, and the reverse primer targeted the *SP3C* gene (5'-TCA TCT TCT TCT GGC TGG CAG-3'), yielding a 1000 bp product (Supplementary figure S01.B). To obtain homozygous transformed plants, the lines were self-pollinated to produce T_2, T_3, and T_4 seeds. Progeny tests were conducted on seedlings using a foliar spray of kanamycin at 500 mg/L. Additionally, *in vitro* progeny tests were performed on 0.5x Murashige-Skoog (MS) medium supplemented with kanamycin at concentrations of 50 µg/mL and 100 µg/mL (Supplementary figure S02). Progeny exhibiting 100% antibiotic resistance were considered to originate from a homozygous parent plant. For all analyses, three independent T_4 transformation events (#8, #9, and #11) were utilized.

3.2. Flowering Time, Rosette Area and Biomass Accumulation

Seeds of Col-0 and T_4 seeds of the selected transgenic events (*SP3C*#8, #9, and #11) were stratified at 4°C for 4 days in the dark. After stratification, the seeds were sown in individual pots containing 60 g of autoclaved substrate and placed in a growth chamber with controlled conditions (21–23°C and a light intensity of 200 μmol m^{-2} s^{-1}). Two experimental groups were established: one group was grown under short-day (SD) conditions (8 h light/ 16 h dark) and other under long-day (LD) conditions (16 h light / 8 h dark). Flowering was recorded daily as soon as the inflorescence reached 0.5 cm, in both SD and LD conditions. Once all plants had flowered (64 days after sowing in SD and 32 days in LD conditions), the rosette area was measured using ImageJ 13.0.6 (USA) software, and the plants were then harvested and oven-dried at 50°C to assess the dry biomass of roots, rosettes, and inflorescences.

3.3. Root Growth Analysis

Seeds of Col-0 and T_4 seeds of the selected transgenic events (*SP3C* #8, #9, and #11) were surface-sterilized with 70% ethanol followed by 2.5% sodium hypochlorite. Approximately 8 seeds were sown 2 cm from the top edge of the square plates containing 0.5x MS media supplemented with 1.5% sucrose. Each transgenic line, as well the genetic control were sown in different plates. The plates were then stratified at 4°C for 4 days in the dark. After stratification, the plates were positioned vertically in a growth chamber under short-day (SD) conditions (8 h light, 21–23°C and light intensity of 200 μmol m^{-2} s^{-1}). Starting 5 days after stratification, root length was recorded daily. 12 days after stratification, the square plates were scanned, and the number of secondary roots was counted, and final root length, as well root growth rate, were measured using ImageJ 13.0.6 (USA) software.

3.4. Seed Germination Assay Under Osmotic Stress

The seed germination assay was *in vitro* conducted with T_4 seeds from each transgenic line, using Col-0 seeds as genetic control. The seeds were surface-sterilized and geometrically sown (25 seeds per Petri dish) on 0.5x MS medium supplemented with 1.5% sucrose, with each dish containing a single genotype. Additional experimental groups were established to assess the effect of NaCl on seed germination by supplementing the medium with three concentrations of NaCl (30, 75, and 150 mM). These concentrations correspond to theoretical osmotic potentials calculated using the van't Hoff equation (-0.149, -0.372, and -0.744 MPa,

respectively) and were adopted from Gonzalez *et al.*, 2024. After stratification, the plates were randomly placed in the growth chamber under controlled SD conditions (8 h light, 21–23°C and light intensity of 300 µmol m^{-2} s^{-1}). Everyday germination was monitored and recorded as soon as the radicle emerged following the method described by Piskurewicz and Lopez-Molina (2016).

3.5. Drought Experiment

Col-0 seeds and T_4 seeds of the selected transgenic events (*SP3C* #8, #9, and #11) were stratified in darkness at 4°C for 4 days. After stratification, pots containing 60 g of autoclaved substrate were divided into quadrants. Seeds from each genetic line were sown in separate quadrants, with each quadrant representing a different genetic line. The plants were then grown in a growth chamber under controlled SD conditions (8 h light, 21–23°C and light intensity of 200 µmol m^{-2} s^{-1}). Drought treatment was applied 25 days after sowing by ceasing irrigation (Wang *et al*., 2018). A well-irrigated group served as the control. After 10 days of drought treatment, plant rosettes were collected, and relative water content (RWC) and metabolic analyses were performed.

3.6. Metabolic Analyses

Plants were grown under controlled SD conditions (8 h light, 21–23°C, and light intensity of 200 µmol m^{-2} s^{-1}). Metabolic analysis was performed using the entire pool of rosette leaves harvested 35 days after sowing, 4 hours after the onset of the photoperiod. The samples were freeze-dried, and methanolic extraction was carried out as described by Lisec *et al*. (2006). For this, 10 mg of the lyophilized sample was dissolved in 700 µL of 100% ethanol, heated to 80°C in a thermomixer, and centrifuged. The supernatant was used for pigment quantification (chlorophylls and carotenoids) as well as the measurement of glucose, fructose, sucrose, amino acids, and proline. The pellet was used to quantify proteins and starch.

3.7. Statistical analysis

For all analyses, three or more biological replicates and two technical replicates were used when necessary for each genotype. A one-way analysis of variance (ANOVA) was performed to examine the differences between genotypes, with Tukey's test applied to identify significant differences. Pearson correlation analysis was conducted with a significance level of $P \leq 0.05$

to explore the relationships between variables. All statistical analyses were performed using GraphPad Prism Software, version 10.0.0 (Boston, Massachusetts USA).

4. RESULTS AND DISCUSSION

4.1. *SP3C* overexpression affects development of *Arabidopsis* in a day length-dependent manner

First, we aimed to characterize the function of the *SP3C* gene as an anti-florigen by measuring the flowering time of *Arabidopsis* overexpressing lines. It is widely known that the balance *TFL1/FT* is strongly regulated by day-length in *Arabidopsis* by influence of *CO* gene, affecting the flowering time of the plants (Freytes *et al.*, 2021). Therefore, the experiment was conducted with groups of plants under two different day-length conditions: short-day (SD) and long-day (LD). The results showed a significant delay in the flowering time of *SP3C*#9 and *SP3C*#11 overexpressing lines under SD conditions (Fig. 1A), confirming the anti-florigenic role of *SP3C* and demonstrating functional complementary with *TFL1* gene in short-day conditions.

Arabidopsis is a facultative long-day plant that flowers earlier under LD than under SD conditions (Osnato *et al.*, 2022). In the experiment, the group of plants under LD conditions began the phase transition approximately 20 days earlier than under SD conditions and showed no differences between the overexpressing lines and Col-0 (Fig. 1B). Under LD conditions, intense light signals increase the transcription of the *CO* gene and activate the photoperiodic pathway of flowering through *FT* upregulation (Turck, 2008). Even though that *SP3C* gene was under constitutive expression, apparently, it was not enough to avoid the photoperiodic pathway of Arabidopsis genetic background. This result suggests that *SP3C* influences flowering response downstream of the *CO* gene. The experiments of Moreira *et al.* (2022) were carried out in cultivated species of tomato, therefore the loss of sensitivity to the photoperiodic pathway could have an effect in the function of *SP3C*. It is essential to measure the expression levels of *CO*, *FT*, *TFL1*, and *SP3C* in order to gain a better understanding of these networks in the transgenic events and their correlation with light signals.

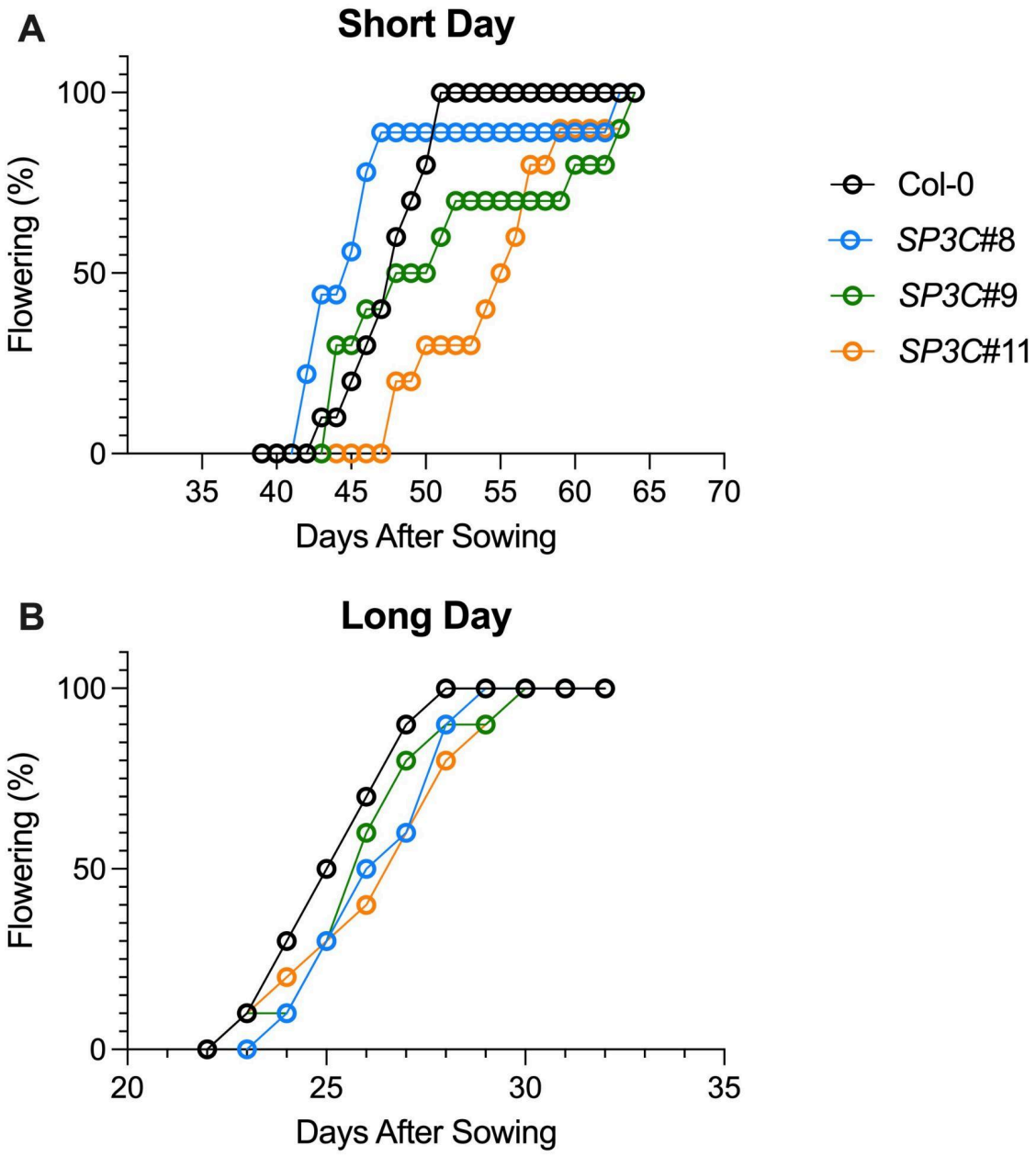

Fig. 1. Flowering time under short-day (A) and long-day (B) conditions. Points represent the cumulative percentage of plants in the reproductive phase defined by an inflorescence length ≥ 5 mm (*n*=10 plants). Black points correspond to the Col-0 line, while colored points represent the overexpressing lines: *SP3C*#8 (blue), *SP3C*#9 (green), and *SP3C*#11 (orange).

We observed that the delayed flowering time in the overexpressing lines under SD conditions was accompanied by a noticeable phenotype, characterized by larger rosettes and leaves compared to Col-0 plants (Fig. 2). This phenotype was consistently present across all three transgenic events, although less pronounced in the *SP3C*#8 lines, which did not exhibit a

delay in the flowering time. However, this phenotype was completely absent in Col-0 plants (Supplementary figure S03). Under LD conditions, it was not possible to visually distinguish any differences among the plants. Therefore, to gain further insights, we characterized the plants grown under both SD and LD conditions by measuring rosette area and biomass accumulation at the end of the experiment (Fig. 2A and B).

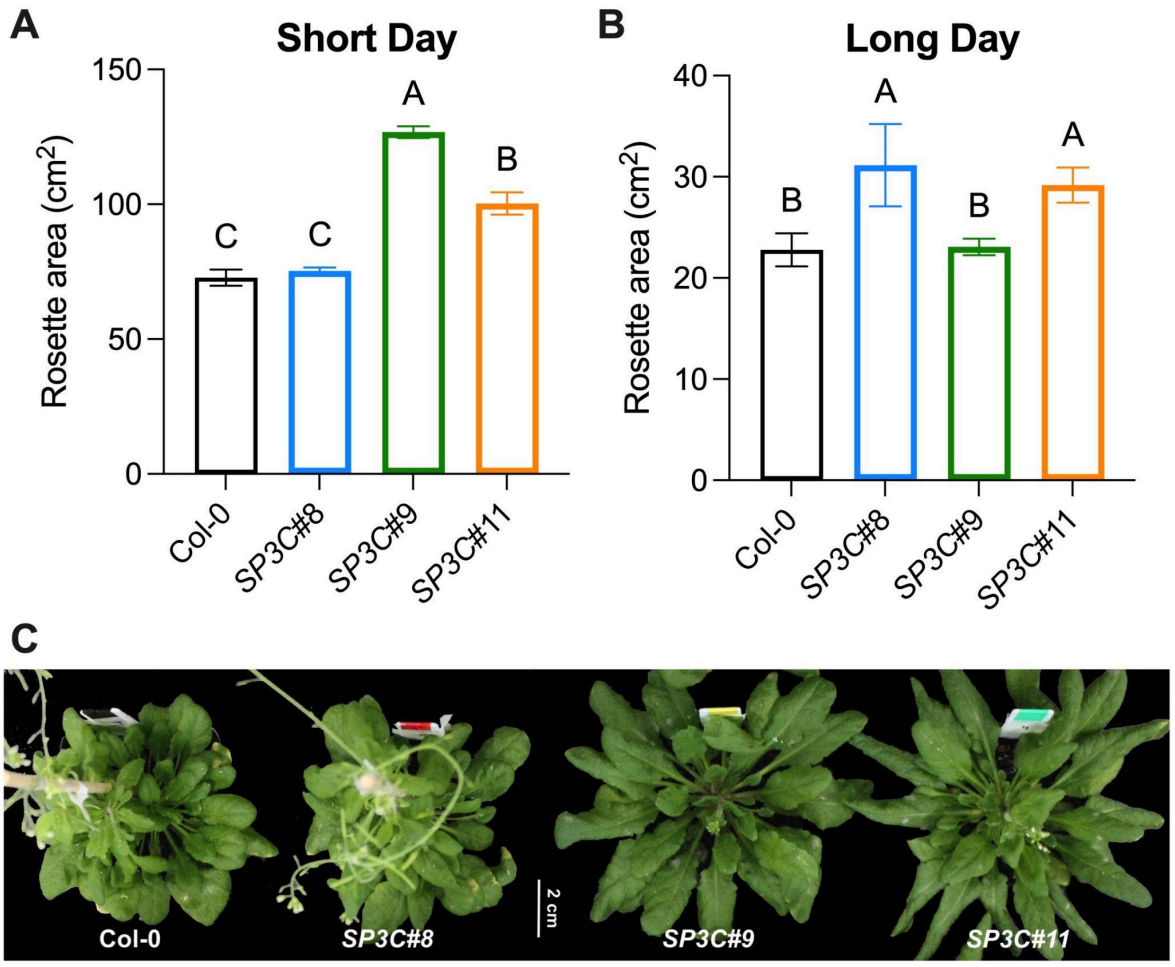

Fig. 2. Rosette area analysis at the end of the experiment. A) Rosette area under short-day and **B)** long-day conditions**.** Bars represent the mean ± SD rosette area in cm² (*n*=5 plants) measured at the end of the experiment, 65 days and 32 days after sowing for short-day and long-day conditions, respectively. Black bars correspond to the Col-0 line, while colored bars represent the overexpressing lines: *SP3C*#8 (blue), *SP3C*#9 (green), and *SP3C*#11 (orange). Capital letters indicate significant differences between the lines according to the tukey's test ($p < 0.05$). **C)** Representative plants of the transgenic lines and Col-0. **D)** The image shows representative rosettes of the plants grown under short-day conditions at the end of the experiment, 65 days after sowing. From left to right: Col-0, *SP3C*#8, *SP3C*#9, and *SP3C*#11.

In *Arabidopsis*, the rosette area is indicative of the plant's life strategy and reflects a greater investment in vegetative tissue, typical of plants with delayed phase transitions (Knapp *et al*., 2022). Under SD conditions, the rosette area of the *SP3C*#9 and *SP3C*#11 overexpressing lines was 75,9% and 38,4% larger than plants of Col-0 (Fig. 2A). As mentioned, this characteristic phenotype was also present in the *SP3C*#8 lines, but did not influence the statistical analysis. Under LD conditions, the differences were less noticeable between the overexpressing lines and Col-0 than under SD conditions (Fig. 2B). It is likely that the rapid development of plants under LD conditions favors the transition to the reproductive stage over the vegetative stage, as this promotes seed production to ensure the accumulation of seed reserves, given the apparent abundance of light. A more detailed study of the inflorescence architecture and length could provide insights into whether the *SP3C* gene plays a role in regulating this balance in LD conditions.

As expected, under SD conditions, the overexpressing lines showed increased biomass accumulation compared to Col-0 (Fig. 3A). In *SP3C*#9 and *SP3C*#11, this increased biomass was principally caused by the root and rosette biomass (Fig. 3C, 3D). The results align with the observed phenotypes during the experiment and support the role of *SP3C* as an anti-florigenic factor suggesting that these plants are prioritizing vegetative tissue over reproductive tissue. However, the *SP3C*#8 line showed a different pattern, accumulating significantly more biomass in the inflorescence compared to Col-0 and the other overexpressing lines (*SP3C*#9 and *SP3C*#11) (Fig. 3B). In *Arabidopsis*, the overexpression of *TFL1* increases inflorescence length by maintaining the SAM in an indeterminate state (Shannon; Meeks-Wagner, 1991). Therefore, this phenotype in *SP3C*#8 may also be influenced by *SP3C* overexpression. Further characterization of the inflorescences is needed to support these findings. Under LD conditions, the Col-0 line exhibited a significant increase in biomass accumulation compared to the overexpressing lines, especially compared to the *SP3C*#11 line (Fig. 3A). However, this increased biomass was predominantly allocated to the inflorescence (approximately 75% of the total biomass) (Fig. 3B).

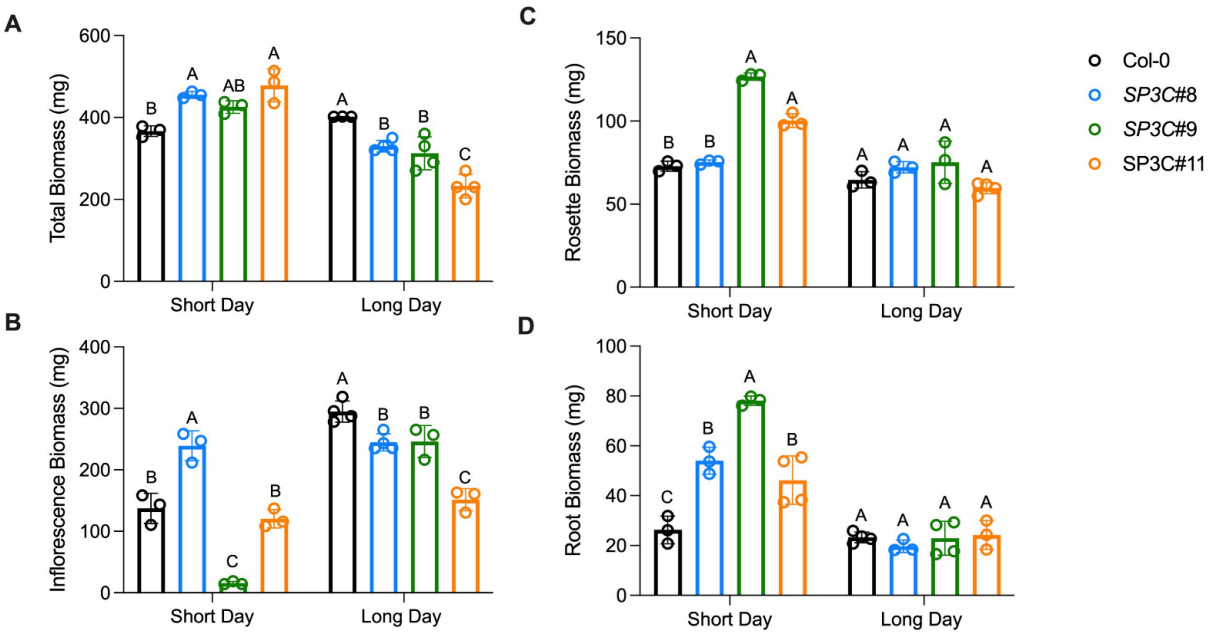

Fig. 3. Biomass accumulation under short-day and long-day conditions. Total biomass accumulation (**A**), and biomass distribution analysis between inflorescence (**B**), rosette (**C**), and root (**D**) of the plants at the end of the experiment. Bars represent the mean ± SD (*n*=5 plants) of dry-weight in mg, 65 days after sowing under SD conditions and 32 days after sowing under LD conditions. Black bars correspond to the Col-0 line, while colored bars represent the overexpressing lines: *SP3C*#8 (Blue), *SP3C*#9 (Green), and *SP3C*#11 (Orange). Capital letters indicate significant differences between the lines according to the tukey's test ($p < 0.05$).

4.2. *SP3C* overexpression influences root length and secondary root formation of *Arabidopsis*

As previously described, under SD conditions, the root biomass of the *SP3C*#8, #9, and #11 overexpressing lines was 1.3, 2.1, and 1.2 times greater, respectively, compared to Col-0. These results align with the findings of Moreira *et al*. (2022) in tomato, where *SP3C* overexpression significantly promoted secondary root formation and enhanced drought stress tolerance. To further investigate the role of *SP3C* in root development in *Arabidopsis*, we conducted an *in vitro* root growth analysis using vertical square plates with nutrient medium. As the biomass accumulation analysis showed no significant difference between the roots of the plants under LD conditions, the experiment was carried out in SD conditions.

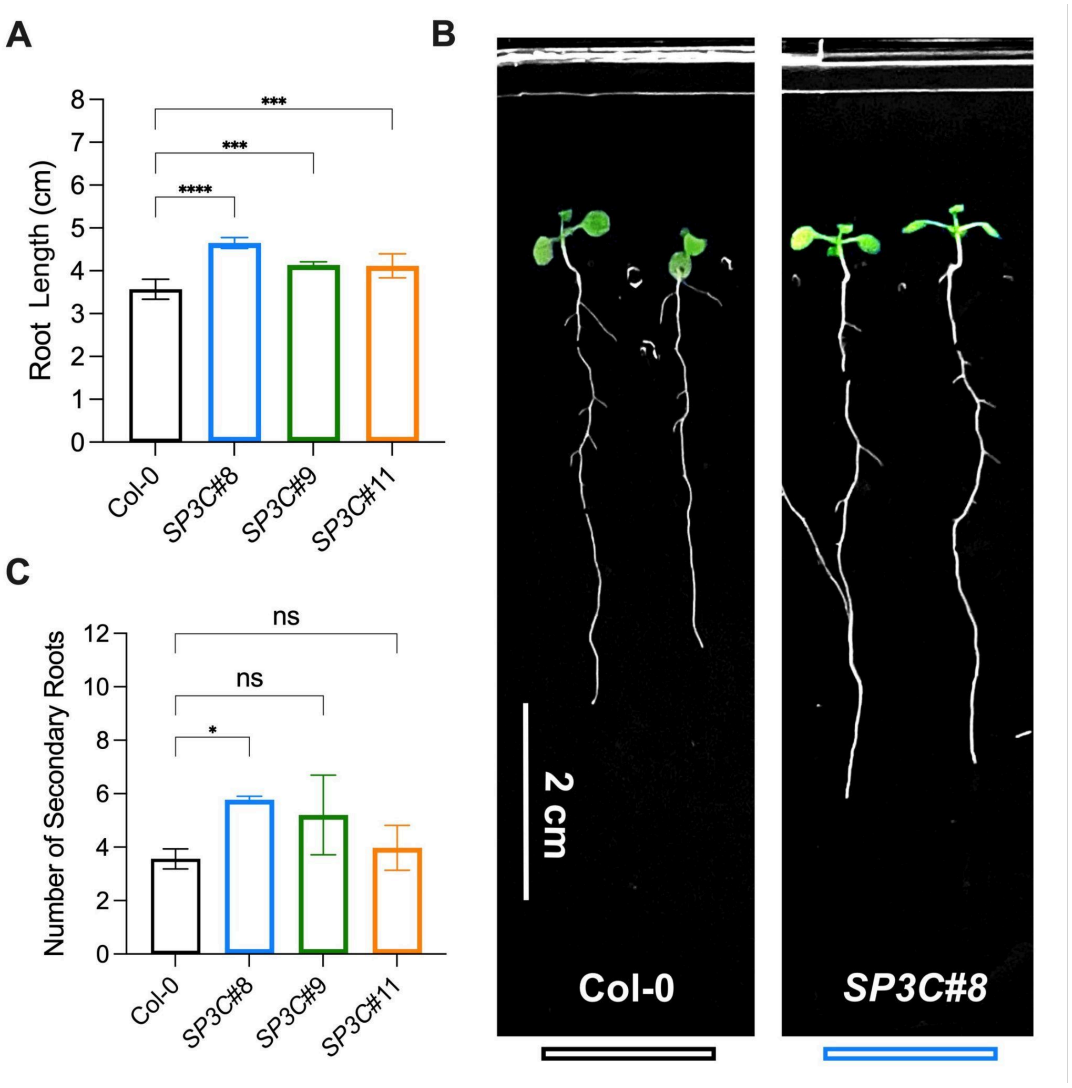

Fig. 4. *In vitro* root growth analysis under short-day conditions. **A)** Root length (cm). **B)** Number of secondary roots. **C)** Representative roots of Col-0 (left) and *SP3C*#8 overexpression line (right). The experiment was conducted *in vitro* using vertical square plates. Data represent the mean ± SD of five or more biological replicates, each containing eight plants, recorded at the end of the experiment (12 days after sowing). Black bars correspond to the Col-0 line, while colored bars represent the overexpressing lines: *SP3C*#8 (blue), *SP3C*#9 (green), and *SP3C*#11 (orange). Capital letters indicate significant differences between the lines according to the tukey's test ($p < 0.05$).

An important increase in root length was observed in the *SP3C*#8 line, with roots approximately 1.5 cm longer than those of Col-0 (Fig. 4A). Additionally, the number of secondary roots was higher in the *SP3C*#8 and *SP3C*#9 transgenic lines, though the difference was modest, with only a few more secondary roots. These findings are consistent with the

phenotype of plants investing more in vegetative tissue. Interestingly, previous studies have shown that *SP* overexpression in tomato plants strongly interacts with auxin transport, primarily influencing the growth habit of the shoot. However, it is likely that *SP* genes also affect auxin homeostasis in the roots (Silva *et al*., 2018).

4.3. *SP3C* overexpression increases seed germination of *Arabidopsis* seeds under NaCl treatment

Seed dormancy and germination are critical stages in the plant life cycle, as the timing of germination directly influences the conditions under which the seedling will develop, ultimately impacting crop productivity (Bentsink; Koornneef, 2008). The germination assay revealed no delayed germination in *Arabidopsis* (Fig. 5A), unlike what was observed previously in tomato plants by Moreira *et al*., 2022. However, *SP3C* overexpression significantly influenced *Arabidopsis* germination performance under NaCl treatments. NaCl is a stress agent that affects seed germination and dormancy by influencing the crosstalk between abscisic acid (ABA) and gibberellin (GA) balance in seeds, typically delaying germination in *Arabidopsis* (Yuan *et al*., 2010).

Under the 30 mM NaCl treatment, the seeds of the overexpressing lines exhibited a significant delay in germination compared to the Col-0 seeds (Fig. 5B). Delayed germination under stress conditions plays a crucial role in protecting the seedling's future fitness, as it enhances water uptake, which is vital for seedling establishment and survival (Penfield, 2017). Curiously, the *SP3C*#8 overexpressing lines exhibited significantly healthier seedlings at the end of the experiment (Fig. 5E) under 75 mM NaCl, in contrast to both Col-0 and the other overexpressing lines. Furthermore, this transgenic event demonstrated an increased germination rate under 75 mM NaCl compared to Col-0 (Fig. 5C). Under 150 mM NaCl overexpressing lines showed increased seed germination percentage compared to Col-0 especially in *SP3C*#8 lines with over 40% of seeds germinating, in contrast to less than 10% germination in Col-0 seeds (Fig. 5D). These findings suggest enhanced NaCl tolerance in *SP3*C#8. Given that NaCl induces both osmotic and salt stress in the MS medium, further experiments are required to dissect the role of *SP3C* overexpression under these two stresses individually (Gonzalez *et al*., 2024).

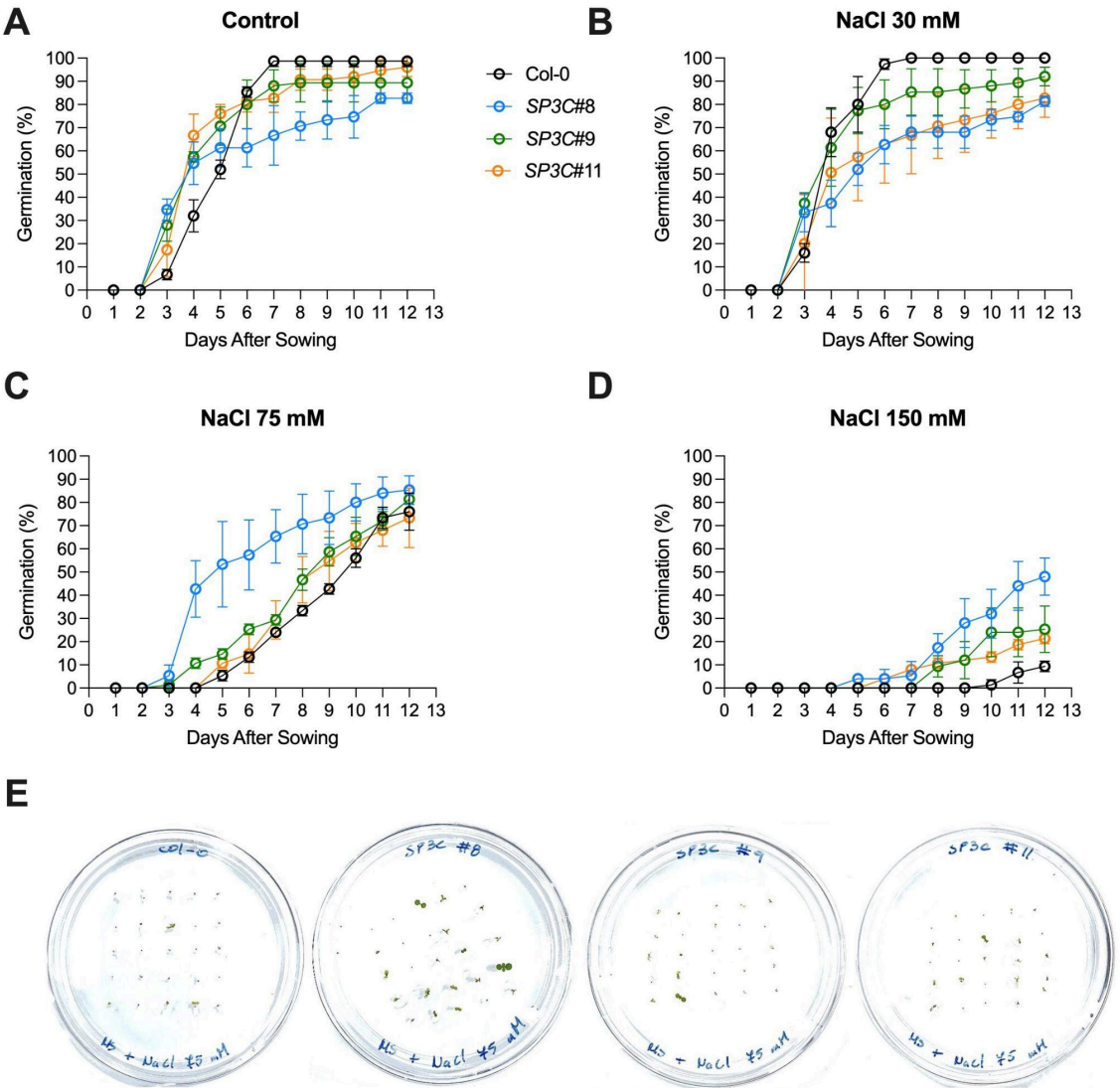

Fig. 5. Cumulative *in vitro* seed germination under NaCl treatments. (A) Germination rate under control conditions. (B) Germination rate under NaCl 30 mM . (C) Germination rate under NaCl 75 mM. (D) Germination rate under NaCl 150 mM. Data points represent daily mean ± SD of germinated seed percentages over 12 days after stratification, based on five biological replicates, each containing 25 seeds. Black points correspond to the Col-0 line, while colored points represent the overexpressing lines: *SP3C*#8 (blue), *SP3C*#9 (green), and *SP3C*#11 (orange). (E) Representative Petri plates of the germination assay under 75 mM NaCl treatment. The images are representative of biological replicates of Col-0 and the transgenic lines, 12 days after stratification, at the end of the experiment.

4.4. *SP3C* overexpression increase leaf starch and influence proline metabolism under severe dehydration

Since the *SP3C* overexpressing lines appeared more vigorous than the Col-0 plants in the germination experiment, and due Moreira *et al*. (2022) demonstrated that *SP3C* influences drought tolerance in tomato plants, we conducted a drought experiment in soil under SD conditions to further explore this pleiotropic role in *Arabidopsis*. The drought experiment was conducted in pots, with the four lines (Col-0 and *SP3C* overexpressing lines) placed in separate quadrants within the same pot minimizing experimental variance. The plants were initially well-watered until 25 days after sowing. At this point, one group was maintained well-watered (control), while the other group was subjected to 10 days without irrigation. After the drought treatment, we collected rosettes to measure relative water content (RWC) and assess metabolic performance.

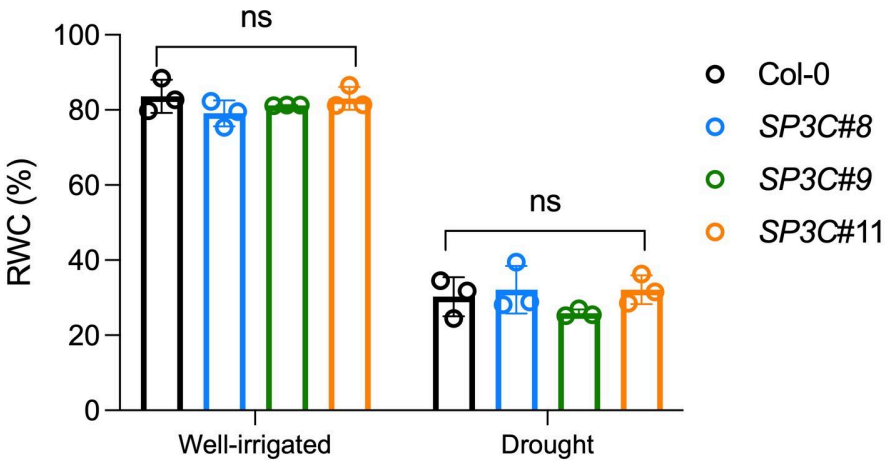

Fig. 6. Relative water content (RWC) of plants in the drought experiment. Bars represent the mean ± SD (n=3) for rosettes of 35-day-old plants grown under short-day conditions subjected to 10 days without irrigation. Black bars correspond to Col-0 plants, while colors bars represent the transgenic lines *SP3C*#8 (blue), *SP3C*#9 (green), and *SP3C*#11 (orange).

Water status analysis revealed that after 10 days without irrigation, rosettes RWC dropped to approximately 25% across all transgenic lines and Col-0 (Fig. 6). Visually, no difference was observed between the phenotypes, maybe related to severe dehydration. However, further evaluation exposed significant differences in metabolism performance between the overexpressing lines and Col-0 plants, especially in sugar metabolism (Fig. 7).

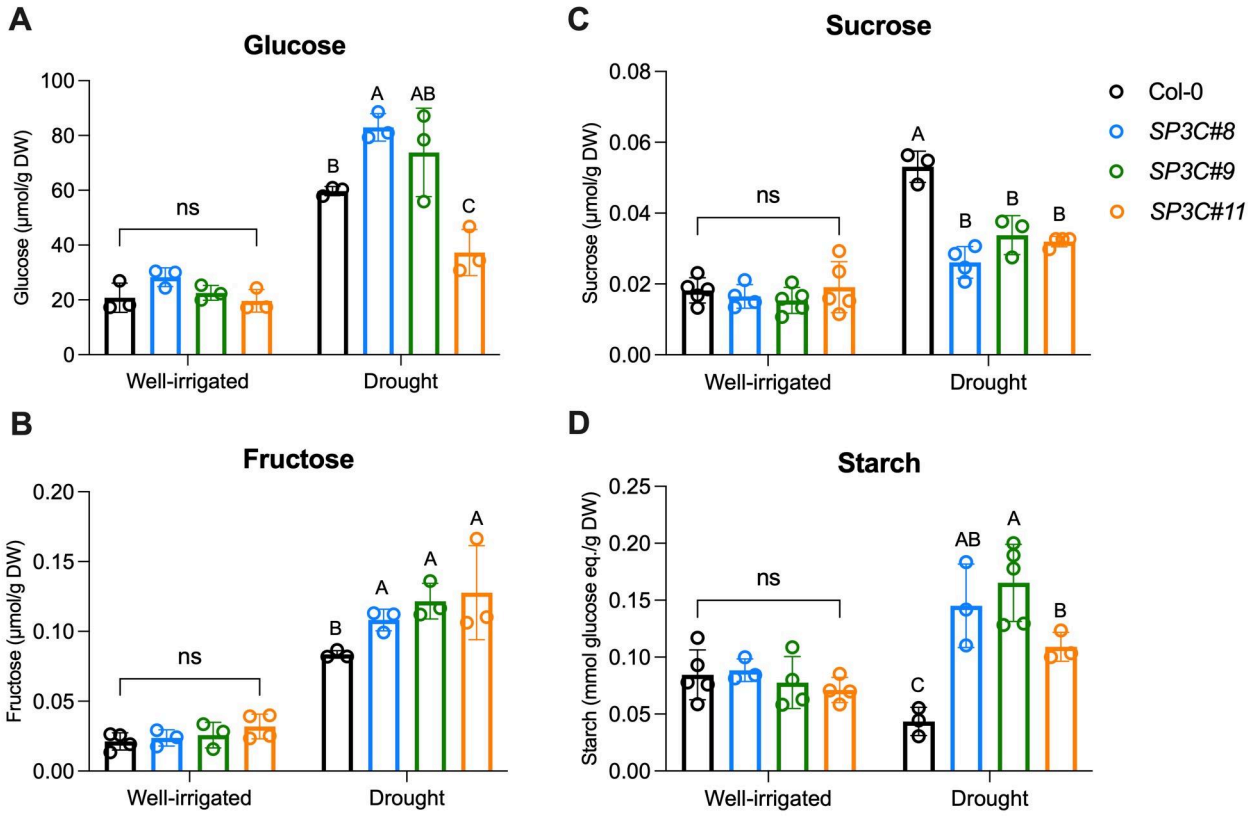

Fig. 7. **Sugar metabolism of plants under severe drought conditions.** Bars represent the mean ± SD (n=3 or more) for samples of leaves of 35-day-old plants grown under SD conditions subjected to 10 days without irrigation collected four hours after the onset of the photoperiods. Black bars correspond to the Col-0 line, while colored bars represent the overexpressing lines: *SP3C*#8 (Blue), *SP3C*#9 (Green), and *SP3C*#11 (Orange). Capital letters indicate significant differences between the lines according to the tukey's test ($p < 0.05$).

Under control conditions (well-watered), none of the *SP3C* overexpression lines exhibited behavior different from Col-0 in any of the analyses related to rosette sugar metabolism, including glucose, fructose, sucrose, or starch. This suggests that the constitutive expression of *SP3C* does not inherently alter central sugar metabolism. However, under drought conditions, the metabolic profiles diverged markedly. Under severe water deficit, Col-0 plants exhibited a 2,2 fold increase in sucrose content accompanied by a decrease in starch content. This inverse relationship is consistent with a phenotype that tends to prioritize carbon translocation to reproductive organs in order to produce seeds and escape the stress episode. On the other hand, this shift of trioses toward sucrose production in Col-0 may also be explained by the osmoprotective properties of this disaccharide, which helps maintain cellular

turgor under water deficit conditions, a behavior previously documented in *Arabidopsis* (Gurrieri et al., 2020).

In contrast, the *SP3C* overexpression lines diverted trioses toward starch synthesis and accumulation in the rosette under severe drought conditions. This suggests that these plants adopted a different carbon allocation strategy, prioritizing storage over translocation. This is coherent with a 'stay-green' strategy in which the plant prioritizes vegetative resilience over rapid reproductive development under water deficit (Thomas & Haworth, 2000). Genotypes that follow this kind of strategy tend to enhance tissue protection, improving their stress tolerance. Moreover, stay-green genotypes have been associated with higher photosynthetic rates and greater accumulation of resources, resulting in better seed quality at the time of flowering (Zheng *et al*., 2009). A clearer understanding of this phenomenon would benefit from the measurement of photosynthetic parameters, as no significant differences were found in any of the photosynthetic pigment analyses (Supplementary Figure S05). In our results, this type of strategy appeared to be activated only under drought conditions, suggesting that *SP3C* interacts with the drought stress response regulatory network of the genetic background. *SP3C*, as a transcription factor, may modulate pathways such as *SnRK1*/*T6P* or repress the expression of *SWEET*/*SUT* sugar transporters, thereby influencing the systemic distribution of assimilates during abiotic stress (Yu *et al*., 2015; Saddhe *et al*., 2020).

Interestingly, although the *SP3C* overexpression lines showed a decrease in the amount of sucrose, an osmotically active compound, this was accompanied by a significant increase in proline content (Fig. 8C). This suggests that proline may partially substitute for sucrose as the primary osmoprotectant under drought conditions. While both compounds serve osmotic functions, proline accumulation also reflects broader stress responses, including reactive oxygen species detoxification and nitrogen recycling. Given that sucrose levels did not increase in *SP3C* lines, the elevated proline may act as a compensatory mechanism to maintain cellular osmotic balance. Proline is a crucial amino acid and osmolyte that plays a key role in plant adaptation to abiotic stress, particularly drought and osmotic stress (Furlan et al., 2020). As an osmoprotectant, proline helps to stabilize cellular structures and maintain turgor pressure under stress conditions by lowering osmotic potential. It accumulates in response to environmental stresses such as drought, high salinity, and extreme temperatures, allowing plants to survive adverse conditions (Mohammadkhani & Heidari, 2008).

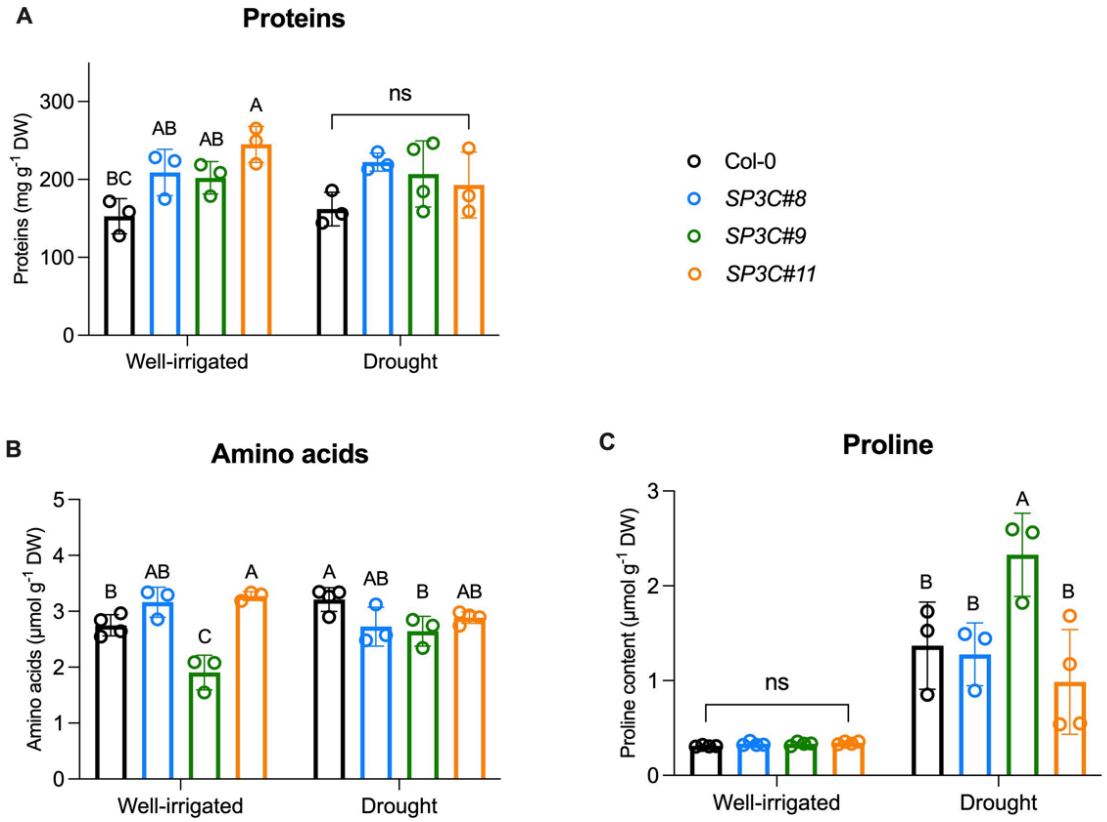

Fig. 8. **Protein (A), amino acids (B) and proline content of the plants of the drought experiment**. Bars represent the mean ± SD (n=3 or more) of protein, amino acids and proline content of leaves of 35-day-old plants grown under short-day conditions collected four hours after the onset of the photoperiods. Black bars correspond to the Col-0 line, while colored bars represent the overexpressing lines: *SP3C*#8 (Blue), *SP3C*#9 (Green), and *SP3C*#11 (Orange). Capital letters indicate significant differences between the lines according to the tukey's test ($p < 0.05$).

In our study, despite the low RWC, a significant increase in proline levels was observed in the *SP3C#9* overexpression line compared to Col-0 plants under severe drought. These findings suggest that *SP3C* overexpression may enhance the plant's ability to cope with drought and/or osmotic stress. This is consistent with the results from the in vitro germination assay, in which transgenic lines showed increased tolerance to NaCl treatments. Altogether, the results reinforce the potential role of *SP3C* in enhancing drought and salt tolerance in *Arabidopsis*, similar to what has been previously observed in tomato plants (Moreira et al., 2022). However, a drought experiment under moderate dehydration conditions could provide more insight into these hypotheses.

Finally, as seen in the bolting time results, flowering delay mediated by *SP3C* in *Arabidopsis* was clearly photoperiod-dependent. Interestingly, the alteration in sugar metabolism was strongly stress-dependent. This suggests that *SP3C* may rely on the photoperiod integration network, while simultaneously interacting with drought-related regulatory pathways. A future experiment that considers both variables, photoperiod and water stress, would be valuable to better understand how these two networks interact under osmotic stress, whether induced by drought or other treatments that reduce water potential.

Arabidopsis follows a drought-escape strategy; its seeds develop inside siliques, which are highly tolerant to water deficiency. In contrast, tomato seeds develop inside fleshy fruits, which have high water demands (Johnson *et al*., 1992). It is plausible that flowering control genes have undergone functional diversification in tomato compared to Arabidopsis, likely as an adaptive response to ensure fruit development under variable environmental conditions. *SP3C* may be one such component of the complex flowering regulatory network in Solanaceae species, particularly in tomato, potentially playing a more prominent role in integrating hydric signals. Moreover, as previously mentioned, domesticated tomato varieties have largely lost sensitivity to photoperiod. This aspect should be considered when investigating how *SP*, *SP3C*, and *SFT* interact in photoperiod-sensitive tomato species or in genetic backgrounds where *SP5G* function is lost.

CONCLUSIONS AND FUTURE PERSPECTIVES

As expected, our results confirm the role of *SP3C* as an anti-florigen, supplementing the function of *TFL1* under SD conditions and delaying the flowering time in Arabidopsis influencing the equilibrium of vegetative/reproductive growth of plants. *TFL1* and *FT knockout* lines could be useful to understand better the interaction of *SP3C* in the genetic background Arabidopsis.

We confirm the pleiotropic functions of the *SP3C* gene as significant differences were obtained in root growth analysis and germination and we suggest a connection between the regulation of flowering and enhanced tolerance to osmotic stress.

As suggested by the severe drought experiment, *SP3C* could influence the sugar metabolism markedly dependent on water status. It is necessary to improve the drought experiment to understand how the progressive drop in RWC influences this effect. Additionally, it could be

interesting to correlate the photoperiod and the flowering time to this disrupted sugar metabolism.

Apparently, overexpressing lines showed an increase in proline content, probably due to the deficiency of sucrose as osmoprotectant. These results could be associated better with salt tolerance to add a new pleiotropic role to the *SP3C* gene.

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SUPPLEMENTARY FIGURES

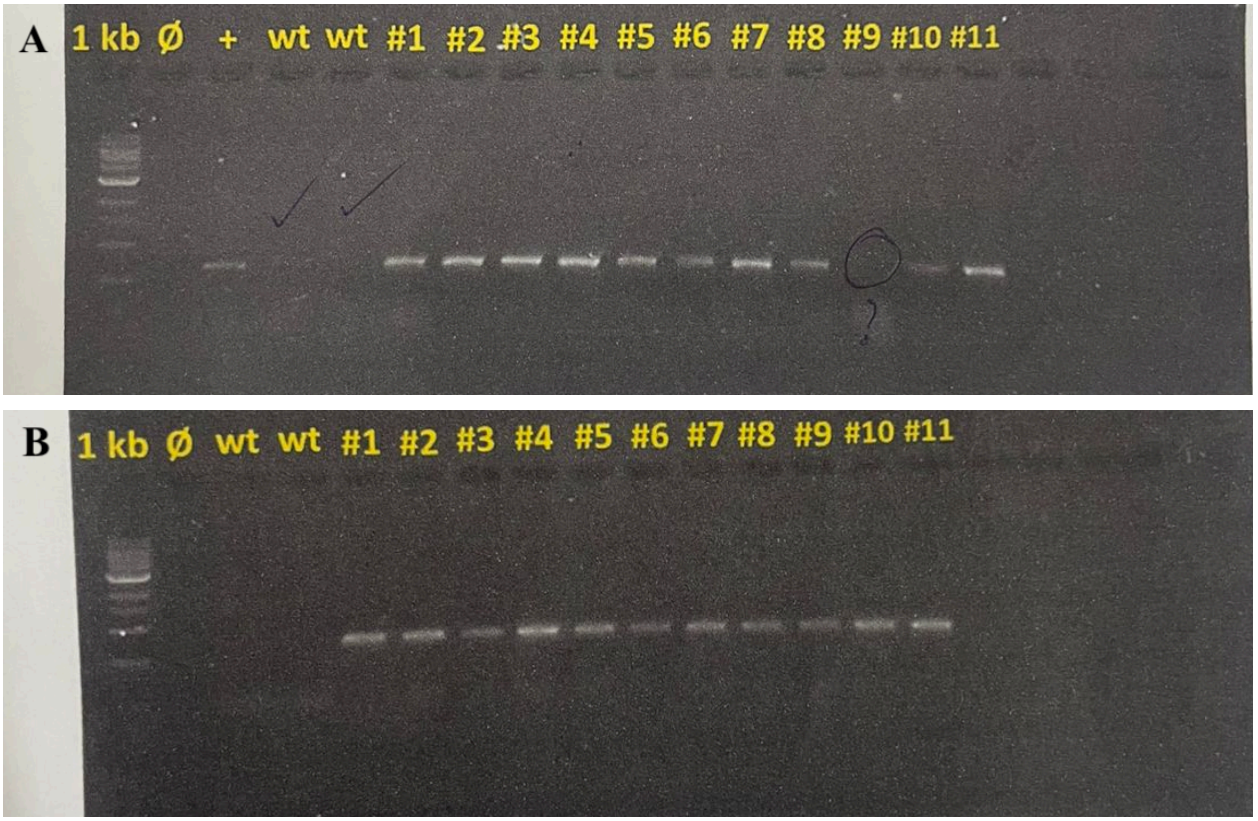

Fig. S01 – A) Amplification of *nptII* gene in leaflets of Arabidopsis T_0 plants, using wild type plant as negative control and purified plasmid as positive control. The *nptII* gene (approximately 700 pb) was amplified using specific primers (Forward: GAG GCT ATT CGG CTA TGA CTG G and Reverse: ATC GGG AGC GGC GAT ACC GTA). The amplified fragment was separated in 1% agarose gel electrophoresis using a TBE buffer stained with ethidium bromide. In the figure, ∅: Water, +, positive control, **wt**: Wild type plants and #1-11 the different selected transgenic events. **B)** To confirm insertion of 35S::SP3C a second PCR was performed using 35S forward primer and SP3C specific primer (Reverse: TCA TCT TCT TCT GGC TGC AG) to obtain a product of 1000 pb approximately. The amplified fragment was separated in 1% agarose gel electrophoresis using a TBE buffer stained with ethidium bromide. In the figure, ∅: water, **wt**: Wild type plants and #1-11 the different selected transgenic events.

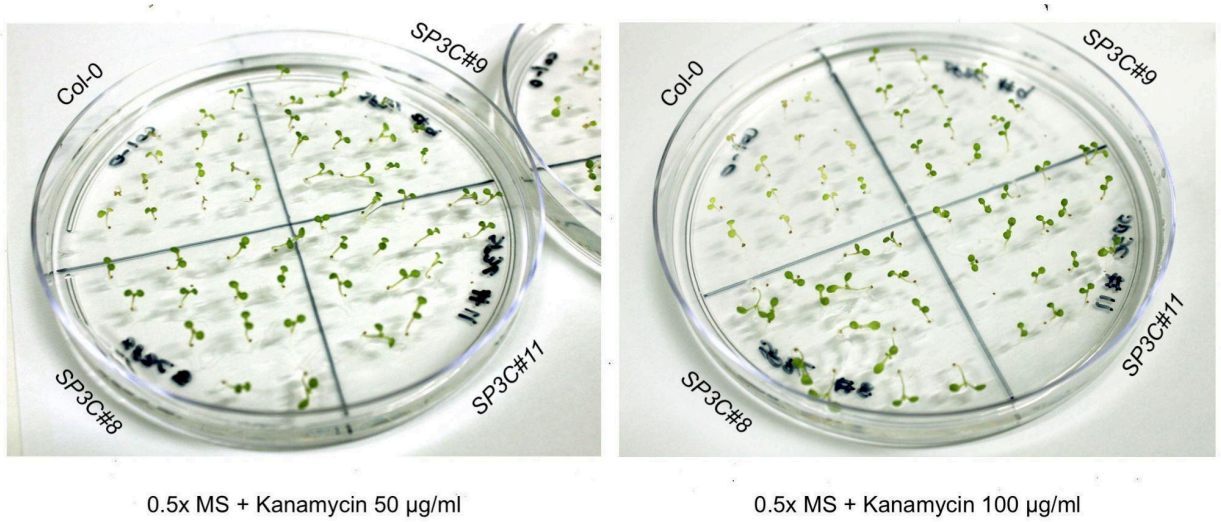

Fig. S02 – *in vitro* selection of T_4 plants on Petri plates containing Kanamycin at 50 µg/ml (left) and 100 µg/ml (right). Col-0 seedlings display signs of Kanamycin toxicity, with more pronounced symptoms at the higher concentration. These plates are representative of three biological replicates, each with 25 seeds from each genotype.

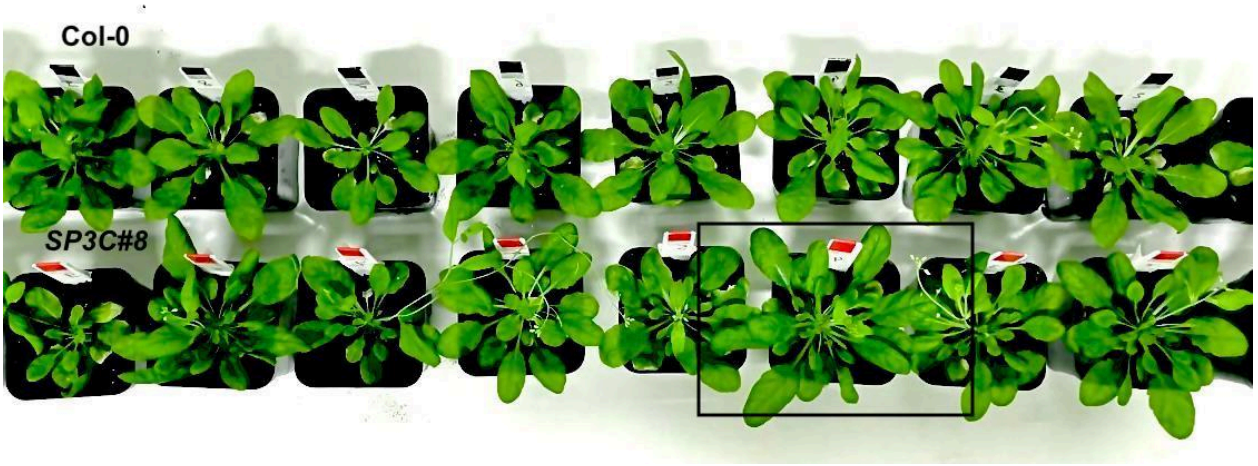

Fig. S03 – Delayed phase transition phenotype was present in *SP3C*#8 lines but completely absent in Col-0. The image shows representative rosettes of the plants grown under short-day conditions at the end of the experiment, 65 days after sowing.

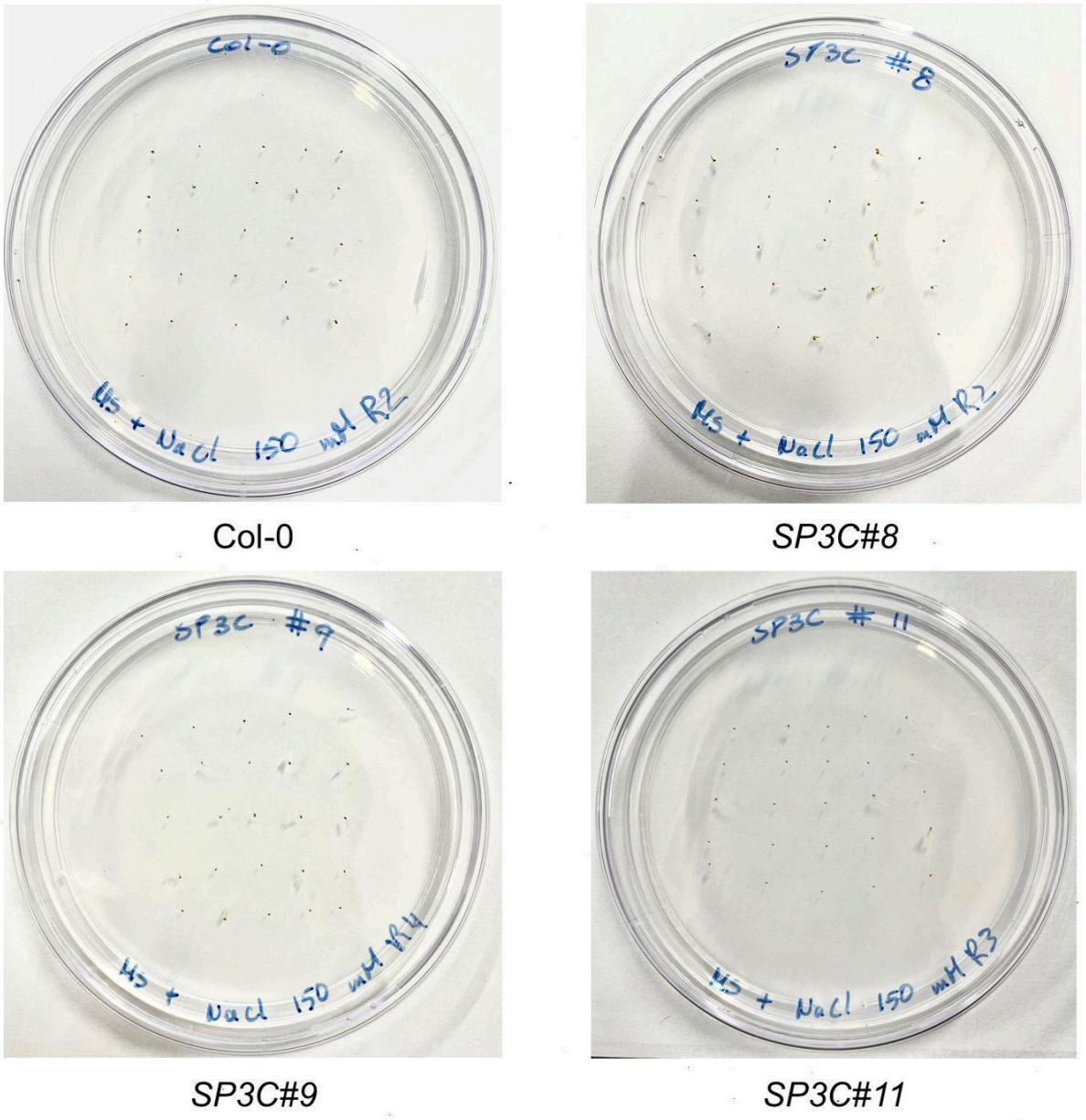

Fig. S04 – Representative Petri plates of the germination assay under 150 mM NaCl treatment. The images are representative of biological replicates of Col-0 and the transgenic lines, 12 days after stratification, at the end of the experiment.

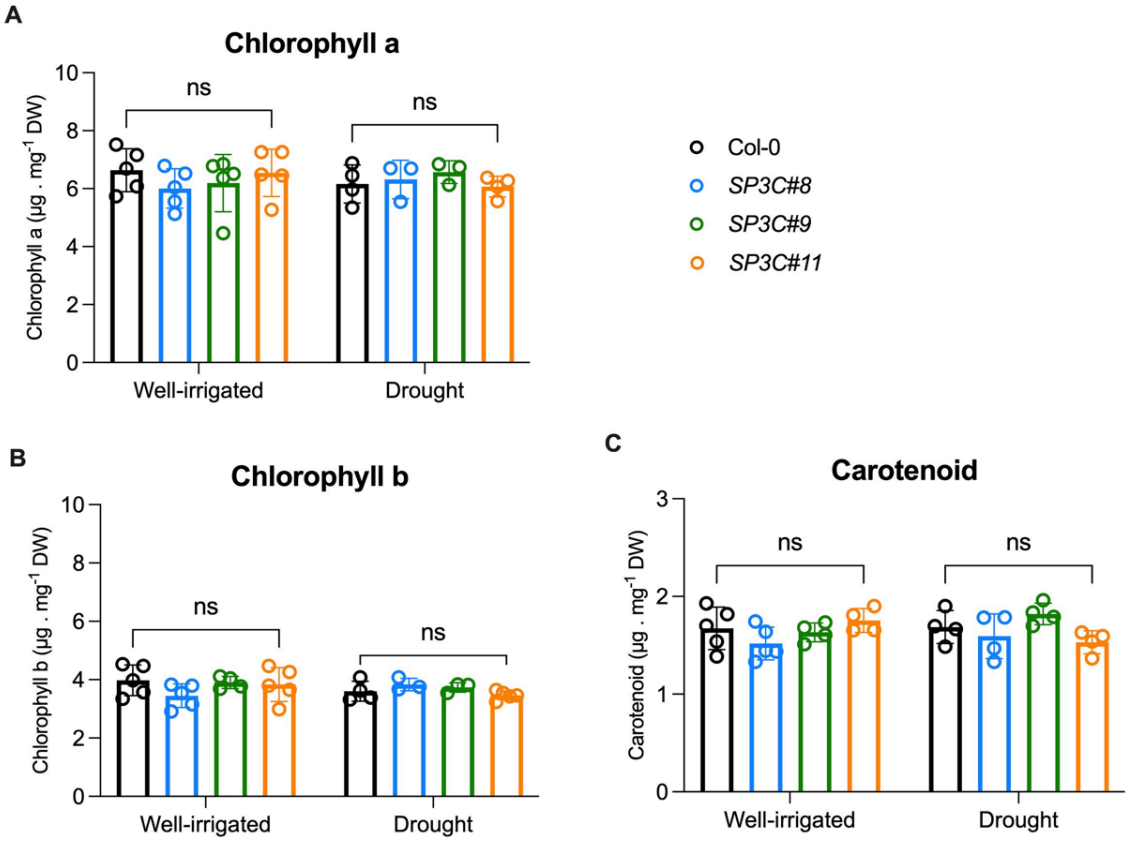

Fig. S05 – No differences were found in photosynthetic pigments quantification between Col-0 plants and overexpression lines *SP3C*#8, #9 and #11. Bars represent the mean ± SD (n=3 or more) of Chlorophyll a (A), Chlorophyll b (B) and Carotenoids (C) content of leaves of 35-day-old plants grown under short-day conditions collected four hours after the onset of the photoperiods. Black bars correspond to the Col-0 line, while colored bars represent the overexpressing lines: *SP3C*#8 (Blue), *SP3C*#9 (Green), and *SP3C*#11 (Orange). ns indicate no significant differences between the lines according to the tukey's test ($p < 0.05$).