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ORIGINAL PAPER

Promoter analysis of the *SPATULA* (*FvSPT*) and *SPIRAL* (*FvSPR*) genes in the woodland diploid strawberry (*Fragaria vesca* L.)

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Abstract
The aim of this study was to identify transcription factor (TF) binding sites and *cis*-regulatory elements (CREs) on the promoters of *FvSPR1-like2* (*SPIRAL*) and *FvSPT* (*SPATULA*) genes in the woodland diploid strawberry (*Fragaria vesca* L.). We identified: (1) MYB59, WRKY25 and WRKY8 TFs which play a role in ethylene signaling; (2) ARF family of TFs which play a role in ARF-mediated auxin signaling on the promoter of *FvSPR1-like2* gene; (3) ARR family of TFs which play a role in cytokinin signaling; (4) ERF family of TFs which play a role in ethylene signaling on the promoter of *FvSPT*. This bioinformatic analysis of TFs and CREs may provide a better understanding of the function of genes involved in, and the mechanism underlying, non-climateric ripening during strawberry fruit maturation.

Keywords Agroinfiltration · *Cis*-regulatory elements · *SPATULA* · *SPIRAL* · Transient gene expression · Transcription factors

Introduction

Members of the *SPR* gene family encode small proteins that contribute to cell elongation by regulating microtubule organization (Nakajima et al. 2004). *SPR* genes in *A. thaliana* are classified into two main groups, *SPR1* and *SPR2* (Bichet et al. 2001; Burk and Ye 2002), and five subgroups, *SPR1-like1* to *SPR1-like5*, all of which have been functionally characterized (Nakajima et al. 2004).

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The *SPR* gene influences the elongation and development of plants at both cellular and organ levels (Furutani et al. 2000; Nakajima et al. 2004). Furutani et al. (2000) induced a mutant *SPR* gene in *A. thaliana* whose roots curved to the right unlike control roots that grew straight. The mutation resulted from the arrangement of cortical microtubules on the opposite side of the optimal direction in epidermal root cells, also effecting helical handedness. Overexpression of the *SPR* gene did not stimulate root skewing since its main function is to maintain the straight elongation of root cells. In addition, the *SPR* gene enhanced the rapid elongation of cells, resulting in the lengthwise enlargement of tissues. Moreover, *SPR* genes interact with cellular molecules to control anisotropic growth (Nakajima et al. 2004).

The SPT transcription factor positively indicates and controls cytokinin output in the medial region of the ovary (Reyes-Olalde et al. 2017a). The *SPT* gene regulates auxin signaling in gynoecium and style-sigma development (Moubayidin and Ostergaard 2014; Schuster et al. 2015). The *SPT* gene is expressed in non-climacteric strawberry (*Fragaria*×*ananassa* Duch.) when treated with auxin and ethylene and is regulated by four ethylene responsive elements (EREs) in the *SPT* promoter region (Tisza et al. 2010).

Transcriptional regulation of gene expression is fundamental to biological processes, such as cell growth, development, differentiation, fruit ripening and responses to

environmental signals (Meshi and Iwabuchi 1995). Given its importance as a transcriptional regulator of genes, the analysis of plant promoters may provide important information that would better guide the construction of biotechnological systems because regulated gene expression systems can increase the function of genetically modified organisms (Corrado and Karali 2009). As internal physiological control regulators, plant hormones also have important roles in the transcriptional regulation of genes, such as development and fruit ripening (An et al. 2020), for example, ethylene and auxin control different steps of the flower-to-fruit transition (Bapat et al. 2010; Kumar et al. 2014; Ziliotto et al. 2012). An antagonistic effect can be observed between ethylene and auxin during tomato fruit ripening (Li et al. 2017).

In this study, tomato (*Solanum lycopersicum* L.) cv. Micro-Tom and tobacco (*Nicotiana benthamiana*) were selected as model plants, as these plants are often chosen in genetic studies to examine and observe differences in gene expression using the green fluorescence protein (GFP) marker gene (Hoshikawa et al. 2019; Reed and Osbourn 2018). *Agrobacterium tumefaciens*-mediated transfer, together with an agroinfiltration (also known as agroinjection) method, was used in this study. Agroinfiltration is an *Agrobacterium*-mediated transient recombinant protein expression method which can be used to avoid labor-intensive and time-consuming methods to produce stable transgenic plants (Hoshikawa et al. 2019). Infiltration is achieved by delivering the *Agrobacterium* with the target genes into extracellular leaf space by physical infiltration (Norkunas et al. 2018). Physical infiltration in this study was performed with a needleless syringe.

The aim of this study was to characterize the *SPT* and *SPR* gene promoters which were isolated from *Fragaria vesca* L., the woodland diploid strawberry, by finding specific motifs. The putative promoter region was identified with the JASPAR 2020 plantae algorithm (Fornes et al. 2020) for TFs as well as a promoter motifs database (http://jaspar.genereg.net/) allowing us to predict the promoter regions of the tomato (*Solanum lycopersicum* L.) cv. Micro-Tom *SPT*, *SPR1-like2* (*MtSPT* and *MtSPR1-like2*), as well as *F. vesca SPT*, *SPR1-like1* and *SPR1-like2* (*FvSPT*, *FvSPR1-like1* and *FvSPR1-like2*) genes, which show selective complementation in *A. thaliana* (Hidvégi et al. 2020). We compared these promoter sequences with promoters of *A. thaliana SPT* and *SPR1-like2* (*AtSPT* and *AtSPR1-like2*) reference genes. Moreover, we used PCR amplification of different lengths of upstream regions of the *FvSPT* and *FvSPR1-like2* coding sequences and insertion of putative promoter fragments into a binary vector (pGWB604) carrying the *sGFP* reporter gene. Tomato and tobacco were agroinjected with the pGWB604 and pGWB405 (CaMV35S::sGFP, as positive control) binary vectors that included a fusion of the promoter deletion lines and the s*GFP* reporter gene. Promoter deletion lines can be used to identify the presence of genetic regulatory elements (TFs or CREs) such as enhancers and silencers in the region upstream of the start codon.

Materials and methods

Plant materials and growth conditions

Diploid strawberry (*Fragaria vesca* 'Rügen') was used as the template to amplify the putative promoter regions of *FvSPT* and *FvSPR1-like2*. Seeds of tomato (cv. Micro-Tom), diploid strawberry and tobacco (*Nicotiana benthamiana*) were sown *ex vitro* in 50 mm Jiffy-7® pots (1 seed/pot). Jiffy pots were placed in a climate room at 22 °C and kept under an 8-h photoperiod at a photosynthetic photon flux density (PPFD) of 37 µmol m^{-2} s^{-1} provided by Biolux tubes (Osram L58W, Markham, Canada). When seedlings formed two fully developed leaves, rooted plantlets were transferred to plastic pots (9 cm) into soil and grown under the same conditions as seedlings. No fertilizers or additional supplements (e.g., pest control agents) were added.

Computational analysis of promoters

The *S. lycopersicum* genome was studied in silico based on the Tomato Genome Consortium (2012) whole-genome sequences for promoters of *MtSPT* (Gene ID: 101,266,791, NC_015439.3) and *MtSPR1-like2* (Gene ID: 101,257,849, NC_015440.3) genes. The *F. vesca* Whole Genome v2.0a1 assembly & annotation (http://www.rosaceae.org) was used to analyze in silico the promoters of *FvSPT* (XM_004287975; LOC101290893), *FvSPR1-like1* (XM_004297177; LOC01307108) and *FvSPR1-like2* (XM_004299243; LOC101309836) genes. We used the *A. thaliana* whole-genome assembly (Swarbreck et al. 2007) as the reference for the promoters of *AtSPT* (BT024676) and *AtSPR1-like2* (BT026462) genes.

The promoters of *FvSPR1-like1*, *FvSPR1-like2* and *FvSPT* genes were isolated and aligned with the *A. thaliana* and *S. lycopersicum* sequences by using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis to find similarities or homologies. The promoter regions of the genes were examined with JASPAR 2020 (Fornes et al. 2020) and PLACE 30.0 (database of plant *cis*-acting regulatory DNA elements; Higo et al. 1999) to determine the transcriptional factor binding sites (TFBS) and CREs to develop promoter deletion lines of promoters of *FvSPR1-like2* and *FvSPT* genes.

PCR amplification of promoter deletion lines

The sequences upstream (from the start codon) of the *FvSPT* gene (3100 bp, XM_004287975; LOC101290893) and the *FvSPR1-like2* gene (2800 bp, XM_004299243; LOC101309836) were amplified. PCR amplification was performed in *FvSPR1-like2* and *FvSPT* promoter genes to yield 500 bp (FvSPR500, FvSPT500), 1000 bp (FvSPR1000, FvSPT1000), 2000 bp (FvSPR2000, FvSPT2000) and 3000 bp (FvSPT3000) fragments. PCR amplification was performed with the GoTaq Long PCR Master Mix (Promega, Madison, WI, USA). Genomic DNA (100 ng) was used as template DNA in a 50-µL PCR mix. The PCR mixture consisted of 25 µL of GoTaq Long PCR Master Mix (2x) and 30 pmol of each primer (Suppl. Table 1). PCR conditions, optimized in-house, were: 95 °C for 2 min followed by 35 cycles at 95 °C for 30 s, 65 °C 1 min (500 bp and 1000 bp promoter deletion lines) or 3 min (2000 bp and 3000 bp promoter deletion lines). Cycling was followed by a final incubation at 72 °C for 10 min. PCR products were separated on 1.0% agarose gels in 1 × TAE buffer (Sambrook et al. 1989) by agarose gel electrophoresis and detected by fluorescence under UV light (302 nm) with a VWR® Smart3 Gel documentation system (VWR International, Radnor, PA, USA) after staining with 0.1% ethidium bromide (IBI Scientific, Dubuque, IA, USA).

TOPO® and gateway® LR cloning

FvSPT500, FvSPT1000, FvSPT2000, and FvSPT3000 from *FvSPT* and FvSPR500, FvSPR1000, and FvSPR2000 from *FvSPR* were cloned by directional cloning using the pENTR™ Directional TOPO® vector based on the Invitrogen pENTR™ Directional TOPO® Cloning Kit manual (Invitrogen, Carlsbad, CA, USA). One Shot® TOP10 chemically competent *Escherichia coli* cells (Invitrogen) were transformed with constructed TOPO vectors according to the One-Shot Chemical transformation protocol (Invitrogen pENTR™ Directional TOPO® Cloning Kit manual, Invitrogen). The *E. coli* was grown on Luria–Bertani (LB) media (Bertani 1951) with 100 mg/mL kanamycin (Duchefa, Haarlem, the Netherlands). To select positive clones of putative promoter regions, colony PCR and agarose gel electrophoresis was applied using the same conditions employed for PCR amplification of promoter deletion lines. Colony PCR used M13 universal primer (Invitrogen pENTR™ Directional TOPO® Cloning Kit manual, Invitrogen). *E. coli* colonies carrying the inserted promoter region were grown overnight on LB plates with 100 mg/mL kanamycin and transferred with toothpicks to 5 mL of LB media containing 5 µL kanamycin (100 mg/mL) and grown on a shaker (140 rpm at 37 °C, overnight). Plasmid DNA from competent *E. coli* was isolated and purified by the PureYield™ Plasmid Miniprep Kit (Promega, Madison, WI, USA) based on the manufacturer's user manual. Gateway® LR Clonase™ II enzyme Mix (Invitrogen) was used for pGWB604 plasmid (Nakagawa et al. 2007; GenBank: AB543113.1) construction as the binary destination vector which contains the *synthetic green fluorescent protein* marker gene (*sGFP*; Niwa 2003). The pGWB405 (Nakagawa et al. 2007; GenBank: AB294429.1) vector, which contains the CaMV35S promoter with *sGFP*, was used as the positive control for transient gene expression. The FvSPR500::pGWB604, FvSPR1000::pGWB604, FvSPR2000::pGWB604, FvSPT500::pGWB604, FvSPT1000::pGWB604, FvSPT2000::pGWB604 and FvSPT3000::pGWB604 vector constructs were built by cloning.

Agrobacterium-mediated transformation

Agrobacterium tumefaciens GV3101 strain (Intact Genomics, Creve Coeur, MI, USA) was incubated in an LB plate with a working concentration of 10 µg/mL gentamycin (10 mg/mL stock; Duchefa) at 28 °C for 2 d. A single colony of *A. tumefaciens* from the LB plate was incubated in 5 mL of liquid LB with 5 µL of spectinomycin (50 mg/mL stock; Duchefa) and 5 µL of gentamycin (10 mg/mL stock) overnight in a MaxQ 4000 Benchtop Orbital Shaker (ThermoFischer Scientific, Waltham, MA, USA) at 140 rpm and 28 °C. Cultures were placed on ice for 30 min then centrifuged for 10 min at 4000 rpm and at 4 °C. The supernatant was discarded, and the pellet was resuspended in 5.0 mL of 20 mM $CaCl_2$ on ice then centrifuged again for 5 min at 4000 rpm and 4 °C. The supernatant was discarded and 1.0 mL of ice-cold 20 mM $CaCl_2$ was added to the pellet in ice water. A 200 µL aliquot as competent *A. tumefaciens* cells was pre-chilled in 1.5-mL microcentrifuge tubes (Eppendorf, Hamburg, Germany). Plasmid DNA (3 µL; 500 ng) was added from the pGWB604 vector containing the promoter region into each tube containing competent *A. tumefaciens* cells and kept on ice for 20 min, placed in liquid nitrogen for 5 min, heat shocked at 37 °C for 5 min, then added to ice for 5 min. Liquid LB media (1.0 mL) was added to each heat-shocked colony and incubated in a shaker at 28 °C and at 140 rpm for 3–4 h. Sample (100–150 µL) was pipetted onto an LB plate supplemented with 10 µg/mL of gentamycin (10 mg/mL stock) and 50 µg/mL of spectinomycin (50 mg/mL stock). Based on the Bergkessel and Guthrie (2013) protocol, colony PCR was performed to confirm the success of transformation using the same conditions used for TOPO® and Gateway® LR cloning.

Agroinfiltration in tomato and tobacco

A single *A. tumefaciens* colony was cultured in 5 mL of LB medium supplemented with 5 µL gentamycin (10 mg/mL stock) and 5 µL spectinomycin (50 mg/mL) overnight at 28 °C on a shaker at 140 rpm. Cultures were transferred to 50 mL of induction medium (10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g Na-citrate, 1 g glucose, 1 g fructose, 4 mL glycerol, 0.12 g $MgSO_4$, 1.95 g MES (10 mM); pH 5.6; Singer et al. 2012) containing 100 µM acetosyringone (Duchefa), which was added after autoclaving (121 °C, 60 min). Cells were incubated in induction medium at 30 °C for 5–6 h at 140 rpm. After incubation, cells were centrifuged at 4000 rpm for 10 min, and then the pellet was resuspended in infiltration medium (10 mM $MgSO_4$, 10 mM MES; pH 5.6; Singer et al. 2012) supplemented with 200 µM acetosyringone. Green and ripening tomato fruits (age: about 60 d after germination; sample number: 20 fruits/vector construct, 2 fruits/plant) and tobacco leaves (age: about 45 d after sowing; sample number: 20 leaves/vector construct, 2 leaves/plant) were agroinjected by using a 1 mL syringe (Z683531; Sigma-Aldrich, St. Louis, MI, USA) with a 0.5 × 1.6 mm needle (Sigma-Aldrich). Infiltration solution was injected (5–6 mm deep) into tomato fruit through stylar apex, while leaves were injected by slightly injuring the epithelium tissue of the abaxial surface. Plants were tested 3 d later with the Phire Plant Direct PCR Kit (ThermoFischer Scientific). The PCR mixture consisted of 10 µL of Phire Plant Buffer (2×), 40 pmol of each primer pair (specific to the *sGFP* gene and the *GlyA* gene of *A. tumefaciens* GV3101), 0.4 µL of Phire Hot Start II DNA Pol and 0.5 µL of diluted plant tissue. PCR conditions were 98 °C for 5 min followed by 40 cycles at 98 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s. Cycling was followed by a final incubation of 72 °C for 1 min. PCR products were separated by gel electrophoresis based on the same protocol that was used for promoter PCR. Only *sGFP*-positive plants were selected for GFP fluorescence and RT-qPCR analysis.

Verification of GFP fluorescence by UV light

GFP fluorescence was verified with a FastGene® blue/green LED flashlight (FG-11; NIPPON Genetics, Tokyo, Japan), which was used to irradiate (excitation: 489 nm; emission: 520 nm) leaves and fruit at the mature red ripening stage (about three days after agroinjection) at a distance of ~ 10 cm from each organ in the dark. To photograph the irradiated leaves, a yellow UV filter (NIPPON Genetics) was mounted to the camera (Nikon Coolpix B500, Tokyo, Japan) lens to filter out blue light, and to allow GFP fluorescence to be visualized. Fluorescence was also verified in controls at the same time. The location of GFP fluorescence was visually assessed and confirmed. Three controls were used for both methods at the same time as the agroinjection into ripe fruits and leaves using infiltration solution: (a) without any *A. tumefaciens*; (b) *A. tumefaciens* without any plasmid; (c) *A. tumefaciens* with a constitutive promoter (CaMV-35S) + *sGFP* in the plasmid.

Quantification of *sGFP* expression by real-time PCR

After confirming the possible presence of the *sGFP* gene using UV fluorescence, tomato fruits and tobacco leaves (two per plant; 20 plants/line) showing fluorescence following UV light detection were picked after 3 d. To measure *sGFP* intensity, RT-qPCR was used (Wang et al. 2004). Total RNA was isolated using Direct-zol™ (Zymo Research, Irvine, CA, USA) with TRIzol reagent based on the manufacturer's protocol. After purifying total RNA, three quality control methods were applied: 1) microcapillary electrophoresis with an Implen n50 spectrophotometer (Implen, Munich, Germany) for preliminary quantification; 2) agarose gel electrophoresis to assess total RNA degradation and potential contamination; 3) Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) to check the quality and quantity of total RNA. cDNA was amplified from 120 ng of total RNA with reverse transcription using the FIREScript RT cDNA Synthesis MIX (Solis BioDyne, Tartu, Estonia). qPCR was performed with the 5 × HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne) on the ABI 7300 real-time PCR system (ThermoFischer Scientific) to detect the intensity of *sGFP* expression. Specific primers (Suppl. Table 1) for RT-qPCR were used to detect *sGFP* and normalizing (reference) genes (*MtGAPDH*: At1g13440, *FvGAPDH*: ID07104 and *NbGAPDH*: At1g12900) which were selected based on the stability of housekeeping gene expression level (Expósito-Rodríguez et al. 2008; Liu et al. 2012, 2020). In the RT-qPCR analysis, we used the $2^{-\Delta\Delta Ct}$ method to quantify the relative changes in gene expression (Livak and Schmittgen 2001). To compare the intensity of *sGFP* gene expression between the positive control (CaMV35S::sGFP) and promoter deletion line::sGFP constructs, gene expression logarithmic fold change (log2LFC) was calculated. The $2^{-\Delta\Delta Ct}$ method and log2LFC were calculated by HTqPCR v3.11 (Dvinge and Bertone 2009) in R software (Gentleman et al. 2004; Huber et al. 2015). The Student's *t* test was performed using ΔΔCt values, and a *p*-value less than 0.05 was considered to be significant. Statistical analyses were conducted in GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Results were exported into Microsoft Excel 365.

Results

Promoter sequence analysis

After comparing of results from JASPAR2020 and PLACE 30.0 data, various putative TFs and CREs were examined in the promoter sequences of *MtSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like1*, *AtSPR1-like2*, *MtSPT*, *FvSPT* and *AtSPT* genes. We identified 222, 364, 117, 186, 323, 473, and 484 TFBS and 473, 645, 248, 30, 548, 733, and 719 CREs in the promoter sequences of *MtSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like1*, *AtSPR1-like2*, *MtSPT*, *FvSPT* and *AtSPT* genes, respectively (TFs: Suppl. Table 2; CREs: Suppl. Table 4).

We compared the different promoter regions (TF and CREs) related to flowering, fruit development and ripening in tomato, *A. thaliana* and *F. vesca* (Suppl. Table 2, Suppl. Table 5). Table 1 shows the frequency of TFBS in the promoter sequences that play a role in flowering and fruit ripening. There were 16, 25, 7, 5, 34, 24 and 29 TFBS in the promoter sequences of *MtSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like1*, *AtSPR1-like2*, *MtSPT*, *FvSPT* and *AtSPT* genes, respectively (Table 1).

Table 2 shows the frequency of CREs in the promoter sequences that played a role in flowering and fruit ripening. There were 11, 25, 6, 1, 27, 26 and 16 CREs in the promoter sequences of *MtSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like1*, *AtSPR1-like2*, *MtSPT*, *FvSPT* and *AtSPT* genes, respectively (Table 2). Based on the PLACE 30.0 database, CREs that were regulated by auxin, ethylene, GA_3 and cytokinin were classification. We identified 1, 8 and 1 CREs that were promoted by ethylene, auxin and GA_3, respectively. Cytokinin did not promote CREs in these promoter regions.

Using the UV lamp, GFP fluorescence was only detected in the leaves of FvSPR2000::pGWB604, FvSPT1000::pGWB604, FvSPT2000::pGWB604, FvSPT3000::pGWB604 and CaMV35S::sGFP (pGWB405) tobacco lines (Suppl. Figure 1f, 1 h, 1i, 1j, 1c), and in the green fruit of FvSPR2000::pGWB604, FvSPT2000::pGWB604, FvSPT3000::pGWB604 and CaMV35S::sGFP (pGWB405) Micro-Tom tomato lines (Suppl. Figure 2c, e, f, b).

Using RT-qPCR, the *sGFP* gene was detected and its expression intensity was measured in the fruits of Micro-Tom tomato and leaves of tobacco plants. In tobacco leaves, the intensity of *sGFP* gene expression (RQ) was 0.072, 0.006, 0.099 and 2.532 in the FvSPR2000::pGWB604, FvSPT1000::pGWB604, FvSPT2000::pGWB604 and FvSPT3000::pGWB604 lines, respectively, based on ΔΔCt values (Suppl. Table 6). The expression logarithmic fold change (LFC) was – 3.8, – 7.29, – 3.33 and 1.34 in the FvSPR2000::pGWB604, FvSPT1000::pGWB604, FvSPT2000::pGWB604 and FvSPT3000::pGWB604 lines, respectively based on a comparison with *sGFP* expression intensity of CaMV35S::sGFP as the positive control (Suppl. Table 6; Fig. 1).

In Micro-Tom tomato fruits, *sGFP* gene expression intensity was 0.026, 0.024 and 1.028 in the FvSPR2000::pGWB604, FvSPT2000::pGWB604 and FvSPT3000::pGWB604 lines, respectively based on the Ct values (Suppl. Table 6). Expression LFC was -5.28, -5.36 and 0.04 in the FvSPR2000::pGWB604, FvSPT2000::pGWB604 and FvSPT3000::pGWB604 lines, respectively (Suppl. Table 6; Fig. 2) based on a comparison with *sGFP* expression intensity of CaMV35S::sGFP as the positive control.

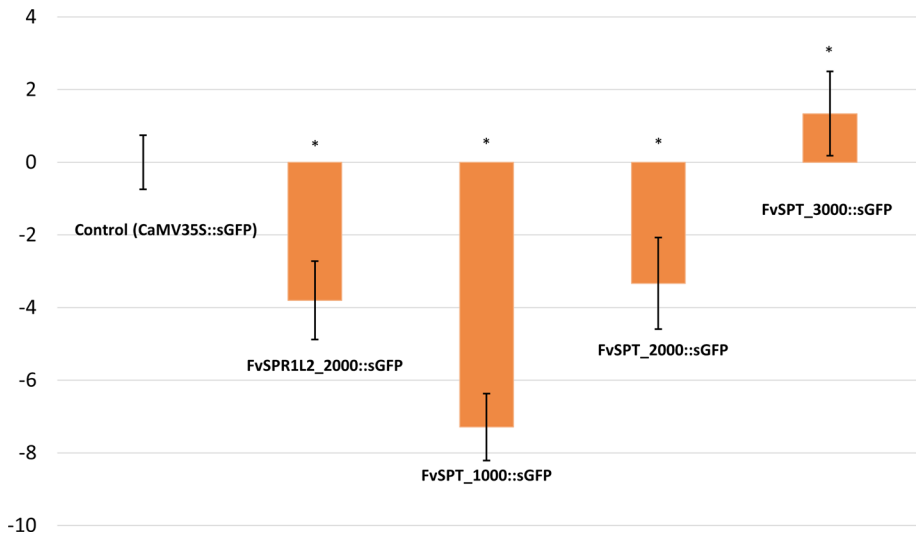

Fig. 1 LFC of *sGFP* in tobacco leaves (control: CaMV35S::sGFP; sample size: 20 measurements per vector construct, total: 80 measurements with three biological replicates). Error bars: standard deviation. * Significant differences compared with the control (Student's *t*-test: $t(38) = 12.96$; $t(38) = 27.51$; $t(38) = 10.17$; $t(38) = 4.38$, $p < 0.05$). Also see Suppl. Table 6

Table 1 Frequency of TFBS which play a role in flowering and ripening processes (based on Suppl. Table 3)

TFBS	Frequency						
	MtSPR1-like2	*FvSPR1-like1*	*FvSPR1-like2*	*AtSPR1-like2*	*MtSPT*	*FvSPT*	*AtSPT*
ARF1		1					
ARF2	2	1	1		1		2
ARF34							1
ARF5		1					
ARF8		1					
ARR10		4	2		3	1	1
ARR11			1		2		
ARR14					2		
ARR18					1		
ARR2	1				1		
ATHB15							1
ATHB20		1			1		1
ATHB23	1	1			6	2	2
ATHB53	1	1			1		2
BEE2						2	2
CAMTA1		1					
CMTA3		2					1
DREB26						1	
EDT1					2	1	
ERF13						1	
FaEOBII						1	
HAT2	1	1		1	1	2	2
KAN1	1	4	2	2	4	2	3
KUA1					2		
MYB124	1						1
MYB59		1			1	1	1
MYB73		1					
OBP3		1					
OsRR22					1		
PIF5	1				1	1	1
RVE1		1					
SGR5							1
SPT						7	2
TGA1A	1		1	1			1
TGA2	1			1			
WRKY25	2	1			2		2
WRKY8	3	1			2	2	2

Compared to the control (CaMV35S::sGFP), FvSPR2000::pGWB604, FvSPT1000::pGWB604 and FvSPT2000::pGWB604 ΔΔCt values were significantly different ($p \leq 0.05$), while FvSPT3000::pGWB604 ΔΔCt values were significantly different ($p \leq 0.05$) for tobacco leaves but not for tomato fruit (Suppl. Table 6).

Discussion

This research focused on the promoter region of *FvSPR1-like2* and *FvSPT* genes, which play an important role in cell development, flowering, and fruit development (Nakajima et al. 2004; Reyes-Olalde et al. 2017b; Hidvégi et al. 2020). Transient expression using agroinfiltration has limitations, such as the exclusive expressed of the marker gene (*sGFP*) in the infiltrated area, while components of the expression cassette (vector construction) and strain or density of the *Agrobacterium* culture might affect the efficiency of gene

Table 2 Frequency of *cis*-regulatory elements (CREs) which play a role in flowering and ripening processes (based on Suppl. Table 5)

CREs	Frequency						
	MtSPR1-like2	*FvSPR1-like2*	*FvSPR1-like1*	*AtSPR1-like2*	*FvSPT*	*AtSPT*	*MtSPT*
Arfat		1			3	2	
Asf1motifcamv	2	5	1		3	4	1
Auxretga1gmgh3		1	1		1		
Cacgcaatgmgh3							1
Cargatconsensus		2					
Catatggmsaur						6	2
Crtdrehvcbf2		2					
D4gmaux28					1		
Erelee4	2	2	1			1	4
Gare1osrep1							1
Gareat	2	6	3			3	2
Ntbbf1arrolb	2	3	1		9	4	4
Sebfconsstpr10a					3	2	
Surecoreatsultr11	3	4		1	8	4	1

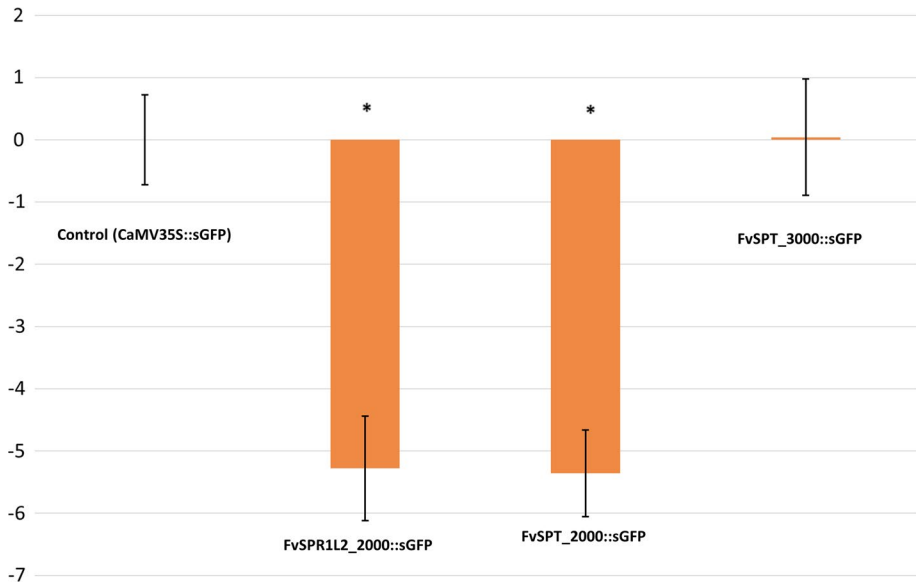

Fig. 2 LFC of *sGFP* in Micro-Tom tomato fruit (control: CaMV35S::sGFP; sample size: 20 measurements per vector construct, total: 60 measurements with three biological replicates). Error bars: standard deviation. * Significant differences compared with the control (Student's *t*-test: $t(38) = 21.34$; $t(38) = 23.90$; $t(38) = 0.13$, $p < 0.05$). Also see Suppl. Table 6

expression (Tyurin et al. 2020). The JASPAR2020 TFBS database has 572 profiles that include different classes and families of TFs in plants based on TF DNA-binding preferences, modeled as position weight matrices (Stormo 2013).

Fruit ripening in strawberry and tomato are controlled by ethylene and can be characterized by their color, ranging from green (unripe) to red (ripe) (Tisza et al. 2010; Li et al. 2017). Auxins retard fruit ripening, and an optimal ethylene-auxin balance can regulate the fruit ripening period (Su et al. 2015). We identified the promoters of the *FvSPR1-like2* and *FvSPT* genes: ARF1 (Ellis et al. 2005), ARF2 (Zhang et al. 2014), ARF34 (Majer et al. 2012), ARF5 (Vidaurre et al. 2007), ARF8 (Nagpal et al. 2005), ATHB15 (Prigge et al. 2005), ATHB20 (Mattsson et al. 2003), ATHB53 (Son et al. 2004), CAMTA1 (Bouché et al. 2002), EDT1 (Cai et al. 2015), HAT2 (Sawa et al. 2002), KAN1 (Hawker and Bowman 2004), KUA1 (Lu et al. 2014), MYB124 (Chen et al. 2015), MYB73 (Kim et al. 2013), OBP3 (Kang et al. 2003), RVE1 (Meissner et al. 2013), SGR5 (Morita et al. 2006), TGA1A (Pascuzzi et al. 1998) and TGA2 (Johnson et al. 2003) sites. Auxin response factors (ARFs) can bind specifically to the DNA sequence 5′-TGTCTC-3′ found in auxin-responsive promoter elements (AuxREs; Majer et al. 2012). We identified ARFAT and SURECOREATSULTR11 CREs that contain ARF binding sequences (Maruyama-Nakashita et al. 2005) on the promoters of the *FvSPR1-like2* and *FvSPT* genes. The AUXRETGA1GMGH3 CRE is a strong binding site for proteins to the AuxRE which regulates ARFs (Guilfoyle et al. 1998).

The AGL42 (Dorca-Fornell et al. 2011), ARR2 (Weirauch et al. 2014), CMTA3 (Bouché et al. 2002), DREB26 (Krishnaswamy et al. 2011), ERF13 (Oñate-Sánchez and Singh 2002), KUA1 (Lu et al. 2014), MYB59 (Li et al. 2006), PIF5 (Khanna et al. 2007), WRKY25 (Li et al. 2011) and WRKY8 (Chen et al. 2013) sites are located on the promoter of *FvSPR1-like2* and *FvSPT* genes. The AGL42 site, which is a MADS-box TF, is involved in the control of flowering time, and promotes flowering at the shoot apical and axillary meristems. Genes that are controlled by AGL42 are regularly expressed in the leaves, flower buds, petals and abscission zone of *A. thaliana* flowers and siliques (Dorca-Fornell et al. 2011). We identified AGL42 on the promoters of *MtSPR1-like2*, *AtSPR1-like2*, *MtSPT*, *FvSPT* and *AtSPT* genes.

ARR1 (Sakai et al. 2001), ATHB34 (Henriksson et al. 2005), BEE2 (Friedrichsen et al. 2002), FUS3 (Tsuchiya et al. 2004), MYB33 (Gocal et al. 2001), MYR2 (Zhao et al. 2011), SOC1 (Lee et al. 2008), SRM1 (Wang et al. 2015) and STZ (Mittler et al. 2006) TFs were found in the promoters of the *FvSPR1-like2* and *FvSPT* genes. The GARE1OSREP1 CRE regulates the gibberellin-responsive element (GARE), including the MYB33 TFBS (Sutoh and Yamauchi 2003).

ARR10 (Hwang and Sheen 2001), ARR11 (Imamura et al. 2003), ARR14 (Mason et al. 2004), ARR18 (Liang et al. 2012) and OsRR22 (Tsai et al. 2012) were located on the promoters of *FvSPR1-like2* and *FvSPT* genes.

The FvSPR500::pGWB604 and FvSPR1000:pGWB604 constructs did not work in tobacco leaves, but FvSPR2000:pGWB604 did. FvSPR2000::pGWB604 also expressed the *sGFP* gene in Micro-Tom fruit. We found ARF1, ARF2, ARF5 and ARF8 sites in the − 1067 to − 1059 bp region of the promoter deletion lines of the *FvSPR1-like2* gene. These sites were not in the − 500 to − 1 bp and − 501 to − 1000 bp regions. The ARF family of TFs play a role in ARF-mediated auxin signaling in the maturation of reproductive organs (Liu et al. 2015), perhaps, explaining why FvSPR2000::pGWB604 was the only construct that induced *sGFP* in tobacco leaves and tomato fruit. The FvSPT1000::pGWB604, FvSPT2000::pGWB604 and FvSPT3000::pGWB604 constructs worked in tobacco leaves, but FvSPT1000::pGWB604 did not work in Micro-Tom tomato fruit. The FvSPR500::pGWB604 construct did not work in Micro-Tom tomato fruit or in tobacco leaves. The FvSPR1000::pGWB604 construct had a lower *sGFP* gene expression intensity than FvSPR2000::pGWB604. This differential expression may have been caused by MYB59, WRKY25 and WRKY8 sites, which are regulated by ethylene (Li et al. 2006, 2011; Chen et al. 2013). The ethylene-auxin interaction might have a role in regulating the promoter of the *FvSPR* gene, as occurs in tomato where there is an antagonistic effect between ethylene and auxin during tomato fruit ripening (Li et al. 2017). The FvSPR1000::pGWB604 construct does not have the MYB59, WRKY25 and WRKY8 sites because these are only found between the −1256 and − 1248 bp, − 1609 to − 1602 and − 1610 to − 1602 regions (Suppl. Table 2), respectively, which do not exist in the FvSPR2000::pGWB604 construct. The FvSPT1000::pGWB604, FvSPT2000::pGWB604 and FvSPT3000::pGWB604 constructs worked in tobacco leaves (Suppl. Figure 1), but the FvSPT1000::pGWB604 construct did not work in Micro-Tom tomato fruit (Suppl. Figure 2).

Conclusion for future biology

In our experiment, we reported CREs specific to various TFs in regions of putative *FvSPT* and *FvSPR1-like2* genes by bioinformatic analysis. The promoter of the *FvSPR1-like2* gene has the following: (1) MYB59, WRKY25 and WRKY8 TFs, which play a role in ethylene signaling; (2) ARF family of TFs, which play a role in ARF-mediated auxin signaling. The promoter of the *FvSPT* gene has the following: (1) ARR family of TFs, which play a role in cytokinin signaling; (2) ERF family of TFs, which play a role in ethylene signaling. The function and names of these sites and elements, as defined in JAPAR2020 and PLACE 30.0 databases, were also identified. The function of TFs and CREs were confirmed with promoter deletion lines and *sGFP* reporter gene constructs in tobacco leaves or tomato fruit by agroinjection.

In recent years, the use of transgenic techniques has led to improvements in many plant species due to identification of a large number of genes. Molecular researchers have made efforts to isolate tissue-specific promoters to increase the added value of transgenes. Transcriptional regulation is a most important goal in the post-genomic era by understanding the transcriptional factors in the promoter regions. There are several databases about analyzing, identifying and characterizing promoters, which currently available from different plant species. With the analyzing progress of promoter and TFs that has been achieved in the agricultural sector through current biotechnological and bioinformatic techniques, might be open a new door to new tissue- and stage-specific promoters in new genetically modified (GM) cultivars.

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Declarations

Conflicts of interest The authors declare no conflicts of interest regarding this paper.

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