Thesis of Doctoral Dissertation

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Gödöllő

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HUNGARIAN UNIVERSITY OF
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Isolation and functional analysis of *SPATULA* and *SPIRAL* genes and promoters of woodland strawberry (*Fragaria vesca* L. cv. Rügen)

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The doctoral school

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Discipline:	**Crop and horticultural sciences**
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Introduction and objectives

In researches conducted at Hungarian University of Agriculture and Life Sciences, Institute of Genetics and Biotechnology, mRNA transcripts were studied from receptacle and achene tissues of strawberry (*Fragaria x ananassa* Duch. cv. Elsanta). Among the transcripts were the *SPATULA* and *SPIRAL* genes. The study of the *SPATULA* and *SPIRAL* genes can be found in many publications, but the actual function of these genes has not been fully clarified yet. In *Arabidopsis thaliana* (L.), the *SPATULA* gene plays a role in the development of all tissues. It encodes a transcription factor (TF) that is a member of the large basic helix-loop-helix (bHLH) family (HEIM *et al.*, 2003, TOLEDO-ORTIZ *et al.*, 2003).

Depending on how ethylene affects their ripening processes, and whether an increase in respiration rate is experienced as their ripening progresses, fruits can be classified into post-ripening (climacteric) and non-post-ripening (non-climacteric) groups.

The *SPATULA* gene of *Arabidopsis thaliana* (L.) (*AtSPT*) plays an important role in the development of the carpel and stigma and is also expressed in tissues where it promotes development and tissue abscission. The *AtSPT* also regulates the size of the leaf and is also shown in the detachment zones of seeds (fruits), developing anthers, embryos, and the skin tissue of the root tips.

The *SPIRAL1* (*SPR1*) and *SPIRAL2* (*SPR2*) genes were first identified in mutant *Arabidopsis* plants in which roots twist to the right instead of the left. The *spr1* mutant plants showed abnormal helical root growth, which was complemented by abnormal hypocotyl development. In root epidermal cells of *spr2* mutants, cortical arrays of microtubules in the basal elongation zone were abnormally helical.

At the genomic level, regulatory elements (transcription factors, enhancers, silencers) that play an important role in transcription regulation can be identified. Transcription factors are proteins that bind to DNA and regulate the transcription of genetic information from DNA to RNA.

Among our objectives was to identify TF binding sites (TFBS) and *cis*-regulatory elements (CREs) in the promoter regions of *Fragaria vesca* L. cv. Rügen *SPATULA* (*FvSPT*), *SPRIAL1-like1* (*FvSPR1-like1*) and *SPIRAL1-like2* (*FvSPR1-like2*) genes, which it was compared with promoter regions of tomato (*Solanum lycopersicum* L. cv. Micro Tom) and *A. thaliana* (L.) *SPATULA* and *A. thaliana* (L.) *SPRIAL* genes. We would like to perform a comparison with *in silico* analysis of the promoter regions in JASPAR and PLACE databases.

Among our other plans was to make deletion lines for the promoters of *FvSPT*, *FvSPR1-like1* and *FvSPR1-like2* genes based on the *in silico* promoter analysis, and by fusing them with *sGFP* (synthetic green fluorescent protein) reporter gene, determined the intensity of the expression of the reporter gene in the tobacco plant leaves (*Nicotiana benthamiana* L.) and tomato berry (*Solanum lycopersicum* L. cv. Micro Tom) as a result of transient expression.

For the functional characterization of *FvSPT, FvSPR1-like1* and *FvSPR1-like2* genes, we planned to perform a complementation test on *Arabidopsis thaliana* (L.) Col-0 *spt* and *spr1-2* mutant plants.

Material and methods

Plant material

The diploid strawberry (*Fragaria veca* L. cv Rügen) was used as a template to amplify the putative promoter regions of *FvSPT, FvSPR1-like1* and *FvSPR1-like2* genes. During the creation of the promoter deletion lines, the *FvSPT, FvSPR1-like1* and *FvSPR1-like2* genes fused with *sGFP* reporter gene were transiently expressed in the leaves of the tobacco plant (*Nicotiana benthamiana* L.) and the fruit of the Micro Tom berry (*Solanum lycopersicum* L. cv. Micro Tom). The *Arabidopsis thaliana* (L.) *spt* and *spr1-2* mutant plants were used for the complementation tests.

Growing conditions for *Arabidopsis thaliana* (L.), strawberry and tobacco plants

The seeds were sown ex vitro in soil in pots with diameter of 6 cm. The *A. thaliana* (L.) plants growing conditions were described by HIDVÉGI *et al.* (2020). The strawberry and tobacco plants growing conditions were described by HIDVÉGI *et al.* (2021).

Identification and amplifying of *SPATULA* (*FvSPT*) and *SPIRAL* (*FvSPR*) genes of *Fragaria vesca* L. cv. 'Rügen' with PCR method

We used *FaSPATULA, FaSPIRAL1-like1* and *FaSPIRAL1-like2* genes for our *in silico* analysis from BALOGH *et al.* (2005) és TISZA *et al.* (2010). The *Fragaria vesca* L. *SPIRAL1-like1* (*FvSPR1-like1*; XM_004297177; LOC01307108), *SPIRAL1-like2* (*FvSPR1-like2*; XM_004299243; LOC101309836), *Arabidopsis thaliana* (L.) *SPIRAL1-like2* (*AtSPR1-like2*; BT024676), *F. vesca* L. *SPATULA* (*FvSPT*; XM_004287975 and AY679615) and *A. thaliana* (L.) *SPATULA* (*AtSPT*; BT026462) genes homology identification and primer design are described by HIDVÉGI *et al.* (2020).

Genomic DNA (gDNA) was obtained from *F. vesca* L. cv. Rügen, the *FvSPT*, *FvSPR1-1* and *FvSPR1-2* genes and their promoters were isolated plant and amplified using the PCR technique according to HIDVÉGI *et al.* (2020).

Cloning of *FvSPT, FvSPR1-like1* and *FvSPR1-like2* genes and promoters into binary vector

The purified PCR products were cloned into the pDONR221 entry vector (Life Technologies, Carlsbad, CA, USA) and then into the pGWB401 binary vector (NAKAGAWA *et al.*, 2007) as described by HIDVÉGI *et al.* (2020). The pGWB401 vector was used to transform *A. thaliana* (L.) *spt* (NASC ID: N857133) and *spr1-2* (NASC ID: N6547) mutant plants (Col) according to HIDVÉGI *et al.* (2020).

Transformation of *Arabidopsis thaliana* (L.) with *Agrobacterium tumefaciens*

The genetic transformation of *spr1-1*/*spr1-2* and spt mutant *A. thaliana (L.)* plants were transformed as reported in HIDVÉGI *et al.* (2020).

Analysis of T_3 and T_4 generations of *Arabidopsis thaliana* (L.) plants with RT-qPCR

The T_3 and T_4 generations of *A. thaliana* (L.) plants were examined using the RT-qPCR (real-time quantitative polymerase chain reaction) method as it is written by HIDVÉGI *et al.* (2020).

Phenotypic analysis of T_3 and T_4 generations of *Arabidopsis thaliana* (L.) plants

In the case of T_3 and T_4 generations of *A. thaliana* (L.) wild-type, mutant and transformant plants, we determined the habitus, root, silique and number of seed/silique (in three biological and technical replicates) (HIDVÉGI *et al.*, 2020).

In silico analysis of promoters

We analyzed the promoters of *S. lycopersicum* L. cv. Micro Tom *SPATULA* (*MtSPT*) (Gene ID: 101,266,791, NC_015439.3) and *SPIRAL1-like2* (*MtSPR1-like2*) (Gene ID: 101,257,849, NC_015440.3) genes, *Fragaria vesca* (L.) *FvSPT* (XM_004287975; LOC101290893), *FvSPR1 -like1* (XM_004297177; LOC01307108) and *FvSPR1-like2* (XM_0042999243; LOC101309836), *A. thaliana* (L.) *AtSPT* (BT024676) and *AtSPR1-like2* (BT026462) genes as described in HIDVÉGI *et al.* (2021).

Amplification of promoter deletion lines using PCR technique

After identifying the regions before the start codon in the case of the promoters of the *FvSPT* (3100 bp) and *FvSPR1-like2* (2800 bp) genes, we prepared the 500 bp (FvSPR500, FvSPT500), 1000 bp (FvSPR1000, FvSPT1000), 2000 bp (FvSPR2000, FvSPT2000) and 3000 bp (FvSPT3000) deletion lines from the regions before the start codon. After the PCR, the PCR products were analyzed as described by HIDVÉGI *et al.* (2021).

TOPO® and gateway® LR cloning

The FvSPT500, FvSPT1000, FvSPT2000 and FvSPT3000 deletion lines from the *FvSPT* promoter region and FvSPR500, FvSPR1000 and FvSPR2000 deletion lines from the FvSPR promoter region were directionally cloned into the pENTR™ Directional TOPO® vector (HIDVÉGI *et al.* (2021). The promoter regions were built into the binary vector pGWB604 (NAKAGAWA *et al.*, 2007) (GenBank: AB543113.1). By cloning, the *FvSPR500::pGWB604*, *FvSPR1000::pGWB604*, *FvSPR2000::pGWB604*, *FvSPT500::pGWB604*, *FvSPT1000::pGWB604*, *FvSPT2000::pGWB604*, and *FvSPT3000::pGWB604* vector constructions have been created (HIDVÉGI *et al.* (2021).

Agrobacterium-mediated transformation of promoter deletion lines

Agrobacterium tumefaciens strain GV3101 (Intact Genomics, Creve Coeur, MI, USA) was used for the transformation according to HIDVÉGI *et al.* (2021). Colony PCR was used to select positive colonies according to the protocol based on the BERGKESSEL and GUTHRIE (2013) study.

Agroinfiltration into tomatoes and tobacco

The *A. tumefaciens* colonies containing the vector construct were agroinjected into tomato berries and tobacco . The transformant plants 3 days after the agroinjection according to HIDVÉGI *et al.* (2021) was selected. Only *sGFP*-positive plants were examined using the RT-qPCR method leaves (HIDVÉGI *et al.* (2021).

Detection of GFP fluorescence with UV light

The existence of GFP fluorescence was identified as described in HIDVÉGI *et al.* (2021).

Quantification of *sGFP* expression by qPCR method

In the case of those plants where the presence of sGFP was successfully detected with UV light, they were also examined using the qPCR method according to WANG *et al.* (2004) and HIDVÉGI *et al.* (2021).

Results and discussion

Promoter sequence analysis

During the promoter sequence analysis, the data were compared from JASPAR2020 and PLACE 30.0 databases. After comparing the data, various hypothetical TFBS and CREs were analyzed in the promoter sequences of the *AtSPR1-like2*, *AtSPT*, *FvSPR1-like1*, *FvSPR1-like2*, *FvSPT*, *MtSPR1-like2* and *MtSPT* genes. We successfully identified 222, 364, 117, 186, 323, 473, and 484 transcription factor binding sites (TFBS) and found 473, 645, 248, 30, 548, 733, and 719 CRE in promoter sequences of the *AtSPR1-like2*, *AtSPT*, *FvSPR1-like1*, *FvSPR1-like2*, *FvSPT*, *MtSPR1-like2*, and *MtSPT* genes.

We compared different promoter regions (TF and CRE) related to tomato flowering, fruit development and ripening in plants *S.* l*ycopersicum*, *A. thaliana* (L.) and *F. vesca* L. The promoter sequences of the *MtSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like1*, *FvSPR1-like1*, *AtSPR1-like2*, *MtSPT*, *FvSPT* and *AtSPT* genes had 16, 25, 7, 5, 34, 24 and 29 TFBS (HIDVÉGI *et al.*, 2021).

The sequences of the *MtSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like2*, *FvSPR1-like1*, *AtSPR1-like2*, *MtSPT*, *FvSPT*, and *AtSPT* genes had 11, 25, 6, 1, 27, 26, and 16, respectively. Based on the PLACE 30.0 database, CREs regulated by auxin, ethylene, GA_3 and cytokinin were classified. We identified 1 CRE for ethylene, 8 for auxin and 1 for GA_3 (HIDVÉGI *et al.*, 2021).

The fruit ripening of strawberries and tomatoes is regulated by ethylene, which determines its color between green (immature) and red (ripe) transitions (TISZA *et al.*, 2010, LI *et al.*, 2017). Auxin and ethylene balance can influence fruit ripening processes (SU *et al.*, 2015). ARF1, ARF2, ARF34, ARF5, ARF8, ATHB15, ATHB5, ATHB5, ATHB53, CAMTA1, EDT1, HAT2, KAN1, KUA1, MYB124, MYB73, OBP3, RVE1, SGR5, TGA1A and TGA2 were identified on promoters of the *FvSPR1-like2* and *FvSPT* genes

(HIDVÉGI *et al.*, 2021). Auxin response factors (ARFs) can bind specifically to 5'-TGTCTC-3' DNA sequence sites along auxin responsive promoter elements (AuxRE) (MAJER *et al.*, 2012). The ARF TFs were first identified in *A. thaliana* (L.) plants (MAYER *et al.*, 1999, ELLIS *et al.*, 2005, NAGPAL *et al.*, 2005, VIDAURRE *et al.*, 2007, ZHANG *et al.*, 2014). We also identified ARFAT and SURECOREATSULTR11 *cis*-regulating elements containing ARF-binding sequences (MARUYAMA-NAKASHITA *et al.*, 2005) on promoters of the *FvSPR1-like2* and *FvSPT* genes. AUXRETGA1GMGH3 CRE is a binding site for AuxRE proteins that regulate ARFs (GUILFOYLE *et al.*, 1998). TFs ATHB, EDT1, HAT2, KAN1, KUA1, MYB, OBP, RVE1, SGR5, and TGA2 associated with maintaining and regulating auxin and ethylene balance were first identified in *A. thaliana* (L.) plants (SAWA *et al.*, 2002, JOHNSON *et al.*, 2003, KANG *et al.*, 2003, MATTSSON *et al.*, 2003, BOWMAN, 2004, HAWKER and BOWMAN, 2004, SON *et al.*, 2004, PRIGGE *et al.*, 2005, MORITA *et al.*, 2006, KIM *et al.*, 2013, MEISSNER *et al.*, 2013, LU *et al.*, 2014, CAI *et al.*, 2015, CHEN *et al.*, 2015) while CAMTA1 TF was identified in rapes (*Brassica napus* L.) (BOUCHÉ *et al.*, 2002) and TGA1A TF in tobacco plants (*Nicotiana tabacum* L.) (PASCUZZI *et al.*, 1998).

We identified AGL42 (DORCA-FORNELL *et al.*, 2011), ARR2 (WEIRAUCH *et al.*, 2014), CMTA3 (BOUCHÉ *et al.*, 2002), DREB26 (KRISHNASWAMY *et al.*, 2011), ERF13 (SINGH *et al.*, 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) on promoters of the *FvSPR1-like2* and *FvSPT* genes. AGL42 is a MADS-box TF that controls flowering time and promotes the flowering process. Genes regulated by AGL42 are most expressed in leaves, flower buds, petals, and beeches of *A. thaliana* (L.) (DORCA-FORNELL *et al.*, 2011). AGL42 TFBS was identified

on promoters of the *MtSPR1-like2*, *AtSPR1-like2*, *MtSPT*, *FvSPT* and *AtSPT* genes.

The ARR1, ATHB34, BEE2, FUS3, MYB33, MYR2, SOC1, SRM1 and STZ TFBS were identified on promoters of the *FvSPR1-like2* and *FvSPT* genes. GARE1OSREP1 CRE regulates gibberellin-responsive elements together with the MYB33 TF (SUTOH and YAMAUCHI, 2003). In addition, ARR10 (HWANG and SHEEN, 2001), ARR11, ARR14, ARR18 and OsRR22 (TSAI *et al.*, 2012) TFBS were identified on promoters of the *FvSPR1-like2* and *FvSPT* genes. The motifs and function of TFs involved in the regulation of gibberellin have been demonstrated primarily in *A. thaliana* (L.) and *Oryza sativa* L. plants by 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) és STZ (MITTLER *et al.*, 2006), 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).

Creation of promoter deletion lines

We successfully identified promoter regions using *in silico* methods, after that the shorter promoter regions were amplified by PCR. The PCR products were cloned into binary vectors as *FvSPR500::pGWB604*, *FvSPR1000:pGWB604*, *FvSPR2000::pGWB604*, *FvSPT500::pGWB604*, *FvSPT1000::pGWB604*, *FvSPT2000::pGWB604* and *FvSPT3000::pGWB604*. Positive *E. coli* transformations were verified by colony PCR with deletion line-specific primers.

The *E. coli* colonies with the fragment of sufficient size have grown during PCR were selected for plasmid isolation to clone deletion constructions

into *A. tumefaciens*. *A. tumefaciens* bacteria were also examined by colony PCR to select positive colonies.

The fluorescence of the *sGFP* gene was detectable only by UV light in tobacco lines *FvSPR2000::pGWB604*, *FvSPT1000::pGWB604*, *FvSPT2000::pGWB604*, *FvSPT3000::pGWB604* and *CaMV35S::sGFP* (pGWB405) (Figure 1). In green berry of tomatoes, sGFP could be identified in the lines *FvSPR2000::pGWB604*, *FvSPT2000::pGWB604*, *FvSPT3000::pGWB604* and *CaMV35S::sGFP* (pGWB405) (Figure 2).

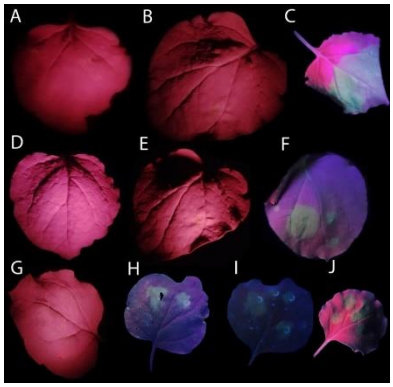

Figure 1: Identification of *sGFP* reporter gene by UV light in tobacco plants. A: *A. tumefaciens* without vector construction, B: Control untreated plant, C: *CaMV35S::sGFP* (pGWB405), D: *FvSPR500::pGWB604*, E: *FvSPR1000::pGWB604*, F: *FvSPR2000::pGWB604*, G: *FvSPT500::pGWB604*, H: *FvSPT1000::pGWB604*, I: *FvSPT2000::pGWB604*, J: *FvSPT3000::pGWB604*

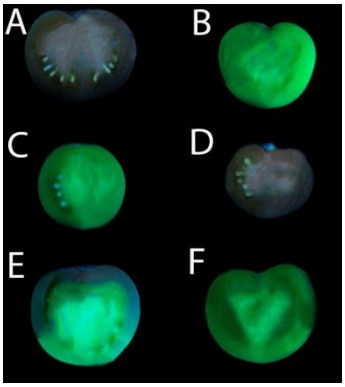

Figure 2: Expression of *sGFP* in berries of a transformant tomato plant illuminated by UV light. A: Control untreated plant, B: *CaMV35S::sGFP* (pGWB405), C: *FvSPR2000::pGWB604*, D: *FvSPT1000::pGWB604*, E: *FvSPT2000::pGWB604*, F: *FvSPT3000::pGWB604*

The RT-qPCR examination of promoter deletion lines

Using the RT-qPCR method, we successfully identified the presence of the sGFP gene, determined the intensity of its expression in berry fruits of tomatoes and leaves of tobacco. The gene expression intensities of sGFP in tobacco leaves were 0.072, 0.006, 0.099 and 2.532 for *FvSPR2000::pGWB604* , *FvSPT1000::pGWB604*, *FvSPT2000::pGWB604* and *FvSPT3000::pGWB604* respectively, compared to the *CaMV35S::sGFP* positive control. The expression logarithmic fold change (LFC) values were -3.8, -7.29, -3.33 and 1.34 for *FvSPR2000::pGWB604*, *FvSPT1000::pGWB604*, *FvSPT2000::pGWB604* and *FvSPT3000::pGWB604*, respectively (Figure 3).

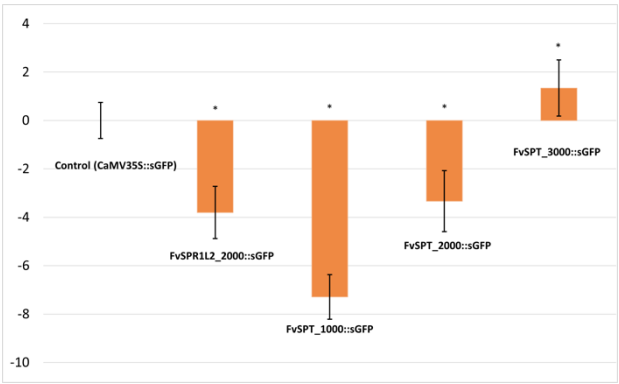

Figure 3: LFC values of the *sGFP* gene detected in tobacco leaves. Values marked with an asterisk (*) were significantly ($p < 0.05$) different from control

The gene expression intensities of sGFP in tomato berry yields were 0.026, 0.024 and 1.028 in the *FvSPR2000::pGWB604*, *FvSPT2000::pGWB604* and *FvSPT3000::pGWB604* lines based on ΔΔCt values. Expression LFC values were -5.28, -5.36 and 0.04, respectively, in the *FvSPR2000::pGWB604*, *FvSPT2000::pGWB604* and *FvSPT3000::pGWB604* lines relative to the *CaMV35S::sGFP* positive control (Figure 4).

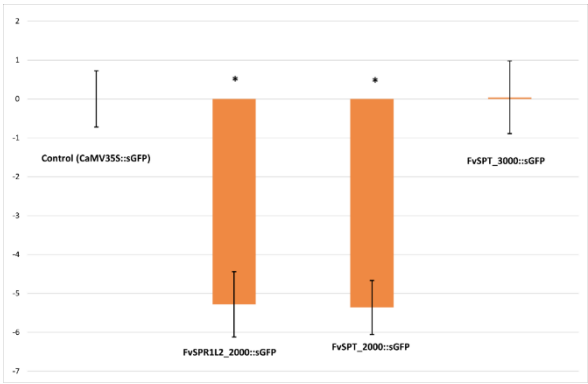

Figure 4: LFC values of the *sGFP* gene detectable in tomato berry. The values marked with an asterisk (*) were significantly ($p < 0.05$) different from the control

The values of the lines *FvSPR2000::pGWB604*, *FvSPT1000::pGWB604* and *FvSPT2000::pGWB604* ΔΔCt values are significantly ($p < 0.05$) compared to control. However, *FvSPT3000::pGWB604* ΔΔCt values were statistically significant ($p < 0.05$) compared to positive control (*CaMV35S::sGFP*) in tobacco plant leaves but not in tomato berries.

The experiment focused primarily on promoters of the *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.*, 2017, HIDVÉGI *et al.*, 2020). Transient expression is determined by the exclusive expression of reporter gene (*sGFP*) in the infiltrated area and the composition of the expression cassette (vector construction), but the *Agrobacterium* strain and its density can also influence reporter gene expression (TYURIN *et al.*, 2020).

The *FvSPR500::pGWB604* and *FvSPR1000::pGWB604* constructions did not work in tobacco leaves, while *FvSPR2000::pGWB604* worked. In the *FvSPR2000::pGWB604* design, *sGFP* is expressed in tomato fruit. We successfully identified ARF1, ARF2, ARF5 and ARF8 TFBS on the deletion line of the promoter of the *FvSPR1-like2* gene from -1067 bp to -1059 bp. These TFBS were not identifiable in deletion lines from -500 to -1 bp and from -501 to -1000 bp. The ARF family of TFBS plays an important role in ARF-directed auxin regulation during fruit ripening (LIU *et al.*, 2015). This may explain why only the *FvSPR2000::pGWB604* design was able to induce expression of *sGFP* in tobacco leaves and tomato berries. The *FvSPT1000::pGWB604*, *FvSPT2000::pGWB604* and *FvSPT3000::pGWB604* designs worked in tobacco leaves, while the *FvSPT1000::pGWB604* construction did not work in tomato berry. The *FvSPT500::pGWB604* design did not work in tomato berries and tobacco leaves. The

FvSPT1000::pGWB604 design had a lower *sGFP* expression than the *FvSPT2000::pGWB604* design. This difference in gene expression intensity may be caused by MYB59, WRKY25 and WRKY8 TFBS, which is regulated by ethylene (LI *et al.*, 2006, CHEN *et al.*, 2013). The ethylene-auxin interaction may play an important role in regulating the promoter of the *FvSPT* gene, causing an antagonistic effect between ethylene and auxin during the ripening of tomato berry crops (LI *et al.*, 2017). The *FvSPT1000::pGWB604* design does not have MYB59, WRKY25 and WRKY8 TFBS because they are only located between -1256 and -1248, -1609 and -1602 and -1610 and -1602, which are only present in *FvSPT2000::pGWB604*. The *FvSPT1000::pGWB604*, *FvSPT2000::pGWB604* and *FvSPT3000::pGWB604* constructions worked in his tobacco leaves, but the *FvSPT1000::pGWB604* construction did not work in tomato berry production.

Complementation test of *A. thaliana* (L.) *spt* and *spr1-2* mutant plants

We successfully isolated the *F. vesca* L. *FvSPT*, *FvSPR1-like1* and *FvSPR1-like2* genes (BALOGH *et al.*, 2005, POLGÁRI *et al.*, 2010), which play an important role in fruit ripening. In our experiment, the mutant plants *A. thaliana* (L.) *spt* and *spr1-2* were complemented by the *SPT* and *SPR* genes of *F. vesca*. *In silico* analyses, promoter regions and genes were identified for *FvSPT*, *FvSPR1-like1* and *FvSPR1-like2* genes that showed homology with the At1g69230 and At4g36930 genes based on the *F. vesca* L. genome sequence (htttp://www.rosaceae.org) (SHULAEV *et al.*, 2011). The homology was 84.03%, 69.45% and 74.24%, respectively, between *FvSPT* (XM_004287975) and *AtSPT* (At4g36930), *FvSPR1-like1* (XM_004297177) and *AtSPR1-like2* (At1g69230), and between *FvSPR1-like2* (XM_004299243) and *AtSPR1-like2* (At1g69230) (Figures 5 and 6).

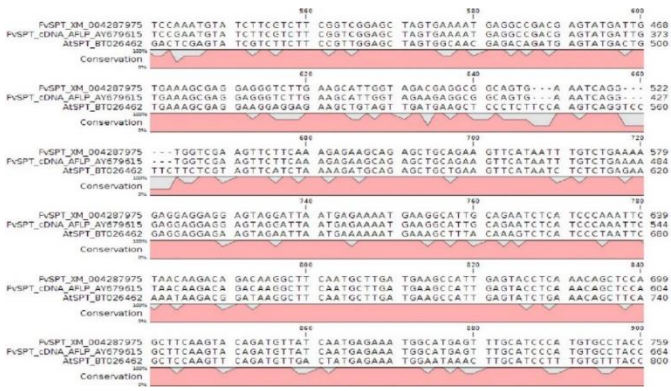

Figure 5: Homology between *FvSPT* (*Fragaria vesca* L. *SPATULA*) (XM_004287975), *FvSPT* cDNA (AY679615) and *AtSPT* (*Arabidopsis thaliana* (L.) *SPATULA*) (BT026462) genes (CLC Main Workbench v7)

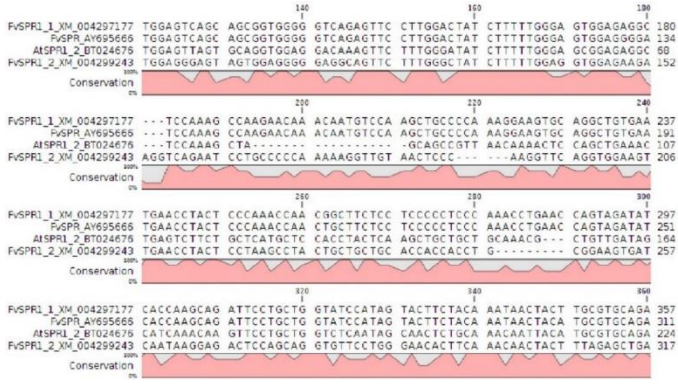

Figure 6: Homology between *FvSPR1-like1* (*Fragaria vesca* L. *FvSPR1-like1*) (XM_004297177), *FvSPR* (*Fragaria vesca* L. *SPIRAL*) (AY695666), *AtSPR1-like2* (BT024676) and *FvSPR1-like2* (XM_004299243) genes (CLC Main Workbench v7)

We successfully amplified the *FvSPT* (6600 bp), *FvSPR1-like1* (9647 bp) and *FvSPR1-like2* (2443 bp) genes and their promoters by PCR. After assembling the vector constructions *FvSPT::pGWB401*, *FvSPR1-like1::pGWB401* and *FvSPR1-like2::pGWB401*, the correct integration of genes and their promoters was verified by colony PCR.

The mutant *A. thaliana* (L.) *spt* and *spr* mutants (60 plants/vector construction) were transformed by floral dip method. The transformed plants were treated with kanamycin selection agent at 3 days, 1 weeks, and 2 weeks after transformation, at concentrations of 100 mg/mL, 200 mg/mL and 400 mg/mL. The *A. thaliana* (L.) plants surviving the selection treatment were also examined by direct PCR method, confirming that the promoter and gene were really present in the plant.

The mean transformation efficiency was 7.6% with repeated floral dip transformation of secondary inflorescences. The phenotypic characteristics of 6-week-old transformant plants were compared with those of wild-type Col-0 *A. thaliana* (L.) plants. The *spt* mutant plants were significantly smaller than the wild type.

Figure 7: Habits of plants of *A. thaliana* (L.) at 8 weeks old. A: Col-0 wild type, B: *spr* mutant, C: *spt* mutant and D: *FvSPT1-like2::pGWB401* complemented plants

The *FvSPT::pGWB604* transformant individual plants were significantly higher than *spt* mutant individual plants, while they were identical to the wild type (Figure 7). This demonstrated that the *spt* mutant plant could be complemented by the *SPT* gene and promoter of the *F. vesca* L. plant.

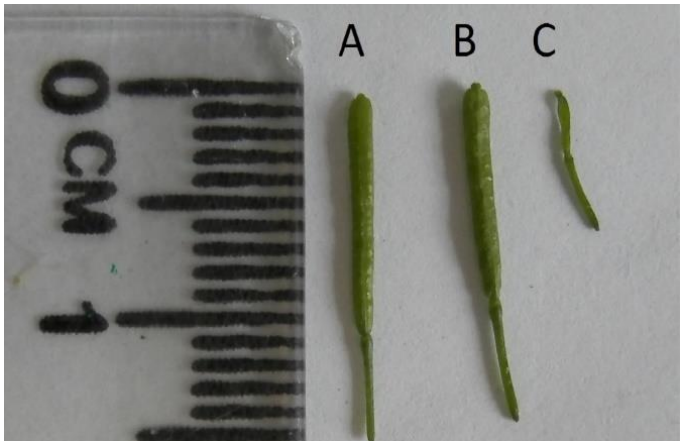

Figure 8: Comparison of the plants of *A. thaliana* (L.) plants. A: Col-0 wild-type plant, B: *FvSPT::pGWB401* complemented plant, C: *spt* mutant plant

The length of the silique (on average 12 siliques/plant individuals), the wild-type, *spt*, *spr* mutant and complemented plants were compared (Figure 8). The mutant plant *spt* had the shortest silique length (3.8 mm), while the wild type and the complemented lines *FvSPT*/*FvSPR1-like1*/*FvSPR1-like2* had significantly longer siliques (12.8 – 13.3 mm). Compared to the *spr* mutant plant, only the complemented plant *FvSPR1-like1* had a significant increase in silique length (Figure 9).

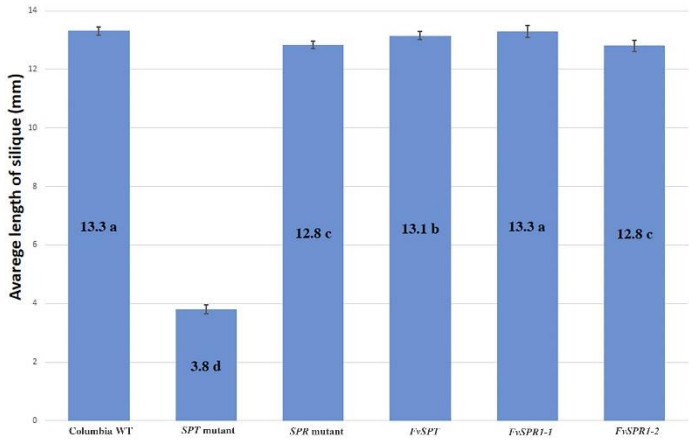

Figure 9: Silique length (mm) of *A. thaliana* (L.) plants. Col WT (wild type): Col-0 wild type, *SPT* mutant: spatula mutant, *SPR* mutant: spiral mutant, *FvSPT*: *FvSPT::pGWB401* complemented Col-0, *FvSPR1-1*: *FvSPR1-like1::pGWB401* complemented Col-0 and *FvSPR1-2*: *FvSPR1-like2::pGWB401* complemented Col-0. The letters next to the numbers in the columns indicate statistically significant differences in individuals compared to plants of the Col-0 wild type in ANOVA ($p < 0.001$) analysis. 80 individuals/experiments/line and three biological replicates.

The *spt* mutant plants produced a lower seed/silique ratio (7.7) than wild type (47.7) (Figure 10). The *spr* mutant plant individuals grew significantly shorter silique types compared to the wild type (Figure 9) and also produced significantly lower seed/silique ratios (37.5) than the wild type (47.7) (Figure 10). The seed number in complemented plant individuals *FvSPT::pGWB401* was, as expected, significantly higher than in the *spt* mutant plant individuals, but significantly less than in the wild type (Figure 10).

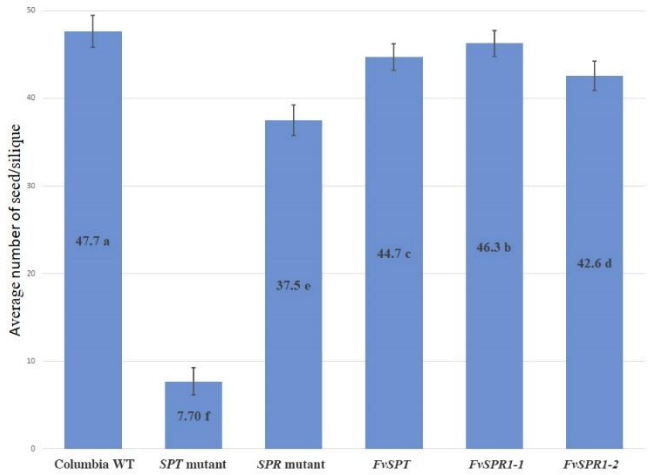

Figure 10: Graph of the seeds/silique average. Columbia WT: Col-0 wild type, *spt* mutant: spatula mutant, *spr* mutant: spiral mutant, *FvSPT*: *FvSPT::pGWB401* complemented Col-0, *FvSPR1-1*: *FvSPR1-like1::pGWB401* complemented Col-0, *FvSPR1-2*: *FvSPR1-like2::pGWB401* complemented Col-0. The letters next to the numbers in the columns indicate statistically significant differences in individuals compared to plants of the Col-0 wildtype in ANOVA ($p < 0.001$) analysis. 80 individuals/experiments/line and three biological replicates.

Complemented plants *FvSPR1-like1::pGWB401* and *FvSPR1-like2::pGWB401* had significantly higher seed number than *spr* mutant plants, but significantly less than the wild plants (Figure 10).

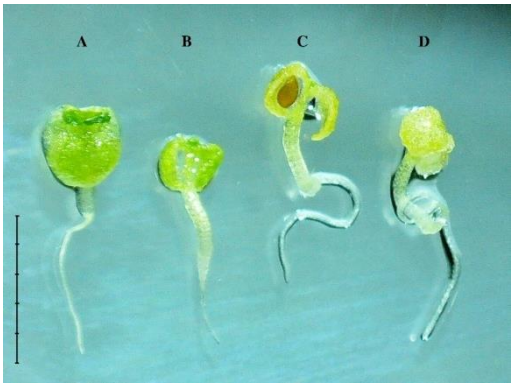

Figure 11: Root abnormalities in 1-week-old *A. thaliana* (L.) plants. A: *FvSPR1-like2::pGWB401* complemented Col-0, B: Col-0 wild type, C: *spr* mutant, D: *FvSPR1-like1::pGWB401* complemented (Bar: 5 mm)

Figure 12: Root abnormalities in 2-week-*old A. thaliana* (L.) plants. A: *spr* mutant, B: *FvSPR1-like1::pGWB401* complemented C: *FvSPR1-like2::pGWB401* complemented Col-0, D: Col-0 wild type (Bar: 5 mm)

Root growth differences between wild and spr mutant plant individuals can be observed in Figures 11 and 12. The *spr* mutation could only be restored by the vector construction *FvSPR1-like2::pGWB401* (Figures 11 and 12). *FvSPR1-like1* plants showed similar helical root growth as the *spr* mutant plant. Current research demonstrates the existence of three recessive *A. thaliana* (L.) spr mutant plants, *spr1-1*, *spr1-2*, and *spr1-3* (NAKAJIMA *et al.*, 2006). For our research, we used the *spr1-2* mutant plants, so it is expected that this mutation can only be complemented by the *FvSPR1-like2::pGWB401*

construct, while the *FvSPR1-like1::pGWB401* construct cannot. This experimentally demonstrates that the *FvSPR1-like1* and *FvSPR1-like2* genes do not have the same function. In the case of complementary *A. thaliana* (L.) plants, the expression intensity of the *FvSPT*, *FvSPR1-like1* and *FvSPR1-like2* genes was verified and measured by RT-qPCR method. The primers are designed for the exon-exon boundary, which multiplies fragments of various sizes from gDNA and cDNA. Our RT-qPCR results confirm that the *FvSPT::pGWB401*, *FvSPR1-like1::pGWB401* and *FvSPR1-like2::pGWB401* constructions work and are expressed in complemented *A. thaliana* (L.) plants.

Our results confirmed that octoploid *F. x ananassa* Duch. cv. *FaSPT* and *FaSPR* genes isolated from Elsanta strawberries by cDNA-AFLP method (BALOGH *et al.*, 2005) show similarities not only with *AtSPT*, *AtSPR1-like1* and *AtSPR1-like2*, but also with diploid strawberries (*F. vesca* L.) *FvSPT*, *FvSPR1-like1*, and *FvSPR1-like2* genes, and are able to complement *A. thaliana* (L.) *spt* and *spr1-2* mutant plants, their phenotypic changes from the wild type, HEISLER *et al.* (2001), where the *spt2* mutation in the *AtSPT2* allele was complemented by the *AtSPT* gene. The literature confirms that the *SPR1-1*, *SPR1-2* and *SPR1-3* mutations cause the same phenotypic changes in root development in the plant *A. thaliana* (L.) (FURUTANI *et al.*, 2000). However, our research showed that the *spr1-2* mutation can only be complemented by the *FvSPR1-like2* gene.

Conclusions and proposals

In our research, we compared promoters *of the AtSPR1-like1, AtSPT, FvSPR1-like1, FvSPR1-like2, FvSPT, MtSPR1-like2* and *MtSPT* genes based on JASPAR2020 and PLACE 30.0 databases.

Through ripening-specific genes, promoter regions (TFBS and CRE) related to flowering, fruit development and ripening were primarily identified. In terms of function, CREs were regulated by the hormones auxin, ethylene, GA_3 and cytokinin. In our research, ARF-specific TFBSs, as well as ARFAT and SUPERCOREATSULTR11 *cis*-regulating elements were identified in the promoter region of the *FvSPR1-like2* gene, which are regulated by auxin. AGL42 TFBS was identified in the promoter regions of the *FvSPR1-like2* and *FvSPT* genes, which, based on data available in the literature, regulates genes expressed in flower buds and petals in *A. thaliana* (L.). The same AGL42 was identified on promoters of the *MtSPR1-like2*, *AtSPR1-like2*, *MtSPT* and *AtSPT* genes showing homology with the *FvSPR1-like2* and *FvSPT* genes.

We identified MYB59, WRKY25 and WRKY8 TFBS on promoters of the *FvSPR* and *FvSPT* genes, which according to the literature are regulated by ethylene. Scientific publications confirm that ethylene-auxin interaction plays an important role during fruit ripening. Our research also showed that the *sGFP* reporter gene fused with deletion lines of the promoter of the *FvSPT* gene did not activate if ethylene-regulated TFBS (MYB59, WRKY25, and WRKY8) were not present in the sequence.

We successfully complemented *spt* and *spr1-2* mutant *A. thaliana* (L.) plants with the *FvSPT* and *FvSPR1-like2* genes. The homology was 84.03% and 74.23% between the *FvSPT* and *AtSPT* genes and between *FvSPR1-like2* and *AtSPR1-like2*, respectively. The success of complementing mutant genes was also ensured by the higher proportion of homology. The average efficiency of the floral dip transformation method was 7.6% with repeated

transformation of secondary inflorescences. According to the available literature, the expected transformation efficiency results were obtained for *A. thaliana* (L.). The transformant plants *FvSPT::pGWB604* showed the same height, seed length and seed number/seed ratio as Col-0 WT (wild type) plants. Only in the case of *FvSPR1-like2::pGWB604* transformant plants was observed the recovery of root growth differences between WT and spr mutant plants. Current research confirms that there are three recessive *A. thaliana* (L.) *spr* mutant plants, *spr1-1*, *spr1-2*, and *spr1-3*. According to the literature, the *spr1-1*, *spr1-2* and *spr1-3* mutations cause the same phenotypic changes in root development, however, our research has shown that the *spr1-2* mutation can be complemented by the *FvSPR1-like2* gene, but not not with the *FvSPR1-like1* gene.

In recent years, transgenic methods and techniques have started developing rapidly through the functional characterization of many genes. Molecular genetic researchers have many opportunities to isolate tissue-specific promoters, which makes the deermination of transgene function more efficient. The most important tool for understanding transcription regulation is the identification of transcription factor bimding sites and *cis*-regulating elements in the promoter region. There are many databases available to identify and understand the regulations of these regions, which aggregate different plant species as references. The identification of new promoters and their regulatory regions is an important area for agricultural companies and researchers, which can create new opportunities for the use of tissue- and cell-specific promoters in the next generation of genetically modified crops.

New scientific results

1. The TFBS and CRE regulatory elements of diploid strawberry (*Fragaria vesca* L.) were identified by *in silico* methods in the promoter region of the *FvSPT*, *FvSPR1-like1* and *FvSPR1-like2* genes and in the promoter regions of the *AtSPT*, *AtSPR1-like2*, *MtSPT* and *MtSPR1-like2* genes. We were able to functionally assign these regulatory elements to regions regulated by the auxin, ethylene, GA_3 and cytokinin hormones, which regulate genes expression in flower buds and petals.
2. By creating promoter deletion lines of the *FvSPT* and *FvSPR* genes, we proved that these promoter regions can express the reporter protein by fusing with the *sGFP* reporter gene, depending on the size of the deletion, in the leaves of the tobacco plant and in the berry fruits of the Micro Tom tomato plant. Using the promoter deletion lines of the *FvSPR* gene, it was demonstrated that the *sGFP* reporter gene does not switch on when TF or CRE elements necessary for regulating the hormone auxin or ethylene are absent in the promoter region.
3. In the case of the mutant plant *A. thaliana* (L.) *spt*, it has been demonstrated that the *FvSPT* gene in diploid strawberry can complement phenotypically occurring lower plant height, silique length and seed number/silique ratio.
4. In the case of the mutant plant *A. thaliana* (L.) *spr1-2*, it has been demonstrated that the diploid strawberry *FvSPR1-like1* gene cannot complement phenotypic changes, while the *FvSPR1-like2* gene is fully capable of complementing phenotypically occurring root developmental abnormality.

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