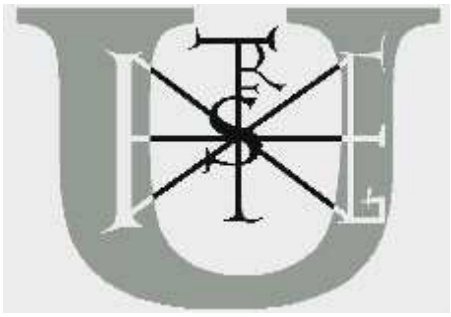

SZENT ISTVÁN UNIVERSITY

Characterization of *SPATULA* gene, encoding a bHLH transcription factor from cultivated strawberry

PhD thesis

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Gödöllő

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Scientific background, aims

Fruits can be divided into two groups - climacterics (e. g.: apple, banana, tomato) and non-climacterics (e.g.: strawberry, citrus, grape) - based on the observation whether they show an increased respiration and a hasty forthcoming ethylene production during the ripening process or not. The ripening regulation role of ethylene is well established in the case of climacteric fruits, but it's function in non-climacterics is not clearly understood. Due to the development of functional genomic studies it seems that ethylene dependent, and ethylene independent (regulated by transcriptional factors) cascades together are operating in both groups. Cultivated strawberry (*Fragaria x ananassa* Duch.) is the most extensively studied model plant for non–climacteric ripening. Despite the intensive projects resulted important advances there are still many questions unanswered concerning strawberry ripening and fruit developmental processes.

Basic-helix-loop-helix (bHLH) transcription factors are regulators involved in essential developmental and physiological processes. The plant bHLH proteins functionally characterized so far act as transcription factors involved in carpel development, phytochrome signaling, anthocyanin biosynthesis and stress responses. Therefore it is an important task to clarify the function of these transcription factors in order to recognize molecular background of physiological processes like fruit ripening, senescence, or fruit development.

Our work was the first report for the characterization of a SPATULA gene encoding for a bHLH transcription factor from the non–climacteric strawberry.

The objectives of our work can be summarized as follows:

1. The isolation and cloning of the ORF (Open Reading Frame) of bHLH transcription factor gene.
2. *In silico* analysis of the known gene sequence in order to identify secondary structures and domains.
3. Determination of gene expression in vegetative and generative tissues.
4. Determination of expression in strawberry leaves, as vegetative tissues in a response to mechanical wounding, auxin, and ethylene treatment.
5. Determination of the expression level in a response to auxin treatment in developing young fruits.
6. Determination of gene expression in a response to ethylene treatment in strawberry fruits at different ripening stage.
7. Silencing of *FaSPT* by using a transient gene expression assay.

Materials and methods

After the cloning of *FaSPT* (isolated from ripe strawberry receptacle), *in silico* analysis were applied using BLASTN, BLASTX, (NCBI, National Center for Biotechnology Information), ClustalW 1.83 programs (Thompson et al. 1994), CLC bio (CLC protein workbench), and Pfam (Sonnhammer et al. 1998) softwares.

FaSPT expression was investigated by qRT-PCR in vegetative and generative strawberry tissues. Changes in gene expression were analyzed also by using qRT-PCR, in a response to hormone treatments (auxin, ethylene) and mechanical wounding in leaf tissues, furthermore, in a response to auxin and ethylene treatments in strawberry fruits at different stages of development and ripening.

In order to brighten the *in planta* function of *FaSPT*, an *Agrobacterium*-mediated transient expression assay was used.

Results

Cloning of *FaSPT* ORF and in silico analysis

We reported the cloning of *SPATULA* ORF (*FaSPT*) from cultivated strawberry. At protein level the gene shared the highest homology with the SPATULA from *Arabidopsis* (AtSPT) which has functionally already been characterized. By using additional *in silico* analysis, a CDRN, Fringe, and serine rich motifs were identified in the amino acid sequence of *FaSPT* (Fig. 1).

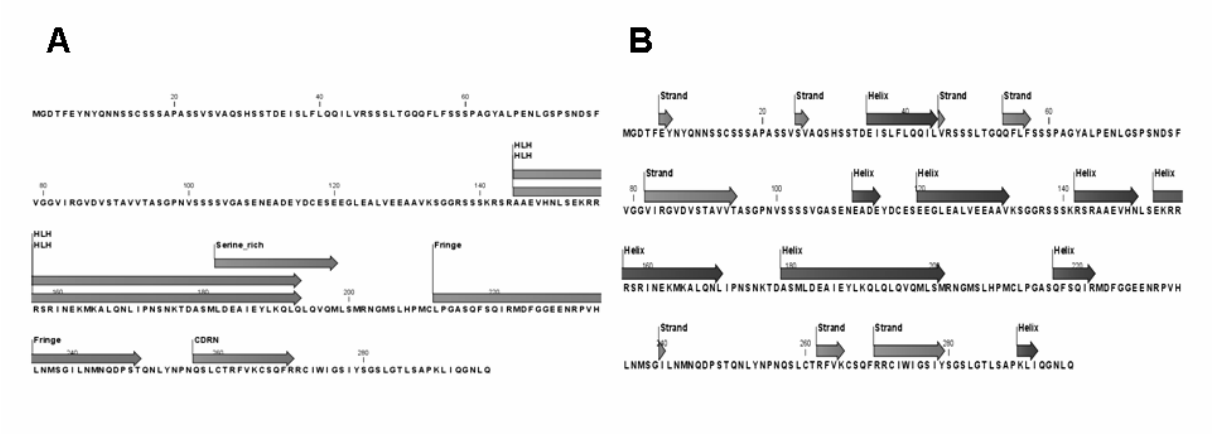

Fig. 1. Serine rich, Fringe and CDRN domains (A), and secondary structure of FaSPT (B). Prediction was performed by CLC protein work bench software (CLC bio).

Gene expression experiments

In order to determine the expression of *FaSPT* at tissue level, qRT-PCR technique was used. We found that *FaSPT* expression varies among different tissues (Fig. 2). A higher level of gene expression was measured in roots, stems, old and young leaves, sepals and petals respectively than in immature receptacles, where a slight continuous increment was detected throughout the three stages (1 cm small green, 2 cm large green, white) of fruit development. There was a sudden increase of the transcript in pink receptacles, then it decreased significantly in the red ones. Differences among expression levels of the receptacles suggest that *FaSPT* expresses constantly in developing and ripening fruits up to the pink stage (Fig. 2).

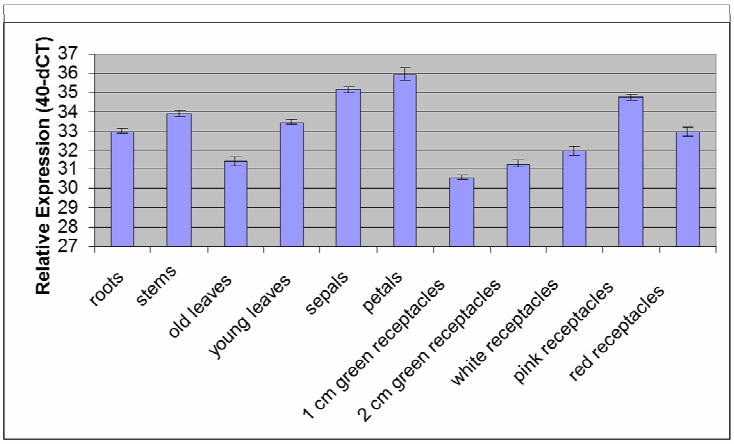

Fig. 2. Relative expression profile of *FaSPT* in various strawberry tissues. Mean values ±SD of three replicates are shown.

It was previously reported that *SPATULA* expresses not only during ripening, but in different vegetative and generative tissues (Heisler et al. 2001), therefore it was worthy to examine its expression in a response to hormone treatments and wounding in strawberry leaves.

It was proven that many key regulators involved in fruit development can also act in leaf developmental process, hereby confirming the evolutionary origin of carpels, as modified leaves (Østergaard 2008).

Auxin affects various processes of plant growth and development, like cell division, extension, vascular tissue differentiaton, lateral root growth, tropism and leaf senescence.

In the promoter region of *FaSPT* a TGA-box was identified 646 bp upstream (Balogh et al. 2005) as a part of an auxin-response element (AuxRE), TGA box is connected with auxin responses, furthermore it serves as binding site of bZIP transcription factors. These findings were suggested a role for the plant hormone auxin on *FaSPT* gene expression.

Our results revealed a decrease in the transcript quantity on the effect of auxin treatment (Fig. 3). After half an hour a significant decrease was detected between the control and auxin treated materials, where the transcript amount was one fifth in the treated leaves compared to the controls. After 2 hours there was a very slight increment, but it was still significantly lower than the control.

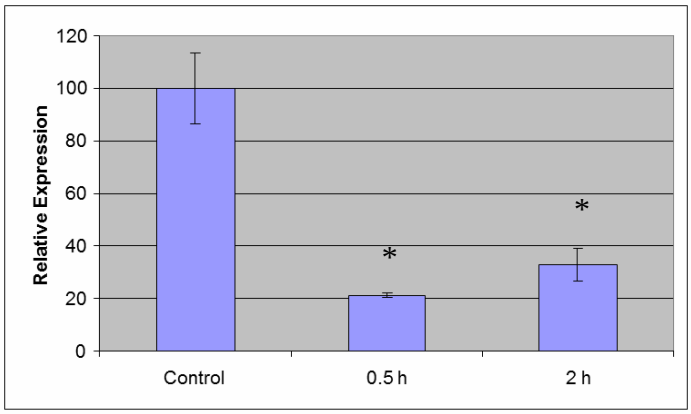

Fig. 3. Relative expression profile of *FaSPT* in strawberry leaves by auxin treatment. Mean values ±SD of three replicates are shown. *P-value < 0.05.

Previous results reported *Arabidopsis* genes with increased expression in a response to auxin which were connected to cell growth and division, while those, which were involved in senescence and stress responses were down-regulated (Bao et al. 2002).

Since four ethylene-response factors (ERE) were found in the promoter region of *FaSPT* (Balogh et al. 2005) thus we supposed a potential role for ethylene on the transcriptional regulation of *FaSPT* in strawberry leaves. In the course of our experiments it revealed, that gene expression decreases after the injection of 20 µl l^{-1} ethylene gas, then a significant decrease occurred after adding 200 µl l^{-1} (Fig. 4).

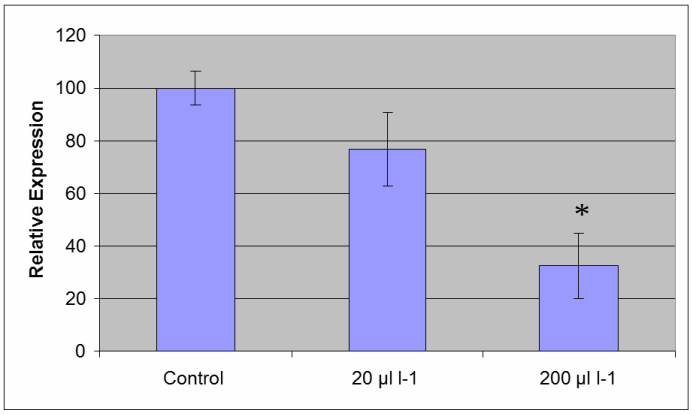

Fig. 4. Relative expression profile of *FaSPT* in strawberry leaves by ethylene treatment. Mean values ±SD of three replicates are shown. *P-value < 0.05.

Pressumably, due to the changes of endogenous ethylene level of vegetative tissues, *FaSPT* showed different expression patterns in old and young leaves (Fig. 2). In the course of ageing, ethylene is proven to have an effect on leaf senescence, while the oldest the tissue is, the highest level of ethylene production is found. We detected a higher decrease of expression as a response to higher amount of ethylene applied. On the basis of this observation we suppose, that *FaSPT* may be involved in senescence connected mechanisms in the case of strawberry leaves.

Many of the bHLH proteins play important role in biotic (bacterial and virus infections) and abiotic (heat stresses, drought, wounding) stress responses (Kiribuchi et al. 2005) by acting transcriptional activators of induced genes. After half an hour a rapid decrease of the transcript amount was detected, and a further decrease occurred after two hours (Fig. 5).

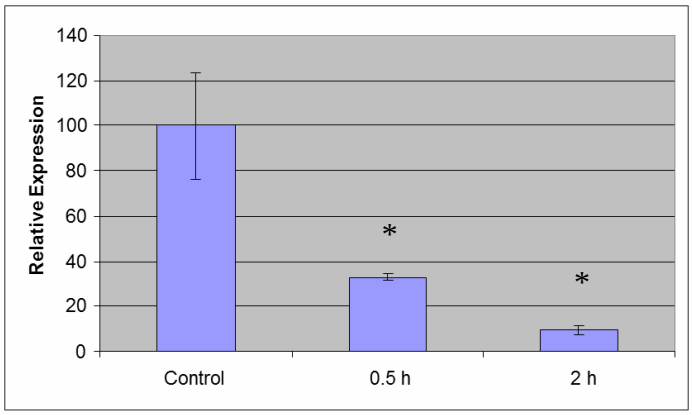

Fig. 5. Relative expression profile of *FaSPT* by mechanical wounding. Mean values ±SD of three replicates are shown. *P-value < 0.05.

Presumably, metabolites released by mechanical wounding could repress *FaSPT* transcription, moreover stress ethylene synthetised by wounding could also influence gene expression. Our result suggest a potential role for *FaSPT* in molecular cascades connected to stress responses.

In order to clarify the role of *FaSPT* during strawberry fruit development, the gene expression level was determined in strawberry receptacles and their achenes at three different (1 cm green, 2 cm green, white) developmental stages (Fig. 6). Moreover by separating achenes from the receptacles, additional data were obtained about the influence of endogenous auxin on developing tissues.

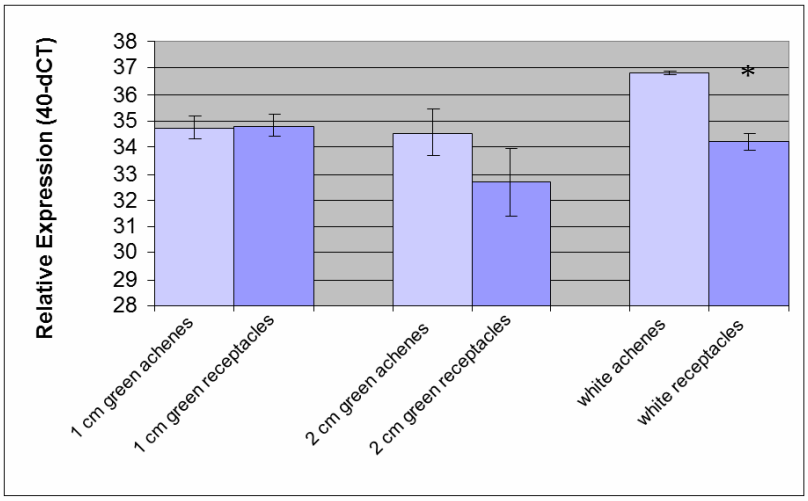

Fig. 6. Expression profile of *FaSPT* in developing achenes and receptacles. Mean values ±SD of the three replicates are shown. *P-value < 0.05.

Analyzing the achene- and receptacle-specific expression almost the same amount of transcript was detected in these tissues of the 1 cm fruit. The reason for this observation is presumably due to the nearly identical hormone levels in both tissues at this developmental stage. By contrast, a sudden decrease of the *FaSPT* expression occurred in the receptacles of the 2 cm fruits compared to their achene tissues where it remained as high as in the 1 cm strawberries. Similar results were obtained examining the white strawberries where the expression level of *FaSPT* was significantly higher in the achenes than in receptacles.

Natural hormone levels in the achenes, the main auxin source are decreasing during fruit developmental processes and consequently the auxin supply for the receptacles also declines triggering the increment of *FaSPT* transcript. The abundance of the transcript in the developing achenes and receptacles of 2 cm green and white strawberries implies a possible role of *FaSPT* in fruit developmental processes.

According to the above mentioned data we expected exogenous auxin treatment to suppress *FaSPT* transcription. Moreover, a TGA-box, as a part of the auxin responsive element (AuxRE) motif, was identified in the *FaSPT* promoter region (Balogh et al. 2005), suggesting that auxin has an impact on *FaSPT* expression.

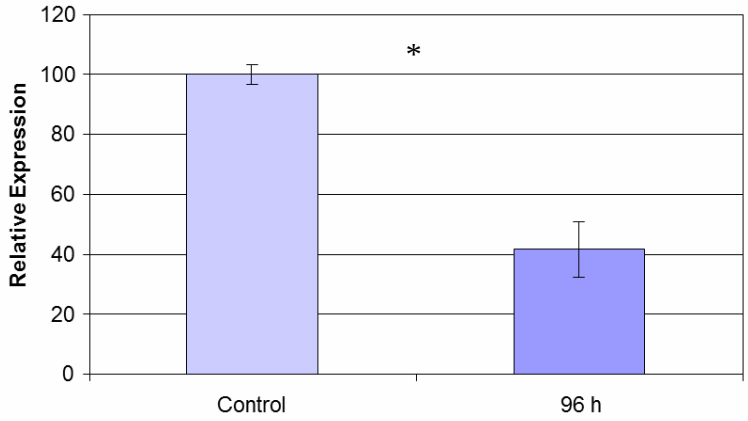

Fig. 7. Relative expression profile of *FaSPT* gene in young green fruits (1 cm) treated by lanolin paste containing 1 mM NAA synthetic auxin in 1% DMSO. Control represents untreated young green fruits which have been treated by the same paste omitting the hormone. All of the fruits were harvested 96 h after treatment. Values were normalized to the controls, arbitrarily set to 100. Mean values ±SD of the three replicates are shown. *P-value < 0.05

Indeed, our experiment revealed that the gene is down-regulated by the 96 h auxin treatment compared to the controls (Fig. 7).

Since four ethylene responsive elements (ERE) were identified in the promoter region (Balogh et al. 2005), we assumed a possible effect of ethylene on the expression of *FaSPT*. To elucidate this assumption, we applied exogenous ethylene treatment on strawberries.
Divergent expression patterns were exhibited in the receptacles at different stages of ripening in response to exogenous ethylene (Fig. 8). After 24 h hormone exposure *FaSPT* did not show any alteration in young green receptacles compared to the control samples. By contrast, a slight increment of transcripts was observed in white ones followed by a very low decrease in the pink strawberries. There were no significant changes found in the mature red ones.

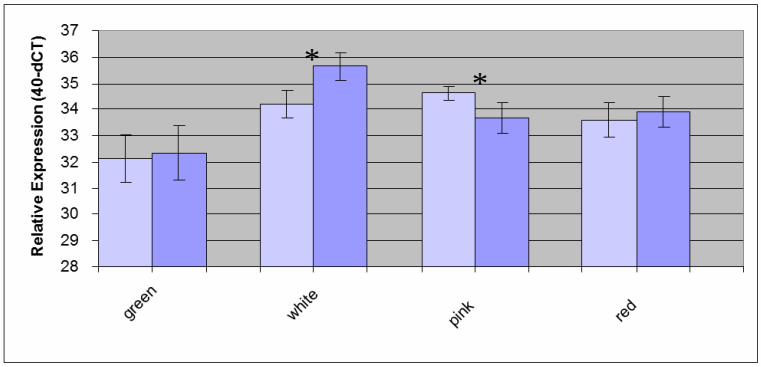

Fig. 8. The effect of the 24 h ethylene treatment on strawberries at different ripening stages (green, white, pink, red). Controls are light grey and the ethylene treated samples are dark grey. Controls represent untreated receptacles. Mean values ±SD of the three replicates are shown. *P-value < 0.05.

These data suggest that ethylene is involved in the regulation of *FaSPT* in white and pink receptacles, but apparently it does not induce its expression in green and mature red strawberries. *FaSPT* expression shows an increasing tendency from the green to white developmental stage while it decreases during ripening (pink and red maturation stages) in the untreated fruits, indicating *FaSPT* involvement in fruit development rather than in ripening.

Silencing of *FaSPT* in young fruits

In order to investigate the function of *FaSPT* in strawberry we applied *Agrobacterium* mediated RNAi to down-regulate its expression, which is an effective method recently developed to demonstrate plant gene functions. A hairpin vector construct carrying a sense-intron-antisense cassette was generated to trigger the silencing of the target gene (Fig. 9).

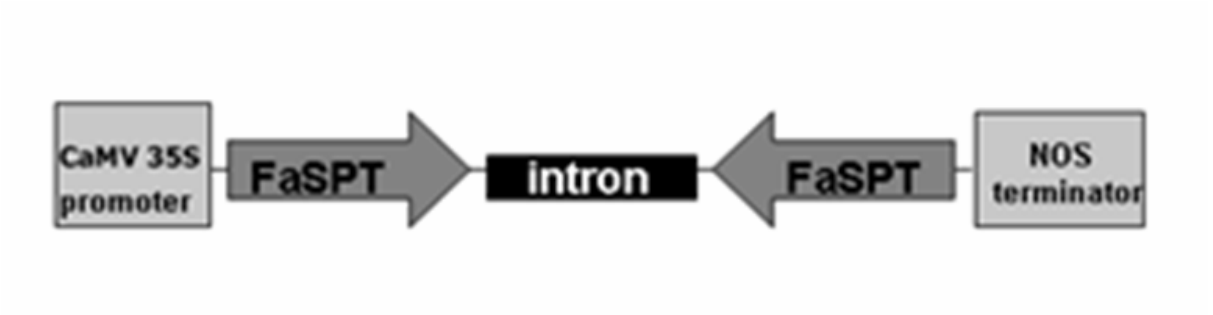

Fig. 9. Schematic figure of the pBIN-FaSPTi vector construct

For the hairpin construct pBIN-FaSPTi, a 399-bp fragment of *FaSPT* clone was amplified and subcloned in sense and antisense orientation into a pBluescript II KS vector (Stratagene, USA) which contained a 597 bp intron sequence (Koscienska et al. 2005). The sense fragment and the intron sequence were cut off together from this construct by an *Xho*I and *Xba*I digestion and ligated into the pBIN20 binary vector. Then the antisense fragment was also cut off by a *Xba*I digestion, and cloned into the pBIN20 construct, resulting a sense-intron-antisense expression cassette. Subsequently, the GV3101 *Agrobacterium tumefaciens* strain was transformed with the pBIN-FaSPTi vector and used for agroinfiltration.

Our results showed that approximately one-fourth of the fruits injected by hairpin vector showed differences in the phenotype compared to the fruits treated with the control construct. Namely, the fruits were reduced in their size and had altered shape compared to the control ones (Fig. 10).

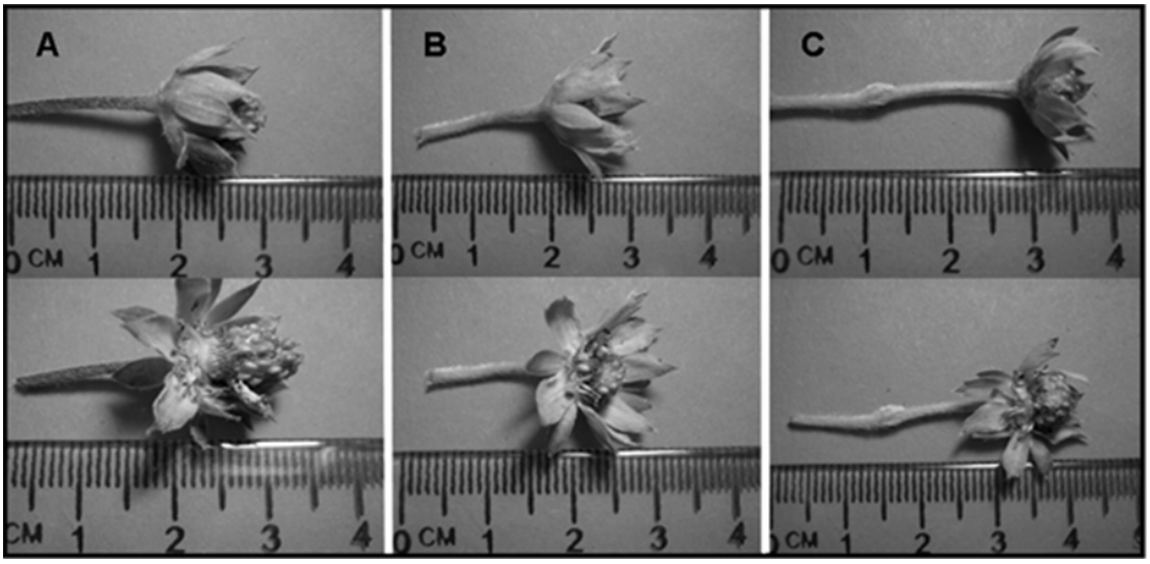

Fig. 10. Down-regulation of FaSPT in developing fruits by transient-gene silencing assay. A, phenotype of the control fruits infiltrated with *Agrobacterium* containing the empty pBIN20 vector, B and C, phenotype of the fruits, injected with the pBIN-FaSPTi construct. Photos were taken 14 days after injection.

While strawberry fruits develop from many separated carpels, there could be a parallelism between the strawberry fruit size reduction and the abnormal phenotype of the *Arabidopsis spt* mutants. Although we confirmed the down-regulation of *FaSPT* by qRT-PCR in fruits 1, 2, and 3 days after agroinfiltration (Fig. 11), we could not detect similar decrease in the amount of transcripts in the fruits (14 days after infiltration) which exhibited the altered phenotype (data not shown). We suppose that the silencing of *FaSPT* occurred at the early stages of fruit development contributing to reduced fruit size, but the gene function was probably restored by the 14th day.

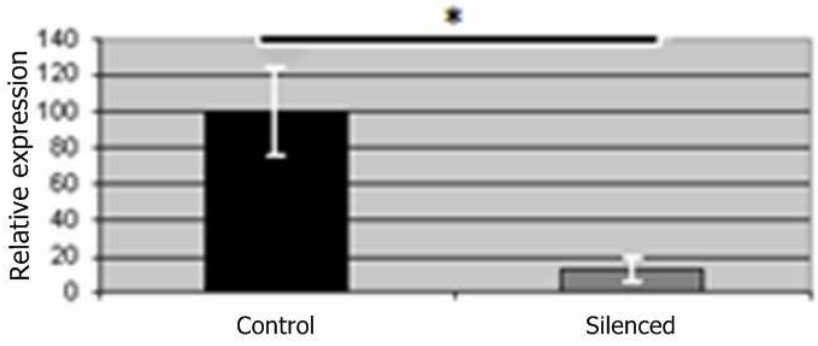

Fig. 11. qRT-PCR analysis of *FaSPT* expression in agroinfiltrated fruits. The cDNAs are derived from control and pBIN-FaSPTi infiltrated fruits 1, 2, 3 days after injection. The reaction was performed using *FaSPT* and *FaGAPDH* gene specific primers. Mean values ±SD of the three replicates are shown. *P-value < 0.05

In our case, since *FaSPT* encodes for an essential transcriptional factor, the early silencing effect was presumably responsible for the resulting defects in fruit development.
Our results may indicate that *FaSPT* is an essential transcriptional factor involved in strawberry fruit developmental processes.

New scientific results

We reported first the study of *SPATULA* encoding a bHLH transcriptional factor from the non-climacteric strawberry fruit. To our knowledge, this gene was characterized only in the model plant *Arabidopsis thaliana* up to now, thus there were very few previous results to take as a basis. The new results of our experiments can be summarized as follows:

We isolated and cloned the ORF region of the gene.
In the view of sequence information, serine rich, Fringe, and CDRN (Cysteine-rich D. radiodurans N terminus) domains were identified on the amino acid sequence of FaSPT.
We determined gene expression in vegetative and generative tissues, and we assessed that *FaSPT* expression was the highest in petals, while the lowest in 1 cm green receptacles.
The level of expression in strawberry leaves was also determined in a response to mechanical wounding, auxin and ethylene treatment. All of these treatments repressed the expression of *FaSPT*.
We assessed that *FaSPT* is repressed in developing young fruits by auxin treatment.
The expression level in strawberries at different ripening stages by ethylene treatment was determined. We found that *FaSPT* was not induced by ethylene in green and red fruits, while there was a very slight increment in the white ones, and a decrease in the pink ones.
Using a transient gene silencing assay, we found that the transcript amount of *FaSPT* decreased, and we detected defects in the shape of young fruits after the 14^{th} day of agroinjection.

Conclusions and suggestions

Although ethylene has no or little effect on the non climacteric ripening of strawberries, the presence of ethylene biosynthetic and signaling key genes are proven in this plant. Presumably, similar to climacterics, ethylene dependent and ethylene independent – regulated by transcriptional factors – molecular cascades are operating in non climacteric fruits, too.

We isolated and cloned a *SPATULA* gene encoding a bHLH transcriptional factor from cultivated strawberry. This gene showed a high homology to a *SPATULA* from *Arabidopsis*, which is well described. It was revealed that this gene plays an important role in carpel development.

Examining gene expression of fruits, we found that it shows an increasing tendency from the green developmental stage until the pink ones, and then it decrease in the red fruits compared to the pinks. This observation suggests that *FaSPT* expresses both in fruit development and ripening, hereby it has obviously a role in this physiological processes.

The gene showed different expression patterns by various treatments. It was repressed in leaves by mechanical wounding, auxin and ethylene treatment. From these results and on the basis of previous works we concluded that *FaSPT* may be involved in processes connected to senescence and stress responses.

Increasing tendency of *FaSPT* expression was detected throughout fruit development in fruits at various ripening stage, namely the prescence of higher amount of transcripts in 2 cm green, and white receptacles and achenes, respectively can suggest the role of *FaSPT* in fruit developmental processes.

Transcription was repressed by 96 h auxin treatment, consequently the exogenous hormone influences *FaSPT* expression.

By ethylene treatments of fruits it was shown that this hormone also regulates *FaSPT* expression in a slight degree in white and pink receptacles, while the gene was not induced in green and mature red strawberries.

Expression studies of different tissues revealed that *FaSPT* shows the highest expression in petals. This data is in correlation with a previous result, where it was determined that *SPATULA* is a key regulator of petal expansion in *Arabidopsis*.

Gene silencing of *FaSPT* based on RNAi was performed in developing fruits using a transient expression assay. Under this effect several fruits carrying defects in their development were found, which can indicate a role in fruit developmental processes. Thus, it is worth considering to perform a stabile plant transformation with this construct in order to obtain better understanding of this gene function. Moreover, complementation test of *Arabidopsis spatula* mutants by an overexpressing construct may elucidate its role in carpel development.

Analysis of *FaSPT* promoter is a task of a following dissertation, dissecting of promoter deletion lines are also being proceed.

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