Flowering Time Regulation by the miRNA156 in the Beet (Beta Vulgaris Ssp. Maritima)

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Research Article

Keywords: Beta vulgaris, miR156, SPL4, SPL9, Flowering pathway, Bolting

Posted Date: September 7th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-865214/v1

Abstract

Successful reproduction of the plants is assumed as a crucial challenge for meeting nutritional needs of growing human population. One of the significant changes in the plant's life cycle is its transition from vegetative to reproductive phase. Obviously, the flowering and bolting processes are influenced by many genetic and physiological factors. In this way, several main regulatory pathways and effective genes have been identified, so far. The age-dependent phenomenon as an effective pathway of reproductive transition phase is influenced by the miR172 and miR156 genes. In the present study, the miR156 sequence was identified in the sugar beet genome. First, the *miR156* gene was cloned into the over-expression construct from the *Beta vulgaris* genome and then, was transferred to the beet explants. After the confirmation of transgenic plants, the transcript level of *miR156* gene, and its target genes (*SPL4* and *SPL9*) were evaluated in transgenic plants related to the control plants. Also, transgenic plants were studied in terms of flower phenotypic development, and root growth characteristics. By evidence evaluation, the research revealed that over-expression of *miR156* played an effective role in reduction of *SPL4* and *SPL9* genes' expression that resulted in flowering suppression in the Sea beet. According to the findings, it had also a suppression effect on the root growth in transgenic beets.

Key Messages

The research confirmed the fact that the overexpression of miR156 had an effective role in the reduction of SPL4 and SPL9 gene expression, and eventually resulted in beet flowering suppression.

Introduction

The plant life cycle consists of several developmental stages in which, the transition from vegetative to reproductive phase is the most important stage, particularly in flowering plants. Successful reproduction of the plants is considered as the major challenge for addressing nutritional needs of the growing human population. This process is influenced by various genetic and physiological factors. Several effective regulatory pathways have been identified in *the Arabidopsis thaliana*, including vernalization, photoperiod, Gibberellin, autonomous, and age-related pathways (Sung and Amasino 2004; Kobayashi and Weigel 2007; Mutasa-Gottgens et al. 2009; Wang et al. 2009; Dohm et al. 2014). Age-related pathway is affected by the miR172 and miR156.

MicroRNAs (miRNAs) as small (16-26 nucleotides) and non-coding RNAs are located at the eukaryotes (Lau et al. 2001; Llave 2002; Reinhart et al. 2002; Ambros 2004; Simpson 2004). The miRNAs are involved in complex cellular processes such as RNA silencing, regulation of the gene expression, and cell death. The regulation of gene expression by the miRNAs is carried out at both transcriptional (changing the chromatin activity within the nucleus) and post-transcriptional levels through transferring to cytoplasm (the target sequence degradation or their translation suppression) (Gandikota et al. 2007; Phillips 2008; Voinnet 2009; Kim et al. 2012; Sun 2012; Wang et al. 2014). In plants, the miR156 controls many developmental and biological regulatory networks such as phase transition, nodulation, fertility,

and development of leaves, flowers and fruits. It is also responsible for regulating *the SPLs*, *WD40*, *TGA1*, *LG1* genes in the plants (Zhou and Luo 2013; Aung et al. 2015).

In Arabidopsis, there are numerous miR156s in the initial phase in which, most of them are responsible for slicing Squamosa Promoter Binding Protein-Like (SPL) transcription factors. The SPL proteins regulate downstream genes by conserving the DNA binding domains. These transcription factors facilitate the transition towards flowering stage by activation of *LEAFY (LFY)*, *FRUITFULL (FUL)*, *SOC1*, *AP1* and *AGL24* genes at the terminal bud (Wei et al. 2012).

Many studies have demonstrated that over-expression of the *miR156* has led to reducing the *SPL3*, *SPL4*, *SPL5* and *SPL9* genes expression, and consequently, delayed flowering in Arabidopsis (Wu and Poethig 2006; Gandikota et al. 2007). The SPL3 and SPL9 are upstream activators for the three genes of *AP1*, *LFY* and *FUL*. These genes are responsible for the transition to meristem identification stage of the plant. Thus, over-expression of them is essential for flowering (Wigge et al. 2005; Yamaguchi et al. 2009; Wang et al. 2009). In addition, two *miR156* targets (SPL9 and SPL10) can trigger the expression of *the miR172* in the leaves, which has similar expression pattern with the miR156 and is accumulated during the maturity (Aukerman and Sakai 2003; Gandikota et al. 2007; Wu et al. 2009). The miR156 has an effective role in reduction of *the SPL9* and *miR172* genes' expression that results in over-expression of *the APETALA2* and flowering suppression by the FT (Jiang et al. 2012; Cho et al. 2012) (Fig. 1).

The miR156 genes are highly expressed in the early stages of the plant development; however, they gradually decline along with increasing the age in Arabidopsis (Voinnet 2009). The miR156 has been successfully conserved among all different species of flowering plants (Voinnet 2009; Huijser and Schmid 2011). The information and function of *the miR156* gene have been studied in several plants. Nevertheless, despite the importance of this gene during the flowering process, no report has been recorded in the sugar beet yet.

The sugar beet (*Beta vulgaris* L.) is herbaceous, dicotyledonous, and biannual plant from Amaranthaceae and is commercially considered as one of the most important flowering crops. In the sugar beet life cycle, the vegetative phase begins with the emergence of a leafy plant and a large root (the main source of the sucrose) in the first year. In the second year, the reproductive phase would be continued by stem elongation (bolting) and flowering after a period of low temperature (Biancardi et al. 2005; Chia et al. 2008). According to previous studies, it is possible to grow the beets in spring (in temperate and cold regions) and autumn cultivations (in tropical and warm regions) over diverse areas of the world. These two cultivations are influenced by the natural resistance of different cultivars among the bolting (El-Mezawy et al. 2002). It could be said that the long and weak lignin stems produced during the first year upon the bolting stage are presumed as the main limiting factors for the cultivation of sugar beet in temperate regions. The plant's exposure to low temperature, and stem emergence, undesirably reduce the sugar percentage, root function and the purity of raw syrup (Mutasa-Gottgens et al. 2010; Pfeiffer et al. 2014; Hébrard et al. 2016). By a full control of the bolting and flowering stages, the sugar beet cultivation is possible in autumn over the northern latitudes (Reeves et al. 2007). This change from the spring to

autumn cultivation causes many positive consequence as well as larger leaf growth in spring, enhancement of the root growth and sugar content, less irrigation and water saving, limited weed growth, and lower sugar beet diseases (Jaggard et al. 2009).

Herby, due to the importance of the sugar beet vegetative growth, the study of flowering processes and involved genes is vital in sugar beet breeding. In the present research, first, the *miR156* gene from the sugar beet genome was isolated; second, the miR156 over-expression construct was transferred to the beet explant; third, the miR156 over-expression effect on target genes was evaluated; and finally, the flowering and root growth variety in transgenic plants were investigated.

Materials And Methods

2.1. In silico search for the miR156 homologue and its target genes in the *B. vulgaris*

The sequences of previously known *miR156* precursor gene of eight different plants (*Ananas comosus*, *Arabidopsis thaliana*, *Camellia sinensis*, *Ceratopteris thalictroides*, *Citrus sinensis*, *Musa AAB*, *Oryza sativa* and *Zea mays*) were compared at the National Center for Biotechnology Information (NCBI) GeneBank and miRBase database. When we started the first part of our research, the sugar beet KWS2320 genotype was the only genome which was annotated in NCBI, so, refseq-genomic sequences of this plant associated with the miR156 precursor sequences from each mentioned plant were identified through the BLASTN. If there were several similar refseq-genomic sequences with the miRNAs precursor sequences, each of them was individually considered. De novo assemblies of the sugar beet refseq-genomic sequences and precursor miRNA sequences from mentioned plants were carried out using the SeqMan Pro NGen (DNAStar, Inc., Madison, WI, USA). After the alignment, common sequences were considered as the miR156 and miR172 precursors in the sugar beet. Verification of the identified sequences was performed using the BLASTN in other plants' genomes. To identify the complete sequence of the genes in the sugar beet genome, various parts of the refseq-genomic sequences containing the gene sequences were thoroughly investigated via alignment. At last, the miRNA sequences were identified in precursor sequences, and were aligned using the MegAlign Pro NGen (DNAStar, Inc., Madison, WI, USA).

For evaluation of main targets of the miR156 in sugar beet, the sequence of identified SPLs (SPL4 and SPL9) genes was first extracted from the NCBI GeneBank. To ensure that these genes are the target sequences, and to identify the restrictions and complementary areas, two psRNATarget (https://plantgrn.noble.org/ psRNATarget) and the RNAhybrid (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid) software programs were used.

2.2. Plant materials and growth conditions

The seeds of *Beta vulgaris* ssp. *Maritima* (collected from the Shoush area of Iran), was obtained from the Sugar Beet Seed Institute (Located at Karaj, Iran), and was used in genetic transformation for all

experiments. The seeds and plants were cultured in the Murashige and Skoog medium, under the long-day conditions with 16 h light/day and 24/16°C day/night rhythm in the growth chamber.

2.3. The gene construct and transformation

A 127-bp stem-loop fragment of BvmiR156 precursor gene was amplified by the polymerase chain reaction (PCR) with the MIRF and MIRR primers (all primer sequences are given in Supplemental Table 1) introducing *the Nco*I and *Bam*HI restriction sites which were designed based on the miR156 precursor identified in *Beta vulgaris* genome. After the sequencing, the *P35S:BvmiR156* was generated by replacing *the Gus* gene with the *BvmiR156* gene between the *Nco*I and *Bam*HI restriction sites in the pGSA1285 vector. The pGSA1285-miR156 construct was sequenced and transformed to the *Agrobacterium tumefaciens* GV107 strain competent cells using the freezing /heating shock method (Sambrook and Russell 2001). After preparing the beet leaf explants (Mirzaei-Asl *et al*., 2010), they were infected for five min and incubated at 25 °C for 48 hours on coculture medium (MS medium and 1 mg/l 6-benzyl aminopurine), followed by incubation in selective medium (MS medium, 1 mg/l 6-benzyl aminopurine, 100-150 mg/l Kanamycin, 200 mg/l Cefotaxime). Thereafter, Kanamycin-resistant shoots were appeared in the selective medium.

2.4. The analysis of transgenic plants

The genomic DNA was extracted from the leaves of both regenerated and non-transgenic plants (control) by the CTAB method (Murray and Thompson, 1980). To confirm the transgenicity of the plants, the PGSAF and PGSAR primers (Supplemental Table 1) designed from the pGSA1285 vector promoter and terminator, were used in PCR. The reaction conditions were included the pre-heating of 94 °C for five min, 35 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s, and the final extension of 72 °C for seven min, for the PCR product sequencing.

2.5. Expression analysis of the miR156 and target genes

The total RNA was isolated from the young leaves of transgenic and non- transgenic plants using the RNX-Plus solution (Sinaclone Co.) according to instruction. Then, it was treated with the RNase-free DNase enzyme (Fermentase Co.) as described in the instruction. Amount of 500 ng RNA was used for the reverse transcription of the miR156, SPL4, and SPL9 by cDNA Synthesis Kit (Fermentase Co.). The RT-PCR reaction was performed with general primers of UB-quinitine (UbiF and UbiR) and actin-8 (ActinF and ActinR). The reaction conditions were included pre-heating of 94 °C for five min, 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, and the final extension of 72 °C for five min.

The quantitative RT-PCR of *the miR156* gene and the target genes (*SPL9* and *SPL4*) were done using the SYBR-Green PCR Mastermix (Amplicon) and an applied biosystem Roche LightCycler 480 instrument. *The Actin 8* gene was used as the internal control. The qPCR reactions were performed with three biological and technical replicates, separately. The PCR conditions were 95 °C for five min followed by 35 cycles at 94 °C for 30 s and 58 °C for 30 s. Transcript levels of the *miR156* and the target genes were

analyzed using relative quantification by the comparative Ct ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). The primers used for the RT-PCR and qRT-PCR are listed in the supplemental Table 1.

2.6. Flower and root development analysis

After confirming the transgenic plants, their exact flowering time was compared with non-transgenic plants to determine the effect of the miR156 over-expression on flowering time. Also, the root development under the miR156 over-expression effect was evaluated in transgenic plants in comparison with non- transgenic plants.

2.7. Statistical Analysis

Triplicate samples were collected for each transgenic line. The statistical analysis of *the miR156*, *SPL4* and *SPL9* genes expression were conducted using one way-ANOVA test after evaluating "the normality residual exam" by the SAS software (SAS Institute, Cary, NC). Also, correlation analysis test was performed using Minitab software. The P values < 0.05 were considered significant.

Results

3.1. Identification of the BvmiR156 and its target genes in *B. vulgaris* genome

Overall, 39 *miR156* precursor sequences (including 12 members of the miR156 family) from eight plant species were isolated from the NCBI GeneBank and miRBase database, and then the mature miRNAs were aligned from the precursor sequences. The number of bases in mature *miR156* varied from 20 to 25 nucleotides in different plants. The most diversity of the *miR156* sequence was detected in *O. sativa* and *C. thalictroides* plants; while the differences among the *miR156* sequence of *A. comosus*, *A. thaliana*, *Camellia sinensis, Citrus sinensis*, *Musa AAB* and *Z. mays* were not considerable at the range of single nucleotide.

After the alignment of the miR156 precursor sequences for each mentioned plant with *Beta vulgaris* Refseq-genomic sequences, the homologous sequences were identified. The highest similarity was observed between the *Beta vulgaris* sequence with accession number of NC_025813 on chromosome two and *A. comosus*, *Camellia sinensis, Citrus sinensis* and *Z. mays* miR156. In the meantime, 127 nucleotides were quite like the *miR156* precursor sequence of *the Camellia sinensis,* and 85-89 nucleotides were completely similar to *the miR156* sequence in other three plants. Also, hairpin structures could be predicted in the identified sequences (Fig. 2 b). The identification of the mature *miR156* sequence in *the miR156* precursor sequences of the sugar beet was performed. The comparison of the identified *miR156* precursor sequences with *A. comosus*, *Camellia sinensis, Citrus sinensis* and *Z. mays* was performed and confirmed that all of them had relatively the same sequences with 21 nucleotides (Fig. 2 a).

3.2. Prediction and validation of the miRNA target sequences

Searching for the *SPL* gene homologues in *Beta vulgaris* genome demonstrated nine *SPL* genes accessions. However, it was found that only five *SPL* genes (XM_010677198, XM_010674387, XM_010669328, XM_010681697, XM_010684453) contained the complementary sequence with *the miR156* gene sequence. Further studies via the psRNATarget and RNAhybrid softwares revealed that the miR156 has 19 complementary nucleotides with 928-947 region of the *SPL4* gene sequence, as well as 1286-1267 region of the *SPL9* gene sequence. It may cause a cleavage in these areas due to the presence of mismatch (Fig. 3). *The SPL4* and *SPL9* gene sequences were then amplified from *B. vulgaris* genome using designed primers.

3.3. Generation of transgenic plants with the BvmiR156b over-expression construct

The presence of the BvmiR156 precursor fragment with 127 bp downstream of CaMV35S promoter was confirmed in over-expression construct through PCR, enzymatic digestion, and sequencing. The selectable marker *Neomycin Phosphotransferase* II gene (*NPT* II) was under the control of the pMAS 2' promoter. The resistant shoot was regenerated after kanamycin selection from the leaf explants infected with the agrobacterium carrying the pGSA-BvmiR156 vector. The regenerated shoots were separated. After transferring to larger plates, the transgenicity of them was confirmed via PCR screening using promoter and terminator-specific primers. Also, the sequencing results of amplified fragments in PCR indicated that the transgene was steadily integrated into the plant genome (Fig. Supplemental 1). In sum, the transgenicity of 150 regenerated shoots could be approved.

3.4. The miR156 expression pattern and its targets

The green leaves from regenerated transgenic plants were used for the quantitative RT-PCR analyses. The level of pre-BvmiR156 transcript was higher in transgenic than the non-transgenic plants. The level of pre-BvmiR156 transcript increased from 12.47% to 38.45% in the transgenic plants compared to the controls (Fig. 4).

For determining the miR156 over-expression effects on its targets' (*SPL4* and *SPL9*) transcript in the Sea beet, the qRT-PCR was also used. The level of the BvSPL9 transcript decreased from 0.80% to 0.23% in transgenic plants compared to the control plants. The results of this experiment also uncovered that the BvSPL4 transcript level decreases from 0.16% to 0.08% in transgenic beets compared to the controls (Fig. 5).

3.5. Morphological characterization of transgenic plants

Monitoring transgenic and non-transgenic plants during 13 months after the inoculation revealed noticeable differences in terms of flowering and rooting abilities. In more detail, it was observed that the control plants were transferred to the reproductive stage through forming flowering stems after seven months; while, transgenic plants were passing the vegetative phase. Nine months later, the transgenic plants were still without any flowering stem; where, non-transgenic plants had several flowering stems and were at the reproductive growth stage. After 13 months of inoculation, none of the transgenic plants

with over-expression of the miR156 were transferred to the flowering stage, and even no flowering stem was shaped in each of them. However, during this period, the flowering and reproductive phase were ended in a great number of non-transgenic plants (Fig. 6).

The evaluation of the *miR156* gene's over-expression effect on the root growth in the Sea beet showed a significant difference between the transgenic and non-transgenic plants. Twelve months after inoculation, all control plants were able to produce root; whereas, no evidences of the root production were observed in the transgenic plants. The commencement of the root production at the end of the month 13^{th} revealed that the root production in plants with *the miR156* gene over-expression has a remarkable delay compared to the control plants (Fig. 7).

Discussion

Although the importance of the miRNAs in the flowering pathway has been studied in many plants, there is no report concerning the effect of these factors on flowering time' regulation in beet plants. According to the results of this study, the impact of *the miR156* gene on the age-related pathway of the sugar beet flowering and bolting was confirmed in the Sea beet plant. Previous studies in eukaryotes suggest that different types of the miRNA are involved in complex processes of gene expression. The miRNAs are quite widespread and properly protected in the plant kingdom, and this probably indicates of the existence of a common ancestor in the early evolution period (Zhang et al. 2006). Moreover, computational methods suggest that one percent of proteins-coding and non-coding genes are consisted of miRNAs in Arabidopsis and Drosophila (Bartel and Chen, 2004;Zhang et al. 2005). The same similarities are also visible in the sugar beet genome which may be approximately a number of 274 miRNA-coding genes among 27,421 genes in the sugar beet genome. These genes are contributed to various mechanisms; nevertheless, none of them has been identified in sugar beet, so far. In our study, in the miR156 case, only the NC-025813 sequence was obtained through the comparison of sugar beet Refseq-genomic with the miRNA sequences in other plants and no other similar sequences were detected in the genome of sugar beet. All recognized miR156 sequences were 21 bp. The sequence identified as the miR156 form of stem-loop structures was very important in executing its functions.

In several research, it has been confirmed that *the miR156* gene does not have any direct effect on flowering procedure and exerts its effect indirectly through other genes such as *the SPL* genes. It should be noted that the action of miR156-SPL network in two stages of flowering stimulation and flowering meristem identification is important (Gao et al., 2016). In Arabidopsis, the number of 11 out of 17 SPL genes have been recognized as the target sequences for the miR156 in which these genes are able to create a junction with the mismatch nucleotides and disrupt the transition process towards the generative phase (Wang et al. 2009). In sugar beet genome, we could confirm five SPL target genes with complementary area for the miR156. Also, the effect of the *miR156* gene expression on the *SPL4* and *SPL9* genes was demonstrated. These genes as the downstream genes of the miR156 are effective on flowering pathway of many plants (Schwarz et al. 2008; Wu and Liu 2009; Yamaguchi et al. 2009).

Over-expression rate of the miR156 in different transgenic plants was varied compared to the control plants. In a study implemented by Aung (2014), three groups of transgenic alfalfa plants were identified in terms of over-expression rate of the miR156. Only in the group with high miR156 expression, the three *SPL* (*SPL6*, *SPL12* and *SPL13*) genes' expression were impressed (Aung and Abdelali Hannoufa 2014). Contrary to that research, and based on the correlation results, it was perceived that over-expression of *the miR156* gene reduced the expression of *the SPL4* and *SPL9* genes in all transgenic plants compared to the control plant.

Considering the *SPL4* gene expression, decreasing the transcript level in transgenic plants was completely in line with the *miR156* gene' over-expression rate, while it was totally different in the *SPL9* gene. It means that the plants with the highest *miR156* gene expression represented the lowest expression of *the SPL4* gene comparing other transgenic plants. Previous studies have shown that the SPL4 gene expression is not affected by environmental conditions, and its expression is constant in both long and short-day conditions (Jung et al. 2011)

These changes probably indicate that the expression of *the SPL9* gene at the long-day conditions is influenced by other factors in addition to the miR156. Various studies have claimed that flowering regulators such as FLC, CO, FT or SOC1 do not have any significant effect on *the miR156* expression in Arabidopsis seedlings. Also, according to previous studies, the FLC expression as a flowering inhibitor, does not affect *the SPL9* gene expression (Michaels and Amasino 2001). It has been shown by researchers that two factors of gibberellin and photoperiod pathway genes are effective on the SPL genes expression (Zhou et al. 2016) . In our experiments, the growth medium of transgenic plants lacked the exogenous gibberellin, and they were cultured under the long-day conditions. Therefore, the SPL9 expression was influenced by the photoperiodic pathway factors in addition to the miR156. Our results are also consistent with the findings of Wang et al (2009). They transformed the transgenic Arabidopsis from the short-day to the long-day conditions and did not find any change in the expression pattern of the miR156. Nonetheless, the *SPL9* gene expression increased in different parts of the lateral and leafy buds (Wang et al. 2009) (Fig. 8).

The flowering time is usually determined by two factors of calendar time and counting leaves' number before the flowering. In our study, after thirteen months of shoot regeneration, none of the transgenic plants with over-expression of *the miR156* gene were able to enter the reproductive phase. Whereas one or several flowering stems was/were created in the control plants and their flowering function was ended during this period.

The effect of the miR156 over-expression on rooting phenotype varies in different plants. Recent studies have uncovered that the miR156 plays an important role in nitrogen fixation and root development in legumes. For example, over-expression of *the miR156* produced more (but smaller) roots in the transgenic rice (Xie et al. 2012). The branching and root length significantly decreased in transgenic *Lotus japonicas* with over-expression of *the miR156* (Wang et al. 2015); whereas, the transgenic alfalfa with *the miR156* over-expression had longer roots and higher number of the nodes (Aung and Abdelali Hannoufa 2014). In

our experiments, over-expression of the miR156 caused one-month delay in the root development of transgenic plants compare to the control plants. It has been shown that the miRNA172 is activated by *the SPL* genes and is targeted by the miR156 and finally inhibits the AP2 (Yant et al. 2010). The studies accomplished by Wu et al. (2009) showed that over-expression of *the SPL3*, *SPL4*, and *SPL5* genes had no effect on the transcriptome level of *the miR172* in Arabidopsis; indicating the fact that these genes do not play any role in the miR156 impact on the miR172. It also demonstrated that the SPL9 is major activator of the miR172 and accelerator of the link between the *miR156* and *miR172* genes (Wu et al., 2009). Thus, these results suggest that probably over-expression of *the miR156* represses the SPL9 and consequently *the miR172* gene expression. Therefore, upregulation of *the AP2* gene leads to the root growth delay in transgenic plants (Fig. 8). It would be also very informative and challenging to determine other factors involved in this pathway.

Declarations

Funding: The research was funded by Bu-Ali Sina University, Hamedan, Iran.

Conflicts of interest: The financial supports of this research were provided by Bu-Ali Sina University budgets.

Availability of data and material: The datasets generated during and/or analyzed through the current study will be available from the corresponding author upon reasonable request.

Authors' contributions: a and b have designed the research. a has conducted the experiment. All authors had contributed to data analysis. a and b have written the manuscript. All authors have read and approved the manuscript.

Consent to participate: All authors are consented to participate in the research.

Consent for publication: All authors are consented for publication of the research.

Acknowledgments

The authors would like to appreciate all supports offered by Bu-Ali Sina University, Hamedan, Iran.

References

1. Ambros V (2004) The functions of animal microRNAs. Nature 431:350–355
2. Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its *APETALA2-like* target genes. PLANT CELL ONLINE 15:2730–2741. https://doi.org/10.1105/tpc.016238
3. Aung B, Abdelali Hannoufa S (2014) Effects of microRNA156 on Flowering Time and Plant Architecture in *Medicago sativa.*

4. Aung B, Gruber MY, Hannoufa A (2015) The MicroRNA156 system: A tool in plant biotechnology. Biocatal Agric Biotechnol 4:432–442. https://doi.org/10.1016/j.bcab.2015.08.002
5. Bartel DP, Chen CZ (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet 5:396–400. doi 10.1038/nrg1328
6. Biancardi E, Campbell LG, Skaracis GN, De Biaggi M (2005) Genetics and Breeding of Sugar Beet. Science Publishers
7. Chia TYP, Müller A, Jung C, Mutasa-Göttgens ES (2008) Sugar beet contains a large *CONSTANS-LIKE* gene family including a *CO* homologue that is independent of the early-bolting (*B*) gene locus. J Exp Bot 59:2735–2748. https://doi.org/10.1093/jxb/ern129
8. Cho SH, Coruh C, Axtell MJ (2012) miR156 and miR390 Regulate tasiRNA Accumulation and Developmental Timing in *Physcomitrella patens*. Plant Cell 24:4837–4849. https://doi.org/10.1105/tpc.112.103176
9. Dohm JC, Minoche AE, Holtgrawe D et al (2014) The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). Nature 505:546–549
10. El-Mezawy A, Dreyer F, Jacobs G, Jung C (2002) High-resolution mapping of the bolting gene *B* of sugar beet. Theor Appl Genet 105:100–105. https://doi.org/10.1007/s00122-001-0859-z
11. Gandikota M, Birkenbihl RP, H??hmann S et al (2007) The miRNA156/157 recognition element in the 3' UTR of the Arabidopsis SBP box gene *SPL3* prevents early flowering by translational inhibition in seedlings. Plant J 49:683–693. https://doi.org/10.1111/j.1365-313X.2006.02983.x
12. Hébrard C, Peterson DG, Willems G et al (2016) Epigenomics and bolting tolerance in sugar beet genotypes. J Exp Bot 67:207–225. https://doi.org/10.1093/jxb/erv449
13. Huijser P, Schmid M (2011) The control of developmental phase transitions in plants. Development 138:4117–4129. https://doi.org/10.1242/dev.063511
14. Jaggard KW, Qi A, Ober ES (2009) Capture and use of solar radiation, water, and nitrogen by sugar beet (*Beta vulgaris* L.). J Exp Bot 60:1919–1925. https://doi.org/10.1093/jxb/erp110
15. Jiang F, Guo M, Yang F et al (2012) Mutations in an *AP2* transcription factor-like gene affect internode length and leaf shape in maize. PLoS One 7:e37040. https://doi.org/10.1371/journal.pone.0037040
16. Jung JH, Seo PJ, Kang SK, Park CM (2011) miR172 signals are incorporated into the miR156 signaling pathway at the *SPL3/4/5* genes in Arabidopsis developmental transitions. Plant Mol Biol 76:35–45. https://doi.org/10.1007/s11103-011-9759-z
17. Kim JJ, Lee JH, Kim W et al (2012) The microRNA156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 Module Regulates Ambient Temperature-Responsive Flowering via FLOWERING LOCUS T in Arabidopsis. PLANT Physiol 159:461–478. https://doi.org/10.1104/pp.111.192369
18. Kobayashi Y, Weigel D (2007) Move on up, it's time for change - Mobile signals controlling photoperiod-dependent flowering. Genes Dev 21:2371–2384. https://doi.org/10.1101/gad.1589007

19. Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294:858–862. https://doi.org/10.1126/science.1065062
20. Llave C (2002) Endogenous and Silencing-Associated Small RNAs in Plants. Plant Cell Online 14:1605–1619. https://doi.org/10.1105/tpc.003210
21. Michaels SD, Amasino RM (2001) Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell 13:935–941. https://doi.org/10.1105/tpc.13.4.935
22. Mirzaie-Asl A, Moieni A, Salmanian AH et al (2010) Effects of hormonal pretreatments and genotype on invitro induction of direct adventitious shoots on Sugar Beet (*Beta Vulgaris* L.) leaves. Iranian Journal of Biology 22:719–729. http://sid.ir/en/journal/ViewPaper.aspx?id=257149 (in persian)
23. Mutasa-Gottgens E, Qi A, Mathews A et al (2009) Modification of gibberellin signalling (metabolism & signal transduction) in sugar beet: Analysis of potential targets for crop improvement. Transgenic Res 18:301–308. https://doi.org/10.1007/s11248-008-9211-6
24. Mutasa-Gottgens E, Qi A, Zhang W et al (2010) Bolting and flowering control in sugar beet: relationships and effects of gibberellin, the bolting gene *B* and vernalization. AoB Plants 2010:1–13. https://doi.org/10.1093/aobpla/plq012
25. Pfeiffer N, Tränkner C, Lemnian I et al (2014) Genetic analysis of bolting after winter in sugar beet (*Beta vulgaris* L.). Theor Appl Genet 127:2479–2489. https://doi.org/10.1007/s00122-014-2392-x
26. Phillips T (2008) No TitleSmall non-coding RNA and gene expression. Nat Educ 1:115
27. Reeves PA, He Y, Schmitz RJ et al (2007) Evolutionary conservation of the FLOWERING LOCUS C-mediated vernalization response: Evidence from the sugar beet (*Beta vulgaris*). Genetics 176:295–307. https://doi.org/10.1534/genetics.106.069336
28. Reinhart BJ, Weinstein EG, Rhoades MW et al (2002) MicroRNAs in plants. Genes Dev 16:1616–1626. https://doi.org/10.1101/gad.1004402
29. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press
30. Schwarz S, Grande AV, Bujdoso N et al (2008) The microRNA regulated SBP-box genes *SPL9* and *SPL15* control shoot maturation in Arabidopsis. Plant Mol Biol 67:183–195. https://doi.org/10.1007/s11103-008-9310-z
31. Simpson GG (2004) The autonomous pathway: Epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. Curr Opin Plant Biol 7:570–574. https://doi.org/10.1016/j.pbi.2004.07.002
32. Sun G (2012) MicroRNAs and their diverse functions in plants. Plant Mol Biol 80:17–36. https://doi.org/10.1007/s11103-011-9817-6
33. Sung S, Amasino RM (2004) Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature 427:159–164. https://doi.org/10.1038/nature02195

34. Voinnet O (2009) Origin, Biogenesis, and Activity of Plant MicroRNAs. Cell 136:669–687. https://doi.org/10.1016/j.cell.2009.01.046
35. Wang J, Huang SK, Zhao M et al (2014) Identification of a circulating microRNA signature for colorectal cancer detection. PLoS One 9:e87451. https://doi.org/10.1371/journal.pone.0087451
36. Wang JW, Czech B, Weigel D (2009) miR156-Regulated SPL Transcription Factors Define an Endogenous Flowering Pathway in *Arabidopsis thaliana*. Cell 138:738–749. https://doi.org/10.1016/j.cell.2009.06.014
37. Wang Y, Wang Z, Amyot L et al (2015) Ectopic expression of miR156 represses nodulation and causes morphological and developmental changes in *Lotus japonicus*. Mol Genet Genomics 290:471–484. https://doi.org/10.1007/s00438-014-0931-4
38. Wei S, Gruber MY, Yu B et al (2012) Arabidopsis mutant *sk156* reveals complex regulation of SPL15 in a miR156-controlled gene network. BMC Plant Biol 12:169. https://doi.org/10.1186/1471-2229-12-169
39. Wigge PA, Kim MC, Jaeger KE et al (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. Science 309:1056–1059. https://doi.org/10.1126/science.1114358
40. Wu G, Park MY, Conway SR et al (2009) The Sequential Action of miR156 and miR172 Regulates Developmental Timing in Arabidopsis. Cell 138:750–759. https://doi.org/10.1016/j.cell.2009.06.031
41. Wu G, Poethig R (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. Development 133:3539–3547. https://doi.org/10.1242/dev.02521
42. Wu X, Liu T (2009) Spectral decomposition of seismic data with reassigned smoothed pseudo Wigner – Ville distribution. J Appl Geophys 68:386–393. https://doi.org/10.1016/j.jappgeo.2009.03.004
43. Xie K, Shen J, Hou X et al (2012) Gradual Increase of miR156 Regulates Temporal Expression Changes of Numerous Genes during Leaf Development in Rice. Plant Physiol 158:1382–1394. https://doi.org/10.1104/pp.111.190488
44. Yamaguchi A, Wu M, Yang L et al (2009) Article The MicroRNA-Regulated SBP-Box Transcription Factor SPL3 Is a Direct Upstream Activator. Dev Cell 17:268–278. https://doi.org/10.1016/j.devcel.2009.06.007
45. Yan Z, Hossain MS, Wang J et al (2013) miR172 Regulates Soybean Nodulation. Mol Plant-Microbe Interact 26:1371–1377. https://doi.org/10.1094/MPMI-04-13-0111-R
46. Yant L, Mathieu J, Dinh TT et al (2010) Orchestration of the Floral Transition and Floral Development in Arabidopsis by the Bifunctional Transcription Factor APETALA2. Plant Cell Online 22:2156–2170. https://doi.org/10.1105/tpc.110.075606
47. Zhang BH, Pan XP, Wang QL et al (2005) Identification and characterization of new plant microRNAs using EST analysis. Cell Res 15:336–360
48. Zhang B, Pan X, Cobb GP, Anderson TA (2006) Plant microRNA: A small regulatory molecule with big impact. Dev Biol 289:3–16. https://doi.org/10.1016/j.ydbio.2005.10.036

49. Zhou B, Fan P, Li Y et al (2016) Exploring miRNAs involved in blue/UV-A light response in *Brassica rapa* reveals special regulatory mode during seedling development. BMC Plant Biol 16:111. https://doi.org/10.1186/s12870-016-0799-z
50. Zhou M, Luo H (2013) MicroRNA-mediated gene regulation: Potential applications for plant genetic engineering. Plant Mol Biol 83:59–75. https://doi.org/10.1007/s11103-013-0089-1

Figures

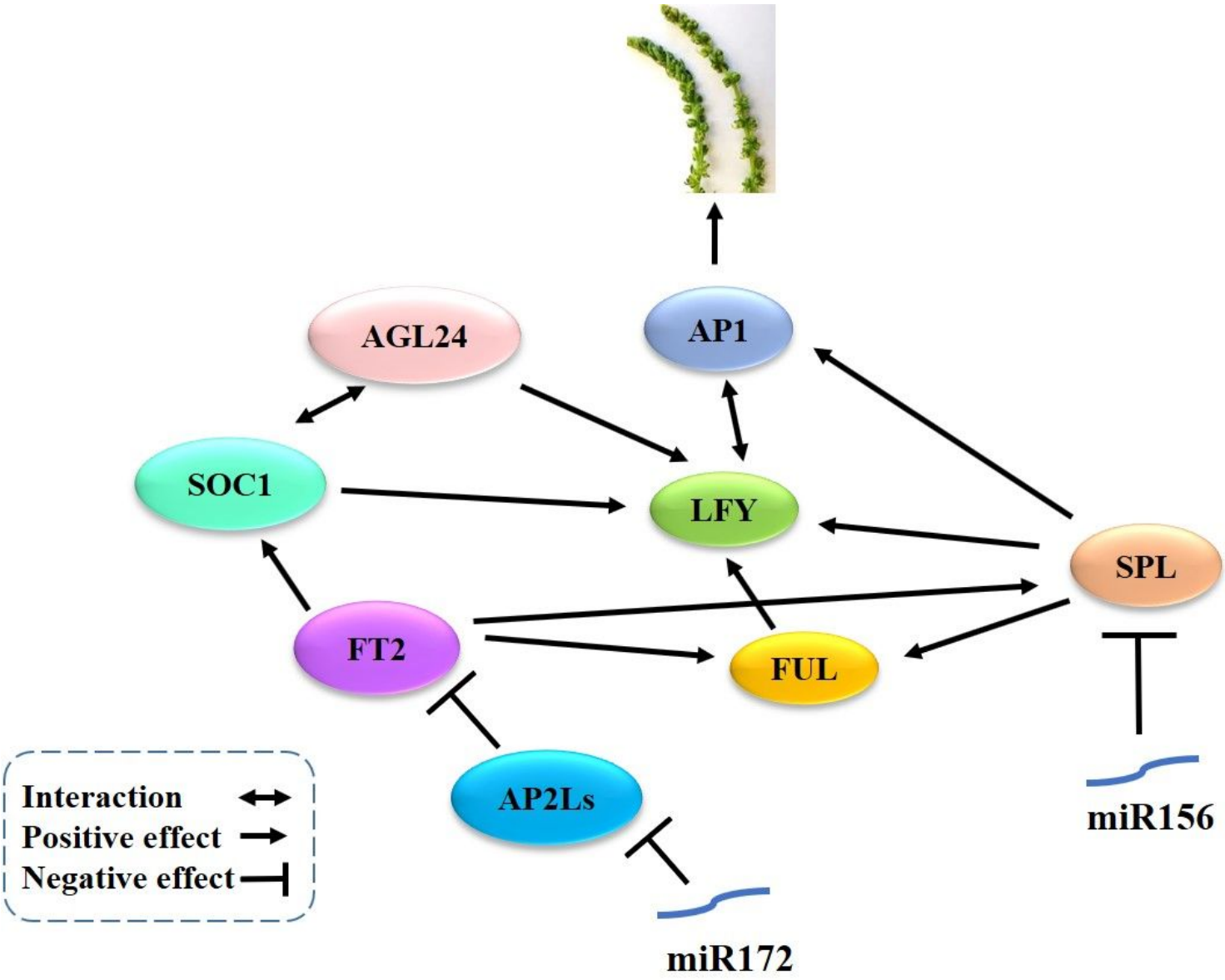

Figure 1

A part of flowering pathway and some genes affected by the miR156 and miR172 in Arabidopsis. In the vegetative stage, the miR156 inhibits the SPL transcription factors. The SPLs activate FUL, LFY and AP1 genes and ultimately stimulate the initiation of the reproductive phase. The miR172 inhibits the function of AP2Ls genes. The AP2Ls inhibit the FT, which is a flowering stimulus

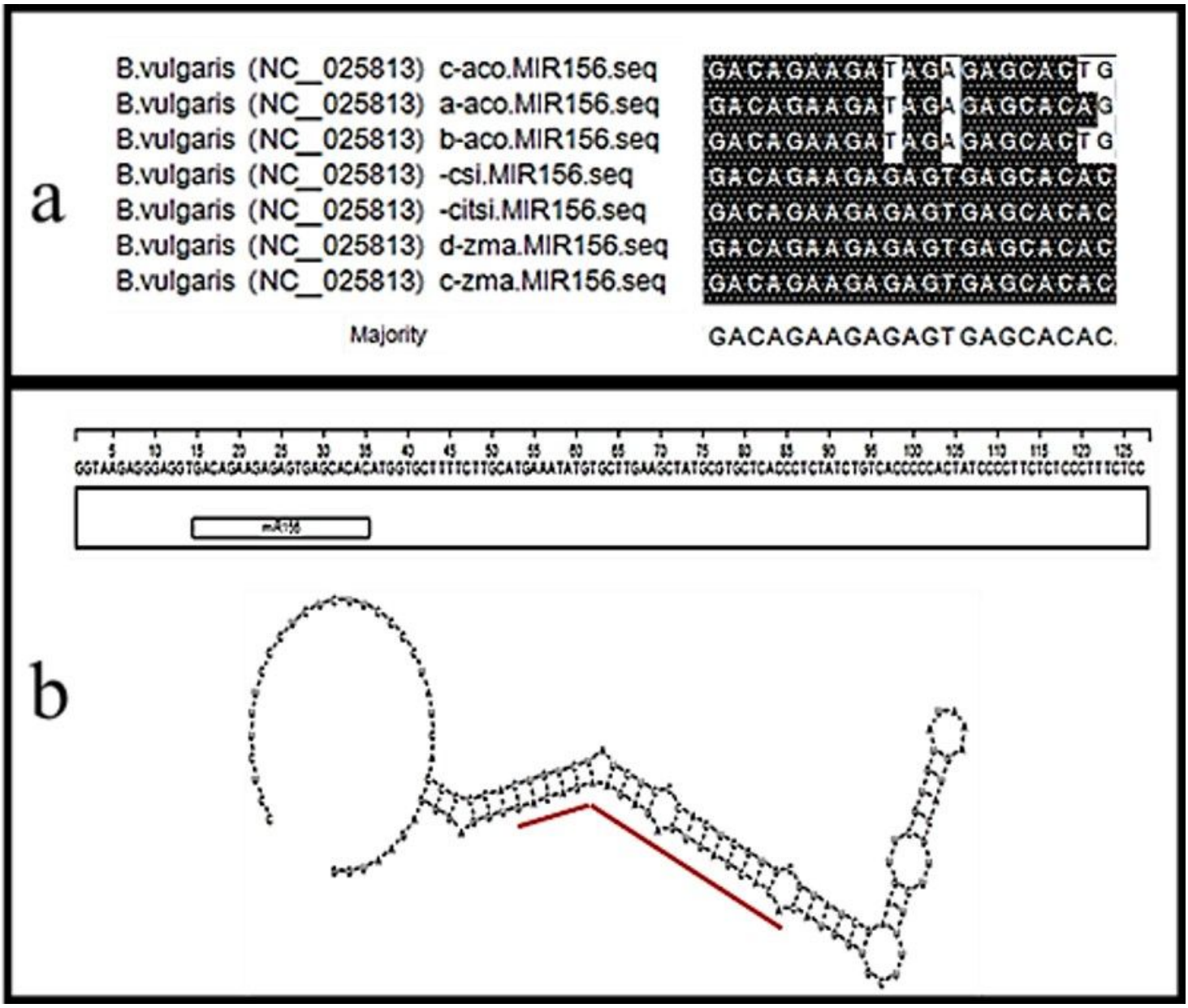

Figure 2

The miR156 sequence, a. Variety of the identified miR156 sequences in the sugar beet like the miR156 sequences of A. comosus (aco), C. sinensis (csi), C. sinensis (citsi) and Z. mays (zma). b. The presence of the miR156 gene in the precursor miR156 sequence and dimensional structure of the precursor miR156

a

miRNA Acc.	Target Acc.	Expect	UPE	Alignment	Target Description	Inhibition	Multiplicity
BvmiR156	BvSPL4	1.0	N/A	miRNA 20 CACGAGUGAGAGAAGACAGU 1 ::::: :::::::::::::: Target 928 UUGCUCCCUCUCUUCUGUCA 947		Cleavage	1

miRNA Acc.	Target Acc.	Expect	UPE	Alignment	Target Description	Inhibition	Multiplicity
BvmiR156	BvSPL9	1.0	N/A	miRNA 20 CACGAGUGAGAGAAGACAGU 1 :::::: :::::::::::::: Target 1267 GUGCUCUCUCUCUUCUGUCA 1286		Cleavage	1

b

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Version: RNAhybrid 2.2
Command line:/vol/bioapps/bin/RNAhybrid
searching
dataset: 1
mde of BvmiR156: -41.100002
Individual hits
----------------------------------------

dataset: 1
target: BVSPL4
length: 1234
miRNA : BvmiR156
length: 19

mfe: -35.7 kcal/mol
p-value: undefined

position  928
target 5' U     C              U 3'
           UGCUC CUCUCUUCUGUCA
           ACGAG GAGAGAAGACAGU
miRNA  3'       U               5'

----------------------------------------
```

```
Version: RNAhybrid 2.2
Command line:/vol/bioapps/bin/RNAhybrid.
searching
dataset: 1
mde of BvmiR156: -41.100002
Individual hits
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dataset: 1
target: BVSPL9
length: 1887
miRNA : BvmiR156
length: 19

mfe: -35.9 kcal/mol
p-value: undefined

position  1267
target 5' G     U              A 3'
           UGCUC CUCUCUUCUGUCA
           ACGAG GAGAGAAGACAGU
miRNA  3'       U               5'

----------------------------------------
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Figure 3

The complementary region confirmation between the miR156 gene sequence and the SPL4 and SPL9 genes sequence in the sugar beet

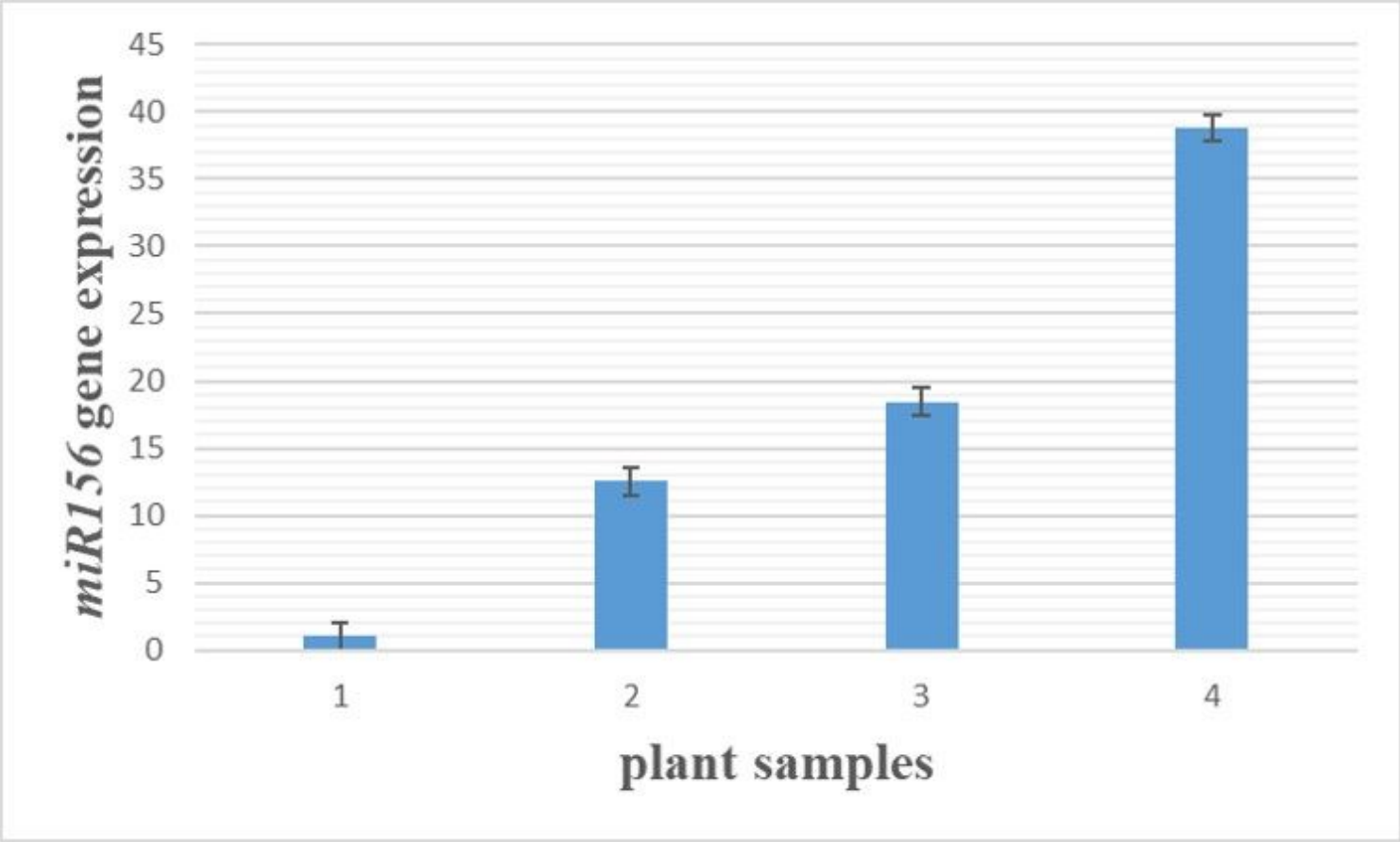

Figure 4

Relative expression changes of the miR156 gene in transgenic plants compared to the control plants. 1: non-transgenic plants, 2-4: transgenic plants Error bar represents standard error. Standard errors indicate the mean proximity of each sample to the mean population

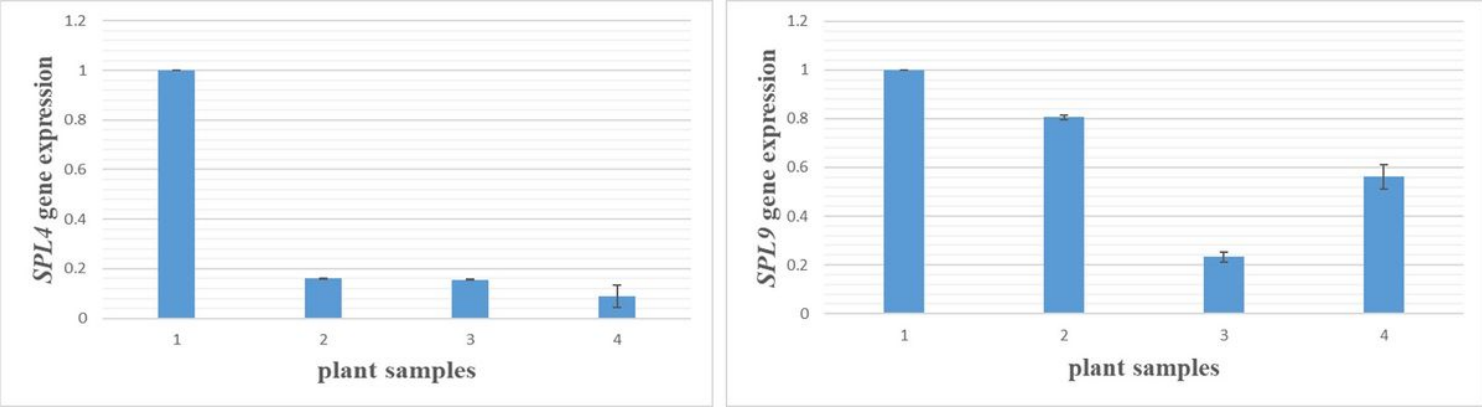

Figure 5

Relative expression changes in the SPL4 and SPL9 genes' expression in transgenic plants compared to non-transgenic plants. 1: non-transgenic plants, 2-4: transgenic plants Error bar represents standard error. Standard errors indicate the mean proximity of each sample to the mean population

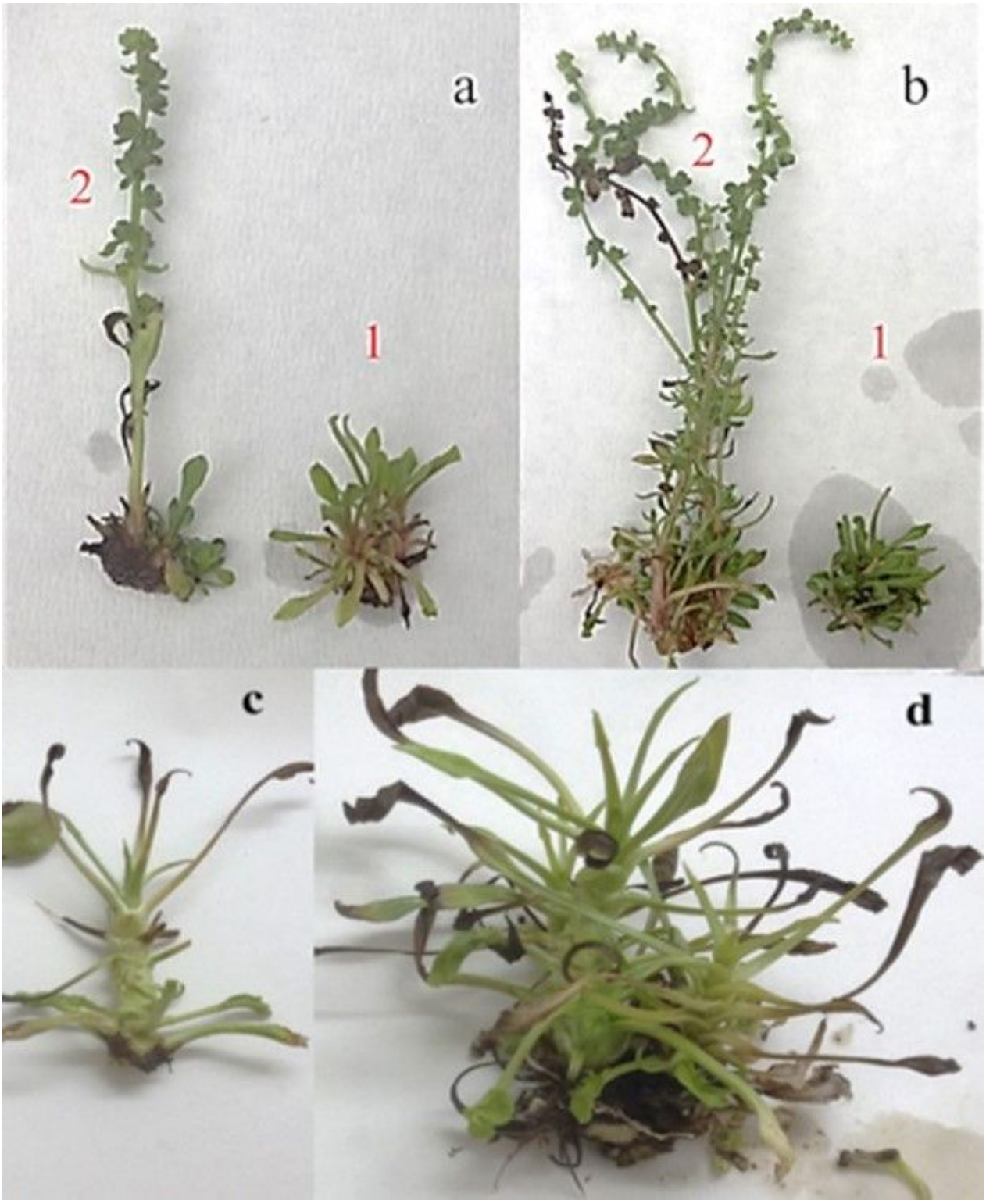

Figure 6

Flowering differences measured in transgenic and non-transgenic plants. a: After seven months, 1: The transgenic plant is in the vegetative growth stage, 2: Non-transgenic plant has entered the reproductive stage; b: After nine months, 1: Transgenic plant is still in the vegetative phase, 2: Non-transgenic plant continues the reproductive phase by reproducing more flowering stems; c and d: After 13 months,

transgenic plants are still in vegetative growth phase with production of extensive leaves and no traces of flowering stem formation are observed

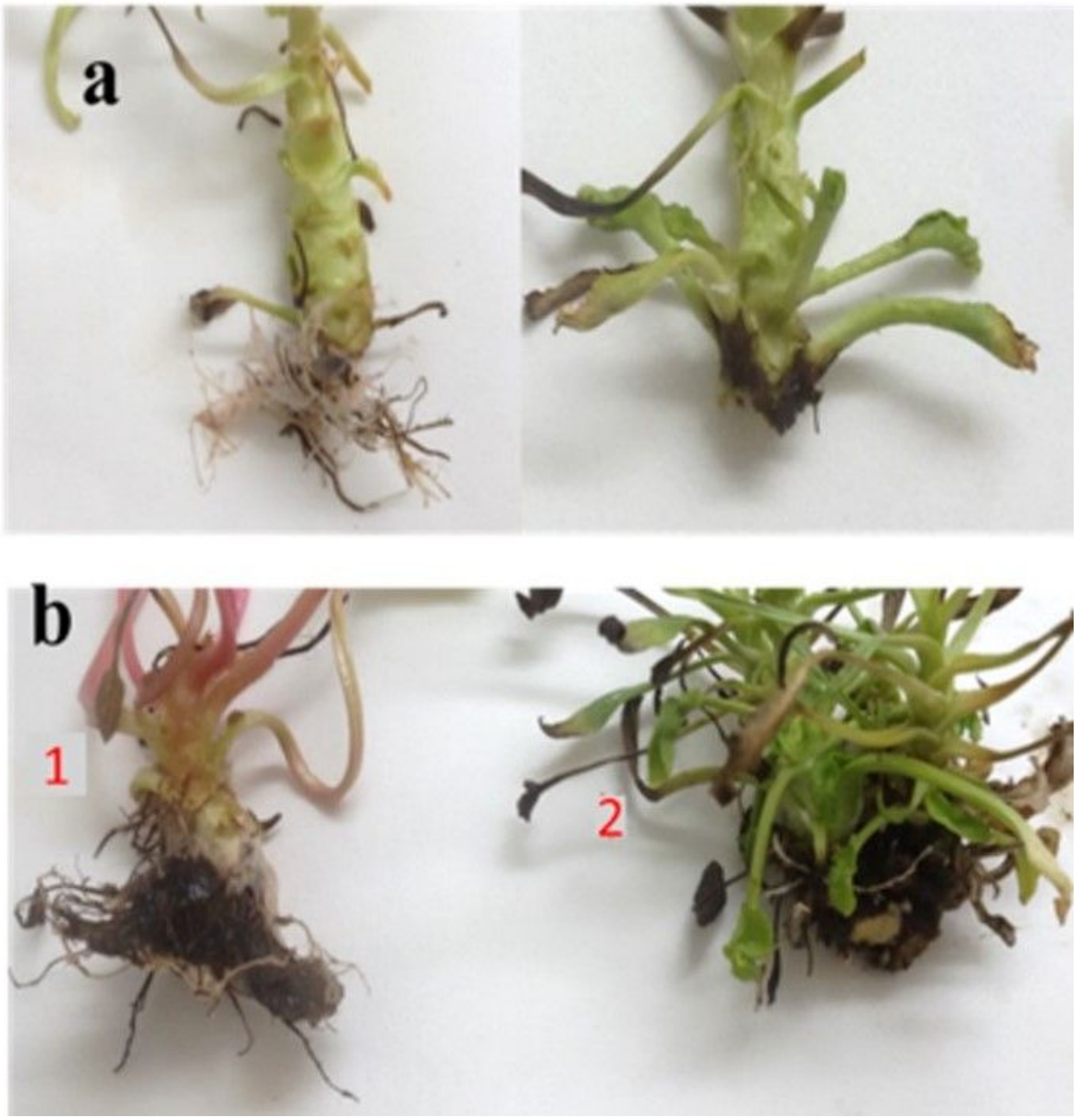

Figure 7

The root growth difference between transgenic and non-transgenic plants. a: After 12 months, 1: The root growth is visible in non-transgenic plant, 2: The transgenic plant has no root; b: After 13 months, 1: The root of non-transgenic plant continues to grow, 2: The transgenic plant shows traces of new roots formation

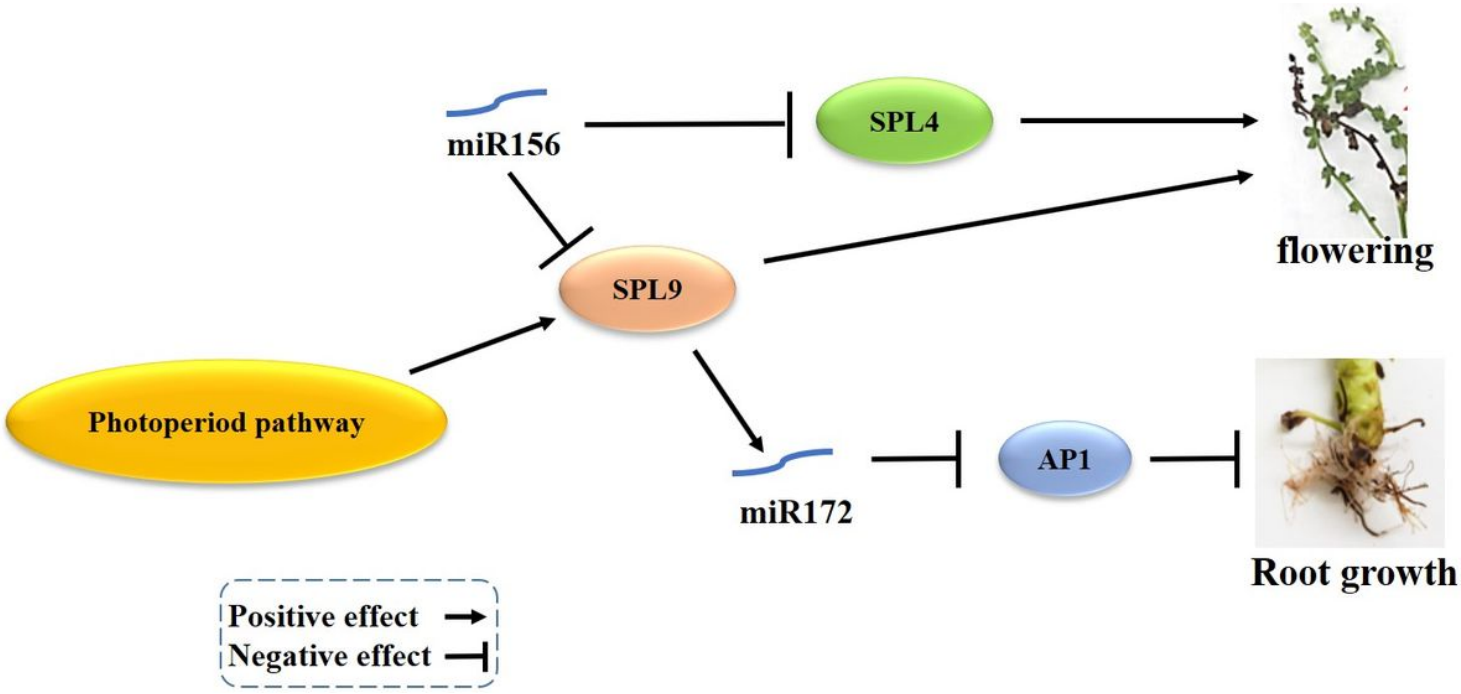

Figure 8

The effect of miR156 gene and its target genes on flowering pathway and root growth in the sea beet plant. The effect of the miR156 on the expression of two SPL4 and SPL9 genes is well verifiable in the beet plant. The SPL4 gene expression is not affected by environmental conditions, while the SPL9 gene expression is affected by gibberellin and photoperiod pathway. The over-expression of the miR156 represses the SPL9 and miR172 genes' expression. It would result in upregulation of the AP2 gene and leads to root growth delay