THE ROLE OF SLMYB GENE IN TOMATO FRUIT DEVELOPMENT

Abdallah A. Hassanin[*], S.S.A. Soliman, T.A. Ismail and M.K.A. Amin

Genet. Dept., Fac. Agric., Zagazig Univ., Egypt

ABSTRACT: The transcription factors regulate the levels of RNA and protein through various processes. Fruit set and fruit development are key process in the life cycle of plants. Fruits provide a suitable environment for the development of seeds and often contribute in the efficient dispersal of mature seeds and also have a high economic impact on the agricultural point of view. The present study aims to characterize the function of SlMYB (*Solanum lycopersicum* MYB) as a candidate gene involved in fruit set process.To achieve that, various methods and techniques were used in this study like in silico analysis, cytolocalization analysis, qPCR , *in situ* hybridization and plant transformation. Results from in silico analysis showed that the deduced protein has 195 amino acids with a conserved MYB DNA-binding domain composed of two conserved repeat (R2R3) characteristic of plant transcription factors. Cytolocalization analysis showed that SlMYB protein was localized in the nucleus and cytoplasm. qPCR results showed that SlMYB gene is expressed only in the reproductive tissues, where the highest level of expression occurs in pollinated flowers. qPCR and *in situ* hybridization results showed that the transcript of SlMYB gene was mainly found in the stamens and ovaries, suggesting that SlMYB may be involved in pollination process and ovary development. To clarify the role of SlMYB gene, gain of function experiment were done by the production of transgenic plants contain the gene of interest overexpressed, characterization of the third generation of transgenic plants showed that the overexpression of the SlMYB gene led to the production of fruit with a large number of seeds. Conversely, silencing of SlMYB gene was done by using virus-induced gene silencing (VIGS) methodology and the silencing led to the reduction of fruit set percentage 13.4%:35.1% in silenced plants and 61.25% in control plants and the production of low number of fruits with very low number of seeds compared to the control plants. To confirm the result of silencing, RNAi methodology was used and the results also showed that the silencing of SlMYB gene led to the reduction of pollen grain fertility and ovary development consequently leading to prevention of fruit set and fruit development. It has been concluded that this gene may involve in flower development and seed formation.

Key words: Fruit development, tomato, MYB genes.

INTRODUCTION

Plant MYBs regulate cell differentiation, organ formation, leaf morphogenesis, secondary metabolism and abiotic stress responses (Ambawat *et al.*, 2013; Qi *et al.*, 2015; Sun *et al.*, 2015; Xu *et al.,* 2015). The function of transcription factors is regulated at the RNA and protein levels through various processes, such as transcriptional regulation, post-transcriptional regulation, post-translational modifications, protein–protein interactions, and controlled protein turnover (Behm-Ansmant *et al.*, 2006; Spoel *et al.*, 2010).

A lot of studies were carried out on tomato fruit development and ripening. The results of those studies led to the identification of specific genes encoding for transcription factors. MADS (MCM1, AGAMOUS, DEFICIENS, SRF) box transcription factors play important roles in the development of higher eukaryotes. Previous investigations has confirmed that MADS box genes play an important role in the regulation of plant development (Itkin *et al.*, 2009; Klee and

* **Corresponding author: Tel. :** +201024005401
  E-mail address: Dr.abdallah4@gmail.com

Giovannoni, 2011; Bemer *et al.*, 2012; Seymour *et al.*, 2013; Fujisawa *et al.*, 2014). The RIN (Ripening-Inhibitor), CNR (clear non-ripening), and NOR (non-ripening) genes have been shown to encode transcriptional regulators and thus likely act to regulate the expression of other genes responsible for ripening phenotypes, including ethylene production (Vrebalov *et al.*, 2002; Giovannoni, 2004; Manning *et al.*, 2006). Other ripening transcriptional regulators have recently been identified *via* transcriptional profiling studies (Alba *et al.*, 2005) and interaction with ethylene synthesis promoters (Lin *et al.*, 2008). Functional studies demonstrated the critical roles of the TAGL1 transcription factor in both early fleshy fruit expansion and later ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009; Pan *et al.*, 2010).

Furthermore, as a negative regulator of maturation of the fruit is SlAP2a that is one of APETALA2/ERF gene family (ethylene responsive element binding protein), loss of function of this gene producing a high yield of ethylene and alters the accumulation of carotenoids (Chung *et al.*, 2010; Dong *et al.*, 2013). Also IAA9 gene has been identified to encode transcriptional repressor of auxin signaling and is a key mediator in fruit set that belongs to the family of proteins Aux/IAA (Wang *et al.*, 2005).

DELLA proteins are conserved repressors of GA signaling that act immediately downstream of the GA receptor to modulate all aspects of GA-induced growth and development in plants (Thomas and Sun, 2004; Griffiths *et al.*, 2006). Recent studies further suggest that DELLA proteins may also restrict plant growth by integrating signals from other hormone pathway and environmental cues (Achard *et al.*, 2003; Fu and Harberd, 2003). GA-induced vegetative growth and floral initiation are repressed by RGA *and* GAI (Dill and Sun, 2001; King *et al.*, 2001). GA-promoted seed germination is mainly regulated by RGL2, although the remaining DELLA genes also play a minor role (Lee *et al.*, 2002; Wen and Chang, 2002; Tyler *et al.*, 2004; Cao *et al.*, 2005; Tyler, 2006). In addition, RGA, RGL1, and RGL2 are involved in flower and fruit development (Yu *et al.*, 2000; Cheng *et al.*, 2004; Tyler *et al.*, 2004).

The first gene encoding a transcription factor in plants was identified; the COLORED1 (C1) locus was found to encode a MYB domain protein required for the synthesis of anthocyanins in the aleurone of maize (*Zea mays*) kernels (Paz-Ares *et al.*, 1987). The functions of MYB proteins have been investigated in numerous plant species such as arabidopsis, maize, rice (*Oryza sativa*), petunia (*Petunia hybrida*), snapdragon (*Antirrhinum majus*), grapevine (*Vitis vinifera* L.), poplar (*Populus tremuloides*) and apple (*Malus domestica*), using both genetic and molecular analyses. A few number of MYB genes have been studied in tomato *e.g.* Lefsm1 (Rivka *et al.*, 2005) that is expressed specifically during early stages of fruit development and the tomato Blind gene that encodes a MYB transcription factor that controls the formation of lateral meristems (Schmitz *et al.*, 2001).

MATERIALS AND METHODS

Molecular and expression studies in this work were done in the Institute of Molecular and Cellular Biology of Plants, University of Polytechnique of Valencia, Spain, the statistical analysis and characterization of the 2nd and 3rd generations of transgenic plants were done in Department of Genetics, Faculty of Agriculture, Zagazig University, also the 4th generation was cultured to confirm the obtained results.

Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom was used in this study. The plants were grown in the greenhouse (24 ± 2°C day and 20 ± 2°C night), using pots of 17 cm diameter filled by coconut fiber. Irrigation with nutrient solution was performed twice every day. The relative humidity ranged from 45-65% during the day and 75-90% at night. To maintain a long-day photoperiod the natural light was supplemented with artificial light by mercury vapor lamps of 400 w.

Nucleic Acids Purification

Extraction of plasmid DNA

For extraction of plasmid DNA 3 ml of overnight culture grown in LB medium supplemented with antibiotic has been used.

Subsequently following the protocol established for extracting and purifying plasmid DNA of the system Rapid Plasmid System ® of Marligen Biosciences, Inc.

Extraction of genomic DNA

Genomic DNA extraction was performed from young leaves (200-300 mg). The leaves were crushed in 500 µL of extraction buffer (0.2 M Tris-HCL pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% *V/V* SDS) and after centrifugation for 5 minutes at 13,000 rpm, 350 µl of supernatant were transferred to an Eppendorf tube containing 350 l of isopropanol (unpublished method). The mixture was centrifuged for 10 min. The supernatant was removed and the pellet was washed with 70% ethanol (*V/V*) 2 times. The pellet was allowed to dry and resuspended in 200 µl of distilled water. Quantification was performed using the NanoDrop spectrophotometer ® ND-1000.

Extraction of total RNA

Total RNA was isolated from 100 mg of plant tissue using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The RNA was quantified by using a spectrophotometer NanoDrop ® ND-1000 at 260 nm. Subsequently the extracted RNA was used for synthesis of cDNA needed for both real-time quantitative PCR and reverse transcription PCR.

Amplification of DNA by polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used to detect the presence of nptII marker gene in transgenic tomato plants from genomic DNA and to isolate different cDNA fragments of SlMYB gene. The amplification reactions were performed in a total volume of 25 µl, from 50 ng of genomic DNA or 5 µl of cDNA in the case of isolation of SlMYB gene. The reaction mixture contained 0.4 µM of each of the specific oligonucleotides, 5 µl of 2 mM dNTPs, 5 µl of 10X reaction buffer, 1.5 µl of 50 mM MgCl2 and 1.25 units of DNA polymerase (Biotools ®). The reactions were run in a Perkin Elmer 2400. The thermocycling conditions used were 95°C 2 min+30-35 cycles (95°C 30 sec, (T)°C 30 sec., 72°C 2 min) +72°C 5 min. In each reaction the annealing temperature (T) was estimated based

on the Tm (estimated melting temperature) of the oligonucleotides. The extension time depends on the length of the amplified fragment, generally used 1 min per kb of expected product.

Expression Studies

Quantitative real time polymerase chain reaction (qRT-PCR)

Experiments of quantitative real time polymerase chain reaction (qRT-PCR) were conducted to analyze gene expression. Oligonucleotides (forward 5'-GGACACTAATT TTCAAGCCTCAAATT-3' and reverse 5'-CATGTTGTCATTTGTTTCATTGAGAA-3') were designed using Primer Express v. 2.0 program (Applied Biosystems ™) taking a small region of 3' end of the gene.

Two µg of total RNA previously treated for 30 min with DNase (Turbo DNA-free from Fermentas ™), were used for cDNA synthesis following the manufacturer's recommendations of the system Prime Script RT reagent kit ® (TaKaRa). This system is based on the action of a reverse transcriptase MMLV (Moloney Murine Leukemia Virus) and an oligo (dT) was used as primer.

In the analysis ACTINA8 (SlACT8) (Martin-Trillo *et al*., 2011), was used as endogenous gene whose expression is constant in all analyzed tissues by using specific primers (forward 5'-CAA GTT ATT ACC ATT GGT GCT-3' and reverse 5'-TGC AGC TTC CAT ACC AAT CAT-3'). Ct comparative calculation is valid when the amplification efficiency of the gene of interest and the endogenous gene was similar. In all gene expression assays the amplification efficiency was between 90 to 100%.

In situ hybridization

Labeled probes were synthesized from a cDNA fragment of 290 bp of the gene SlMYB cloned into the vector pCR8/GW/TOPO TA (Invitrogen™, USA). The fragments were amplified by PCR with oligonucleotides specific to the vector M13 (forward 5'- GTAAAACGA CGGCCAGT-3' and reverse 5'- AACAGCTA TGACCATG -3') and then purified from a 1% agarose gel and used as templates for each probe. Probe was transcribed in "antisense"

respect to the T7 promoter of the vector, and a "sense" probe was generated as a negative control against nonspecific hybridization.

Flowers were taken in early stages of development, before anthesis, and were immersed in a fixing solution of paraformaldehyde 4% in 0.1 M phosphate buffer, pH7.2 containing Triton X-100 (0.1%). Three vacuum pulses of 5 minutes each were used to assist penetration of the fixing solution, then the fixing solution was changed by fresh one and kept at 4°C for 16 hours followed by dehydration steps and subsequent embedding in Jung Historesin (Leica). Histological sections of 8 μm thickness were made using a Microm microtome HM330, and then placed on slides treated with poly-l-lysine sections on slides were deparaffinized by treating with Histo-Clear®, then rehydrated and washed. For hybridization, 50 μl of probes diluted in hybridization buffer were placed on each slide and then slides are covered and incubated overnight in a moist chamber at 50°C. After hybridization slides were washed and then incubated with anti-Dioxigenin-AP, Fab fragments Roche® followed by detection buffer which has been replaced by 50 ml of detection buffer containing the substrate (75 μl of NBT (100 mg/ml) and 75 μl of BCIP (50 mg/ml) from Roche®), incubation in darkness longer than 48 hr., until the signal was observed. Finally the reaction was stopped by changing the detection solution with water. The samples were dried, mounted with Merckglass (Merck ®) and visualized under microscope.

Virus-induced gene silencing (VIGS)

Tomato plants used for silencing experiments should be around 7-8 days old when the true leaves have not yet appeared. 1 μl from the glycerol stock of *Agrobacterium tumefaciens* (strain C-58) harboring pTRV1, pTRV2-PDS and pTRV2-host target gene was grown in LB liquid medium supplemented with 100 μg/ml of kanamycin and 100 μg/ml of rifampicin and incubated with shaking at 28°C for 2 days. The kanamycin selects for the pTRV plasmid while the rifampicin selects for the *Agrobacterium*. Silencing of PDS will cause the plants to photobleach and is used as a control for silencing efficiency. After 2 days 100 μl of each preculture was transferred to a new 5ml LB

liquid medium with the previous mentioned antibiotics and incubated overnight at 28°C. The cells were harvested by centrifugating for 20 minutes at 3000 rpm. Every pellet was resuspend in 20 ml MES1X + acetosyringone (stock 200 ml: 20 ml MES10X + 2ml MgCl2 1M + 180 Ml water + 200 μl acetosyringone 100 mM) and then incubated 1.5-2 hour at room temperature. Bacterial suspension was prepared with an O.D.600 of (0.2-0.8) for each bacterial culture. Final concentration of 0.05 of each bacterial suspension was calculated and prepared. The cultures containing the pTRV1 and the pTRV2 vector (or the pTRV2 containing the gene of interest) were mixed in a 1 to 1 *V/V*. Also the same was done with pTRV2-PDS control. A 1 ml needless syringe was used to infiltrate the bacterial suspension into both cotyledons of the seedlings. Infiltrated seedlings were kept under greenhouse conditions.

Transformation of bacterial cells

For transformation of bacterial cells 40 μl aliquot of competent cells was thawed on ice, to which was added 1 μl of the plasmid carrying the construct of interest. The strain of *E. coli* TOP10 was transformed following the chemical transformation protocol provided by the manufacturer (Invitrogen ®). In the case of the *E. coli* (strain DH10B), *A. tumefaciens* (strain C58) and *A. tumefaciens* (strain LBA4404) transformation was performed by electroporation. In all cases the transformed bacterial cells were resuspended in 800 μl of LB medium without antibiotics, and incubated under constant stirring for 1 hr., at 37°C for *E.coli* and 2 hr., at 28°C for *A. tumefaciens*. After incubation without selection pressure, bacterial cells were plated on LB medium supplemented with selective pressure to select transformed cells.

Plant transformation

To generate 35S: SlMYB transgenic plants, forward 5'-ATGGATAAAATATGCAACTCT CAAG-3' and reverse 5'-ATCTCCATTAAGCA ATTGCA-3' primers were used to amplify the complete cDNA sequence of SlMYB gene. This sequence was then cloned into pK2GW7 binary vector in sense orientation under the transcriptional control of the cauliflower mosaic virus 35S promoter and the T35S terminator. On the other hand the same was done to generate

RNAi-SlMYB transgenic plants by using forward 5'- GAACGGATAACGAGATAAAA -3' and reverse 5'- ATCTCCATTAAGCAATTGCA -3' primers to amplify 290 bp from the 3' end of SlMYB cDNA as a specific region to this gene. This sequence was then cloned into pK7GWIWG2 binary vector in sense and antisense orientation under the transcriptional control of the cauliflower mosaic virus 35S promoter and the T35S terminator (Fig. 1). Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation according to Ellul *et al.* (2003), and transformed lines were first selected on kanamycin (100 mgl1) and then analyzed by PCR, the presence of kanamycine resistance gene by using 5'-GACAAGCCGTTTTACG TTTG-3' and reverse 5'-GATACTTTCTCGGC AGGAG-3' primers as specific primers to the gene of kanamycin resistance and the number of T-DNA insertions according to the segregation ratio of dominant kanamycin resistance gene.

Evaluation of ploidy level

The ploidy level of obtained transgenic plants was measured by flow cytometric quantification of nuclear DNA content of cells using young leaves of acclimated transgenic plants according to Smulders *et al.*, 1994. The tissue was finely minced in a petri dish on which was added 200 µl of nucleus extraction buffer (Partec). The resulting liquid was passed through a nylon mesh of 50 microns and 800 µl of a dye solution containing 1 mg/l of fluorochrome DAPI (4,6-diamino-2-phenyl - indole) (DAPI staining solution, Partec) were added, DNA from isolated nuclei was measured using a flow cytometer Partec PAS-II ploidy Analyser. The result showed in a graph represented the data of nuclear DNA content (on the horizontal axis) and the counted number of nuclei of each type (in the vertical axis). The peak corresponding to a DNA content equal to 2n (diploid) was used for calibration.

Cytolocalization analysis

Agroinfiltration experiments were performed on *N. benthamiana* grown in the greenhouse. Plants were grown for 6-8 weeks before infiltration. Binary plasmids (pEarleyGate 101 and pEarleyGate 104) were recombined with complete cDNA of SlMYB gene to be under the control of 35S promoter and fused to YFP gene. Binary plasmids were transformed into *Agrobacterium tumefaciens* strain C58 and incubated for 2 days on LB medium supplemented with kanamycin, rifampicin and gentamicin 50 mg/l to select for transformed *Agrobacterium* cells. For agroinfiltration the transformed *Agrobacterium tumefaciens* strain C58 were incubated for 2 days in LB medium supplemented with the same antibiotics. The optical density at 600 nm was measured and suspensions were made for co-infiltrations with a final OD600 of 0.3 in 2ml of infiltration solution (200 µl MES, 20 µl $MgCl_2$, 4 µl acetosyringone, 1776 µl distilled water) for each plant. Agroinfiltration of young leaves of *N. benthamiana* was done as described by Feller *et al.* (2006). Two to three days later, infiltrated leaf areas were excised and localization of green fluorescent protein (YFP) was determined by confocal laser scanning microscopy.

Data analysis

Statistical analyses were performed using Microsoft Excel. If two observations are described as different this means that their difference was determined to be statistically significant ($P < 0.05$) by the performance of Student's t-tests.
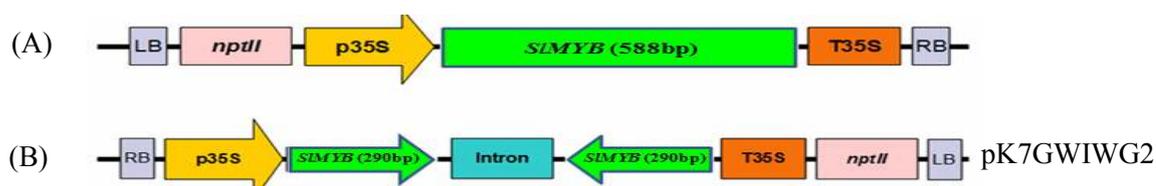

Fig. 1. Schematic representation of the constructions used for transformation of tomato. (A) The construction of overexpression (35S: SlMYB). (B) Construction of silencing (RNAi-SlMYB)

RESULTS AND DISCUSSION

In Silico Analysis of SlMYB Gene

The transcript identified in the genomic analysis corresponds with the unigen SGN-U576253 and was renamed as SlMYB (*Solanum lycopersicum* MYB). The genomic sequence of SlMYB gene was analysed in silico and includes the 588 bp coding sequence in three exons (133 bp, 130 bp, 325 bp respectively from 5´ to 3´) flanked by a 5´untranslated region (5´ UTR) of 96 bp and a 3´ untranslated region (3´ UTR) of 223 bp and contains two introns (292 bp and 2431 bp, respectively from 5´ to 3´) (Fig. 2). The deduced protein sequence produces a typical R2R3 MYB protein of 195 amino acids with a calculated molecular weight of 22.42 kDa.

Bioinformatics analysis of the protein sequence identified the presence of two domains of R2R3 MYB protein and each domain contain a helix-turn-helix (HTH) motif (Fig. 3). Type R2R3 MYB proteins are transcription factors which play an important role in eukaryotic cells, as they regulate different pathways of transduction signal and control processes such as development and programmed cell death. This domain allows the interaction or binding to DNA, RNA or other proteins.
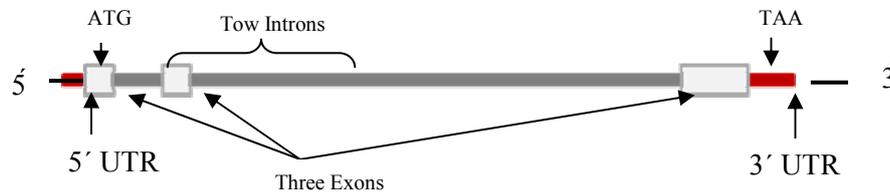
Fig.2. The genomic SlMYB gene structure
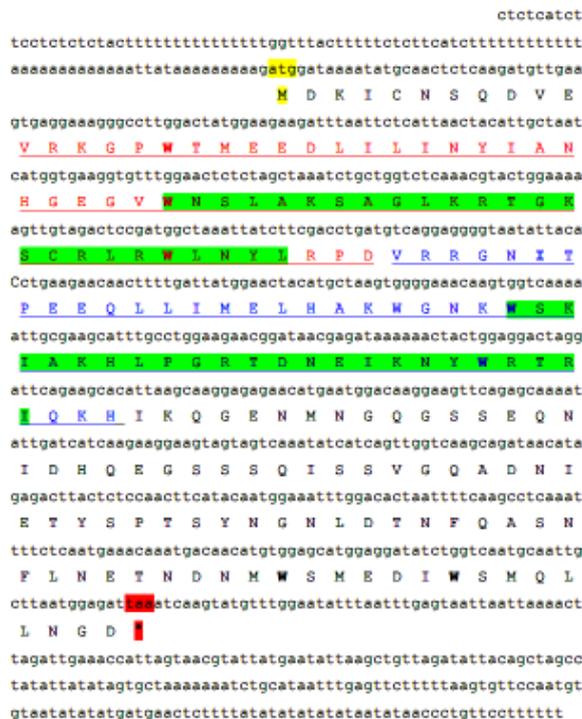
Fig. 3. (A) Nucleotide and protein sequence of SlMYB. Start codon is indicated in yellow while stop codon is indicated in red. R2MYB domain (53aa) is indicated in red and the blue indicates the R3MYB domain (51aa). Green color indicates the DNA binding domain (helix-turn-helix (HTH) motifs). Tryptophan and isoleucine residues are highlighted in bold

Note: aa (amino acid)

MYB proteins are characterized by a highly conserved DNA-binding domain (the MYB domain). This domain generally consists of up to four imperfect amino acid sequence repeats (R) of about 52 amino acids, each forming three α–helices. The second and third helices of each repeat build a helix–turn–helix (HTH) structure with three regularly spaced tryptophan and isoleucine (or hydrophobic) residues, forming a hydrophobic core in the 3D HTH structure (Ogata *et al.,*1996). The third helix of each repeat is the ''recognition helix'' that makes direct contact with DNA and intercalates in the major groove (Jia *et al.,* 2004). During DNA contact, two MYB repeats are closely packed in the major groove, so that the two recognition helices bind cooperatively to the specific DNA sequence motif.

Alignment was performed using homologous proteins ClustalW0 program. A highly conserved region corresponding to the R2 and R3 MYB domains. The presence of the conserved tryptophan and isoleucine residues within every MYB domain have been also identified (Fig. 4).

The alignment of the complete sequences of the 15 MYB proteins of different plant species that are similar to SlMYB, a dendrogram was generated using Uniprot database (http://www.uniprot.org). Fig. 5 shows the degree of similarity between the deduced protein and the other plant MYB proteins of different plant species. The SlMYB protein has a degree of similarity with proteins from the same family (Solanaceae) ranged between 83% - 86% while the degree of similarity with homologous proteins from other families ranged between 70% - 78%.

Although the MYB gene superfamily comprises one of the largest families of transcription factors known to date, the functional roles of most plant MYB genes are largely unknown. Interestingly, plant R2R3 MYB genes have a highly conserved N-terminal MYB domains, but have dramatically divergent C-terminal regions (Fig. 4). Recently, conserved motifs within the C-terminal regions of paralogous and orthologous MYB genes have been identified (Stracke *et al*., 2001). These conserved motifs may reflect constraints upon the functions of related Myb proteins.

Cytolocalization of SlMYB Gene

In eukaryotes, transcription factors are transcribed in the nucleus but are then translated in the cytoplasm of the cell. Many proteins that are active in the nucleus contain nuclear localization signals that direct them to the nucleus. But, for many transcription factors, this is a key point in their regulation (Whiteside and Goodbourn, 1993). To examine the subcellular localization of SlMYB, the constructions p35S:: SlMYB-YFP and p35S::YFP–SlMYB were generated by using pEarleyGate 101(C-YFP-HA) and pEarleyGate 104 (N-YFP) as a destination vectors. Agroinfiltration into *N. benthamiana* leaves shows that YFP fluorescence was detected in both cases in the cytoplasm and the nucleus which indicate that the gene of interest works as transcription factor (Fig. 6).

Analysis of SlMYB Gene Expression Pattern

qRT-PCR analysis

SlMYB gene expression was assessed at different developmental stages and plant organs by quantitative real-time PCR (qRT-PCR). To determine the expression of SLMYB gene, two oligonucleotides were designed to amplify a fragment of 52 bp at the 3´end of the gene, which is intended to detect only the candidate gene expression; because this region is specific to SlMYB as MYB genes are divergent in the 3´ end (Fig. 4). The cDNA was reverse transcribed from RNA extracted from 7 days old seedling (from apical meristem and root), young leaves from adult plants and flowers in various stages of development (flowers 6, 4, 2 days before anthesis, flowers at anthesis and flowers 2, 4 days after anthesis).

It has been showen a very low levels of transcript in vegetative tissues (apical meristems, leaves and roots) compared to the high level observed in reproductive tissues. During the development of the flower, the highest level of expression was detected in flowers at anthesis (Fig. 7A). This expression pattern suggests that this gene is required during flower development.

In order to confirm the role of SlMYB gene in flower development the expression of the gene was analyzed in the different flower organs at the anthesis stage; it was observed that the gene is expressed in all the organs and the highest level of gene expression is localized in stamens (Fig. 7B).

CLUSTAL O (1.1.0) multiple sequence alignment

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SlMYB_SOLLC     --------------MDK------IC-NSQDVEVRKGPWTMEEDLILINYIANHGEGVWNS 39
G9BIM3_NICAT    --------------MDKK------PC-NSQDVEVRKGPWTMEEDLILINYIANHGEGVWNS 40
A7Y1T9_NICLS    --------------MDKK------PC-NSQDVEVRKGPWTMEEDLILINYIANHGEGVWNS 40
F5CSS3_9SOLA    --------------MDKK------PC-NSQDAEVRKGPWTMEEDLILINYIANHGEGVWNS 40
F5CSS2_9SOLA    --------------MDKK------PC-NSQDAEVRKGPWTMEEDLILINYIANHGEGVWNS 40
A8VPW5_VITVI    --------------MDKK------PC-NSQDAEVRKGPWTLGEDLILINYIANHGEGVWNS 40
MYB05_ANTMA     --------------MDKK------PC-NSQDVEVRKGPWTMEEDLILINYIANHGEGVWNS 40
MYB40_ANTMA     --------------MDKK------PC-NSHDVEVRKGPWTMEEDLILINFISNHGEGVWNT 40
A9ZMI2_GENTR    MANSPTPIDIEMNINNNNSK---AACNSGDVQVRKGPWTMEEDMILINYIANHGEGVWNS 57
A9ZMI3_GENTR    --------------MDKKGSN---INSSSKDPEVRKGPWTMEEDLILINYIANHGEGVWNS 44
Q0PJJ5_SOYBN    --------------MDK--K---LGNTSHDPEVRKGPWTMEEDLILITYIANHGEGVWNS 41
Q0PJJ4_SOYBN    --------------MDKK--Q---QCKTSQDPEVRKGPWTMEEDLILMNYIANHGEGVWNS 42
Q0PJJ6_SOYBN    --------------MDKK--Q---LCNTSQDPEVRKGPWTMEEDLILINYIANHGEGVWNS 42
Q70RD2_GERHY    --------------MEKR------PNNSSQDVEVRKGPWTMEEDLILINYIANHGEGVWNS 41
tr|At3g27810    --------------MEKRGGGSSGGSGSSAEAEVRKGPWTMEEDLILINYIANHGDGVWNS 47
                                       ..      .* : :********: **:**: .*:**:****:
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A7Y1U0_NICLS    LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 100
SlMYB_SOLLC     LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEQLLIMELHAKWGNKWSKIAKHLPGR 99
G9BIM3_NICAT    LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 100
A7Y1T9_NICLS    LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 100
F5CSS3_9SOLA    LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 100
F5CSS2_9SOLA    LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 100
A8VPW5_VITVI    LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITDEEQQLIMELHAKWGNRWSKIAKHLPGR 100
MYB05_ANTMA     LARSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKTLPGR 100
MYB40_ANTMA     IARSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 100
A9ZMI2_GENTR    LARSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 117
A9ZMI3_GENTR    LARSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 104
Q0PJJ5_SOYBN    LAKAAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 101
Q0PJJ4_SOYBN    LAKAAGLKRNGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 102
Q0PJJ6_SOYBN    LAKAAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 102
Q70RD2_GERHY    LARSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 101
tr|At3g27810    LAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLLIMELHAKWGNRWSKIAKHLPGR 107
                :*:;***** ************************ *** ;********** ******* ****
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A7Y1U0_NICLS    TDNEIKNYW-RTRIQKHIKQAENMNGQAANSEQNDH-----QE-GSSSHMSSAG------ 147
SlMYB_SOLLC     TDNEIKNYW-RTRIQKHIKQGENMNGQGSSEQNIDH-----QE-GSSSQISSVGQAD--- 149
G9BIM3_NICAT    TDNEIKNYW-RTRIQKHIKQAENMNGQAASHEQNDH-----QE-GSSSHMSSAG------ 147
A7Y1T9_NICLS    TDNEIKNYW-RTRIQKHIKQAENMNGQAANSEQNDH-----QE-GSSSHMSSAG------ 147
F5CSS3_9SOLA    TDNEIKNYW-RTRIQKHIKQAETMNNGQAASSEQNDH-----QE-ACTSQMSNGPNDN--- 150
F5CSS2_9SOLA    TDNEIKNYW-RTRIQKHIKQAETMNNGQAASSEQNDH-----QE-ACTSQMSNGPNDN--- 150
A8VPW5_VITVI    TDNEIKNFW-RTRIQKHIKNAETFTAQSSDQT---H-----DQ-ATTSQV---MGAA--- 144
MYB05_ANTMA     TDNEIKNYW-RTRIQKHMEQGDQSSSTTFNNGQMNLDHSCNDQ-ASSSQMSACGPVVDHT 158
MYB40_ANTMA     TDNEIKNYWNRTRIQKHIKQAEASFIGHINPE-----HS-NEQ-ASTSLLSSSCHAD--- 150
A9ZMI2_GENTR    TDNEIKNFW-RTRIQKQMKHVENSSVGQSSEQTEAPSTS--HE-ASTA----AAATATTA 169
A9ZMI3_GENTR    TDNEIKNFW-RTRIQKHMKQAENYAGQSSSEQNERV-----AS-TSMQTSAATAPHE--- 154
Q0PJJ5_SOYBN    TDNEIKNYW-RTRIQKHLKQASSSFQQQSSNSEIIYH----PQ-ACTSQVS-------TM 148
Q0PJJ4_SOYBN    TDNEIKNYW-RTRIQKHIKQAEN-FQQQSSNNSEIND----HQ-ASTSHVS-------TM 148
Q0PJJ6_SOYBN    TDNEIKNYW-RTRIQKHIKQAEN-FQQQISNNSEIND----HQ-ASTSHVS-------TM 148
Q70RD2_GERHY    TDNEIKNYW-RTRIQKHIKQVENFSGQTSSGQNDQA-----TM-S-STQT---CNVV--- 147
tr|At3g27810    TDNEIKNFW-RTRIQKYIKQSDVTTTSSVGSHH---SSEINDQAASTSSH----NVFCTQ 159
                ******:*;* ****** ::. .
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A7Y1U0_NICLS    --PTETYSPTS--YSANIDTTFQGP-FL-----------------TETNDNIWSMEDIWS 185
SlMYB_SOLLC     --NIETYSPTS--YNGNLDTNFQASNFL-----------------NETNDNMWSMEDIWS 188
G9BIM3_NICAT    --PTETYSPTS--YSANIDNTFQGP-FL-----------------TETNDNIWSMEDIWS 185
A7Y1T9_NICLS    --PTEAYSPTS--YSANIDTTFQGP-FL-----------------TETNDNIWSMEDIWS 185
F5CSS3_9SOLA    -TIDQTYSPTS--YSGNVDTFQAGPNFL-----------------TEANDNMWSMEDIWS 190
F5CSS2_9SOLA    -TIDQTYSPTS--YSGNVDTFQAGPNFL-----------------TEANDNMWSMEDIWS 190
A8VPW5_VITVI    -HVADSYSPPS--YPANLEAFPGF--SS-----------------AESNDNFWTMEDIWS 182
MYB05_ANTMA     AVDQSSYSPHS--FNGNDHTFQ--APFP-----------------TDQSNDNMWSMEDFWS 198
MYB40_ANTMA     -HAVESYSSS---FNGNMGNNVQYPNHF-----------------PTESNDYFWSMEDLWS 190
A9ZMI2_GENTR    HDAVEAYSPQS--YTANMESFQ--NPFP-----------------PDQSNDNMWSMEDLWS 209
A9ZMI3_GENTR    -LMMETQSPPSN-YTMDHLPEAFQNPFP-----------------TESNDNLWSMEDLWS 195
Q0PJJ5_SOYBN    AQPIETYSPPS--YQGMLDPFS-I-QFP----T-NPHHSSCCTNDDD-NNNYWSMEDIWS 198
Q0PJJ4_SOYBN    AEPMEMYSPPC--YQGMLEPFS-T-QFP----TINFDQSSCCTNDNN-NINYWSMEDSWS 199
Q0PJJ6_SOYBN    AEPMETYSPPF--YQGMLEPFSSI-QFP----TINFDQSSCCTNDNNNSINYWSMEDIWS 201
Q70RD2_GERHY    -NPMETYSPPY--D--NFQNFS--GPFP-----------------TETNENMWSMEDLWS 183
At3g27810       DQAMETYSPTPTSYQHTNMEFN-YGNYSAAAVTATVDYPVPMTVDDQTGENYWGMDDIWS 218
                . *                                     :      *  *:* **
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A7Y1U0_NICLS    -MQLLNGD- 192
SlMYB_SOLLC     -MQLLNGD- 195
G9BIM3_NICAT    -MQLLNGD- 192
A7Y1T9_NICLS    -MQLLNGD- 192
F5CSS3_9SOLA    -MQLLNGD- 197
F5CSS2_9SOLA    -MQLLNGD- 197
A8VPW5_VITVI    -MQLLNGD- 189
MYB05_ANTMA     -MQLLNGD- 205
MYB40_ANTMA     -MQLPNNGG 198
A9ZMI2_GENTR    -MNLLN--- 214
A9ZMI3_GENTR    -LHLLN--- 200
Q0PJJ5_SOYBN    -MQLANY-- 204
Q0PJJ4_SOYBN    -MQLLNGD- 206
Q0PJJ6_SOYBN    -MQLLNGD- 208
Q70RD2_GERHY    -MQLLNSE- 190
At3g27810       SMHLLNGN- 226
                :.* *
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Fig. 4. Alignment of the deduced R2R3 MYB protein type from *Solanum lycopersicum* with proteins from other species of plants. Protein alignment was generated using ClustalW0. The red line indicates the R2 MYB domain (55aa) and the blue line indicates the R3 MYB (51aa). Tryptophan and isoleucine residues are indicated in red and blue boxes in both R2 and R3 domain, respectively
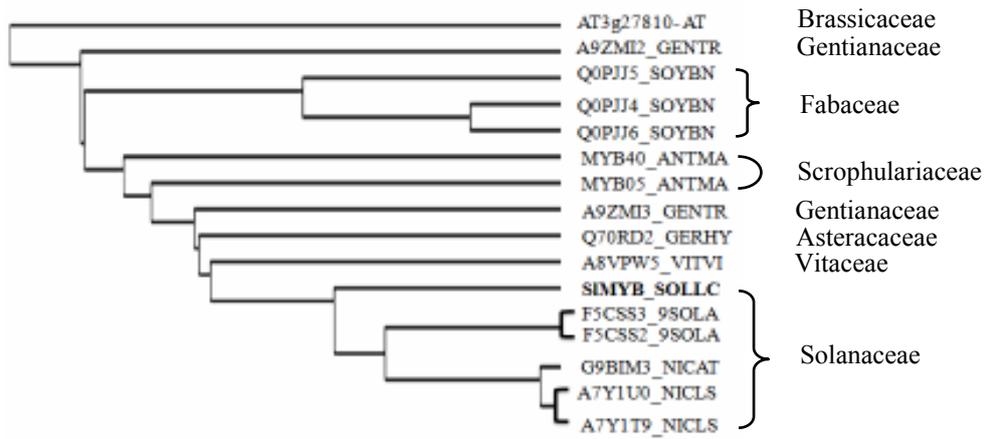
Fig. 5. Phylogenetic tree displaying the similarity of SlMYB (in bold) to other R2R3 MYB proteins. The tree was constructed using Uniprot database

(A) (B)
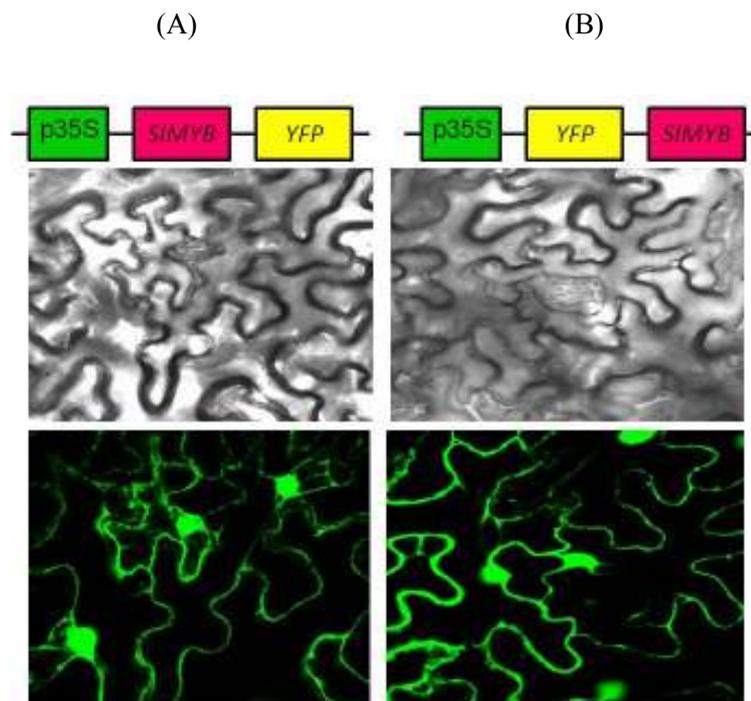
Fig. 6. Cytolocalization of SlMYB

A. *Nicotiana benthamiana* leaf cells agroinfiltrated with SlMYB-YFP in pEarleyGate 101 (C-YFP-HA) vectors

B. *Nicotiana benthamiana* leaf cells agroinfiltrated with SlMYB-YFP in pEarleyGate 104 (N-YFP) vectors. Both of them show green fluorescence in the nucleus and the cytoplasm.

.

A

Seedling stage   Flowering

B
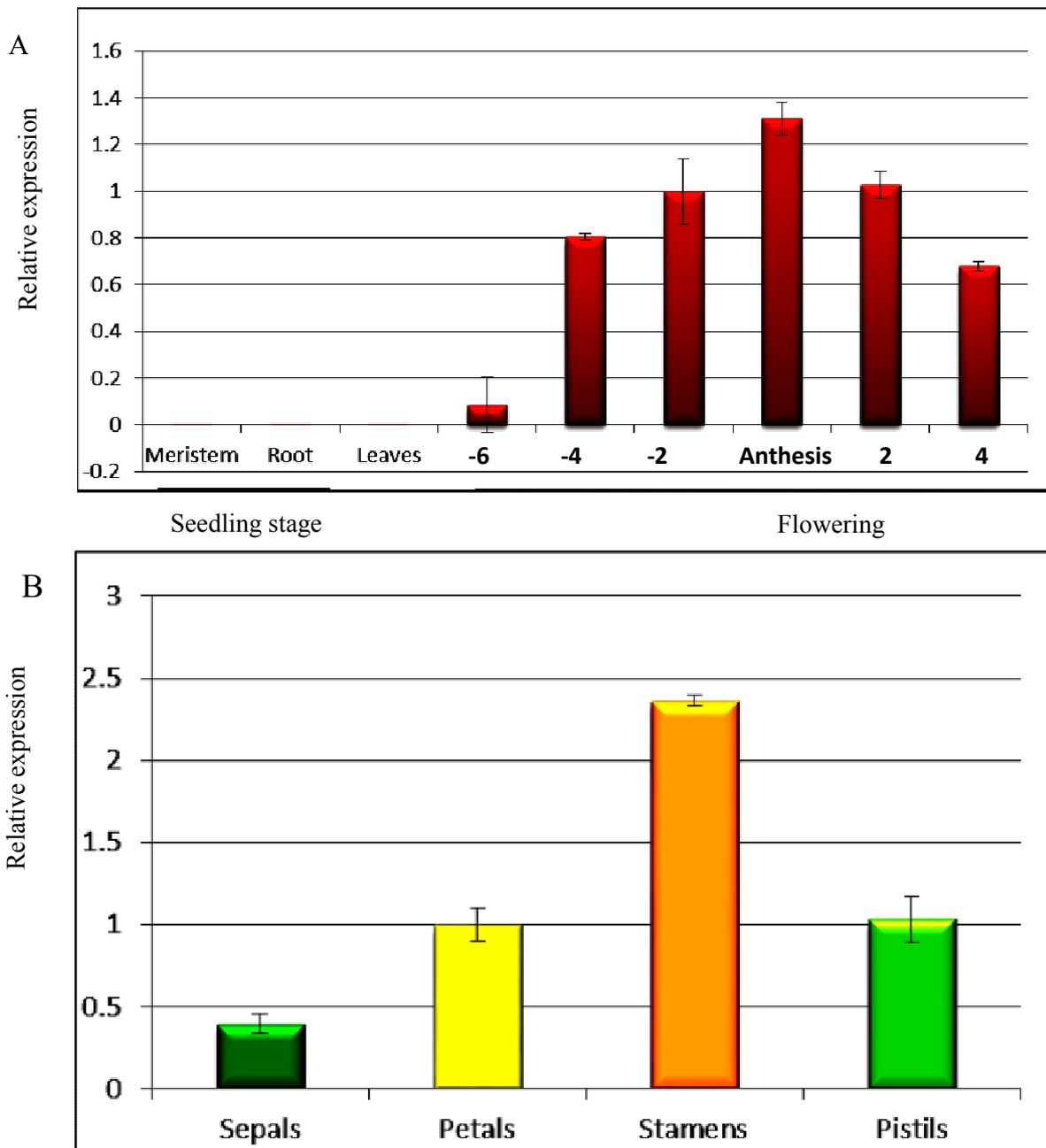
Fig. 7. **Relative expression of SlMYB gene in wild type tomato cv. Micro-Tom. A. Transcript level in the apical meristem and root of 7-day-old seedlings, adult plant leaves, flowers of six, four and two days before anthesis, flowers at anthesis and flowers two and four days after anthesis. Expression levels were relating to the expression in the flowers two days before anthesis (-2). B. Expression level in flower organs in flowers at anthesis. The valus are relative to the expression level in petals. The level of gene expression was quantified by qRT-PCR using specific primers and the SlACT8 endogenous gene as a normalizer**

The homologous gene from Arabidopsis (At3g27810) has similar expression in different plant parts and in flower organs. This gene encodes a member of the R2R3-MYB transcription factor gene family, induced by jasmonate and involved in jasmonate response during stamen development

In situ hybridization analysis

To determine the expression of SlMYB gene in flower tissues, *in situ* hybridization was performed using an RNA probe labeled with dioxygenin. The probe was synthesized from a fragment of 290 bp corresponding to the 3' region of the gene, cloned in antisense direction against T7 promoter. The evaluation was performed in reproductive tissues, and sections were obtained from floral meristem, flowers 6 days befor anthesis, flowers 4 days befor anthesis, flowers 2days before anthesis and flowers at anthesis. *In situ* hybridization showed that the expression of SlMYB gene appeared in all tested stages except floral meristem. Results showed that SlMYB transcript is located in the stamens and carpels (Fig. 8). In particular signal within the ovary is located on ovules. This suggests that the gene may participate in ovules development and located in stamen cells which suggest that the gene may be involved in stamens development.

Gain of function (overexpression) of SlMYB gene increases the number of seeds in tomato fruits

For the overexpression of the complete cDNA of SlMYB gene (588 bp) was amplified by PCR by designing a pair of specific primers that amplify the sequence from ATG to the stop codon. Ovaries and stamens cDNA libraries of tomato (cv. Micro-Tom) were used as a template in PCR. The complete cDNA was inserted by recombination reaction into the destination vector pK2GW7 to be under the control of constitutive 35S promoter (Fig. 1 materials and methods) generating the construction 35S: SlMYB.

This construct was used to genetically transform tomato plants of Micro-Tom cultivar. *A. tumefaciens* LBA4404 strain was used for transformation and cotyledons explants were used as plant material. Regenerated explants without inoculation with *A. tumefaciens* were obtained to be used as a control in subsequent analysis. It was obtained 2 primary transgenic shoots for the construction of overexpression 35S:: SlMYB (Fig. 9). The transgenic plants were analyzed by flow cytometry to assess ploidy level and they proved to be diploid (2n). The presence of a 583 bp fragment corresponding to the nptII gene was detected in the plants indicating that the resulted plants have the overexpression construction. Three generations were cultivated to obtain the homozygous plants. Relative expression of SlMYB gene was measured in flowers at anthesis in T3 homozygous lines showed that the gene of interest is overexpressed (Fig. 10A). The phenotype of T3 plants growing under the greenhouse conditions was analyzed. These plants showed no differences in the vegetative development. But the fruits of these plants had a large number of seeds (Fig. 10D). The difference in the number of seeds between the T3 plants, control plants was statistically significant (Fig. 10B).

Numerous R2R3-MYB proteins have been characterized by genetic approaches and found to be involved in the control of plant-specific processes in Arabidopsis including regulation of cell fate and identity (Jakoby, 2008; Li, 2009), regulation of developmental processes such as anther development and/or functionality (Millar and Gubler, 2005; Zhang, 2007; Cheng, 2009; Mandaokar and Browse, 2009) and controlling male germ cell division and differentiation (Brownfield *et al*., 2009).

Loss of Function (Silencing) of SlMYB Gene Lead to the Reduction of Fruit Set and Production of Fruits with Low Number of Seeds

Stable silencing

To generate the construct to obtain stable silencing pair of primers were designed (materials and methods) that amplify a fragment of 290 bp corresponding to the 3´region of the coding sequence of SlMYB gene. This fragment was chosen as a gene specific fragment. The fragment was recombined in the silencing vector pK7GWIWG2 containing the 35S promoter
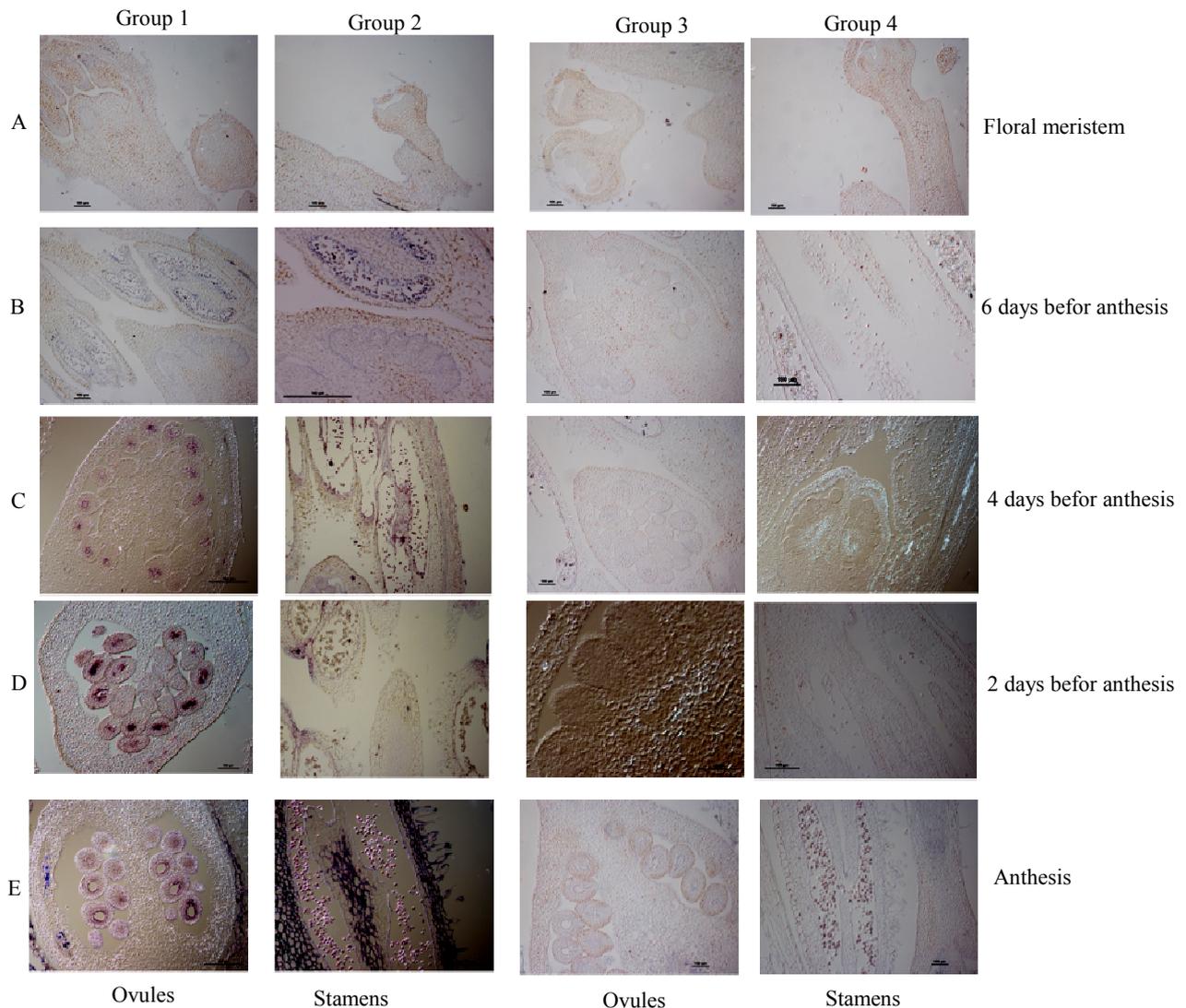
Fig. 8. Transcript location of SlMYB gene in tomato flowers cv. Micro-Tom by *in situ* hybridization. (A) Expression of SlMYB gene in floral meristem. (B) Expression of SlMYB gene in flowers 6 days befor anthesis. (C) Expression of SlMYB gene in flowers 4 days befor anthesis. (D) Expression of SlMYB gene in flowers 2 days befor anthesis. (E) Expression of SlMYB gene in flowers at anthesis. Groups 1, 2 indicate the expression of SlMYB gene in ovules and stamens, respectively; Groups 3, 4 indicate the control in ovules and stamens, respectively. Bars in all photos are 100 μm.
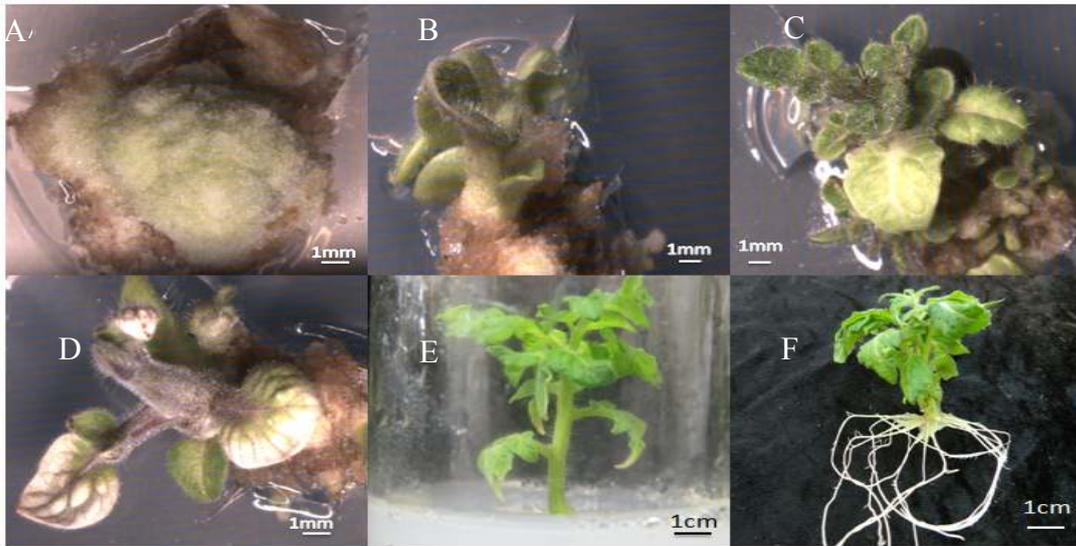
Fig. 9. 35S::SlMYB T1plants, organogenic response of cotyledon explants inoculated with *A. tumefaciens*. (A) Organogenic callus after 2 weeks of culture. (B-D) Organogenic callus with buds begin to form the first adventitious shoots. (D) Adventitious bud after 6 weeks of culture. E. Individual plants on rooting medium. (F) Transgenic rooted plant, ready for acclimatization in the greenhouse, after 12 weeks of culture.
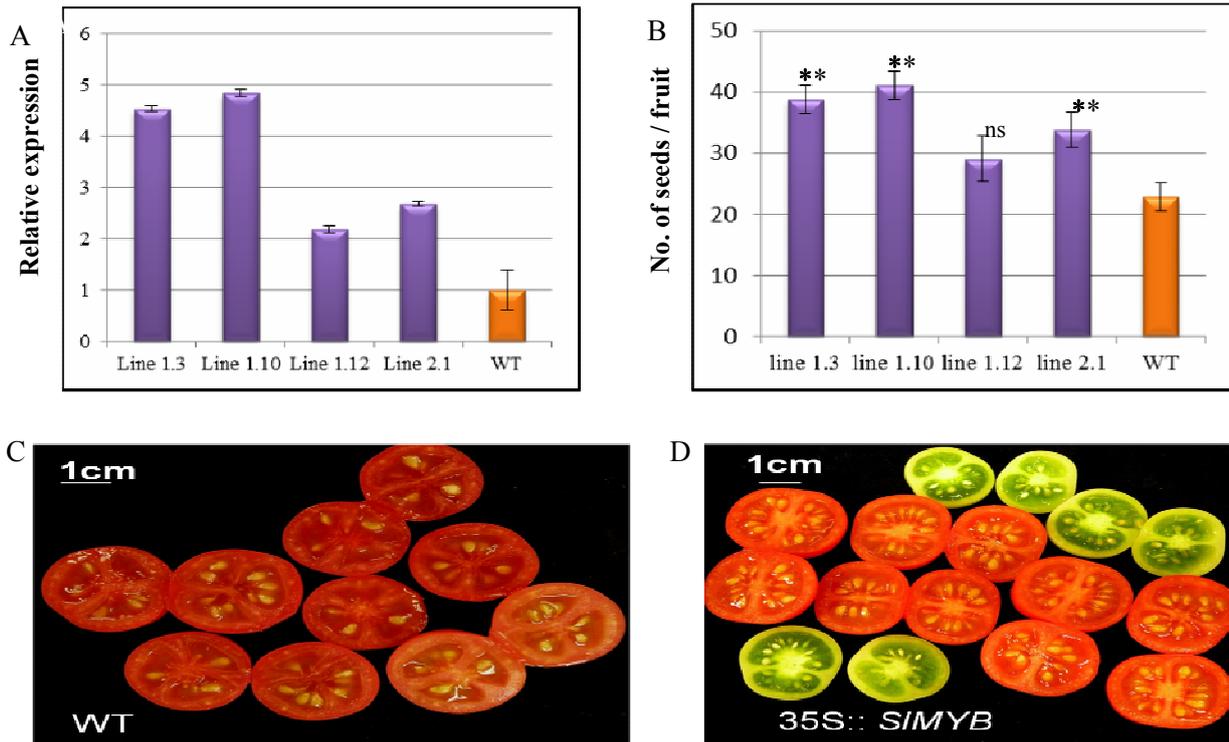
Fig. 10.A. Relative Expression of SlMYB gene in flowers at anthesis of T3 lines and wild type tomato cv. Micro-Tom. The values are relative to the expression level in WT. The level of gene expression was quantified by qRT-PCR using specific primers and the SlACT8 endogenous gene as a normalizer. B. Number of seeds of T3 homozygous plants and wildtype. C. Fruits morphology of nontransgenic plants (WT). D. Fruits morphology of T3 transgenic lines of 35S::SlMYB overexpression.

Note: In figure B the presented data is the Mean values of 20 fruits (4 fruits x 5 plants/line and standard errors,

after two inverted Gateway cloning sites separated by an intron. Thus, SlMYB cDNA fragment was cloned into the vector pK7GWIWG2 twice, in direct and inverted form, separated by intron (Fig. 1 materials and methods) generating the construction RNAi-SlMYB which used to genetically transform tomato plants Micro-Tom cultivar (the same as mentioned in the gain of function section), 4 independent plants were obtained for this construction. The transgenic plants were analyzed by flow cytometry to assess ploidy level, only one of them was diploid (2n). Relative expression of SlMYB gene was measured in flowers at anthesis in T1 plant and showed that the gene of interest is completely scilenced (Fig. 11F). T1 plant showed no differences in the vegetative development. The stable silencing of SLMYB gene leads to the reduction of pollen grain fertility as found in pollen grain germination experiment (2.91% in RNAi pollen, 60.77% in control pollen) (Fig. 11 D, E), consequently the prevention of fruit set and fruit development (Fig. 11A). To check the effect of SLMYB on the development of flower ovary, RNAi plants were used as female and wild type plants were used as male plants in a hybridization experiment, the resulted fruits were seedless fruits (Fig. 11 G, H) indicating that the silencing of the gene of interest leads to the undevelopment of the ovules which insure that the SLMYB gene is involved in fruit development and seed formation.

Transient silencing

In the case of the transient silencing, the method of virus induced gene silencing (VIGS) was used. The PTRV2-GW was used as a destination vector where a 290 bp fragment corresponding to the 3' region of the coding sequence of SlMYB gene was recombined. A mixture of *Agrobacterium* cultures containing (pTRV1 + pTRV2 - SlMYB) were infiltrated onto the cotyledons and lower leaves of 3-week-old tomato cv. Micro-Tom plants. As a positive control the expression of the endogenous phytoene desaturase gene (PDS) in Micro-Tom tomato was suppressed. A mixture of *Agrobacterium* cultures containing pTRV2, carrying tomato PDS (pTRV2-PDS) and pTRV1 were infiltrated onto the cotyledons and lower leaves of 3-week-old tomato plants. These plants developed a photo-bleached phenotype in the upper leaves 10 days post-agro-infiltration Fig. 12A shows the agroinfiltration of pTRV-PDS resulted in the PDS silencing phenotype in five out of five tomato plants (100% efficiency), while the agroinfiltration of pTRV-SlMYB resulted in the SlMYB silencing phenotype in 7 out of 10 tomato plants (70% efficiency). The relative expression of SlMYB was measured in flowers at anthesis by using qPCR. The analysis showed low levels of expression in silenced plants compared to a normal level in control plants (Fig. 12 G). The phenotype resulting from SlMYB silencing was the reduction of fruit set percentage (13.4%:35.1% in silenced plants and 61.25% in control plants) (Fig. 12E, F) and the production of low number of fruits with very low number of seeds compared to the control plants (Fig. 12 D, H) compared to a normal number of seeds in negative control plants.

It was demonstrated in several studies that VIGS *via* the pTRV system is a fast and highly effective tool for gene downregulation in tomato (Liu *et al.*, 2002; Burch-Smith *et al.*, 2004; Dong *et al.*, 2007). The PDS gene was chosen as a positive control to test the ability of the system to induce gene silencing because it allows the detection of symptoms in different plant organs such as stems, leaves, pedicels, sepals, petals and also in anthers (Hileman *et al.*, 2005).

The 290 bp fragment that was used for silencing seems to be specific to the gene of interest suggesting that the obtained phenotypes by silencing is specific to the SlMYB.
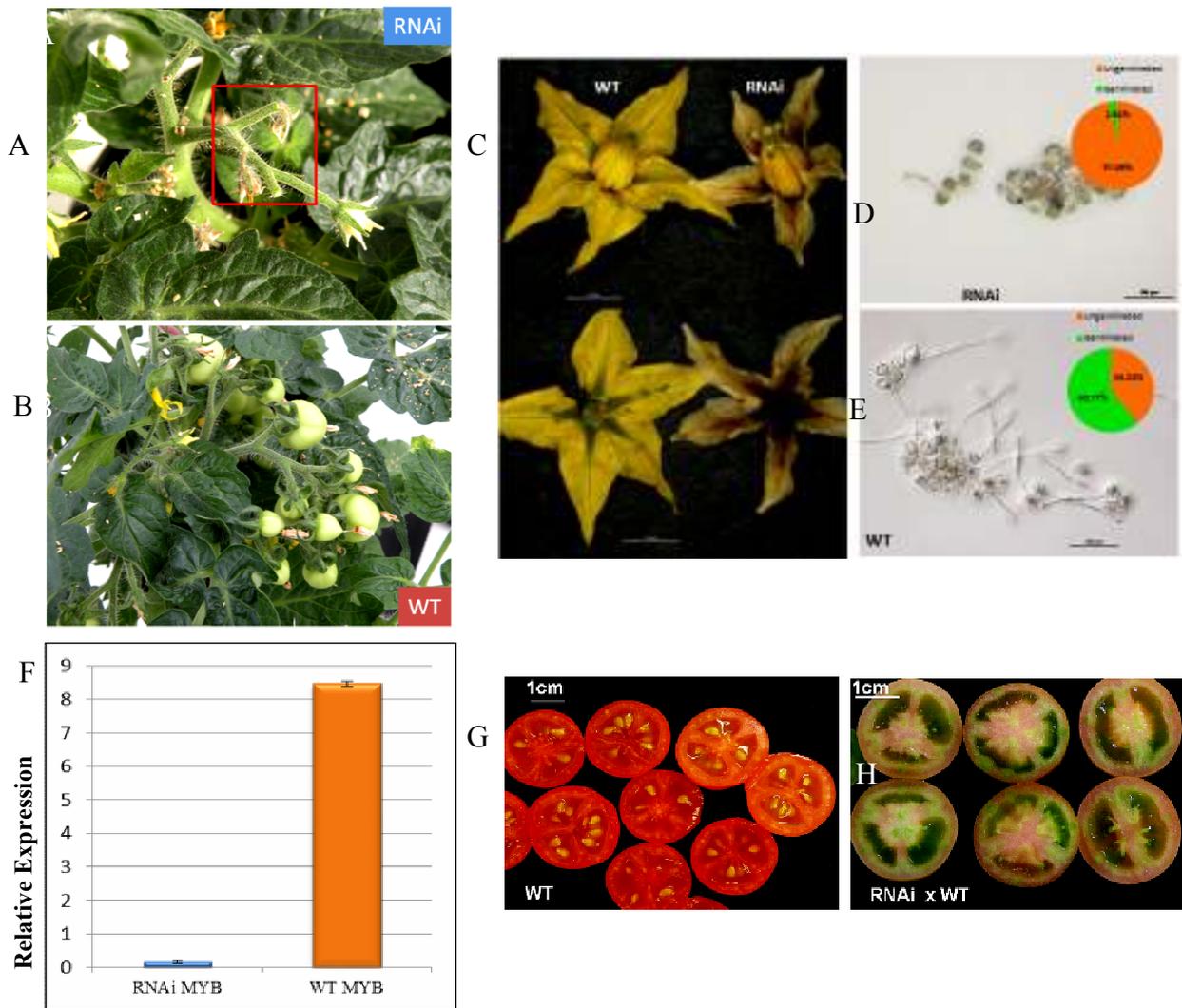
Fig. 11. Morphological characterization of SlMYB gene silencing in tomato plants by RNAi method. (A) Morphology of RNAi plant showing the death of flowers as a result of SLMYB gene scilencing. (B) Morphlogy of control plant with normal fruit set. (C) Morphology of flowers in RNAi and control plants. (D) Percentage of pollen grains germination RNAi plant. (E) Percentage of pollen grains germination RNAi plant. (F) Relative Expression of SlMYB gene in flowers at anthesis of T1 RNAi line and wild type tomato cv. Micro-Tom. (G) Fruits of control plant with normal seeds. (H) Fruits resulted from hybridization of RNAi plants with control plant showing undeveloped ovules
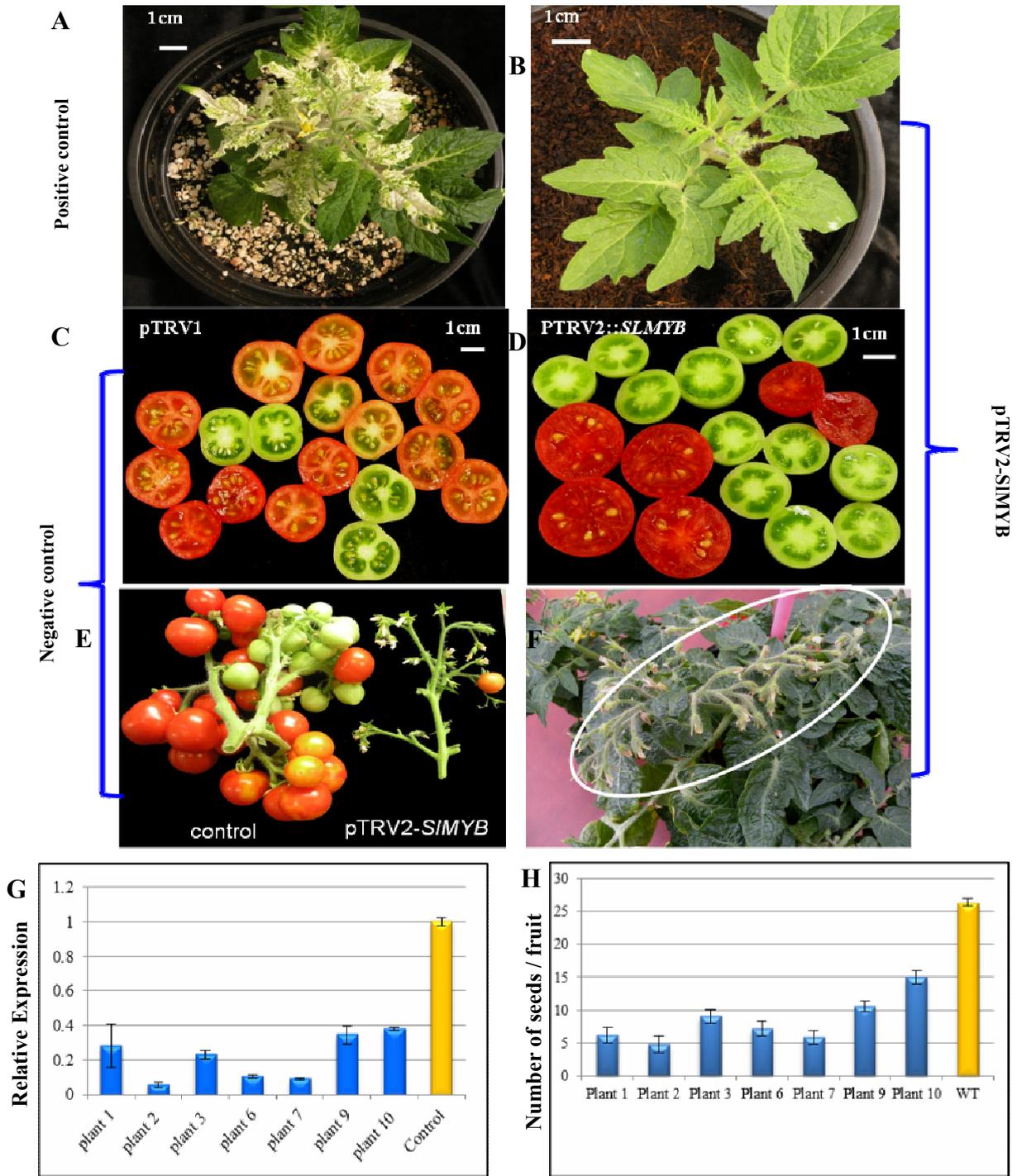
Fig. 12. Morphological characterization of SlMYB gene silencing in tomato plants by VIGS method. (A) Tomato plant cv. Micro-Tom 10 days post-agro-infiltration with PDS gene (control). (B) Infiltrated plant using pTRV1 and pTRV2-SlMYB gene. (C) Fruits of negative control plants with normal number of seeds (D) Fruits of silenced plants fruits with very low number of seeds.(E, F) The phenotype resulting from SlMYB silencing indicating the percentage of fruit set reduction. (G) Relative Expression of SlMYB gene in flowers at anthesis of VIGS plants and wild type tomato cv. Micro-Tom. (H)Number of seeds in VIGS plants and wild type tomato cv. Micro-Tom

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دور الجيـــن SLMYB فـــي تطـــور ثمـــرة الطمـــاطـــم

عبدالله عبدالعظيم حسانين – ســعيد ســعد ســليمان
طارق أبو المحاسن إسماعيل ـ ممدوح كامل على أمين

قسم الوراثة ـ كلية الزراعة ـ جامعة الزقازيق ـ مصر

تنظم عوامل النسخ مستوى الـ RNA والبروتين من خلال عمليات متنوعة، يعتبر تكوين وتطور الثمرة عملية مفصلية فى دوره حياه النباتات حيث توفر الثمار البيئة المناسبة لتكوين وانتشار البذور الناضجة وأيضاً للثمار تأثير وأهميه اقتصاديه من وجهه النظر الزراعية، تهدف الدراسة الحالية إلى توصيف وتحديد وظيفة الجين SLMYB كأحد الجينات المرشحة أن يكون لها دور في عمليه عقد وتطور الثمار فى الطماطم، ولتحقيق هذا الهدف تم استخدام طرق وتكنيكات متنوعة مثل التحليل باستخدام الحاسب الآلي، تحليل الموقع تحت الخلوى، تفاعل البلمرة المتسلسل الكمى، التهجين فى ذات الموقع والتحوير الوراثي للنبات، أوضحت نتائج التحليل باستخدام الحاسب الآلى أن هذا الجين يشفر لبروتين مكون من ١٩٥ حمض أمينى والذي يحتوى على سلسله ارتباط مع الـ DNA مكونه من تتابعين مميزين للبروتين الذى يعمل كعامل نسخ. تحليل الموقع تحت الخلوى أوضح أن البروتين الناتج من الجين موضع الدراسة يعبر عن نفسه فى كل من نواه وسيتوبلازم الخلية، نتائج تفاعل البلمرة المتسلسل الكمى أوضحت أن الجين موضع الدراسة يعبر عن نفسه فقط فى الأنسجة التناسليه حيث أن أعلى مستوى تعبير للجين حدث في الأزهار التى حدث بها تلقيح، نتائج تفاعل البلمرة المتسلسل الكمى والتهجين فى ذات الموقع أظهرت أن التعبير الجينى للجين SLMYB وجد بصوره أساسية فى متوك ومبايض أزهار الطماطم والذى من خلاله يمكن القول بأن الجين موضوع الدراسة يشترك فى عمليه التلقيح وتطور المبيض، ولتوضيح دور الجين SLMYB تم عمل زيادة فى التعبير الجينى لهذا الجين عن طريق إنتاج نباتات محوره وراثيا تحتوى على هذا الجين فى صوره تعبير فائقة وأظهر توصيف نباتات الجيل الثالث من النباتات المحورة وراثيا أن زيادة التعبير الجينى للجين SLMYB أدت إلى إنتاج ثمار بها عدد كبير من البذور، وعلى العكس من ذلك تم عمل اسكات لفعل الجين بطريقه الـ VIGS والتى من خلالها يتم وضع جزء من الجين مع جزء من جينوم الفيرس حتى يحفز النبات على إنتاج RNA مضاد لجينوم الفيرس والذى بدوره يؤدى لإسكات الجين موضع الدراسة، وكانت نتيجة عمل إسكات الجين موضع الدراسة (بطريقه الـ VIGS وهى طريقه مؤقتة) أدى ذلك بدوره إلى تقليل نسبه عقد الثمار (٣٥,١% : ١٣,٤%) مقارنه بنسبه العقد فى نباتات الطراز البرى ٦١,٢٥% كما أدى إلى إنتاج ثمار بها عدد قليل جدا من البذور . ولتأكيد نتيجة الجين تم عمل اسكات لفعل الجين بطريقة الـ RNAi (وهى طريقه دائمة) وقد أدى ذلك الى تقليل خصوبة حبوب اللقاح وتطور المبيض والذى ادى بدوره إلى إعاقة عمليه العقد وتطور الثمار والذى يمكن من خلاله اعتبار أن هذا الجين مهم فى عمليه تطور الزهرة وتكوين البذور.