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Comparative Analysis of Differential Gene Expression in Wild-type and $^{12}C^{5+}$ Ion Beam-induced Abnormal Flower Mutant of *Solanum villosum* by Tomato cDNA Macroarray

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ABSTRACT

The differential expression of genes in an abnormal floral organ mutant of *Solanum villosum*, T-5, was compared with those in wild-type (W-T) plants at the pre-anthesis bud stage using a tomato cDNA macroarray. Genes whose expression was three-fold higher or lower in the mutant than in wild-type plants were considered to be up- or down-regulated, respectively. Of 11520 genes, differential expression of genes was observed in a total of 122 genes out of which 45.9% were down-regulated while 54.1% were up-regulated in the mutant. The functional distribution of differentially expressed genes included cellular biological processes potentially associated with floral patterning such as regulation of metabolism, transcription, cellular communication and signal transduction mechanisms, systemic interaction with the environment and tissue/organ differentiation based on the annotated catalogue of Munich Information Center for Protein Sequences (MIPS). Down-regulated genes with potential effects on floral organ specification included homologues of *Sepallata 1* (*SEP1*), *Agamous* (*AG*), *HUA1* and *Circadian Clock Associated 1* (*CCA1*). With the identification the genes with putative transcription factor activity in the control of T-5 floral organ identity, we have moved closer to a complete understanding of the underlying factors and the culprit gene responsible for the differences in W-T and T-5 mutant.

Keywords: floral organ structure, gene expression, macroarray, mutation, transcription factor activity, temperature sensitivity

INTRODUCTION

Genetic and molecular analyses of floral organ identity mutants in *Arabidopsis thaliana* and *Antirrhinum majus* led to the proposal of the famous ABC(D)E model (Bowman and Meyerowitz 1991; Coen and Meyerowitz 1991; Pelaz *et al*. 2000; Ditta *et al*. 2004; Soltis *et al*. 2007; Causier *et al*. 2010) where overlapping activities of regulatory genes in classes A and E specify the sepals, A, B and E specify the petals, B, C and E specify the stamens while C and E specify the carpels. The D-class was taken up by genes that regulate ovule formation (Colombo *et al*. 1995). Mutations that affect the normal expression of genes responsible for the A-, B-, C- or E-functions result in a floral organ identity shift (Soltis *et al*. 2007; Causier *et al*. 2010).

We have isolated a novel *S. villosum* mutant with abnormal floral organs (T-5) after seed irradiation with 20 Gy $^{12}C^{5+}$ ions (Ojiewo *et al*. 2006). While the wild-type (W-T) *S. villosum* flowers are complete with 5 sepals, 5 petals, 5 stamens and a single pistil, the T-5 mutant flowers consist of numerous large leaf-like sepals in all 4 floral whorls from early-winter to mid-spring (Ojiewo *et al*. 2006). From late-spring through early-summer, some of the largely vegetative floral buds differentiate with a few distorted stamens, which abort one or two days after anthesis, making the flowers stamen-less. In addition, whorl 2 organs maintain the general appearance of petals, but have sepaloid features: petals have greenish tips and margins. In summer, there seems to be an iteration of the floral programme resulting in the production of new floral buds from the centre of each flower making the inflorescence indeterminate. In autumn, the floral organ structure is partially restored, with all the floral whorls present. All these pleiotropic effects begin with leaf-like flower buds and differentiation starts two weeks after the flower bud formation. In addition, T-5 leaves are darker in colour than the W-T leaves (Ojiewo *et al.* 2006).

When grown under controlled conditions in the growth chamber with a constant day temperature of 30°C and low night temperature of 10°C, 75% of the T-5 mutant flowers had only leaf-like organs, while the rest were indeterminate. Under high night temperature conditions (30°C), 65% of the flowers were indeterminate; the rest were sepaloid. Setting the day/night temperatures at 30/20°C resulted in 13% floral organ differentiation but all the flowers were stamen-less, thus infertile. The optimum temperatures for floral structure and fertility restoration were between 20-25°C (day) and 15-20°C (night) (Ojiewo *et al.* 2007a).

In the model plants, A-function mutants have carpels in place of sepals and stamens in place of petals, B-function mutants have sepals in place of petals in the second whorl and carpels in place of stamens while C-function mutants have petals in place of stamens and another flower in place of carpels in a repeated pattern, resulting in indeterminate flowers (Soltis *et al*. 2007; Causier *et al*. 2010) Mutation in one E-function gene does not affect the phenotype, but flowers of plants with mutation in three E-function genes are indeterminate with all the whorls converted into sepals (Pelaz *et al*. 2000) while quadruple mutants develop leaves in all whorls (Ditta *et al*. 2004).

Although the pleiotropic nature of the T-5 mutant complicates direct comparison with any single phenotype in the model plants, it seems plausible to argue that B- or C-function could have been affected. In this study, a tomato cDNA macroarray composed of 11520 EST clones was used to compare the differential gene expression in pre-anthesis flower buds of W-T and T-5 *S. villosum* plants. The objectives of this study were to:

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1) investigate the global relationship among the gene;
2) expression patterns in the two genotypes at pre-anthesis stage of flower buds;
3) use genebank information on molecular functions and corresponding physiological processes of differentially expressed genes to explain the differences between T-5 mutant and the W-T *S. villosum* plants.

MATERIALS AND METHODS

Plant material and RNA extraction

Seeds of W-T and T-5 *S. villosum* were sown on April 1, 2007 in cell flats (30 cm × 60 cm) filled with vermiculite. After germination, the seedlings were fertigated daily with half-strength of a complete nutrient solution containing 8 mM NO_3^-, 0.7 mM NH_4^+, 0.7 mM $H_2PO_4^-$, 4 mM K^+, 1 mM SO_4^{2-}, 2 mM Ca^{2+}, and 1 mM Mg^{2+}. Micronutrients were supplied at full-strength and consisted of 54 μM Fe^{2+}, 46 μM BO_3^{3-}, 9 μM Mn^{2+}, 8 μM Zn^{2+}, 0.3 μM Cu^{2+} and 0.1 m μMoO_4^{2-}. The pH and electrical conductivity (EC) of the solution were 6.5 and 1.25 mS cm^{-1}, respectively. The seedlings were transplanted to plastic pots at three-leaf stage and further to Wagner pots at six-leaf stage. Flower buds were harvested at pre-anthesis stage (**Fig. 1**) using liquid nitrogen-cooled forceps. They were then placed in small holed polythene bags and immediately immersed into liquid nitrogen. Total RNA was extracted from frozen material using QIAGEN RNeasy Plant Mini Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer's protocol and instructions.

Probe preparation and hybridization

Macro-array membranes composed of two nylon membranes (80 to 125 mm each) by a MICROGRID II Robotic workstation (Beckman Instruments, Inc., Fullerton, CA) (Takahashi *et al*. 2006) and consisting of 11520 non-redundant ESTs were supplied by the Japanese Solanaceae Genomics Project (JSOL). The list of EST clones is available online MiBase MicroTom tomato database (http://www.kazusa.or.jp/jsol/microtom/). Total RNA was reverse transcribed to synthesize (α^{33}P)dCPT-labelled cDNA probes using the SuperScript First-strand System for RT-PCR (Invitrogen) (Ishihara *et al*. 2004). The nylon membranes were pre-hybridized at 65°C for 2 h with 8 ml of 0.5 M Church's phosphate (Church and Gilbert 1984) buffer (Na_2HPO_4; pH 7.2) containing 1 mM EDTA, 7% sodium dodecyl sulphate (SDS) and 8 μl of poly(dA) solution (1 μg/μl). Heat-denatured ^{33}P-labeled cDNA was mixed with 8 ml of Church's phosphate hybridization buffer containing 8 μl poly(dA) solution. The pre-hybridization solution was discarded, the labelled cDNA was added to each membrane set in the hybridization bag and hybridization was performed for 20 h at 65°C. After incubation, the membranes were washed once with 1X SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS at 65°C for 15 min, and twice with 0.1X SSC containing 0.1% SDS at 65°C for 30 and 40 min. For detection, the membranes were wrapped with plastic film and exposed to imaging plates (BAS-IP SR 2040; Fuji Photo Film, Tokyo, Japan) for 72 h.

Data analysis

The signals on the imaging plates were scanned with BAS-1800II (Fuji Photo Film, Tokyo, Japan) and quantified using the Array Vision 5.1 software (Imaging Research Inc., Ontario, Canada). The log-transformed signal intensities were normalised (Obayashi *et al*. 2004). Background noise was corrected for as follows:

$$ESTspot = \left\{ \frac{(\mathrm{RAW}_{\mathrm{ESTclones}} - \mathrm{vBG}_{\mathrm{membrane}})}{\mathrm{median}(\mathrm{RAW}_{\mathrm{ESTclones}})} \right\} \quad [1]$$

where *ESTspot* is the established sequence tag spot intensity; $RAW_{ESTclones}$ is the set of raw data of EST clones; *vBGmembrane* is the virtual background of the membrane calculated as:

$$vBGmembrane = 0.8 \times \min(RAWall\ spots) \quad [2]$$

Fig. 1 The structure of pre-anthesis floral buds at the time of sampling and RNA extraction. While the floral whorls of W-T buds (**A**) already show signs of appearing, the T-5 mutant floral buds are largely sepaloid (**B**).

where *RAWall spots* is the set of raw data of all spots on the membrane. The coefficient 0.8 has long been established and optimized for macroarray system by estimation of the fluctuation scales for weak signals (Obayashi *et al*. 2004).

T-tests were performed on the log-transformed expression values to identify the genes with similarly altered expression patterns in five replicate experiments. The mean of the normalized value of the signal intensity for each gene in the five replicate experiments was adopted as the expression value of the gene. The ratio of induction or suppression of each gene was calculated by dividing the expression value of T-5 by that of W-T. Values less than 1 were transformed to -1/ratio and considered suppression. Genes were considered to be differentially expressed if the T-5 value was three-fold higher (up-regulated) or lower (down-regulated) than the W-T.

The putative function of each gene product was determined using The Institute for Genomic Research (TIGR) tomato genome index (http://compbio.dfci.harvard.edu) with cross references to the GeneBank of the National Center for Biotechnology Information/National Institutes of Health (http://www.ncbi.nlm.nih.gov), Uniprot/Swissprot (http://www.uniprot.org), Solanaceae Genome Project Network (SGN) Tomato unigene (http://www.sgn.cornell.edu), Arabi Protein (http://www.arabidopsis.org) and Rice Protein (http://rapdb.dna.affrc.go.jp). The differentially expressed genes were categorized according to cellular biological processes in which they are involved, such as metabolism, cellular transport, response to stimuli, defence, translation and regulation of transcription based on the annotated catalogue for functional distribution of genes of the Munich Information Center for Protein Sequences (MIPS) (MIPS FunCat; http://mips.gsf.de/proj/funcatDB/search_main_frame.html).

RESULTS AND DISCUSSION

The scatter plot of the signal strength of each spot (gene) is shown in **Fig. 2**. Spots were distributed linearly (y = 0.94) and the correlation coefficient (R^2) was 0.89. With very little variation between the five selected replicates, it is assumed that the macroarray technique provided authentic and reliable gene expression data. A total of 122 out of 11520 genes exhibited a differential expression pattern, of which 66 (54.1%) were up-regulated (**Table 1**) while the remaining 56 (45.9%) were down-regulated (**Table 2**) in the T-5 mutant as compared to the W-T *S. villosum*.

The functional classification of protein products was based on the MIPS annotated catalogue for functional distribution of genes (MIPS FunCat), a hierarchically structured classification system that puts genes involved in more than one protein products into several categories (Ruepp *et al*. 2004). The majority of the differentially expressed genes are involved in sub-cellular localization, with 26.2 and 28.8% of the up-regulated and down-regulated genes, respectively, based on MIPS FunCat (**Table 3**). The influence of this category of genes on the morphological differences between the two genotypes is unclear. Several differentially

Table 1 List of induced genes and the level of induction in the pre-anthesis T-5 *S. villosum* mutant flower buds.

JSOL clone number/name[a]	Product annotation/Putative function[b]	Relative expression value (Mean ± SE)[c]		Induction fold[d]
FA21BB10	leucine rich-repeat (LRR)	1.0 ± 0.3	19.5 ± 2.1	19.1
LC12DD10	no suitable match	1.5 ± 0.3	22.2 ± 2.2	15.1
FA25DH08	ATP-binding	3.2 ± 0.4	45.0 ± 1.4	14.1
FA13AF04	unnamed protein product	1.0 ± 0.1	13.3 ± 0.8	12.8
FA05AB12	putative WRKY transcription factor 30	2.5 ± 0.1	25.3 ± 1.6	10.1
LC09AC03	no suitable match	3.0 ± 0.2	20.6 ± 2.6	6.9
LC16DG09	no suitable match	0.7 ± 0.1	4.6 ± 0.4	6.9
LC19CF09	DNAJ heat shock N-terminal domain-containing protein	1.9 ± 0.3	12.7 ± 1.0	6.8
LC22BB03	40S ribosomal protein S5	5.9 ± 0.8	39.7 ± 3.8	6.7
LC05BC04	no suitable match	7.0 ± 1.0	45.4 ± 5.0	6.5
LC13DB07	no suitable match	7.3 ± 1.3	43.9 ± 0.8	6.0
FA05BD07	actin filament binding	6.2 ± 0.2	37.5 ± 2.8	6.0
LB12AF08	ferredoxin-1, chloroplast precursor	1.6 ± 0.1	9.4 ± 1.4	5.9
LC01DD10	no suitable match	5.4 ± 0.8	31.2 ± 1.8	5.8
FA13BF02	Peptidase family M48	4.0 ± 0.6	21.5 ± 1.2	5.4
FA35BA01	no suitable match	2.8 ± 0.2	14.7 ± 1.4	5.2
FA11DB10	heparanase-like protein 3 precursor	1.0 ± 0.2	5.3 ± 0.5	5.2
LC17CE08	no suitable match	1.7 ± 0.2	8.5 ± 0.7	5.1
LC04CE03	RuBisCO activase 1; chloroplast precursor	1.6 ± 0.2	7.8 ± 0.8	5.0
FA25BB06	unnamed protein product	4.7 ± 0.6	22.3 ± 1.1	4.8
FA08CC09	no suitable match	6.0 ± 0.9	28.0 ± 3.3	4.7
FA22CE07	xyloglucan endotransglucosylase-hydrolase XTH9	7.3 ± 0.9	33.3 ± 1.9	4.6
LA21DD10	no suitable match	1.5 ± 0.2	6.9 ± 0.8	4.6
FB14CG01	no suitable match	3.4 ± 0.9	14.7 ± 1.0	4.3
LC17BC12	benzylalcohol acetyltransferase	7.8 ± 0.2	33.1 ± 1.8	4.3
FA18DC10	fructokinase 3	1.2 ± 0.3	4.7 ± 0.3	4.0
LC06CC03	no suitable match	14.9 ± 1.2	58.6 ± 5.0	3.9
LC21AA11	transcription factor, homeobox 2 protein	2.3 ± 0.1	9.0 ± 0.9	3.9
LC05AH12	proton pump interactor	13.5 ± 1.0	52.8 ± 3.6	3.9
FA24BH07	ATP binding protein	1.4 ± 0.2	5.2 ± 0.7	3.8
FA33AF07	no suitable match	1.4 ± 0.1	5.3 ± 0.7	3.8
LC18AE03	no suitable match	5.8 ± 0.2	21.7 ± 1.3	3.7
LC17CE07	auxin repressed dormancy associated protein	5.8 ± 0.6	21.3 ± 1.5	3.7
LB14AG01	no suitable match	5.1 ± 0.9	18.8 ± 1.8	3.7
LB12DH12	cytokinesis-related Sec1 protein (KEULE)	4.1 ± 0.1	15.0 ± 1.3	3.6
FA03CC08	EIN3-like protein	6.6 ± 0.6	22.9 ± 1.8	3.4
LC03CA07	putative elicitor-responsive Dof protein	5.5 ± 0.4	18.8 ± 1.9	3.4
LC13BH12	no suitable match	14.1 ± 0.4	48.3 ± 2.3	3.4
LC09BH06	metallothionein-like protein	12.5 ± 0.6	42.9 ± 2.3	3.4
LC04CE09	no suitable match	5.1 ± 0.3	17.4 ± 0.2	3.4
LC09AH06	D12 oleate desaturase	9.6 ± 1.3	32.5 ± 0.9	3.4
LB01DE01	histone H3.2 protein	11.9 ± 1.5	40.1 ± 2.1	3.4
FA33DD04	no suitable match	0.7 ± 0.1	2.4 ± 0.2	3.4
LC01BC12	unknown protein	6.6 ± 0.8	22.1 ± 1.1	3.4
FA09DD07	putative serine carboxypeptidase II-3 precursor	3.6 ± 0.6	12.0 ± 0.6	3.4
LC21BE08	zinc transporter	11.5 ± 0.7	38.5 ± 2.6	3.4
LC09DE06	Syntaxin 81 (AtSYP81)	14.4 ± 0.9	46.4 ± 3.1	3.2
FA14BE05	no suitable match	2.8 ± 0.3	8.9 ± 1.1	3.2
FA25DH07	no suitable match	6.9 ± 0.6	22.0 ± 1.2	3.2
LC02BG05	no suitable match	4.3 ± 0.4	13.7 ± 1.0	3.2
FA10BD12	glutamine amidotransferase/cyclase	1.8 ± 0.3	5.8 ± 0.9	3.2
FA04CG03	putative protein of unknown function	6.7 ± 0.8	21.0 ± 2.1	3.2
FA30DE01	no suitable match	2.2 ± 0.6	6.8 ± 0.8	3.1
LC06DC01	no suitable match	14.1 ± 1.0	44.4 ± 3.5	3.1
FA16CF04	no suitable match	4.5 ± 0.8	13.9 ± 1.4	3.1
LC13AE02	lipase class 3 family protein	10.2 ± 0.9	31.6 ± 0.5	3.1
FA30DD04	ADP-RIBOSYLATION FACTOR 1	1.7 ± 0.1	5.2 ± 0.6	3.1
LA27DH02	negative regulator of systemic acquired resistance	5.2 ± 0.3	15.9 ± 0.7	3.1
LA28AH12	no suitable match	0.5 ± 0.1	1.5 ± 0.9	3.0
LC01BD08	no suitable match	14.9 ± 1.3	45.2 ± 3.1	3.0
LC09BF03	unnamed protein product	4.1 ± 1.5	12.3 ± 0.6	3.0
LC07DD06	no suitable match	1.6 ± 0.2	4.7 ± 0.9	3.0
FA26BF07	myosin-like protein	2.3 ± 0.8	6.8 ± 1.0	3.0
LC09BB06	PAPS-reductase-like protein	6.2 ± 0.1	18.6 ± 1.5	3.0
FA06AH05	Lycopene beta cyclase	3.8 ± 1.0	11.4 ± 1.0	3.0

[a]Japanese Solanaceae Genomics Project (JSOL) numbers were assigned using the MiBASE Micro-Tom database (http://www.kazusa.or.jp/jsol/microtom/).

[b]Putative function of each gene product was determined by BLASTN or BLASTX homology search using The Institute for Genomic Research (TIGR) tomato genome index (http://compbio.dfci.harvard.edu) with cross references to the GeneBank of the National Center for Biotechnology Information/National Institutes of Health (http://www.ncbi.nlm.nih.gov), Uniprot/swissprot (http://www.uniprot.org), SGN Tomato unigene (http://www.sgn.cornell.edu), Arabi Protein (http://www.arabidopsis.org) and Rice Protein (http://rapdb.dna.affrc.go.jp)

[c]The average of the normalized value of the signal intensity for each gene in five replicate experiments was adopted as the expression value of the gene. One-way analysis of variance of the expression values was performed to identify the genes having similar altered expression patterns in four replicate experiments. The gene in which expression level was significantly different between T-5 and W-T was selected using Fisher's least significant difference procedure ($P < 0.05$).

[d]Induction fold of each gene in T-5 and W-T *S. villosum* was calculated by the expression value for T-5 divided by that of W-T. Then, when the value of gene expression increased more than 3-fold in the T-5 mutant plant, the gene expression was identified as induced.

Table 2 List of genes with suppressed expression and the level of suppression in the pre-anthesis T-5 *S. villosum* mutant flower buds.

JSOL clone number/name[a]	Product annotation/Putative function[b]	W-T	T-5	Suppression fold[d]
LC08DG12	unknown protein	5.0 ± 0.1	0.6 ± 0.1	8.6
FA11AF09	cupin family protein	6.4 ± 0.2	1.0 ± 0.2	6.5
FA19BF02	L-allo-threonine aldolase-related protein	8.8 ± 0.1	1.7 ± 0.1	5.2
FA16BD05	unknown protein	4.7 ± 0.1	1.0 ± 0.1	4.6
FA35AE06	floral homeotic protein HUA1	1.7 ± 0.1	0.4 ± 0.1	4.4
FA27DH04	pectinesterase-1 precursor	23.1 ± 0.6	5.3 ± 0.6	4.4
LA16DA11	60S ribosome protein L19-like	48.2 ± 1.0	11.1 ± 1.0	4.3
FA30AG12	putative plasma membrane intrinsic protein	3.8 ± 0.1	0.9 ± 0.1	4.1
LA23AG04	40S ribosomal protein S3a-like	14.3 ± 0.5	3.5 ± 0.5	4.0
LC10BB09	polygalacturonase non-catalytic	3.4 ± 0.2	0.8 ± 0.2	3.9
LA19DC05	enolase (2-phosphoglycerate dehydratase)	30.7 ± 0.6	7.8 ± 0.6	3.9
FA36AF06	T-complex protein 1 subunit epsilon	14.1 ± 0.7	3.6 ± 0.7	3.9
FA36AC05	mandelonitrile lyase	6.8 ± 0.3	1.8 ± 0.3	3.8
LA13CA05	putative lipid acyl hydrolase	7.6 ± 0.4	2.0 ± 0.4	3.8
FA54DG01	40S ribosomal protein SA (p40)	19.3 ± 1.2	5.2 ± 1.2	3.7
FB02AB08	serine carboxypeptidase S28 family protein	44.6 ± 2.1	12.3 ± 2.1	3.6
FA11BC06	malate dehydrogenase	3.3 ± 0.2	0.9 ± 0.2	3.6
FA14BC10	cysteine protease	4.6 ± 0.1	1.3 ± 0.1	3.6
LC13BE09	ATP-dependent RNA helicase-like protein	1.0 ± 0.1	0.3 ± 0.1	3.6
FB12DC07	unknown protein	14.3 ± 0.1	4.0 ± 0.1	3.6
LA18CB01	60S ribosomal protein L5	6.0 ± 0.4	1.7 ± 0.4	3.6
FA12BC05	inositol-3-phosphate synthase	17.6 ± 0.8	5.0 ± 0.8	3.5
LB04BD12	40S ribosomal protein S6	4.1 ± 0.1	1.2 ± 0.1	3.5
FA51AD08	DNA-binding protein	19.2 ± 1.0	5.5 ± 1.0	3.5
FA02DE12	elongation factor 1-gamma 2	20.8 ± 0.5	6.0 ± 0.5	3.5
LA19BB08	no suitable match	27.6 ± 0.8	7.9 ± 0.8	3.5
FB08BB08	ubiquitin	18.7 ± 1.1	5.4 ± 1.1	3.5

[a]See **Table 1**
[b]See **Table 1**
[c]See **Table 1**
[d]Suppression fold of each gene in T-5 and W-T *S. villosum* was calculated by the expression value for T-5 divided by that of W-T. Ratios of <1 were transformed to –1/ratio.Then, when the value of gene expression decreased more than 3-fold in the T-5 mutant plant, the gene expression was identified as suppressed. Genes with expression suppression below 3.5 are not shown.

Table 3 Classification of induced and suppressed genes according to their putative functions based on MIPS Functional Catalogue (MIPS Funcat).

Mips No.	Functional category	Distribution of genes			
		Induced genes		Suppressed genes	
		No. of entries	(%)	No. of entries	(%)
01	Metabolism	16	12.3	6	4.8
02	Energy	4	3.1	3	2.4
04	Storage Protein	0	0.0	1	0.8
10	Cell cycle and DNA processing	4	3.1	1	0.8
11	Transcription	3	2.3	4	3.2
12	Protein synthesis	2	1.5	16	12.8
14	Protein fate (folding, modification and destination)	13	10.0	6	4.8
16	Protein with binding function of cofactor requirement	18	13.8	23	18.4
18	Regulation of metabolism and protein function	4	3.1	1	0.8
20	Cellular transport, transport facilities and transport routes	6	4.6	7	5.6
30	Cellular communication, signal transduction mechanism	2	1.5	0	0.0
32	Cellular rescue, defence and virulence	2	1.5	6	4.8
34	Interaction with the environment	5	3.8	6	4.8
36	Systemic interaction with the environment	2	1.5	1	0.8
40	Cell fate	1	0.8	0	0.0
41	Development (systemic)	1	0.8	3	2.4
42	Biogenesis of cellular components	4	3.1	1	0.8
45	Tissue differentiation	1	0.8	0	0.0
47	Organ differentiation	0	0.0	2	1.6
70	Subcellular localization	34	26.2	36	28.8
77	Organ localization	1	0.8	1	0.8
99	Unclassified proteins	7	5.4	1	0.8

expressed genes fell into categories with potential roles in the determination of T-5 phenotype, such as regulation of metabolism, transcription, cellular communication and signal transduction mechanisms, systemic interaction with the environment and tissue/organ differentiation. Some genes coded for proteins with unknown, hypothetical or ill-defined functions. These are indicated as 'no suitable match' from the genebank data or as 'unclassified proteins' based on MIPS FunCat. This category could probably be novel genes in *S. villosum* that are not yet described in other species.

Some genes exhibited high up- or down-regulation in the T-5, but initial EST search on MiBase and further BLAST search did not reveal functions that could link them either directly or indirectly to any role in the formation of normal W-T flower or temperature-sensitive dynamics of T-5 floral organs. Three of the down-regulated genes with putative transcription factor activity – *HUA1* (hybridizing with clone number FA35AE06), *Sepallata 1 (SEP1)* homologue (hybridizing with clone number FA36AA06) and

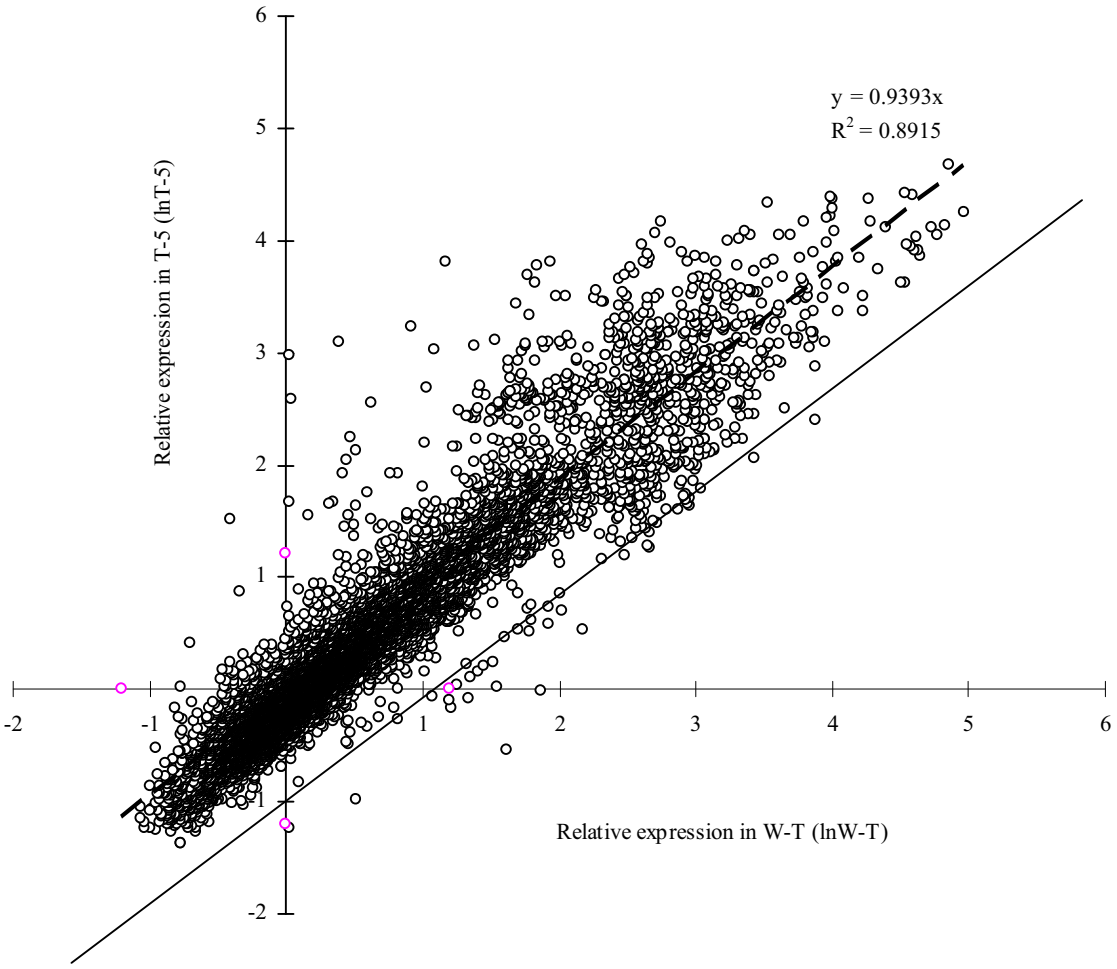

Fig 2 Correlation between the signal strength of the spots in the W-T and those in the T-5 mutant. Dotted line denotes the regression line. Two solid lines delimit spots with a signal intensity more than or less than 3-fold in relation to the regression line.

Table 4 Differentially expressed genes with putative roles in flower development and floral organ specification.

JSOL clone number/name	Product annotation/Putative function	Domain	Cellular function	Physiological process	Suppression (fold)
FA35AE06	Floral homeotic protein HUA1	Putative zinc finger	RNA binding	cell fate determination	4.4
FA36AA06	Developmental protein SEPALLATA1 (SEP1)	MADS-box	transcription factor activity; DNA binding	-flower development -ovule development	3.2
FA24AA05	Floral homeotic protein AGAMOUS (TAG1)	MADS-box	transcription factor activity; DNA binding	-carpel development -stamen development -maintenance of floral organ identity	3.1
LC17DD01	Circadian clock associated1 (CCA1)	MYB family	transcription factor activity; DNA binding	-regulation of circadian rhythm -regulation of transcription	3.0

Agamous (AG) homologue (hybridizing with clone number FA24AA05) – have more direct roles in floral organ patterning and were probably affected at a point upstream or downstream in the floral organ determination pathways (**Table 4**). The ABCE genes regulating the floral organ development encode transcription factor complexes that activate or inhibit specific target genes for the formation of sepals (A- and E-function genes), petals (A-, B- and E-function genes), stamens (B-, C- and E-function genes) and carpel (C- and E-function genes) (Bowman and Meyerowitz 1991; Coen and Meyerowitz 1991; Pelaz *et al*. 2000; Ditta *et al*. 2004; Soltis *et al*. 2007; Causier *et al*. 2010). In a hypothesis paper, we suggested that the protein complex transcription factors may operate by binding to two CArG-box sequences of a target promoter, either activating or repressing expression of the downstream *S. villosum* gene (Ojiewo *et al*. 2007b). For example, in petals, the putative heterodimer SvAP3-SvPI was proposed to bind to one CArG box as SvAP1-SvSEP heterodimer binds to a second CArG box. In this study, the expression patterns of a number of gene homologues with putative transcription factor activities were down-regulated in the T-5 mutant as compared to the W-T *S. villosum* (**Table 4**).

The down-regulation of E-function SEP 1 homologue (clone number FA36AA06) in the T-5 *S. villosum* mutant, suggests a potential suppressed functionality of the gene or another gene in the *SEP* pathway. While the W-T plants have perfect flowers (**Fig. 3A**), the near 'vegetative' flower buds of the *S. villosum* T-5 mutant in as temperatures rise (**Fig. 3B**) closely mimic the Arabidopsis ABC triple loss-of-function mutants (Bowman and Meyerowitz 1991) or *sep1 sep2sep3sep4* quadruple loss of E- function mutant (Ditta *et al*. 2004). In both mutants, all floral organs are transformed into leaf-like organs. The purely sepaloid indeterminate phenotype of T-5 mutant as the day temperature rises above 25°C (**Fig. 3D**) resembles that of *Arabidopsis* E (*sep1sep2 sep3*) (Coen and Meyerowitz 1991) where, only sepals are produced and the flowers form new floral buds from the central meristematic region.

The down-regulation of the C-function AG homologue (clone number FA24AA05) suggests a loss of functional interaction of an AG hormologue in *S. villosum* (*SvAG*)

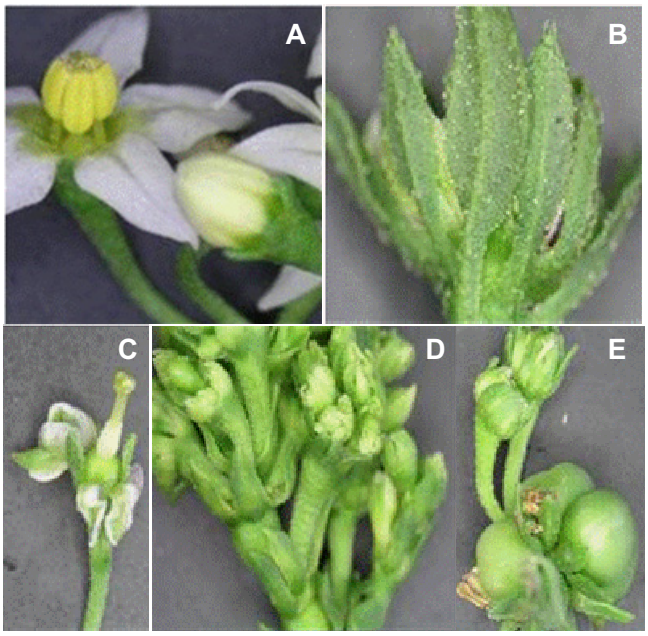

Fig. 3 Structure of *S. villosum* wild-type (**A**) flower and T-5 mutant flowers in late-winter to early-spring (**B**), mid- to late-spring (**C**), summer (**D**) and autumn (**E**).

with B or E genes (**Fig. 3D**). In *Arabidopsis*, loss of function of AG results in the homeotic conversion of reproductive organs into perianth organs. The stamen-less phenotype of T-5 mutant under high temperature (**Fig. 2C**) is similar to that of thermo-sensitive loss-of-B-function mutants of *Antirrhinum*, *def101* (Schwarz-Sommer *et al*. 1992) and *Arabidopsis*, *ap3-1* (Bowman *et al*. 1989) grown under restrictive temperatures. Like *def101* and *ap3-1*, T-5 is temperature sensitive (Ojiewo *et al*. 2007a). However, unlike the two thermo-sensitive model plant mutants, T-5 has several 'floral phases', making its floral dynamics unique.

HUA1 homologue (clone number FA35AE06) down-regulated in the *S. villosum* T-5 mutant is associated with *AG*. Recessive mutations in *HUA1* together with *HUA2* exhibit the floral homeotic phenotype of ag-4 mutant (Chen *et al*. 1999; Prunet *et al*. 2009). The double mutant, *hua1hua2* exhibits weak stamen-to-petal and carpel-to-sepal transformations, that indicates the partial loss of C function in the flower. *HUA1*, together with other genes (*HUA2*, *HEN2* and *HEN4*) promote the production of AG mRNA by facilitating efficient splicing or by preventing alternative polyadenylation, thus regulating *AG* expression (Cheng *et al*. 2003). A temperature-sensitive splicing defect resulting in unstable interaction between *S. villosum* HUA1 microRNA homologue and that of other HUA gene homologues and inhibiting the production of AG mRNA homologue could be responsible, at least in part, for the *S. villosum* T-5 phenotypes.

Under controlled conditions, T-5 flowers are generally sepaloid at low growth chamber (10°C) and greenhouse (<15°C) night temperature, indeterminate at high growth chamber (30°C) and greenhouse (>25°C) night temperatures, stamen-less at day/night temperatures of 30/20°C and its structured and fertility are restored to near normal between 20-25°C (day) and 15-20°C (night) (Ojiewo *et al*. 2007a). We have reported that the temperature condition during plant growth is as important as that during and after pollination in a thermo-sensitive tomato mutant T-4 whose fertility is restored in autumn but not in spring (Masuda *et al*. 2007). Thus, although the temperature conditions in spring and autumn are more or less similar, it may not be surprising that the floral morphology of the T-5 *S. villosum* mutant in the two seasons are different, given the sharply contrasting preceding conditions in winter and in summer.

Gene interaction with other genes or with the environment can result in a complex system when mutation is not controlled at specific loci. While genetic engineering procedures can be used to induce specific mutations at target gene loci, microdosimetric and radiobiological studies have revealed that high LET (linear energy transfer) radiation, such as ion particles, could produce double-strand DNA breaks (Blakely and Kronenberg 1998) and multiple mutations at unspecified loci within the same genome system (Shikazono *et al*. 2001). Therefore, it seems plausible that multiple mutations could be responsible for the T-5 mutant phenotype.

Having shown that there are differences in the expression patterns of genes with potential role in floral patterning, and thus the differences between the flowers of T-5 plants and those of W-T plants, it is now essential to isolate, clone and sequence these genes for further expression analyses. Further studies to establish the exact structures of the putative transcription factor complexes and the target genes they control during the development of floral organ identity of the W-T flowers and how these are affected in the T-5 mutant flowers will be necessary. Isolation of the MADS-box orthologues determining floral organ identity in *S. villosum* and the analysis of the effects of temperature on their expression or activity in the T-5 mutant is also important to completely unravel the mystery surrounding the thermo-sensitive floral dynamics.

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