DOI: 10.1007/s10535-016-0655-x BIOLOGIA PLANTARUM 61 (1): 55-63, 2017

Mobilization of the *Tetu1* transposable element of *Helianthus annuus*: evidence for excision in different developmental stages

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Abstract

The *tubular ray flower* (*turf*) mutant of sunflower is characterized by a switch of ray flowers from zygomorphic to near-actinomorphic disc flowers. In sunflower, floral symmetry of ray and disc flowers is specified by the activity of members of a *CYCLOIDEA* (*CYC*) gene family. The *turf* mutant is generated by the insertion of a CACTA-like transposable element (TE), named *Transposable element of turf1* (*Tetu1*), in the coding sequence of the *HaCYC2c* gene. The TE insertion changes the reading frame of *turf-HaCYC2c* for the encoded protein and leads to a premature stop codon. *Tetu1* is a non-autonomous version of a CACTA TE carrying the minimum sequences necessary for transposition in the presence of autonomous elements in the sunflower genome. In the previous analysis, performed in more than 11 000 plants homozygous for the *turf-HaCYC2c* allele, the absence of chimerism and the segregation rate of derived-progenies from reverted phenotypes suggest that *Tetu1* transpositions are restricted to a time shortly before and/or during meiosis. Here, we report the analysis of F_5 and F_6 progenies, derived from an F_4 progeny of the cross *turf* × *Chrysanthemoides2*, where plants with a chimeric inflorescence were detected. *Tetu1* showed active excision in all progenies taken into consideration and named High Frequency of *Tetu1* Transposition (HFTT). Within a total of 449 plants, *Tetu1* excision generated a 13.81 % of non-chimeric revertants but also a 5.12 % of plants with somatic sectors of variable size in the outmost whorl of the inflorescence. These unexpected results suggest variations in tissue specificity and time of TE excision. The excision of *Tetu1* was confirmed by DNA molecular screening of non-chimeric and chimeric revertants and transcription analysis of the *HaCYC2c* gene. In HFTT progenies, sequence analyses excluded significant DNA changes with respect to the original *Tetu1* transposon as well as to the adjacent 5'- and 3'-TE regions. Genetic and epigenetic regulatory mechanisms were proposed to explain the time and frequency of *Tetu1* transposition in HFTT progenies.

Additional key words: CACTA, common sunflower, floral symmetry, *CYCLOIDEA* gene, transposable element.

Introduction

Floral symmetry is a characteristic that has given rise to innumerable variations in floral shape (Busch and Zachgo 2009). Actinomorphic (polysymmetric) flowers are considered ancestral for angiosperms, and zygomorphic (monosymmetric) flowers have evolved several times independently from actinomorphic ancestors (Preston and Hileman 2009, Carlson *et al.* 2011). The inflorescence of *Helianthus annuus* (sunflower) is composed of both zygomorphic ray (sterile) and actinomorphic disc (hermaphrodite) flowers (Çetinbas and Ünal 2012). In this species, symmetry of ray and disc flowers is specified by the activity of members of a *CYCLOIDEA* (*CYC*) gene family (Chapman *et al.* 2008, 2012, Fambrini *et al.* 2011, Tähtiharju *et al.* 2012). These genes encode for plant-specific transcription factors (TFs) belonging to a TCP family (Cubas *et al.* 1999). These TFs, characterized by a non-canonical bHLH motif called TEOSINTE-BRANCHED1/CYCLOIDEA/PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1,2 (TCP) domain, can be divided into two classes, I and II (Martín-Trillo and Cubas 2010, Uberti-Monassero *et al.* 2013, Li 2015). Class II TCP TFs has an additional motif rich in arginine, called R domain (Cubas *et al.* 1999). The class II subfamily is further divided in two lineages: CINCINNATA-like and CYC/TEOSINTE BRANCHED1 (TB1) (or ECE). The CYC/TB1 lineage is specific of angiosperms and underwent two major duplication events just before the radiation of the core eudicots that

Submitted 25 February 2016, *last revision* 31 March 2016, *accepted* 22 April 2016.
Abbreviations: *Chry2* - *Chrysanthemoides2*; CpG - 5'-Cytosine-phosphate-Guanine-3'; *CYC* - *CYCLOIDEA*; HFTT - High Frequency of *Tetu1* Transposition; ORF - open reading frame; RF - ray flowers; TB1 - CYC/TEOSINTE BRANCHED1; TCP - TEOSINTE-BRANCHED1/CYCLOIDEA/PROLIFE-RATING CELL NUCLEAR ANTIGEN FACTOR1,2; TE - transposable element; *Tetu1* - *Transposable element of turf1*; TIR - terminal inverted repeat; *turf* - *tubular ray flower*; WT - wild type.
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gave rise to the CYC1, CYC2, and CYC3 clades (Preston and Hileman 2009, Martín-Trillo and Cubas 2010, Uberti-Monassero *et al.* 2013). Analysis of different species showed that most of the *CYC2* clade genes are specifically expressed in dorsal or dorsal plus lateral regions of developing flowers (Costa *et al.* 2005, Damerval *et al.* 2007, Preston and Hileman 2009, Citerne *et al.* 2013), and functional data demonstrate a key role for *CYC2* genes in the evolution of zygomorphic flowers (Luo *et al.* 1996, 1999, Wang *et al.* 2008). By contrast, zygomorphic ray flowers in gerbera display *CYC2* clade gene activities primarily in a ventral region and is connected with organ fusion and development of a large ligule (Broholm *et al.* 2008, Tähtiharju *et al.* 2012, Juntheikki-Palovaara *et al.* 2014). In sunflower, when the *HaCYC2c* gene is ectopically expressed, disc flowers switch from actinomorphic to zygomorphic, and the inflorescence looks like an ornamental chrysanthemum (Chapman *et al.* 2012, Fambrini *et al.* 2014b). When the *HaCYC2c* gene is interrupted by the insertion of a CACTA-like transposable element (TE), named *Transposable element of turf1* (*Tetu1*) or by the insertion of retrotransposons, ray flowers switch from zygomorphic to near-actinomorphic disc flowers (Fig. 1*A*); this trait is typical of *tubular ray flower* (*turf*) and *tubular-rayed* (*tub*) mutants (Fambrini *et al.* 2011, 2014a, Chapman *et al.* 2012). These data suggest that *CYC2*-like genes have played a key role in the evolution of the complex inflorescence structure of sunflower.

In addition, flowers arranged in the outmost whorl of the *turf* mutant (*i.e.*, tubular-like ray flowers) maintain their positional identity because they are bigger than the wild type (WT) polysymmetric disc flowers (Fig. 1*A*). Nevertheless, *turf* plants produce hermaphrodite tubular-like ray flowers (Berti *et al.* 2005, Mizzotti *et al.* 2015). The site of *Tetu1* insertion is in the basic region of the TCP motif of *HaCYC2c*, 427 nucleotides after the start codon (Fambrini *et al.* 2011). The TE integration changes the reading frame of *turf-HaCYC2c* for the encoded protein and leads to a premature stop codon. Transposons CACTA belong to class II of TEs, and they generally move by a cut-and-paste mechanism in which the transposon is excised from one location and reintegrated elsewhere, a process that involves the transposase enzyme encoded by the TE (Wicker *et al.* 2003, Bennetzen 2005, Feschotte and Pritham 2007, Lisch 2013, Oliver *et al.* 2013, Buchmann *et al.* 2014). A full-length CACTA TE consists of two terminal inverted repeats (TIRs) bordering two open reading frames (ORFs), one encoding a transposase and the other, called ORF2, encoding a protein of an unknown function (Buchumann *et al.* 2014). The first and last 5 bp of the TIRs consist of the highly conserved CACTA and TAGTG motifs, respectively, hence the name of the TE. *Tetu1* is a non-autonomous version of a CACTA TE carrying the minimum sequences necessary for transposition in the presence of autonomous elements (Fambrini *et al.* 2011). The excision of *Tetu1* restores a WT inflorescence, but the occurrence of some inflorescences with different degrees of phenotype ranging from the WT to *turf* has been related to footprints generated by imperfect excisions of *Tetu1* (Fambrini *et al.* 2014a).

The large genome size of sunflower and the huge presence of repetitive elements (Ungerer *et al.* 2006, Giordani *et al.* 2014) have hindered, until now, the complete genome sequencing in this species. As TEs form an extensive component of the genome, previous studies have generally focused on the contribution to TE families in the sunflower evolution (Vukich *et al.* 2009a,b, Cavallini *et al.* 2010, Staton *et al.* 2012, Gill *et al.* 2014), whereas a role of naturally active TEs operating as mutagens to generate various spontaneous mutations (*e.g.*, *Tetu1*) are less well investigated (Chapman *et al.* 2012, Fambrini *et al.* 2014a,b).

The excision of CACTA elements usually occurs in somatic and germinal plant cells (Roccaro *et al.* 2007, Xu *et al.* 2010). In wide analyses, performed in inflorescences of more than 11 000 plants homozygous for the *turf-HaCYC2c* allele, the lack of visible sectors in reverted plants and the segregation rate of derived-progenies from reverted phenotypes suggest the hypothesis that *Tetu1* excision is restricted to a time shortly before and/or during meiosis (Fambrini *et al.* 2007, 2014a). Surprisingly, in an F_4 progeny derived from a self-fertilized *turf* plant (*i.e.*, homozygous for the *turf-HaCYC2c* allele) of an F_3 progeny of the cross *turf* × *Chrysanthemoides2* (*Chry2*, Fambrini *et al.* 2014a) along with non-chimeric (NC) reverted plants, a plant with a chimeric (C) inflorescence (*i.e.*, a sector of near-normal zygomorphic ray-flowers in a *turf* background) recently occurred. This observation suggests an unusual somatic *Tetu1* excision. The timing of *Tetu1* activity has an important bearing on the determination of the excision rate and is of interest *per se* as one of the properties of this TE system. To gain further insight on the behavior of the CACTA TE *Tetu1*, we performed a genetic and molecular analysis in populations derived from these progenies.

Materials and methods

Plants: All sunflower (*Helianthus annuus* L.) populations were grown at the Department of Agriculture, Food and Environment, the Experimental Station of Rottaia, Pisa, Italy. The somatic and germinal reversion frequency of the *turf* to the WT phenotype was evaluated in F_5 and F_6 progenies derived from self-pollinated plants (*i.e.*, homozygous for the *turf-HaCYC2c* allele) of the F_4 progeny of the crosses *turf* × *Chry2* where in 2013 we detected non-chimeric WT inflorescences and one chimeric inflorescence, suggestive of germinal and somatic *Tetu1* excision, respectively. The *Chry2* inbred line contains an insertion (1 034 bp) of a small non

autonomous CACTA (SNAC; Wicker *et al.* 2003), named *Tech2*, in the promoter region of *Chry2-HaCYC2c*, 558 bp upstream the start codon (Fambrini *et al.* 2014b). This insertion can alter *HaCYC2c* expression, and it has been extensively evaluated (Chapman *et al.* 2012, Fambrini *et al.* 2014b). Within the F_4 and F_5 progenies of the cross *turf* × *Chry2*, several *turf* plants were selfed. Progenies F_5 and F_6 were grown during a time lapse of two years, from 2014 to 2015. A total of 11 progenies were evaluated for the presence of chimeric or non-chimeric revertant plants. In addition, 16 non chimeric revertant plants were self-fertilized and a χ^2-test was used for testing the goodness-of-fit of observed and expected frequencies of different phenotypic classes in the progenies. Exhaustive information on growth conditions can be found in Fambrini *et al.* (2007).

Isolation of DNA and PCR-screening of genotypes: DNA was extracted from leaves of 30-d-old plants of the WT (*TURF/TURF*; *TT*), *turf* mutant (*turf/turf*; *tt*), non-chimeric reverted phenotypes (*TURF/turf*; *Tt*NC), ray flowers (RF) of WT inflorescences (RF*TT*), normal ray flowers of non-chimeric reverted inflorescences (RF*Tt*NC), normal ray flowers of chimeric reverted inflorescences (RF*Tt*C), tubular-like ray flowers (TF) of *turf* mutant inflorescences (TF*tt*), and tubular-like ray flowers of chimeric reverted inflorescences (TF*tt*C). A *NucleoSpin® Plant II* DNA extraction and purification kit was used according to the manufacturer's instructions (*Macherey-Nagel GmbH & Co*., Düren, Germany). The gene *HaCYC2c* and the *Tetu1* CACTA TE were previously isolated from the WT (Chapman *et al*. 2008, 2012) and sunflower *turf* mutant (Fambrini *et al*. 2011), respectively (GenBank accession numbers: HE604335 and HE604336). In the inbred line TURF, 5'- (1 498 bp) and 3'- (2 828 bp) regions flanking the *HaCYC2c* coding DNA sequence were also isolated (Fambrini *et al*. 2011). Sequence information from the above mentioned amplification products was used to isolate, from the reverted phenotypes, the *Tetu1* TE as well as the region upstream the start codon and the region downstream the stop codon of the *HaCYC2c* gene. In addition, the primer combination CYC10 (forward, 5'-CAGTGTAAAGCT TAAGAGGGGTGG-3')/CYC11 (reverse, 5'-CCACTT CTCGGCACTAGTTGATTGG-3') was used for genotype screening identifying homozygous *TURF/TURF* and heterozygous (*TURF/turf*) materials (*i.e.*, originated by excision of *Tetu1*). In the *turf* genome (*turf/turf*), the PCR conditions were unable to amplify the ample sequence (7 179 bp) spanning from CYC10 to CYC11 (Fig. 1 Suppl.). The primer combination RAG19 (forward, 5'-GGTTGCATTTGAGTGTAACAAAGG-3')/CYC11 amplified a product of 1 259 bp. The primer RAG19 is placed in the sequence of *Tetu1*, and therefore, the PCR product was expected only in homozygous (*turf/turf*), and heterozygous (*TURF/turf*) genotypes (Fig. 1 Suppl.). The PCR conditions for both the primer combinations were: 95 °C for 4 min and 30 cycles (30 s at 94 °C, 30 s at 62 °C, and 60 s at 72 °C) with a final extension of 7 min at 72 °C.

Gene expression analysis: To analyze *HaCYC2c* transcription, the total RNA was extracted from corollas of ray flowers of WT inflorescences (RF*TT*), normal RF of non-chimeric reverted inflorescences (RF*Tt*NC), normal RF of chimeric reverted inflorescences (RF*Tt*C), tubular-like ray flowers of *turf* mutant inflorescences (TF*tt*), and tubular-like ray flowers of chimeric reverted inflorescences (TF*tt*C). The total RNA was extracted and purified as previously described (Fambrini *et al.* 2014a). The total RNA (4 μg) was used with a *Superscript III* pre-amplification kit (*Thermo Fisher Scientific*, Waltham, MA, USA) to produce the first strand cDNA. Polymerase chain reactions were performed using gene-specific primers for *HaCYC2c*. To normalize the amount of RNA in each sample, amplification of the constitutively expressed sunflower *β-actin* transcript (*β-actin*) was carried out. Primers were designed to amplify a 745 and 154 bp fragments for *HaCYC2c* and *β-actin*, respectively. The number of PCR cycles was chosen in the exponential range of amplification. The primers CYC10 and 14R (reverse, 5'-TCGCCTTCTTGGACTTGGTAAAGAGCC AAT-3') were used for *HaCYC2c*; the primers ACTF (forward, 5'-AGGAAATCACGGCTCTTGCCC-3') and ACTR (reverse, 5'-CCTGACTCGTCATATTCACCC-3') were used for *β-actin*. Conditions for PCR were 95 °C for 3 min, 26 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 7 s, and 5 min at 72 °C for *β-actin*; 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s, and 5 min at 72 °C for *HaCYC2c* (modified from Fambrini *et al.* 2014a). The PCR products were separated by electrophoresis on a 1.5 or 2.0 % (m/v) Tris-acetate-EDTA (TAE)-agarose gel, visualized with a *Gel Red™* nucleic acid stain (*Biotium,* Hayward, CA, USA) under UV radiation and cloned as previously described (Fambrini *et al.* 2014a).

***In silico* analysis of sequences**: Several clones were sequenced on both strands. The chosen sequences were aligned using *ClustalW v. 1.7* (Thompson *et al*. 1994) and the *Kalign* multiple sequence alignment from *EMBL-EBI* (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/msa/). The *MethPrime*r tool was used for 5'-C-phosphate-G-3' (CpG) islands (CGIs) prediction in the *Tetu1* sequence (Li and Dahiya 2002).

Results and discussion

The phenotypic analysis carried on F_5 and F_6 progenies of the cross *turf* × *Chry2*, expressing the *turf* phenotype, allowed to estimate the reversion frequency from the *turf* to the WT or near WT phenotype (Fambrini *et al.* 2014a).

Tetu1 showed an active excision in all progenies (100 %) taken into consideration (Table 1). In previous analyses, a lower percentage of reversion, ranging from 25.0 to 66.67 %, was detected for progenies of different crosses as well as in the *turf* mutant line (Fambrini *et al*. 2007, 2014a). Getting more in detail into the fraction of revertant plants (taking into consideration the sum of individuals of all populations), 18.93 % of plants showed *Tetu1* excision (Table 1). In particular, 62 non-chimeric plants (13.81 %), likely germinal TE excision, and 23 chimeric revertants (5.12 %), likely somatic TE excisions, were detected (Table 1). Together with non-chimeric revertants, showing a WT-like inflorescence (Fig. 1*B*), non-chimeric inflorescences with intermediate ray flowers were also detected (Fig. 1*C*). This latter individuals are likely reminiscent of imperfect *Tetu1* excisions as previously demonstrated (Fambrini *et al.* 2014a). In addition, in most progenies (*i.e.*, 9/11) chimeric inflorescences were detected either on the main axillary inflorescence (Fig. 1*D,E,F*) or on secondary or tertiary axillary inflorescences (Fig. 1*G,H,I*). In these populations, the various chimeric morphs [*i.e.*, the position and ampleness of the reversion of tubular-like to normal ray flowers (Fig. 1 and Fig. 2 Suppl.)] suggest a large variability in the developmental time of *Tetu1* somatic transposition. In the last years, during the analysis of more than 11 000 plants, chimeric inflorescences were undetected, and the reversion

Fig. 1. Inflorescence morphology after germinal and somatic excision of the *Transposable element of turf1* (*Tetu1*) in *Helianthus annuus*. Typical inflorescence of the *tubular ray flower* (*turf*) mutant (*A*). Reversion of the *turf* to normal plants for supposed germinal excision (*B*). Supposed germinal excision of the *Tetu1*: the footprints generated by transposition originated an intermediate inflorescence (*C*). Primary (*D,E,F*), secondary (*G,H*), and tertiary (*I*) inflorescences with somatic sectors, suggestive of *Tetu1* excision occurred throughout the ontogeny of plants.

Table 1. The excision frequency of the *Transposable element of turf1* (*Tetu1*) CACTA-like transposon in F_5 and F_6 progenies derived from the cross *turf* × *Chry2* of sunflower. Germinal and somatic reversions are established for non-chimeric and chimeric plants, respectively. The reversion percentage in progenies and total plants are indicated in brackets.

Number of progenies	Number of progenies with reversion	Number of total plants	Number of plants with reversion	Germinal reversion, non chimeric plants	Somatic reversion, chimeric plants
11	11 (100 %)	449	85 (18.93 %)	62 (13.81 %)	23 (5.12 %)

Table 2. Segregation analysis from 16 reverted plants (non-chimeric plants). In brackets there are mutant plants with somatic reversion (chimeric plants) or with an intermediate phenotype, suggestive of imperfect excision of the *Transposable element of turf1* (*Tetu1*). Underlined are χ^2 corresponding to $P < 0.05$.

Progeny	Number of plants	Normal	Mutant	χ2 (3:1)	*P*
1	43	34	9	0.379	0.50 - 0.70
2	29	22	6 (+1)	0.038	0.80 - 0.90
3	48	36	12	0	1
4	63	51	12	1.190	0.20 - 0.30
5	49	37	11 (+1)	0.007	0.90 - 0.95
6	69	58	11	3.019	0.05 - 0.10
7	44	30	12 (+2)	1.091	0.20 - 0.30
8	90	74	11 (+5)	2.504	0.10 - 0.20
9	98	82	15 (+1)	3.932	0.05 - 0.02
10	51	36	13 (+2)	0.529	0.40 - 0.50
11	96	73	23	0.056	0.80 - 0.90
12	61	46	15	0.005	0.90 - 0.95
13	33	30	3	11.000	< 0.001
14	62	47	14 (+1)	0.021	0.80 - 0.90
15	42	33	7 (+2)	0.286	0.50 - 0.70
16	28	22	5 (+1)	0.191	0.60 - 0.70
Pooled	906	711	179 (+16)	5.741	0.002 - 0.001
Sum of 16 χ^2				24.248	
Heterogeneity				18.507	0.20 - 0.30

frequency was lower, ranging from 1.21 to 6.09 % (Fambrini *et al*. 2007, 2014a). For this reason, the progenies here reported were named High Frequency of *Tetu1* Transposition (HFTT). Notably, the reversion percentage is an underestimate of the *in vivo* excision frequency of *Tetu1* from its genomic location. In fact, footprints generated by imperfect TE excisions could generate premature truncated versions of HaCYC2c protein leading to phenotypes indistinguishable by the *turf* mutant (Fambrini *et al*. 2014a).

The data of the genetic analysis, conducted in 16 progenies derived from self-fertilized non-chimeric plants, are reported in Table 2. In two progeny samples, the χ^2 values are consistent to $P < 0.05$ while most of the χ^2 (14 progenies) are not significant. However, the segregating progenies are small and each χ^2 may be weakly informative. The analysis of the pooled samples indicates a significant χ^2 value (5.741, with $P < 0.02$). The reason is a deficit of the mutant phenotype in most progenies (13). This persisting tendency might have been strong enough to lead to rejection of the 3:1 hypothesis. Heterogeneity is 18.507 and with 15 degrees of freedom (*d.f.*), $P = 0.20$ - 0.30, verifying the obvious consistency in the sample ratios (Table 2). We suggest that, in the pooled population, the χ^2 significance originated by supposed germinal (non-chimeric) reversion occurred in the *turf* plants. Likely, these reverted plants, with an inflorescence similar to the WT normal plants, were classified as the normal phenotype. In fact, in the pooled population, the expected mutant plants are 226.5 (observed: 179 + 16 = 195), and with a frequency of 13.81 % of germinal reversion (Table 1), the hypothetical *turf* population will be composed by 195 + (226.5 × 0.1381) = 226.27965 plants not different to the expected mutant population for a monogenic 3:1 segregation. This hypothesis is corroborated by the presence of 16 chimeric plants in segregating progenies, suggestive of somatic reversions (Table 2).

In revertant plants, a PCR approach was used for the molecular screening of *Tetu1* excision with the primer combinations CYC10/CYC11 and RAG19/CYC11 (Fig. 2 and Fig. 1 Suppl.). In the (*TT*) ray flower corollas (RF*TT*), the only primer combination that gave a PCR product was CYC10/CYC11 (Fig. 2). By contrast, in tubular-like ray flowers of the mutant (TF*tt*) and tubular-like ray flowers of chimeric reverted inflorescences

(TF*tt*C), only the RAG19/CYC11-PCR product was obtained. As expected, from heterozygous (*Tt*) material (RF*Tt*NC and RF*Tt*C) originated by excision of *Tetu1* at a different time of plant development, both PCR products were amplified (Fig. 2). These results are consistent with an active movement of the TE confirming that reversion of the *turf* to WT phenotype was linked to *Tetu1* excision and not to specific genomic conditions related to the hybrid nature of HFTT progenies (Fambrini *et al.* 2011, 2014a).

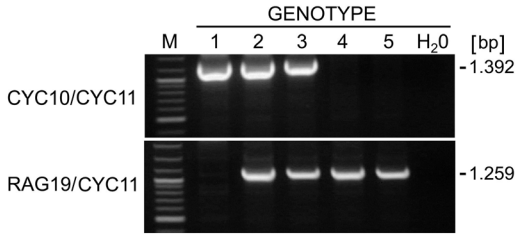

Fig. 2. Molecular screening for the excision of the *Transposable element of turf1* (*Tetu1*). A PCR approach was used to evaluate the *HaCYC2c* genomic region in corollas of the following samples: ray flowers (RF) of wild type inflorescences (RF*TT*; 1); normal RF of non-chimeric (NC) reverted inflorescences (RF*Tt*NC; 2); normal RF of chimeric (C) reverted inflorescences (RF*Tt*C; 3); tubular-like ray flowers (TF) of *turf* mutant inflorescences (TF*tt*; 4); TF of chimeric reverted inflorescences (TF*tt*C; 5). Distilled water (H_2O) was used as a negative control. The size of amplified PCR products is indicated *on the right*. Primer combinations are indicated *on the left*. M - *GeneRuler DNA ladder mix* (*Thermo Fisher Scientific*, Waltham, MA, USA).

In the DNA of a reverted non-chimeric plant, the primer combination CYC10/CYC11 amplified a PCR-product of 1 392 bp (Fig. 3*A* Suppl.). The *Kalign* alignment of this reverted non-chimeric sequence to the whole *turf-HaCYC2c* (*tt*) sequence shows that, except for the *Tetu1* insertion, the two allelic sequences were identical (Fig. 3*B* Suppl). This result suggests a perfect *Tetu1* excision in the reverted non-chimeric plant (Fambrini *et al.* 2011, 2014a).

We analyzed the transcription pattern of *HaCYC2c* in ray flowers of WT inflorescences (RF*TT*), normal ray flowers of non-chimeric reverted inflorescences (RF*Tt*NC), normal ray flowers of chimeric reverted inflorescences (RF*Tt*C), tubular-like ray flowers of *turf* mutant inflorescences (TF*tt*), and tubular-like ray flowers of chimeric reverted inflorescences (TF*tt*C). As expected, *HaCYC2c* was specifically transcripted in RF*TT*, RF*Tt*NC, and RF*Tt*C samples while tubular-like ray flowers of the mutant (TF*tt*) as well as of TF*tt*C displayed no *HaCYC2c* transcription (Fig. 3). In tubular-like ray flowers, the large *Tetu1* insertion prevented the amplification of the *HaCYC2c* PCR-product (Fambrini *et al.* 2011).

One major unresolved point is the real mechanism contributing to the activation of the TE transposition in eukaryotes. There are many factors known to influence TE activity, including abiotic and biotic stresses, environmental changes, the concentration of element-specific transposase, the size, structure, content, and organization of the element, and the presence of regulatory proteins and/or small RNAs (Feschotte and Pritham 2007, García Guerreiro 2012). Class II TEs, including CACTA superfamily elements, display both germinal and somatic excisions (Groose *et al.* 1988, Palmer *et al.* 1989, Carpenter and Coen 1990, Luo *et al.* 1991, Xu *et al.* 2010). The absence of detectable somatic sectors in the inflorescences of all homozygous *turf* populations previously analyzed and the segregation rate of progenies derived from self-fertilized revertant plants (Fambrini *et al.* 2007, 2014a) suggest a tight developmental control of *Tetu1* excision, likely restricted to a time shortly before and/or during meiosis. Indeed,

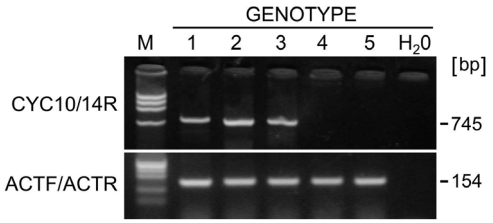

Fig. 3. The transcription pattern of *HaCYC2c*. The following samples were used: ray flowers (RF) of WT inflorescences (RF*TT*; 1); normal RF of non-chimeric (NC) reverted inflorescences (RF*Tt*NC; 2); normal RF of chimeric (C) reverted inflorescences (RF*Tt*C; 3); tubular-like ray flowers (TF) of *turf* mutant inflorescences (TF*tt*; 4); TF flowers of chimeric reverted inflorescences (TF*tt*C; 5). Distilled water (H_2O) was used as a negative control. The size of amplified PCR products is indicated *on the right*. Primer combinations are indicated *on the left*. M = *PhiX 174 DNA HaeIII digest DNA ladder* (*BioLabs,* Ipswich, MA, USA).

tissue specificity is a frequent type of developmental regulation observed with TEs, and a typical example is the germline-specific TE excision regarding *P* and *hobo* elements of *Drosophila melanogaster* (Laski and Rubin 1989, Calvi *et al.* 1991) and *Tc1* of *Caenorhabditis elegans* (Emmons and Yesner 1984). In plants, a mechanism of germinal excision has been reported for *TamI*, a CACTA TE inserted in the ORF of the *Antirrhinum majus CYC* gene (Carpenter and Coen 1990). A tight control of germinal TE excision has been also revealed for the insertion of *Tam4*, a defective TE, in the first exon of the *nivea* locus of *Antirrhinum majus* (Luo *et al.* 1991). As Luo *et al*. (1991) suggested for the behavior of *Tam* TE families, the TURF progenitor line might contain either repressors of somatic excision or activators of germinal excision of transposons belonging to the *Tetu1* family. Notably, the data presented above show that a dramatic change of *Tetu1* transposition occurred in the HFTT progenies. In particular, the large variability of normal or near-normal sectors established afterward TE excision suggests that the strict control of tissue specific excision and timing were lost in these progenies. Several studies indicate that specific regions in the TEs can hugely modify their excision stage. For

example, somatic and germinal excision activities of the *Arabidopsis* transposon *Tag1*, a member of the *hAT* superfamily, are controlled by distinct regulatory sequences within *Tag1* (Liu *et al.* 2001). It is also noteworthy that mutations in *Ds* elements or in unlinked genes have been found to reduce germinal but not somatic excision of *Ac/Ds*, indicative of an *ad hoc* control of both processes (Eisses *et al.* 1997, Giedt and Weil 2000). Moreover, intrinsic properties of the element-flanking DNA influence the ability of *piggyBac* transposable elements to be remobilized and that all of the information responsible for position-dependent variation in transposable element activity lies within 1 kb from the terminal inverted repeats of the element (Esnault *et al.* 2011). We showed that within the resolution limits of our analyses, in the HFTT progenies, no significant DNA changes in the *Tetu1* sequence as well as in 5' and 3' regions adjacent to the TE were detected (Fig. 4 Suppl.; Fambrini *et al.* 2011). Nevertheless, we cannot exclude the occurrence of ectopic recombination generating structural DNA changes in regions proximal to *Tetu1* in the HFTT progenies, but not analyzed in this study [*e.g.,* variation in frequency of transposase (Brutnell and Dellaporta 1994), or with activators of TE mobilization].

It is unknown if the density of TE elements in a chromosomal region changes the tissue specificity as well as the excision time. The *Chry2* inbred line crossed with *turf* contains a small non autonomous CACTA (*Tech2*) in the 5'-promoter region of the *Chry2-HaCYC2c* gene (Fambrini *et al.* 2014b). However, within the HFTT progenies, PCR analyses demonstrated the absence of *Tech2* (data not shown). Thus, the *turf* progenies here analyzed were only homozygous for *Tetu1* and we can exclude that the mobility of *Tetu1* was influenced by the SNAC *Tech2*.

Several studies have demonstrated that the transposition frequency of TE can drastically increase with a change in the genetic background (Alleman and Freeling 1986, Comai *et al.* 2003, Wang *et al.* 2006). Therefore, the regulation of TE transposition rate in germinal and somatic cells may be affected by specific crosses (Liu and Crawford 1998). This genomic stresses can occur, for example, during crosses between individuals that have high genetic differences as *turf* and *Chry2*. In *Drosophila*, crosses between different strains and/or species can produce these types of stresses, and a great number of examples of TE mobilization through genetic crosses have been reported (García Guerreiro 2012). Analogously in plants, hybridization and allopolyploidy have often been associated with the regulation of transposition of TEs (Liu and Crawford 1998, Comai *et al.* 2003, Wang *et al.* 2006, Petit *et al.* 2010, Sarilar *et al.* 2013). However, most efforts have been performed on retrotransposon behavior in response to allopolyploidy (Comai *et al.* 2003, Wang *et al.* 2006, Petit *et al.* 2010, Sarilar *et al.* 2013), whereas there are few examples in model species on the influence of hybridization on mobility, tissue specificity, and regulation of excision time of class II TEs (Liu and Crawford 1998). Therefore, HFTT progenies could be useful to understand the effects of genomic environment on genetic and epigenetic regulation of TE mobility in an important crop.

In conclusion, molecular mechanisms underlying the change in the behavior of *Tetu1* in the frequency and excision time during plant development remain to be determined. Mechanisms that trigger transposition in hybrids are poorly understood, but it is well known that when two different genomes combine to form a zygote, it must respond to a massive shift in regulatory mechanisms due to this 'genomic shock' (Comai *et al.* 2003, Wang *et al.* 2006, Petit *et al.* 2010, Sarilar *et al.* 2013). Therefore, we suppose that a modification of the epigenetic control of *Tetu1* transposition could be a consequence of the cross *turf* × *Chrysanthemoides2*, with putative effects about tissue specificity and time of excision. Earlier, McClintock (1958) described the first CACTA in maize, *Suppressor-Mutator* (*Spm*), and its complex regulation (McClintock 1961). She discovered that *Spm* could switch back and forth between an "inactive" form and an active form what she called "changes of phase" now known as result of methylation (Fedoroff and Banks 1988, Schläppy *et al.* 1993). The analysis of *Tetu1* TE for the CpG island prediction by the *MethPrimer* tool (Li and Dahiya 2002) revealed 3 CpG island located with a GC percent > 50 (Fig. 5 Suppl.) which are putative targets for methylation. Genomic stresses, such as hybridization, can induce methylation changes (Dong *et al.* 2006, Verhoeven *et al.* 2010); the effect of these changes could be observed in some progenies of the cross *turf* × *Chry2*. Similarly, the genetic background of the inbred lines involved could have an influence on TE activity *via* RNA interference pathways. Genomes have defence systems that avoid TE proliferation and one of the most widely studied systems is the RNA-mediated silencing system controlled by small RNAs in animals and plants (Michalak 2009, García Guerreiro 2012, Lisch 2013, Oliver *et al.* 2013). After hybridization, changes in tissue specificity and time of *Tetu1* excision may result from a differential ability of small RNAs in their capabilities to suppress their TE target.

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