Gene 549 (2014) 198–207

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Excisions of a defective transposable CACTA element (*Tetu1*) generate new alleles of a *CYCLOIDEA*-like gene of *Helianthus annuus*

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ARTICLE INFO

Article history:
Received 3 January 2014
Received in revised form 7 July 2014
Accepted 8 July 2014
Available online 18 July 2014

Keywords:
CACTA transposable element
CYCLOIDEA
Helianthus annuus
Multiple allelism
Mutant
Sunflower

ABSTRACT

tubular ray flower (*turf*) is a sunflower mutant that caught attention because it bears actinomorphic ray flowers, due to the presence of an active, although non-autonomous CACTA transposon (*Tetu1*) in the TCP domain of a *CYCLOIDEA*-like gene, *HaCYC2c*, a major regulator of sunflower floral symmetry. Here, we analyzed its excision rates in F_3 population deriving from independent crosses of *turf* with common sunflower accessions. Our results suggest that the excision rate, ranging from 1.21 to 6.29%, depends on genetic background; moreover, the absence of somatic sectors in inflorescences of revertant individuals analyzed (182) and genetic analyses suggests a tight developmental control of *Tetu1* excision, likely restricted to germinal cells. We individuate events of *Tetu1* excision through molecular analysis that restore the wild type (WT) *HaCYC2c* allele, but even transposon excisions during which footprints are left. All mutations we detected occurred at the TCP basic motif and cause a change in ray flower phenotype. In particular, we selected five mutants with a one-to-four amino acid change that influence the capacity of reproductive organ development and ray flower corolla shaping (*MUT-1*, *-2*, *-3*, *-4*, *-5*). Revertant alleles not affecting *turf* phenotype (i.e. reading frame mutations) have also been identified (*MUT-6*). In all mutants, Real-time quantitative PCR (qPCR) experiments revealed variations of the steady state level of *HaCYC2c* mRNA. *MUT-1* and *MUT-4* showed a significant *HaCYC2c* down-regulation with respect to WT. A large variation within the biological replicates of *MUT-2*, *MUT-3* and *MUT-5* was detected and not significant differences in transcription levels between mutants and WT were observed. We detected low steady state level of *HaCYC2c* mRNA both in *turf* as in *MUT-6*. A three dimensional (3D) structure prediction tool let us predict an incorrect folding of the TCP protein already after a single amino acid deletion. This in turn is detectable as the restore of traits that are not peculiar of WT ray flowers, such as male fertility. Our analysis of an active TE sheds light on the TCP motif of the *HaCYC2c* gene and suggests that *Tetu1* may be useful to obtain new natural mutants and for transposon tagging in different inbred lines of sunflower.

1. Introduction

Sunflower (*Helianthus annuus* L.), apart from being one of the most important crops providing food to humans, is a model plant to study flower symmetry regulation because flowers with radiate symmetry and flowers with bilateral symmetry are found in the same inflorescence (Berti et al., 2005; Chapman et al., 2008, 2012; Fambrini et al., 2011, 2014; Tähtiharju et al., 2012). *H. annuus* belongs to the Asteraceae family, which is one of the largest families of flowering plants. It is a radiate species with a head-like inflorescence (capitulum) that looks like a giant single flower, considered as the innovative and successful trait of Asteraceae (Gillies et al., 2002). Wild type (WT) *H. annuus* inflorescence is composed of parastichies of hermaphrodite flowers with actinomorphic symmetry (disk flowers), surrounded by a whorl of zygomorphic sterile flowers (ray flowers). Zygomorphy has been associated with increased speciation rates (Preston and Hileman, 2009).

Recently, it has been found that symmetry of ray and disk flowers in sunflower is mainly ruled by a *CYCLOIDEA* (*CYC*) gene, named *HaCYC2c*, one of the ten *CYC*-like genes found in sunflower genome (Chapman et al., 2008). These genes encode for plant-specific transcription factors (TFs) belonging to TCP family. The acronym stands for the first three identified members: *TEOSINTE BRANCHED1* in *Zea mays*, *CYCLOIDEA* in

Abbreviations: bp, base pair(s); BiFC, Bimolecular Fluorescence Complementation; *Chry2*, *Chrysanthemoides2*; CMT3, CHROMOMETHYLASE3; *CYC*, *CYCLOIDEA*; DDM1, DNA METHYLATION1; EMSA, Electrophoretic Mobility Shift Assay; *En/Spm*, *Enhancer/Suppressor–mutator*; MET1, METHYLTRANSFERASE1; MUT, Mutant; *niv*, *nivea*; nt, nucleotide(s); qPCR, Real-time quantitative RT-PCR; SD, Standard Deviation; siRNAs, small interfering RNAs; sub-TRs, sub-terminal repetitive regions; TEs, transposable elements; TIRs, terminal inverted repeats; *Tetu1*, *Transposable element of turf1*; TSD, target site duplication; *tub*, *tubular-rayed*; *turf*, *tubular ray flower*; WT, wild type; 3D, three dimensional.

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http://dx.doi.org/10.1016/j.gene.2014.07.018
0378-1119/

Antirrhinum majus, and *PROLIFERATING CELL FACTOR 1* and *2* in *Oryza sativa* (Cubas et al., 1999). These TFs are characterized by a non-canonical bHLH domain of *circa* 60 residues called TCP domain (Aggarwal et al., 2010; Cubas et al., 1999; Martín-Trillo and Cubas, 2010) and are considered a novel family of plant TFs, basically involved in cell cycling and growth. Phylogenetic studies based on TCP domain consensus sequences let the family be divided into two classes, I and II. Class II TCP TFs have an additional domain, called R domain, which is *circa* 20 residues long motif rich in arginine (Cubas et al., 1999). Phylogenetic and sequence analysis let the class II to be split into two clades, the *CYC/TB1*-like and the *CINCINNATA*-like clades. The first clade is further divided into *CYC1*, *2* and *3* sub-clades which have evolved due to a series of duplication events (Martín-Trillo and Cubas, 2010; Preston and Hileman, 2009). Analysis of different angiosperm species showed that most of the *CYC2* clade genes are specifically involved in the control of floral symmetry (Citerne et al., 2013; Costa et al., 2005; Damerval et al., 2007; Luo et al., 1996, 1999). Concerning the sunflower, when *HaCYC2c* is ectopically expressed, disk flowers switch from actinomorphic to zygomorphic, and the inflorescence looks like a chrysanthemum (Chapman et al., 2012; Fambrini et al., 2014). When the gene is, instead, interrupted by the insertion of a non-autonomous CACTA-like transposable element (TE) (Fambrini et al., 2011) or by the insertion of retrotransposons (Chapman et al., 2012), ray flowers switch from zygomorphic to actinomorphic, resembling disk flowers; this trait is peculiar of *tubular ray flower* (*turf*) and *tubular-rayed* (*tub*) mutants (Berti et al., 2005; Chapman et al., 2012; Fambrini et al., 2007, 2011; Fick, 1976). In *turf*, flowers arranged in the outmost whorl (i.e. tubular-like ray flowers) maintain their positional identity because they are bigger than the WT polysymmetric disk flowers but achieved hermaphrodite features (Berti et al., 2005). *turf* plants produce tubular-like ray flowers in which stamen filaments, anthers, style and ovule display some developmental defects, but morphological and genetic analysis demonstrate that both male and female organs are functional (Berti et al., 2005).

We focused our attention on *turf* mutant plants. As we said before, *turf* mutation is due to the insertion of a non-autonomous CACTA TE, named *Transposable element of turf1* (*Tetu1*) (Fambrini et al., 2011), that creates a perfect three bp (ATA) target site duplication (TSD). The site of *Tetu1* insertion (5787 bp) is in the basic region of the TCP motif, 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. CACTA or *En/Spm* (referring to the first element described in maize, *Enhancer/Suppressor–mutator*; Peterson, 1953), belong to class II of TEs, which move via a DNA intermediate and whose excision and reintegration require an enzyme known as transposase (Bennetzen, 2005; Feschotte and Pritham, 2007; Lisch, 2013; Oliver et al., 2013). The name 'CACTA' refers to the flanking terminal inverted repeats (TIRs), which are normally 10–28 bp long and terminate in a conserved 5′-CACTA-3′ motif (Wicker et al., 2003) and are necessary to let the transposase enzyme work properly (Lewin, 1997). In addition, its characteristic subterminal repetitive regions (sub-TRs) of 10–20 bp units, repeated in direct and inverted orientations, are essential for the transposition (Craig et al., 2002; Frey et al., 1990; Schiefelbein et al., 1988). *Tetu1* is classified as non-autonomous because it lacks a transposase coding sequence but still it can be excised thanks to the action of other *trans*-active transposases (Fambrini et al., 2011, 2014). These TEs are potent mutagens generating variability primarily by their insertion into host DNA (Bennetzen, 2005; Feschotte and Pritham, 2007; Morgante et al., 2007). In addition, because they employ an error-prone cut and paste mechanism, class II elements are also subjected to frequent readjustments. Footprints or DNA sequence rearrangements left at the donor site after transposition provide clues to the repair mechanisms of DNA (Weil and Kunze, 2000). The excision of *Tetu1* restores a WT inflorescence but, during some preliminary studies on small mutant populations, we witnessed the occurrence of plants that bear ray flowers similar to WT ones, ray flowers with a phenotype that resembles hybrid characteristics between ray and disk flowers, further on indicated as intermediate, and ray flowers typical of *turf* mutants (Fambrini et al., 2007, 2011). A possible explanation to the occurrence of inflorescences with different degrees of phenotype ranging from WT to *turf* might rely on imperfect excisions of *Tetu1*. The excision of CACTA elements usually occurs in both somatic and germinal plant cells (Roccaro et al., 2007; Xu et al., 2010). The absence of chimerism at inflorescence level and the segregation rate of derived-progenies from reverted phenotypes let us speculate that *Tetu1* excision is limited to germinal cells. Although the sunflower genome has been extensively analyzed for the presence of repetitive elements (Cavallini et al., 2010; Gill et al., 2014; Staton et al., 2012; Ungerer et al., 2006; Vukich et al., 2009a,b), few naturally active TEs have been identified so far, making this an interesting field of research.

Starting from all these statements we extended our analysis to larger sunflower populations of *turf* mutants and to populations obtained by crossing *turf* with different WT inbred lines. Here, a total of 29 distinct excisions at the *HaCYC2c locus* were molecularly analyzed and new alleles and the corresponding mutants were isolated. We demonstrated that *Tetu1*, regardless of the genetic background, is excised with tight developmental control and its excision generates different footprints on the TCP domain of *HaCYC2c* resulting in different phenotypes.

An extensive study on biochemical properties and structure of HaCYC2c TF has been performed using *Arabidopsis thaliana* TCP4 as a model. As for the canonical bHLH domain, the basic region interacts with the major groove of target DNA and the HLH region is required for dimerization (Aggarwal et al., 2010). The amino acidic modifications due to imperfect excision of *Tetu1* gave us the possibility to study natural occurring mutations in their genomic environment, without the need of artificial techniques. Understanding the connection between modification at the level of amino acidic sequence of *HaCYC2c* TCP domain and ray flowers phenotype as well flower sex determination add some knowledge about this key sunflower gene and, moreover, this new data could be exploited to fulfil the general characterization of TCP TFs.

2. Materials and methods

2.1. Plant material and growth conditions

The reversion frequency of *turf* to WT phenotype was evaluated in four different experimental populations: (i) 162 self-pollinated progenies (8045 plants) of *turf* mutant (Berti et al., 2005); (ii) twenty-four F_3 progenies (1072 plants) obtained from self-pollinated F_2 *turf* plants derived by the cross *turf* × EF2; EF2 is an inbred line taken from the genetic collection of the Department of Agriculture, Food and Environment, University of Pisa, Italy, which displays lemon-coloured corolla of ray and disk flowers (Fambrini et al., 2010); (iii) fourteen F_3 progenies (691 plants) obtained from self-pollinated F_2 *turf* plants derived by the cross *turf* × *Chrysanthemoides2* (*Chry2*); *Chry2* is a mutant with zygomorphic disk flowers (Fambrini et al., 2003, 2014; Fick, 1976); (iv) eighteen F_3 progenies (509 plants) obtained from self-pollinated F_2 *turf* plants derived from the cross *turf* × *white cream*; *white cream* is a mutant characterized by a peculiar pollen colour previously described by Fambrini et al. (2010). A total of 218 progenies (10,317 plants) were analyzed.

All sunflower populations were grown at the Department of Agriculture, Food and Environment, Experimental Station of S. Piero a Grado, Pisa, Italy. Progenies from self-pollinated *turf* mutants were grown during a time lapse from 2005 to 2013; F_3 progenies derived from the crosses *turf* × EF2 and *turf* × *white cream* were grown from 2010 to 2013, instead, the F_3 progenies derived from the crosses *turf* × *Chry2* were grown in 2005, 2006, 2012 and 2013.

Twenty-nine revertant plants were self-fertilized and a chi-square test was used for testing the goodness-of-fit of observed and expected frequencies of different phenotypic classes in the progenies.