Molecular genetic basis of flower colour variegation in *Linaria*

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Summary

To identify transposons that may be of use for mutagenesis we investigated the genetic molecular basis of a case of flower colour variegation in *Linaria*, a close relative of the model species *Antirrhinum majus*. We show that this variegation is attributable to an unstable mutant allele of the gene encoding dihydroflavonol-4-reductase, one of the enzymes required for anthocyanin biosynthesis. This allele carries an insertion of a transposon belonging to the CACTA family (Tl1, Transposon Linaria 1) which blocks its expression thus conferring an ivory flower colour phenotype. Tl1 is occasionally excised in dividing epidermal cells to produce clonal patches of red tissue on the ivory background, and in cells giving rise to gametes to generate reversion alleles conferring a fully coloured phenotype. This finding may open the way for targeted transposon-mutagenesis in *Linaria*, and hence for using this genus in comparative genetic studies.

1. Introduction

Although transposons have been identified in numerous species as DNA segments with particular structures, in only a few cases has it been possible to detect phenotypic consequences of their activity. Such consequences are particularly evident in the case of plant transposons which, when inserted in pigmentation genes, give rise to unstable mutant alleles conferring variegated colour phenotypes (Bonas *et al.*, 1984; Fedoroff *et al.*, 1984; Kroon *et al.*, 1994; Inagaki *et al.*, 1994; Chopra *et al.*, 1999). In maize, *Antirrhinum* and *Petunia*, detailed analysis of alleles of this type has led to the use of transposons as tools for systematically generating mutants (Carpenter & Coen, 1990; Luo *et al.*, 1991; van Houwelingen *et al.*, 1998; May & Martienssen, 2003). An advantage of transposon mutagenesis is that it offers the prospect of isolating genes from unstable alleles or, conversely, selecting for mutants carrying transposon insertions or transposon-induced deletions in known genes (Koes *et al.*, 1995; Ingram *et al.*, 1997; Davies *et al.*,

1999; Galego & Almeida, 2002; May *et al.*, 2003). Furthermore, transposition can in some instances be exploited to genetically mark clones of cells, hence for lineage analysis or for studying cell autonomy in gene action (Dawe & Freeling, 1990; Dudley & Poethig, 1993; Carpenter & Coen, 1995; Hantke *et al.*, 1995; Vincent *et al.*, 1995; Rolland-Lagan *et al.*, 2003).

The possibilities above have been exploited for detailed studies on the evolution of genetic and developmental mechanisms incorporating comparisons of mutants in diverse, distantly related, model species. For example, site-directed selection for transposon insertions has been used to investigate the role of *Antirrhinum* counterparts of the *Arabidopsis Ap*2 gene in flower development (Keck *et al.*, 2003). Equivalent tools are, however, unavailable for comparisons between *Antirrhinum* and its close relatives. Thus, as an exploratory step towards expanding transposon mutagenesis to a genus closely related to *Antirrhinum*, we have investigated the genetic molecular basis of a case of flower colour variegation in *Linaria*, a member of the *Antirrhinum* tribe (Olmstead *et al.*, 2001; Vargas *et al.*, 2004). We show that such variegation results from activity of a CACTA transposon inserted in the gene encoding dihydroflavonol-4-reductase and that Tl1 transposes in cells giving rise to gametes.

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Fig. 1. Flower colour phenotypes in *Linaria*. A fully red inflorescence is shown on the left and variegated inflorescence on the right.

2. Materials and methods

(i) *Origin of plants*

The *Linaria* plants considered here (Fig. 1) were found in a garden population of unknown origin. Many morphological and colour variants occurred in the population, which could possibly comprise more than one species or species hybrids. Assigning individuals to particular species was therefore problematic. One possibility was that plants with variegated colour belonged to or comprised part of *Linaria maroccanna*, a species from which popular garden varieties are derived. These plants had reniform seed with distinct transverse ridges and a style with lateral lobes bearing discrete stigmatic areas, all traits common to *L. maroccanna* and a few other species (Sutton, 1988). However, it was unclear whether capsules had conspicuously unequal loculi, a trait which, together with those above, is common to only *L. maroccana* and *L. gharbensis*.

(ii) *Primers*

The following primers were used:

i1: AA(A/G)(C/A)GNCA(C/T)ACNGA(C/T)CCN-GGNACNAT(A/C/T)AC
i2: TTNACNGC(T/C)TG(G/A)TG(G/A)TCNGC-(G/A)TT(T/C)TT
p1: GGNTT(C/T)AT(A/C/T)GGN(A/T)(G/C)NT-GG(T/C)TNGTNATG
p2: AA(G/A)ATGACNGGNTGGATGTA(C/T)TT

p3: GGTCACTGGAATCGGTTTCATCG
p4: GTGGCTATACCATTCGTGCAAC
p5: CTCATGACCAGCCATGAGCCAA
p6: CTGCCAACGTGACGAACTTCAC
p7: CCACAATAACATGTCCGTATC
p8: TGCAACTCACAATTAGCT
p9: CCGTTGGCTCCAGTAACGCAT
p10: GCGATACTAGGCAACAAGTTC
Tam1-3′: CCAGAGCCCAATTTTGTTGTAGTG

The positions and orientations of primers p1 to p9 are indicated with arrowheads in Fig. 2*C*.

(iii) *Molecular analysis*

cDNA Segments of L*PAL* and L*INC* were isolated by RT-PCR using RNA extracted from red inflorescences and degenerate primers designed according to aligned sequences of genes encoding dihydroflavonol-4-reductase (p1 and p2; 1 and 2 in Fig. 2*C*) and flavonol-3-hydroxylase (i1 and i2) from various species. A genomic segment containing the right end of Tl1 and its flanking L*pal* sequence was isolated by PCR amplification of DNA from L*pal*-1 homozygotes using an L*PAL*-specific primer, p3, and a primer for the 3′ end of the *Antirrhinum* transposon Tam1 (Tam1–3′) at low annealing temperature (55 °C). A genomic segment comprising 478 bp of the right end of Tl1 and its flanking L*pal*-2 region was then obtained by inverse PCR using p4 and p5 on DNA cut with *Sau*3A prior to ligation. Using the same approach on DNA from fully red revertants
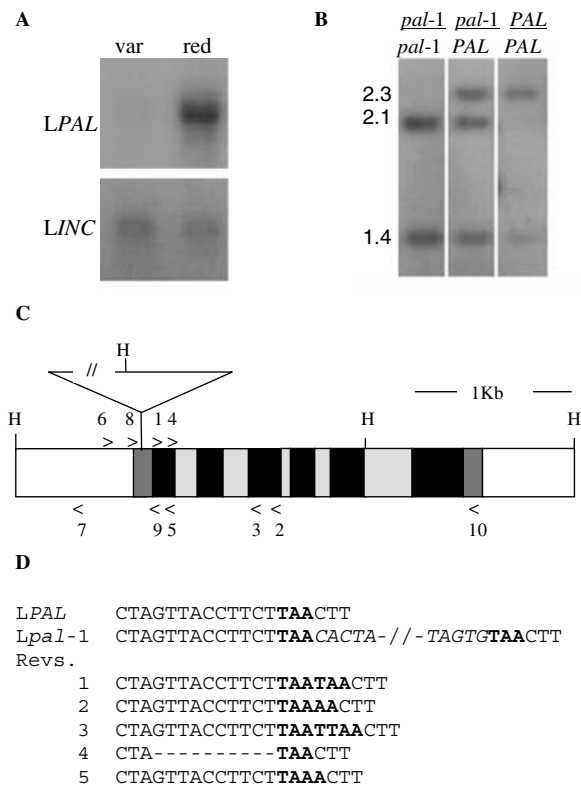
A

var	red

L*PAL*

L*INC*

B

pal-1 / pal-1	pal-1 / PAL	PAL / PAL

2.3
2.1

1.4

C

D

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LPAL      CTAGTTACCTTCTTAACTT
Lpal-1    CTAGTTACCTTCTTAACACTA-//-TAGTGTAACTT
Revs.
     1    CTAGTTACCTTCTTAATAACTT
     2    CTAGTTACCTTCTTAAAACTT
     3    CTAGTTACCTTCTTAATTAACTT
     4    CTA---------TAACTT
     5    CTAGTTACCTTCTTAAACTT
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Fig. 2. Molecular analysis of colour variegation. (*A*) Northern blot of RNA from inflorescences of a plant with variegated color (var) and a fully red revertant (red) probed with L*PAL* and L*INC*. The two plants were sibs derived from a cross between variegated plants. The same result was obtained in all of four independent comparisons with pairs of variegated and revertant sibs. (*B*) Southern blot of DNA from genotypes indicated above the lanes. DNAs were cut with *Hind*III and probed with a full-length cDNA segment of L*PAL*. Sizes of fragments in kilobases are indicated on the left. L*pal*-1/L*PAL* was derived from a cross between two homozygotes for L*pal*-1. The L*PAL*/L*PAL DNA* was from a red segregant in a family derived from crossing two independent revertants. (*C*) Structure of the L*PAL* gene. Transcription is from left to right with UTRs in dark grey, coding region in white and introns in light grey. The triangle represents Tl1 and the arrowheads indicate primers used in this work. H indicates sites cut by *Hind*III. (*D*) Sequences of L*pal* alleles in the vicinity of the Tl1 insertion site. The TSD is in bold and Tl1 ends in italic. Reversion alleles (Revs.) 2, 3 and 4 are germinal events. Sequence 5 is from a large somatic sector. Sequence 1 was found in four independent germinal events and in two large somatic sectors.

resulted in amplification of a segment spanning the site of Tl1 excision and a further 453 bp to its left. A segment containing 342 bp of the left end of Tl1 was then obtained by inverse PCR using p6 and p7 on DNA from L*pal*-1 homozygotes cut with *Sau*3A prior to ligation. The L*PAL* transcription start site was determined by 5′RACE using p3 and p5. A nearly full length cDNA was then obtained by 3′-RACE using p8 as specific primer. A genomic segment spanning the transcribed region was amplified using p8 and p10.

Segments described above were cloned in pBluescript SK (Stratagene) and sequenced automatically (Stabvida, Oeiras). Segments from reversion alleles, carrying Tl1 excision footprints, were obtained by PCR using p5 and p6. Sequences of these segments were then determined directly on PCR products using p9.

Sequences for L*PAL*, Tl1 and L*INC* have been deposited in the GenBank Data Library under accession nos. EF517133, EF517134 and EF517135, respectively.

3. Results and discussion

(i) *Germinal instability of flower colour*

Plants with variegated colour had ivory flowers and green stems with clonal patches of red and purple cells respectively, a phenotype very similar to those conferred by some unstable alleles of pigmentation genes in *Antirrhinum* (Coen & Carpenter, 1986; Schwarz-Sommer *et al.*, 2003; Fig. 1). Typically, insertion of a transposon in a pigmentation gene blocks gene expression giving non-pigmented tissue. However, the transposon is occasionally excised from the gene in dividing cells, hence giving clones of pigmented cells on the non-pigmented background. In addition, the transposon can excise in cells giving rise to gametes and therefore a few plants with wild-type phenotype (revertants) can be recovered in progenies of homozygotes for the unstable allele. To test whether the apparent instability in *Linaria* occurred in germinal tissue we examined pigmentation phenotypes in progenies from crosses between plants with variegated colour (these plants were self-incompatible). In nearly 700 progeny from five crosses, 10 plants had fully red flowers and purple stems whereas the remaining ones had the parental phenotype. Thus, by analogy to *Antirrhinum*, an explanation for the variegated colour phenotype and genetic behaviour of *Linaria* plants was that they carried an active transposon in a pigmentation gene.

(ii) *Lpal-1: an unstable mutant allele of the gene encoding dihydroflavonol-4-reductase*

In *Antirrhinum*, red colour depends on structural genes encoding enzymes required for anthocyanin synthesis, and on regulatory genes that control the expression of structural genes (Almeida *et al.*, 1989; Goodrich *et al.*, 1992; Schwinn *et al.*, 2006). Null mutants for structural genes are in general devoid of anthocyanin pigment in all plant parts whereas regulatory mutants show altered colour patterns relative to wild-type. In *Linaria*, except for the clonal patches of pigmented cells, all plant parts lacked red pigment, suggestive of mutation in a structural gene.

Null mutations affecting enzymes required at early steps of the anthocyanin synthesis pathway in *Antirrhinum*, such as chalcone synthase, result in albino flowers whereas mutations affecting late steps give ivory flower colour identical to that in *Linaria* (see Coen *et al.*, 1989). Late steps include those requiring flavonol-3-hydroxylase and dihydroflavonol-4-reductase, the products of the *INCOLORATA* (*INC*) and *PALLIDA* (*PAL*) genes respectively (Martin *et al.*, 1985, 1991; Coen *et al.*, 1986).

To determine whether variegated plants were mutant at the counterparts of either *INC* or *PAL* (L*INC* or L*PAL*) we probed Northern blots of inflorescence RNA from variegated and fully red sibs derived from the crosses described above with cDNA segments for the L*INC* or L*PAL* genes (Fig. 2A). L*INC* mRNA was detected in both variegated and red inflorescences whereas L*PAL* mRNA was detected in red but not in variegated inflorescences, suggesting that mutation at L*pal* caused colour variation. Consistent with this and with L*pal* carrying a transposon insertion that could excise in germinal tissue, Southern analysis showed that progeny with variegated colour derived from the crosses above were homozygous for an L*pal* allele (L*pal*-1) whereas all their fully red sibs tested (six from the five families) were heterozygous for L*pal*-1 and putative reversion alleles (Fig. 2B).

(iii) *Lpal-1 carries a CACTA transposon (Tl1, Transposon Linaria 1) in the 5′-UTR*

Transposons of two classes, distinguishable according to the sequences of their terminal inverted repeats (TIR) and sizes of their target site duplications (TSD), give rise to unstable alleles in *Antirrhinum*. One class includes Tam3 (the same family as Ac/Ds in maize; Hehl *et al.*, 1991) and the other class includes a number of transposons, such as Tam1, which together with other plant transposons (e.g. Spm in maize) belong to a family known as CACTA, after a common sequence motif at their TIRs (Nacken *et al.*, 1991; Kunze & Weil, 2002). To determine whether L*pal*-1 carries a transposon of one of these classes we amplified DNA from L*pal*-1 homozygotes using primers for L*PAL* and for the ends of either Tam3 or Tam1. This resulted in amplification of DNA with a Tam1 primer. Full characterization of the L*pal*-1 allele then showed that it carries a CACTA transposon (Tl1, Transposon Linaria 1) in the 5′UTR, 44 bp upstream of the translation initiation codon and 29 bp downstream of the transcription start site (Fig. 2C). Similarity searches showed that the 342 bp sequence of the left end of Tl1 has over 60% identity with Tam1, Tam2 and Tam4, all Antirrhinum CACTA transposons.

To test further whether Tl1 was responsible for L*pal*-1 instability, we took advantage of the property of imprecise transposon excision (Saedler & Nevers, 1985; Coen *et al.*, 1986, 1989). Duplications of short sequences at host loci generated on integration (TSD) can be retained intact or in diverse modified forms following excision. In addition, transposon excision can generate deletions adjacent to the TSD. As is typical for CACTA transposons, Tl1 was flanked by a 3 bp TSD in L*pal*-1 (the trinucleotide TAA, which had a single copy in plants with red flowers of unknown relatedness to the variegated plants; Fig. 2D). In germinal reversion alleles and in single large somatic red clones from which DNA could be extracted we found no cases with a single TAA sequence, six independent cases in which both copies of the TSD were retained intact, three cases with modified TSDs and one case in which a 10 bp deletion was generated adjacent to the TSD (Fig. 2D). Presumably, these small sequence modifications did not have a large effect on L*PAL* expression given their location in the 5′UTR.

(iv) *Concluding remarks*

In conclusion, by applying knowledge derived from the analysis of unstable pigmentation mutants in *Antirrhinum* to a case of flower colour variation in *Linaria* we have trapped an active copy of a transposon belonging to the CACTA family in the gene encoding dihydroflavonol-4-reductase. This may open the way for developing a system to select for transposon insertions in *Linaria* counterparts of *Antirrhinum* genes such as, for example, those controlling flower morphology. The two genera have readily comparable flowers with similar structures and overall dorsoventral patterns (Cubas *et al.*, 1999; Gübitz *et al.*, 2003). However, they differ in details of petal size and shape as well as in some conspicuous traits. The most obvious is a long spur in the ventral petal of *Linaria* which is thought to be the natural counterpart of ventral petal outgrowths arising in some gain-of-function *Antirrhinum* mutants (Golz *et al.*, 2002). Thus, *Antirrhinum* and *Linaria* might provide a useful pair of close relatives for investigating origins of both subtle and large morphological variations.

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