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TECHNICAL ADVANCE

Stable two-element control of *dTph1* transposition in mutator strains of *Petunia* by an inactive *ACT1* introgression from a wild species

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Summary

The high copy *dTph1* transposon system of *Petunia* (Solanaceae) is one of the most powerful insertion mutagens in plants, but its activity cannot be controlled in the commonly used mutator strains. We analysed the regulation of *dTph1* activity by QTL analysis in recombinant inbred lines of the mutator strain W138 and a wild species (*P. integrifolia* spp. *inflata*). Two genetic factors were identified that control *dTph1* transposition. One corresponded to the *ACT1* locus on chromosome I. A second, previously undescribed locus *ACT2* mapped on chromosome V. As a 6-cM introgression in W138, the *P. i. inflata act1*^{S6} allele behaved as a single recessive locus that fully eliminated transposition of all *dTph1* elements in all stages of plant development and in a heritable fashion. Weak *dTph1* activity was restored in *act1*^{S6}/*ACT2*^{S6} double introgression lines, indicating that the *P. i. inflata* allele at *ACT2* conferred a low level of transposition. Thus, the *act1*^{S6} allele is useful for simple and predictable control of transposition of the entire *dTph1* family when introgressed into an ultra-high copy W138 mutator strain. We demonstrate the use of the *ACT1*^{W138}/*act1*^{S6} allele pair in a two-element *dTph1* transposition system by producing 10 000 unique and fixed *dTph1* insertions in a population of 1250 co-isogenic lines. This *Petunia* system produces the highest per plant insertion number of any known two-element system, providing a powerful and logistically simple tool for transposon mutagenesis of qualitative as well as quantitative traits.

Keywords: transposon, *Petunia*, regulation, mutagenesis, two-element, *dTph1*.

Introduction

Understanding the molecular basis of natural genetic and phenotypic diversity within and among plant species is one of the key challenges of plant genetics. Causal links between phenotype and genotype are usually pinpointed by a combination of high-resolution mapping, insertion mutagenesis and various genomic techniques. These methods are hard to implement for the majority of plant species other than the common models, leaving many striking natural genetic phenomena unexplored and unexplained.

Arguably, the primary limiting factor to access exotic genetic systems is efficient mutagenesis with retrievable insertions, such as endogenous transposable elements. Good examples are systems like *Antirrhinum* and *Petunia*, in which endogenous transposons play key roles as gene cloning vectors in the absence of extensive genomic resources (Carpenter and Coen, 1990; Koes *et al.*, 1995; Schwarz-Sommer *et al.*, 2003; Vandenbussche *et al.*, 2003). In the *Petunia* genus, natural variation includes complex floral pollination syndromes and a gradient of genetic hybridization barriers among its species (Ando *et al.*, 2001; Griesbach *et al.*, 1999; Quattrocchio *et al.*, 1999; Stuurman *et al.*, 2004). Such characters are hallmarks of flowering plants but have eluded molecular analysis due to a lack of suitable models. Insertion mutagenesis would be a fruitful entry into their mechanisms and evolution, which justifies a re-evaluation of the commonly practised tagging methodologies and their possible improvement.

The *dTph1* transposition system of *Petunia* (Gerats *et al.*, 1990) occupies a unique position among plants, being the highest copy (up to 200) *Ds*-type transposon known, and among the smallest (284 bp) of its kind to retain transposition ability. The level of mutagenesis caused by *dTph1* can be very intense, generating large numbers of *de novo* mutations (Koes *et al.*, 1995; Vandenbussche *et al.*, 2003; Van Houwelingen *et al.*, 1998). In the *P. hybrida* line (W138) most commonly used for transposon mutagenesis, *dTph1* is the foremost cause of unstable mutations (Van Houwelingen *et al.*, 1998). Although other elements transpose in W138, such as d*Tph*2 (Van Houwelingen *et al.*, 1998) and d*Tph*4 (Renckens *et al.*, 1996), they provide only a small fraction of the mutagenic activity. Transposon display techniques have successfully linked individual *dTph1* elements to segregating mutations, harnessing their exceptional mutagenic potential (De Keukeleire *et al.*, 2001; Stuurman *et al.*, 2002; Tobena-Santamaria *et al.*, 2002).

One remaining drawback of *dTph1*-based transposon mutagenesis is that it has not been reduced to a simple two-element system of practical utility, although genetic evidence suggests that regulation of transposition may be genetically simple (Huits *et al.*, 1995; Wijsman, 1986). Instead, the commonly practised method is continuous inbreeding with actively transposing lines. For full application of *dTph1* to a wide range of phenotypic effects including mild allelic variants, it is important that insertions be stabilized to allow unambiguous assessment and/or quantification of phenotypes. Continuous transposition would hamper this, because of the genetically mosaic structure of the plants, the occurrence of footprint alleles, and the accumulation of new insertions in the background. Active *dTph1* strains produce many mutant progeny that are not discernible due to tagged mutations but lead to very high mutational variance and a steady reduction in the fitness of inbred progeny. As a result, the traditional method works well only with phenotypes of strong effect, to the exclusion of (natural) variation that is of degree rather than of kind. Both types of mutational variation could be analysed if transposition can be shut down at will (Norga *et al.*, 2003; Mackay, 2004).

Classical genetic analyses have shown that *dTph1* transposition requires at least one activator (*ACT1*, formerly called *Bi*) that occupies a stable position on chromosome I (Huits *et al.*, 1995; Wijsman, 1986). Although the molecular nature of this locus remains unknown, it might represent a remnant of the autonomous version (*Tph1*) that has lost its cis-requirements for excision but not its transposase function. It is also not known what causes the difference between active *ACT1* and inactive *act1* alleles. It has been speculated that inactive *act1* could represent an epigenetically silent allele from wild *Petunia* species that was reactivated to *ACT1* upon ancient interspecific crosses (Huits *et al.*, 1995). Clear evidence for these ideas is lacking.

The observation of a stable activator is promising, in that it may in principle allow control of the *dTph1* system by segregating *ACT1* in and out of a high copy *dTph1* background. For this to be useful, the inactive form of *ACT1* must first be introgressed as a single locus into W138 and shown to be the only factor required for full and heritable inactivation of all *dTph1* elements. In addition, introgression is essential to reduce genetic variance upon crossing. The currently available *act1* lines, such as *P. hybrida* W162 (Wijsman, 1986), have largely unknown genomic compositions, and may not be sufficiently isogenic to W138 in order to be useful for stabilizing crosses. Divergent crosses would cause segregation of polygenic systems, potentially interfering with detection of phenotypes associated with transposon insertions (Norga *et al.*, 2003).

Here, we aimed at reducing *dTph1* to a simple two-element tagging system. We applied a quantitative trait locus mapping strategy to determine the minimal requirements for *dTph1* transposition in W138 and to breed near-isogenic lines that differ in transposition activity. A simple two-element system results based solely on an *ACT1*/*act1* allele pair, which will undoubtedly be helpful for tagging and cloning of the many mutations that *dTph1* produces. It will also allow for identifying quantitative phenotypes possibly associated with the great many insertions in introns, regulatory sequences, redundant genes, etc. which do not eliminate gene function but may produce the kind of variation seen in natural species of *Petunia*.

Results

Recombinant inbred lines show genetic variation for dTph1 *transposition*

The most frequently used mutator strain in *Petunia* is the *P. hybrida* line W138, which contains a *dTph1* insertion in the *AN1* gene for anthocyanin biosynthesis (Spelt *et al.*, 2000), along with 150–200 additional insertions elsewhere in the genome (De Keukeleire *et al.*, 2001). The $an1^{W138}$ allele causes white flowers with red and pink revertant spots and sectors due to *dTph1* excision (Figure 1a).

To identify genetic factors that control the frequency of *dTph1* excision from $an1^{W138}$, we monitored the *AN1* sectoring pattern in backcross recombinant inbred populations (BILs) of a wild species (*P. integrifolia* spp. *inflata*, Figure 1b) with W138 as recurrent parent. *Petunia i. inflata* is wild type for *AN1*. BILs were derived by crossing W138 to *P. i. inflata* and backcrossing the F_1 hybrid as a female to W138. Each of 120 BC1 individuals were self-fertilized for five generations to produce BC1F5 recombinant inbred lines, termed WI-BILs. In the initial BC1, the theoretical population average of *P. i. inflata* genome segments equals one-fourth of the total genome composition, which should be

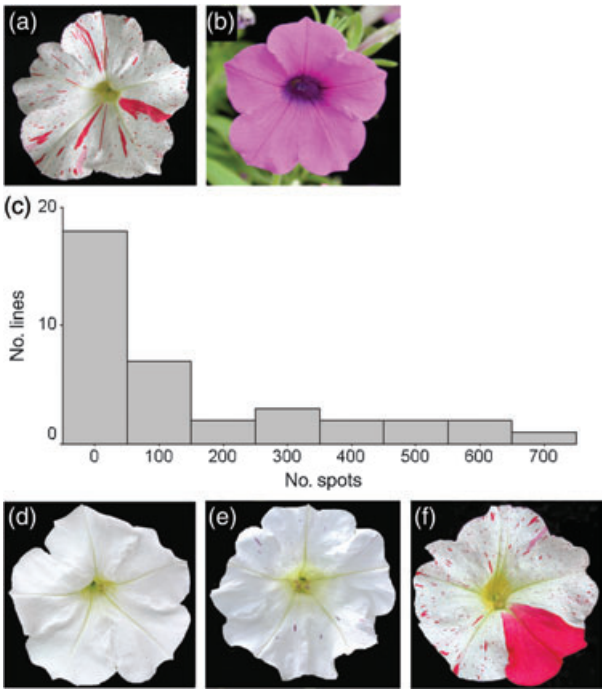

Figure 1. (a) W138 flower in front view. Red sectors reveal excision of *dTph1* from the *an1*^{W138} allele.
(b) Flower of *Petunia integrifolia* spp. *inflata* in front view.
(c) Frequency distribution of sector numbers resulting from excision of *dTph1* from *an1*^{W138} in the WI-BIL population, pre-selected for *an1*^{W138} homozygous genotypes. The number of spots is counted for a single petal.
(d) WI-BIL line WI92, showing a complete absence of *dTph1* excision.
(e) WI-BIL line WI11, showing low frequency of *dTph1* excision.
(f) WI-BIL line WI17, showing a high frequency of *dTph1* excision, resembling W138.

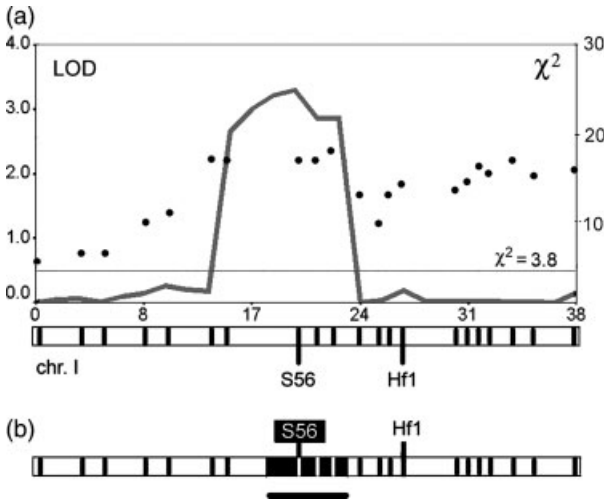

Figure 2. (a) Chromosome I QTL map for frequency of *dTph1* excision events per flower from *an1*^{W138} in the WI-BIL population. The likelihood profile as obtained by CIM is given as a solid grey line. Single marker association scores obtained by Kruskal–Wallis mapping are given as black dots. The AFLP-based chromosome map is below the image, with black tick marks indicating the position of markers. The marker S56 is closest to the QTL likelihood maximum. The *x*-axis is given in cM, the *y*-axis in LOD units (left axis) or chi-squared values (right axis). The horizontal line at $\chi^2 = 3.8$ gives the significance threshold of a chi-squared distribution with 1 df. For interval mapping, a significance threshold based on permutation tests cannot be reliably estimated from non-normally distributed data, and is therefore not given. *Hf1* = flavonoid 3′, 5′-hydroxylase.
(b) Chromosome I structure of the *act1*^{S6} introgression line W5. The black, underlined segment with white *tick* marks denotes *Petunia i. inflata*-derived chromosomal material. White with black tick marks denotes W138.

preserved through the generations of inbreeding to reach final 3:1 ratios in favour of W138. Of the initial 120 BC1 plants, 75 were maintained as lines to produce the final WI-BIL population. The remaining lines were lost due to accumulating inbreeding depression or as yet undefined sterilities (Stuurman *et al.*, 2004).

In 75 BILs, 37 lines homozygous for the *an1*^{W138} allele were obtained. This number significantly deviates from the 3:1 expectation ($\chi^2 = 26.3$, df = 1, $P = 0.000$) in the used population structure. Analysis of tightly linked AFLP markers indicated that segregation at the *AN1* locus was distorted in favour of the *P. i. inflata* allele (data not shown). We quantified the number of spots on a single petal for all 37 lines. As shown in Figure 1(c), sectoring intensities varied significantly. The range of activity was large, from zero transposition (Figure 1d, no spots) to weak activity (Figure 1e) and strong activity (Figure 1f). Patterns of spotting intensities were heritably stable in lines of the WI-BIL population (data not shown) and therefore represent variation of genetic origin. The variation in spotting intensity did not follow a normal distribution and was skewed towards low transposition. This suggests that the genetic variation may be controlled by one or a few major loci with modifiers. It must be noted that spotting intensities in pure lines of W138 can also be quiet variable, possibly representing epigenetic fluctuation. Some of the variation in the WI-BILs may be of this type as well.

A major QTL for dTph1 *transposition frequency maps to the* ACT1 *locus*

To gain further insight into the nature of genetic variation for transposition frequencies, we proceeded with quantitative trait loci (QTL) mapping. Quantitative sectoring data were used to localize QTL on linkage maps of the seven *Petunia* chromosomes that have been constructed previously with AFLP and gene markers in the same BIL population (Stuurman *et al.*, 2004).

We mapped transposition QTLs by composite interval mapping as well as by single marker association using a Kruskal–Wallis rank test procedure. In the WI-BILs, both methods identified a single QTL on chromosome I (Figure 2a). Single marker associations did not well resolve the QTL, as expected. Composite interval mapping mapped it about 6 cM away from the gene *Hf1* (flavonoid 3′,

5′-hydroxylase, Figure 2a). The QTL had a negative effect on excision frequency and explained 34% of total phenotypic variance. It must be noted that only a fraction of all WI-BILs (37 lines) is $an1^{W138}$ homozygous and thus informative for scoring *dTph1* excision. Given its straightforward detection in this modest number of lines, the effect of the chromosome I QTL is likely to be major.

Judging from the complex distribution of spotting patterns in the recombinant inbred lines and the residual unexplained variance (66%), control of *dTph1* excision is genetically quite complex. One interpretation is that a major QTL controls an on–off response for *dTph1* transposition. In the presence of the active QTL allele, several (or many) factors in the genetic background may modify transposition patterns and frequencies to account for the 66% of residual variation. Additional loci could not be identified with this initial QTL screen. This may be due to the low statistical power of a small sample size (37 informative lines).

We conclude that an easily detectable locus on chromosome I is responsible for a decrease in transposition frequency in the WI-BILs. The wild parent of this population (*P. i. inflata*) carries the negative allele, and is therefore expected to be a low *dTph1* transposition genotype. Previous work in *Petunia* (Huits *et al.*, 1995) has identified a genetic factor (*ACT1*) on chromosome I that controls *dTph1* transposition, which mapped about 6 cM away from *Hf1* in some crosses. It is very likely that our QTL corresponds to *ACT1*. Therefore, we coin the *P. i. inflata* QTL allele $act1^{S6}$, and the W138 allele $ACT1^{W138}$.

act1^{S6} *is a stable, recessive locus of major effect in near-isogenic lines*

To study $act1^{S6}$ in more detail, near-isogenic lines were produced that substitute $ACT1^{W138}$ for $act1^{S6}$ in a W138 genetic background. A single, stable white WI recombinant inbred line was selected that carried half of *P. i. inflata* chromosome I encompassing $act1^{S6}$, and a small segment of chromosome VI. A backcrossing scheme (W138 recurrent parent) with marker mapping and phenotypic selection was applied to recover an introgression line that carried a part of the maximum likelihood interval for *ACT1* as identified by QTL mapping, but lacked all other *P. i. inflata* markers across the genome (Figure 2b). A single representative plant was inbred for three generations to produce a homozygous introgression line W5.

All plants of line W5 ($n = 50$) and all flowers on these plants ($n = 500$) were fully white and did not reveal a single red revertant sector (Figure 3a). The same was true for all white flowering plants across the generations of inbreeding that led to line W5. PCR genotyping for *AN1* revealed that W5 was homozygous for the *dTph1* insertion allele $an1^{W138}$, as expected (Figure 3b). The lack of excision from $an1^{W138}$ in W5 was further confirmed by PCR (Figure 3b). In W138, excision of *dTph1* is easily seen as the appearance of PCR products that correspond to the wild type *AN1* gene (R27 allele), in addition to products that reveal the *dTph1* insertion allele. In contrast, W5 revealed only the insertion allele, with excision not detectable.

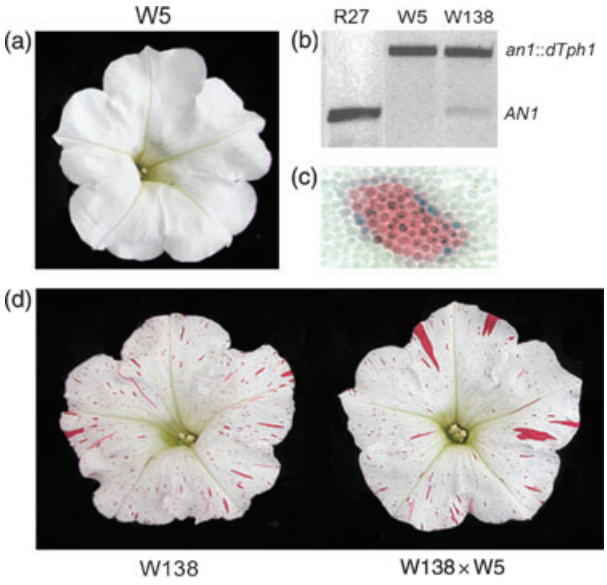

Figure 3. (a) The $act1^{S6}$ introgression line W5 is completely *dTph1* transposition deficient at the $an1^{W138}$ locus. Shown is a flower of line W5, in front view. Note the complete absence of red revertant sectors, due to the homozygous presence of an inactive $act1^{S6}$ allele.
(b) Inactivity of $act1^{S6}$, demonstrated by PCR analysis of *AN1*. The line R27 is a progenitor of W138, carrying a wild type *AN1* allele. Allelic forms are given on the right, distinguishing the $an1^{W138}$ insertion allele (*an1::dTph1*), and an excision allele (*AN1*). Note the appearance of the excision allele in W138, but not in W5.
(c) The $an1^{W138}$ insertion allele can sporadically revert in W5. Closeup of a small, red-revertant sector on an $act1^{S6}$ homozygous F_2 individual derived from the near-isogenic cross W138 × W5. The sector is about 41 cells large.
(d) The $ACT1^{W138}$ allele is dominant over $act1^{S6}$. Frequency of *dTph1* excision from $an1^{W138}$ is comparable in W138 (left image) to that of the F_1 hybrid of a cross of W138 to W5 (right image).

To obtain more accurate information about the stability of $act1^{S6}$, we grew 1250 F_2 families ($n = 12$) from 1250 F_1 hybrids from a cross of W5 to W138. In F_1 hybrids, we observed restoration of $an1^{W138}$ excision patterns to levels reminiscent of pure W138 (Figure 3d), making W5 × W138 hybrids indistinguishable from W138 in terms of sector intensity. We have not quantified differences in timing of transposition, which may lead to differences in sector sizes. However, should such differences exist, they must be subtle because they have never stood out to draw attention. In F_2 offspring, randomly selected from the 1250 F_2 families, we observed perfect 3:1 (75 spotted versus 20 white) segregation patterns ($\chi^2 = 31.8$, df = 1, $P = 0.000$).

To test the spontaneous reversion frequency of $act1^{S6}$, 1250 $act1^{S6}$ homozygous, fully white flowering individuals were selected from the 1250 F_2 families. Visual inspection of

approximately 6000 flowers for the occurrence of red revertant sectors revealed two single flowers that carried a tiny sector of a few cells (Figure 3c). As only two small sectors were observed in a sample of around 6000 flowers, spontaneous reversion can be estimated to be very low and might approach the level where non-excision-related mutations might be involved.

We conclude that the *act1*^{S6} introgression behaves as a fully recessive Mendelian locus. Furthermore, *act1*^{S6} is stably inactive across generations and causes a near-absolute elimination of *dTph1* excision from the *an1*^{W138} allele.

A second locus, ACT2*, confers weak transposition activity*

Although of major effect, *ACT1* does not explain the majority of phenotypic variation in spotting patterns in the inbred line population. Some of this unaccounted variation may be due to modifier loci, epigenetic phenomena, epistatic interactions, etc. that could not be detected by our initial QTL mapping for main effects at single QTL loci.

The WI-BIL inbred population was searched for individual lines that carried homozygous *act1*^{S6} alleles (as judged by a contiguous string of *P. i. inflata* derived AFLP markers covering the entire maximum likelihood interval of the *ACT1* locus) and instability of *an1*^{W138} was assessed. Thirteen such lines were identified, of which three were unstable (for example WI11 and WI46, Figure 4a). The intensity of sectoring was weak relative to W138, often restricted to a single revertant spot per flower. Thus, *act1*^{S6} confers transposition deficiency in the majority of lines (10/13 = 77%), but not in all. The low-intensity sectoring phenotypes were heritably stable (not shown), indicating that the lines were fixed for genetic factors that control a low level of *dTph1* excision in an *act1*^{S6} genetic background.

Because QTL analyses for main effects did not reveal evidence for such genetic factors, a test for pair-wise epistatic interactions between *ACT1* (approximated by AFLP marker S56, see Figure 2a) and the rest of the genome was performed. Initial tests analysed quantitative data with numbers of spots per flower, which yielded no significant results. Subsequently, data were reduced to an all or none response (spotting present or absent) to eliminate variation in sectoring intensity and to concentrate analysis only on whether transposition occurred at all. A highly significant interaction was detected with AFLP locus S114, on chromosome V.

To quantify the effect of S114 on transposition the locus was incorporated into a general linear model (GLM), which also included *ACT1* and an interaction term between S114 and *ACT1*. The results are given in Table 1. S114 did not have a significant main effect, confirming initial QTL mapping. However, the interaction between S114 and *ACT1* was highly significant ($F = 8.766$, df = 1, $P = 0.006$).

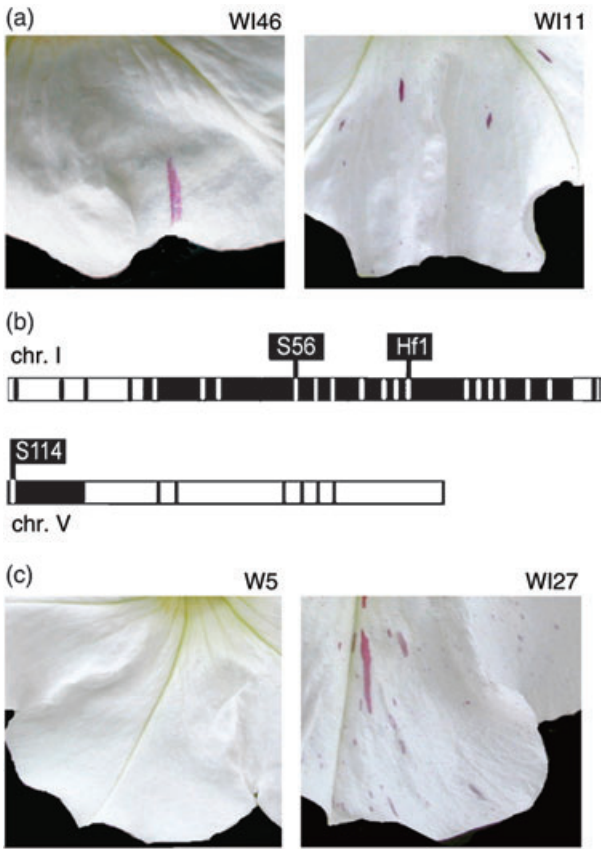

Figure 4. (a) Weak *dTph1* transposition activity from *an1*^{W138} occurs in *act1*^{S6} homozygotes. Shown are two examples of *act1*^{S6} homozygous WI-BIL inbred lines with distinct *an1*^{W138} sectoring intensities. WI46 shows infrequent small sectors, often limited to a single small spot per petal, whereas WI11 gives revertant *AN1* sectors at a somewhat higher frequency.
(b) Graphical genotypes of chromosomes I and V in the *act1*^{S6} homozygous line WI27. Chromosomes are represented as horizontal bars. White denotes W138, black *Petunia i. inflata*. All other chromosomes in this line were pure W138.
(c) *ACT2* partially restores *dTph1* activity in *act1*^{S6} backgrounds. Note the difference in spotting between the *act1*^{S6} single introgression line W5 and *act1*^{S6}/*ACT2*^{S6} double introgression (WI27). Also note the greyish-red colour of the sectors, which is due to the presence of a homozygous *P. i. inflata* allele at *Hf1*.

The simplest interpretation of these results is that S114 defines a second locus for *dTph1* activation, and that its (small) effect can only be detected in the absence of *ACT1*^{W138}. We coin the locus *ACT2.* The *P. i. inflata* allele at the locus is defined as *ACT2*^{S6}, the W138 allele as *act2*^{W138}. The *ACT2*^{S6} allele confers weak transposition activity. The *act2*^{W138} allele is apparently not active, judging from the essentially complete absence of *dTph1* excision from *an1*^{W138} in the *act1*^{S6} introgression line W5.

The distribution of *ACT2*^{S6} over all 13 *act1*^{S6} homozygous BIL lines was inspected, as summarized in Table 1. Its association with *dTph1* transposition in this subset of *act1*^{S6} homozygotes is highly significant (Mann–Whitney test,

Table 1 Contribution of *ACT1* and *ACT2* to variation in *dTph1* transposition

	Whole WI-BIL population[a]				*act1*^{S6} homozygotes[b]			
Genotype	*F*	df	*P*	R^2	Spotted	White	U^f	*P*
[c]S56	24.2	1	0.000	0.40	0	9	4.5	0.004
[d]S56 × S114	8.77	1	0.006	0.13	3	1		
[e]S114	1.43	1	0.24	0.01	–	–		

[a]Contribution of *ACT1*, *ACT2* and their interaction to a multiple linear regression model, predicting transposition from the single and combined genotypes.
[b]Distribution of spotted and white phenotypes over *act1*^{S6} homozygous genotypes and *act1*^{S6}/*ACT2*^{S6} homozygous genotypes.
[c]Homozygous genotype, synonymous to *act1*^{S6}.
[d]Interaction term for S56 and S114.
[e]Homozygous genotype, synonymous to *ACT2*^{S6}.
[f]Mann–Whitney test.

$U = 4.5$, $P = 0.004$) despite the limited sample size, which confirms that *ACT2*^{S6} co-segregates with instability at *an1*^{W138}. The association does not appear complete, however. In the one line of the group *ACT2*^{S6}/*act1*^{S6} that was not unstable, *ACT2*^{S6} might have been eliminated by a recombination event that could not be detected due to the paucity of available markers at the locus. Otherwise, regulation of transposition may be even more complex than that suggested by the current two-locus model.

Analysis of genome-wide graphical genotypes revealed that the line WI27 contained only the *P. i. inflata* allele at *ACT2*, in addition to a chromosome I introgression encompassing S56 (*act1*^{S6}; Figure 4c). Therefore, this line can be regarded as a double introgression of *act1*^{S6} and *ACT2*^{S6}. The effect of *ACT2*^{S6} becomes apparent by comparing the single and double introgression lines W5 and WI27 respectively (Figure 4d). *ACT2*^{S6} restores *dTph1* excision from *an1*^{W138}.

act1^{S6} *introgression inactivates the entire* dTph1 *family throughout development*

We addressed whether the *act1*^{S6} introgression line W5 would be suitable for two-element control of *dTph1* transposition. It is formally possible that *ACT1* affects *dTph1* transposition at some loci only, including *an1*^{W138} and some others, such as *dfr*C::*dTph1* (Huits *et al.*, 1995). In addition, the activity of *ACT1* may not be the same in all stages of plant development, which could lead to elimination of excision in petals (as monitored by *an1*^{W138}), but not in leaves or other tissues.

To investigate the activity of other *dTph1* elements, we compared the accumulation of new *dTph1* insertions through generations of W138 and W5 by transposon display (Van den broeck *et al.*, 1998) on leaf tissue. In six self-progeny of a single W138 plant, at least 36 new insertions were identified (Figure 5a). These insertions represented single bands unique to one sibling, and are thus novel without ambiguity. In contrast, the W5 line did not accumulate any new insertions (Figure 5a). Many other bands showed segregation patterns that originated from pre-existing heterozygous insertions in the parent plants (Figure 5a). Inbreeding of W5 generated a pattern of increasing homozygosity for all *dTph1* elements. The result was a homogeneous family with few remaining heterozygous d*Tph1* insertions, in which no new insertions arose (Figure 5b). It must be noted, however, that transposon display may not detect weak transposition activity and rare transposition events in small somatic sectors.

To further quantify *dTph1* excision levels in W5 for insertions alleles other than *an1*^{W138} and in a third stage of development, we scored revertant sectors in plants containing a *dTph1* insertion in the *ALB1* gene, which is required for chlorophyll accumulation in the cotyledons. The *alb1*::*dTph1* mutation arose spontaneously in W138. Because cotyledons are embryonic tissue, assaying excision from *alb1*::d*Tph1* reveals *dTph1* activity during embryogenesis. In plants homozygous for *ACT1*^{W138} and *alb1*::*dTph1*, revertant sectors in cotyledons were frequent and ranged in size from small sectors to entire cotyledons (Figure 5c). In contrast, in seedlings double homozygous for *act1*^{S6} and *alb1*::*dTph1* revertant sectors or cells were never observed ($n = 98$, Figure 5c).

We conclude that *ACT1* controls transposition of all *dTph1* elements during the entire life cycle of the plant and that *act1*^{S6} eliminates *dTph1* transposition essentially completely.

Segregation of act1^{S6} *creates stable tagging populations*

Among the most significant advantages of having a single activator on a fixed chromosomal position in a high-copy *dTph1* genetic background is the ability to trans-activate a large number of *dTph1* elements that can subsequently be stabilized at once by segregating the activator out.

To assess the feasibility and efficacy of this approach, we generated 1250 F_1 plants from the near-isogenic cross W5 × W138. To produce F_1 hybrids, a single W5 plant was used as either a father or a mother in crosses to a single W138 plant. To maximize the number of *dTph1* positions, we used a W138 line that was relatively distant from W5. Because F_1 hybrids were generated from a single parental individual, transposition frequencies can be measured from the appearance of new PCR fragments in transposon display of F_2 progeny, without interference of pre-existing insertions from distinct parental origin. In addition, seed capsules were kept separate to enable any flower-specific transpositions in the W138 parent to be traceable through the progeny of the cross. A schematic pedigree structure is given in Figure 6(a).

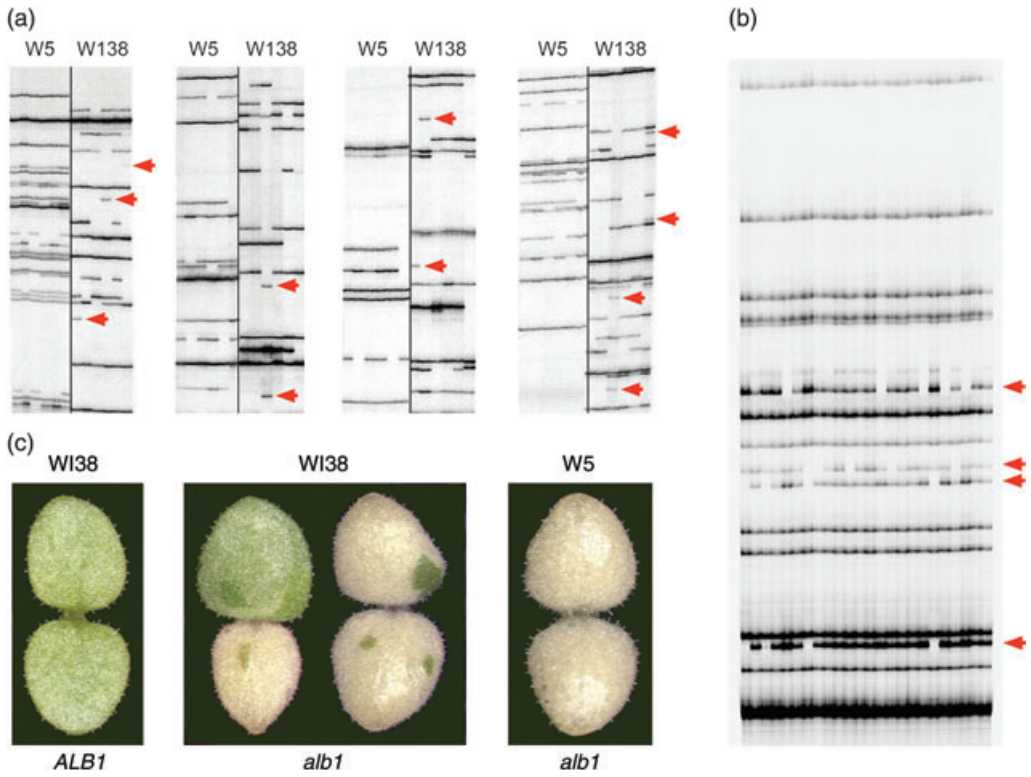

Figure 5. (a) The *act1*^{S6} allele eliminates transposition of the entire *dTph1* family. Shown are fragments of transposon display gels with a single selective base (from left to right, A, C, G, T) on DNA from leaf tissue. Each gel contains six W5 full siblings, and six W138 full siblings. Note that new insertions (unique bands, red arrows) are seen exclusively in W138 progeny.
(b) Inbreeding with W5 leads to increasing homozygosity on *dTph1* loci, without occurrence of new insertions. Shown is a part of a transposon display gel (C selective) with 22 full W5 siblings after three generations of inbreeding. Note that the majority of bands occur in all samples, with some (red arrows) segregating as a consequence of remaining heterozygosity in the parent. Bands unique to one plant are absent.
(c) The *act1*^{S6} allele eliminates transposition of *dTph1* during embryogenesis, monitored from instability at the *alb1::dTph1* locus. *ALB1* wild types have green cotyledons (left image), whereas *alb1::dTph1* gives rise to albino cotyledons with revertant sectors in the presence of *ACT1*^{W138} (W138, middle image), but not in the presence of a homozygous *act1*^{S6} (W5, right image).

Each of 1250 F_1 plants was visually inspected for the intensity of *AN1* sectoring, which was in the large majority of cases comparable to pure W138. All 1250 F_1 plants were self-fertilized and each of the resulting 1250 F_2 families ($n = 12$ individuals per family) was scored for the segregation of spotted and white flowering phenotypes. In all families, near 3:1 patterns were observed (data not shown). Of each family, a single white F_2 segregant was selected, yielding a population of 1250 F_2 individuals that should have accumulated independent, germinally transmitted *dTph1* insertions that originated from transpositions in the F_1 generation.

To count the number of novel insertions, we used transposon display on a subset ($n = 16$) of F_2 individuals (Figure 6b). These individuals originated from F_1 siblings that came from a single seed capsule of the original cross between parental plants. New insertions are defined as those arising in the F_1 generation, and must thus be unique bands in transposon display on F_2 progeny. Figure 6(b) shows transposon display with one single selective base, thus representing a quarter of all insertions. From a full analysis, we conclude that the 16 F_2 individuals carried on average eight germinally inherited novel *dTph1* insertions per individual plant. Similar results were obtained for other F_2 plants derived from different seed capsules (data not shown). Therefore, if we extrapolate to the entire population of 1250 F_2 plants, the total number of new insertions amounts to approximately 10 000.

Discussion

We have aimed here to map and characterize genetic factors that control the *dTph1* transposon system in *Petunia*, and to create a two-element system for mutagenesis programmes. Using a quantitative approach to analyse excision frequencies from an indicator locus (*an1*^{W138}), we localized two QTLs. One QTL coincided with the previously described *ACT1* locus on chromosome I (Huits *et al.*, 1995). A second, previously undescribed QTL (*ACT2*) was detected on chromosome V, and conferred partial transposition activity in a genetic background of the inactive *act1*^{S6} allele. Comparison

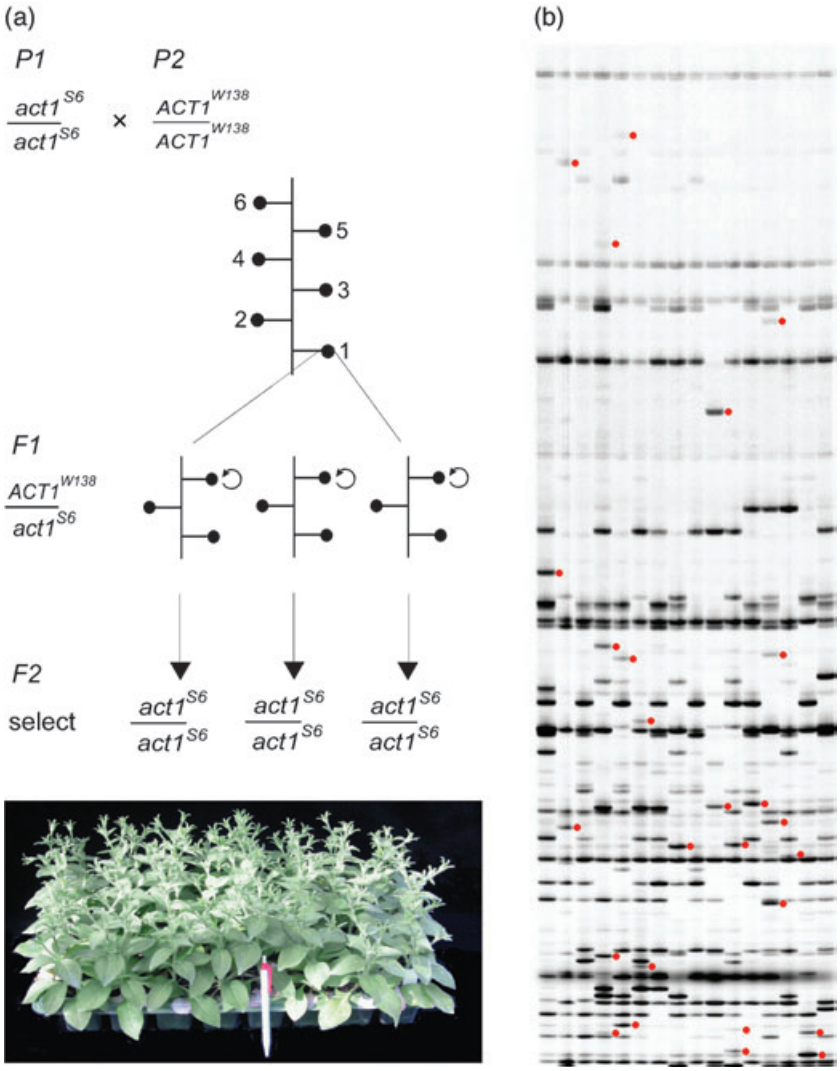

Figure 6. (a) Genetic scheme to derive stable *dTph1* transposon insertions in isogenic lines. Parental genotypes (P1 = W5; P2 = W138) are crossed to produce individual seed capsules (numbered 1–6) containing F_1 hybrid seed. First-generation hybrid plants (F_1) are grown from each single capsule (example shown for capsule 1 only). F_1 flowers are self-fertilized intraflorally (curved arrows). F_2 plants are grown from each F_1 individual separately, and selected phenotypically for absence of *ACT1* activity (*act1*^{S6}) by scoring instability of *an1*^{W138}. From each F_1 individual, a single *act1*^{S6} homozygous F_2 plant is grown to maturity in flats with small pots (see image). F_2 individuals are self-fertilized for maintenance. For a sense of scale, note the pen in front of the flat, which contains F_2 plants at seed-harvesting stage.
(b) Stabilized F_2 lines contain multiple new insertions. Shown is a fragment of a transposon display gel (C-selective) containing 16 F_2 lines derived from a single F_1 seed capsule. New insertions are recognized as single bands, unique to a single plant (red dots).

of QTL data and introgression lines of *act1*^{S6} and *ACT2*^{S6} shows that *dTph1* transposition is controlled mainly by *ACT1*, which behaves essentially as an on–off switch for the entire *dTph1* family. Excision frequencies in our inbred line population varied widely among lines, and not all variation can be explained by *ACT1* and *ACT2*. The control systems that govern *dTph1* transposition are likely to be more complex than predicted by a two-locus model, and might involve a variety of mechanisms each of comparatively small effect.

From analyses of *act1*^{S6} in the introgression line W5, it appears that *ACT1* controls the entire *dTph1* family in all developmental stages. It is notable that some other small transposable elements (e.g. d*Tph3*) do not transpose in W138, whereas in other backgrounds they do (Kroon *et al.*, 1994; Van Houwelingen *et al.*, 1998). This suggests that *ACT1* is not a general (host) factor required for transposition *per se* but specifically controls *dTph1* and perhaps some other elements, such as the highly related *dTph4* (Renckens *et al.*, 1996). Such properties can be expected from, for example, a transposase. The same argument may hold for *ACT2*, which might correspond to a weak transposase source. Alternatively, *ACT2* might modify *ACT1*, rather than being a *dTph1* trans-activator itself. It remains unresolved what determines the difference in activity of the *ACT1* locus in genotypes such as W138 and *P. i. inflata*. As suggested previously (Huits *et al.*, 1995), the locus maybe under epigenetic control from which it might occasionally be lifted, for example after interspecific crosses. *ACT2* might play a role in this process by affecting the activity level of *ACT1*.

Because the copy number of *dTph1* can be very high in some strains of *Petunia*, it is interesting that no other trans-activators or autonomous *Tph1* elements have been described in either *P. hybrida* or in several wild *P. axillaris* and *P. integrifolia*. In addition, it is remarkable that *ACT1* maps on similar locations in various *Petunia* genotypes and is

generally inactive in wild species (Huits *et al.*, 1995). It may be that in natural populations *Tph1* has been low-copy for long periods of time, and that a single autonomous element mutated into *ACT1* before the split between the major species in the *Petunia* genus. The origin of the mutations may have been a loss of cis-requirements for excision but not transposase function. Molecular cloning of *ACT1* is required to settle this issue. From our observation that the *P. i. inflata* allele of *ACT2* is the active one, the general inactivity of *ACT1* in various wild *Petunia* accessions (Huits *et al.*, 1995) does not necessarily imply that *dTph1* transposition is fully impaired. In fact, we have found that natural *P. axillaris* populations can contain large numbers of *dTph1* elements on many distinct genomic positions (J. Stuurman, unpublished data). It would be interesting to investigate whether $ACT2^{S6}$ is mobile, as this would indicate a role as a transposase source and active autonomous element.

Our analyses provide several parameters for judging the utility of the $act1^{S6}/ACT1^{W138}$ allele pair as an activation system for two-element *dTph1* tagging. Most importantly, *ACT1* occupies a stable position in all genotypes analysed thus far, making it a single locus that can be segregated in a simple Mendelian fashion. The active allele appears completely dominant over the inactive allele. The intensity of *AN1* instability in W5 × W138 F_1 hybrids is essentially equal to that in pure W138. The average number of new insertions in stable white flowering F_2 individuals (about eight per plant) is comparable to that found in W138 progeny. With about 200 *dTph1* elements per diploid genome, this number translates into a germinal transposition frequency of approximately 5%. De Keukeleire *et al.* (2001) found larger average insertion frequencies in the line W137, which is a direct derivative of W138 (Wijsman, 1986). It is possible that among W138 individuals and their progeny, there is variability in activity of the population of *dTph1* elements, which may be caused by the non-autonomous elements themselves (genomic position, methylation status, etc.), or by some variation in *ACT1* activity. The latter has been suggested to occur in line W87 (isogenic to W138), which presumably contains a weak allele of *ACT1* (Wijsman, 1986).

In comparison with other endogenous plant transposon systems (*Ac*, *Mu*, *Spm*, *Tam1*, etc.) the latter are often characterized by multiple autonomous and non-autonomous elements dispersed over the genome (Kunze *et al.*, 1997). Creating lines with a single activator, if at all possible, involves backcrossing to low-copy strains and thus dilution of copy number and tagging efficiency (e.g. Lisch *et al.*, 1995). With *ACT1*/*dTph1* in the W5-W138 nearly isogenic pair of *Petunia* this is not the case, resulting in a highly effective two-element transposition system. When compared with heterologous two-element tagging systems, which are based on mostly single transgenic insertions (Maes *et al.*, 1999), the undiluted high-copy number of *dTph1* translates into a gain of mutagenic efficiency of almost an order of magnitude. For a population with 50 000 stable insertions, a *Petunia* system would require the production of about 5000 F_2 plants, which is not logistically expensive. It has often been claimed that single-copy heterologous insertions are advantageous for cloning tagged genes, providing simple ways to associate mutant phenotypes with a particular transposable element. In *Petunia*, *dTph1* has been associated with virtually all unstable mutations in W138, indicating that interference of other element families is minimal. In addition, high-resolution techniques such as transposon display can resolve large families into single elements with Mendelian properties. It must be noted that maintenance of stabilized *dTph1* populations by inbreeding will reduce the copy number by half over time, due to random segregational loss of insertion alleles.

The application of the W5 introgression line for stabilizing *dTph1* elements in transposon mutagenesis presents an important advantage over the conventional use of *dTph1* in continuously inbred active lines. One of the main advantages of a two-element system is that phenotypic effects of stable insertions can be assessed in replicated trials with inbred lines derived by single seed descent (mutant inbred lines, MILs). This would allow identification and cloning of QTL by transposon tagging, where the basic problem consists of identifying tagged alleles with mild phenotypic effects relative to non-genetic variation. In *Drosophila*, large numbers of quantitative mutations are being identified by *P* element mutagenesis (e.g. Norga *et al.*, 2003), a number of which affect genes that correspond to positionally cloned natural QTL. This approach reconciles transposon mutagenesis and quantitative trait analysis (Mackay, 2004). A two-element *Petunia* tagging system should identify many mutants with quantitative phenotypes, which may be associated with insertions in regulatory sequences, introns, redundant genes, etc. Identification of subtle effects associated with insertions requires extensive replication of genotypes, making the logistic effectiveness of the *Petunia* system a clear advantage.

A *Petunia* system along the lines described here provides rich genetic variation for many different traits, qualitative or quantitative. As a model Solanaceae, the *Petunia* system may serve to build insertion resources. This family includes several important crops (tomato, potato, tobacco, pepper, eggplant, etc.) for which no high-efficiency tagging systems have been described and for which the development of such systems is hampered by the necessity for introducing heterologous insertion elements as well as the generally large size of these crop plants. It is well conceivable that conservation of gene function is considerable among Solanaceae, and that the *Petunia* tagging system would be a valuable addition to the efforts paid to its nearest relatives (SOL initiative, http://www.sgn.cornell.edu/). It can serve as a testing platform to identify functions for sequences obtained from the genome sequencing efforts in tomato.

For such applications, a stabilized resource would have clear advantages.

Experimental procedures

Plant material and genotypes

The line W138 is a standard laboratory strain that is maintained by inbreeding. The WI-BIL backcross recombinant inbred lines including their derivation and linkage map have been described before (Stuurman *et al.*, 2004).

Construction of the W5 introgression line used a backcross of W138 (recurrent parent) to the line WI92 (from the WI-BIL population). WI92 is a fully white flowering plant carrying segments of chromosome I and VI. In the first-generation backcross progeny, AFLP marker analysis was performed on those plants most similar in phenotype to W138. The line W5 was selected and inbred. Phenotypically, W5 is very similar to W138, with some distinctions in leaf surface area and sepal size that cosegregate with *act1*.

All genotypic information on all WI-BIL inbred lines was obtained from a database of graphical genotypes in the computer program GGT (Van Berloo, 1999). This database is available upon request.

All plants were grown in greenhouses under standard conditions.

QTL analysis and statistics

Quantitative data on spotting intensities were obtained by counting the number of individual spots on digital images of a single petal of all BIL lines. Composite interval mapping (CIM) was performed using the computer program QTL Cartographer (Basten *et al.*, 1994, 2002). The population type was SF7 (seven self-generations), which allows for some level of residual heterozygosity. Cofactors were selected using forward/backward (Pin-out = 0.01) regression in model 6 of Cartographer. The exclusion window was reduced stepwise down from 10 cM, until the localization and LOD scores were optimized. Single marker Kruskal–Wallis rank test procedures were performed on every non-redundant marker on the linkage maps. Dominant AFLP markers were selected along chromosomes with significant associations to set the significance levels at $P < 0.05$ ($\chi^2 = 3.84$, df = 1).

Genome-wide searches for pairwise interactions among all AFLP loci were performed using the computer program MapManager QTXb17 (Manly *et al.*, 2001), at a significance level of $P = 10^{-6}$. Main effects of QTL and pairwise interactions were analysed using a GLM with closely linked markers as proxies for QTLs. Markers and interactions were taken as significant if they contributed to the overall model fit at $P < 0.01$. This procedure takes account of our BC1F5 population structures, which result in unequal group sizes for alternative genotypes (3:1, unless distorted). All standard statistical analyses were performed using SPSS 11.0 for Windows.

DNA methods and transposon display

All molecular technique followed standard methods. PCR analysis for *dTph1* excision from $an1^{W138}$ was performed using primers *an1*F (5′-cccgttatctacagcagtagtg-3′) and *an1*R (5′-gagaaacttcagctgcagtttg-3′).

AFLP analyses were performed as described by Vos *et al.* (1995).Transposon display, as a modification of the protocol originally described by Van den broeck *et al.* (1998), was performed essentially as regular AFLP (Vos *et al.*, 1995), except that *Mse*I was used as the sole restriction enzyme. After ligation of *Mse* adapters, templates were preamplified with primer out12 (5′-ccagcattgacacccttcg-3′) and Mse+0. Reactions were diluted 200 times, and reamplified with out13 (5′-gcagtgtaaattttgcgcaaa-3′) and Mse+1, which carries a one-base extension of A, C, G or T. Primers out13 were 5′ labelled either with P^{33} and run on standard sequencing gels, or with IRD700 fluorescent dyes and run on a LICOR 4300 DNA analyser (LI-COR Biosciences, Lincoln, NE, USA).

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