A four-element based transposon system for allele specific tagging in plants – Theoretical considerations

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The two-element transposon constructs, utilizing either Ac/Ds or Spm/dSpm, allow random tagging of genes in heterologous model species, but are inadequate for directed tagging of specific alleles of agronomic importance. We propose the use of Ac/Ds in conjunction with Spm/dSpm to develop a four-element system for directed tagging of crop-specific alleles. The four-element based construct would include both Ds and dSpm along with relevant marker genes and would function in two steps. In the first step dSpm(Ds) stocks (a minimum of two) would be crossed to a line containing transposases of Spm and unlinked integrations would be selected from segregating population by the use of a negative selection marker to develop stocks representing integration of dSpm(Ds) at a large number of locations in the genome. Selections would be made for a line in which dSpm(Ds) shows partial or complete linkage to the allele of interest. In the second step selected line would be crossed to a line containing Ac transposase to induce transpositions of Ds element to linked sites thereby exploiting the natural tendency of Ds element to jump to linked sites. Unlinked jumps of dSpm(Ds) and linked jumps of Ds could be monitored by appropriate marker genes. The proposed model would allow tagging of allele of interest in chromosome addition lines and also help in the efficient use of genic male sterility systems for hybrid seed production by tightly marking the fertility restorer gene with a negative selection marker.

1. Introduction

Crop species carry a large number of agronomically important alleles that are usually present dispersed in many cultivars, accessions and land races. Very extensive work has been done in the last few decades on the transmission genetics of crop-specific alleles that confer resistance to biotic and abiotic stresses. Molecular analysis of such alleles could be of tremendous significance for stabilization breeding of crop species. Of particular interest are alleles that have been transferred from alien relatives to crop species (Jiang *et al* 1994). In many cases such alleles are tightly linked to loci that have deleterious effects on the yield of crop plants. Molecular characterization of such alleles would allow separating the allele of interest (AI) from bad linkage and provide the possibility of introducing such genes into crop species by genetic transformation. Crop-specific alleles could be isolated either by map-based cloning (Arondel *et al* 1992; Martin *et al* 1993) or by transposon-mediated tagging (Jones *et al* 1994). We concern ourselves in this paper with transposon-mediated gene tagging.

Transposable elements have the ability to transpose in the genome and thereby create mutations at the site of insertion (McClintock 1948, reviewed by Fedoroff 1989). Genes tagged by a transposable element can be isolated by using the tag as a probe (reviewed by Sundaresan 1996). Transposable elements have been used for tagging a number of genes from maize and snapdragon using their native transposable element(s) Ac/Ds, Spm/dSpm, Mu in case of maize and Tam3 in case of snapdragon as tags (Fedoroff *et al* 1984; Martin *et al* 1985, reviewed by Walbot 1992). While Ac and Spm are autonomous elements, Ds and dSpm are their defective homologues that cannot transpose on their own, but would transpose if Ac (in case of Ds, Fedoroff 1983) or Spm (in case of dSpm, Masson and Fedoroff 1989) or their respective transposases(TPases) (Coupland *et al* 1988; Frey *et al* 1990) are available in *trans*. The auto-

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nomous element Ac, has been introduced into a number of plant species (Baker *et al* 1986; Van Sluys *et al* 1987; Yoder *et al* 1988; Knapp *et al* 1988) and has been shown to transpose in these heterologous systems. However, it is difficult to stabilize the mutations created by insertion of Ac and transpositions cannot be regulated. Despite these limitations a few genes have been tagged using Ac as a single-element system (Lawrence *et al* 1993; Whitham *et al* 1994; James *et al* 1995).

Some important methodological improvements have been made to improve the procedure of gene tagging. Marker genes have been deployed to monitor excision and integration of the transposable element in heterologous systems. Two-element systems viz., Ac/Ds (Hehl and Baker 1989) and Spm/dSpm (Masson and Fedoroff 1989), have been used in which TPase source (from Ac or Spm) is stabilized by clipping its border sequences. Consequently clipped Ac or Spm cannot transpose on their own but would induce transposition of Ds or dSpm, respectively in *trans*. The frequency of transposition has been improved by using a strong promoter i.e., CaMV35S to drive the expression of TPases (Grevelding *et al* 1992; Cardon *et al* 1993; Honma *et al* 1993). Further the two element systems can be improved by using herbicide resistance conferring genes as markers so as to allow efficient field selection of plants in which transpositions have occurred (Phogat S, Burma P K and Pental D, unpublished results). The most recent advance has been the use of a negative selectable marker (*iaaH* gene that converts non-toxic a naphthalene-acetamide, NAM to auxin, a naphthalene acetic acid, NAA) in conjunction with a two-element system based on Ac/Ds (Sundaresan *et al* 1995). The incorporation of *iaaH* in the constructs allows selection for unlinked transposition events, and this negative selection marker can also be used to segregate the TPase source in subsequent generations after transposition has taken place so as to stabilize the Ds element at the mutated locus.

The methodological improvements described above have allowed random tagging of a few genes that produce scorable phenotypes (Aarts *et al* 1993; Bancroft *et al* 1993; Chuck *et al* 1993; Springer *et al* 1995; Bishop *et al* 1996). All these genes have been tagged by a random tagging approach in which any interesting gene with a distinct phenotype was tagged and characterized. Directed tagging by the two-element system in crop plants has been achieved only for one gene, *Cf-9* from tomato that confers resistance to infection by the fungus *Cladosporium fulvum* (Jones *et al* 1994).

We discuss in this paper the shortcomings of the existing models for directed gene tagging and project a model based on the use of four-element constructs that would alleviate the difficulties faced in the use of current models. The proposed four-element system has the potential to make transposon-mediated tagging of crop-specific alleles feasible and much simpler than possible with the earlier models. The four-element system would also allow exploitation of genic male sterility for heterosis breeding in any crop species.

2. Theoretical considerations

2.1 *Limitation of two-element systems for directed allele tagging in crop species*

Directed tagging essentially involves three steps: (i) Introduction of a single-copy Ds or any other impaired element in different lines such that the population would represent the presence of Ds at large number of locations on all the chromosomes, (ii) linkage of the Ds element to the AI and (iii) tagging of the AI by transposition of Ds into the AI.

Both Ac/Ds and Spm/dSpm tend to transpose into linked sites (Greenblatt 1984; Dooner and Belachew 1989; Sundaresan 1996). Unlinked transpositions are at far lower frequencies than the linked ones. The bias towards linked transposition by both Ac/Ds and Spm/dSpm elements allows directed tagging of only those AIs that are linked to the Ds or dSpm element (James *et al* 1995).

The use of a negative selectable marker allows enrichment for unlinked transpositions (Sundaresan *et al* 1995), but the frequency for unlinked transpositions into a particular unlinked site would be very low (calculated to be around 6×10^{-7} in case of tomato, van der Biezen *et al* 1994). This strategy also suffers from the limitation that once stocks are developed with Ds element present at reasonable frequency on each chromosome, subsequent jumps of Ds cannot be detected. Consequently the propensity of transposable element to insert in linked sites cannot be fully exploited for successful directed gene tagging.

The strategy of generating a large number of single-copy Ds or dSpm carrying transgenic stocks so as to get an integration close to the AI is an arduous task as transformation protocols for important crop species (except members of Solanaceae family) are difficult and inefficient (reviewed by Christou 1996). We assessed, by 10^4 runs (at 95% confidence level) of a random hit generation computer program the number of transgenics that will have to be produced, to have a random hit (either 1, 2, 3, 4, 5, 10 or 20 on each of the chromosomes) in some of the major crop species (table 1). As in general one in ten genetic transformants carry single-copy integration and each line should only have a single insertion thereby a large number of primary transformants will have to be produced. For example in rice, even with the best possible transformation system, only one in six genetic transformants carries single-copy integration (Hiei *et al* 1994), around 1750 primary transformants will have to be produced to have a representation of four single-copy Ds inserts on each chromosome.

2.2 *The four-element transposon constructs for directed allele tagging*

The inadequacies of the existing models for tagging crop-specific alleles can be alleviated by using constructs that are based on the utilization of four elements – dSpm, Ds and the TPase encoding genes of Spm and Ac (figure 1). Monitoring of integration and excision could be done by herbicide resistance conferring markers, PAT for resistance to phosphinothricin (DeBlock *et al* 1987) and modified ALS for resistance to imidazolinones (Sathasivan *et al* 1990). IAAH converts non-toxic NAM to auxin, NAA and could be used as a negative selection marker (Bancroft *et al* 1992). The four-element system would allow tagging of genes in two steps. In the first step, a minimum of two dSpm(Ds) (each one with a single copy insert) lines would be crossed to a line carrying Spm TPases and unlinked integrations would be selected based on the absence of the negative selection marker from the segregating population to develop stocks representing integration of dSpm(Ds) at large number of locations in the genome. These newly generated lines could be marked for the location of the dSpm(Ds) element in the chromosomes by RFLP analysis, and can then be used for directed allele tagging. For directed tagging, selection would be made for a line in which dSpm(Ds) shows partial or complete linkage to the AI. In the second step, the lines showing partial or complete linkage to the AI would be crossed to a stock containing Ac TPase to induce transposition of Ds element into the target site.

2.3 *A four-element system for hybrid seed production*

In majority of plants genic male sterility (GMS) arises due to a single recessive gene (Duvick 1966). It is not possible to maintain a male sterile (ms/ms) plant as these cannot be selfed. Maintenance of male sterile plant is, however, possible by crossing these plants to a heterozygous fertile plant (MS/ms). This cross leads to progeny that segregates for fertile and sterile plants in 1 : 1 ratio. Weeding out the fertile plants is not possible until they come to flowering and this is a major limitation for utilization of GMS for large scale hybrid seed production.

The model described above with some modifications can be used for hybrid seed production using the GMS. This modified four-element system (figure 2) would harbour a dominant negative selection marker i.e., bacterial cytochrome P450 that catalyzes activation of sulphonylurea proherbicide, R7402, to herbicide (O'Keefe *et al* 1994) placed along with PAT within the Ds borders. This construct would be used for tight-marking of the dominant homologue (MS) of the recessive allele (ms). Tight-marking of MS gene would be feasible by the outlined two step procedure (figure 2). A line harbouring MS linked to both a dominant negative selectable marker as well as a positive selectable marker would serve as the maintainer line of the male sterile line. A cross between such a maintainer line and the male sterile line would give rise to a mix of fertile and sterile plants (1 : 1) in the F1. However, the fertile plants can be weeded out at the seedling stage by spraying the precursor compound that would be converted to herbicide by the negative selectable marker (O'Keefe *et al* 1994; Kriete *et al* 1996). The male sterile plants can be crossed to any good combiner for hybrid seed production (figure 2).

3. Discussion

The four element system described in this paper will provide the following advantages over the existing systems for gene tagging:

Table 1. The number of single-copy transgenics in major crop plants, that will have to be generated to have a representation of Ds element on all the chromosomes in order to tag an allele of interest by the two-element system.

	Number of single copy transgenics to be generated[a,b]					
No. of hits per chromosome	Pea ($2n$ = 14)	Corn ($2n$ = 20)	Rice ($2n$ = 24)	Rapeseed ($2n$ = 38)	Soybean ($2n$ = 40)	Wheat ($2n$ = 42)
1	75	118	145	250	261	277
2	105	160	199	323	355	372
3	132	196	242	401	430	455
4	155	233	288	483	504	532
5	179	266	324	544	572	606
10	283	417	510	835	884	931
20	472	688	835	1361	1441	1515

[a]These numbers were deduced on the basis of 10^4 runs of a random hit generation programme (at 95% confidence limit) for different number of hits given in column I.
[b]With an expectation of one out of ten transgenics being single-copy, the number of primary transgenics that will have to be generated would increase by 10-fold over the values given in the table.

(i) Only two transformed lines are needed to initiate the gene tagging programme. Transformation techniques in most of the cereal and legume crops are adequate to produce such small number of transformed plants (Christou 1996).

(ii) The dSpm borders could be deployed for the first transposition event instead of Ds borders because transpositions of dSpm occur throughout the plant development, as late as in the gametes (Aarts *et al* 1995) whereas in case of Ds early transpositions occur (Sundaresan *et al* 1995). The use of dSpm would allow screening of a fewer number of F1s for selection of large number of plants with unlinked transposition events. However, some of the recent work from our lab (unpublished) and from other labs (Tissier *et al* 1999) show that dSpm has very poor frequency of unlinked transpositions in *Arabidopsis*. Therefore, the use of dSpm or Ds for the first step of unlinked jumps will have to be experimentally tested.

(iii) The marker system viz., PAT and ALS for integration and excision, respectively would make this system versatile and self-sufficient for tagging since PAT conferring resistance to phosphinothricin is a marker for both *in vitro* and in field selections, and modified ALS conferring resistance to imidazolinones can be used as an excision marker at the field level. However, other selection markers like *bxn* conferring resistance to bromoxynil (Stalker *et al* 1988) and ALS conferring resistance to sulphonylureas can also be used for field level selections. The use of a negative selection marker (i.e., *iaaH*) would facilitate the selection of non-linked transposition. Other negative selectable marker genes that convert benign precursor compounds to herbicides (O'Keefe *et al* 1994; Kirete *et al* 1996; Tissier *et al* 1999) would be more useful because these can be used for field selection.

(iv) The negative selectable markers described above can also be used along with the transposase source to segregate out the transposase once the transposition of Ds has occurred (Bancroft *et al* 1992; Sundaresan *et al* 1995).

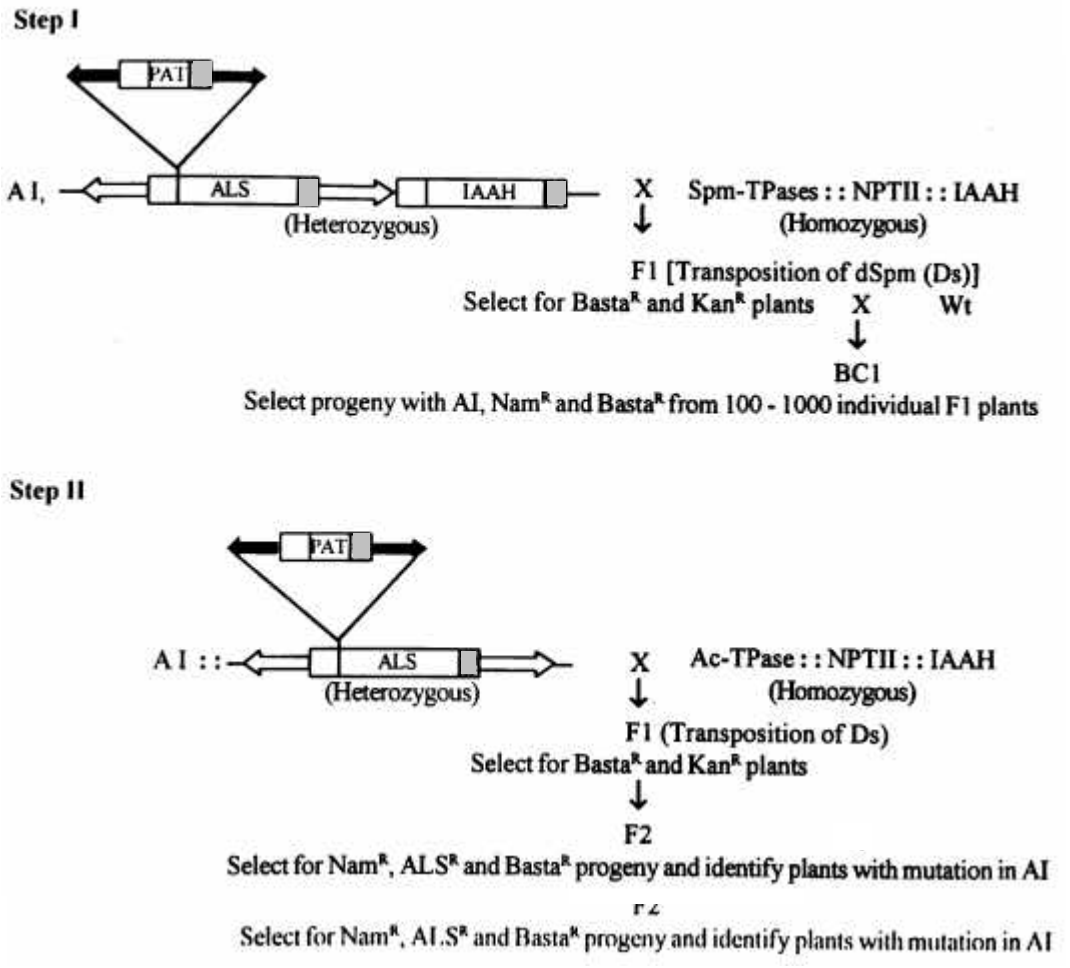

Figure 1. A two-step model for the use of the proposed four-element system that envisages the use of transposable elements dSpm and Ds and their respective transposases to tag an allele of interest (AI). Monitoring of integration and excision could be done by herbicide resistance conferring markers (i.e., PAT for resistance to phosphinothricin and ALS for resistance to imidazolinones) for easy field selection. IAAH is proposed as a negative selection marker, but could be replaced with other marker genes that convert benign precursors to lethal herbicides and therefore, would be more appropriate for field selection. Step ⇨ would lead ➡ to the production of lines in which dSpm(Ds) is integrated at a number of sites on the chromosomes. Subsequent to that, these lines would be tested for linkage of dSpm(Ds) to the AI. ( ), dSpm; ( ), Ds; (□), Promoter; (■), pA signal.

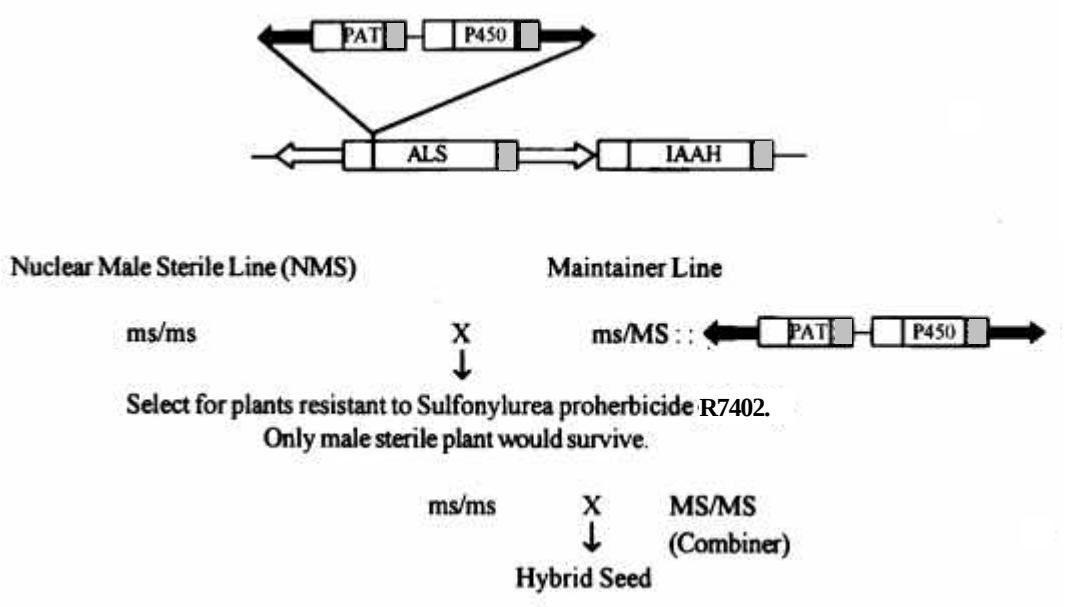

Figure 2. A four-element model with a modification of including a negative selection marker i.e., P450 (converts non-toxic pro-herbicide R7402 to toxic sulphonylurea) within the Ds element along with a positive selection marker (PAT for resistance to phosphinothricin) for heterosis breeding utilizing genic male sterility (GMS). The dominant marker PAT would allow tight linkage of the Ds element to the fertility restorer (MS) gene of GMS, following the steps depicted in figure 1. Such a line would serve as a maintainer line for the GMS line. A cross between GMS and its maintainer line would give rise to a mixed population of fertile and sterile plants. Fertile plants would be killed by the use of the negative selection marker. Sterile plants could be used for producing hybrid seed.

(v) The AIs in crop species lie scattered in a large number of breeding lines and it is difficult to introduce these genes simultaneously in a master line to make two-element based tagging feasible. Tagging of such AIs would be possible by the four element system. The dSpm(Ds) containing stock could be crossed with any breeding line harbouring the AI, subsequent to which this hybrid can be crossed with the Spm transposase containing stock to allow scattering of dSpm(Ds) element. These lines can then be screened for linkage of the AI with the dSpm(Ds), followed by tagging of the AI.

(vi) The model would allow tagging of alleles from cultivars that are amenable to transformation but can also be used to tag AIs from any cultivars or accessions that are recalcitrant to transformation by crossing dSpm(Ds) stock with any genetic stock carrying the AI.

(vii) The wild relatives of crop species carry many interesting alleles that could be transferred to crop species for yield stabilization. However, many extensive and laborious efforts have not yielded the desired results since the AI is linked to character(s) that have a deleterious effect on the yield of crop plants. These important AIs could be tagged by the four-element system and can then be introduced into the variety of choice by genetic transformation thereby, avoiding linkage drag. Alternatively, the AI could be tagged by crossing alien addition lines with dSpm(Ds) stocks, selecting for distant jumps to the alien chromosome and finally tagging the gene by inducing transposition of Ds.

(viii) The proposed four-element system would allow exploitation of genic male sterility for hybrid seed production. Heterosis breeding utilizing genic male sterility is an exciting proposition as at least one male sterility locus has been found in virtually every diploid and polyploid plant species (Duvick 1966).

We conclude that the proposed four-element model would have major impact on molecular characterization of crop-specific alleles and could also allow heterosis breeding using genic male sterility.

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