Chromosomal localization of cotransformed transgenes in the hexaploid cultivated oat *Avena sativa* L. using fluorescence *in situ* hybridization

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Fluorescence *in situ* hybridization (FISH) was used to localize two transgenes (*gus* and *bar*), carried on plasmids pACT-1F and pUBA, respectively, on mitotic metaphase squashes of T1 plants of the cultivated hexaploid oat *Avena sativa* L. cotransformed by microprojectile bombardment of embryogenic callus. Among the eight progeny analysed by FISH in each of two lines, we detected plants null, hetero- and homozygous for the two genes in one line, and plants null and heterozygous for the two genes in the other line. Our results demonstrated that in the two independent transformation events, the *gus* and *bar* genes had inserted in the same position relative to each other. In each transformation event, the insertions occurred on D satellite (SAT) chromosomes bearing a C genome translocation.

Keywords: *Avena*, cotransformation, FISH, oat transformation, physical mapping.

Introduction

Although transfer of foreign DNA and the production of transgenic plants is now routine in most crop species, the processes involved in the integration, stability and expression of transgenes are not well understood. It has been suggested, for example, that the genomic sequences flanking the transgenic inserts may have an effect on the level and variability of transgene expression (Peach & Velten, 1991), and that the physical position of transgene integration may also be involved in the phenomenon of transgene silencing (Register *et al.*, 1994; Srivastava *et al.*, 1996; Zhang *et al.*, 1996; Pawlowski *et al.*, 1998). Similarly, it is not clear if transgenes integrate into particular genomes, chromosomes or chromosome regions or whether some vectors integrate more readily than others. There has been speculation that there may be 'hot spots' for the integration of transgenes, or that an initial insertion event may act as a 'hot spot' for subsequent integrations (Kohli *et al.*, 1998). Even if such 'hot spots' exist, the genes will not necessarily function as expected after integration.

Current protocols for producing transgenic plants rely on the simultaneous integration of selectable markers in

addition to the gene of interest, so that transformed tissues can be identified and isolated during the early stages of plant regeneration from the target tissues. The physical location of such multiple gene integrations could have a profound effect on the speedy recovery of the desired genes in a plant breeding programme especially if the inserts are on different chromosomes. This paper reports the physical localization of two independent cotransformation events in the oat variety Melys, and discusses the results in relation to some of these issues and their possible implication with regard to breeding genetically modified oat varieties.

Materials and methods

Plant material

Transgenic plants of *Avena sativa* L. var Melys were obtained by particle bombardment of primary embryogenic callus derived from immature embryos, using a combination of two separate plasmids: pUBA, which carries the *bar* gene under control of the maize ubiquitin promoter, first exon and first intron (Toki *et al.*, 1992), and pACT1-F, which carries the *gus* gene under control of a partially deleted rice actin 1 promoter and first intron (McElroy *et al.*, 1990). The plants were

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self-pollinated and the T1 progenies of two lines, D1.3 and D6.1, were used for fluorescence *in situ* hybridization (FISH) analysis of the transgenes.

Polymerase chain reaction

DNA was extracted according to Yu & Pauls (1994). The reaction mixture for polymerase chain reaction (PCR), total volume 30 μ L, consisted of 5 μ L DNA, 1 $\times$ reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM nucleotides, 0.2 μ M primers and 1 unit Taq polymerase (Boehringer Mannheim). The primers were 5'-TgCAC-CATCgTCAACCAC-3' and 5'-ACAgCgACCACgCT-CTTg-3' for *bar*, leading to the amplification of a 0.31-kb fragment, and 5'-TAGCgggACTTTgCAAgTg-3' and 5'-gTTTTTgCAGCAGAAAAGCC-3' for *gus*, leading to the amplification of a 1-kb fragment. The reaction was performed by a Perkin Elmer DNA thermal cycler 480. A first cycle with 2 min denaturation (94°C), 30 s annealing (60°C) and 1 min extension (72°C) was followed by 39 cycles of 1 min denaturation, 30 s annealing and 1 min extension with 5 s auto-extension/cycle. A time delay of 5 min at 72°C ended the reaction. The same conditions were used for both sets of primers. Amplified products were analysed by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized under an ultraviolet lamp.

Southern blots

Total genomic DNA was extracted according to Robbins *et al.* (1991) except that the extraction buffer consisted of 100 mM Tris, 50 mM ethylenediaminetetraacetic acid (EDTA), 500 mM NaCl, pH 8.0, the first two centrifugations were carried out at 27 000 *g* and the final DNA pellet was resuspended in 500 μ L Tris-EDTA. Polysaccharides were precipitated after the phenol/chloroform extraction by first adding 0.05 vol. of 5 M NaCl, then 0.35 vol. of ethanol dropwise with continuous mixing (Michaels *et al.*, 1994). After 20 min incubation on ice, the polysaccharides were spun down in a microcentrifuge, the supernatant transferred to a fresh tube and the DNA precipitated with isopropanol. The quantity of total extracted DNA was determined using a Hoefer® DyNA Quant 200 fluorometer (Pharmacia Biotech).

Fifty micrograms of DNA was digested with *Hind*III, *Xho*I or *Eco*RI in a total volume of 400 μ L, precipitated and resuspended in 30 μ L sterile distilled water. A 25- μ g sample was loaded on a 1% agarose gel in 0.5 $\times$ Tris-borate-EDTA buffer. After transfer on a nylon membrane, DNA was hybridized with a *bar* or *gus* probe, digoxigenin-labelled by PCR.

Chromosome squash preparation

Seedling germination, root-tip fixation, enzyme treatment of root tips and chromosome squash slide preparations were carried out as described by Leggett & Markhand (1995). Eight seedlings of the resultant T1 progeny of both the D1.3 and the D6.1 lines produced mitotic root-tip squashes of sufficient quality for use in FISH.

Probe DNA and *in situ* hybridization

The pACT-1F plasmid has a size of approximately 6.4 kb, whereas the pUBA plasmid has a size of approximately 5.5 kb. The rDNA clone pTa71 contains the wheat-derived 18S.26S gene repeat sequence (Gerlach & Bedbrook, 1979). Total genomic DNA of the CC genome diploid species *A. eriantha* was extracted according to the method of Dellaporta *et al.* (1983).

Labelling of plasmids and *in situ* hybridization and detection were essentially as described by Leggett & Markhand (1995) except that plasmids pUBA and pACT1-F, the pTa71 clone, and the total CC genomic DNA were sheared via sonication and labelled with the Cyanine dye CY3-dCTP and FluorX-dCTP (Amersham) by nick translation.

Slides were probed either singly or dually with labelled pUBA/pACT1-F with or without labelled total genomic DNA from the CC genome diploid species *A. eriantha*, and with or without labelled pTa71 (see Results). No blocking DNA was used in any of the hybridization procedures and slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI).

Sites of hybridization were visualized using a Leitz Laborlux fluorescence microscope and photographed on Kodak Ektachrome 400HC slide film and digital images were captured on a CCD camera using Biovision software and assembled in Adobe Photoshop.

Results

Screening of transgenic progenies and molecular analysis

Embryogenic calli of oats (var Melys) were cotransformed by particle bombardment with pUBA (Toki *et al.*, 1992) and pACT1-F (McElroy *et al.*, 1990) plasmids containing the *bar* and the *gus* genes. Plants from 17 independent transformation events were regenerated and the T1 inbred progeny of two of the transformants containing either high (D1.3) or low (D6.1) copy numbers of both genes were chosen for use in this study. A PCR screen for the presence of the *bar*

Fig. 1 PCR analysis of the 10 T1 progenies of plant D1.3. (a) Amplification using primers specific to the *bar* gene. (b) Amplification using primers specific to the *gus* gene. (c) Untransformed plant; 1–10, T1 progenies of D1.3; P, plasmid.

and *gus* genes in 10 progeny plants of line D1.3 revealed that seven of them were positive for both genes (Fig. 1), whereas in line D6.1, 35 progeny plants were screened and 29 were positive for both genes. In neither case were any plants found carrying only one of the transgenes, showing no evidence of two independent insertion sites in these two primary transformants.

Southern analyses were performed on progeny plants of D1.3 and D6.1 in order to estimate the number of copies of each gene (Fig. 2). The restriction enzymes used were *Hind*III, which restricts pUBA at one site but does not restrict the pACT1-F plasmid; *Xho*I which restricts at one site on both plasmids, and *Eco*RI which restricts either side of the *bar* gene in pUBA, generating a 1.5-kb fragment and at one site in pACT1-F. When hybridized with the *bar* probe, the *Hind*III digest of the T1 progeny of plant D1.3 showed seven bands of equal intensity, whereas the *Eco*RI digest indicated that there were three or four complete copies of the expected 1.5-kb fragment, as judged by the comparison of the intensity of the signal with the intensity of the signal given by the plasmid, plus eight rearranged copies, the 2.5-kb fragment counting as two copies according to the strength of the signal (Fig. 2a). Two of the higher molecular weight bands of the *Eco*RI digest were not as intense as the others, which may be caused by incomplete digestion or by fragments which contain only part of the *bar* gene. The *Xho*I digest confirmed that there are at least nine copies of the *bar* gene. Unfortunately when the membrane was hybridized with the *gus* probe the negative control seems to have been contaminated with pACT1-F DNA because a band of the same size as the

digested plasmid (6.4 kb) appeared (Fig. 2b). Therefore the bands of similar size which appear in other lanes cannot be taken into account. The *Eco*RI digest suggests that there are at least nine copies of the *gus* gene, the 4.4 kb band corresponding to two copies as judged by its intensity compared to the intensity given by the two copies of the plasmid, and the two faint lower bands of 2.3 and 3.0 kb being partial copies of the gene. Although pACT1-F has no *Hind*III restriction site, at least six fragments hybridized with the *gus* probe, indicating that pACT1-F and pUBA plasmids are probably alternately integrated as concatamers into the genome of D1.3, or that pACT1-F is inserted at more than one site.

When hybridized with the *bar* probe the *Hind*III digest of the T1 progeny of plant D6.1 showed three bands of equal intensity, whereas the intensity of the signal observed in the *Eco*RI lane suggests that there are two or three complete copies of the gene when compared to the signal given by the plasmid (Fig. 2a). The *Eco*RI digest suggests that there are two copies of the *gus* gene. As with D1.3, at least three fragments hybridized with the *gus* probe, following *Hind*III digestion (Fig. 2b), again indicating alternate integration of pACT1-F and pUBA plasmids into the genome of D6.1.

In situ hybridization

In all chromosome squash preparations where the labelled pTa71 rDNA probe was used, six sites of hybridization to the nucleolar organizing regions (NOR) of the three pairs of satellite chromosomes were observed as expected in hexaploid oat (Rajhathy, 1963).

Fig. 2 Southern analysis of progeny of plants D1.3 and D6.1. (a) Blot hybridized with a *bar* probe. (b) Blot hybridized with a *gus* probe. CX, *Xho*I digest of an untransformed plant; H, *Hind*III; X, *Xho*I; E, *Eco*RI; PX, *Xho*I digest of pUBA and pACT1-F at the dose of two copies per haploid genome; MW, molecular weight markers, with size indicated in kilobases between the two figures.

When labelled total genomic DNA from the diploid species *A. eriantha* was included in the hybridization mixture, the results were as described by Leggett & Markhand (1995) for monosomics IV, V and XVII, indicating five C/AD and two AD/C translocations in addition to five intact CC genome chromosomes and nine intact A/D genome chromosomes. Because of the high homology of the rDNA among all oat genomes, in the absence of blocker or rDNA specific probe, the labelled total genomic *A. eriantha* DNA hybridized to all NOR regions as well as to the C-genome specific regions.

Initially, we probed slides with a hybridization mixture which contained both the pUBA and pACT1-F labelled probes in addition to labelled pTa71. However, when we detected the signal from both probes at the same site (Fig. 3a,biii) it was decided to re-probe some slides with each labelled plasmid in turn, with or without

labelled pTa71 and with or without labelled total genomic DNA from the CC genome diploid *A. eriantha*. This was undertaken in order to be certain that the dual signal observed was from the two probes independently and not a confounding effect of the probes giving a background signal at both wavelengths.

Two of the eight plants of line D1.3 (D1.3/2 and D1.3/3) analysed by FISH produced no hybridization signal, indicating that the transgenes were not present in these plants, which concurred with the results of the PCR reactions. Of the six remaining D1.3 plants so analysed, four revealed two sites of hybridization on a single chromosome, heterozygous (Fig. 3a,b), and two revealed sites of hybridization on two chromosomes, homozygous, using either of the labelled plasmids (Fig. 3c,d). As above, the presence (but not the dose) of the transgenes confirmed the results of PCR. The discrepancy between the PCR results and those of FISH

with regard to plant D1.3/10 (null detected by PCR, but positive by FISH) is almost certainly caused by the PCR failing to amplify the segment. We did in fact observe a very faint band on the PCR gel for the *gus* amplification, which does not appear on the photograph (Fig. 1b). In the six D1.3 plants where a signal was detected, the site of integration of the inserts was distally located on the

long arm of a SAT chromosome which also carries a C genome translocation. The point of insertion of both transgenes was shown to be identical (Fig. 3a,b), on the centromere side of the C genome translocation, but close to it (Fig. 3c,d).

The identity of this chromosome was established from the presence of the pTa71 site on the short arm of the

Fig. 3 (a) Mitotic metaphase squash of line D1.3, probed with labelled pUBA (green) and pACT1-F (red) showing that both transgenes have integrated at the same site (arrowhead). (b) The transformed chromosome from (a) enlarged, showing the independent signal from: (i) the pUBA probe (green); (ii) the pACT1-F probe (red); and (iii) the combined signals from both probes. (c) Mitotic metaphase squashes of line D1.3, probed with labelled pTa71 showing six sites (red) of hybridization at the NORs of the satellite chromosomes. (d) The same cell as in (c) but labelled with total genomic DNA from the CC genome diploid *A. eriantha* (green) and labelled pUBA containing the *bar* gene (red) illustrating the transgene in the homozygous condition on chromosomes 13D (arrowed). (e) Partial mitotic metaphase squash of line D6.1 probed with labelled total genomic DNA from the CC genome diploid *Avena eriantha* (red) and labelled pUBA containing the *bar* gene (green spots) on chromosome 3D (arrowed). (f) Mitotic anaphase of D1.3 probed with labelled pTa71 showing five green sites of hybridization at the NORs (white arrows) in the left half of the cell and seven (white arrows) in the right half of the dividing cell. Both chromatids carrying labelled pUBA containing the *bar* gene (red) can be seen in the right half of the cell (yellow arrows). All cells are counterstained with DAPI and the bar represents 10 μ m.

chromosome(s) in conjunction with the signal produced by the labelled total genomic DNA from the *A. eriantha*, which hybridized to the C genome translocation at the terminal end of the long arm of the chromosome (Fig. 3c,d). Of the two SAT chromosomes bearing a C genome translocation in *A. sativa*, this one has the larger translocation and according to Chen & Armstrong (1994) is a group 3 chromosome, or chromosome 13D according to the classification of Linares *et al.* (1998).

Of the eight D6.1 plants studied by FISH analyses, three produced no hybridization, indicating that the transgenes were not present in these plants. The remaining five plants revealed a single site of hybridization indicating that the transgenes were present in the heterozygous condition. As with the D1.3 plants, the site of integration of the inserts was distally located on the long arm of a SAT chromosome carrying a C genome translocation. However, in this case, the hybridization signal was detected within the translocated C genome segment (Fig. 3e). As with D1.3, the point of insertion of both transgenes was shown to be the same. Initially, we thought that the transgenes had inserted into the same chromosome (13D) as in D1.3, but detailed examination of many cells (not illustrated) revealed that the C genome translocation in this case was the smaller of the two, identifying the chromosome as a group 3 chromosome (Chen & Armstrong, 1994), or

chromosome 3D according to the classification Linares *et al.* (1998).

The size of the inserts detected in D1.3 and D6.1 was calculated from Southern analysis. In the high copy number line D1.3, it was relatively easy to visualize the inserts by standard FISH techniques as they contained nine to 12 copies of pUBA, giving a minimum size of 50 kb and a maximum of 66 kb, together with nine to 11 copies of pACT1-F, giving a minimum of 58 and a maximum of 70 kb. However in line D6.1 the hybridization signal was weak for both probes. The larger of the two inserts (two to three copies of pUBA giving an insert size of 11–17 kb) could be seen without signal integration via computer software, whereas the smaller of the two inserts (two copies of pACT1-F giving an insert size of 13 kb) could only be visualized after integrating digital images 30 + times. Whilst recognizing that the degree of hybridization can vary between different experimental batches, it is evident from these observations that we are reaching the limits of detection using the experimental protocol outlined here, and that in the future it may be necessary to modify the protocol to include amplification cycles to detect smaller inserts.

A single mitotic anaphase cell which was encountered in one of the D1.3 plants during the screening procedure (Fig. 3f) is worthy of recording, in that it was observed to have five SAT chromatids in one half of the dividing cell (visualized via the pTa71 probe) and seven in the other. The latter half of the cell also contained two sites of hybridization from the pUBA labelled probe on two of the SAT chromatids, whereas none was present in the other half.

Discussion

As described in the results, five of the 16 plants produced no hybridization signal when probed with labelled pUBA and pACT1-F, confirming in plants D1.3/2 and D1.3/3 the results of the PCR analysis, that is that the transgenes were not present. This finding is important because it could be argued (as in other, similar reports) that the hybridization signal observed was not that of the insert, but simply that of the plasmid or a plasmid-like sequence present on the relevant oat chromosome(s). However, we believe that the positive and negative PCR analysis and *in situ* results reported here for the D1.3 plants provide very strong evidence that the sites of hybridization we detected were of the plasmid and insert(s). The ratio of presence:absence of the transgenes as determined by PCR analysis for D1.3 and D6.1 progeny (Table 1) indicates that plants with and without the inserted genes do not differ significantly from the expected 3:1 Mendelian segregation. It was fortuitous that the inserts occurred on chromosomes D3

Table 1 Segregation of T1 progeny plants obtained from self-pollination of D1 and D6 transgenic plants

Constructs	Parent plant	T1 plants analysed (<i>n</i>)	Genotype of T1 plants based on PCR results				Ratio	χ^2 †	<i>P</i> ‡
			bar ⁺ gus ⁺	bar ⁺ gus ⁻	bar ⁻ gus ⁺	bar ⁻ gus ⁻			
pUBA/pACT1-F	D1.3	10	7	0	0	3	3:1	0.13	> 0.7
pUBA/pACT1-F	D6.1	35	29	0	0	6	3:1	1.15	> 0.20

†Based on PCR results.

‡*P* = χ^2 probability with 1 d.f.

and D13 because these two chromosomes are among the few chromosomes of *A. sativa* which can be fairly readily identified.

In line D1.3 both transgenes (*bar* and *gus*) appear to have integrated at the same site (Fig. 3a,b). Similarly, in line D6.1 both transgenes appear to have integrated at the same site, although on a different chromosome to line D1.3. The integration of multiple genes at the same site could have implications for the development and subsequent release of genetically modified (GM) varieties of oat, if this is the rule and not the exception. Before the release of a GM crop, it is highly desirable to remove selectable marker genes, most particularly where the selectable gene confers herbicide resistance, as in our case with the *bar* gene. The close proximity of insertion of the genes we have demonstrated here will inevitably mean that the genes will cosegregate and it will be difficult to break the linkage. However, for genes other than marker genes, the lack of segregation of multiple gene inserts for desirable characters would be invaluable and a bonus to the breeder precisely because they do not segregate.

Kohli *et al.* (1998) presented evidence that in rice, an initial site of integration may provide a 'hot spot' for subsequent integration of successive transgenic molecules at the same locus. Our observations that the *bar* and *gus* genes have integrated at the same site in *A. sativa* provide some support to that of Kohli *et al.* (1998). However, the absence of separate integration events at different sites in the genome for the two cotransformed genes and the presence of multiple copies of both genes at a single site in two independent transformation events may also suggest that homologous recombination between plasmids could have occurred at some point during the transformation, possibly between the two ampicillin-resistance genes on either plasmid.

It is also of interest that the sites of integration of the transgenes we describe here involve SAT chromosomes. In their work on transgene inserts in amphidiploid tobacco, Moscone *et al.* (1996) traced the 'multipurpose silencing locus 271' to a SAT chromosome (one of three such pairs, two of which are in the S subgenome), which

they designated T3. Similarly, Pedersen *et al.* (1997) reported that, of the 10 chromosomes identified as containing various integrated transgenes, four were SAT chromosomes, three in barley lines and one in a wheat line. Integration into four triticale lines, however, did not involve a SAT chromosome. The authors suggest that rDNA regions which are highly active during the cell cycle, are likely to be more susceptible to the incorporation of DNA. However, of the four inserts shown to be on SAT chromosomes, only one is actually within the NOR, two are in the opposite arm, the fourth being in the same arm as the NOR but distal to it.

The integration of pUBA and pACT1-F into SAT chromosomes in both the lines we have reported here could, of course, be pure chance (about 29% would integrate into SAT chromosomes if integration were random), as could those incidences of transgene insertion in the other crop species outlined above involving SAT chromosomes. With the small data sets presently available, it is speculative to suggest otherwise. As further transgenes are physically mapped in different accessions and genera using different vectors, delivery systems and regeneration protocols, a clearer picture will emerge with regard to apparent 'hot spots'. An alternative explanation for 'hot spots' could be that the selection pressure exerted on the target tissue ensures that only regenerating callus tissue in which the selectable marker gene inserts into a chromosome region where it is active, survives. Clearly such a situation might, falsely, lead one to presume that certain chromosomes or chromosome regions act as preferential sites for integration.

Pawlowski & Somers (1998) have demonstrated that transgenic DNA integrated into the hexaploid oat genome is frequently interspersed by host DNA. It is therefore noteworthy that we did not detect multiple sites of hybridization in any of the plants examined. It is likely that the interspersed regions of host DNA are so small that the detection protocol used here was not able to resolve such interspersions. It is also possible, however, that the transgenic insertions reported here did not contain any interspersed host DNA sequences.

The single anaphase we observed to have a distorted separation of the SAT chromosomes and hence the transgenes, could be of significance. If this was not a unique event, it could have an effect on the transmission and segregation of the transgenes and the fertility of subsequent progeny, because such unequal mitotic cell divisions could lead to the formation of aneuploid gametes during ensuing meiotic divisions. Such misdivisions could contribute to the non-Mendelian segregations reported by some researchers (Pawlowski *et al.*, 1998). Further research into the observations we have made, in transformations involving different varieties and different vectors, will help identify methodologies for a more efficient transformation system in oats.

Finally, we have demonstrated that FISH analyses with transgene inserts enable us to discriminate between homozygous and heterozygous plants containing one or more inserted gene at the seedling stage. This will be a great asset to breeding programmes involving transgenic plants because it will dispense with time-consuming and often very expensive progeny testing.

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