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Gene targeting in *Arabidopsis*

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Summary

Precise modification by gene targeting (GT) provides an important tool for studies of gene function *in vivo*. Although routine with many organisms, only isolated examples of GT events have been reported for flowering plants. These were at low frequencies precluding reliable estimation of targeting efficiency and evaluation of GT mechanisms. Here we present an unambiguous and straightforward system for detection of GT events in *Arabidopsis* using an endogenous nuclear gene encoding protoporphyrinogen oxidase (PPO), involved in chlorophyll and heme syntheses. Inhibition of PPO by the herbicide Butafenacil results in rapid plant death. However, the combination of two particular mutations renders PPO highly resistant to Butafenacil. We exploited this feature for selection of GT events by introducing the mutations into the PPO gene by homologous recombination. We have estimated the basal GT frequency to be 2.4×10^{-3}. Approximately one-third of events were true GT (TGT) leading to the anticipated modification of the chromosomal PPO copy. The remaining events could be classified as ectopic GT (EGT) arising by modification of vector DNA by the chromosomal template and its random integration into the *Arabidopsis* genome. Thus the TGT frequency in our experimental setup is 0.72×10^{-3}. In view of the high efficiency of *Arabidopsis* transformation, GT experiments of a reasonable size followed by a PCR screen for GT events should also allow for modification of non-selectable targets. Moreover, the system presented here should contribute significantly to future improvement of GT technology in plants.

Keywords: homologous recombination, gene targeting, protoporphyrinogen oxidase (PPO).

Introduction

In cells of muticellular eukaryotes, foreign DNA tends to integrate randomly even when it contains sequences identical to chromosomal DNA. Nevertheless, experiments allowing for rapid assessment of the ratio of random integration to integration directed by homologous recombination have fostered progress in the choice of recipient cells and in improving targeting vectors. Today, gene targeting (GT) is routine in mouse embryonic stem cells (Capecchi, 1989) and has been extended recently to *Physcomitrella* (Schaefer and Zryd, 1997), *Drososphila* (Rong and Golic, 2000), sheep (McCreath *et al.*, 2000) and human somatic cells (Hanson and Sedivy, 1995; Sedivy *et al.*, 1999). In contrast, the first GT events in flowering plants were reported 12 years ago (Paszkowski *et al.*, 1988) but this technology is still inefficient and has limited reproducibility. Over the years, a number of reports have claimed successful targeted modification of transgenic target loci in plants (Halfter *et al.*, 1992; Offringa *et al.*, 1990; Paszkowski *et al.*, 1988; Reiss *et al.*, 2000; Risseeuw *et al.*, 1995) but only a single example of predicted chromosomal modification was revealed as a homozygote in the progeny (Risseeuw *et al.*, 1995). Similarly, numerous trials of GT-mediated modification of endogenous genes (Kempin *et al.*, 1997; Lebel, 1994; Lee *et al.*, 1990; Miao and Lam, 1995) have resulted in only one example of an *Arabidopsis* plant with an anticipated gene disruption (Kempin *et al.*, 1997). In cases of minor sequence modification, RNA/DNA oligonucleotides, probably exploring the mismatch repair mechanism (Beetham *et al.*, 1999; Zhu *et al.*, 1999; Zhu *et al.*, 2000) may be a useful substitute for GT by homologous recombination. However, both the efficiency and precision of this method need further improvement

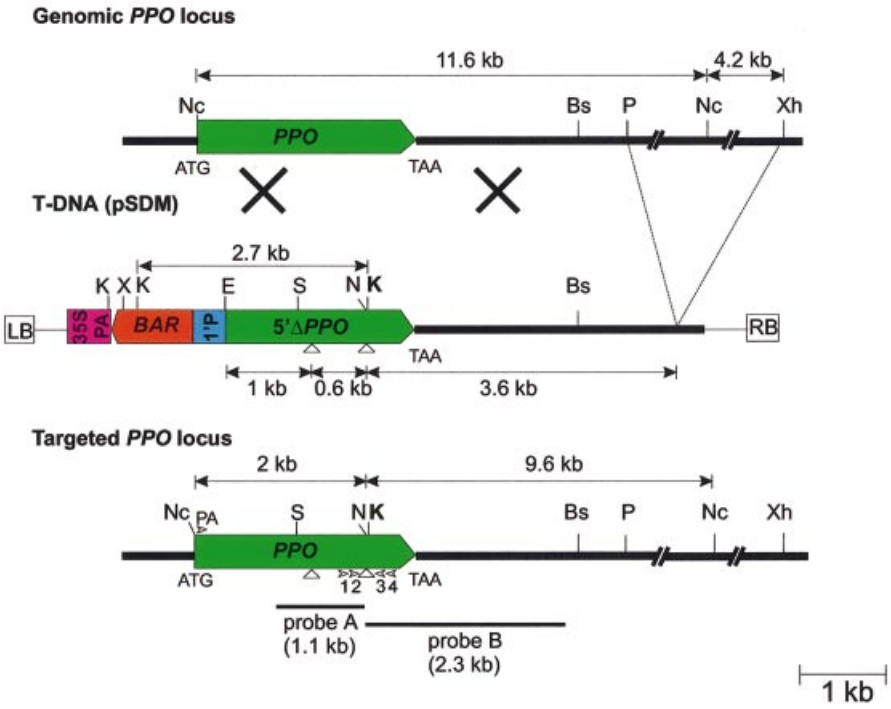

Figure 1. Experimental design for targeted modification of the *Arabidopsis PPO* locus. The *PPO* coding region is indicated as a black box marked *PPO*. The thick lines represent flanking plant genomic DNA. The two mutations conferring Butafenacil resistance are marked by open triangles below the *PPO* coding region (middle and lower panel). The truncated 5′ΔPPO of the T-DNA is linked to the *BAR* resistance gene, which is flanked by 1′ promoter (1′pr) and the polyadenylation signal of the Cauliflower Mosaic Virus (35S PA). The sizes of fragments produced after *Nco*I and *Kpn*I digestion and of those in the homologous region used for gene replacement are also indicated. The positions of primers (PA, 1, 2, 3, 4) used for PCR and of probes A and B used for Southern analysis are shown in the lower panel. Abbreviations are RB, right border; LB, left border; Bs, *Bsiw*I; E, *Eco*RI; K, *Kpn*I; N, *Nla*III; Nc, *Nco*I; P, *Pac*I; S, *Sac*I; X, *Xba*I; Xh; *Xho*I. The newly introduced *Kpn*I site is indicated in bold.

(Dewitt, 2000; Hohn and Puchta, 1999). In various GT experiments using different plants and transformation systems, a wide range of GT frequency was recorded (from 10^{-3} to 10^{-6}) (for review see Vergunst and Hooykaas, 1999). As usually only one or few GT events were found with any given experimental setup, these estimates of GT frequencies should be treated with extreme caution. Therefore, the systematic optimization of this technology in plants is in urgent demand.

Optimization requires a rapid and inexpensive way of identifying GT events without a background of ambiguous clones. Although deletion constructs of selectable transgenes could serve this purpose, endogenous targets probably better reflect the natural situation. The locus meeting most of the above criteria, which has been used in past targeting experiments, is the acetolactate synthase (ALS) gene, the product of which is the target of sulfonylurea herbicides (Lee *et al.*, 1990). However, several single base-pair changes in the ALS coding sequence render the product and thus transgenic plants herbicide resistant. Thus, under stringent herbicide selection, spontaneous mutations result in a relatively high background level of herbicide resistance.

Here we report an experimental system in *Arabidopsis* that allows reproducible recovery of GT events and provides basic knowledge required for further GT improvements. This novel assay meets the requirements for efficient and unambiguous recovery of GT events directed towards an endogenous gene, *PPO (*protoporphyrinogen oxidase). PPO catalyses oxidation of protoporphyrinogen IX to protoporphyrin, the last common step in heme and chlorophyll biosynthesis (Beale and Weinstein, 1990). In plants, mitochondrial and plastid isoforms are known (Lermontova *et al.*, 1997; Martinge *et al.*, 1989). The PPO protein residing in chloroplasts (cPPO) is the target of several herbicides (e.g. Butafenacil), which competitively block the enzyme (Duke *et al.*, 1991). After inhibition of cPPO, accumulating protoporphyrinogen IX leaks to the cytoplasm where it is rapidly oxidized to protoporphyrin by non-specific peroxidases. In the light, this generates reactive singlet oxygen, which results in rapid cell damage. Two simultaneous mutations introduced into *Arabidopsis PPO* cDNA expressed as a transgene render plants highly resistant to PPO-inhibiting herbicides (Volrath *et al.*, 1997). Since both mutations need to be introduced simultaneously to obtain a high level of Butafenacil resistance, the frequency of spontaneous resistance is expected to be very low and, indeed, has never been observed. This system was developed as a GT assay in *Arabidopsis* for the determination of the basic frequency and the molecular characterization of GT events.

Results and discussion

The targeting vector used in this study (pSDM, Figure 1) contains a T-DNA with a 5.6 kb fragment of the *Arabidopsis PPO* gene (ecotype *Wassilewskija*) modified by two mutations conferring Butafenacil resistance

Table 1. Summary of targeting experiments (EGT, ectopic gene targeting; TGT, true gene targeting)

Experiment	No. of infiltrated plants	No of seeds selected on Butafenacil (10^6)	No. of recombinants	Type of targeting	
				TGT	EGT
1	300	1.0	2		2
2	280	0.27	2	1	1
3	300	0.8	3	2	1
4	250	1.2	1		1
5	280	0.952	1		1

(Volrath *et al.*, 1997). One mutation is a single bp change at codon 305 (TCA to TTA) leading to an amino acid change, S to L. The second one is at codon 426 which leads to one amino acid change Y to M and generates a novel restriction site *Nla*III (CTAC to CTAG). The *PPO* sequence encoding 119 amino terminal amino acids, comprising part of the chloroplast signal peptide and a nucleotide-binding motif required for enzyme activity, was deleted, thereby inactivating the *PPO* gene on the targeting vector. The vector also contains a hybrid *BAR* gene construct (Mengiste *et al.*, 1997) as a marker for transformation (Figure 1). To facilitate molecular analysis of the recombination events, an additional sequence change was introduced into the eighth intron of *PPO*, generating a *Kpn*I site. Recovery of herbicide-resistant plants was only possible after homologous recombination between the T-DNA and the chromosomal genomic *PPO* locus in the homology regions 1 kb upstream of the first mutation and 3.6 kb downstream of the second mutation (Figure 1).

Five independent populations of 250–300 *Arabidopsis* plants (1410 in total) (Table 1) were transformed by vacuum infiltration with *Agrobacterium* (Bechtold *et al.*, 1993) containing the T-DNA construct depicted on Figure 1. The progeny seedlings were grown in soil and were sprayed with Butafenacil. Herbicide selection yielded 10 resistant individuals from a total of 4.2×10^6 plated seeds. Amplification using primer PA, which anneals to the 5′ part of the *PPO* gene absent in the targeting vector, and primer 4 yielded a PCR product of 2100 bp (Figures 1 and 2). This fragment was then used as a template for nested PCR using primers 1 and 4 or 2 and 3 (Figure 2) that flank the *PPO* region predicted to contain the novel *Nla*III and *Kpn*I sites after homologous recombination between vector and target. The PCR products were analyzed by restriction digestions with *Nla*III and *Kpn*I. The presence of both restriction sites in approximately half of the PCR products can be explained by homologous recombination at one target locus, creating a heterozygous situation in primary transformed (T1) plants. These patterns were indeed found in DNA products derived from all Butafenacil-resistant individuals (Figure 2 and data not shown).

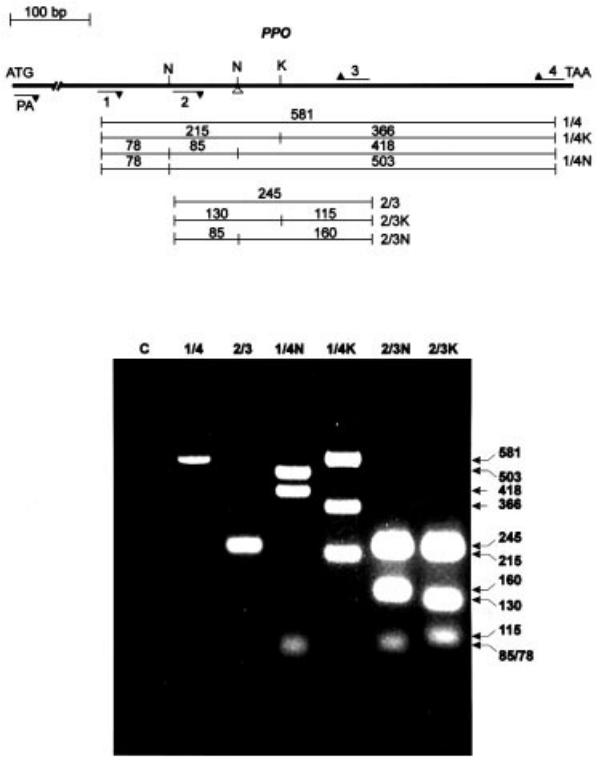

Figure 2. Example of an initial DNA analysis of a Butafenacil-resistant clone by restriction of PCR-amplified fragments. The upper panel is a diagram showing the *PPO* coding region, the expected sizes of the PCR-amplified fragments, and their endonuclease digestion products. The positions of primers are indicated. One of the two mutations is indicated by an open triangle. Two PCR were performed using 1 + 4 or 2 + 3 as primers and a 2100 bp fragment amplified from a leaf extract by primers PA + 4 (see upper panel and Figure 1) as the template. The expected sizes (in bp) of the amplified fragments (1/4 and 2/3) and their digestion products (N for *Nla*III and K for *Kpn*I) are shown below the *PPO* coding region. The lower panel is the analysis by gel electrophoresis of the fragments described above. C indicates the control for PCR, where H_2O was used instead of DNA. The fragment sizes are indicated at the right of the gel.

PCR and Southern blot analysis (data not shown) of the T1 plants suggested that the gain of resistance was indeed due to homologous recombination between the targeting vector and a chromosomal copy of the *PPO* gene. However, examination of the T1 generation could not

distinguish between the anticipated modification of chromosomal *PPO (*or true gene targeting, TGT) and ectopic gene targeting (EGT) resulting from conversion of vector DNA by the chromosomal *PPO* copy and its ectopic integration. In T1, TGT or EGT would lead to expression of the resistant form of the *PPO* gene and results of PCR and Southern blot analysis of either event would be also similar. Only analysis of the progeny of primary resistant recombinants (T2) can distinguish between TGT and EGT events, since the T2 would segregate individuals homozygous for the modified copy of the chromosomal *PPO* locus only in the case of TGT. In addition, genomic DNA analysis of random T2 plants would allow establishment of linkages of foreign DNA inserts and the chromosomal *PPO* locus in the cases of multiple T-DNA integration.

Nine selected T1 plants were fertile and their selfed progeny showed Mendelian segregation of the herbicide-resistance phenotype as a single dominant trait (data not shown). The genomic DNA of random T2 plants was examined by Southern blotting of the *PPO* locus defining three TGT and six EGT events. Representative examples of T2 Southern blot analysis for TGT and EGT events are provided in Figures 3 and 4, respectively. The DNA digested simultaneously with *Nco*I and *Kpn*I was hybridized with either probe A or probe B (Figure 1). The *Kpn*I restriction site introduced into the targeting vector separates the two probes. With both probes, a band of 11.6 kb was the signature of the wild-type *PPO* gene. In addition, new 2 and 9.6 kb bands expected after GT events between the genomic locus and the T-DNA were detected with probes A and B, respectively (Figure 1).

For the representative TGT (Figure 3), the following T2 genotypes with respect to the *PPO* locus were recovered: five heterozygotes (Figures 3a, b, lanes 1, 2, 5, 8, 9), three homozygotes for the modified *PPO* gene (Figures 3a, b, lanes 3, 4, 10) and three with the wild-type locus (Figures 3a, b, lanes 6, 7, 11). In addition to the targeted modification of the *PPO* gene, two ectopic transgenic inserts were detected as additional bands of 2.7 kb with probe A (Figure 3a) or 7.7 and 3.6 kb with probe B (Figure 3b). The two transgene loci are not linked to each other or to the *PPO* gene. They segregated easily and one plant homozygous for the modified *PPO* gene and free of additional foreign DNA was recovered already in the T2 (Figure 3a–c, lane 3). Further TGTs were documented for two additional lines after a similar series of Southern blot hybridizations (data not shown). Unlinked T-DNA insertions were also detected in these lines and segregated away from the modified *PPO* locus. The modifications of *PPO* and the resistance to Butafenacil were stably transmitted to progeny (data not shown). Furthermore, sequence analysis of *PPO* locus confirmed the presence of the introduced mutations only, proving the precision of the gene replacement event (data

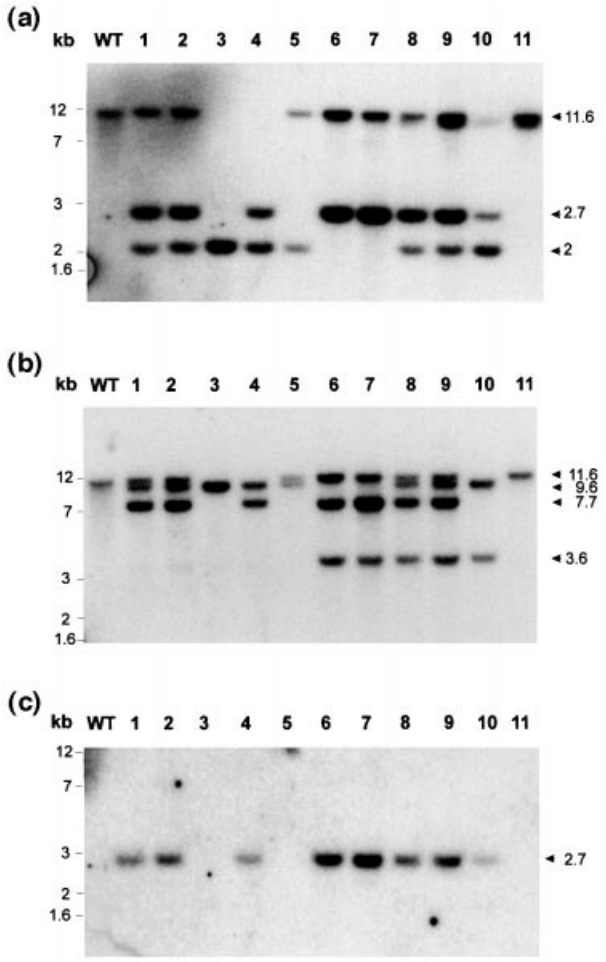

Figure 3. Southern blot analysis of 11 T2 plants from the same T1 candidate (TGT1). WT, *Wassilewskija* wild-type DNA and lanes 1–11 DNA of the T2 plants. Genomic DNA cleaved with *Nco*I and *Kpn*I and restriction products were separated by electrophoresis on an 0.8% agarose gel stained with ethidium bromide. The membrane was then hybridized to ^{32}P-labeled probe A (a), probe B (b)or the BAR gene (c).The positions of bands of interest are presented at the right of the blot by black arrows and the positions of the relevant bands of 1 kb size markers are indicated at the left.

not shown). Plants homozygous for the modified *PPO* locus are phenotypically indistinguishable from wild type (Figure 4), suggesting that there is no significant alteration in the activity of the gene and/or the enzymatic properties of its protein product.

Southern blot analysis of the T2 progeny of the remaining six Butafenacil-resistant plants revealed EGT events. All T2 individuals (approximately 15 per line) still contained the 11.6 kb band indicative of the wild-type copy of the *PPO* gene (representative blots of two T2 populations are shown in Figure 5). The DNA of T2 individuals of line EGT-1 digested with *Nco*I and *Kpn*I and hybridized to probe A gave just two bands of 11.6 and 2 kb (Figure 5a). The 2 kb band indicative of the modified *PPO* gene could

Figure 4. Targeted modification of *PPO* gene does not affect plant growth. Eight-week old *Arabidopsis* plants are presented. (a) wild type (*PPO*/*PPO*) (b) heterozygous (*PPO*/*ppo*) and (c) homozygous (*ppo*/*ppo*).

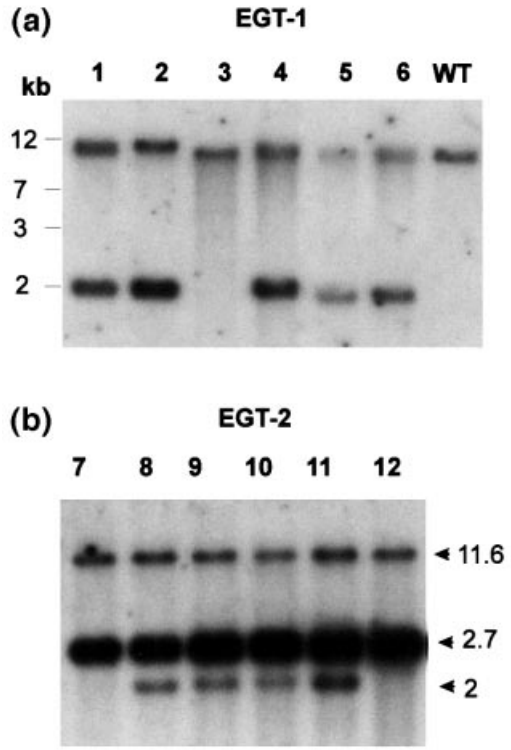

Figure 5. Southern blot analysis of six T2 plants derived from two candidates, (a)EGT-1 and (b)EGT-2. Genomic DNA was digested by *Nco*I/*Kpn*I and the membrane was hybridized with probe A. WT, DNA of *Wassilewskija* wild type. Bands of interest are indicated by black arrows. The positions of the relevant bands of 1 kb size marker are also presented.

be segregated from the *PPO* locus (Figure 5a, lane 3), which suggests that incoming T-DNA was extended (converted) using the 5′ end of the genomic copy of *PPO* as a template, followed by random integration of the modified T-DNA. Thus, the homologous recombination event restored function at the truncated Butafenacil-resistant form of the *PPO* gene carried on the T-DNA but the chromosomal copy remained unaltered. As in EGT-1, the T-DNA in the EGT-2 line (Figure 5b) was also most probably extended by a gene conversion event restoring the 5′ deleted part of *PPO* and this was followed by random insertion of the modified vector elsewhere in the genome. In contrast to EGT-1, however, additional random insertions of the original T-DNA, visualized by a 2.7-kb band labeled with probe A, were detected in the genome of EGT-2 (Figure 5b). Events analogous to EGT-2 were identified in four further EGT lines. Hybridization of the blots with probe B confirmed the occurrence of the T-DNA conversion in all these lines (data not shown).

To determine the targeting efficiency in relation to transformation frequency, a subset of seeds was subjected to PPT selection, resistance to which should be conferred by random integration of the *BAR* gene residing on the targeting construct (Figure 1). The transformation frequency was uniform throughout the five independent experiments at 0.1% ($\pm$ 0.011). Considering all 10 GT events, we estimated a basal targeting frequency of 2.4×10^{-3}, which is rather high compared with other data from flowering plants. However, since two-thirds of targeting events were of the EGT type, the TGT frequency was estimated to be 0.72×10^{-3}. Both types of event were observed previously but their individual rates were not determined. The proportion of TGTs is encouragingly high and the absolute frequency of approximately one in 1500 transgenic individuals provides a good starting point for further optimization of GT methodology. This number of transgenic lines can be exceeded easily in a single medium-sized *in planta* transformation experiment. It remains to be determined whether other site-directed modifications by GT, such as large insertions for gene disruption or sequence replacement, can occur at frequencies similar to the modifications at the *PPO* gene presented here. This is likely to be the case, assuming that TGT uses a double crossover mechanism within homologous DNA flanking the *PPO* region containing the two mutations and

the novel *Kpn*I site (Figure 1). Such a prediction is also in agreement with the number of screened transgenic clones (750) required for recovery of a single *ALG5* gene disruption in *Arabidopsis* (Kempin *et al.*, 1997) although the isolated event recovered may not be highly informative in this respect.

The introduction of mutations into the *PPO* gene could also involve gene conversion using extrachromosomal T-DNAs or ectopically inserted T-DNA copies as templates, possibly involving the synthesis-dependent strand annealing (SDSA) process (Nassif *et al.*, 1994). Gene conversion by SDSA or a similar mechanism has been proposed for double-strand break repair in plants (Gorbunova and Levy, 1997; Puchta, 1998). The level of sequence divergence between the targeting vector and chromosomal target could influence the efficiency of this pathway.

Remarkably, all but one recombinant clone had additional ectopically integrated T-DNAs. This could indicate that *Arabidopsis* has a particularly high competence for integration of foreign DNA linked to competence for GT or that these copies might be somehow involved in the modification of the chromosomal *PPO* copy. A surprisingly similar situation was found previously in a unique TGT-modified tobacco line obtained after *Agrobacterium*-mediated GT. This line also contained additional randomly integrated copies of the targeting T-DNA (Risseeuw *et al.*, 1995).

The gene targeting system described here should directly permit experimental examination of parameters influencing GT frequencies, such as modification of targeting vectors, *Agrobacterium* strains, and preconditioning of recipient cells or plants. *Arabidopsis* genotypes could also be screened for increased GT competence (Bhatt *et al.*, 1998; Hanin *et al.*, 2000; Mysore *et al.*, 2000; Xiao and Peterson, 2000). Furthermore, the results presented here should increase confidence in using this technology in *Arabidopsis* as the *Agrobacterium*-mediated transformation is an efficient method to generate large number of transformants required for screening of GT events.

Experimental procedures

Construction of the targeting vector

A DNA fragment of 15 781 bp containing 5′-deleted *PPO* was isolated from a genomic library (*Arabidopsis thaliana* ecotype *Wassilewskija*) of Stratagene, La Jolla, USA. The two *PPO* mutations were introduced using the Quick-Change site-directed mutagenesis kit (Stratagene). The *Kpn*I site was introduced by PCR into the *PPO* gene fragment containing the eighth intron (Figure 1). The resulting construct was further deleted by removal of a 10.5 kb *Xho*I-*Pac*I fragment (Figure 1). The final construct, called pSDM, has a T-DNA of 5.6 kb.

Agrobacterium *strain*

The *Agrobacterium tumefaciens* strain C58ClRif^{R} containing the Ti plasmid pGV3101 (Van Larebeke *et al.*, 1974) was transformed with pSDM as described (Höfgen and Willmitzer, 1988) The selection of transformed bacteria was carried out on plates containing rifampicin at 100 μg/ml, gentamycin at 25 μg/ml and kanamycin at 50 μg/ml.

Arabidopsis *transformation*

Agrobacterium-mediated transformation of *Arabidopsis* adult plants was performed by the inflorescence infiltration method (Bechtold *et al.*, 1993).

Butafenacil selection

The selection was performed on soil-grown, 5- to 6-day-old-seedlings. Seedlings were sprayed with a 1500 nM Butafenacil solution (Tomlin, 2000) supplemented with 0.01% silwet L-77 (Lehle Seeds, TX, USA). Spraying was repeated three times within 10 days. For T2 segregation, selection was carried out on aseptic germination medium supplemented with Butafenacil at 50 nM. Transformation efficiency was estimated after Basta selection as described previously (Mengiste *et al.*, 1997).

Southern blot analysis

DNA extracted from leaves of seedlings using the nucleon Phytopure extraction kit (Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. After endonuclease digestion and electrophoresis on a 0.8% agarose gel, DNA fragments were blotted to nylon membranes (Hybond N, Amersham Pharmacia Biotech). The membranes were hybridized to ^{32}P-labeled probes (shown on Figure 1) as described before (Church and Gilbert, 1984).

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