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WEED SCIENCE

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A detoxification gene in transgenic *Nicotiana tabacum* confers 2,4-D tolerance

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Transgenic *Nicotiana tabacum* with tolerance to 2,4-D has previously been produced using a bacterial 2,4-D-dioxygenase gene (*tfdA*) driven by the 35S promoter of cauliflower mosaic virus. Using promoters from the *Pisum sativum* plastocyanin gene (*petE*) and an *Arabidopsis thaliana* histone gene (H4A), we demonstrate that similar protection from 2,4-D can be obtained in transgenic *N. tabacum* by targeting expression of *tfdA* to either meristematic tissues or chloroplast-containing tissues. As with the 35S promoter constructs, the plants are tolerant but not completely resistant; very young seedlings in particular are only slightly protected. However, the levels of tolerance observed could offer a useful degree of protection from accidental spray drift.

Nomenclature: 2,4-D; *Nicotiana tabacum* L., tobacco; *Pisum sativum* L., pea.

Key words: Crop injury, herbicide resistance, targeted gene expression, transgenic plants, promoter.

The broadleaf herbicide 2,4-D has had a long history of use in agriculture and continues to be widely used in many countries for weed control in fallow fields and cereal crops. In Australia, susceptible crops such as *Gossypium hirsutum* L. (cotton) have been damaged by 2,4-D spray drift or by residual herbicide in the soil after treatment of fallow.

The gram-negative soil bacterium *Alcaligenes eutrophus* produces a set of plasmid-encoded enzymes that form a pathway for the catabolism of 2,4-D (Don et al. 1985). The first enzyme of the pathway, encoded by the gene *tfdA*, is an α-ketoglutarate–dependent dioxygenase (Fukumori and Hausinger 1993). This enzyme catalyses the conversion of 2,4-D to 2,4-dichlorophenol, which is approximately 100 times less phytotoxic than 2,4-D (Lyon et al. 1989). The coding sequence of the *tfdA* gene has previously been used in transcriptional fusions with the cauliflower mosaic virus 35S promoter (Guilley et al. 1982) to generate 2,4-D–tolerant transgenic *Nicotiana tabacum* and *G. hirsutum* plants (Bayley et al. 1992; Lyon et al. 1989, 1993; Streber and Wilmitzer 1989).

The most damaging effects of 2,4-D are concentrated around the shoot apex and the young developing leaves and flowers, i.e., sink tissues to which the herbicide is actively translocated. At the low doses resulting from accidental spray drift, young leaves and flower buds take on a characteristic elongated and twisted shape; at higher doses, the leaves, flowers, and shoot meristems are killed. The 35S promoter used in previous reports to drive the expression of the *tfdA* gene in transgenic plants gave expression in nearly all plant parts with elevated levels of expression around vascular tissues (Odell et al. 1985). This generalized expression of the dioxygenase was sufficient to give plants a degree of tolerance but not complete resistance to 2,4-D.

The initial aim of this study was to determine whether improved protection could be obtained by more precise targeting of the enzyme to only those tissues that are most susceptible to 2,4-D. A histone gene promoter from *Arabidopsis thaliana* (L.) Heynh. (mouse-ear cress) (Atanassova et al. 1992; Chaboute et al. 1987) was selected for this purpose. This promoter has been shown to drive high levels of β-glucuronidase (GUS) gene (Jefferson et al. 1987) expression in transgenic *A. thaliana* in the primary shoot and root meristems, axillary and lateral root meristems, and in flower buds, particularly in the developing ovules and embryos (Atanassova et al. 1992).

The second approach to improving 2,4-D tolerance was to employ the *Pisum sativum* plastocyanin gene (*petE*) promoter (Last and Gray 1989), which is active in chloroplast-containing tissues and has given severalfold higher GUS expression than the 35S promoter when compared in transgenic *N. tabacum* leaves (Pwee and Gray 1993).

Eleven of the 13 most serious weeds affecting the Australian *G. hirsutum* industry (Charles 1991) are dicots and can be controlled by 2,4-D. These include *Xanthium occidentale* Bert. (Noogoora burr), *Xanthium spinosum* L. (spiny cocklebur), *Ipomoea lonchophylla* J.M. Black (peach vine), *Haloragis glauca* Lindl. (grey raspwort), and *Polymeria longifolia* Lindl. (peak downs curse). If sufficient improvements could be made to the 2,4-D tolerance of *G. hirsutum*, 2,4-D could be applied for postemergence control of broadleaf weeds.

Materials and Methods

Plasmid Constructions

Two new herbicide tolerance constructs were made using the *tfdA* coding region described in Lyon et al. (1989), except that the translation start was mutated to introduce an *NcoI* site (CCATGG) for subsequent ligation with the promoter fragments. Similar constructs with a *NcoI* site at the start codon of the GUS gene were also constructed to confirm the tissue-specific expression patterns of the promoters used for generating herbicide-tolerant plants. The first pair of constructs (pH4AtfdA-nos and pH4AGUS-ocs) (Figure 1) employ the *A. thaliana* histone H4A promoter. The H4A promoter (−603 to +3 relative to the translation start) was

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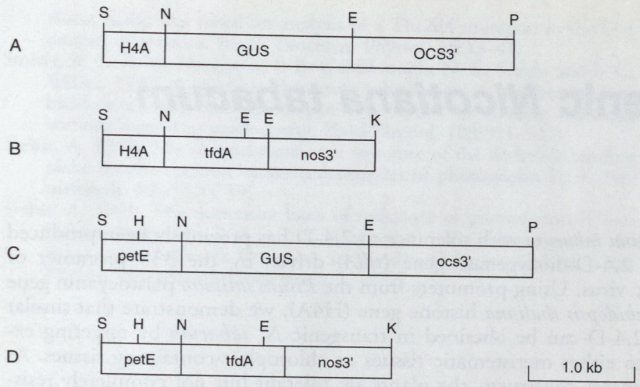


FIGURE 1. Schematic maps of the *tfdA* and GUS constructs driven by the histone and plastocyanin promoters, incorporating 3′ regulatory regions of the nopaline (nos) or octopine (ocs) synthase genes. The regions shown are cloned within the multiple cloning sites of pUC18 (*tfdA* constructs) or pUC118 (GUS constructs) in the following plasmids: (A) pH4AtfdA-nos, (B) pH4AGUS-ocs, (C) pPetEGUS-ocs, (D) pPetEtfdA-nos. E indicates an *EcoRI* site; H indicates a *HindIII* site; K indicates a *KpnI* site; N indicates an *NcoI* site; P indicates a *PstI* site; and S indicates an *SalI* site.

amplified by polymerase chain reaction from *A. thaliana* (ecotype 'Columbia') DNA using standard procedures. Mismatched primers were employed to introduce an *NcoI* site at the translation start codon and a *SalI* site at the 5′ end of the amplified fragment, which was then cloned into pGEM5Z(−) cut with *SalI* and *NcoI* to give pGEMH4A. The 960 bp *BamHI* fragment containing the *tfdA* coding region was subcloned into the *BamHI* site of pJKKmf(−) (Kirschman and Harris Cramer 1988). The resulting plasmid was cut with *NcoI* and *PstI* and ligated with the *NcoI*/*NsiI* fragment of pGEMH4A to generate pGEMH4AtfdA. The promoter and coding region from this plasmid were excised as a *HindIII*/*SmaI* fragment and subcloned into *HindIII*/*SmaI*-cut p35SKN100 (J. C. Walker, unpublished data), a derivative of pCN100 (Howard et al. 1987), to give pH4AtfdA-nos (Figure 1).

To make the corresponding GUS construct, the GUS coding region and 3′ ocs termination sequence was excised as a *XhoI*/*SalI* fragment from pKIWI101 (Janssen and Gardner 1989). This was subcloned into the *SalI* site of JKKmf(−), such that the *SalI* site was regenerated 5′ to the *NcoI* site of the GUS start codon to give pJKKIWIGUS-ocs. To insert the *petE* promoter, the *SalI*/*NcoI* fragment of pGEMH4A was then ligated with *SalI*/*NcoI*-cut pJKKIWIGUS-ocs. The *BamHI*/*HindIII* fragment of the resulting plasmid was then subcloned into pUC118 (Vieira and Messing 1987) to give pH4AGUS-ocs (Figure 1). Plasmids pH4AtfdA-nos and pH4AGUS-ocs were linearized with *HindIII* and cloned into the *HindIII* site of *Agrobacterium tumefaciens* binary vector pGA470 (An et al. 1985).

The second pair of gene constructs (pPetEtfdA-nos and pPetEGUS-ocs) (Figure 1) employed the *P. sativum* plastocyanin gene (*petE*) promoter. The −784 *petE* promoter from pKHn2 (Pwee and Gray 1993) carried on an *XbaI*/*SnaBI* fragment was subcloned into *SmaI*/*XbaI*-cut pUC118. An *NcoI* site was added at the translation start codon by oligonucleotide mutagenesis, and the promoter was excised from the resulting plasmid with *SalI*/*NcoI*. Replacement of the *SalI*/*NcoI* fragments of the H4A constructs with this fragment generated the *petE* constructs as shown in Figure 1.

The binary vector pGA470 (An et al. 1985) was modified by introducing an *XhoI* linker at the end-filled *HindIII* site to yield pGA470X. Plasmids pPetEtfdA-nos and pPetEGUS-ocs were linearized with *SalI* and cloned to the *XhoI* site of pGA470X. DNA manipulations were carried out using standard procedures (Sambrook et al. 1989).

Confirmation of Gene Expression

All four binary constructs were introduced by a triparental mating into *A. tumefaciens* strain AGL1 (Chaudhury and Signer 1989), which was then used to infect *N. tabacum* cultivar 'Wisconsin 38' (W38) using kanamycin sulfate as the selective agent, as described previously (Lyon et al. 1989). Five to six independent primary transformants were selected for each gene construct and were analyzed further. GUS-containing transgenics were analyzed in the T_0 and T_1 generations by staining seedlings and sections of larger plants with X-Gluc[1] as described in Jefferson et al. (1987). After staining, the chlorophyll was removed by soaking the seedlings first in ethanol : water (1 : 1 by vol) for 30 min., then in four changes of 100% (by vol) ethanol over 6 h.

Selection of True-Breeding Lines

Primary transformants containing *tfdA* constructs were self-pollinated. The resulting segregating T_1 seeds were germinated on plates of plant tissue culture medium (MS medium) (Murashige and Skoog 1962) containing 300 mg L^{-1} kanamycin sulfate to select lines, which gave a 3 : 1 ratio of kanamycin-resistant to kanamycin-susceptible progeny, indicating that the transgene had integrated at a single locus. Several T_1 plants were self-pollinated and T_2 seeds were germinated on kanamycin plates to determine which of the T_1 lines bred true for the kanamycin resistance gene and were likely to be homozygous for the transgenes. Lines in which all seedlings were kanamycin-resistant were selected for the 2,4-D spraying trial.

Spray Treatments

T_2 seeds of kanamycin-resistant lines and untransformed controls were germinated in potting compost. Plants were allowed to grow without transplantation to two developmental stages (approximately 30 and 300 mg fresh weight, respectively) corresponding to about 2-cm and 5-cm rosette diameters. They were then sprayed to run-off with various concentrations of 2,4-D ranging from 0 to 1,000 mg L^{-1} (free acid form dissolved in acetone : water [1 : 1 by vol]) using a hand-operated sprayer. Six seedlings from each line were sprayed with each concentration at each developmental stage. Fourteen d after treatment, each plant was cut at ground level, and individual whole shoots were weighed to determine the fresh weight increase as a measure of plant survival and growth after 2,4-D treatment.

A separate spraying trial was performed to rank some of the different transgenic lines with respect to their 2,4-D tolerance. Between 50 and 100 homozygous T_2 seedlings of each line were allowed to grow to a mean rosette diameter of about 2 cm with 2 to 4 seedlings per pot, taking care to ensure that all plants were at approximately the same de-

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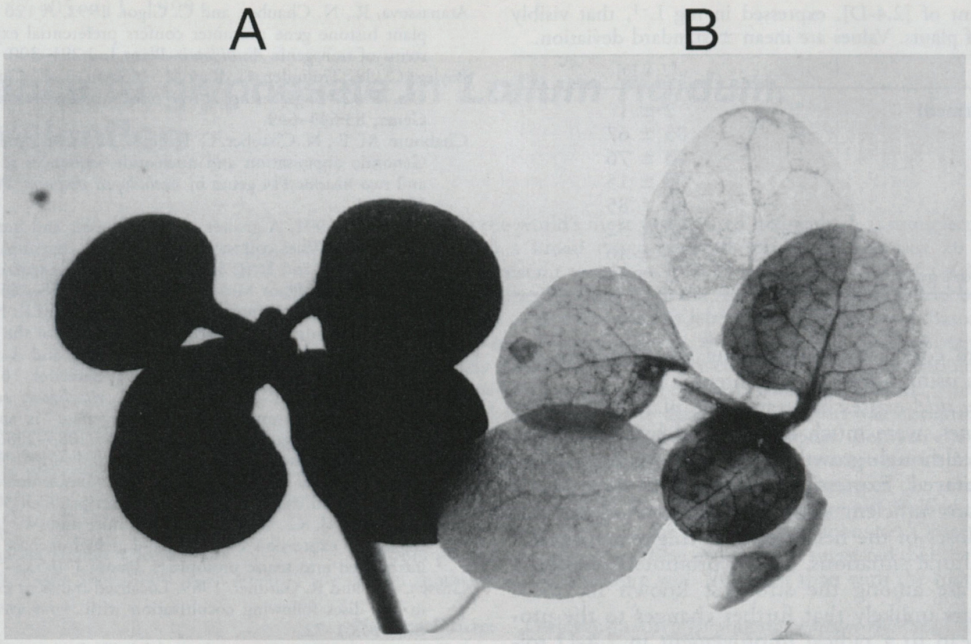


FIGURE 2. Histochemical localization of GUS expression in transgenic *Nicotiana tabacum* seedlings expressing the petEGUS construct (A) or the H4AGUS construct (B). All dark areas in the photograph are the indigo product derived by the action of β-glucuronidase on X-Gluc.

velopmental stage. Plants were sprayed with a range of 2,4-D concentrations from 0.005 to 330 mg L^{-1} produced by making a series of fourfold dilutions of a stock solution in acetone : water (1 : 1 by vol). Four d after spraying, the plants were visually inspected for symptoms of 2,4-D damage, and the numbers of damaged and undamaged plants were recorded.

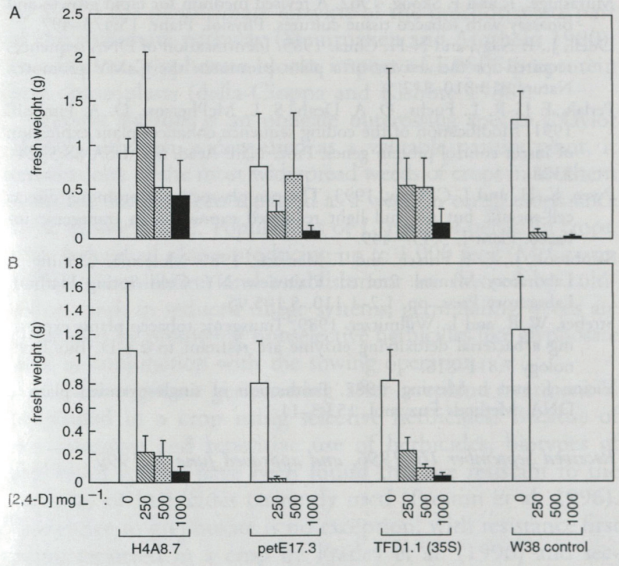


FIGURE 3. Fresh weight of plant after 2,4-D spray treatment of homozygous single copy T_2 seedlings at two developmental stages: (A) plants at 300 mg fresh weight, and (B) plants at 30 mg fresh weight. Error bars are one standard deviation.

Results and Discussion

Plants transformed with the H4AGUS construct showed intense staining only at the apical and root meristems (Figure 2B) in agreement with the result reported for *Arabidopsis* (Atanassova et al. 1992). Older H4AGUS-expressing plants showed a very low level of X-Gluc staining in mature leaves, whereas the petEGUS plants showed intense staining. The petEGUS construct gave plants whose expression was confined to green tissues, e.g., young and old leaves and flower buds. These results are consistent with those reported previously (Perlak et al. 1991). As both promoters gave the expected pattern of GUS reporter gene expression, it was expected that the similar constructs with the *tfdA* coding region instead of the GUS coding region would result in identical distributions of the 2,4-D dioxygenase.

The results of the spraying trial for a typical set of transformants are shown in Figure 3. Although the level of variation was relatively high, it was clear that these transformants showed enhanced tolerance of 2,4-D. However, very young T_2 plants, even of the relatively tolerant line H4AtfdA8, were severely retarded in their growth at 2,4-D concentrations as low as 250 mg L^{-1}.

From the results of the spraying trial using a range of dilutions of the herbicide, graphs were plotted to show the number of plants visibly damaged vs. 2,4-D concentration. From each graph, it was possible to estimate the 2,4-D concentration at which 50% of plants would be visibly damaged. Some of the values obtained are shown in Table 1. The results suggested that some of the transgenic lines may be 20- to 50-fold less sensitive to 2,4-D than the controls without *tfdA* genes. However, the high level of variation (standard deviation sometimes larger than the mean) pre-

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TABLE 1. Amount of [2,4-D], expressed in mg L^{-1}, that visibly damages 50% of plants. Values are mean ± standard deviation.

Plant line	[2,4-D]
W38 (untransformed)	2 ± 1
TFD1.1 (35S)	85 ± 67
TFD1.26 (35S)	73 ± 76
petE17.3	26 ± 15
petE4.5	80 ± 85
H4A5.2	98 ± 32
H4A8.7	64 ± 46
petEGUS (no tfdA gene)	3 ± 2

cluded any firm conclusions concerning the difference between the lines using different promoters.

Many of the *tfdA*-expressing transgenic plants, including the 35StfdA lines, were much less affected by 2,4-D than control plants, although growth rates were significantly reduced when sprayed. Expression of the 2,4-D dioxygenase therefore appears sufficient to confer tolerance of, but not resistance to, doses of the herbicide that might be encountered in agricultural situations. As the promoters employed in this study are among the strongest known for dicot plants, it appears unlikely that further changes to the promoter would lead to a radical improvement in 2,4-D tolerance. Codon optimization (e.g., Perlak et al. 1991) of the *tfdA* gene might enhance tolerance levels. The levels of tolerance obtained with the constructs described here could possibly be effective in protecting a crop from accidental spray drift damage. The H4A promoter gave two of the most 2,4-D-tolerant of the transgenic lines (H4AtfdA8 and H4AtfdA5), making it worthy of consideration for use at a commercial level in crops such as *G. hirsutum.* The precise targeting of expression with this promoter could also minimize any (hypothetical) yield penalty that might be associated with expression of the transgene. Rigorous field testing will be required to confirm the agronomic usefulness of this technology.

Source of Material

[1] 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, Progen Industries, 2806 Ipswich Road, Darra, Qld 4076, Australia.

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