Development of 2,4-D-resistant transgenics in Indian oilseed mustard (*Brassica juncea*)

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Transgenic lines resistant to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were developed in mustard (*Brassica juncea*), a major oilseed crop grown in more than six million hectares of land in North India. The developed construct contained the *tfdA* gene, encoding the enzyme 2,4-D monooxygenase, cloned downstream to the 35S promoter along with a leader sequence from RNA4 of alfalfa mosaic virus (AMV leader sequence), for improved expression of the transgene in plant cells. Southern analysis of T0 transgenics confirmed six out of 24 transgenics to be single copy events, from both the flanks of T-DNA. Selfed progeny derived from single copy *tfdA* lines germinated normally and rooted in medium containing 2,4-D at concentrations as high as 2.5 mg l^{-1} compared to the wild-type seedlings which did not root even at a concentration of 0.5 mg l^{-1}. The *tfdA* transgenic lines were also sprayed with commercially available 2,4-D herbicide at concentrations ranging from 10 to 1000 mg l^{-1} under field conditions. Wild type plants were affected by levels as low as 10 mg l^{-1} and were completely killed at a concentration of 50 mg l^{-1}. The four transgenic lines tested in the study were resistant to herbicide concentration of 500 mg l^{-1}. The available transgenic lines can be used for testing the potential of 2,4-D in weed control including the control of parasitic weeds (*Orobanche* spp) of mustard and for low-till cultivation of mustard.

TRANSGENIC approach has been used for improvement of *B. juncea* in the area of hybrid seed production[1,2], herbicide resistance[3] and oil quality[4]. The *bar* gene conferring resistance to herbicide phosphinothricin has been successfully deployed in *B. juncea* either alone[3] or in constructs containing *barnase* and *barstar* genes[1,2]. In both cases, *bar* gene was used as a selectable marker and enabled both *in vitro* and field-level selection of transgenic plants. However, phosphinothricin and its commercial product Basta, are expensive herbicides and their availability is rather limited in India. It will therefore be useful to test the use of 2,4-dichlorophenoxyacetic acid (2,4-D) as a herbicide for selection of transgenic plants.

2,4-D and several related phenoxy compounds have been used extensively for more than 50 years to control broadleaf weeds. It is a post-emergence, translocatable herbicide and specific only to broadleaf plants. Soil organisms that degrade 2,4-D were identified more than forty years ago[5] and the multi-enzyme pathways for 2,4-D degradation have subsequently been demonstrated in several bacterial genera[6–8]. The first gene (*tfdA*) involved in the 2,4-D degradation pathway of soil organism *Alcaligenes eutrophus*, encodes 2,4-D monooxygenase enzyme, which converts 2,4-D into less toxic 2,4-dichlorophenol and glyoxylate by cleavage of the aliphatic side chain[9]. Transgenic plants resistant to 2,4-D have been developed in tobacco as a model system[10,11] and in cotton[12,13].

In this communication, we report the development of 2,4-D-resistant transgenics in Indian mustard (*B. juncea* cv. Varuna), an agronomically important broadleaf crop, by introduction of *tfdA* gene. The single copy transgenic lines obtained were grown in a containment net-house and tested for their resistance to 2,4-D herbicide. Limited small scale trials showed that the transgenic lines were resistant to ten times higher level of herbicide tested compared to the wild-type cultivar Varuna tested under field conditions. The *tfdA* gene could provide an additional herbicide resistance field marker for use along with gene to be introgressed in transgenic crop, e.g. in the *barnase–barstar* system.

A binary vector containing the *tfdA* gene (864 bp; obtained from plasmid pBRL7) under the transcriptional control of CaMV35S promoter was developed (Figure 1 *a*). The start codon of *tfdA* gene was changed from GTG to ATG by PCR-based site-directed mutagenesis. Further, an AMV leader[14] and consensus Kozak[15] sequence were incorporated for enhanced expression of the transgene in plants. The 35S–AMV–*tfdA*–35SpA expression cassette thus constructed was finally cloned in binary vector[16] pPZP200 (ref. 16), which also contained a nosPr–*npt*II–ocspA [neomycin phosphotransferase gene (*npt*II) driven by nos promoter and carrying ocs poly A signal at 3′ end] cassette as an additional marker gene cloned within the lox sites (Figure 1 *a*). The above binary vector was transformed into disarmed *Agrobacterium tumefaciens* strain GV3101 by electroporation[17] and used for genetic transformation.

Genetic transformation of hypocotyl explants derived from *Brassica juncea* cv. Varuna was carried out following the protocols described earlier[1,3]. In two transformation experiments, around 700 explants were infected with disarmed *Agrobacterium* strain GV3101 containing the binary plasmid with *tfdA* gene. Shoots regenerated from the hypocotyl explants at an overall transformation frequency of 8.5 and 11.2% in two different experiments (Table 1). Transgenics were rooted in kanamycin-containing medium and maintained *in vitro* as nodal cultures. Forty independent transgenic plants were transferred to soil during October–November in a containment net-house as per the guidelines of the Department of Biotechnology, New Delhi.

Southern analysis of putative transgenics was carried out by isolating total genomic DNA from leaves of T0-

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grown plants using the CTAB method[18]. A 2.3 kb nosPr–*npt*II–ocspA fragment and a 200 bp *tfdA* fragment were used as probes for analysis of copy number on left and right border flanks of T-DNA respectively (Figure 1 *a*). Of the 24 transgenics subjected to analysis by Southern hybridization, six were found to be single copy integration events at both the T-DNA flanks. A representative Southern blot is shown in Figure 1 *b*. All the T0 transgenics with single copy insertion were selfed and backcrossed for their maintenance.

The T1 seeds of a representative *tfdA* line (*tfdA* 1.32) were germinated on medium containing varying concentrations (0, 0.5, 1.0, 2.5 and 5 mg l^{-1}) of 2,4-D (Sigma) along with non-transformed control Varuna (Figure 2). Both transformed and non-transformed lines showed normal germination and expansion of cotyledons up to four days. However after the fourth day, seedlings of non-transformed plants and null-segregants showed poor hypocotyl elongation on 0.5 mg l^{-1} of 2,4-D concentration. At higher concentrations of 2,4-D (> 1.0 mg l^{-1}) no hypocotyl elongation was observed even up to 10 days. Elongation of roots was inhibited even at the lowest 2,4-D concentration that was tested (0.5 mg l^{-1}). The roots remain stunted and had high density of root hair.

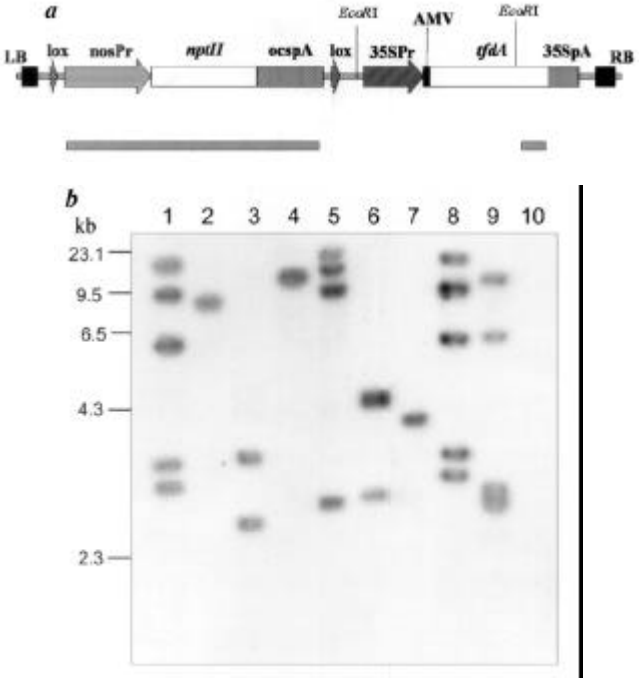

Figure 1. ***a***, Schematic representation of T-DNA region of the *tfdA* construct showing the location of *Eco*RI site used for genomic DNA digestion along with the probe fragments used for the Southern analysis (hatched boxes). ***b***, Southern blot of T0 transgenics showing the T-DNA integration pattern for left T-DNA border when probed with nosPr–*npt*II–pA cassette. DNA (10 µg) was digested with *Eco*RI and electrophoresed on a 0.8% agarose gel. DNA fragments were transferred onto nylon membrane (Hybond N +) and hybridized with the probe DNA labelled with ^{32}P*a*–dCTP using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Transgenics in lanes 2, 4 and 7 represent single copy events. Transgenics in lanes 1 and 8 represent the same transformation event. Lane 10 is untransformed negative control.

The selfed progeny of heterozygous *tfdA* line, *tfdA* 1.32, with single copy insertion of the T-DNA segregated in 3 : 1 ratio at the level of seed growth (data not shown). The progenies with transgene showed proper hypocotyl elongation at all the concentrations of 2,4-D that were tested (Figure 2). These seedlings also rooted properly in media containing 2,4-D up to a concentration of 2.5 mg l^{-1}. However at the concentration of 5 mg l^{-1} of 2,4-D, root elongation of transgenic plants was delayed.

For determining the 2,4-D toxicity at the field level, T1 backcrossed progenies of representative single copy lines, viz. *tfdA* 1.4, *tfdA* 1.9, *tfdA* 1.32 and *tfdA* 2.1 were transplanted in the field along with non-transformed Varuna as a control. After 3–4 weeks of germination, seedlings were sprayed with different concentrations (10, 20, 50, 100, 200, 500 and 1000 mg l^{-1}) of herbicide 2,4-D (isopropyl ester; commercially available as Hammer 38, Pusti Agrochemical Pvt Ltd, India). The effect of 2,4-D spray on the survival of plants was analysed over a period of two weeks. Wild type plants and segregants without the *tfdA* gene were affected by 2,4-D levels as low as 10 mg l^{-1} and were completely killed at 50 mg l^{-1} and higher levels. Such sensitive plants showed wilting of leaves 24 h after the spray and were killed within 10–12 days. All the four transgenic lines tested in the current study were resistant to 10 times the concentration of 2,4-D (i.e. 500 mg l^{-1}), at which the wild-type plants get completely killed. Two lines *tfdA* 1.32 and *tfdA* 2.1 were resistant to 2,4-D herbicide even at a concentration of 1000 mg l^{-1} (data not shown). The backcrossed progeny of transgenic lines tested, segregated in 1 : 1 ratio of resistant to sensitive plants, thereby confirming the single copy status of the transgene (Table 2). Resistant plants in the segregating progeny of transgenic lines were confirmed for the presence of *tfdA* gene by tissue-PCR[19] using *tfdA* gene-specific primers (data not shown).

In the transgenic lines developed in this study, nosPr–*npt*II–ocspA cassette, used as the selectable marker for *in vitro* selection of T0 transformants, has been cloned within

Table 1. Transformation of *Brassica juncea* cv. Varuna using *tfdA* construct

No. of explants	No. of transformants	Transformation frequency (%)	No. of plants taken to field	No. of plants analysed for copy number	No. of single copy events
352	30	8.5	20	14	3
352	42	11.2	20	10	3

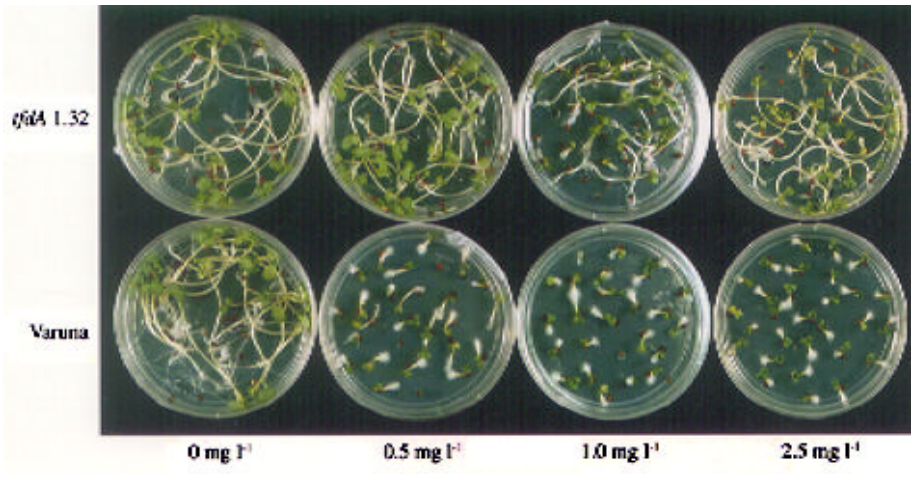

Figure 2. Seed germination of T1 selfed progeny of line *tfdA* 1.32 on MS medium supplemented with various concentrations of 2,4-D. Untransformed seeds of cultivar Varuna were also tested for germination as a control.

Table 2. 2,4-D spraying analysis on BC1 progenies of *tfdA* transgenic lines of *B. juncea*

Transgenic line	2,4-D at 50 mg l^{-1} S	R	chi-square[a]
tfdA 1.4	34	31	0.14
tfdA 1.9	10	11	0.05
tfdA 1.32	30	35	0.55
tfdA 2.1	37	31	0.53
Varuna	78	0	nt

S, sensitive; R, resistant; nt, not tested; [a]chi-square was performed at 95% confidence limit ($P < 0.05$) to determine the goodness-of-fit.

the lox sites. After development of homozygous lines, the marker gene can be removed using lox/*cre* system of recombination[20]. The efficiency of lox/*cre* system for marker removal in mustard is being tested at present in the laboratory. Our limited field experiments have shown that *tfdA* gene could be used as an effective field level selection marker allowing the use of a cheaper and more readily available herbicide 2,4-D in place of a herbicide like 'Basta'. It would be interesting to check the efficacy of 2,4-D to control weeds in mustard including the root-parasite *Orobanche* spp. under field condition. With the availability of resistant lines, it will be also possible to see if the 2,4-D-resistant transgenics could be useful for low-till cultivation of mustard both for moisture conservation and for development of two-crop systems in the rain-fed and low-irrigation areas of North India.

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Validation of multi-channel scanning microwave radiometer on-board Oceansat-I

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Sea surface temperature (SST), sea surface wind speed (WS) and columnar water vapour (WV) derived from Multi-frequency Scanning Microwave Radiometer (MSMR) sensor on-board IRS-P4 (Oceansat-I) were validated against the *in situ* measurements from ship, moored buoy (MB), drifting buoy (DB) and autonomous weather station (AWS). About 1400 satellite *in situ* match-ups were used for the validation of SST and WS, while only 60 match-ups were available for the validation of WV. Therefore specific humidity, Q_a was used as a proxy for validating WV.

The drifting buoy SSTs showed good correlation with the satellite values ($r = 0.84$). The correlation of MB SSTs was better during night when the WS varied between 0 and 10 m/s. During the day, correlation peaked for higher wind speeds (> 10 m/s). MB ($r > 0.80$) was relatively better than AWS ($r \sim 0.70$) and ship ($r < 0.50$) for validating satellite-derived WS. Daytime winds exhibited better correlation with satellite values when measured from ocean platforms (MB and ship), but the winds measured from land-based platforms (AWS) were closer to satellite values during night-time. Q_a values consistently showed higher correlation with satellite values during night-time. The low root mean square deviation (RMSD) of DB SST (1.17°C) and MB WS (1.52 m s^{-1}) is within the achievable accuracy of the microwave sensor when validated with data collected over the tropical Indian Ocean. The RMSD of Q_a (1.81 g kg^{-1}), however, falls much beyond the attainable accuracy of the microwave sensor.

IRS-P4 (Oceansat-I) launched on 26 May 1999 carried an all-weather capable Multi-frequency Scanning Microwave Radiometer (MSMR) payload besides Ocean Colour Monitor (OCM). Details of these payloads are described elsewhere[1]. Channels of MSMR are suitable to retrieve sea surface temperature (SST), sea surface wind speed (WS), columnar water vapour (WV) and cloud liquid water on a global scale. The 6.6 GHz band, suitable for the retrieval of SST and WS (along with 10 GHz), was an improvement over the contemporary sensors (SSM/I, SSMR, etc.).

Weak microwave radiation in these channels makes the spatial resolution more coarse, but is still useful for supplementing high-resolution, cloud-sensitive AVHRR data[2]. Microwave radiometers can measure SST with an accuracy[3,4] of ± 1.5°C, which is much coarser than the one (± 0.5°C) attainable from thermal infrared sensors[5]. Wentz *et al.*[6] showed that passive microwave radiometers can measure WS with an accuracy of approximately 2 m s^{-1}, which is as good as the scatterometer and altimeter-derived WS. Retrieval accuracy of WV from Nimbus 5 microwave spectrometer was shown[7] to be 0.2 g cm^{-2} and it improved substantially to 0.07 g cm^{-2} when derived from SSM/I on-board DMSP satellite[8].

The objectives of this satellite validation exercise are to (a) identify the best frequency combinations to derive SST, WS and WV from satellite sensors by comparing with *in situ* data, (b) identify suitable *in situ* platform for validating satellite-derived SST, WS and WV, (c) study the role of WS in the satellite SST validation and (d) understand the accuracy of MSMR measurements.

The MSMR geophysical data product supplied by the National Remote Sensing Agency consists of 24 h data and has been generated on three different grid sizes, viz. 150 km (grid I), 75 km (grid II) and 50 km (grid III). Details of the grids are given elsewhere[1].

The sea truth data for SST, WS and WV were collected for two years from sensors mounted on various platforms

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