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A PLANT TRANSFORMATION VECTOR CONTAINING THE GENE *dehD* FOR THE DEVELOPMENT OF CULTIVARS RESISTANT TO MONOCHLOROACETIC ACID

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ABSTRACT

A *dehalogenase D* gene (*dehD*) capable of degrading monochloroacetic acid (MCA) has been previously isolated from *Rhizobium* sp. RC1 and characterized. The 804-bp *dehD* gene was inserted into pCAMBIA under the control of the cauliflower mosaic virus 35S promoter and designated pCAMdehD, with a total size of 10,592 bp. The pCAMdehD was introduced into tobacco (*Nicotiana benthamiana*) via *Agrobacterium*-mediated transformation. The integration and expression of *dehD* in *N. benthamiana* was confirmed by PCR and reverse transcription PCR, respectively. MCA-resistant transformants were selected in tissue cultures containing 60 µg/L MCA. Analysis of plants using a leaf-painting assay revealed that transgenic *N. benthamiana* was resistant to 4.0 g/L MCA compared with 2.0 g/L for non-transformed controls. The use of *dehD* could thus be advantageous for herbicide-resistant plant breeding systems, and it is also a suitable marker gene for plant-transformation studies. To the best of our knowledge, this is the first report detailing transgenic *N. benthamiana* engineered with *dehD* from *Rhizobium* sp. RC1.

Key words: Binary vector, *dehD* gene, Herbicide resistant, Monochloroacetic acid, Tobacco.

INTRODUCTION

Weed infestation is a major problem for agricultural activities, and invasive plants negatively impact production through competition for nutrients, moisture (Singh and Yadav, 2012), and light, thereby reducing crop yields (Gressel, 1999) as well as the quality of produce. Moreover, invasive plants can render pastures virtually unproductive. Weed management, which encompasses invasive plant control activities, labor expenses, and herbicide use, can cost up to billions of dollars per year worldwide. Worldwide industrialization and rapid economic development have increased the price of farm labor, which has necessitated the development of cost-effective herbicides. As a result of increased preference for herbicides to control weeds, herbicides now constitute approximately 50% of worldwide sales of agricultural chemicals (herbicides and pesticides) and as much as 60–70% of sales in the United States, Australia, and Germany (Varshney and Mishra, 2008).

Herbicides are routinely applied in present-day agricultural production (Milosevic and Govedarica, 2002). However, repeated and intensive use of herbicides has some drawbacks, including environmental pollution, e.g., through surface run-off that leaches into deep soil strata and ground water or adsorption of herbicides in soil (Michaelidou *et al*., 2000), potential effects on human and animal health (Milosevic and Govedarica, 2002), and the evolution of herbicide-resistant weeds (Vencil *et al*., 2012). Since the beginning of 2012, 372 unique, herbicide-resistant weed biotypes have been confirmed in the top 19 agricultural-based countries. The United States, Australia, and Canada recorded the highest numbers of herbicide-resistant weeds, with 139, 60, and 52 biotypes, respectively (Vencil *et al*., 2012). Thus, it is important to investigate alternative weed control methods that could overcome such disadvantages as well as improve crop yields and productivity.

The transgenic crops that are resistant toward specific herbicides are developed through biotechnological methods due to the potentially harmful effects of herbicide. Herbicide resistance in selected plants involves the addition of a gene encoding an enzyme that detoxifies the herbicide or an altered form of an enzyme targeted by the herbicide. Thus, bacterial genes that are able to degrade toxic compounds are inserted into plants to render a new generation of cultivars insensitive to herbicides. Apart from using bacterial genes, synthetic genes can also be used in crops, such as genes that provide resistance to bromoxynil, as reported by Taghipour (2013), and to the herbicide Basta and Buster (Zang *et al*., 2009).

Rhizobium sp. RC1 was isolated by Berry *et al*. (1979), and the 798-bp *dehalogenase D* gene (*dehD*) was then cloned (Cairns *et al*., 1996). This dehalogenase cleaves the carbon-halogen bond of a specific enantiomer of each of D-2-chloropropionate and monochloroacetic acid (MCA) and has been further characterized (Huyop *et al*., 2008; Huyop and Sudi, 2011). According to Hill *et al*. (1999), DehD is a group I dehalogenase, and its structure and function were later resolved by Sudi *et al*. (2014a, b).

Broad-spectrum herbicides, such as MCA and D-2-chloropropionate, are effective at killing a wide variety of weeds. Unfortunately, they also kill valuable crops and thus can cause substantial agricultural losses. One solution to this problem is developing herbicide-resistant plants. The application of technologies that render herbicide-resistant plants has been reported in many plants and crops, such as tobacco (Wang *et al*., 2003), potato (Choi *et al*., 2007), and carrot (Chen and Punja, 2002).

Here we describe the production of a plant transformation vector for the development of MCA-based herbicide-resistant tobacco cultivars using *dehD* as a selectable marker. The resistance of transgenic *N. benthamiana* cultivars to MCA was confirmed at the tissue-culture level, and its efficacy at resisting herbicidal effects was evaluated using a leaf-painting assay.

MATERIALS AND METHODS

Preliminary test of MCA toxicity against non-transformed *N. benthamiana*: The sensitivity of *N. benthamiana* at the plant tissue-culture level to the toxic effects of MCA (Fluka) was measured by testing shoot regeneration from leaf pieces cultured on MS medium (Murashige and Skoog, 1962) containing different concentrations of MCA (20, 40, 60, 80 and 100 µg/L). Whole-plant sensitivity to MCA was measured by spraying the leaves with different concentrations of MCA (1.0, 2.0, 3.0 and 4.0 g/L).

Plant materials, plasmid constructs and transformations: Transformations were performed using *N. benthamiana* that was cultivated on MS medium. Cultures were kept in a programmable growth chamber (25°C, 16/8 h light/dark) for 8 weeks.

The *dehD* was amplified by PCR using pUC57dehD as the DNA template. The upstream primer DehDREF (5 -CGCGCCATGGATGATAGAT-3) and downstream primer DehDRER (5 -GATGTCACGGTCACCCTATG-3) were designed with flanking sequences containing specific sites for cleavage with the restriction enzymes *Nco*I and *BstE*II (underlined nucleotides), respectively. Amplified *dehD* was cloned into pCAMBIA 1305.2 that had been cut with *Nco*I and *BstE*II. The binary vector pCAMBIA 1305.2 carries the *-glucuronidase* (*GUS*) gene as a reporter driven by the cauliflower mosaic virus 35S promoter and terminates with the *Agrobacterium* nopaline synthase poly-A terminator sequence. It also contains the selectable marker genes *hygromycin phosphotransferase* (*hyg)* and *kanamycin (kan^r)*. In this study, the vector was modified by incorporating *dehD* and removing the GUS gene (Fig. 1). The resulting *dehD*-inserted plasmid was designated pCAMdehD. The construct was then transformed into *A. tumefaciens* strain LBA4404 as described by Sambrook *et al*. (1989), followed by *Agrobacterium* mediated transformation of tissue cultured *N. benthamiana* leaf discs (Bhatti and He, 2009).

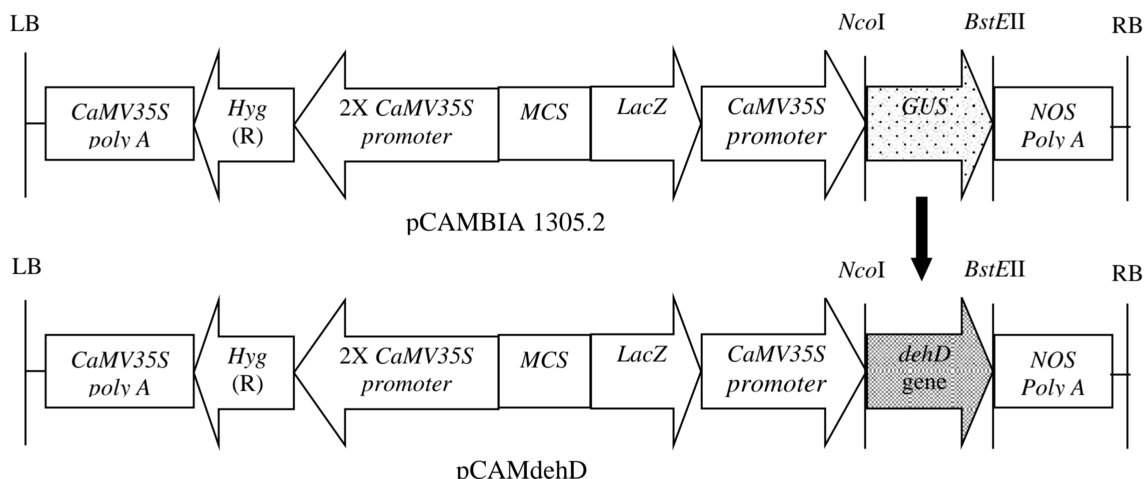

Figure 1. Schematic representation of the plant transformation vectors. (A) The *GUS* gene was excised out of binary vector pCAMBIA 1305.2 using the *Nco*I and *BstE*II restriction enzymes. (B) *dehD* was inserted into the vector.

Restriction enzyme, PCR, and sequencing of the recombinant vector were performed to confirm the integration of full-length *dehD*. The binary vector pCAMdehD was introduced into *N. benthamiana* leaf discs by *Agrobacterium*-mediated transformation as described by Bhatti and He (2009). Regeneration of shoots was carried out on MS selection medium containing 6-benzylaminopurine (5 mg/L) and hygromycin (40 µg/mL) or MCA (60, 80, or 100 µg/L). Shoots were cut from the calli and transferred to MS selection medium containing naphthalene acetic acid (0.1 mg/L) and hygromycin (40 µg/mL) or MCA (60, 80, or

100 µg/L). Upon root formation, the plants were transferred to soil.

Confirmation of transgenic *N. benthamiana*: Putative transgenic *N. benthamiana* were tested for the presence of *dehD*. In addition, *dehD* expression was determined qualitatively using reverse transcription PCR (RT-PCR). In total, 10 putative T_0 transgenic *N. benthamiana* were randomly selected and used for DNA and RNA isolation. DNA and RNA from non-transformed *N. benthamiana* plants were also isolated and used as controls. Total genomic DNA samples from transformed and non-transformed *N. benthamiana* plants were extracted from fresh leaves using Plant DNAzol Reagent (Invitrogen); similarly, total RNA samples were extracted using Trizol Reagent (Invitrogen). Spectrophotometric assays were conducted to determine the quality and quantity of the isolated DNA and RNA. A Quantitect Reverse Transcriptase kit (Qiagen) was used to synthesize cDNA from the RNA samples.

The genes *hpt* (primers hptF: 5'-GAACATCGCCTCGCTCCAG-3' and hptR: 5'-GACCTGCCTGAAACCGAACTG-3') and *dehD* (primers dehDF: 5'-ATGATAGATCTTCCGAGGCA-3' and dehDR: 5'-CTATGGCAGTAGACTGGATTC-3') were amplified to confirm the integration of these transgenes in transformed plants. RT-PCR was performed on cDNA using primers dehDF and dehDR to confirm *dehD* expression in transformed plants. PCR and RT-PCR were each performed in a total volume of 25 µL containing of 20–100 ng of plant genomic DNA or cDNA, PCR Master Mix (Promega), 1.0 µM for each forward and reverse primer, and distilled water. PCR and RT-PCR conditions were as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C or 57°C for *dehD* or *hpt*, respectively, for 2 min, and extension at 72°C for 2 min, with final extension at 72°C for 5 min. PCR products were used for sequence analysis and confirmed by comparison with the *dehD* sequence obtained from the NCBI database under accession number CAA63793.1.

A leaf-painting assay was used to test putative T_1 transgenic *N. benthamiana* planted in soil for resistance to MCA. MCA in water (1.0, 2.0, 3.0, 4.0 g/L) was applied to the leaves of T_1 transgenic tobacco plants.

RESULTS

Preliminary evaluation of MCA toxicity in non-transgenic *N. benthamiana* in tissue cultures and in whole plants: At plant tissue cultures, explants of *N. benthamiana* were grown in the appropriate MS medium supplemented with various concentrations of MCA. At 0–40 µg/L MCA, green calli and shoots were obtained from the leaf pieces, whereas at 60 µg/L MCA only a small number of calli without shoots was obtained where as at 80–100 µg/L MCA, no calli were observed (data not shown). Therefore, an MCA concentration above 60 µg/L was used for selection in plant transformation experiments. In whole plants, spraying leaves with 1.0 g/L MCA resulted in no symptomatic effects, whereas at 2.0 g/L and above the leaves turned yellow and developed brown spots. Thus, at concentrations above 2.0 g/L, MCA had toxic effects in whole plants.

Preparation of DNA inserts containing *dehD* for ligation into pCAMBIA 1305.2: An amplified 804-bp DNA band, representing *dehD*, was observed on a DNA gel (Fig.2A). pCAMBIA 1305.2 was double digested with *Nco*I and *BstE*II restriction enzymes, resulting in the two band pattern (Fig. 2B). The top band (9,788 bp) represented the DNA vector and the bottom represented the GUS gene (approximately 2,133 bp). The latter was then removed to allow the 804-bp *dehD* to be inserted into the vector. Before ligation, both the vector and insert were checked on DNA gels as shown in Fig. 2C.

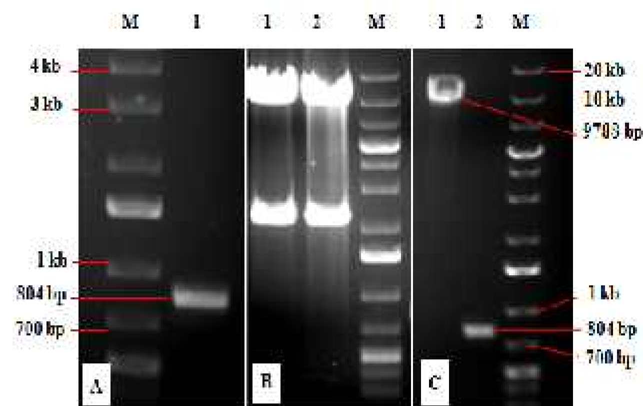

Figure 2. *dehD* and pCAMBIA 1305.2 fragments preparation. (A) DNA containing *dehD* for ligation into pCAMBIA 1305.2. Lane M: Gene Ruler 1 kb Plus DNA ladder; lane 1: amplified *dehD* (804 bp). **(B)** Digestion of pCAMBIA 1305.2 with both *Nco*I and *BstE*II. Lanes 1–2: digested pCAMBIA 1305.2 resulting in two bands; lane M: Gene Ruler 1 kb Plus DNA ladder. **(C)** pCAMBIA 1305.2 (after removing the GUS gene) and the DNA insert (*dehD*). Lane 1: vector digested with *Nco*I and *BstE*II (pCAMBIA 1305.2); lane 2: DNA insert (*dehD*); lane M: Gene Ruler 1 kb Plus DNA ladder.

Ligation of *dehD* into the pCAMBIA 1305.2 plant transformation vector: The plasmid construct was double digested with *Nco*I and *BstE*II, and the presence of the 804-bp fragment confirmed that the *dehD* DNA had been inserted into the construct (Fig. 3). The construct was designated as pCAMdehD.

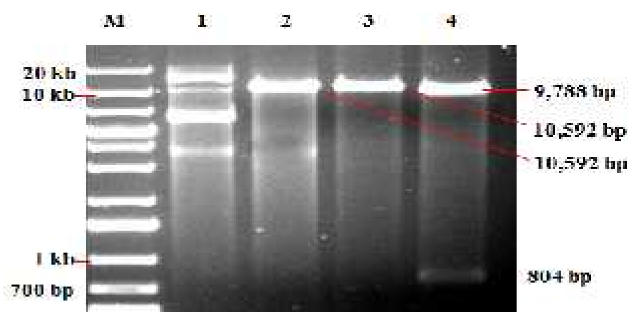

Figure 3. Digestion of pCAMdehD to confirm the presence of *dehD*. Lane M: Gene Ruler 1 kb Plus DNA ladder; lane 1: undigested pCAMdehD; lane 2: pCAMdehD digested with *Nco*I; lane 3: pCAMdehD digested with *BstE*II; lane 4: pCAMdehD digested with both *Nco*I and *BstE*II.

Sequence analysis of the 804-bp *dehD* fragment from pCAMdehD: The *dehD* gene from pCAMdehD was confirmed by comparing its sequence with the original *dehD* sequence taken from the NCBI database [Accession number CAA63793.1]. The results showed that it was 100% identical to the original sequence, indicating that PCR amplification of *dehD* did not introduce any mutations.

PCR analysis of pCAMdehD: A PCR analysis was carried out, using primers dehDF and dehDR, to re-confirm the presence of *dehD* in the pCAMdehD construct. The amplified *dehD* was 798 bp, as expected (Fig. 4).

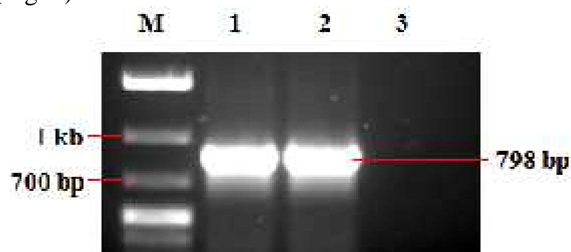

Figure 4. PCR amplified *pCAMdehD* from two different *E. coli* colonies. Lane M: Gene Ruler 1 kb Plus DNA ladder; lane 1: PCR amplification from bacterial colony 1; lane 2: PCR amplification from bacterial colony 2 ; lane 3: negative control (PCR without pCAMdehD as the template).

Analysis of *N. benthamiana* transformed with pCAMdehD and resistance to MCA: pCAMdehD carried both the hygromycin resistance gene and *dehD*, either of which could be used as a selectable marker. Transformed explants of *N. benthamiana* were selected on MS medium containing MCA and hygromycin as selective agents. The results suggested that the transformed cells were resistant to MCA (60 µg/L) and hygromycin (40 µg/mL), both separately and together. *dehD* expression may have resulted in the degradation of MCA in the cells allowing for the development of resistant shoots and roots. Shoots and roots were allowed to develop in plant tissue culture medium for 8 weeks before the transformants were transferred to soil for further analysis. To confirm the integration of *dehD* into the chromosomal DNA of transformed *N. benthamiana*, 10 selected transgenic plants were subjected to PCR, RT-PCR, and leaf-painting analysis, as presented below.

Analysis of transformed and non-transformed *N. benthamiana*, and the integration of *dehD* into the chromosomal DNA: To verify the presence of *dehD*, total genomic DNA samples of transformed and non-transformed 12-week-old *N. benthamiana* were extracted from fresh leaves and analyzed with PCR using dehDF/R and hptF/R primer pairs. Specific bands for *hpt* (370 bp, Fig. 5A) and *dehD* (798 bp, Fig. 5B) were observed in all of the tested transformed plants, whereas no amplification occurred with DNA templates from non-transformed plants.

***dehD* expression analysis:** The molecular analyses strongly indicated the integration of *dehD* into the chromosomal DNA of the 10 tested samples. *dehD* expression was quantified via RT-PCR using *dehD* cDNA as the reverse transcription template and *dehD*-specific PCR primers (Fig. 6). A *dehD*-specific band (798 bp) was observed in all of the transformed plants, whereas cDNA from non-transformed control plants did not yield an amplification product.

Leaf-painting assay: This assay was performed using T_1 transgenic *N. benthamiana* to determine their resistance to MCA. Four different concentrations of MCA (1.0, 2.0, 3.0, and 4.0 g/L) were sprayed on the leaves of the T_1 transgenic and non-transformed (control) plants, and plants were photographed at day 7. An MCA concentration of 1.0 g/L did not affect transformed or non-transformed plants, but leaves of non-transformed plants started to show necrosis at 2.0 g/L (Fig. 7). Transformed plants did not show necrosis up to 3.0 g/L MCA, but necrosis was observed at 4.0 g/L. Thus, the leaf-painting analysis suggested that *dehD* was indeed expressed in *N. benthamiana*, and the results were in agreement with those of RT-PCR.

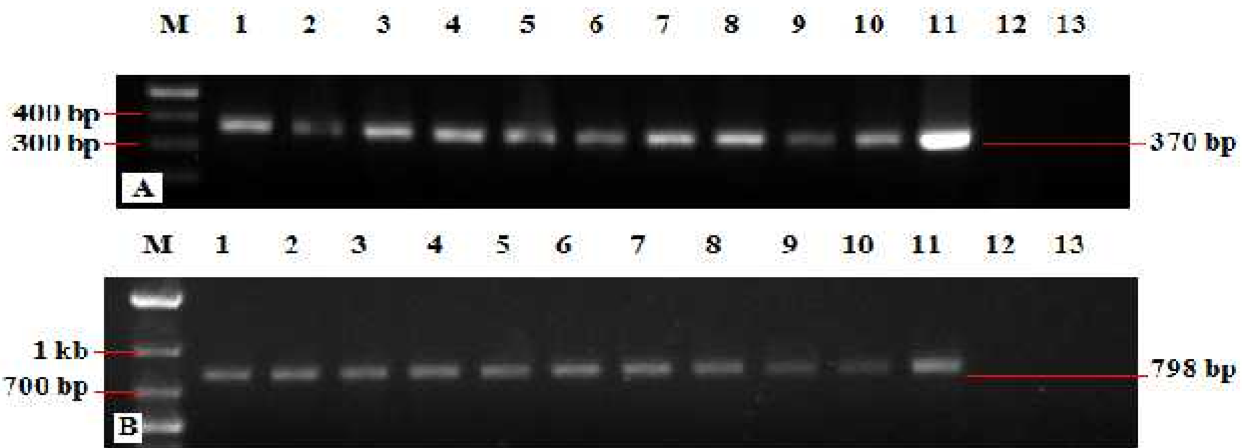

Figure 5. PCR of *hpt* and *dehD* (A) Amplification of *hpt*. Lane M: 1 kb Plus DNA O'Gene ladder; lanes 1–10: putative transgenic tobacco; lane 11: positive control (pCAMdehD); lane 12: negative control (PCR without template); lane 13: untransformed tobacco. **(B)** Amplification of *dehD*. Lane M: 1 kb Plus DNA O'Gene ladder; lanes 1–10: putative transgenic tobacco; lane 11: positive control (pCAMdehD); lane 12: negative control (PCR without template); lane 13: untransformed tobacco.

Figure 6. RT-PCR of *dehD*. M: 1 kb Plus DNA O'Gene ladder; lane 1: untransformed tobacco; lanes 2–11: putative transgenic *N. benthamiana*.

Figure 7. Four-week-old and transgenic T_1 *N. benthamiana* and control wild-type plants photographed 7 days after MCA spraying. (A) Transgenic (left) and wild-type (right) untreated plants. **(B)** Transgenic (left) and wild-type (right) plants treated with 2.0 g/L MCA. **(C)** Transgenic (left) and wild-type (right) plants treated with 4.0 g/L MCA.

DISCUSSION

Among the variety of binary vectors available for plant transformation, pCAMBIA 1305.2 is particularly useful because it is compact, with a pBR322 origin for high-copy replication in *E. coli*, and it stably replicates in *Agrobacterium* (Sarangi *et al.*, 2011). A prominent feature of this vector is its stability when used for plant genetic transformations. Moreover, pCAMBIA 1305.2 contains a kanamycin resistance gene for selection during bacterial transformation and a hygromycin resistance gene for selection during plant transformation.

In this study, the GUS sequence in the pCAMBIA 1305.2 vector was replaced with *dehD* to develop MCA herbicide–resistant cultivars. *dehD* can also be used as a selectable marker for herbicide resistance selection in plant genetic transformations. Only plant cells can integrate the selectable marker gene, and they will survive when grown on a MS medium containing the antibiotic hygromycin and herbicide MCA. A similar study transformed *dehE* into tobacco,

and plants showed resistance to 2,2-DCP (Kaya *et al*., 2013). Thus, *dehD* can be useful as selectable marker in plant transformation systems.

The mechanism of gene transfer from *A. tumefaciens* to host plant cells involves several steps: bacterial colonization, induction of the bacterial virulence system, generation of the T-DNA transfer complex, T-DNA transfer, and integration of the T-DNA into the plant genome. The integration and enhancement of gene expression in the plant genome greatly depends on the promoter that is fused with the 5 end of the gene of interest (Rao, 2014). In that study, the cauliflower mosaic virus promoter and the *Agrobacterium* nopaline synthase transcriptional terminator were used, which together promote high-level gene expression in transgenic plants (Lee and Gevin, 2008).

The *dehD* gene fragment (798 bp) was detected in all of the independent selected lines and in the positive control pCAMdehD overexpression vector. *dehD* was not found in non-transformed plants (Fig. 5A). PCR amplification of *hpt* was used to confirm the presence of the transgene. *hpt* encodes hygromycin phosphotransferase, which imparts resistance to the antibiotic hygromycin. Transgenic plants that carry *hpt* are able to survive on MS medium containing hygromycin at 40 µg/mL. PCR analyses for *hpt* are widely used to screen selected transformed plants that are resistant to hygromycin. Our results showed that *hpt* (370 bp) was detected in all 10 independent transgenic lines, but *hpt* was not found in non-transformed tobacco (Fig. 5B). *dehD* can convert MCA into glycolic acid. When *dehD* expression was controlled by the constitutive cauliflower mosaic virus 35S promoter, an active dehalogenase D enzyme was produced in plant cells, and transgenic plants were completely resistant to MCA. RT-PCR revealed that *dehD* was expressed in all 10 independent transgenic lines, whereas no expression occurred in non-transformed tobacco.

MCA is readily taken up by plant cells, probably by active transport because its relatively small dissociation constant makes it unlikely that a specific receptor exists for this molecule. MCA's mode of action is not well characterized, but it may exert its toxicity by attenuating the mitochondrial citric acid cycle via the formation of halocitrate and the subsequent inhibition of aconitase (Frank *et al*., 1994). MCA also acts by interfering with the photosynthesis system, causing a decrease in the transpiration rate and in total chloroplast area. Higher MCA doses can cause necrosis and contact injury (Sutinen *et al*., 1997) and may also influence the enzymes responsible for xenobiotic metabolism in plants, inhibiting peroxidation activity and oxidative metabolism (Schroder *et al*., 1996).

The *dehD* expression resulted in resistance to MCA. High MCA levels resulted in contact injuries that manifested as rapidly formed brown spots on control leaves, and these were much reduced in the transformants, indicating that enough degradation activity was present to reduce this very rapid effect.

Control plants sprayed with lower levels of MCA (2.0 and 3.0 g/L) showed yellowing owing to the systemic action of the herbicide, whereas the transformants did not display this symptom. This indicated that *dehD* expression resulted in the detoxification of the herbicide within plant cells, which prevented this systemic effect.

Many studies have been published on the transformation of tobacco with herbicide resistance genes, but only a few studies involved dehalogenase genes, and there are no reports on the transformation of tobacco with *dehD* from *Rhizobium* sp. RC1. In our study, we transformed *N. benthamiana* to express *dehD* that originated from *Rhizobium* sp. RC1. This research provides a possible manner to improve the herbicide resistance of crops to the wide-spectrum herbicide MCA, which could have significant economic and environmental implications.

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