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Developing herbicide tolerant transgenic plants for sustainable weed management

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ABSTRACT

The use of herbicide is important in the modern integrated weed management system. However, herbicide also can kill valuable crops and cause significant losses in agricultural activity. One of the solutions to this problem is by developing herbicide resistant plant for instance using *dehD* gene from Rhizobial system. In this study, we developed transgenic tobacco plants that were resistant to monochloroacetic acid (MCA) herbicide. A gene from *Rhizobium* sp. coding for a dehalogenase D (dehD) capable of degrading monochloroacetic acid (MCA) has been previously isolated, characterised, cloned and sequenced. The coding sequence was fused with a cauliflower mosaic virus 35S promoter and introduced into tobacco plants by *Agrobacterium*-mediated gene transfer. In plant transformation experiments, the gene was shown to confer tolerance to MCA at a tissue culture level where MCA could be used to select for transformants. Integration and expression of the *dehD* gene in regenerated plants was confirmed by PCR analyses and Reverse Transcriptase PCR, respectively. The Chi Square analyses of five transgenic plants (T_1), suggested that the *dehD* gene was segregated according to Mendelian 3:1 ratio. These findings showed that transgenic *N. benthamiana* plant resistant to MCA herbicide was successfully produced. The mode of action of dehD enzyme is not known but they probably affect many enzyme pathways. *dehD* gene has several advantages as a marker in plant breeding and genetic studies. In our knowledge, this is the first experimental data of transgenic *N. benthamiana* engineered with *dehD* gene originated *Rhizobium* sp.

Key words : *dehalogenase D* gene, herbicide resistant, monochloroacetic acid, *Nicotiana benthamiana*, transgenic plant.

1. INTRODUCTION

Weed infestation is a major problem for agricultural activities and these invasive plants directly affect production through competition for nutrients, moisture and light that reduce crop yields to below economic levels, reduce quality of the produce and can render pastures virtually unproductive [1]–[3]. Weed would cost billions in economic losses every year. According to Weed Science Society of America (2016), United State and Canada loses $43 billion annually in their corn and soybean crops industries [4]. The advent of worldwide industrialization and fast economic development, have boost the cost of farm labor, hence increasing the necessity for cost-effective chemical weed control using herbicides. The increase preference for herbicides for control weed have resulted in worldwide herbicide market which grew by 39% between 2002 and 2011 and it is projected to grow by another 11% by 2016 [5].

Intensive use of herbicides has been associated with a number of drawbacks such as environmental pollution through surface run-off that leach into deep soil strata and ground water, or adsorption of herbicides in soil [6], human and animal health issues and most troubling is the evolution of herbicide-resistant weeds [7]–[9]. As of the beginning of the year 2012, a total of 372 unique, herbicide-resistant weed biotypes have been confirmed in the top 19 countries with intensive agriculture. The United States, Australia and Canada recorded the highest number of herbicide resistant weeds of 139, 60 and 52 biotypes, respectively [9]. Therefore, it is pertinent that alternative methods that would overcome such disadvantages, improve crop yields and productivity would be of significant advantage.

Given the harmful implications of herbicides, development of transgenic crops that are resistant toward specific herbicides using biotechnological method is timely. Herbicides resistance in selected plants involves the addition of a gene coding for an enzyme that detoxifies the herbicide, or encodes for an altered form of an enzyme targeted by the herbicide. In this context, bacterial genes able to degrade toxic compounds are inserted into plants to render new generation of cultivars insensitive to herbicides [10], [11]. Crops displaying resistance to bromoxynil and the herbicide Basta and

following transformation of a synthetic bxn and bar gene, respectively, were reported [10], [12]–[14].

Current study will focus using the bacterial genes encodes for production of dehalogenases that cleavage the carbon-halogen bond of the active component of herbicides such as the D-enantiomers monochloropropionate (D-2CP) and monochloroace-tate (MCA). The dehalogenase D (DehD) previously isolated from Rhizobium sp. RC1 that was shown to act specifically on D-2- chloropropionate (D-2CP) and monochloroacetate (MCA) [15]–[17].

Broad-spectrum herbicide such as monocloroacetic acid (MCA) is effective at killing a wide range of weeds. Unfortunately, they also kill valuable crops and cause significant losses in agricultural activity. One of the solutions to this problem is by developing herbicide resistant plant using *dehD* gene from Rhizobial system. An application of herbicide resistant plant technology has been reported on many plants and crops such as wheat, tobacco, rice, soybean and canola [18]–[22]. In this study we successfully produced plant transformation vector for development of herbicide MCA resistant tobacco cultivar *Nicotiana benthamiana* using the *dehD* gene as herbicide resistance gene and in the same time as a selectable marker gene. The transgenic *N. benthamiana* cultivars resistant towards the herbicide MCA were obtained and its efficacy in resisting herbicide effects was then evaluated.

2. MATERIALS AND METHODS

Use either SI (MKS) or CGS as primary units. (SI units are strongly encouraged.) English units may be used as secondary

2.1 Development and confirmation of binary vector pCAMdehD

The *dehD* gene was introduced into pCAMBIA 1305.2 under the control of the Cauliflower Mosaic Virus 35S (CaMV35S) promoter and transformed into *Agrobacterium tumefaciens*, which further used for transformation in *N. benthamiana*.

2.2 Development and molecular confirmation of *N. benthamiana* transgenic plants.

The putative transgenic plants were regenerated on three selective Murashige and Skoog (MS) medium; MS supplemented with MS supplemented with 40 µg/mL hygromycin, MS supplemented with 60 µg/L MCA, MS supplemented with 40 µg/mL hygromycin and 60 µg/L MCA to analyse the efficiency of *N. benthamiana* transformation.

The tissue culture generated putative transgenic plants were confirmed by PCR and the expression analysis of confirmed transgenic plants was performed by reverse-transcriptase PCR (RT-PCR). To identify the purity of isolated genomic DNA and gene insertion in transgenic plants, primer sets were designed as shown in **Table** 1.

Table 1: Primer sets used for PCR and RT-PCR

Gene	Primer sequence (5' to 3')
NAD5	Forward: 5'-TAGCCCGACCGTAGTGATGTTAA-3' Reverse: 5'-ATCACCGAACCTGCACTCAGGAA -3'
dehD	Forward: 5'-ATGATAGATCTTCCGAGGCA-3' Reverse: 5'-CTATGGCAGTAGACTGGATTC-3'
hpt	Forward: 5'- GAACATCGCCTCGCTCCAG -3' Reverse: 5'-GACCTGCCTGAAACCGAACTG -3'

2.3 Mendelian Inheritance Pattern

Both putative transformed *N. benthamiana* seeds (from T_0 plants) and wild type tobacco seeds (as negative control) were used to confirm Mendelian inheritance pattern for T_1 plants. They were surfaced sterilized and were placed on selective medium MS supplemented with 60 µg/L MCA. For each line, approximately 100 seeds were used. The number of surviving plantlets on MS selective medium was counted after 6 weeks and then Chi- Square Analysis were performed to test the Mendelian Inheritance Pattern. The germinated seeds were assumed as resistant plantlets, while non-germinated seed were assumed as susceptible plantlets.

3. RESULTS AND DISCUSSIONS

3.1. Development and confirmation of binary vector pCAMdehD

A *dehD* gene encoding dehalogenase enzyme that has capability to degrade monochloroacetic acid (MCA) was isolated and characterized from *Rhizobium* sp. RCI. In this study, *dehD* gene was used as herbicide resistance gene and selectable marker gene in *N. benthamiana* plant transformation. The 798 bp *dehD* gene was inserted into pCAMBIA 1305.2 under the control of the Cauliflower Mosaic Virus 35S (CaMV35S) promoter and designated as pCAMdehD, with a total size of 10,592 bp. The expression cassette containing *dehD* gene shown in **Figure 1**.

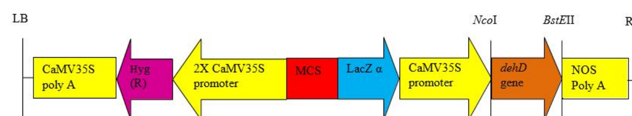

Figure 1. Schematic representation of expression cassette containing *dehalogenase D* gene

The binary vector pCAMdehD was then transformed into *A. tumefaciens*. Antibiotic streptomycin (100 µg/mL) and

kanamycin (50 µg/mL) were used for bacterial selection. PCR colony (to detect the presence of *dehD* gene) was performed on single colony and expected band 798 bp was observed on gel electrophoresis (**Figure 2**). Restriction enzymes analyses were performed to verify the extracted plasmid pCAMdehD, and expected band were observed on gel (**Figure 3**). Plasmid pCAMdehD was send to First Base Laboratory Sdn. Bhd. (1st BASE) for sequencing analysis. The sequencing result was analyzed by using BLASTn (Nucleotide Basic Local Alignment Search Tool). BLASTn result had shown dehD sequence from pCAMdehD was 100% identical to sequence of *Rhizobium* sp. *dehD*, indicating the integration of full length of *dehD* gene and the transformation did not introduce any mutation.

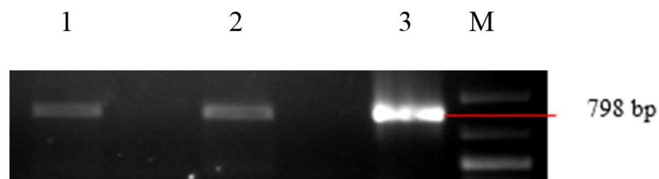

Figure 2: PCR amplified pCAMdehD from two different *A. tumefaciens colonies*, lane 1: colony 1; lane 2: colony 2; lane 3: positive control (plasmid pCAMdeD); lane M: Gene Ruler 1kb Plus DNA ladder (Thermo Scientific).

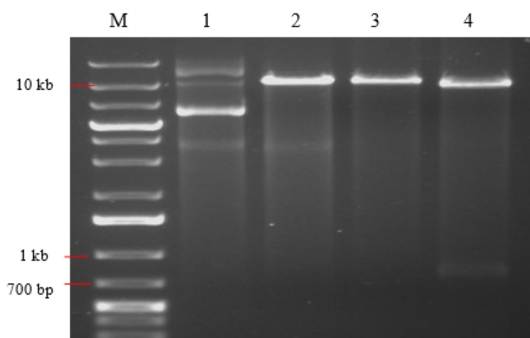

Figure 3: Digestion of pCAMdehD to confirm the presence of *dehD* gene. Lane M: Gene Ruler 1kb Plus DNA ladder (Thermo Scientific); lane 1: undigested plasmid; lane 2: digested with NcoI (10,592 bp); lane 3: pCAMdehD digested with BstEII (10,592 bp); lane 4: pCAMdehD double digested with *Nco*I and *BstE*II (804 bp and 9,788 bp).

3.2 Development and molecular confirmation of *N. benthamiana* transgenic plants.

In this study, we used the *Agrobacterium*-mediated transformation method for the generation of transgenic plant. Four to five weeks old leaves of *N. benthamiana* plants were used as explants in genetic transformation and analysis of *dehD* gene. Genetic transformation was performed by *Agrobacterium* strain LBA 4404 harboring pCAMdehD through leaf disc method. The putative transgenic plants were regenerated on three selective MS medium; MS supplemented with 40 µg/mL hygromycin, MS supplemented with 60 µg/L MCA, MS supplemented with 40 µg/mL hygromycin and 60 µg/L MCA. **Figure 4** showed transformed *N. benthamiana* leaf disks on MS supplemented with 60 µg/L MCA.

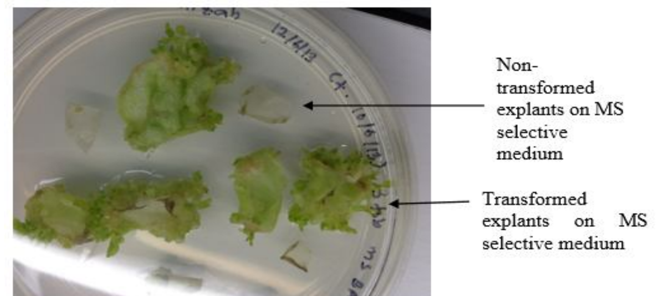

Figure 4: Transformed *N. benthamiana* leaf disks on MS supplemented with 60 µg/L MCA.

Figure 5 shows the *N. benthamiana* genetic transformation. **Table 2** shows the efficiency transformation of putative transformant on selective MS medium. The callus was observed after 2-3 weeks after *Agrobacterium* infection, and then followed by shoots emergence. Control plants did not initiate any callus or shoots or roots and they died in 1- 2 weeks after *Agrobacterium* infection.

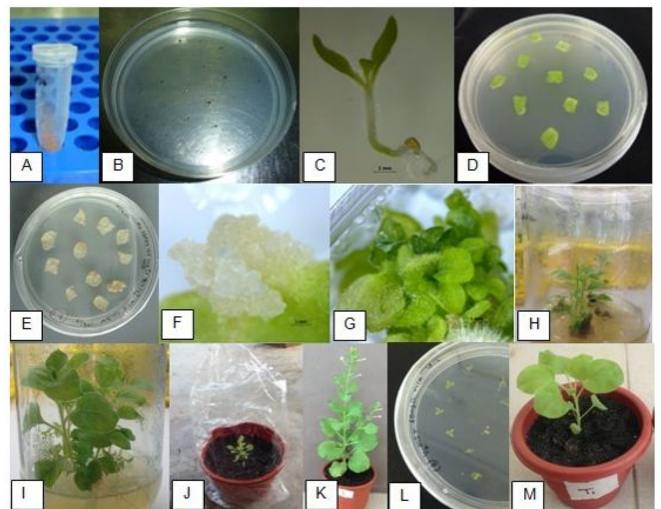

Figure 5: An overview of *N. benthamiana* genetic transformation. (A). *N. benthamiana* seed. (B) *N. benthamiana* seed culture on ½ MS media after sterilization step. (C). 1 week seedling of *N. benthamiana*. (D) Leaf disk of *N. benthamiana* after infected with *A. tumefaciens* that contained pCAMdehD plasmid. (E) Negatives control transformation, non-transformed *N. benthamiana* cultured on selective media. (F) Callus formed on 1 month infected leaf disk. (G, H) Shoot regeneration from callus on infected leaf disk. (I) Root induction. (J) Acclimatization step. (K) T_0

Transgenic plant. (L) Seed from T0 transgenic plants cultured on ½ MS media supplemented with 60mg/L MCA. (M) T_1 transgenic *N. benthamiana* plant.

Table 2: Efficiency of *Agrobacterium*-mediated transformation of *N. benthamiana* on selective MS medium

	Selective agent in MS media	Total explants	Putative transformed regenerants	Transformation efficiency (%)
1	40 µg/mL hygromycin & 60 µg/L MCA	300	147	49
2	40 µg/mL hygromycin	300	171	52
3	60 µg/L MCA	300	150	50

The putative transgenic plants were initially screened for the NAD5 gene (endogenous gene in plant) to check the purity of isolated *N. benthamiana* genomic DNA qualitatively. NAD5 gene amplification results showed that all genomic DNA obtained was pure and suitable to use in PCR amplification analyses to detect the presence of the transgene in transformed *N. benthamiana* plants (**Figure 6**). Then, PCR analyses for *hpt* (**Figure 7**) and *dehD* (**Figure 8**) gene were done to verify the integration of transgene *dehD* gene in transformed plants. According to PCR analyses results, *dehD* gene present in all transformed plants. The *dehD* gene expression was further validated by reverse-transcriptase (RT-PCR) analysis (**Figure 9**). The gel observation had shown all transformed plant has positive PCR result, verified the expression of the *dehD* gene in transformed *N. benthamiana* plants.

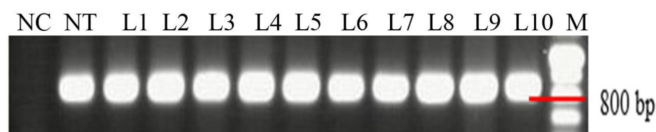

Figure 6: PCR for NAD5 gene. M: 1kb Plus DNA O'Gene (Fermentas); L1-10:putative transgenic tobacco; Lane NC: negative control (ddH2O); Lane NT: non- transformed tobacco; M: 100 bp marker ladder (Promega).

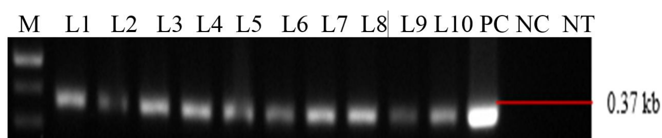

Figure 7: PCR for hpt gene. M: 1kb Plus DNA O'Gene (Fermentas); L1-10: putative transgenic tobacco; Lane PC: positive control (pCAMdehD plasmid); Lane NC: negative control (ddH_2O); Lane NT: non-transformed tobacco.

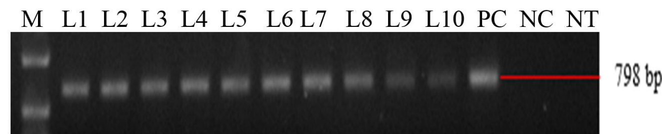

Figure 8: PCR for *dehD* gene. M: 1kb Plus DNA O'Gene (Fermentas); L1-10: putative transgenic tobacco; Lane PC: positive control (pCAMdehD plasmid); Lane NC: negative control (ddH2O); Lane NT: non-transformed tobacco.

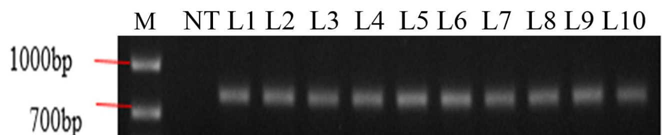

Figure 9: PCR for *dehD* gene on cDNA. M: 1kb Plus DNA O'Gene (Fermentas); Lane NT: non-transformed tobacco; Lane 1-10: putative transgenic tobacco.

In this study, *dehD* gene expression controlled by the constitutive cauliflower mosaic virus 35S promoter. An active dehalogenase D enzyme produced in plant cells, and transgenic plants were completely resistant to MCA. RT-PCR revealed that *dehD* gene expressed in all 10 independent transgenic lines. Whereas no expression occurred in non-transformed *N. benthamiana*, demonstrating that there was no transcription from endogenous gene in the *N. benthamiana* plants.

Differences detected at the transcript level may be due to T-DNA insert locations, random mutations following transformation or direct effects of the recombinant protein itself, or a combination of these [23]. As a constitutive promoter, CaMV35s promoter helps to express the genes anywhere in the plant at all the time [24]. This promoter is active expressed the transgene in most plant tissues and throughout the developmental stages of plants [25].

In plant, dehalogenase activities can convert MCA into glycolic acid. In green plants there is an active enzyme which oxidizes glycolic acid to glyoxylic acid, however, in crude plant saps the glyoxylic acid does not accumulate in appreciable amounts but is further utilized. The major end-products from glycolic acid utilization in plants are glycine and serine.

The pathway for the formation of serine from glycolic acid is presumably through the intermediates, glyoxylic acid and glycine. The lower activity in the β-carbon of serine may be due to inactive glycine in the leaf or it may indicate the functioning of some other pathway. Besides that, several functions for glycolic acid in plants have recently been proposed. The oxidation of glycolic acid to glyoxylic acid is a reversible system capable of functioning as a terminal oxidase in plants. Glycolic acid has been shown to be closely associated with the carbon cycle in photosynthesis. It serves as a carbon source for glycine and serine synthesis and thus probably functions between a 2-carbon fragment of the carbon

cycle in photosynthesis and these amino acids [26]–[28]. It's shown that this transgenic plant has the ability to mineralize the herbicide; which is the major challenging and limitation in herbicide resistance and phytoremediation plant development.

3.3 Mendelian Inheritance Pattern

With the intention of determining the segregation pattern of transferred gene in T_1 transgenic *N. benthamiana* plants, the seeds from randomly selected 5 lines were germinated in selective media containing MS supplemented with 60 µg/L MCA. For each line approximately 100 seeds were used and the numbers of survivors in selective media were counted.

In this study, probabilities were greater than 0.05 and the results indicated that the transgenic tobacco plants produced by *Agrobacterium*-mediated transformation method were genotypically and phenotypically stable in the progenies. The values obtained from experiments correlate with the 3:1 Mendelian ratio. **Table 3** shows transgenic lines T3 had highest percentage of seed germination (79.6 %). The expected ratio for Mendelian inheritance is 3:1 (75 % : 25 %) and these data was analyzed by Chi-Square Analysis. **Table 4** shows the results of Chi Square analyses with p ($\chi^2 1 \leq 3.841$) = 0.3597, proved that in all five transgenic lines, the *dehD* gene was segregated according to Mendelian 3:1 ratio.

Table 3. Percentage of germinated seed of transgenic *N. benthamiana*

Transgenic Lines	Total no of seeds	No of germinated seeds	No. of non germinated seeds	Percentage of germinated (%)
T1	92	67	25	72.8
T2	99	73	26	73.7
T3	93	74	19	79.6
T4	99	75	24	75.8
T5	97	70	27	72.2

Table 4: Mendelian Inheritance Analysis of T_1 progeny, p($\chi^2_{0.3597}$) ≤3.841. O= observed, E= expected.

Lines	Categories	O	E	(O-E)^2/E	χ^2
1	Resistant	67	69	0.0580	0.232
	Susceptible	25	23	0.1739	
2	Resistant	73	74.25	0.0210	0.084
	Susceptible	26	24.75	0.0631	
3	Resistant	74	69.75	0.2590	1.036
	Susceptible	19	23.25	0.7769	
4	Resistant	75	74.25	0.0076	0.303
	Susceptible	24	24.75	0.0228	
5	Resistant	70	72.75	0.1040	0.416
	Susceptible	27	24.25	0.3119	

Mendelian inheritance assay showed that in all five lines the gene was segregated according to Mendelian 3:1 ratio with with p ($\chi^2 1 \leq 3.841$) = 0.3597. It can be concluded here the results showed that transgene inheritance followed Mendelian laws. The 3:1 segregation ratio for monochloroacetic acid resistance would also come from a single copy of the *dehD* gene. In most cases, the transformed genes behave as a single dominant locus exhibiting normal Mendelian segregation [29]–[31]. Transgenic line T_2 showed highest percentage of seed germination (79.6%) and higher than expected percentage (75%). It may be related to multiple copy number of transgene integration into *N. benthamiana* plant cell, which is multiple copy number of insertion has been reported in transgene tobacco, wheat and chickpea transformed through *Agrobacterium* mediated transformation [32]–[34].

5. CONCLUSION

We have successfully developed herbicide resistant *N. benthamiana* plants which were confirmed at various plant development stages i.e. at seed germination and mature leaves. The findings represent important information about the potential of *dehD* gene to be used to develop transgenic resistant plant to broad spectrum herbicide MCA, and the important role of *dehD* gene as dominant selectable marker gene in herbicide resistant plant technology and plant breeding technology and eventually contribute to a development of sustainable weed management system.

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