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Original Research Paper

Development of transgenic *Brassica napus* plants with *AtATM3* gene to enhance cadmium and lead tolerance

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ABSTRACT

Four popular genotypes of *Brassica napus*, cv. BARI Sarisha-8, BARI Sarisha-13, Binasarisha-4, and Binasarisha-8 were subjected to cadmium (Cd) and lead (Pb) stress to identify a genotype with enhanced tolerance of Cd and Pb. The goal was to use the selected genotype for genetic engineering, thereby improving traits related to heavy metal tolerance and accumulation to remediate polluted agricultural soils. The genotype, BARI Sarisha-8 with superior tolerance to both Cd and Pb stresses, was chosen for genetic engineering with *Arabidopsis thaliana* ABC transporter of the mitochondrion 3 (*AtATM3*) gene. The transformation process involved co-culturing the cotyledon explants with *Agrobacterium* strain GV3101 carrying a binary vector containing the hygromycin phosphotransferase (*HPT*) gene as a selectable marker and the *AtATM3* gene. Transformation efficiency was significantly enhanced with a two-day co-cultivation period on shoot induction medium composed of MS medium, α-naphthaleneacetic acid (0.5 mg/L), and 6-benzyladenine (3 mg/L), supplemented with acetosyringone (20 mg/L), along with a four-day delay in exposing the explants to the selective agent hygromycin. Hygromycin-resistant shoots were obtained by employing a three-step selection process. The refined protocol resulted in a transformation efficiency of 15.38%. Polymerase chain reaction (PCR) analysis confirmed the integration of *AtATM3* and *HPT* genes into the host genome in all recombinant plants. Transgenic *B. napus* plants expressing the *AtATM3* gene exhibited notable improvements in tolerance, demonstrating a 1.4- to 1.7-fold increase in Cd tolerance and a 1.3- to 1.5-fold increase in Pb tolerance compared to the wild type (non-transformed) under both Cd and Pb stress conditions.

Keywords: *AtATM3, Brassica napus*, co-cultivation, heavy metals, hygromycin, transgenic plants

INTRODUCTION

Across the globe, large portions of agricultural soil are polluted by heavy metals (Cd, As, and Pb, etc.), stemming from human and natural activities, including industrial operations and agricultural practices (Nagajyoti et al., 2010). In Bangladesh, soil is generally polluted with heavy metals (HMs) and metalloids due to geological factors. Apart from that, recent advances in industrialization have become another key issue for rising heavy metals in cultivable land and leafy-vegetables cultivated in sewage-irrigated zones throughout the country (Islam et al., 2018; Proshad et al., 2018). This heavy metal deposition in agricultural soils is becoming a matter of growing concern because of the associated risks to food safety. Regardless of the source of the heavy metals in the soil, high levels can cause soil quality deterioration, crop yield loss, and poor agricultural product quality; pose serious risks to ecosystem and human health (Rahman et al., 2012; Islam et al., 2016).

Brassica crops have recently piqued the interest of researchers due to their high biomass production with additional economic value and their great capacity to translocation and collect various metals and metalloids such as As, Pb, Se, Cd, Cu, and Zn from contaminated soils, making them a promising candidate in the phytoremediation process (Clemente et al., 2005; Eapen & D'souza, 2005; Gasic & Korban, 2007). *Brassica* species exhibit efficient phytoremediation activities such as phytostabilization, phytovolatilization, and phytoextraction, and also possess physiological processes helping in transportation, absorption, and sequestration of harmful metals into low-activity cellular organelles. Additionally, *Brassica* species feature a proficient mechanism that reduces

oxidative damage caused by reactive oxygen species overproduction (Bortoloti & Baron, 2022).

Many genes and protein families, including HM transporters like ATP-binding cassette transporters, have been characterized in recent years in relation to their function in detoxifying heavy metals (Kim et al., 2006). The overexpression of the *AtATM3* gene improved Cd and Pb tolerance and accumulation by uplifting the synthesis of Fe-S clusters and facilitating their transfer from the mitochondria to the cytosol (Kim et al., 2006). Earlier, the *AtATM3* gene was successfully inserted into *B. juncea* and was found to improve heavy metal tolerance (Bhuiyan et al., 2011a). Considering that, the goal of this study was to find the genotype(s) of *B. napus* plants that are more impervious to heavy metals (Cd and Pb) and can extract more heavy metals in their harvestable segments. Additionally, to increase heavy metal phytoremediation by developing fast-growing and high-biomass producing transgenic *B. napus* plants with *AtATM3* via *Agrobacterium*-mediated transformation approach in order to clear up contaminated agricultural soils and minimize heavy metal impacts in food chain.

MATERIALS AND METHODS

Seed materials

Four extensively grown varieties of *B. napus* namely BARI Sarisha-8, BARI Sarisha-13, Binasarisha-4, and Binasarisha-8 were used, which are recognized for their excellent yield and oil content.

Heavy metal stress screening of *B. napus* genotypes

The seeds were sterilized following a recently developed standard procedure for *B. napus* (Dina et al., 2019), which entailed soaking them in 70% ethyl alcohol (2 min), 10% Clorox (10 min), and distilled water (3 min). Sterilized seeds were placed on the ½ strength MS (Murashige & Skoog, 1962) media containing various concentration of heavy metals: $CdCl_2$ (0.1-0.3 M $CdCl_2$) and $Pb(NO_3)_2$ (0.1-1.25 M), and grown in culture room under controlled environmental conditions (photoperiod: 16/8 h light/dark using a light intensity of 115 mmol $m^{-2}s^{-1}$; temperature 25±2°C; relative humidity 65-75%). After a specific period of time (7 days), seedling development was assessed to determine their tolerance to heavy metals. At each harvesting period, plants were meticulously rinsed with distilled water and then utilized for the assessment of various parameters. The root lengths of the seedlings were measured on a metric scale, and the fresh weight or biomass of the pooled seedlings was determined on an electric balance.

Standardization of *in vitro* plant regeneration protocol of *B. napus* cv. BARI Sarisha-8

According to the procedure outlined (Dina et al., 2019), several factors including media combinations, explant age, explant type were tested once again in the laboratory in order to achieve high frequency shoot regeneration of *B. napus* cv. BARI Sarisha-8.

Genetic transformation of *B. napus* cv. BARI Sarisha-8

***Plasmid vector construction with heavy metal relevant genes*:** The information of genes responsible for HM tolerance and accumulation was collected from the website NCBI (gene ID: 835939) and plasmid vector with the gene (*AtATM3*) was constructed (Bhuiyan et al., 2011a) (Fig. 1). The media was prepared based on MS medium containing 3% sucrose. The medium's pH was set to 5.7 before incorporating 10 g/L agar, followed by autoclaving at 121°C for 15 min. acetosyringone (20 mg/L) and anitibiotics [filter sterilized hygromycin (0-20 mg/L and cefaotaxime (300 mg/L)] were added into after it had cooled to 50 to 60°C following autoclaving. Seeds of selected genotype of *B. napus* were germinated in ½ strength MS media then the cotyledons including 1-2 mm petioles were carefully excised from 4-day old seedlings and were used them as explants in transformation experiments. To determine the effect of hygromycin concentration on shoot regeneration, cotyledon explants were placed on medium of shoot induction (MS medium added with 3 mg/L BA containing 0.5 mg/L NAA and 20 mg/L acetosyringone with different concentrations of hygromycin (5-30 mg/L). A solitary colony of *A. tumefaciens* strain GV3101 harboring both *HPT* and *AtATM3* genes was cultured in liquid YEP medium supplemented with antibiotics, and it was incubated at 28°C with continuous agitation at 150 rpm overnight. The bacteria having OD600 of 0.6-0.8 were harvested by centrifuging at 5,000 rpm for 2 minutes and then delicately resuspended in 15 ml of *Agrobacterium*-inoculation medium. The experiments for shoot regeneration and *in vitro* rooting of regenerated shoots were performed (Dina et al., 2019). Thirty putative transgenic and WT plants were

evaluated by the PCR to confirm the presence of transgenes. DNA was extracted from the leaves of plants according to the protocol by Edwards et al. (1991). The primers pairs utilized for amplification of DNA were 5'-TCA CTT ACC ACC TTC GCT GC-3'(forward) and 5-CGA CTG TCA TCT GGC CAT TC-3' (reverse) for *AtATM3* gene; and 5' GTA CTT CTA CAC AGC CAT CGA TC-3' (forward) and 5'-CAT GTG TAT CAC TGG CAA ACT GT-3' (reverse) for *HPT* gene. The mixture volume of PCR reaction was 20 µl which contained of SUN PCR blend (10 µl), distilled sterile water (7 µl), genomic DNA (10 ng), and each primer 1µl (10 pmol). Thermocycling was done as follows: one 5 minutes step at 94°C, following by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, with a final 5-minute extension at 72°C. The amplified products were separated by 1% agarose comprising 0.5 µg/ml ethidium bromide. The primers were developed to target the junction between the plasmid's non-transferable vector sequences and the T-HPT DNA's gene in order to identify bacterial DNA contamination in plant samples. The region would not be amplified by PCR in samples containing the T-DNA-inserted plant chromosome. The primer 5'-TGA TGG GCT GCC TGT ATC GA-3' (forward) and 5'-CAT GTG TAT CAC TGG CAA ACT GT-3' (reverse) was used, resulting in a 939-bp PCR product. PCR cycling conditions described above were utilized for this reaction.

Fig. 1 : T-DNA region of the pCambia1302-AtATM3 vector. LB, T-DNA left border, CaMV35S poly A, cauliflower mosaic virus 35S terminator, *HPT*, hygromycin phosphotransferase gene. CaMV35S Pro, cauliflower mosaic virus 35S promoter. AtATM3, *Arabidopsis thaliana* ABC transporter of the mitochondrion 3. nos poly A, nopaline synthase terminator. RB, T-DNA right border

T_1 seed segregation

Putative transgenic *B. napus* plants were self-pollinated and the seeds of each plant were collected separately and designated as T_1 progeny of respected transgenic lines. About 50-55 seeds from each line were germinated on ½ MS agar medium containing 30 mg/L hygromycin. The non-germinated seeds and brown seedlings were considered sensitive and 2-week-old green and healthy seedlings were scored as resistant.

Cd and Pb tolerance by WT and *AtATM3*-transgenic lines

The T_1seeds of six independent transgenic plants (#1, #3, #4, #5, #6, and #7) and WT were grown for 7 days in ½ MS agar medium with (01.5 M Cd or 1 M Pb) or without heavy metals. The culture conditions were same as described above. Seven days after culture, fresh weights of 10 seedlings for each treatment were measured to examine level of tolerance. Three replicates were employed for each treatment.

Statistical analysis

The mean and standard deviation were computed using Microsoft Excel 2010 software. One-way analysis of variance (ANOVA) was used to assess tabulated data, and statistical differences between means were estimated using Duncan's Multiple Range Test (DMRT) using the statistical software R program.

RESULTS AND DISCUSSION

Screening of *B. napus* genotypes against Cd and Pb stress

As the tolerance of metals is a key plant characteristic required for phytoremediation purpose, the study was aimed to develop transgenic *B. napus* plants with *AtATM3* gene. The *AtATM3* gene is known for its role in enhancing heavy metal tolerance and accumulation through the biogenesis of Fe-S clusters and iron homeostasis (Bhuiyan et al., 2011a).Before embarking the heavy metal tolerance experiments, optimal concentrations of heavy metal were determined by BARI Sarisha-8 of *B. napus* seedlings treated with several concentrations of $CdCl_2$ and $Pb(NO_3)_2$ (Fig. 2A and B). This is crucial because elevated levels of heavy metal concentrations could lead to the death of all seedlings, rendering no data for comparison with control findings (seedlings treated without heavy metal). Similarly, the levels cannot be too low, otherwise the Cd- or Pb-treated seedlings would grow just as well as the control plants (without Cd- or Pb-treated seedlings), with no tolerance difference observed. The goal was to identify an optimal concentration of Cd and Pb that would significantly impact the seedlings without causing their death.

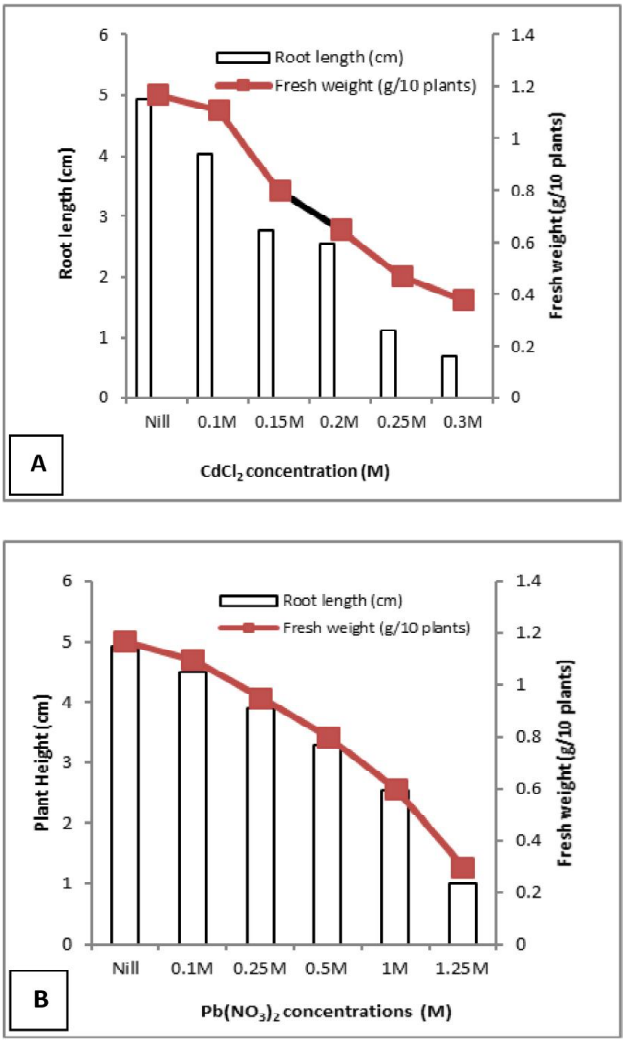

Fig. 2 : A. Standardization of $CdCl_2$ concentration on growth of *B. napus* cv. BARI Sarisha-8, seedlings were grown in ½ MS media + various concentrations of $CdCl_2$ for 7 days; B. Standardization of $Pb(NO_3)_2$ concentration on growth of *B. napus* cv. BARI Sarisha-8, seedlings were grown in ½ MS media + various concentrations of $Pb(NO_3)_2$ for 7 days

In the present study, the root length and fresh weight of seedlings reduced on increasing heavy metal concentration [0.1-0.3 M $CdCl_2$ and 0.1- 1.25 M $Pb(NO_3)_2$]. When no stress was applied, after 7 days the roots length was 4.93 cm/plant and the fresh weight was 1.17 (g/10 seedlings). When root length was 2.78 cm and fresh weight was 0.8 g, the dosage of 0.15 M dramatically lowered both parameters by nearly 50% when compared to the control. Additionally, when the concentration was increased to 0.3 M, the growth was rigorously inhibited by almost 80% when the root length was 0.68 cm and the fresh weight was 0.38 g (Fig. 2A). After considering the effect of various concentrations, we determined the suitable concentration of Cd as 0.15 M $CdCl_2$.

Similar approach was followed to identify suitable concentration for Pb stress. Seedlings were exposed to $Pb(NO_3)_2$ with various concentrations of 0.1 M, 0.25 M, 0.5 M, 1 M, and 1.25 M. Fig. 2B presents the details performance data of seedlings on various lead concentrations. Around 50% reduction on growth was observed in root length and fresh weight at the dose of 1 M, which was 2.78 cm and 0.6 g, respectively. However, the growth was reduced nearly 80%, when the concentration was maximum 1.25 M, *i.e.* the root length was only 1 cm and fresh weight was 0.3 g. Upon considering the effect of various Pb concentration, we determined the suitable concentration at 1 M $Pb(NO_3)_2$.

Then the seedlings of all genotypes were exposed to heavy metal stress following the standardized concentrations which was 0.15 M $CdCl_2$ for Cadmium and 1 M $Pb(NO_3)_2$ for lead. In terms of cadmium stress, seedlings were grown in ½ MS media supplemented with 0.15 M $CdCl_2$ for 7 days. The growth response was positive for all *B. napus* genotypes for 7 days screening, however, among them BARI Sarisha-8 was most prominent. The root length of BARI Sarisha-8 was 2.55 cm and the fresh weight was 0.8 g/10 plants. Following the performance, the suitable genotypes were ranked respectively; BARI Sarisha-13 (root length 2.31 cm and fresh weight 0.53 g), Binasarisha-8 (root length 1.65 cm, fresh weight 0.45 g), and Binasarisha-4 (root 1.45 cm and fresh weight 0.39 g) (Fig. 3A).

In terms of lead stress, BARI Sarisha-8 was also most prominent. The root length of BARI Sarisha-8 was 2.55 cm and the fresh weight was 0.60 g/10 plants. Following the performance, genotypes were ranked respectively: BARI Sarisha-13 (root length 2.2 cm, fresh weight 0.53 g), Binasarisha-8 (root length 1.6 cm, fresh weight 0.45 g), and Binasarisha-4 (root length 1.4 cm and fresh weight 0.4 g) (Fig. 3B).

After evaluating both cadmium and lead stress on *B. napus*, we found BARI Sarisha-8 showed better growth. This was found to be the most tolerant cultivar to both Cd and Pb stress than other cultivars possibly due to a better capacity to transport Cd and Pb in their vacuolar sink. As per evaluation, BARI Sarisha-8 showed most suitable performance for Cd and Pb phytoremediation which was aligned to our objective.

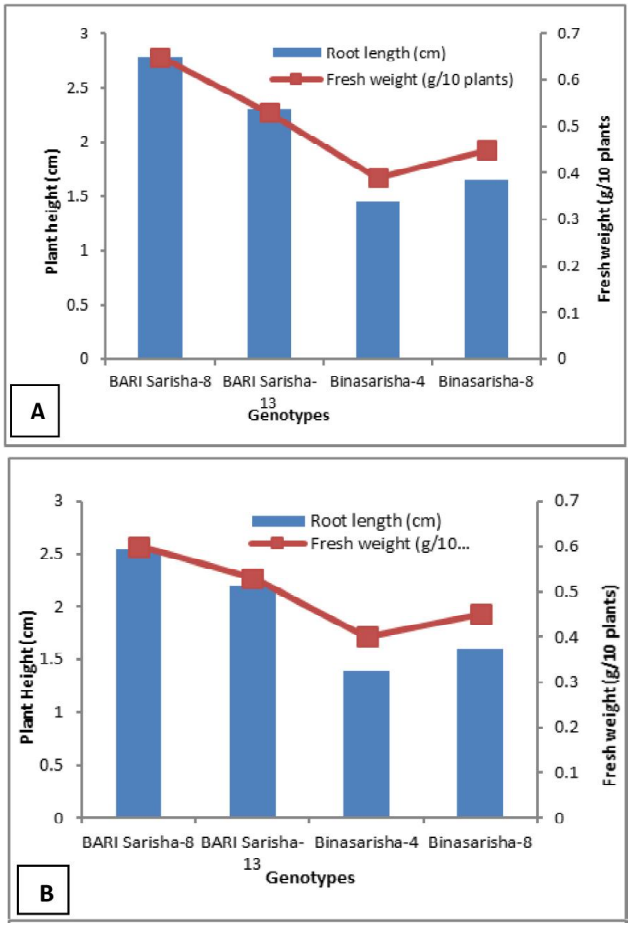

Fig. 3 : A. Genotypic variation of *B. napus* against Cd(II) stress, seedlings were grown in ½ strength MS media + 0.15 M $CdCl_2$ for 7 days; B. Genotypic variation of *B. napus* against Pb(II) stress, seedlings were grown in ½ strength MS media + 1 M $Pb(NO_3)_2$ for 7 days

Therefore, we chose BARI Sarisha-8 for transgenic plant development.

Optimal hygromycin concentration determination

In *B. napus*, protocols based on cotyledon and hypocotyl explants were commonly used due to the high plant regeneration potential (Liu et al., 2015; Dina et al., 2019). However, for specific *B. napus* cv. BARI Sarisha-8, Dina et al. (2019) revealed explants from cotyledon responded better, therefore, in the present study, cotyledon explants were utilized for genetic transformation of cv. BARI Sarisha-8.

Following genetic transformation, selective agents are widely applied to successfully screen transgenic plants. An appropriate selection agent at an optimal dose can effectively restrict non-transformed tissue growth while increasing the acquisition of transgenic plants (Hlozakova et al., 2014; Liu et al., 2015). Therefore, we tested hygromycin as a selective agent for transgenic cotyledon explants of *B. napus* cv. BARI Sarisha-8 which is accepted method to screen effectively the transgenic *Brassica* plants.

Cotyledon explants were cultured on shoot-induction medium containing various concentrations (0, 5, 10, 15, 20, and 30 mg/L) of hygromycin. Explant was grown in medium with no hygromycin (0 mg/L) was considered as control where highest percentage (73.33%) of shoot regeneration was observed (Fig. 4). However, increasing hygromycin concentration sequentially on medium showed declining response of shoot regeneration frequency. Upon increasing concentration 5 mg/L sequentially, the shoot regeneration was reduced approximately 25%. Following that, only 8.3% of the explants shoots were regenerated in 15 mg/L hygromycin and there was no shoot regeneration with 20 mg/L or greater concentrations of hygromycin. Consequently, for the primary transgenic shoot selection, 15 mg/L hygromycin was chosen. Interestingly, despite the fact that hygromycin sensitivity differs amongst *Brassica* species, our results were aligned to the results of Bhuiyan et al. (2011b). The hygromycin concentration was then raised to 20 mg/L for the subsequent sub-culturing procedures. Other important steps involved to reduce false positives and select transgenic shoots, which can be done by increasing quantity of the selective agent while decreasing the glucose supply in the medium (Bhalla & Singh, 2008). Therefore, hygromycin concentrations were raised to 30 mg/L for the shoot-elongation and root-induction procedures in order to completely eradicate false-transgenic shoots.

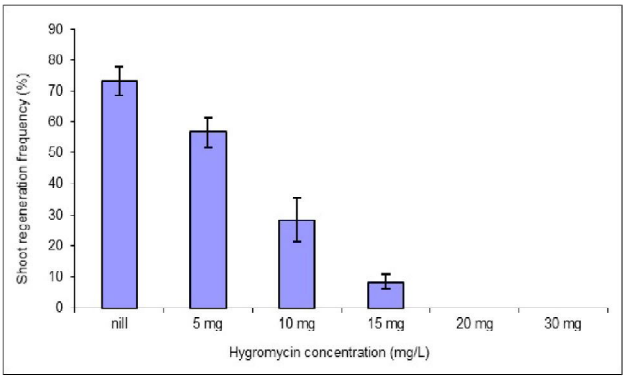

Fig. 4 : Effect of hygromycin on shoot regeneration of *B. napus* cv. BARI-Sarisha-8. Values represent mean ± SD (n = 2)

Table 1 : Effect of various co-cultivation duration of cotyledon explants of *B. napus* cv. BARI Sarisha-8 on efficiency of transformation

Co-cultivation duration (day)	Explants tested (No.)	Explants regenerated shoots (No.)	Transformation efficiency (%)
0	100	0	0
1	145	0	0
2	195	30	15.38
3	120	2	1.6
4	124	0	0

Regeneration of transformants

For high efficiency transformation, the co-cultivation period is quite important. Cotyledon explants are extremely delicate and were discovered to be quickly rendered necrotic. For high transformation efficiency, we examined several co-cultivation times, ranging from 0 to 4 days. The maximum transformation efficiency was found to be with co-cultivation lasting two days. The transformation efficiency was only 1.6% on 3-day co-cultivation period and later it was 0% (Table 1). More than two days of co-cultivation found to be harmful since the plant tissue perished and produced no or very few transgenic shoots.

Also, we studied the impact of delayed exposure of the explants to 15 mg/L hygromycin on the effectiveness of transformation for up to 6 days. In this scenario, prolonging the transfer of explants into selection media after 3 days to 5 days co-cultivation improved transformation efficiency (Table 2). Delaying explants exposure to the selection agent improves the proliferation and recovery of transgenic cells, which give rise to transgenic plants (Bhalla & Singh, 2008). According to Visser et al. (1989) the transformation efficiency was greatly improved when the selection agent was applied to transgenic potato plants afterwards. Bhuiyan et al. (2011a) reported, that it is helpful for plant species with a propensity to regenerate quickly in tissue culture.

A three-step hygromycin selection strategy was applied that had previously been effectively developed by Bhuiyan et al. (2011a) to increase the transformation efficiency. Initially briefly exposed the explants to 15 mg/L of hygromycin (Fig. 5A), and then increased that concentration to 20 mg/L in the subsequent subculture using shoot-induction medium. Eventually, the hygromycin concentrations in the shoot-elongation and root-induction media were increased to 30 mg/L. Based on observations, it was discovered that both untransformed andtransformed explants may regenerate shoots when given initial low amounts of hygromycin. Nevertheless, the higher hygromycin concentrations (20–30 mg/L) in the following stages are probably going to accelerate up the division of transformed cells, while, slowing down the division of untransformed cells. After 2 weeks to 4 weeks of growth on selection media, resistant shoot buds to

Table 2 : Effect of various delayed exposure of the explants of *B. napus* cv. BARI Sarisha-8 to hygromycin (15 mg/L) on efficiency of transformation

Period (day)	Explants tested (Total No.)	Explants regenerated shoots (No.)	Transformation efficiency (%)
0	132	0	0
1	100	0	0
2	155	0	0
3	157	1	0.6
4	135	3	2.2
5	195	30	15.38
6	98	0	0

hygromycin developed on the cut edges of cotyledonary petioles (Fig. 5B). Most of the initially green shoots changed to white or purple when hygromycin was added to the shoot-elongation medium at high doses (30 mg/L). Green shoots were taken, grown in SIM for a longer period of time, and then transferred to the rooting medium (Fig. 5C). Roots began to grow from all of the shoots after 10-15 days of culture. With a transformation efficiency of 15.38%, 30 putatively transgenic shoots were generated. Putative transformants were introduced to soil, acclimated for five days in a controlled environment, and then released into the wild (Fig. 5D). Every transformant displayed expected shape and blooming characteristics.

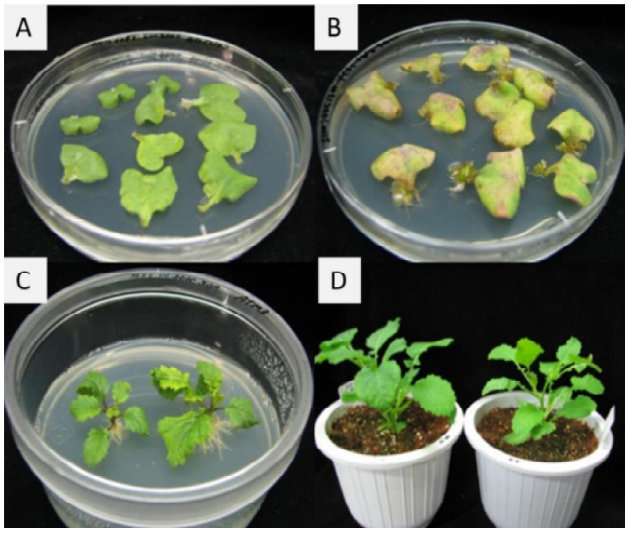

Fig. 5 : *In vitro* regeneration of shoot and transformation of *B. napus* cv. BARI Sarisha-8 from cotyledon explants. A. After 5 days in SIM + Cef 300 mg/L; B. Shoot induction in SIM + Hyg (15 mg/L) + Cefotaxime (300 mg/L; C. Shoot elongation and rooting in MS + NAA (0.1 mg/L) + Hyg (30 mg/L) + Cef 300 mg/L; D. Potted transgenic *B. napus* cv BARI sarisha-8 plants (acclimatized)

Transgene presence confirmation by PCR

To validate the incorporation of the transgenes into the *B. napus* host genome, all hygromycin tolerant plants were subjected to PCR test with primers specific for *AtATM3* and *HPT*. As a positive control, a plasmid encoding the *AtATM3* gene was used, and a normal plant was used as a negative control. Positive controls include the 500-bp band representing the HPT fragment and the 712-bp band representing the *AtATM3* fragment. A 712-bp band and a 500-bp band were found in all transgenic plants except line 2, as expected, whereas, no PCR bands were discovered in the WT genome (Fig. 6). The positive integration of the *AtATM3* gene into the BARI Sarisha-8 genome has been observed in several plants, indicating successful stable transformation.

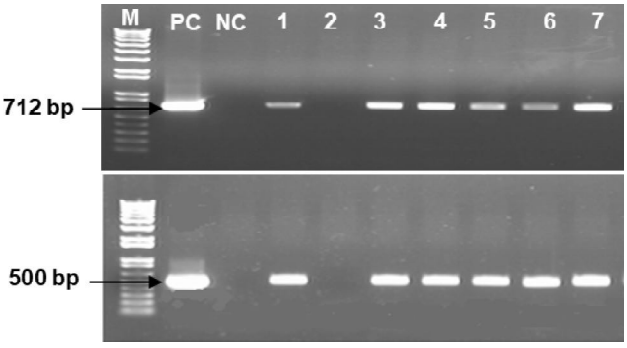

Fig. 6 : Detection through PCR of *AtATM3* (upper panel) and *HPT* (lower panel) genes in transformed shoots of *B. napus* cv. BARI sarisha-8, M: molecular DNA size marker (1 kb plus ladder), PC: plasmid containing *AtATM3* gene as a positive control, NC: normal plant as a negative control, 1, 3, 4, 5, 6, and 7 represent independent transgenic lines whereas line 2 represents false transgenic shoot

T_1 seed segregation analysis

The transgenic lines along with WT (non-transformed) were maintained natural environment until seed setting. T_1 seeds were grown in media containing 30 mg/L hygromycin in which WT showed some initiation of germination but turned yellow and dried up, whereas, the transgenic seeds survived as green and healthy seedlings. All the transgenic lines showed about 3:1 Mendelian fashion on hygromycin containing media (Table 3), which indicated that a single-copy of T-DNA was stably integrated in transgenic *B. napus* plants.

Enhanced Cd and Pb tolerance by transgenic *B. napus* plants

Compared with wild-type, the transgenic lines grew better both in 0.15 M $CdCl_2$ and 1 M $Pb(NO_3)_2$; leaves of them were greener and broader, roots were longer (data not shown), and fresh weight was 1.4- to 1.7-fold higher in Cd stress and 1.3- to 1.5-fold higher for Pb stress (Fig. 7). In the ½ medium (control), the phenotypes of transgenics were similar to that of WT plants. In medium containing Pb, comparatively enlarged cotyledons were found in both WT and transgenic lines. The increased tolerance of heavy metals in *AtATM3* transgenic lines occurred due to

Table 3 : Segregation of hygromycin-resistant plants in the self-pollinated T_1 progeny of transformed *B. napus* cv. BARI Sarisha-8. ÷2:in accordance with the expected Mendelian ratio of 3:1 at α=0.05. #1, #3, #4, #5, #6, and #7 represent six independent *AtATM3*-transgenic lines

Plant line	No. of seeds tested	No. of seedlings		÷² value	Probability ($÷2_{(0.05)}$ = 3.84)
		resistant	sensitive		
WT	50	0	50	-	-
#1	55	37	18	1.6875	<0.05
#3	50	38	13	0.5052	<0.05
#4	51	37	14	0.15384	<0.05
#5	48	38	10	0.5052	<0.05
#6	48	34	14	0.40336	<0.05
#7	45	35	10	2.008	<0.05

active involvement of the *AtATM3* gene, which produced Fe-S clusters and transports these cluster to the cytoplasm. This process ultimately led to a decrease in free-Fe within the mitochondria.

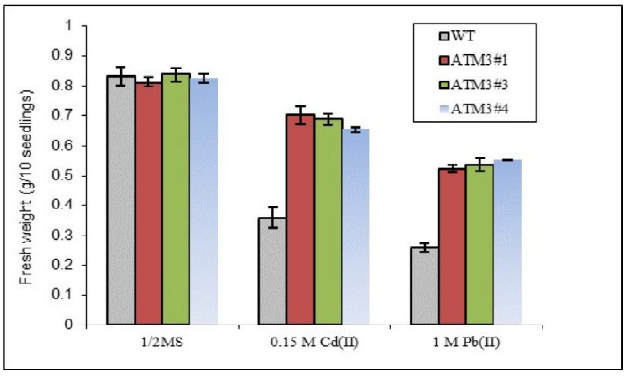

Fig. 7 : Cd and Pb tolerance analysis of transgenic lines of *B. napus* cv. BARI Sarisha-8. Data are expressed as mean ± SD (*n* = 3)

CONCLUSION

In this study, *B. napus* cv. BARI Sarisha-8 identified as a highly resistant *B. napus* genotype to Cd and Pb stress. An efficient protocol for *Agrobacterium*-mediated transformation, introducing the *AtATM3* gene into cv. BARI Sarisha-8 for heavy metal (Cd and Pb) was developed. Cotyledon explants were co-cultured with *Agrobacterium*, and optimizing with a 2-day co-cultivation on shoot induction medium and a 4-day delay in exposing explants to hygromycin significantly improved transformation efficiency (15.38%). PCR analysis confirmed *AtATM3 and HPT* gene integration. These transgenic lines exhibit heavy metal resistance, promising economic benefits through phytoremediation, reducing heavy metal levels in contaminated soils and the food chain.

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