Research Article

Genetic Transformation of Tobacco *Serine Acetyltransferase* 4 (*NtSAT4*) gene in *Brassica napus* L.

Hala Rajab[1], Muhammad Sayyar Khan[1*], Safdar Hussain Shah[1] and Syed Mehar Ali Shah[2]

[1]Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar, Khyber Pakhtunkhwa, Pakistan, Postal code 25130; [2]Department of Plant Breeding and Genetics, The University of Agriculture Peshawar, Khyber Pakhtunkhwa, Pakistan, Postal code 25130.

Abstract | Defense compounds containing sulfur, play vital roles in the plant's survival under abiotic and biotic stresses. Cysteine (Cys) is the first organic molecule in the cell containing reduced sulfur and acts as a precursor for many sulfur-containing compounds including Glutathione (GSH). GSH functions to protect the plants against different forms of stresses. In this study, the feedback-insensitive *serine acetyltransferase* (*SAT*); a rate-limiting enzyme for Cys biosynthesis from tobacco i.e. *NtSAT4* was successfully cloned into three types of overexpression constructs i.e. *pBinAR_NtSAT4* (targeted to cytosol), *pBinAR-TKTP_NtSAT4* (targeted to plastids) and *pBinAR-SHMT_NtSAT4* (targeted to mitochondria). For stable transformation of *B. napus* floral-dip and tissue culture based approaches were tested using different formulations of phytohormones for calli, shoots and roots induction in a variety of genotypes. While false positive lines were obtained in the result of floral-dip method, the tissue culture based method led to successful regeneration of shoots on Murashige and Skoog (MS) media containing BAP 3 mgL^{-1} and developed roots in ½ MS media supplemented with IBA 3 mgL^{-1} in case of OSCAR cultivar. Successful selection of transgenic lines was achieved using selective antibiotic kanamycin 50 mgL^{-1}. *NtSAT4* gene integration into the plants genome was confirmed by PCR using the *NtSAT4* gene specific primers. The positive transgenic lines were successfully acclimatized to soil and glass house conditions. The developed transgenic lines have the potential to overproduce high levels of cysteine and glutathione for enhanced stress tolerance especially against heavy metals.

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***Correspondence** | Muhammad Sayyar Khan, The University of Agriculture Peshawar; **Email:** sayyar@aup.edu.pk
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Introduction

Increasing industrialization and rapidly growing world populations have brought many challenges in the form of pollutants, which pose serious threats to our environment, agriculture and human health (Gairola et al., 1992; Mazess and Barden, 1991). Although some of the heavy metals like zinc (Zn), iron (Fe), copper (Cu), molybdenum (Mo), cobalt (Co) and manganese (Mn) are nutritionally essential in trace amounts, their excess is potentially harmful (Keeling et al., 2003). Exposure of plants to such toxic elements like Cadmium (Cd), Arsenic (As), Lead (Pd) and mercury (Hg) proved to have acute and chronic toxic effects on the plants' health and productivity (Divrikli et al., 2006). Due to their strong affinity with the soil, they cannot be removed by normal cropping or rain water so they remain to be non-biodegradable (Tandi et al., 2004). On worldwide level, Cd sedimentation ranged between 0.03-1 mg/

kg while on average the level of Cd in soil in various regions in Pakistan ranged from 0.02 to 184 mg/kg (Muhammad et al., 2011; Perveen et al., 2012).

Various conventional methods used to clean up these environmental pollutants are laborious and economically not feasible particularly in developing countries. The use of phytoremediation to clean up the environment through plants has been reported to be 10-100 folds cheaper and easier to be implemented (Pilon-Smits, 2005), though, plants' tolerance and their accumulation to such pollutants is a limitation.

The use of *Brassica napus* (canola) for phytoextraction has gained interest for cleaning the lands polluted with heavy metals (Van Ginneken et al., 2007). The species of Brassicaceae family has exhibited an extraordinary hyperaccumulator ability to heavy metal accumulation (Ebbs and Kochian, 1997). *B. napus* was reported to be one of the most suitable plants and can act as one of the most powerful phytoaccumulator to be used in heavy metal contaminated areas to clean up the environment (Angelova et al., 2017). Thus transformation of oilseed rape via *Agrobacterium tumefaciens* to manipulate and enhance its tolerance to heavy metals bears great potential. The transformation of *B. napus* via tissue culture is a laborious but most efficient plant transformation method (Moloney et al., 1989). Among the various direct DNA transfer methods, *Agrobacterium* transformation via tissue culture is considered to be a cost effective technique delivering the specific gene of interest and avoiding the possibility of random vector sequences and foreign DNA integration into the plant genome (Bhalla and Singh, 2008; Nielsen, 2003; Rommens et al., 2004).

The accumulation of heavy metals or xenobiotics triggers the abiotic defense response of plants to form Glutathione (GSH). GSH is one of the most important antioxidant thiol compound which functions in sequestering, chelating these harmful substances in the plants and thus protects them against oxidative stresses including drought and heavy metals (Farago and Brunold, 1994; Foyer and Noctor, 2005; Mullineaux and Rausch, 2005; Rüegsegger and Brunold, 1992). Therefore, one of the promising approaches to enhance the tolerance potential of the plants against abiotic stresses is the genetic manipulation of GSH biosynthesis in plants (Pilon-Smits and Pilon, 2002). To achieve elevated *in planta* GSH contents, various genes in the assimilatory sulfate reduction pathway have been overexpressed in different plant species. *Serine acetyltransferase* (*SAT*, EC 2.3.1.30) which catalyses the acetylation step of *L*-serine along with the acetyl-CoA is one of the rate limiting enzyme in this context. *SAT* forms a complex with *O-acetylserine-(Thiol) lyase* (*OAS-TL*), which is essential for its activity (Bogdanova and Hell, 1997; Droux et al., 1998; Kredich, 1996). Cys synthesis is tightly regulated by the feedback inhibition of *SAT* by Cys itself. A Cys-insensitive *SAT* isoform, was successfully screened by a cDNAs encoding Cys-insensitive *SAT* isoform from *Nicotiana tabacum* (Wirtz and Hell, 2003). In this context, *NtSAT4* from tobacco was found to be feedback insensitive compared to other *SAT* isoforms causing a strong GSH production in *E. coli* host. The Cys-insensitive *SAT4* differs from the other *SATs* by a 3 amino acid deletion at position 380 of the conserved C-terminal end and an insertion of 2 Ser and Cys residues at position 190 of the variable region. The expression of this isoform in bacteria resulted in 50-fold increase of Cys and in turn increases GSH. This *NtSAT4* has proved to exhibit complete insensitivity up to 0.6 mM for Cys (Wirtz and Hell, 2003).

In this study, *NtSAT4* gene was successfully cloned and subsequently overexpressed in *B. napus* plant via tissue culture method using cotyledonary explants. The expression of *NtSAT4* was targeted to cytosol, plastid and mitochondria. The aim of the study was to obtain stable *B. napus* transformants overexpressing *NtSAT4* to enhance the potential of the plants against heavy metals via overproduction of glutathione.

Materials and Methods

Cloning of NtSAT4 gene in overexpression constructs

NtSAT4 gene to be used as probe was amplified from a cDNA clone (kindly provided by Professor Ruediger Hell, Centre for Organismal Studies, Heidelberg University) using the following specially designed primers.

Forward Nt*SAT4*: 5′-GATCCCATGTCTATAAC-TACGTTAAATTTCT-3′
Reverse Nt*SAT4*: 5′- AGCTAAATTACATAAT-CAGACCACTCA-3′

The PCR amplified fragment was then cloned into pCAP primary vectors (pCAP_*NtSAT4*) with the help of suitable restriction enzymes. Antibiotic-

resistant clones were selected for plasmid extraction and further screening of the positive clones was carried out through sequencing. The following primers were used for sequencing.

Forward 35S: 5′-GCAAGTGGATTGATGTGA-TATC-3′
Reverse Nt*SAT4*: 5′-GCC TAC CAT TTG ACC ATAA TTT ATG-3′

After confirmation, the *NtSAT4* fragment, restricted from the pCAP vector was gel purified and successfully cloned into a binary vector pBinAR (Wirtz and Hell, 2003) to produce the cytosolic construct (*pBinAR_NtSAT4*). The *NtSAT4* gene expression in this binary vector is driven by 35S promoter. For the mitochondrial targeting, the *NtSAT4* fragment, restricted from the pCAP vector was gel purified and cloned into the binary vector *pBinAR-SHMT* to produce the mitochondrial overexpression construct (*pBinAR-SHMT_NtSAT4*). Similarly, for the plastid targeting, the gel purified restricted fragment was cloned in to *pBinAR-TKTP* containing the plastidic transit peptide signal to get the plastidic overexpression construct (*pBinAR-TKTP_NtSAT4*). The positive clones from each construct were confirmed via sequencing.

Plant material

The *B. napus* seeds of genotypes Abasyn-95, Durr-e-Nifa, Nr-1, Nr-6, Nr-7 and 'Oscar' were used as the starting material for the study. The seeds of cultivar OSCAR were available at the Institute of corresponding author whereas seeds of all other lines were kindly provided by Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan.

Plant transformation via floral dip method

The *B. napus* genotypes 'Abasyn-95' and 'Durr-e-Nifa' were subjected to transformation by Clough and Bent (1998) floral dip method and grown under long day in the glass house at 25 °C with 16 hrs light and 8 hrs dark conditions. A colony of transformed *AGL1* with *NtSAT4* gene was allowed to grow overnight in 5ml LB media with antibiotics rifampicin 25 mgL^{-1}, carbenicillin 50 mgL^{-1} and kanamycin 50 mgL^{-1}. This was subcultured in a 250 ml LB medium and grown to an OD_{600} of 0.8-1.2. The bacterial was harvested at 6000×g at 4 °C for 15 min. The *Agrobacterium* pellet was resuspended in transformation medium (sucrose 50 gL^{-1} and Silwett L-77, 200 μlL^{-1} acetosyringone) to an OD_{600} 0.8-1.2. The plant's floral buds were placed in the transformation solution and dipped for about 20 sec, then kept in dark for 24 hrs and later shifted to long day conditions in the glass house to for seed setting.

Plant transformation via tissue culturing

Plant growth conditions: The *B. napus* genotypes Abasyn-95, Durr-e-Nifa, Nr-1, Nr-6, Nr-7 and Oscar seeds were grown in long day conditions i.e., 16h/8h light/dark at 25 °C with a light intensity 600-3300 lux and humidity 50%. Seeds were surface sterilized and placed on ½ MS media (Murashige and Skoog, 1962) and allowed to germinate in dark or dim light ~660 Lux for 4-5 days.

Transformation using hypocotyls explants: The hypocotyls were removed and cut from the germinated seedlings and pre-cultured for two days. These were infected with transformed strain of *Agrobacterium* harbouring *NtSAT4* genes without or with transit peptides for plastids and mitochondria, for an hour in liquid MS media containing 500 µl an overnight inoculum, acetosyringone 100 µM and sucrose 20%. The media was then replaced with a fresh MS media and allowed to co-cultivate for 48 hrs (Wallbraun et al., 2009). The hypocotyls were transferred to selection MS media containing the suitable hormones (Table 1) and carbencillin 300 mgL^{-1} to induce callus formation. The developed calli were then shifted to regeneration media to develop green shoots in MS media supplemented with hormones (Table 2), kanamycin 25 mgL^{-1}, carbenicillin 300 mgL^{-1} and kept to grow under long day conditions.

Table 1: *Formulations of growth regulators for callus induction media.*

Callus induction media	Auxin mgL^{-1}	Cytokinin mgL^{-1}
CIM 1	NAA 4	BAP 0.4, Kinetin 0.4
CIM 2	NAA 3	BAP 0.3, Kinetin 0.3
CIM 3	2, 4-D 3	BAP 0.3
CIM 4	2, 4-D 0.3	BAP 3
CIM 5	2, 4-D 0.3	BAP 0.2
CIM 6	2, 4-D 0.6	BAP 0.2
CIM 7	2, 4-D 1	BAP 0.2
CIM 8	NAA 0.2	BAP 0.75

Transformation using cotyledon explants: The cotyledons of the genotype 'Oscar' were cut from the seedling and infected with MS media containing

Agrobacterium culture harbouring *NtSAT4* gene without or with transit peptides for plastids and mitochondria at OD_{650nm}=0.05 with addition of acetosyringone 100 µM) and regenerated as described by Bhalla and Singh (2008). The cotyledons were dipped into the infection medium in a petri plate for 30 sec and then transferred to a solid co-cultivation MS medium containing MES 0.5 gL^{-1}, 20 g sucrose, pH 5.8, phytagel 4 gL^{-1}, BAP 0.75 mgL^{-1}, NAA 0.2 mgL^{-1}, GA_3 0.01 $mgL^{-1}$and $AgNO_3$ 30 µM and kept in dark for 48 hours. The explants were transferred for callus induction to the same MS media plates with the addition carbencillin 500 mgL^{-1} for one week. The calli were further transferred to shoot induction MS media containing MES 0.5 gL^{-1}, 20 g sucrose, pH 5.8, phytagel 4 gL^{-1}; supplemented BAP 3 mgL^{-1}, NAA 0.2 mgL^{-1}, GA_3 0.01 mgL^{-1}, $AgNO_3$ 30 µM, carbencillin 500 mgL^{-1} and kanamycin 50 mgL^{-1} for four weeks under long day conditions. The shoots were transferred to rooting media supplemented with MS media, MES 0.5 gL^{-1}, sucrose 10 gL^{-1}, IBA 1-3 mgL^{-1}, phytagel 4 gL^{-1} and kept to develop roots for 2 weeks. The rooted plants were removed, roots washed with luke warm water to remove traces of media and inserted into soil and kept covered to acclimatize under high humidity. Once the plants had acclimated to the glass house conditions, these were uncovered and grown to set flowers. Each plant's flowers were covered by perforated plastic bags to ensure self fertilization.

Table 2: *Combination and concentration of growth regulators for shoot induction media.*

Shoot induction	Media Cytokinin mgL^{-1}	Auxin mgL^{-1}	Gibberellin mgL^{-1}
SIM 1	BAP 3	NAA 0.3	--
SIM 2	BAP 0.5	2, 4-D 2	--
SIM 3	BAP 3	NAA 0.5	--
SIM 4	BAP 1	IBA 0.01	GA3 0.01
SIM 5	BAP 3, Coconut water 10%	NAA 0.5	--
SIM 6	BAP 3, Zeatin 1	NAA 0.5	--
SIM 7	Zeatin 3	NAA 0.5	--
SIM 8	Zeatin 4	NAA 0.5	--
SIM 9	BAP 4	NAA 0.4	--
SIM 10	BAP 3	NAA 0.2	GA3 0.01

Extraction of gDNA from leaves and Polymerase chain reaction

The gDNA from *B. napus* leaves was extracted by CTAB method according to Rogers and Bendich (1989). For testing the integration of *NtSAT4* gene, the extracted genomic DNA was used as template against the following two different *NtSAT4* gene specific primer pairs NtSAT4_F: GCGCACCCATTCCTGAAAAG and NtSAT4_R: TCTGTGAGCACCCCTAGGAA, generating a product of ~250 bp; NtSAT4 F1: GTCTATAACTACGTTAAATTCTG and NtSAT4 R1: CATAATCAGACCACTCATATG, generating a product of ~900 bp.

RNA extraction and reverse transcription PCR (RT-PCR)

Approximately 50 mg of ground leaf material was used for extraction of total RNA with the peq GOLD total RNA Kit. cDNA was synthesized from 500 ng of RNA with the RevertAid ™ Minus First Strand cDNA Synthesis Kit according to the manufacturer's protocol. cDNA was analyzed for the expression of *NtSAT4* through semi-quantitative PCR using *NtSAT4* gene specific forward primer: TAGGGGTGCTCACAGAGGAT and reverse primer: GTCCTCGCCGGAACTTTCTT.

Results and Discussion

Production and characterization of NtSAT4 overexpressing oilseed rape lines via floral dip method

Brassica napus was transformed using three constructs that target the feedback-insensitive isoform of the rate limiting enzyme *Serine acetyltransferase (SAT)*, i.e. *NtSAT4* from tobacco under the 35S promoter either without transit peptide targeting the cytosol (Figure 1A) or with plastid transit peptide (TKTP) (Figure 1B) and mitochondrial signal sequence (SHMT) (Figure 1C) as described in materials and methods section. Sequencing analysis was carried out for the confirmation of each construct (Supplementary Figure 1).

B. napus cultivars 'Abasyn-95' and 'Durr-e-NIFA' were transformed with *Agrobacterium tumefaciens* containing each of the three constructs by floral dip method. Seeds (F2 generation) from about 200 independent putative transgenic lines carrying the three constructs were analyzed by genomic PCR using *NtSAT4* (NtSAT4 F1 and R1) specific primers. Out of 200 independent lines, 60 showed positive results for the gene specific primers (Figure 2).

The positive lines were selected and analyzed for their transcript levels using reverse transcription PCR

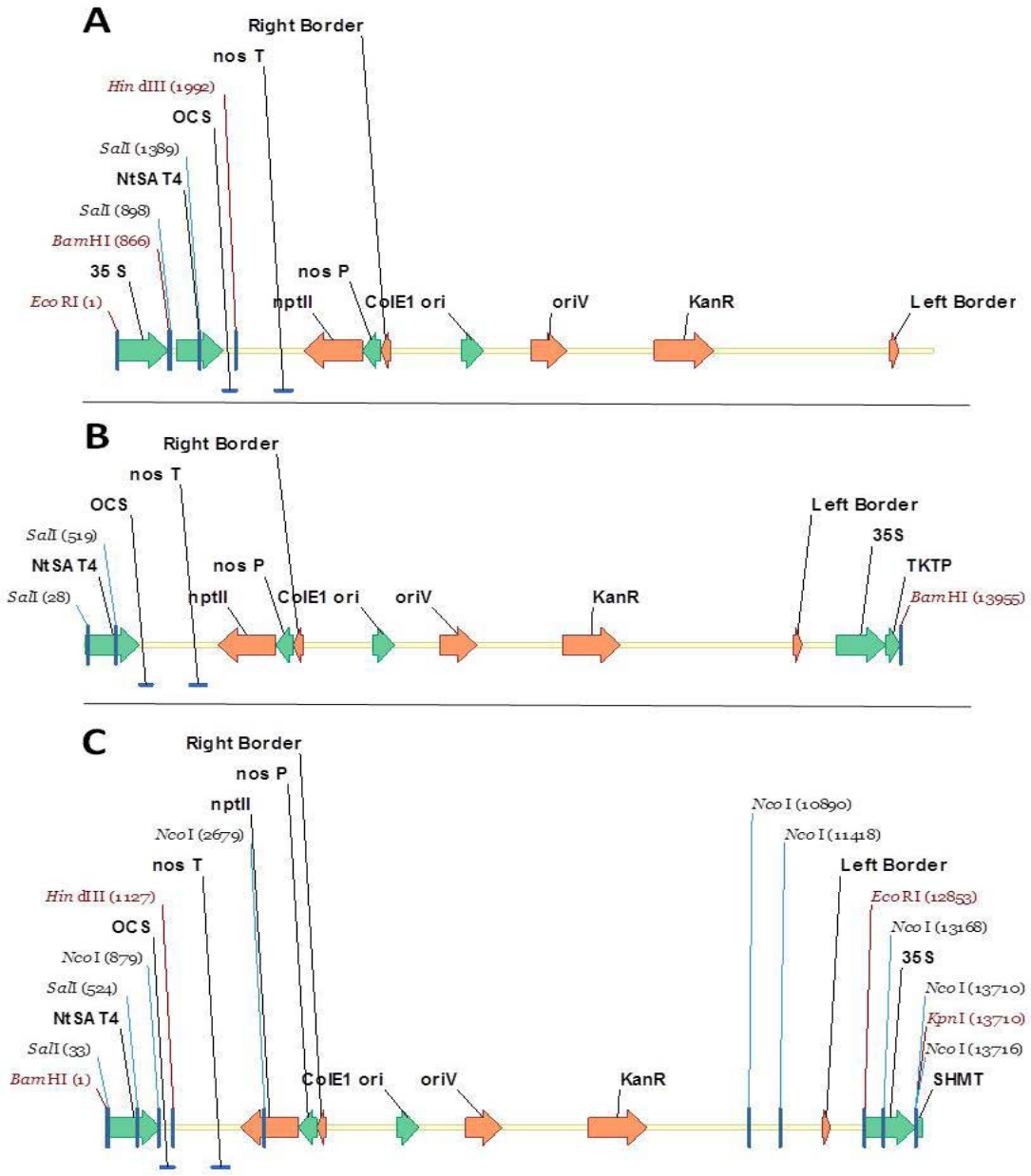

Figure 1: *Overexpression construct of NtSAT4 targeting the three compartments; Expression vectors of the NtSAT4 gene is put under the 35S promoter in each of (A) Cytosolic targeted pBin-AR-NtSAT4 13717 bp; (B) Plastid targeted pBin-AR-TKTP-NtSAT4 13959 bp; (C) Mitochondrial targeted pBin-AR-SHMT-NtSAT4 13818 bp.*

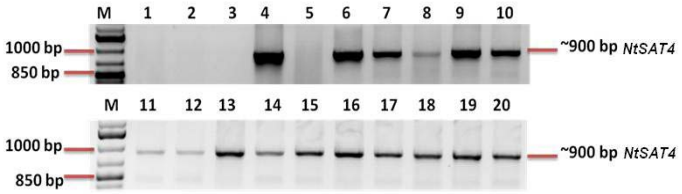

Figure 2: *Genomic PCR characterization of the putative transformed NtSAT4 overexpression lines; PCR result (0.9 Kb) using genomic DNA against NtSAT4 gene specific primers. Lane M: DNA ladder 1kb plus; Lanes 1,2,3: wild-type B. napus as negative control; Lane 4: positive control; Lanes 5-20: examples of putatively transformed plants with the cytosolic; plastidic and mitochondrial NtSAT4 constructs.*

(RT-PCR) analysis. In comparison to negative and positive controls, the analysis of cDNAs with *NtSAT4* specific primers from these selected lines showed no clear overexpression of the gene of interest (Data not shown). Therefore, it became necessary at this stage to have an alternate procedure to obtain stable transformants from oilseed rape.

Generation of tissue cultured transformed B. napus lines via Agrobacterium infection

Transformation of hypocotyls explants: In order to select the most suitable line(s) among the local genotypes for our transformation experiments, seeds of *B. napus* genotypes i.e., Nr.1, Nr.2, Nr.6 and Nr.7 along with cultivars Durr-e-NIFA and Abasyn-95, were surface sterilized and grown *in vitro* on half-MS media for seed germination. The hypocotyls of the germinated seedlings were cut in small pieces

and kept on callus induction media with different composition for efficient callus induction. The data from these experiments showed great variations among genotypes in terms of producing calli on each of the callus induction media. Among these eight compositions of callus induction media (CIM) as described in Table 1; CIM 1 i.e. MS media containing hormones NAA 4 mgL^{-1}, BAP 0.4 mgL^{-1} and Kinetin 0.4 mgL^{-1}, showed maximum induction of callus. The response to induce callus is highly genotype dependent as evident from the responses of different genotypes in the same media composition. Among the tested lines, NIFA Nr.6 and NIFA Nr. 2 lines were the most responsive genotypes in terms of callus induction capacity (Figure 3A). All lines showed significant variation in terms of callus induction with specifically Durr-e-NIFA genotype producing the lowest number of calli.

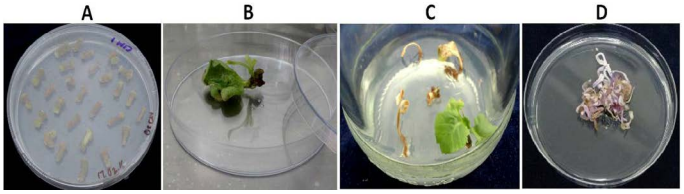

Figure 3: *Development of transgenics through tissue culturing using hypocotyls explants; (A) Hypocotyls after infection growing on callus induction medium; (B) Shoot developed from the green callus outgrowth into small shoot and leaf; (C) The shoots turning brown and plants don't develop roots; (D) Bleaching and purple coloration of shoots outgrowth in the presence of Kanamycin as a selective marker. Scale bar= 3cm.*

Among the different shoots induction media (SIM) compositions SIM1 i.e. MS media containing NAA 0.3 mgL^{-1} and BAP 3 mg L^{-1} showed highest efficiency in shoot induction followed by SIM 4 (Figure 3B and C) as described in Table 2.

Similar to callus induction, response of different genotypes to regeneration also showed high dependency upon the genotype with varying responses to similar media composition. Here again NIFA Nr.6 and NIFA Nr. 2 proved to the best among tested genotypes in terms producing regenerated shoots followed by NIFA Nr. 1. The rest of genotypes showed varying responses to shoot induction with Durr-e-NIFA variety showing the lowest. Based on these findings, we subsequently used these most responsive genotypes i.e. NIFA Nr.1, NIFA Nr.2 and NIFA Nr.6 (Ahmad et al., 2016) in our transformation experiments to develop *NtSAT4* overexpression lines.

Although in the absence of *Agrobacterium* pressure, NIFA Nr.1 and NIFA Nr.6 performed well in terms of producing calli and regeneration but after transformation presumably due to selection pressure the calli producing capabilities of both lines were significantly reduced compared to non-transformed conditions. The calli from both lines were very recalcitrant and the shoots outgrowths from these calli turned purple presumably due to anthocyanin production and bleached subsequently (Figure 3D). The already reported transformation and tissue culture responsive variety Oscar (Belide et al., 2013; Bhalla and Singh, 2008; Zhang et al., 2005) was therefore included in the subsequent experiments.

Transformation of cotyledons explants

B. napus cultivar "Oscar" was further used to be transformed with the three *NtSAT4* constructs according to Bhalla and Singh (2008) with some modifications. The cotyledons were cut, infected by *Agrobacterium* harboring the constructs, and then kept for co-cultivation and callus induction in MS media containing the respective hormones. The explants' cut ends showed swellings, indicating the onset of callus induction (Figure 4A).

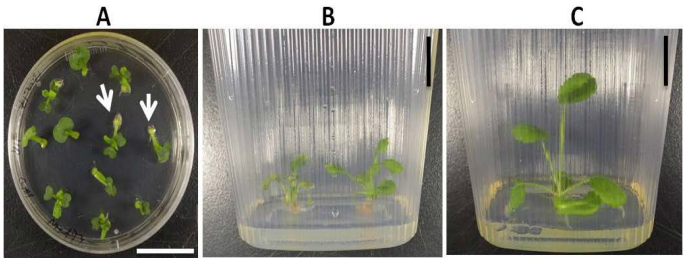

Figure 4: *Callus and shoot formation from cotyledon explants following infection with Agrobacterium AGl1 strain harboring NtSAT4 gene; (A) Callus induction of cotyledons after infection with Agrobacterium harboring NtSAT4 gene, growing in MS medium containing BAP 0.75 mgL^{-1}, NAA 0.2 mgL^{-1} and GA3 0.1 mgL^{-1} grown at 25 °C under long day conditions. White arrows indicate the callus formation in the cotyledons; (B) Side-view of emerging shoots on shoot initiation medium (MS medium, BAP 3 mgL^{-1}, NAA 0.2 mgL^{-1} and GA3 0.1 mgL^{-1} and kanamycin 25 mgL^{-1}); (C) Plants surviving selection in MS media, BAP 0.0125 mgL^{-1} and kanamycin 50 mgL^{-1}. Scale bar= 3cm.*

The calli were shifted to shoot induction MS media with optimized hormone concentrations and allowed to regenerate directly for 2 to 4 weeks under controlled long day conditions (Figure 4B). Fully developed shoots (Figure 4C) on shoot outgrowth MS media for 2 weeks, developed roots on root induction media in 2-3 weeks (Figure 5A).

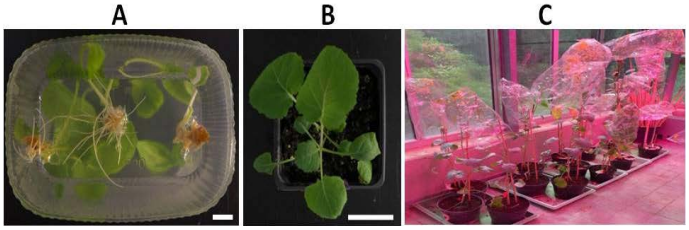

Figure 5: *Root induction and acclimatization of plants in soil under controlled conditions; (A) Plantlets developed roots on half-MS media with IBA 3 mgL^{-1} (bottom view); (B) Plants transferred to soil to acclimatize under controlled long day conditions at 25 °C and kept covered to ensure high humidity. Scale bar = 3cm; B) Positive expressing NtSAT4 transgenic plantlets transferred to soil to acclimatize under controlled long day conditions at 25 oC and high humidity. Scale bar = 3cm; (C) Developed transgenic plant with early floral buds covered with perforated plastic bags to ensure self fertilization and seed setting.*

Acclimatization of the plants to soil and glass house conditions

The plants with well developed root were removed from the media, washed to remove traces of media and placed into soil in covered small pots to ensure high humidity (Figure 5B). The plants well acclimated to soil conditions were uncovered for few days. These were then shifted to the glass house for the development of stable transgenic plants for flowering and seed setting (Figure 5C).

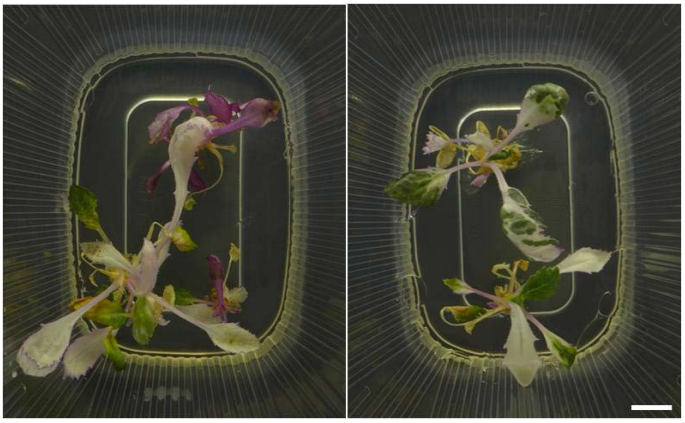

Figure 6: *Top view of supposedly non-transgenic plants on selection media; Non-transformed plants showed anthocyanin production and ultimate bleaching on long term exposure to kanamycin on MS media. Scale bar= 3cm.*

Transformation efficiency and survival rate

A total of about more than 580 explants were successfully grown through tissue culture procedure undergoing infection, co-cultivation, callus induction and shoot regeneration. There were 76% successfully regenerated shoots and 9% lost under kanamycin selection. However, 13% of the explants were lost due to contamination. The non-transgenic plants turned white or purple showing anthocyanin production due to stress, eventually died and later discarded (Figure 6).

*Selection of positive gDNA integrated **NtSAT4** lines from F1 generation*

Samples from the leaf of plantlets growing on transformation media containing kanamycin 50 mgL^{-1} were screened for their gene integration by genomic DNA PCR analysis. The leaf material was used to extract DNA with CTAB method by PCR using the specific gene *NtSAT4* primers NtSAT4_F/ NtSAT4_R for amplification. Out of 312 plants tested for gDNA, only 110 plants showed positive signal (250 bp) for *NtSAT4* gene, indicating that only 35% of the total plants were stably transformed (Figure 7).

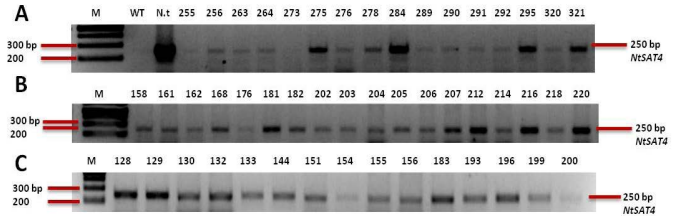

Figure 7: *Analysis of gDNA PCR for the F1 transgenic samples run on 1.5 % agarose; Lanes; M: 1kb plus ladder; WT: B. napus wild-type (negative control); N.t: Nicotiana tabacum wild-type (positive control); The gels show the samples corresponding to different compartments; A: cytosolic lines gDNA samples; B: Plastidic lines; C: Mitochondrial lines.*

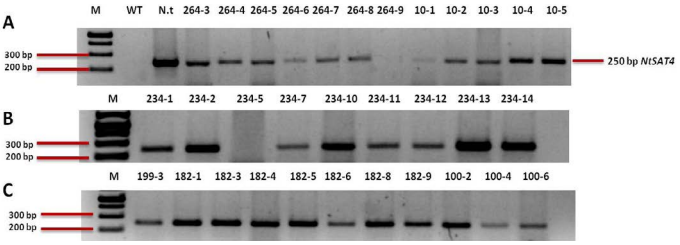

Figure 8: *Analysis of gDNA PCR for the F2 transgenic samples run on 1.5 % agarose; Lanes; M: 1kb plus ladder; WT: B. napus wild-type (negative control); N.t: Nicotiana tabacum wild-type (positive control); Agarose gels result show the ands for samples corresponding to different compartments; A: cytosolic lines gDNA samples; B: Plastidic lines; C: Mitochondrial lines.*

*Selection of positive gDNA integrated **NtSAT4** lines from F2 generation*

The positive expressing lines from F1 generation seeds were grown in small soil pots for further analysis. Leaf sample from F2 growing plants were used for gDNA extraction by CTAB method and analyzed for their *NtSAT4* gene integration by PCR using the specific gene primers (expected product 250 bp). Among the plants positive lines selected from F1 generation, 64% showed positive gene integration through gDNA PCR in their F2 generation. The representative lines showing positive PCR signal for successful *NtSAT4* gene integration targeted to different compartments have been shown (Figure 8). Thus, the positive signal

from these lines confirmed successful integration of *NtSAT4* gene and stable transformation which is transferred to next generations.

B. napus in our study was transformed with the *NtSAT4* gene via floral dip method which showed no positive expression of the gene by their transcript analysis. One possibility that has been reported to be associated with the floral dip method in plant species other than *Arabidopsis* is that *Agrobacteria* cells carrying the transgene may have survived the antibiotic selection procedure in intercellular spaces of the plant organs (Davis et al., 2009). Upon genomic DNA extraction, the genomic DNA from plants is somehow contaminated by DNA from the *Agrobacteria* cells, and therefore gives false positive results in case of genomic DNA PCR analysis. The *B. napus* in our study was thus transformed via tissue culture by selecting the cultivar OSCAR reported to be one of the suitable genotypes for tissue culture propagation (Belide et al., 2013; Bhalla and Singh, 2008; Zhang et al., 2005). The shoot induction was successful at NAA 0.2 mgL^{-1} and BAP 3 mgL^{-1} using cotyledons which was also reported in many studies for *B. napus* (Bhalla and Singh, 2008; Petrie et al., 2012; Zhang and Bhalla, 2004). However, different concentrations of BAP such as 4.5 mgL^{-1} have been used in case of cotyledon explants regeneration (Mashayekhi et al., 2008). Roots were induced by IBA @ 3 mgL^{-1} which produced 100% successful root formation for shoots grown in ½ MS media containing 10 gL^{-1} sucrose. The same media composition was also used in other studies with 5 mgL^{-1} IBA (Cardoza and Stewart Jr, 2003), IBA 10 mgL^{-1} (Cegielska-Taras et al., 2008), NAA 0.2 mgL^{-1} (Wang et al., 2006). In our study cotyledons transformation showed an efficiency of 35%, while in other studies *B. napus* Oscar showed higher efficiencies like 68% (Zhang et al., 2005), in SLM046 cultivar 4.7% (Mashayekhi et al., 2008) and 4-25% efficiency (Cardoza and Stewart Jr, 2003); however, *B. rapa* showed an efficiency of 9% (Wahlroos et al., 2003). The great variations observed in terms of transformation efficiencies for above mentioned genotypes indicate that apart from transformation procedures, the transformation efficiencies are highly dependent on genotypes. The transformation method adopted in this study led to development of a large number of directly regenerated false positive lines presumably due to less effective selection pressure. The comparatively low transformation efficiencies observed for cultivar OSCAR in our study could therefore be attributed to the less effective selection in our method compared to Zhang et al. (2005).

The plants with developed roots were successfully acclimatized to soil conditions were transferred to the glass house for flowering and seed setting. The seeds of this F1 generation were germinated in soil and screened through gDNA PCR and the lines showing positive signal were selected and grown for the second generation (F2). The F2 generated plants were also tested for their genomic integration of *NtSAT4* gene and several lines showed positive signal for each of the targeted compartmental lines showing positive integration of *NtSAT4* gene in the OSCAR genome and thus successful stable transformation.

Conclusions and Recommendations

Successful stably transformed three kinds of *NtSAT4* (*Nicotiana tabacum serine acetyltransferase 4*) overexpressor *Brassica napus* lines targeted to cytoplasm, plastids and mitochondria were developed. Following initial selection by selection marker, the independent lines from each compartment were further characterized for *NtSAT4* gene integration by testing their gDNA by PCR using the *NtSAT4* gene specific primers. The transgenic lines shall be tested for the *NtSAT4* expression and SAT activity. Furthermore, the promising lines may be tested against heavy metals and oxidative stresses.

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Novelty Statement

Previous attempts to enhance *in planta* cysteine and GSH contents via overexpressing the rate limiting enzyme of cysteine biosynthesis i.e. *serine acetyltransferase* (*SAT*) showed limited success because of the strong feedback inhibition of *SAT* by cysteine. To overcome this technical challenge, feedback insensitive isoforms of SAT from tobacco i.e. *NtSAT4* was used in this study which has been reported to exhibit complete insensitivity up to 600 µM for Cys compared to feedback inhibition of SAT by 1.8 µM Cys in water melon, 2.9 µM Cys in *Arabidopsis* and 7.2 µM Cys in Spinach. Moreover, since Cys biosynthesis take place not only in the plastids, but also in the cytosol and mitochondria, therefore in this study the expression of *NtSAT4* was targeted to these three

compartments of the cell.

Author's Contributions

HR conducted the research, MSK designed the project and experiments, HR and MSK wrote the first draft of the manuscripts. SHS, SMAS helped in supervision and critical revision of the data. All authors read and approved the final manuscript.

Supplementary Material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.sja/2019/35.4.1224.1233

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