Research Article

Evaluating the Performance of Genetically Engineered *Serine acetyltransferase* 4 *(NtSAT4)* Overexpression *Brassica napus* L. Lines under Xenobiotics Exposure

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Abstract | Glutathione (GSH) is a powerful antioxidant thiol compound that is critical for the detoxification of xenobiotics in plants. The genetic manipulation of GSH biosynthesis-related genes is considered a prime strategy to achieve higher *in planta* GSH contents. In this study, stably transformed *Brassica napus* lines harboring the feedback-insensitive isoform of *Serine acetyltransferase* (*SAT*), a rate-limiting enzyme for cysteine (Cys), and GSH biosynthesis, were subjected to H_2O_2, metolachlor, and atrazine-induced oxidative stress. The overexpression of the *NtSAT4* gene from *Nicotiana tobacco* under 35S promoters in various compartments of the cell, which includes cytosol, plastids, and mitochondria in transgenic lines, resulted in enhanced tolerance in terms of lesser wilting and pigment discoloration to induced stress compared to non-transformed plants. In terms of approximate percentage damage, under 14% H_2O_2stress,30-60% of the leaf area turned necrotic in the single overexpression lines compared to 95% damage in the wild-type plants. Whereas, the least amount of damage (10-20%) was observed in the double overexpression lines. When subjected to 24 µM metolachlor, the wild-type leaf discs were fully necrotic, whereas the single overexpression lines exhibited 20-60%, and the double overexpression lines showed only 15-20% necrosis. The data suggested that overexpression of *NtSAT4* is a promising strategy for improved stress tolerance against these xenobiotics in *B. napus*.

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Introduction

Glutathione (GSH) is critical in protecting plants against several stresses, including heavy metals and xenobiotics. Their accumulation in plants triggers the abiotic defence response resulting in the production of GSH, which in turn sequesters/chelates these harmful substances to protect them (Liedschulte *et al*., 2010; Rajab *et al*., 2020; Foyer and Noctor, 2005; Mullineaux and Rausch, 2005). Conjugation of xenobiotics of different structures with GSH is a common detoxification pathway in higher plants. These GSH conjugates are catalyzed by GSH S transferases (GSTs) (Bártíková *et al*., 2015; Dasari *et al*., 2017). This conjugation generally occurs in the cytosol and is subsequently deposited in the

vacuoles of plants and fungi to avoid toxicological effects (Verbruggen *et al*., 2009; Tong *et al*., 2004). Previous research has demonstrated that GSH availability is correlated with xenobiotic (herbicide) resistance (Nakka *et al*., 2017; Syguda *et al*., 2018; Ye *et al*., 2019; Dixon *et al*., 2002). Its availability is a critical factor in determining cell sensitivity and under standing herbicide selectivity (Marrs, 1996). Since conjugation of xenobiotics with GSH or its oligomers phytochelatins results in the fast depletion of cytosolic GSH pools, thereby, any such perturbation that causes the depletion of GSH can severely weaken a plant's defense against xenobiotics. Therefore, the genetic manipulation of GSH biosynthesis in plants is a very promising approach to enhancing the plant's tolerance potential against abiotic stresses (Pilon-Smits and Pilon, 2002; Rajab *et al*., 2020; Xiang *et al*., 2018; Khan *et al*., 2021). For this reason, the assimilatory sulfate reduction pathway has been genetically manipulated by overexpressing different rate-limiting genes to elevate *in planta* GSH in different plant species (Pilon-Smits *et al*., 1999; Rajab *et al*., 2020; Xia *et al*., 2018).

Cys availability is rate-limiting in GSH production; overexpression of a rate-limiting gene in the Cys synthesis pathway *ATPS1* (*ATP sulfurylase*) has resulted in elevated GSH contents (Pilon-Smits *et al*., 1999). The GSH overproducer transgenic lines also showed improved tolerance to atrazine (Flocco *et al*., 2004). *Serine acetyltransferase* (*SAT*), which catalyzes the acetylation of *L*-serine, is one of the rate-limiting enzymes in the production of Cys. However, Cys synthesis is tightly regulated, and *SAT* activity is feedback inhibited by Cys itself. A Cysteine insensitive isoform of *SAT* from *Nicotiana tabacum* (*NtSAT4*) has been explored to be completely insensitive to Cys up-to 0.6 mM (Wirtz and Hell, 2003). The expression of this isoform in bacteria has resulted in elevated Cys and GSH contents.

The usage of *Brassica napus* L. for phytoextraction has gained importance in cleaning polluted lands (Van Ginneken *et al*., 2007; Zhang *et al*., 2018). It is a powerful phyto accumulator of heavy metals for environmental cleanup (Angelova *et al*., 2017). We have recently demonstrated that overexpression of *Brassica napus* L. with the feedback insensitive *SAT* (*NtSAT4*) in different subcellular compartments has resulted in up to 3.5-fold higher free Cys and 5.3-fold higher GSH contents. Overproduction of GSH in these lines led to improved tolerance against oxidative stress with hydrogen peroxide (H_2O_2) and the heavy metal cadmium (Cd) in overexpression lines (Rajab *et al*., 2020). These lines also translocated significantly greater amounts of iron (Fe) from roots to shoots. Since GSH-dependent GST-mediated detoxification is important in the bio transformations of several xenobiotics in plants to minimize their potential phytotoxicity (Bartha *et al*., 2014; Vontas *et al*., 2001), we tested the performance of these *NtSAT4* GSH overproducer lines under selected herbicides stress in this study.

Materials and Methods

Plant material

The seeds of the single and double overexpression *NtSAT4* transgenic *B. napus*lines recently produced at the Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar, were the starting material for this study. The seeds of the cultivar Oscar were used as a starting material for this project.

Plant growth conditions

The seeds of wild-type (Oscar) and single and double overexpression *NtSAT4* transgenic lines plants were surface sterilized as mentioned before by (Syed *et al*., 2023), then treated at cold temperatures (2 days at 4 °C) to break dormancy. The seeds were then germinated on Murashige and Skoog (MS) media (half-strength) on Petri plates for five days. The plantlets were then shifted to the soil in pots and allowed to acclimatize in the greenhouse. DNA was extracted (Rogers and Bendich, 1989) from the seedlings, and they were analyzed through PCR. The PCR-positive plants were allowed to grow and later used in the experiment.

Exposure of leaf discs to H_2O_2 and other xenobiotics

About 8-10 leaf discs from young leaves of single overexpressor *NtSAT4* transgenic lines (P, M, and C), double overexpressors, and wild-type plants were subjected to 14% H_2O_2 for 24 hours (Rajab *et al*., 2020) and one set of leaf discs was kept as control and only subjected to distilled water. The plants were also subjected to xenobiotics such as herbicides, metolachlor, and atrazine. Leaf discs were exposed to 24 µM metolachlor stress for 24 hrs. While in the case of atrazine stress, whole *NtSAT4* transformed and wild-type plantlets were subjected to 12 µM and 24 µM for 48 and 24 hours, respectively, in quarter-

strength MS media. The control set of plants was kept in quarter-strength MS media. The performance of the *NtSAT4* single and double overexpressor lines and wild-type plants was arbitrated by comparing the degree of chlorosis or wilting. Leaf damage in response to the above-mentioned stresses was also documented by taking into account the percentage of the total leaf area damaged in response to a particular stress in transgenic lines.

Results and Discussion

Performance of the NtSAT4 overexpression lines under H_2O_2 stress

In biological sciences, the leaf disc methodology is an established approach to documenting the response of plants to reactive oxygen species exposure (Rajab *et al.*, 2020; Liedschulte *et al.*, 2010). To essay the response of single and double overexpressor *NtSAT4* lines and wild-type plants, the leaf discs taken from these lines grown under glasshouse conditions were exposed to14% H_2O_2. The leaf discs were kept at room temperature under light conditions for 24 hours. It was noted that the *NtSAT4* (single and double) overexpressor lines exhibited lesser loss of pigments (chlorosis) as compared to the wild-type leaf discs after oxidative stress (Figure 1). The C264 and M193 showed lesser chlorosis as compared to the P230 amongst the single overexpressor lines and hence showed better performance. The double overexpressor *NtSAT4* lines (M193×P230 and M193×C264) also performed better as compared to the wild-type leaf discs. Furthermore, amongst the transgenic lines, the double overexpressor lines exhibited distinctly reduced chlorosis as compared to *NtSAT4* single overexpressor lines. Numerical data in the form of approximate percentage damage showed 95% of the leaf area turned necrotic in the wild-type when exposed to 14% H_2O_2; the single overexpressor lines showed 30-60% damage, whereas the least amount of damage was exhibited by double overexpressor lines (10-20%). The overall result of this experiment concluded that all the *NtSAT4* transgenic lines showed lesser chlorosisas compared to the wild-type plants and hence showed moretolerance toH_2O_2 oxidative stress. Additionally, the double overexpressor *NtSAT4* lines performed slightly superior to the single overexpression lines.

Performance of the overexpression lines under herbicide metolachlor stress

In another leaf disc assay, the leaf discs from *NtSAT4* transgenic and wild-type greenhouse-grown plants were exposed to herbicide metolachlor. The leaf discs were subjected to 24 µM metolachlor and kept at room temperature under light conditions for 24 hours. The transgenic (single and double overexpression) lines indicated lesser chlorosis as compared to the non-transgenic leaf discs (Figure 2). In terms of percentage damage, the wild-type leaf discs showed 100% necrosis, whereas the single overexpressor lines exhibited 20-60%, and *NtSAT4* double overexpressor lines revealed 15-20% necrosis.

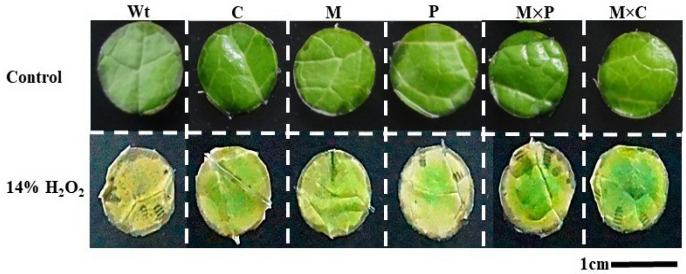

Figure 1: *Effect of 14% H_2O_2 on the leaf discs of NtSAT4 single and double overexpression lines. Leaf discs from three compartment-specific single overexpressor lines cytosolic (C), mitochondrial (M), and plastidic (P) and double overexpressor lines mitochondrial×plastidic (M×P) and mitochondrial×cytosolic (M×C) lines and wild-type plants treated with 14% H_2O_2 for 24 hours. Scale bar = 1 cm.*

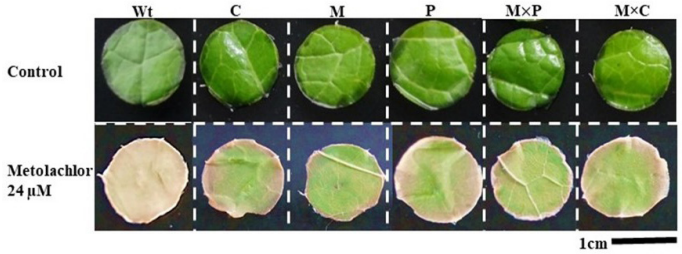

Figure 2: *Effect of metolachlor on the leaf discs of NtSAT4 single and double overexpression lines. Leaf discs of single overexpressor lines cytosolic (C), mitochondrial (M), and plastidic (P) and double overexpressor lines mitochondrial × plastidic (M×P) and mitochondrial × cytosolic (M×C) lines and wild-type plants exposed to 24µl metolachlor. Leaf discs were kept under continuous light for 24 hours at room temperature. Scale bar = 1 cm.*

Performance of the NtSAT4 overexpression lines under herbicide atrazine stress

To determine the effect of another xenobiotic on transgenic plants as compared to non-transgenic plants, four-week-old plantlets kept in growth chambers in hydroponic media were subjected to aherbicide known as atrazine. The transgenic (single and double overexpressor) lines and wild-type plants were distributed in three sets, one set was kept as control and subjected to 1⁄4 strength MS media without the herbicide, next set was exposed to 24 µl atrazine for 24 hours and the last set was

exposed to 12 µl of atrazine for 48 hours. The single overexpression lines M193 and P230, and the double *NtSAT4*overexpression lines containing M193×C264 and M193×P230 exhibited higher tolerance compared to the wild-type plants in terms of lesser wilting (Figure 3). Among the *NtSAT4* transgenic lines, M193 (single overexpressor line) and M×C (double overexpressor line) showed lesser wilting to both concentrations of atrazine stress.

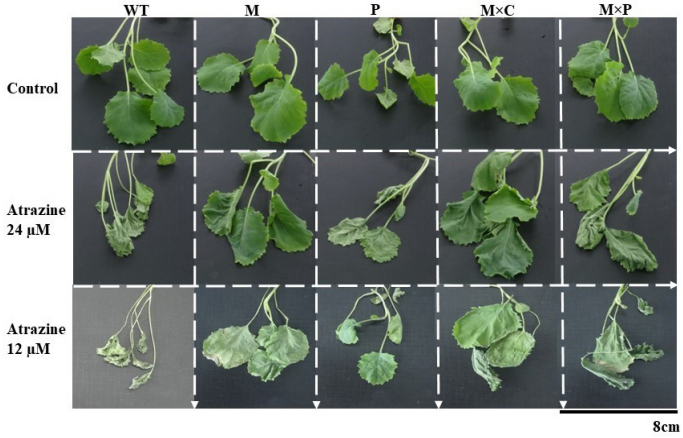

Figure 3: *Effect of the herbicide atrazine on NtSAT4 single and double overexpressor lines and wild-type. One-month-old non-transformed (Wt), NtSAT4 single overexpressor lines mitochondrial (M) and plastidic (P) and double overexpressor lines mitochondrial × cytosolic (M×C) and mitochondrial × plastidic (M×P) plantlets grown on 1/4-strength MS hydroponic media. Plants were treated with control (1/4-strength MS), 12 µl atrazine for 48 hrs, and 24 µl for 24 hours. These were kept under control conditions in the growth room under continuous light. Scale bar = 8cm.*

Response of single and double overexpressionNtSAT4 lines to xenobiotics-induced oxidative stress

Heavy metals and herbicides such as metolachlor and atrazine have very harmful effects on plants and, ultimately, human health. Recently, it has been shown that genetically engineered plants can exhibit more tolerance to these xenobiotics as compared to their respective wild-type plants. These transgenic lines also showed an ability to remove these contaminants from the environment. After uptake from their surroundings via roots and shoots, these herbicides and heavy metals are converted to non-toxic forms through different biochemical pathways. These toxic metabolites chelate with GSH, amino acids, and sugars and are subsequently sequestered in the cell vacuoles (Linacre *et al.*, 2003; Cao *et al.*, 2019; Chandrakar *et al.*, 2020; Asare *et al.*, 2023).

These toxic environmental stimulants result in the production of reactive oxygen species (ROS), such as hydroxyl radicals, H_2O_2, and superoxide radicals, which are extremely damaging to the cell components and biochemical pathways (Ozougwu, 2016; Juan *et al.*, 2021; O'Kane *et al.*, 1996). The hydrogen peroxide produced triggers the plant's defense mechanisms, and hence these toxic oxides are sequestered in the vacuole (Smirnoff and Arnaud, 2019; Sachdev *et al.*, 2021; Foyer *et al.*, 1997). The leaf disc assessment in biological sciences is a widely accepted approach in documenting plants responses to reactive oxygen species exposure (Rajab *et al.*, 2020; Liedschulte *et al.*, 2010). To document the antioxidant response of *NtSAT4* single and double overexpression lines, their leaf discs were subjected to different xenobiotics. The leaf discs from single and double overexpression lines and wild-type plants were subjected to 14% H_2O_2 stress for 24 hours at room temperature under 16 hours of light conditions. The leaf discs of all the single overexpression lines exhibited lesser chlorosis as compared to leaf discs from wild-type plants. The double overexpressor lines M193×P230 and M193×C264 also performed significantly better as compared to the wild-type plants. Moreover, both the double overexpressor lines showed distinctly lesser pigment discoloration as compared to their single overexpressor counterparts as well. This better tolerance to ROS may be attributable to the higher potential for GSH production in the double overexpression transgenic lines. GSH is an important antioxidant and protects the cells from the damaging effects of ROS produced during different stresses by sequestrating them from the system (Gill and Tuteja, 2010; Hasanuzzaman and Fujita, 2011; Navari-Izzo *et al.*, 1997).

To determine the response of *NtSAT4* single and double overexpressor lines to organic contaminants such as herbicides, they were subjected to metolachlor and atrazine. The leaf discs were exposed to 24 µM metolachlor for 24 hours under light conditions at room temperature. The transgenic lines, both single and double overexpressors, exhibited lesser bleaching of pigments as compared to wild-type leaf samples. One-month-old *NtSAT4* transgenic lines and wild-type plants were also subjected to another herbicide known as atrazine. Two concentrations of atrazine 12 µM for 48 hours and 24 µM for 24 hours were applied to the plants in hydroponic media. Both the single overexpressor lines (M193 and P230) and double overexpressor lines demonstrated significantly lesser wilting compared to the wild-type plants. GSH acts as a substrate of PCs biosynthesis, and PCs are

involved in defense mechanisms of detoxification of toxic compounds (Liedschulte *et al*., 2010; Zhu *et al*., 1999). *N. tabacum StGCL-GS* overexpressors lines showed improved tolerance to herbicide paraquat-induced oxidative stress compared to the wild-type leaf discs (Liedschulte *et al*., 2010). Transgenic plants exhibited better tolerance to herbicides when expressed with P450 genes (Gorinova *et al*., 2005; Zheng *et al*., 2022; Ohkawa *et al*., 1998). Transformed *Oryza sativa* overexpressing *CYP1A1*, *CYP2B,* or *CYP2C19* genes detoxify different herbicides (Kawahigashi *et al*., 2002). Whereas the *pIKBACH* transgenic plants detoxify many herbicides as these transgenics overexpress all three P450s (Kawahigashi *et al*., 2003, 2006). The leaf disc experiments indicated that *NtSAT4* single and double overexpressing *B. napus* lines can potentially be used for phytoremediation of xenobiotics, including hydrogen peroxide, heavy metals, and herbicides from the environment.

Conclusions and Recommendations

The overexpression of *NtSAT4* in single and double-overexpressing *B. napus* lineshas resulted in enhanced stress tolerance against H_2O_2, metolachlor, and atrazine-induced oxidative stresses. In terms of H_2O_2-inducedoxidative stresses, the double overexpression lines performed better compared to the single overexpression lines. The transgenic lines may be tested against a combination of heavy metals and oxidative stresses under different growth conditions.

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Novelty Statement

Unlike the previous approach for enhancing *in planta* GSH contents, we have addressed the issue of feedback inhibition by overexpressing the feedback-insensitive isoforms of SAT from tobacco, i.e., *NtSAT4,* to overproduce *in planta* GSH. Moreover, since Cys biosynthesis takes place in three subcellular compartments, therefore in this research, the expression of *NtSAT4* was targeted to the mitochondria, plastids, and cytosol of the cell.

Authors contribution

Fariha Qahar: Conducted the research and wrote the first draft of the manuscript.
Muhammad Sayyar Khan: Designed the project and experiments.

Conflict of interest
The authors have declared no conflict of interest.

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