Exploring the role of potato trithorax proteins (StSDG4 and StATX1) in the *de novo* shoot regeneration in *Nicotiana benthamiana* and *Arabidopsis thaliana*

A Thesis Submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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Date: April, 2025

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From May 2024 to Mar 2025

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled **Exploring the role of potato trithorax proteins (StSDG4 and StATX1) in the *de novo* shoot regeneration in *Nicotiana benthamiana* and *Arabidopsis thaliana*** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Megha S** at Indian Institute of Science Education and Research under the supervision of **Prof. Anjan K Banerjee**, Department of Biology, during the academic year 2024-2025.

13/03/25

Prof. Anjan K Banerjee

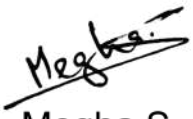
Committee:

Prof. Anjan K Banerjee

Dr. Krishanpal Karmodiya

Declaration

I hereby declare that the matter embodied in the report entitled **"Exploring the role of potato trithorax proteins (StSDG4 and StATX1) in the de novo shoot regeneration in *Nicotiana benthamiana* and *Arabidopsis thaliana*"** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER) Pune, under the supervision of Prof. Anjan K Banerjee, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.



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Abstract

Regeneration is a fundamental characteristic feature that enables plant to propagate, repair, replace tissue, and adapt to the environment. Understanding regeneration is only achievable through elucidating the function of underlying genes and their interplay mechanistically governing the process of differentiation, dedifferentiation, the primary steps of plant regeneration. Preliminary results from the lab show that the two Potato trithorax genes, *StSDG4* and *StATX1* are involved in regeneration response. This is due to the fact that shoot regeneration efficiency is found to be severely reduced and delayed in *Agrobacterium*-mediated plant transformation events., a process that is usually quite faster in potato *de novo* shoot regeneration. In this study, we hypothesize that these trithorax proteins could have conserved functions in other plants. To understand this further, two model plants – *Nicotiana benthamiana* and *Arabidopsis thaliana* – were selected for exploring the effects of *StSDG4* and *StATX1* genes in the de novo shoot regeneration process. In this aspect, overexpression transgenic lines of *N. benthamiana* were generated through *Agrobacterium*-mediated plant transformation. During the transformation stage and in the latter regeneration assays, a delayed and less efficient frequency of shoot regeneration is observed in the two overexpression lines in *N. benthamiana* . Similarly, preliminary experiments in *A. thaliana* indicated that in the processes of line generation using *Agrobacterium* mediated plant transformation I have observed a less transformation efficiency for overexpression lines of these two genes as well as these genes having some role in phenotypic traits affecting development. This study brings us one step closer to understanding the mechanism of regeneration and shows that the potato trithorax genes, *StSDG4* and *StATX1* may have a conserved role in mediating regeneration across species.

Acknowledgments

I would like to express my deep gratitude towards my thesis supervisor, Prof. Anjan. K. Banerjee for his valuable mentorship and guidance, without which I could not have made it this far. I would like to thank my TAC member, Dr. Krishanpal Karmodiya for following up with my project and providing suggestions and directions. I would like to thank Shikha Singh for giving me appropriate suggestions and for helping me with the conceptual understanding. I am also thankful to Amey Bhide for his constant support and suggestions during my entire stay in this lab. I would also like to extend my appreciation to Ishfaq, Arati, Nikita, Mohit, Kanishka, Debasmita, Rachana, Jyoti, Bahnishikha, Drishti, Vaishnavi and Varad. Finally, I would like to thank my father, mother, and friends for always being there for me.

Contributions

Contributor name	Contributor role
Shikha Singh, Anjan K Banerjee	Conceptualization Ideas
Shikha Singh	Methodology
	Software
Megha S, Shikha Singh	Validation
Megha S	Formal analysis
Megha S	Investigation
Anjan K Banerjee	Resources
Megha S	Data Curation
Megha S	Writing - original draft preparation
Megha S, Shikha Singh, Anjan K Banerjee	Writing - review and editing
	Visualization
Anjan K Banerjee	Supervision
Anjan K Banerjee	Project administration
	Funding acquisition

Introduction

‘If there is no regeneration then there is no life and on the other side if everything got regenerated then there is no death so ultimately regeneration lies between these two extremes’(Goss et al. 2013). Regeneration is a fundamental process that either repairs or replaces damaged or lost tissues, which maintains an organism's functional and structural integrity(Brockes, J. P., & Kumar, A. et al. 2008). The word regeneration makes people think about regeneration in hydra, salamander, planaria, etc. It is quite fascinating for the whole scientific world how these regenerations take place (mechanism) and to what extent.

Although all these processes are for survival, the mechanisms behind them are different. For example, hydra show a whole-body regenerative mechanism; salamanders show limb and organ regeneration; planaria show regeneration from small fragments; and plants show almost a mix of all these: tissue, organ, and whole-body regeneration (Echeverri and Zayas et al. 2018). The mechanism behind regeneration varies across species, and the regenerative capacity also varies across species(Alvarado et al. 2004). Few organisms like hydra, salamander, zebrafish, etc, have high regenerative capacity, while few others like lizards, deers, newts, etc, have moderative regenerative capacity(Bosch et al. 2007, Tanaka et al. 2011, Poss et al. 2002). Meanwhile, mammals, birds, etc., have low regenerative capacity. In one kingdom itself, regeneration can vary widely from high to low. For example, in plants, bryophytes and some gymnosperms have high regeneration, angiosperms have moderate regeneration, and woody trees have low regeneration capacity.

In plants, regeneration is a fundamental characteristic feature that enables the plant to repair itself, propagate vegetatively, and adapt to the environment. Unlike animals, plants have a unique ability to regenerate from a single cell to an organ due to its totipotency, which is the ability to regenerate from a single cell to an entire organism(Zhang et al. 2020). Besides totipotency, its meristematic activity (meristems are actively dividing cells that can repair and regrow organs), dedifferentiation, and redifferentiation (mature cells can revert to less specialized cells and differentiate again into new tissue) are also relevant(Jurgen et al. 2002,Ben et al. 2005). There are 2 types of plant regeneration: somatic embryogenesis and *de novo* organogenesis. In somatic embryogenesis, the development of embryos takes place from somatic cells

(that are non reproductive cells) which can later develop into mature complete plants (like embryo formation from carrot tissue culture). It is used in genetic engineering to get genetically uniform plantlets. In *de novo* organogenesis, the development of shoots or roots takes place from existing tissue or cells. It is widely used in micropropagation and plant transformation techniques(Ikeuchi, Momoko, et al. 2016, Ibáñez, Sergio, et al. 2020)

Here, we study *de novo* organogenesis in detail for plant transformation application. Regeneration in plants helps in agriculture and horticulture in micropropagation (mass propagation of crops through tissue culture) and clonal propagation of high-yield or disease-resistant plant varieties. In forestry, it can regenerate plants through tissue culture, which improves afforestation efforts. In genetic engineering and biotechnology, it is used to generate transgenic plants like Bt cotton(Kumria, Ratna, et al. 2003). Finally, it can even help in propagation and conservation of rare and endangered species by regeneration techniques(Shukla, Mukund R., et al. 2012).

Regeneration in plants can be done by vegetative propagation, leaf and stem regeneration, or by regeneration from callus culture. In vegetative propagation, plants regenerate directly from vegetative parts such as rhizomes, tubers, stolons, etc. Potato tubers sprouting new shoots is an example of this. In leaf and stem regeneration, plantlets are produced asexually from a leaf or stem, like how Bryophyllum develops new plantlets from its leaf notches. In callus culture, explants (hypocotyl, leaves, etc) after creating an injury(/wound) are incubated on a callus-induction media (CIM) with a high auxin-to-cytokinin ratio to induce cell dedifferentiation and formation of a pluripotent cell mass called callus. Subsequently, the explants are then transferred to a shoot-induction media (SIM) with a high cytokinin-to-auxin ratio for *de novo* shoot organogenesis from the callus (Park, Ji-Sun et al. 2023). Here it is understood that wounding is a key factor necessary for cellular reprogramming(Park, Ji-Sun et al. 2023).

Plant regeneration, as understood till now, is a complex process influenced by multiple factors that can be classified broadly into environmental, hormonal, genetic, and epigenetic categories. Environmental factors that affect regeneration are temperature, light, water availability, etc. The temperature at which plants develop will

influence the efficiency of the developmental process and, in turn, the health of the coming generation. Too much fluctuation in temperature might affect the whole mechanism and process of regeneration itself. Like temperature, light is also a critical factor that affects various physiological and developmental processes in tissue culture outcomes.

Phytohormones play a crucial role in regeneration. As mentioned earlier, balance and concentration of hormones, especially auxin and cytokinin, are necessary for regeneration. A high auxin to cytokinin ratio needs to be kept for the induction and proliferation of callus. A high cytokinin to auxin ratio must be supplemented for shoot induction in the media during culture(Duclercq, Jérôme, et al. 2011). Along with this, gibberellic acid is also necessary for shoot growth at a particular concentration. Altogether, the optimum level of phytohormones is fundamental for regeneration.

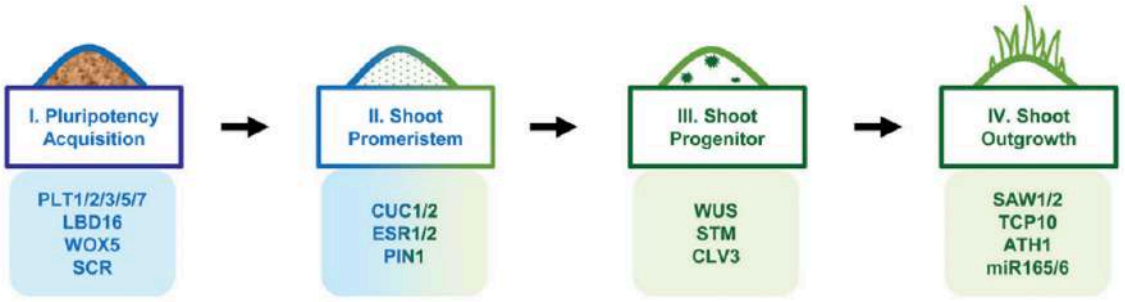


Figure 1: Stages of *de novo* shoot organogenesis. It shows hormonal as well as genetic factors especially regeneration markers and hormonal gene markers involved in regeneration in each stage. Blue background indicates auxin-rich callus-inducing medium (CIM), and green background represents cytokinin-rich shoot inducing medium (SIM)(Shin et al.2020)

The inherent genetics of the plant significantly affect its regenerative capacity. As mentioned earlier, variation among species and even among cultivars (that is, its change in genotype) will lead to different regenerative capacities(Park, Ji-Sun et al. 2023).

Regeneration is orchestrated by a network of molecular markers that regulate gene expression and cellular reprogramming. It is mainly through the transcriptional modulation of various meristematic and embryonic regulators. Markers include *Lateral organ boundaries domain(LBD), auxin response factors(ARF), cytokinin response regulators(ARR), WUSCHEL(WUS), Wuschel related homeobox(WOX), shoot meristemless(STM), cup-shaped cotyledon(CUC), PLETHORA(PLT),* etc. After wounding in the stage of callus formation, expression of *LBD* genes are upregulated by

WOX and *ARF* transcription factors (Fan et al. 2012; Liu et al. 2014). The four *LBD* genes downstream of ARFs, *LBD16*, *LBD17*, *LBD18*, and *LBD29*, are rapidly and dramatically induced by CIM in multiple organs(Mingzhu Fan et al. 2012).

Auxin is one of the most important phytohormone which plays a role in this process by enhancing the expression of WOX genes and activating ARF proteins. During SIM, cytokinin-induced WUS is crucial for the transition of callus cells to shoot identity. The expression of WUS is upregulated by type-B ARABIDOPSIS RESPONSE REGULATOR (ARR) transcription factors, whose expression rises with cytokinin signaling activation. Additionally, auxin-induced genes such as WOX5, WOX11, LBD16, LBD18, and PLT3 contribute to callus formation in CIM and the development of a shoot promeristem with the potential for shoot regeneration.(Fan et al. 2012; Liu et al. 2018; Ikeuchi et al. 2019). Meanwhile, *STM, CUC1*, and *WUS* are the key regulators of shoot regeneration from callus.

From previous literature, it is known that various epigenetic factors regulate this regeneration process. These factors include members involved in DNA methylation, histone modification, and chromatin remodeling. They differentially regulate gene expression to control the regeneration processes. Histone methylation can either activate or repress gene transcription, depending on which precise amino acid is modified and the number of methyl groups attached. Histone lysine methylation (HKM) is one of the significant epigenetic marks that is catalyzed by histone lysine methyltransferases (HKMTs).

Most of HKMTs contain a conserved domain consisting of 130–150 amino acids with methyl transferring activity. This domain is named SET based on the three histone lysine methyltransferases: Suppressor of protein-effect variegation 3–9 [Su(var) 3–9], Enhancer of zeste [E(z)], and Trithorax (trx), which were originally identified in Drosophila(Zhou et al. 2020). The currently known HKMTs include SET domain group (SDG) proteins containing a conserved SET domain, as well as DOT1p and DOT1L, which are exclusive of the SET domain(Avramova et al. 2002). In plants, however, only SDG proteins are considered to serve as HKMTs. The trimethylation of lysine 4 on histone H3 (H3K4me3) is a prominent histone modification that is dogmatically associated with gene activity. But more recently, it has also been linked to gene repression. Transcript levels of many genes related to root and shoot development and

flowering are affected by dynamic H3K4me3 levels, as are those for several stress-responsive and stress memory-related genes(Smith et al. 2021).

Trithorax and polycomb members, which work antagonistically, regulate histone modifications by methylating lysines of histone with the help of their SET domain(Grossniklaus et al. 2007). From previous studies from our lab we found many trithorax members might be involved in another important plant development event i.e. tuberization. Among these two important members are SDG4 and ATX1. *AtSDG4* has shown its role in reproductive development, mainly pollen tube growth(J.A. Cartagena et al. 2007). It is observed that the overexpression of *SDG4* has been linked to pleiotropic development defects, indicating that precise regulation of *SDG4* expression is needed for plant development.

AtATX1 is involved in development and, additionally, stress response in plants. It has an influential role in floral organ identity(Alvarez-Venegas, Raul, et al. 2003), root development(Napsucialy-Mendivil, Selene, et al. 2014), and secondary cell wall formation through its histone methyltransferase activity and subsequent modulation of gene expression(Wang, Xianqiang, et al. 2021). In root development, *ATX1* is essential for maintaining root apical meristem RAM activity and facilitates the transition to the elongation zone. *ATX1* is also known to deposit trimethylation marks on H3K4me3, which is crucial for various developmental processes, including the regulation of genes involved in secondary cell wall biosynthesis(Wang, Xianqiang, et al. 2021) .

In summary, both *ATX1* and *SDG4* are integral to the epigenetic regulation of gene expression in *Arabidopsis thaliana*. From literature it is observed that other trithorax members like *ATX4*(Figure 2) and *ATXR2*(Figure 3) in *A. thaliana* are involved in *de novo* shoot organogenesis by either influencing shoot identity genes or by regulating cytokinin metabolism genes respectively.

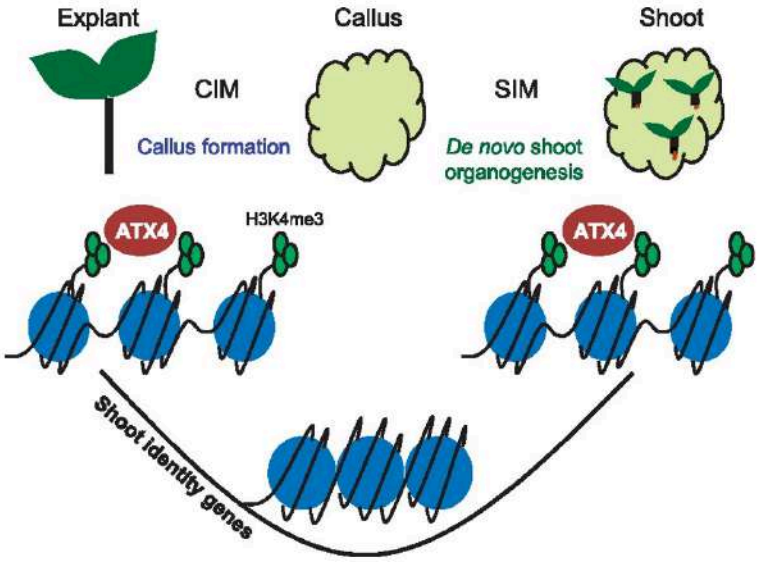


Figure 2: Proposed model showing how *ATX4* is involved *de novo* shoot organogenesis in *A. thaliana*. During the transition from leaf to callus, expression of *ATX4* is reduced to suppress shoot identity and promote root identity establishment. Then it is reactivated during shoot regeneration to establish shoot identity. (Lee, Kyounghee, et al. 2019)

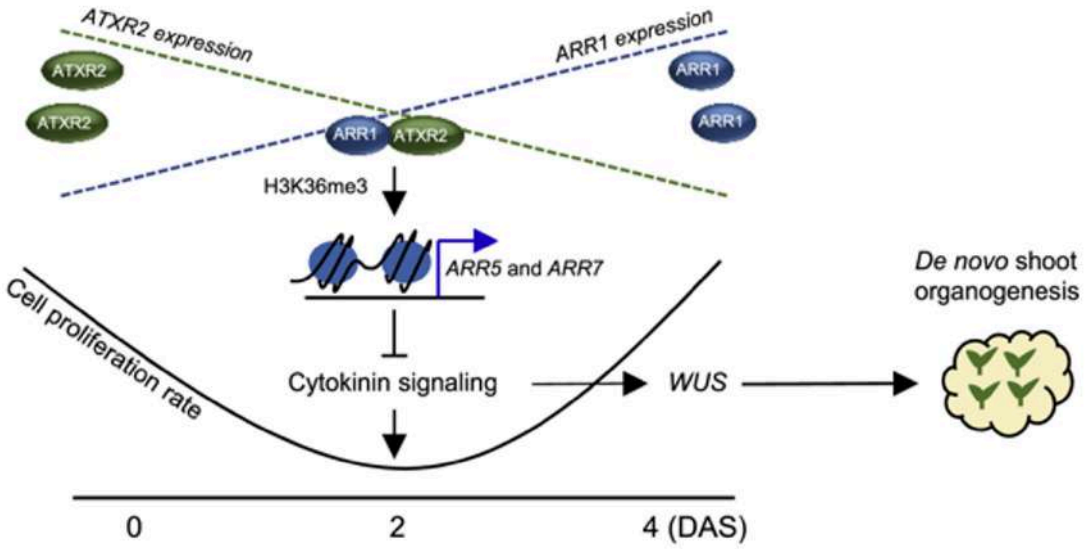


Figure 3: Proposed model showing how *ATXR2* is involved in shoot regeneration in *A. thaliana*. In SIM, *ATXR2* gene is downregulated whereas *ARR1* gene is induced; both of these will transiently interact in the early stages of shoot regeneration. This *ATXR2-ARR1* complex directly binds to *ARR5* and *ARR7* promoters and catalyzes H3K36me3 deposition to activate their expression which ultimately represses *WUS* expression. This results in the cease in cell proliferation that may ensure proper cell fate transition.(Lee, Kyounghee, et al. 2021)

Previous studies from our lab suggest the role of trithorax members (*StSDG4* and *StATX1*) in potato tuber development. While generating stable transgenic lines to further characterize their role in this process, we found that these genes might be involved in the *de novo* shoot regeneration. In *de novo* shoot regeneration assay which was performed in the lab using the *Agrobacterium* mediated plant transformation in leaf explants of *Solanum tuberosum ssp andigena*, overexpression constructs showed delayed shoot regeneration compared to antisense as well as vector control(Figure 4).

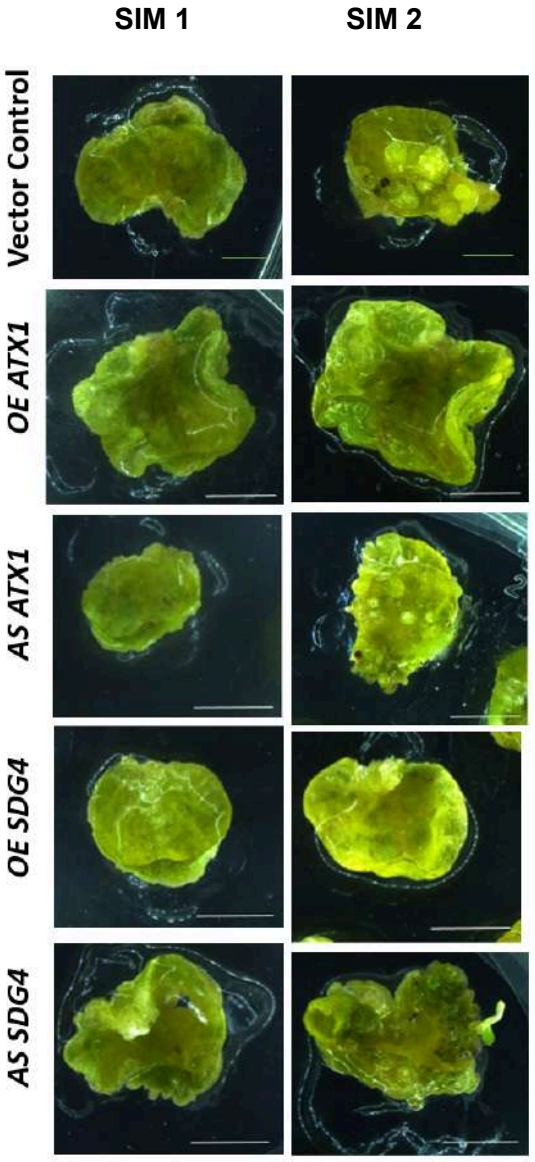


Figure 4: Images of explants during de novo shoot regeneration in *Solanum tuberosum ssp andigena* . Wounded leaves were incubated on CIM and SIM for overexpression and antisense of *StSDG4* and *StATX4* with respect to vector control (*pBI121*). CIM - callus induction media, SIM - shoot induction media (*Singh et. al*. 2025 unpublished data)

Thus, we aim to delineate the role of potato trithorax members *StSDG4* and *StATX1* in *de novo* shoot regeneration process. To study whether the role of these genes in the *de novo* shoot regeneration is conserved, we selected two model plants, *Nicotiana benthamiana* and *Arabidopsis thaliana*. *Nicotiana benthamiana*, known as benth or benthi, is a species of *Nicotiana* native to Australia that belongs to the Solanaceae family. It is a close relative of tobacco. It is mainly used in research for infiltration experiments, pharming of monoclonal antibodies, vaccine development, etc. *Arabidopsis thaliana* is a small weed plant from the Brassicaceae family native to Eurasia and Africa. *A. thaliana* is a popular model plant in plant biology and genetics. It is used in plant growth and developmental studies, hormonal regulation studies, stress-related studies, genetic and epigenetic research, agricultural and biotechnological applications, space biology, and environmental studies. It was the first plant to have its genome sequenced, so it is mostly considered a reference model plant. The regeneration capacity varies widely across species.

Based on the literature survey as well as the preliminary data obtained from our experiments of trithorax proteins in potato, it indicates that these proteins might have a conserved role in other species as well. In this study, we aim to understand the effect of these two trithorax proteins in the de novo shoot regeneration process among the two model plants *N. benthamina* and *A. thaliana.*

Objectives

1. To generate overexpression lines of StSDG4 and StATX1 (Potato trithorax proteins) in *Nicotiana benthamiana* and *Arabidopsis thaliana*

2. To generate promoter lines of *StSDG4::GUS-pBI101* and *StATX1::GUS-pBI101* and the marker assessment in *N. benthamiana*

3. Comparative analysis of *de novo* shoot regeneration frequency among these model plants followed by SAM marker assessment

Materials and Methods

Wild type seeds were sterilized and plated in MS medium(Murashige and Skoog's (MS) media supplemented with 2% sucrose (w/v)) containing plates. Once it is 2 weeks around, the seedlings are transferred into magenta boxes containing MS medium and grown in the culture room for around 4 weeks in long day condition which will be ready for *Agrobacterium* mediated plant transformation. Then molecular confirmation of putative lines will be done after subculture using genomic DNA PCR, RT PCR and qRT PCR. From those lines, 2 overexpression lines as well as wild type and vector control were subjected to regeneration assay. For tracking microscopy was done in the Leica S8 Apo stereomicroscope in a 7 days interval and no. of shoots were counted to find out the regeneration efficiency. Explants were also collected in a 7 day interval to check the expression of regeneration markers in it(Figure 5).

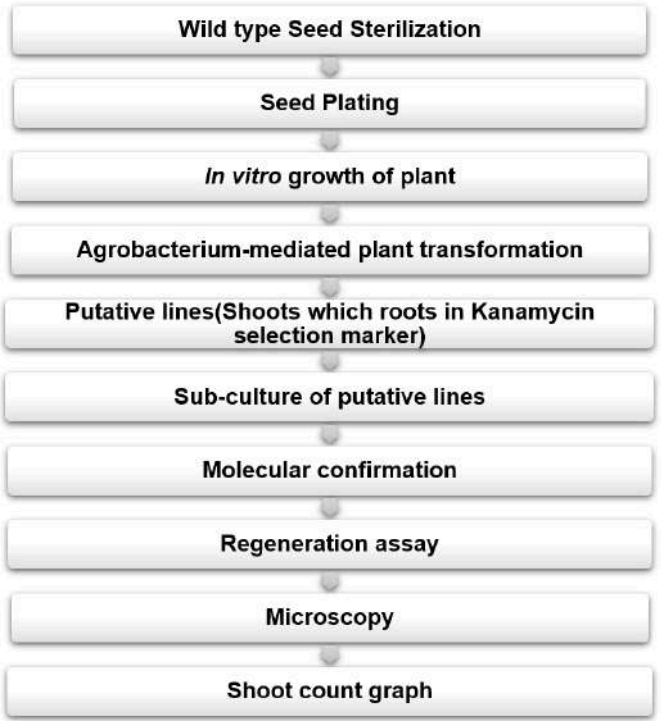


Figure 5.: Flow chart illustrating the process of *de novo* shoot regeneration in *N. benthamiana*.

Wild type seeds were sterilized and plated in MS medium(Murashige and Skoog's (MS) media supplemented with 0.8% sucrose (w/v)) containing plates. Once it is 2 weeks around, the seedlings are transferred into soil and subjected to

Agrobacterium mediated plant transformation using floral dip method. Seeds from those plants were collected, dried, sterilized and plated in MS plates with 10 mg/L

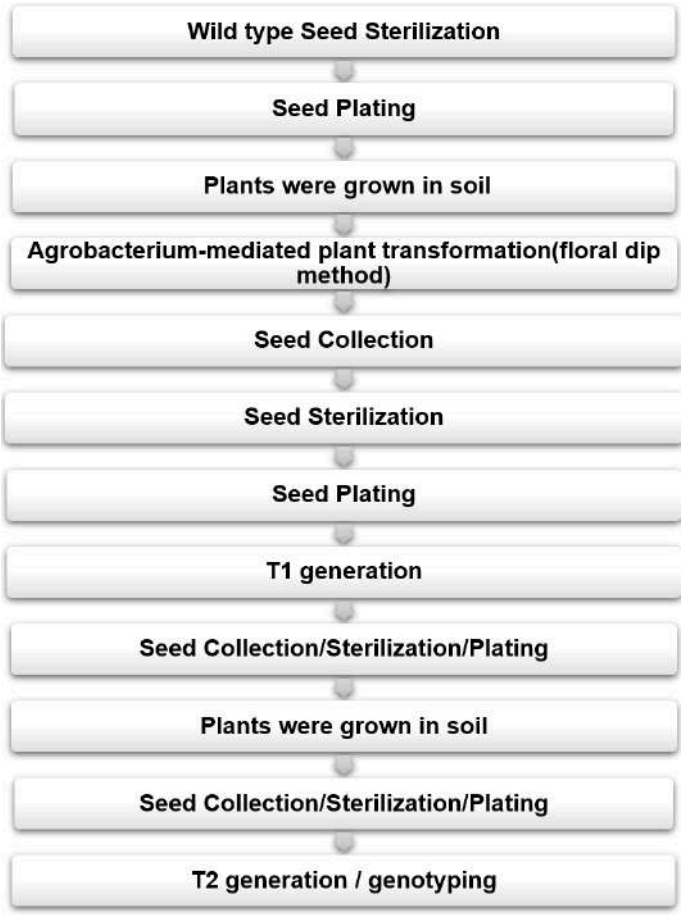


Figure 6: Flow chart illustrating transgenic line generation in *A.thaliana*

kanamycin and transformants were again grown in soil(T1 generation). Seeds from these plants are collected, dried, sterilized and plated in MS plates with 10 mg/L kanamycin which is the T2 generation which is then subjected to molecular confirmation using genomic DNA.(Figure 6)

Plant growth conditions

In vitro plates after transformation as well as regeneration assay were maintained in a plant growth incubator at 24°C under long-day (LD; 16 h light/8 h dark) conditions. *In vitro* plants as well as plates for seed germination were kept in the plant

culture room under long-day conditions. Soil grown plants were kept in the greenhouse under long-day conditions maintaining around 24°C.

Seed sterilization protocol

Approximately 300 seeds were transferred to 1.5ml MCT (each tube will go into 2 plates). Add around 700ul of 70% ethanol in the tube then vortex for 2-3 seconds and keep for 2 minutes. Remove that ethanol and add 20% bleach in it and vortex for 2-3 seconds and keep for 1- 1.5 minutes. After removing bleach, wash with sterile water 3 times each 3-4 minutes. Repeat the whole process again and wash around 5 times with sterile water. Store it in 4°C till plating it on MS plates.

Constructs

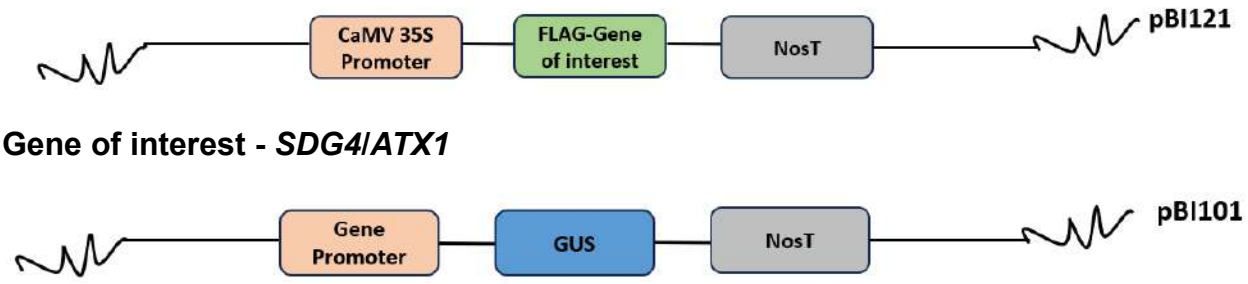


Figure 7: Design of constructs subjected for regeneration assay.

Agrobacterium-mediated plant transformation in *A. thaliana*

Grow *A. thaliana* plants in pots in the greenhouse/growth chamber (3 plants in 1 small pot). Cut the first bolts of inflorescence to get proliferation of many secondary inflorescences from axillary buds of the rosette. Allow the plants to make many immature flower clusters. Remove the siliques on the plant before taking it for transformation. Do not water the plant 2 days prior to transformation. Prepare the pre-inoculum of *agrobacterium* strain harbouring the gene of interest in a binary vector. For pre-inoculum, inoculate the glycerol stock culture from plate into 5 ml Luria Bertani

(LB) media with 10 mg/L of Kanamycin and 25 mg/L of Rifampicin. Incubate culture at 28°C for 12-24hrs. Inoculate 2ml of the pre- inoculum into 200 ml LB with Kan and Rif. Incubate culture at 28°C for 16-24hrs. The cells can grow till the stationary phase (OD ~ 1.5-2.0). Pelletize *agrobacterium* cells by centrifugation at 6000 rpm for 15 min at room temperature and resuspend the pellet in 30ml of 5%(wt/vol) sucrose solution. Bring the plants with many young flower buds into the growth chamber room and label the constructs name properly. Verify that there are no siliques formed, as it will contain wild-type seeds. Add 0.04% silwet L-77(12ul of silwet for 30ml culture) into bacterial culture in sucrose solution just before dipping the floral buds. Use gloves for protection as Silwet L-77 is toxic. with the help of a dropper, drop the culture into floral buds till it seems wet (10 sec). Make sure that all floral buds are properly dipped in the culture. Cover each pot containing plants with an autoclave bag which is closed at both ends and the whole set of plants with black bag so that high light/temperature will not get affected. After 16-24 hrs, put holes in the autoclave bag (try to put it near the basal part) and remove black bag. After one day change the autoclave bag into one which is open from both sides but still for 3-5 days fold and keep the top end. Make sure after those days it is open properly and water in the tray for around 15 days. (Try to keep the plants in the growth chamber if not shift it to the greenhouse). Once siliques turned brown collect the seeds in 50 ml falcons and keep open till it gets dried.

Agrobacterium-mediated plant transformation in *N.benthamiana*

Grow *N.benthamiana* plants *in vitro* for 3-4 weeks. Three days before the transformation experiment, prepare LB media and grow the *Agrobacterium*-containing construct of your choice on solid plates with required antibiotics (48 hrs). On day three, pick up a colony and grow in a falcon containing 10 ml of LB medium with required antibiotics overnight (Primary culture). On the next day that is the day of transformation, check the OD of the primary culture. Incubate secondary culture in fresh 5-10 ml media without antibiotics and adjust the OD to 0.2. Grow for a period of 2-4 hrs. Check the OD again and it should be between 0.8 – 1.0. While the culture is growing, harvest one *N. benthamiana* leaf and remove the mid vein as well as the

boundary of the leaf and make small pieces of 0.5 sq.cm. A total of 30-35 leaf discs will suffice for an efficient transformation. Put the pieces in 30ml of liquid MS media in a sterile petri plate to avoid desiccation. Add ~500ul of agro culture (OD: 0.8 – 1.0) to leaf discs. Shake the plate for 20 - 25 mins to evenly distribute the culture. Collect all the leaf discs and blot with sterile whatman paper. Culture them on co-cultivation medium for 48 hrs in dark. After 48 hrs, harvest all the leaf discs and wash them in sterile water thrice if required. Then re-blot with sterile whatman paper and put them in a regeneration medium. Incubate the plates in an incubator (long-day condition). Continue incubation of leaf disc for a period of 10 – 15 days.Prepare fresh regeneration medium again and transfer all the shoot masses to fresh medium. Repeat this process as and when required. Cut the shoots from callus mass and put them on root inducing medium containing antibiotics which will be putative positive lines.

RNA isolation - Trizol method

Take 100mg of crushed tissue and add 1 ml of RNAiso plus (Vortex thoroughly). Keep it overnight at -80 ℃. Add 300µl chloroform, invert and mix it 2-3 times. Incubate at room temperature for 15 mins. Centrifuge at 12,000 rpm for 15 mins at 4 ℃. Transfer the upper aqueous layer (Supernatant) carefully in a 1.5ml centrifuge tube. Don't disturb the interphase. Add 500µl of Isopropanol, invert mix 2-3 times. Incubate at room temperature for 15 mins. Centrifuge at 12,000rpm for 10 mins at 4 ℃. Discard the supernatant. Wash the pellet with 500µl of 70% ethanol. Centrifuge at 12,000rpm for 2 mins at 4 ℃. Discard supernatant and keep precipitated pellets for air dry. Add 40µl of DEPC-treated water once the pellet becomes transparent. Incubate at 55 ℃ for 15 mins. Check concentration using nanodrop. Load on 1.2% Agarose gel.

Genomic DNA ISOLATION- CTAB METHOD

Take 100mg of crushed tissue. Add 1 ml of CTAB(1X) buffer along with 5µl of mer-capto ethanol to the tube. Vortex mix. Incubate in water bath at 60 ℃ for 1-2 hour. Invert mix in between. Take the sample out once incubation is done. Centrifuge at 12,000rpm for 10 mins at 25 ℃. Take upper aqueous level in fresh 2ml tube, Add 25µl

of RNase A solution. Incubate in water bath at 37℃ for 30 mins. After Incubation add equal volume of chloroform : Isoamyl alcohol(24:1). Vortex thoroughly. Centrifuge the mix at 12,000rpm for 5 mins at 4 ℃. Transfer upper aqueous phase to 1.5ml tube. Precipitate DNA by adding 700µl of chilled isopropanol. Incubate at -20 for 15 mins. Centrifuge at 14,000rpm for 10 mins at 4C.Discard the supernatant. Wash the pellet with 500µl of 70% ethanol. Centrifuge at 12,000rpm for 2 mins at 4 ℃. Add 40µl of Milli-Q water once the pellet becomes transparent. Incubate at 55℃ for 15 mins. Check concentration using nanodrop. Load on 1% Agarose gel.

Regeneration assay

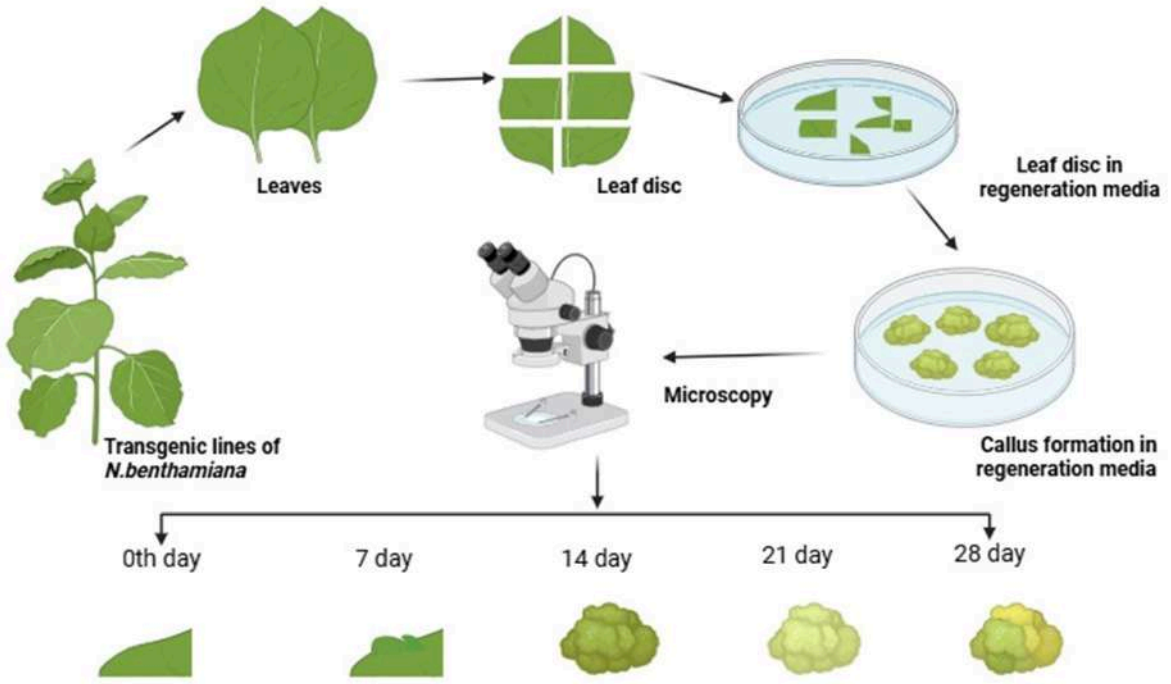


Figure 8: Schematic representation of regeneration assay in *N. benthamiana* (***Created with BioRender.com*** *Megha S et al. unpublished image)*

Grow *N.benthamiana* transgenic lines *in vitro* for around 4 weeks. Harvest one *N. benthamiana* leaf and remove the mid vein as well as the boundary of the leaf and make small pieces of 0.5 sq.cm. A total of 50 leaf discs will be sufficient for assay. Then transfer those leaf discs of transgenic lines as well as control into regeneration media. Incubate the plates in incubator (conditions). Continue incubation of leaf disc for

a period of 10 – 15 days.Prepare fresh regeneration medium again and transfer all explants to fresh medium. Repeat this process as and when required.(Figure 8) Count the number of shoots as well as track those explants using stereozoom and harvest 3 explants in every 7 day interval for expression level check (qRT for regeneration marker genes)

GUS assay

Explants were put in β-glucuronidase (GUS) staining assay (protocol adapted from JEFFERSON *et al.* 1987). It was kept overnight in 37 ℃ in the dark. After that remove the GUS solution, and add 30% ethanol and keep it for 2-3 hrs, then remove that and add 70% ethanol and keep for another 1-2 hrs and last wash with 100% ethanol till chlorophyll is cleared(approx 2hrs).

qRT PCR

Using RNA isoplus, total RNA was isolated from tissue. One microgram of RNA was taken for cDNA synthesis using MMLV reverse transcriptase kit and Oligo dT. For gene expression analysis, reverse transcription quantitative PCR(RT qPCR) was done using SYBR green as fluorophore on a CFX96 Real-Time System (Bio-Rad) using gene-specific primers.

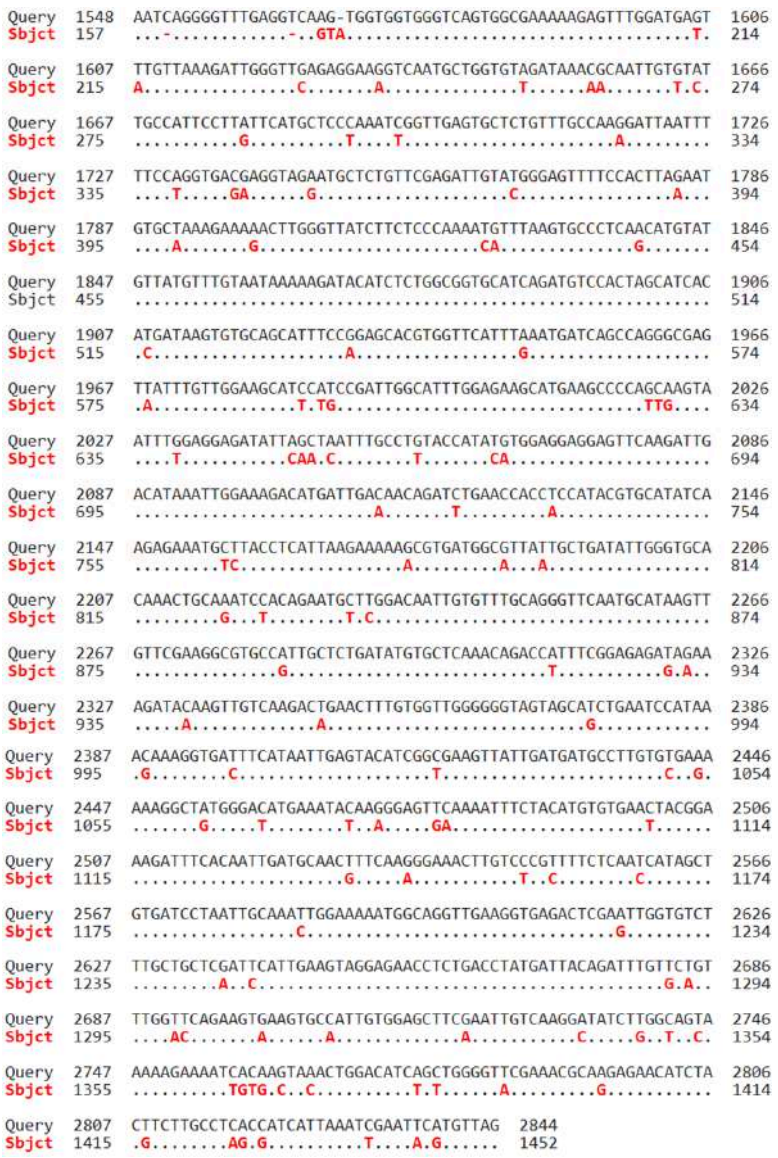
Primer list for PCR, RT PCR, qRT PCR

Vector specific primers	Kan Scr FP	CTACCCGTGATATTGCTGAA
	NosT Scr RP	GCAACAGGATTCAATCTTAAG
Gene specific primers	StSDG4 qRT FP	TAAAGATTGGGTTGAGAGGAAG
	StSDG4 qRT RP	AATCCTTGGCAAACAGAGCACTC
	StATX1 qRT FP	CCCTGTTATTGGTGGTGCTA
	StATX1 qRT RP	GTTTGAGCACTGAATGCAAG
Gene specific primers	AtSDG4 KO LP	TGGTGAACTGTGGAAACCTAG
	AtSDG4 KO RP	TGCAGTTTTTGGTACCAAAGG
	AtATX1 KO LP	ACCGGAGAACTTGTTAGACCG
	AtATX1 KO RP	TGCAGTTTTTGGTACCAAAGG

Table 1: List of primers used for confirmation and expression analysis.

Results

In silico data showing the similarity between *StSDG4* and *NbSDG4*



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Query  1607  TTGTTAAAGATTGGGTTGAGAGGAAGGTCAATGCTGGTGTAGATAAACGCAATTGTGTAT  1666
Sbjct  215   A..............C.......A.............T......AA.......T.C.  274

Query  1667  TGCCATTCCTTATTCATGCTCCCAAATCGGTTGAGTGCTCTGTTTGCCAAGGATTAATTT  1726
Sbjct  275   ..........G.........T....T....................A.........  334

Query  1727  TTCCAGGTGACGAGGTAGAATGCTCTGTTCGAGATTGTATGGGAGTTTTCCACTTAGAAT  1786
Sbjct  335   ....T.....GA......G....................C................A...  394

Query  1787  GTGCTAAAGAAAAACTTGGGTTATCTTCTCCCAAAATGTTTAAGTGCCCTCAACATGTAT  1846
Sbjct  395   ....A.......G.......................CA.............G.......  454

Query  1847  GTTATGTTTGTAATAAAAAGATACATCTCTGGCGGTGCATCAGATGTCCACTAGCATCAC  1906
Sbjct  455   ............................................................  514

Query  1907  ATGATAAGTGTGCAGCATTTCCGGAGCACGTGGTTCATTTAAATGATCAGCCAGGGCGAG  1966
Sbjct  515   .C.....................A.................G...................  574

Query  1967  TTATTTGTTGGAAGCATCCATCCGATTGGCATTTGGAGAAGCATGAAGCCCCAGCAAGTA  2026
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Query  2087  ACATAAATTGGAAAGACATGATTGACAACAGATCTGAACCACCTCCATACGTGCATATCA  2146
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Query  2147  AGAGAAATGCTTACCTCATTAAGAAAAAGCGTGATGGCGTTATTGCTGATATTGGGTGCA  2206
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Query  2207  CAAACTGCAAATCCACAGAATGCTTGGACAATTGTGTTTGCAGGGTTCAATGCATAAGTT  2266
Sbjct  815   .........G...T........T.C..................................  874

Query  2267  GTTCGAAGGCGTGCCATTGCTCTGATATGTGCTCAAACAGACCATTTCGGAGAGATAGAA  2326
Sbjct  875   ..............G............................T...........G.A..  934

Query  2327  AGATACAAGTTGTCAAGACTGAACTTTGTGGTTGGGGGGTAGTAGCATCTGAATCCATAA  2386
Sbjct  935   .....A.............A............................G............  994

Query  2387  ACAAAGGTGATTTCATAATTGAGTACATCGGCGAAGTTATTGATGATGCCTTGTGTGAAA  2446
Sbjct  995   .G........C.....................T.........................C..G.  1054

Query  2447  AAAGGCTATGGGACATGAAATACAAGGGAGTTCAAAATTTCTACATGTGTGAACTACGGA  2506
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Sbjct  1115  ......................G.....A...........T..C........C.......  1174

Query  2567  GTGATCCTAATTGCAAATTGGAAAAATGGCAGGTTGAAGGTGAGACTCGAATTGGTGTCT  2626
Sbjct  1175  ................C.............................G.........  1234

Query  2627  TTGCTGCTCGATTCATTGAAGTAGGAGAACCTCTGACCTATGATTACAGATTTGTTCTGT  2686
Sbjct  1235  .........A..C.....................................G.A..  1294

Query  2687  TTGGTTCAGAAGTGAAGTGCCATTGTGGAGCTTCGAATTGTCAAGGATATCTTGGCAGTA  2746
Sbjct  1295  ....AC.......A......A.............A...........C.....G..T..C.  1354

Query  2747  AAAAGAAAATCACAAGTAAACTGGACATCAGCTGGGGTTCGAAACGCAAGAGAACATCTA  2806
Sbjct  1355  ..........TGTG.C..C...........T.T......A.........G...........  1414

Query  2807  CTTCTTGCCTCACCATCATTAAATCGAATTCATGTTAG  2844
Sbjct  1415  .G........AG.G..........T....A.G......  1452
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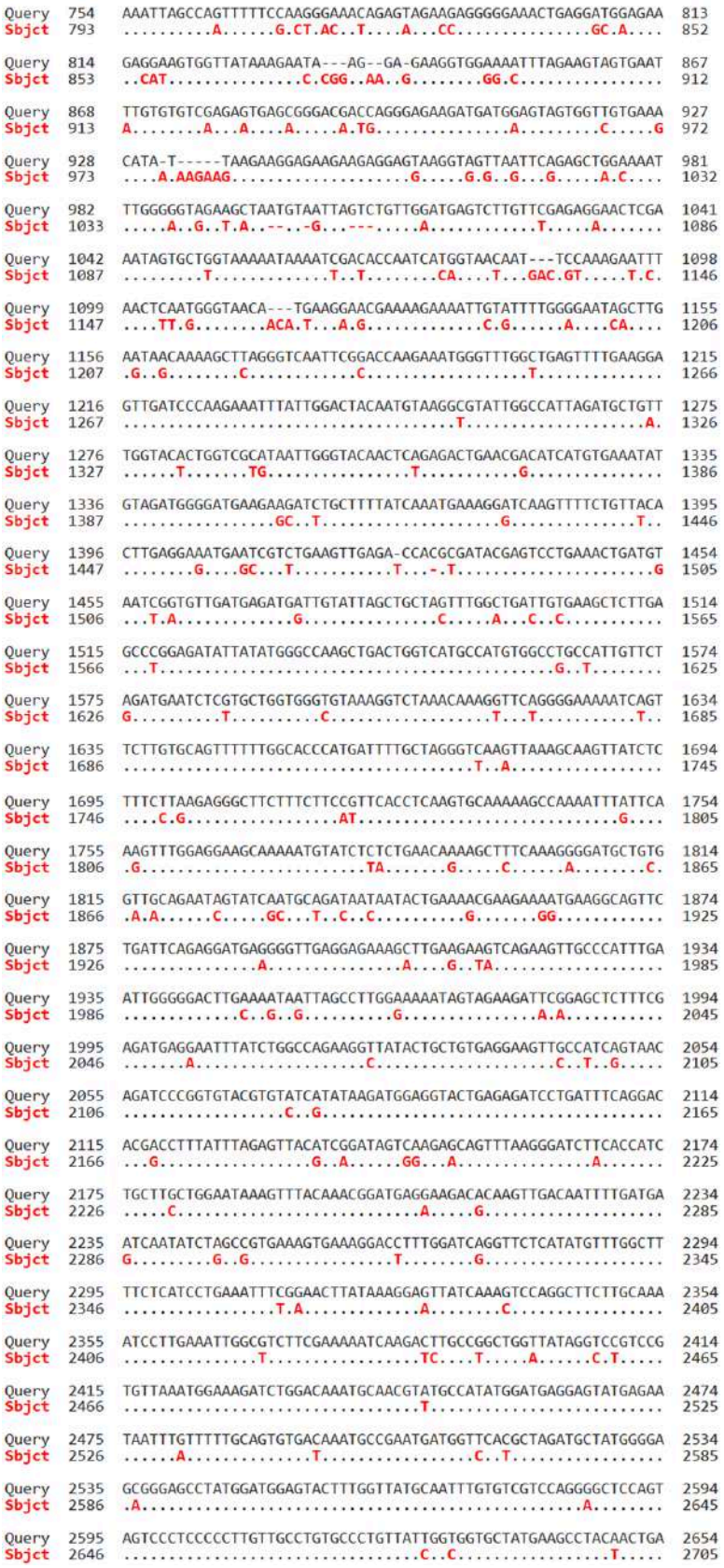
Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
1757	1757	46%	0.0	91.14%	1452	Query_4971543

Figure 9: *In silico* data showing the percentage similarity and coding sequence alignment between *StSDG4* (Query shown in fig) and *NbSDG4* (Sbjct shown in fig).

In silico data showing similarity between *StATX1* and *NbATX1*



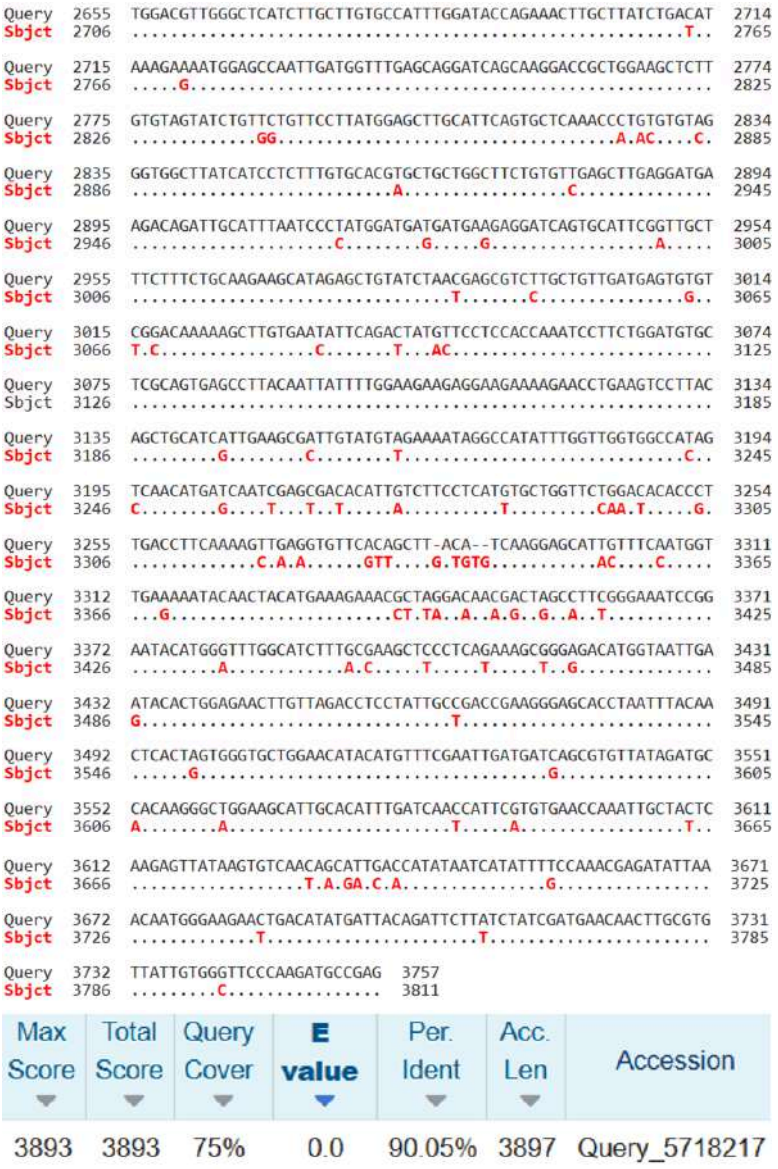


Figure 10: *In silico* data showing the percentage similarity and coding sequence alignment between *StATX1*(Query shown in fig) and *NbATX1*(Sbjct shown in fig).

Part 1: Regeneration assay for overexpression lines of *StSDG4* and *StATX1* in *Nicotiana benthamiana*.

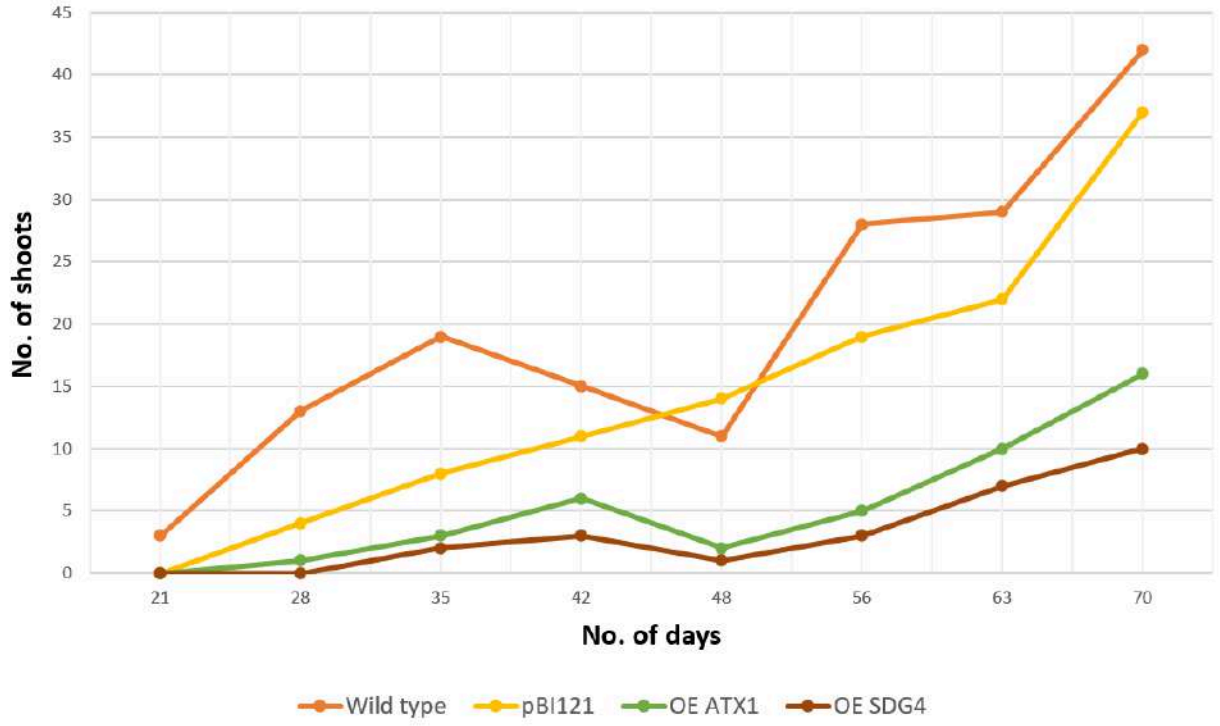


Figure 11: Graph showing number of shoots developed in an interval of 7 days from ~ 50 leaf discs after transformation in overexpression *StSDG4* and overexpression *StATX1* with control as empty vector (*pBI121*) and wild type in *N. benthamiana.* It is observed that there is a delay of about 1-2 weeks for first shoot formation and 3-4 weeks for subsequent increases in shoots in overexpression *StSDG4* and overexpression *StATX1* with respect to control in all 3 rounds of transformation done.

N. benthamiana is one of the best model plants to generate transgenic lines due to its high regenerative capacity. In the process of transgenic line generation of *StSDG4* and *StATX1* in *N. benthamiana* with respect to pBI121(as vector control), it was observed that there is a delay in shoot formation more than that the no: of shoot formed was also too low (Figure 11, Table 2). From the graph, a delay of about 1-2 weeks for first shoot formation and 3-4 weeks for subsequent increase in shoots was observed after transformation in all 3 rounds, which proves *SDG4* and *ATX1* have some sort of significant role in regeneration.

Constructs	No. of explants	Regeneration efficiency (in %) - 21 days	Regeneration efficiency(in %) - 35 days	Regeneration efficiency(in %) - 49 days	Regeneration efficiency(in %) - 63 days
Wild type	55	5.4	34.5	20	71
pBI121(vector control)	50	0	16	28	44
35S::FLAG StSDG4_pBI121	54	0	3.7	1.8	12.9
35S::FLAG StATX1_pBI121	57	0	5.26	3.50	17.5

Table 2: Regeneration efficiency in *N. benthamiana* after A*grobacterium* mediated plant transformation in overexpression *StSDG4*, overexpression *StATX1*, empty vector (*pBI121*) and wild type as control. It is seen that regeneration efficiency is low in these overexpression line compared to controls

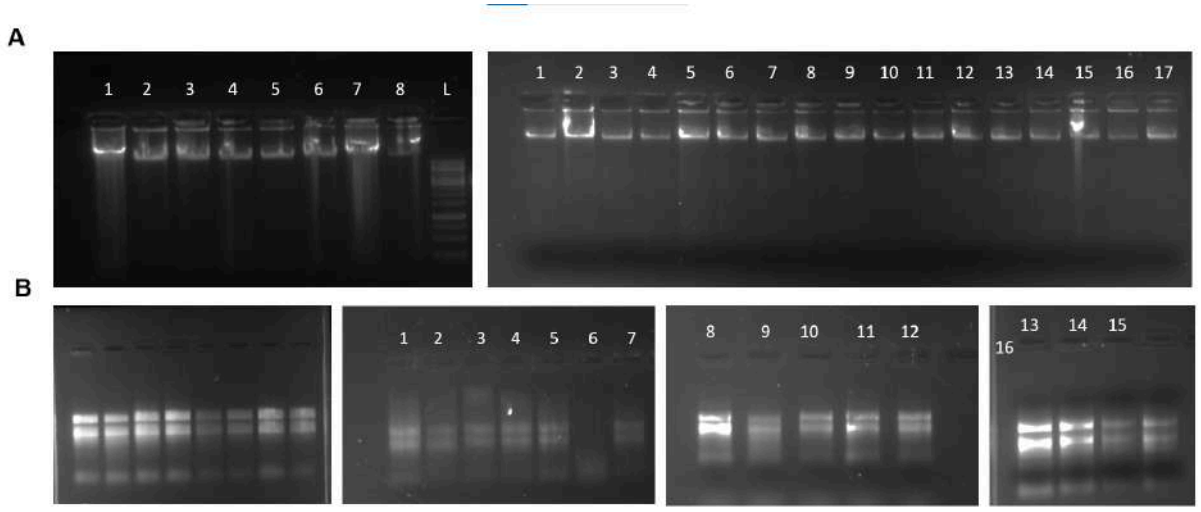


Figure 12: (**A**) Agarose gel showing isolated genomic DNA using CTAB method for putative overexpression lines of *StSDG4* and *StATX1*, and vector control lines.(**B**) Agarose gel image showing RNA isolated from putative overexpression of *StSDG4* and *StATX1*, and vector control lines for molecular confirmation.

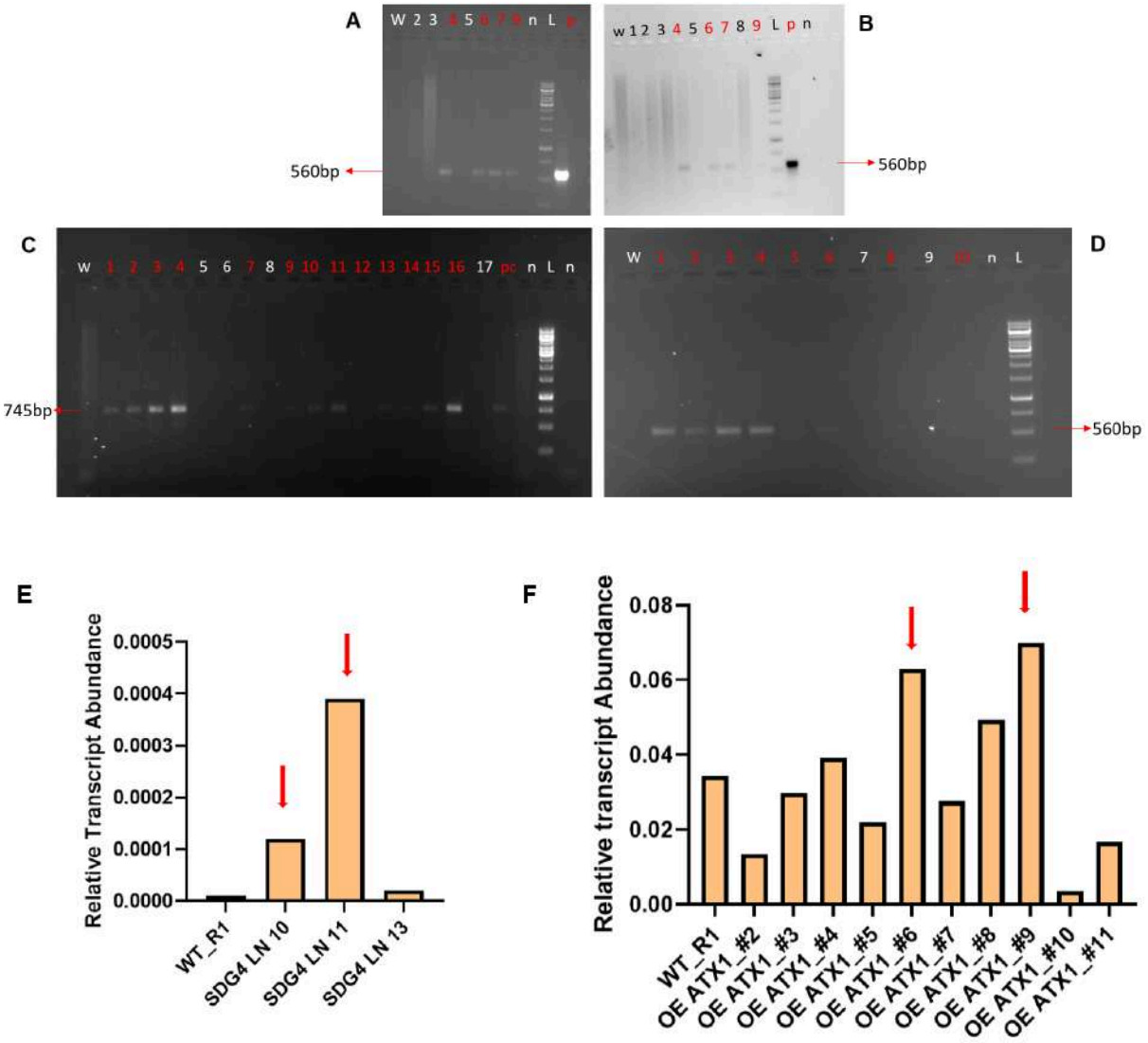


Figure 13: Molecular confirmation of transgenic lines in *N. benthamiana*. From genomic DNA PCR for overexpression *StSDG4* lines(**A**) line number 10, 11, 12 and 13 are positive with no band in wild type and negative control with vector specific primers, overexpression *StATX1* lines(**C**) line number 3, 4, 5, 6, 7,8,9 and 10 are positive with no band in wild type and negative control with vector specific primers and for vector control(*pBI121*) lines(**C**) line number 1, 2, 3, 4 and 8 are positive with no band in wild type and negative control with vector specific primers. From RT PCR for overexpression *StSDG4* lines(**B**) in line no 10, 11, 12 and 13 are positive with no band in wild type and negative control with vector specific primers, overexpression *AtATX1* lines(**D**) in line number 2, 3, 4, 5, 6, 7, 9 and 11 are positive with no band in wild type and negative control with vector specific primers. For qRT PCR of overexpression *StSDG4* lines(**E**) in line number 10, 11 and 13 overexpressed compared to wild type with gene specific primers and for overexpression *StATX1* lines(**F**) in line number 4, 6, 8 and 9 overexpressed compared to wild type with gene specific primers. Those lines with red arrows were the ones taken for regeneration assay. The lanes labeled in red is positive, and those with white is negative with 1kb ladder

After subsequent rounds of transformation and subculture of those shoots, very few were rooted in antibiotic selection, which was considered as putative positive overexpression lines. These lines were molecularly confirmed through genomic DNA and RNA. From genomic DNA PCR for overexpression *StSDG4* lines(Figure 13A); line number 10, 11, 12 and 13, for overexpression *StATX1* lines(Figure 13C); line number 3, 4, 5, 6, 7, 8, 9 and 10, and for vector control(pBI121)(Figure 13C): line number 1, 2, 3 and 4 are positive with no band in wild type and negative control. From RT PCR for overexpression *StSDG4* lines(Figure 13B); line number 10, 11, 12 and 13, for overexpression *StATX1* lines(Figure 13D); line number 2, 3, 4, 5, 6, 7, 9 and 11 are positive with no band in wild type and negative control. From qRT PCR for overexpression *StSDG4* lines(Figure 13E); line number 10, 11 and 13 and for overexpression *StATX1* lines(Figure 13F); line number 4, 6, 8 and 9 show increased relative transcript abundance compared to wild type which proves those are overexpression lines.

After performing a regeneration assay for confirmed overexpression lines of *StSDG4* and *StATX1,* it too shows a huge delay in callus as well as shoot formation. It is observed that compared to controls in *StSDG4* the leaf disc delays callus formation(Figure 14) by 1-2 weeks as well as shoot formation(Figure 15, Table 3) was not observed even after 2 months of regeneration assay and the colour of leaf disc started pale yellowing which is not a sign of shoot acquisition. It is also observed that compared to controls, in the StATX1 overexpression line; the leaf disc doesn't delay in callus formation(Figure 14), but shoot formation(Figure 15, Table 3) was not observed even after one and half months of regeneration assay. There is a chance of shoot formation as the colour of the leaf disc is still greenish with callus proliferation.

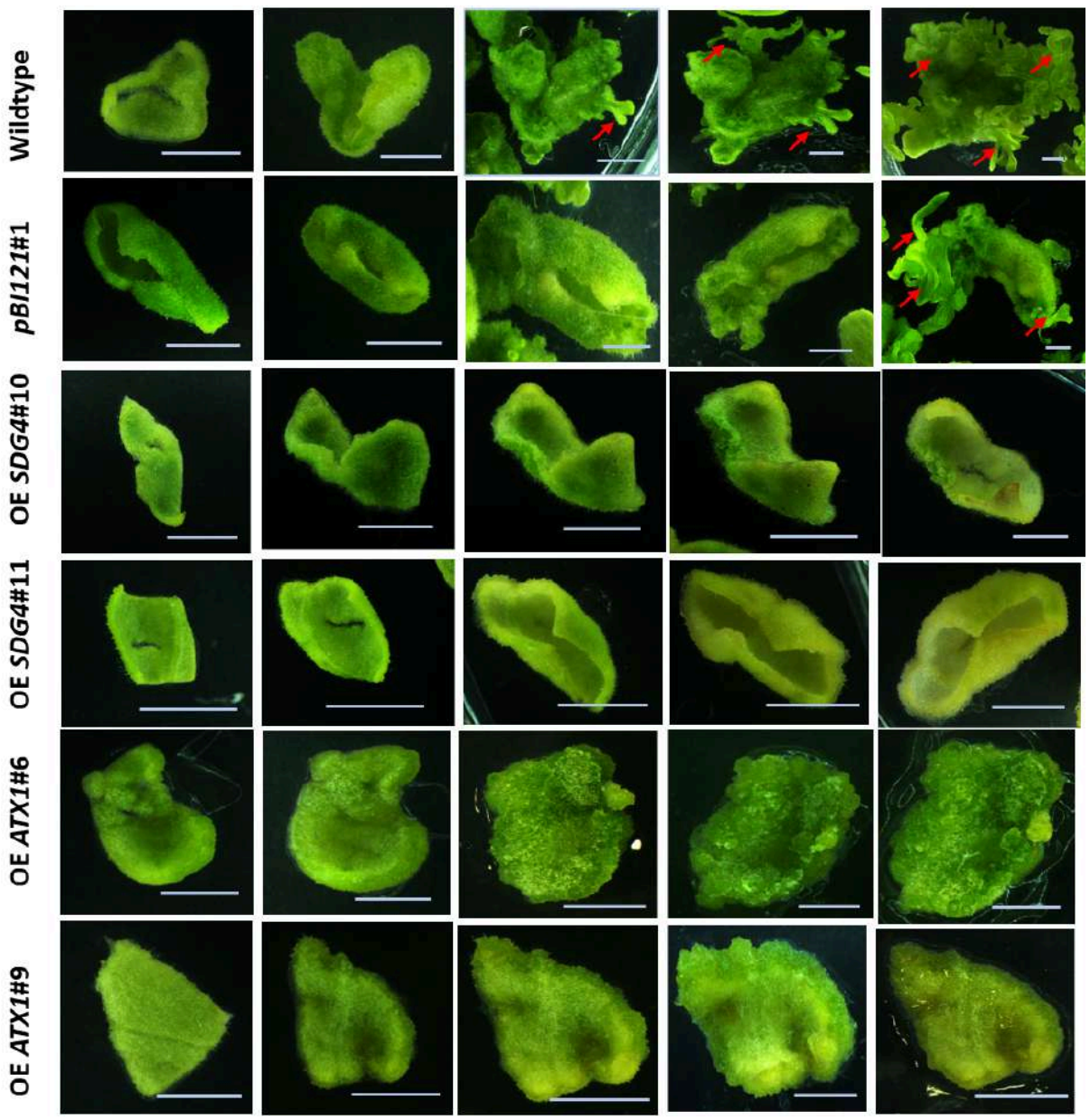


Figure 14: Images of leaf disc during *de novo* shoot regeneration in *N. benthamiana.* Wounded leaf discs were incubated on regeneration media for confirmed overexpression lines of *StSDG4* and St*ATX1*, vector control, and wild type. It shows that there is a huge delay in callus as well as shoot formation in *StSDG4* and St*ATX1* overexpression compared with vector control and wild type. The red arrow shows the regenerated shoots.(Scale is in the range of 1cm - 2cm)

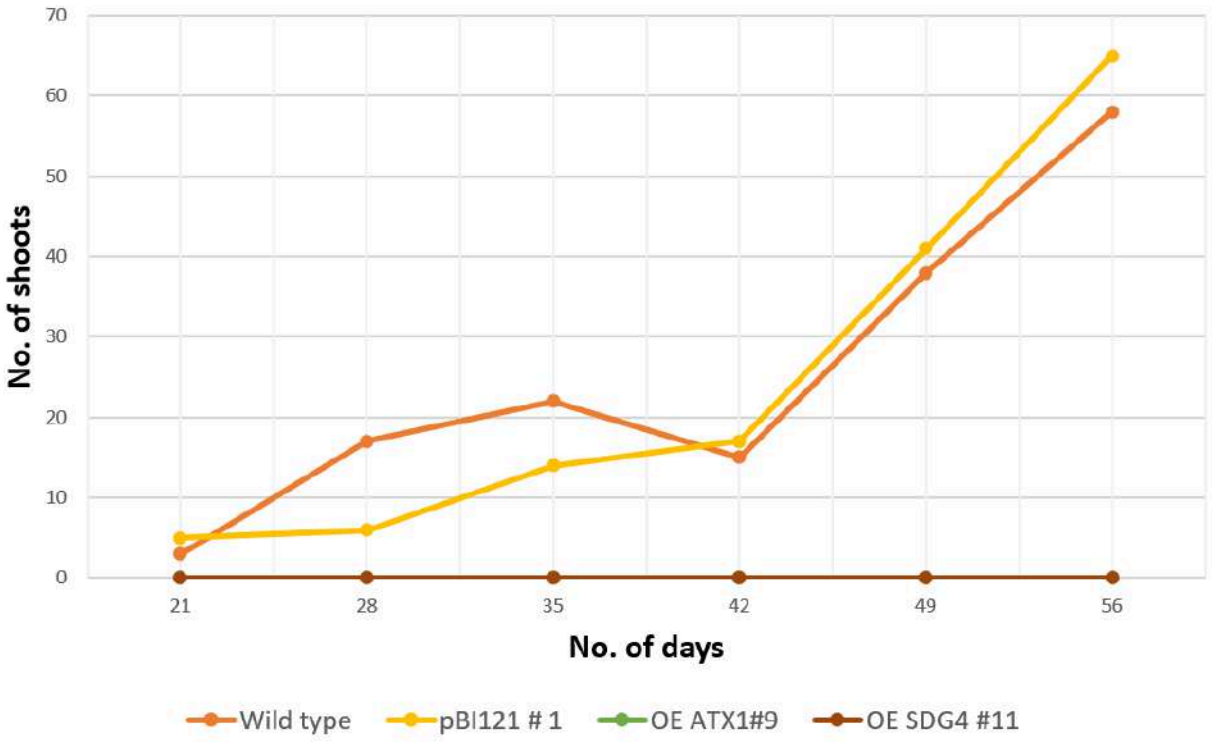


Figure 15:Line graph showing the number of shoots counted in every 7-day interval from almost 50 leaf discs after regeneration assay in overexpression *StSDG4* and overexpression *StATX1* with vector control (*pBI121*) and wild type in *N. benthamiana.* It is observed that there is a huge delay in shoot regeneration(no shoots regenerated from leaf disc even after 2 months) in overexpression *StSDG4* and overexpression *StATX1* with respect to control in regeneration assay

Constructs	No. of explants	Regeneration efficiency (in %) - 21 days	Regeneration efficiency(in %) - 35 days	Regeneration efficiency(in %) - 63 days
Wild type	50	6	44	73.4
pBI121(vector control)	53	9.4	26.4	89
35S::FLAG StSDG4_pBI121	52	0	0	0
35S::FLAG StATX1_pBI121	49	0	0	0

Table 3: Regeneration efficiency in *N. benthamiana* after regeneration assay conducted in overexpression of *StSDG4*, overexpression of *StATX1*, vector(*pBI121*) lines and wild type as control.

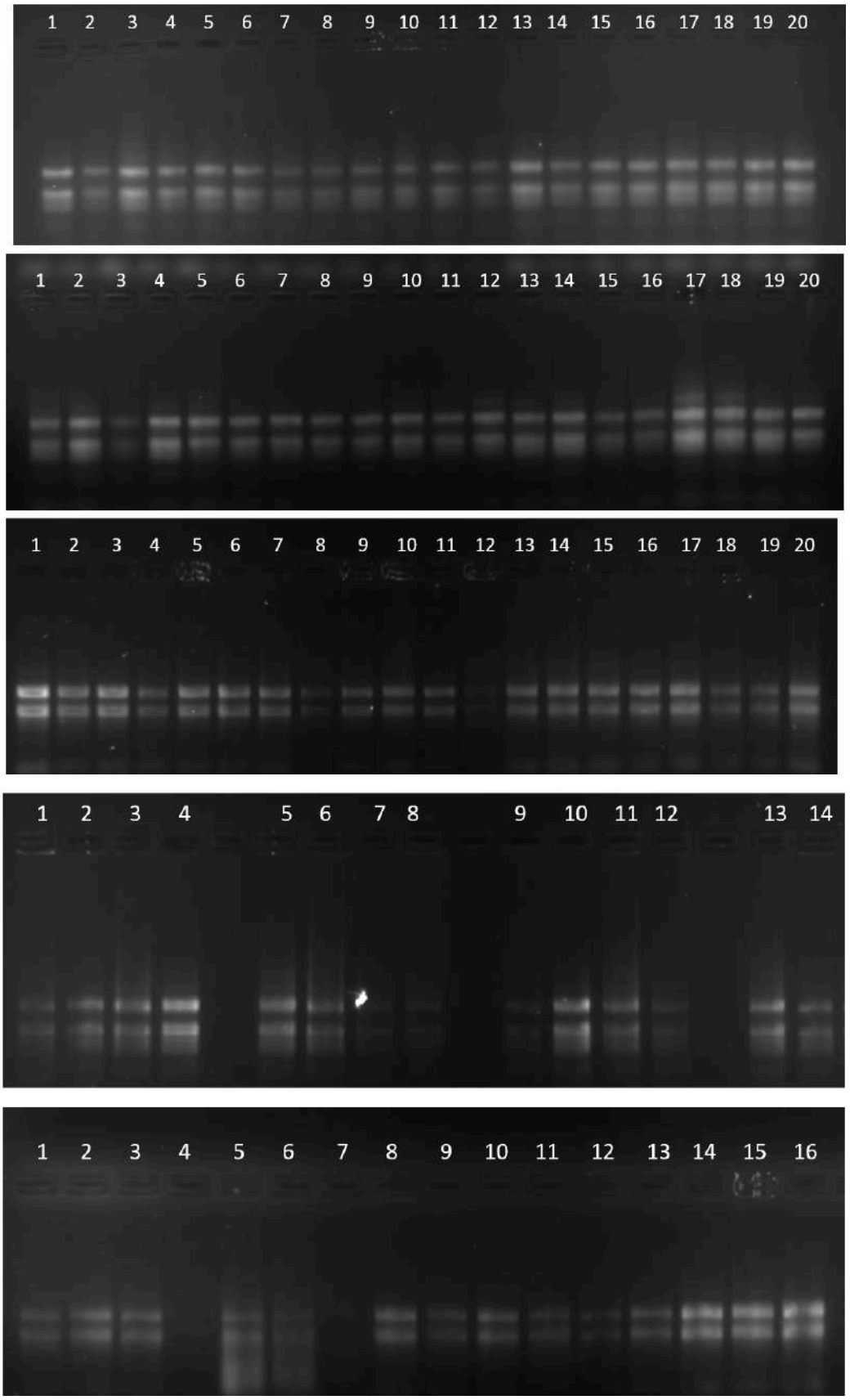


Figure 16: Agarose gel showing isolated RNA for checking expression levels of regeneration markers from tissue harvested after the assay.

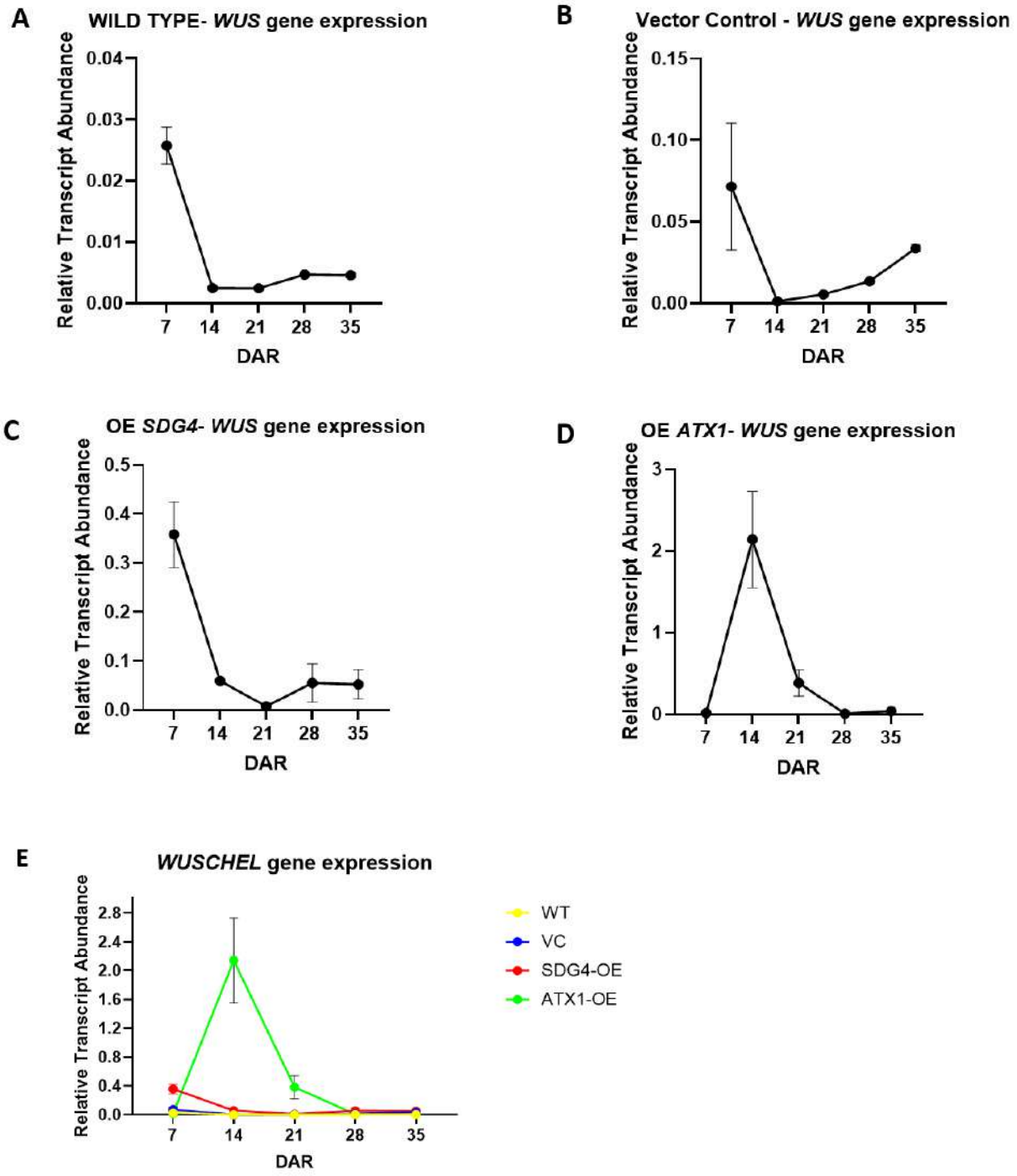


Figure 17: Quantitative analysis of *WUSCHEL* gene expression in different stages of explant (leaf discs) subjected to *de novo* shoot regeneration in *N. benthamiana* . *WUS* gene expression in different stages of explant subjected to *de novo shoot regeneration* in Wild type(**A**), vector control(**B**), overexpression SDG4(**C**), overexpression ATX1(**D**) and combined graph(**E**). [mean ± SD, n = 3 biological replicates, technical replicate] DAR - Days after regeneration

In wild type(Figure 17A), *WUS* expression is highest at day 7, then reduces and stays the same afterwards. In vector control(Figure 17B) on the other hand, *WUS* expression is higher than wild type, but remains the same trend where it is highest on day 7, and then reduces and stays low for the rest of the assay. In the case of the overexpression lines of *StSDG4* and *StATX1*, *WUS* expression is significantly high throughout. In *StSDG4*(Figure 17C), the trend is similar to wild type and vector control, where it starts high and drops to a lower level and slightly increases by day 35. In the case of *StATX1*(Figure 17D), *WUS* expression is significantly low compared to others, but climbs to a higher value comparable to the *StSDG4* line by day 14, then drops to a lower value and slightly increases by day 35.

Part 2: Regeneration assay in promoter lines of *StSDG4* and *StATX1* in *Nicotiana benthamiana*

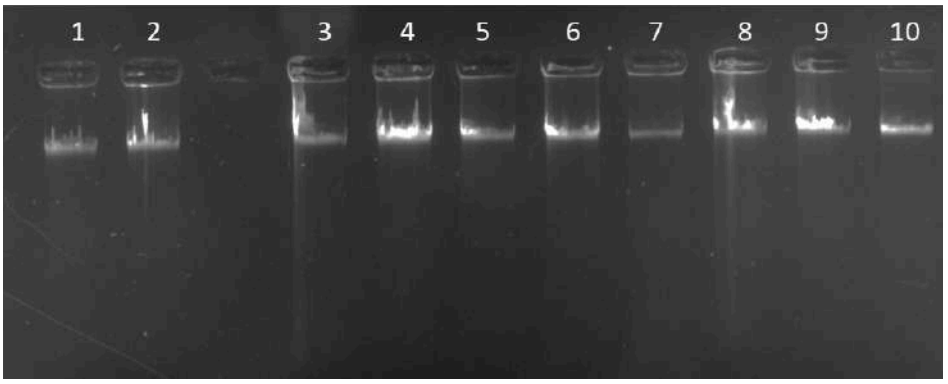


Figure 18: Agarose gel showing isolated genomic DNA isolated using CTAB method from putative promoter lines of *StSDG4* and *AtATX1*.

In one round of transformation itself, the required amount of shoots was produced for promoter lines of *StSDG4* and *StATX1*, which were confirmed by molecular analysis. In genomic DNA PCR(Figure 19), for *StSDG4* promoter line number 1, 2, 3, 5, 6, 7 and 8; for *StATX1* promoter line number 1 is positive with no band in wild type and negative control.

Figure 19: Molecular confirmation of promoter lines in *N. benthamiana*. From genomic DNA PCR for promoter *StSDG4* lines line number 1, 2, 3, 5, 6, 7 and 8 are positive with no band in wild type and negative control with vector specific primers and promoter *AtATX1* lines line number 1 is positive with no band in wild type and negative control with vector specific primers. The lanes labeled in red are positive, and those with white are negative with a 1Kb ladder.

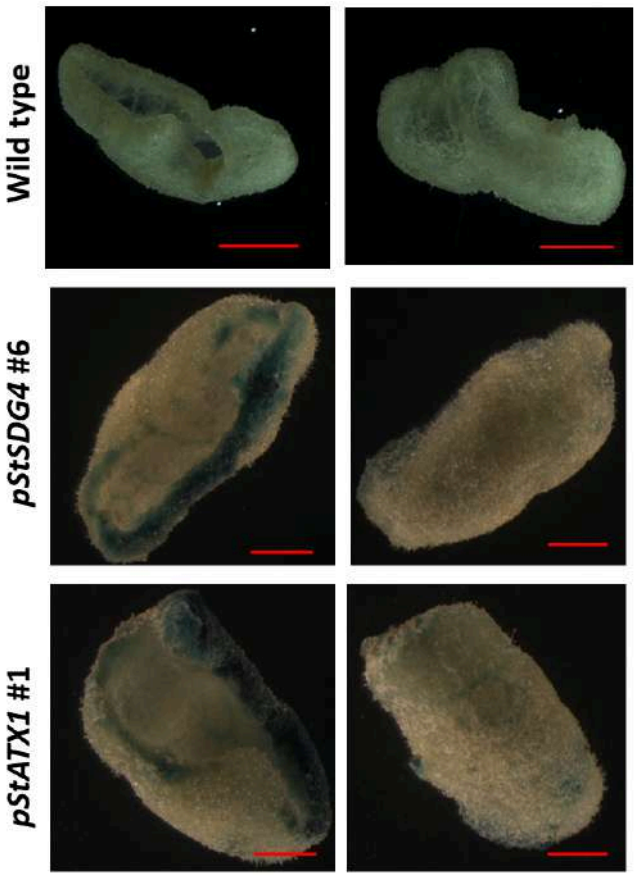


Figure 20: GUS activity was evident in leaf discs of promoter lines of *StSDG4* and *StATX1* compared to wild-type. The transgenic promoter reporter line *StSDG4* and *StATX1*, while doing regeneration assay(3 weeks) when infused with GUS staining solution, showed a GUS expression in the wounded portion where callus started proliferating.

While performing GUS assay in a leaf disc that had undergone regeneration experiments of promoter *StSDG4* and promoter *StATX1* in *N. benthamiana*, it is observed that expression was there in callus (Figure 20).

Part 3: Regeneration assay for overexpression of *StSDG4* and *StATX1* in *A.thaliana;* and knockout of *AtSDG4* and *AtATX1* in *A.thaliana*

Constructs	No: of rounds of transformation	No: of seeds germinated	No: of transformants	Transformation efficiency
35S::FLAG StSDG4_pBI121	2	1200	2	0.16
35S::FLAG StATX1_pBI121	3	1000	0	0
pStATX1:: FLAG StATX1	3	1200	5	0.41
pBI121(vector control)	3	300	40	13.33
pStSDG4	1	100	18	18
pStATX1	1	100	23	23

Table 4: Transformation efficiency in *A. thaliana*. It is observed that transformation efficiency is very low in overexpression *StSDG4* and *StATX1* in *A. thaliana* compared to the vector and *St*Promoter of these genes.

StSDG4 and *StATX1* overexpression transgenic lines were generated in the reference model plant, *A. thaliana*, through 2 - 3 rounds of transformation. It was observed that compared to vector control as well as promoter line generation, transformation efficiency was excessively low for overexpression *StSDG4* and *StATX1* in *A. thaliana*(Table 4). It's noticed that even the promoter driving this gene plays a role in regeneration because for *StATX1* with *35S* promoter, there were no transformants, but with the native promoter of *ATX1,* it was observed that very few were transformed(Figure 21). Survival of the transformants in soil was difficult, especially in the case of the *StSDG4* overexpression line.

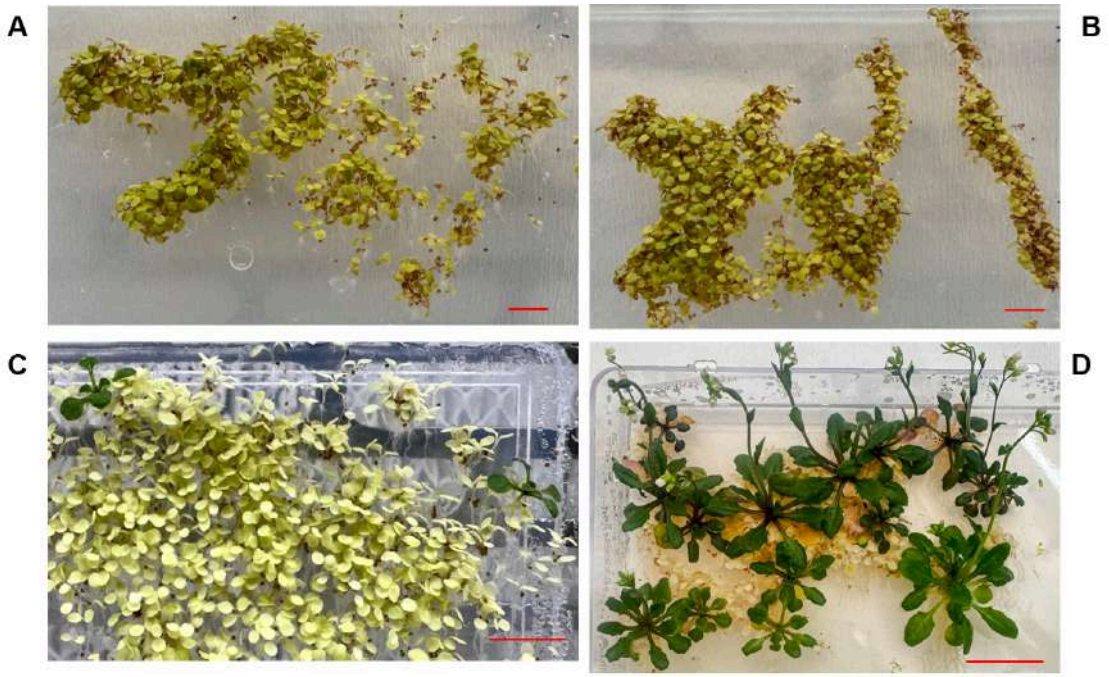


Figure 21: Plates showing seedlings from the seeds collected after *Arobacterium*-mediated plant transformation, which is subjected to antibiotic selection marker for differentiating transformants and non transformants in *A. thaliana.* It is observed that the number of transformants is 0 in overexpression *StSDG4*(**A** - 2 weeks old) and *StATX1* (**B** - 2 weeks old) compared to vector control(**C** - 3 weeks old) and promoter lines(**D** - 4 weeks old) in *A. thaliana*. After multiple rounds of seed plating, very few transformants were found in overexpression *StSDG4* and *StATX1* in *A. thaliana.*

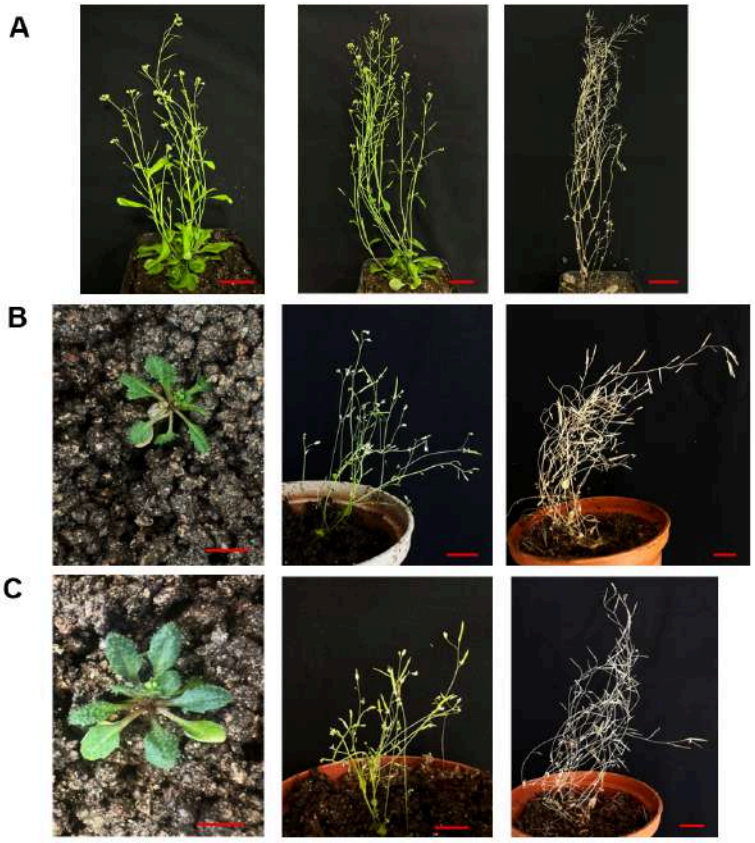


Figure 22: Stages of T1 generation overexpression *StSDG4*(**C**), *StATX1*(**B**), and vector control(*pBI121*)(**A**) line in *A. thaliana*. It is observed that the phenotype of these overexpression lines are affected compared to vector control lines. Overexpression lines show retarded growth with less proliferated shoot as well as weak stem. (The stage of plants for vector control is 3 weeks, 5 weeks, and 7 weeks; for overexpression *StSDG4*, it is 1 week, 5 weeks, 7 weeks, and for overexpression *StATX1*, it is 1 week, 5 weeks, 7 weeks in soil).

Very few of the T1 generation overexpression *StSDG4* and *StATX1* of *A. thaliana* only survived in soil whose phenotype was affected compared to vector control T1 generation. From Figure 22, *StSDG4* overexpression as well as *StATX1* overexpression lines show less proliferated plants with weak stem and retarded height compared with vector control lines in *A. thaliana*.

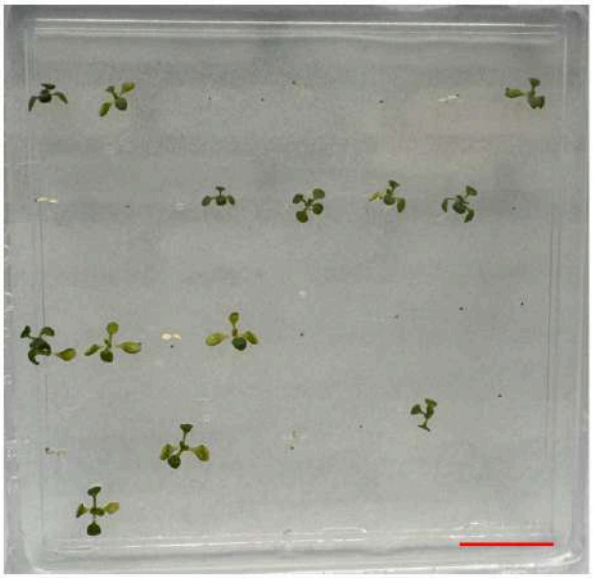


Figure 23: *A. thaliana* plate showing T2 generation overexpression *StSDG4* lines from T1 generation overexpression *StSDG4* line number 2

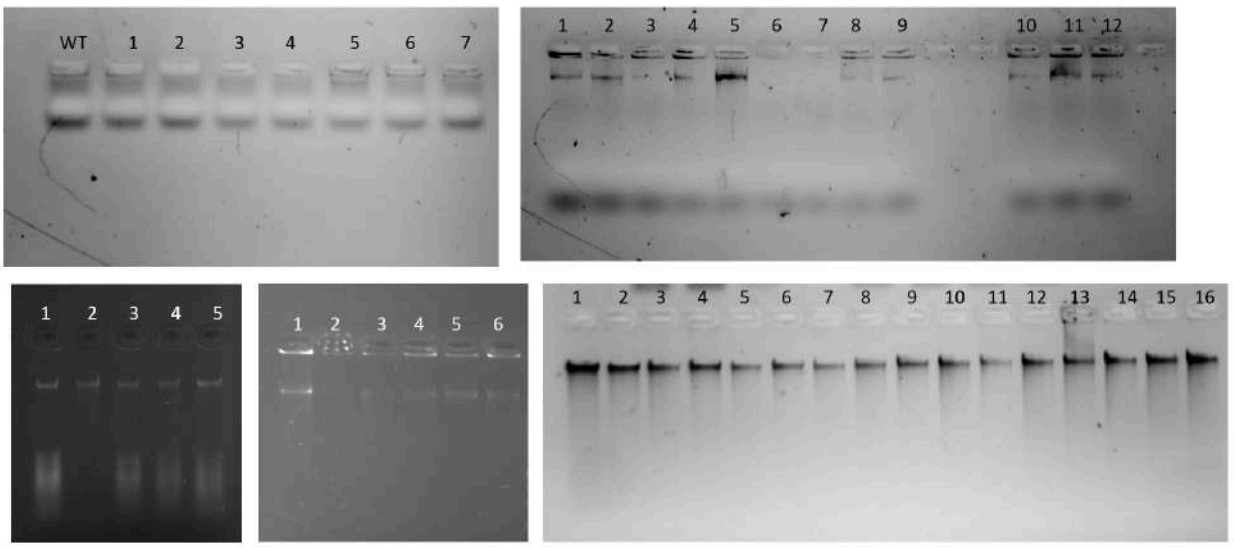


Figure 24: Agarose gel showing genomic DNA isolated using CTAB method for molecular confirmation of *AtSDG4* and *AtATX1* knockout lines.

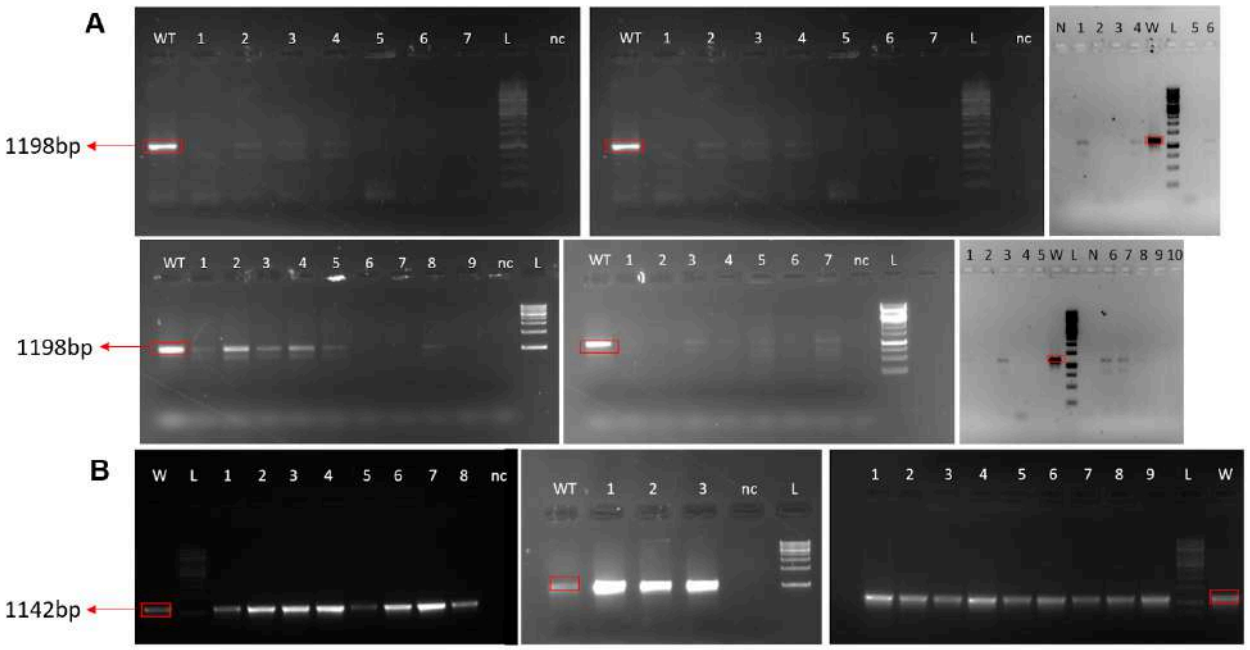


Figure 25: Molecular confirmation of knockout lines of *AtSDG4* and *AtATX1* in *A. thaliana.* From genomic DNA PCR for knockout lines of *AtSDG4* line(**A**) no 1, 2, 3, 4, 5 and 8 are heterozygous with single band in wild type and no band in negative control but knockout lines of *AtATX1*(**B**) shows single band at same size of wild type(not even heterozygous) with no band in negative control. 1 kb ladder is used as a reference.

Figure 26: *A. thaliana* plate showing T4 generation *AtSDG4* knockout lines from T3 generation *AtSDG4* knockout line number 3

Out of 100 seeds received from ABRC, genotyping was done after plating and genomic DNA isolation to figure out which all lines are homozygous as they have provided T3/T4 generation *AtSDG4* and *AtATX1* knockout lines seeds. In the case of *StSDG4*, almost 70% germinated, out of which all were heterozygous (Figure 25A), but for *StATX1*, only 10-12% germinate, and all showed a similar pattern as wild type (Figure 25B).

Following these, seeds were collected from this generation also and were plated(Figure 26) to see if it is homozygous, but the few which were genotyped show heterozygous as well as no amplification at all, which is inconclusive. (According to the generation number, it should be putative homozygous)

Discussion

The mechanism behind the regeneration of lost limbs and organs is one of the most sought-after answers in the whole of biology. In animals, almost all phyla show regeneration up to some level(Alvarado et al. 2004). Plants, on the other hand, exhibit excellent potential to regenerate, which is evident in totipotency, meristematic growth, and asexual reproduction(Echeverri and Zayas et al. 2018). Thus, plants are a perfect model organism to study the genetic mechanisms leading to regeneration. Our lab has preliminary data on *SDG4* and *ATX1*, which hint at the fact that the two *Solanum tuberosum* trithorax genes influence *de novo* shoot regeneration.

The objective of this project was to probe the conserved functions of the *Solanum tuberosum* trithorax members *SDG4* and *ATX1* using two model plants, *Nicotiana benthamiana* and *Arabidopsis thaliana*. To study whether the role of these genes in the *de novo* shoot regeneration is conserved, we planned to generate overexpression lines of these two genes in these model plants and look at the effect it has on regeneration.

Out of the two, overexpression lines for *N.benthamiana* were generated. During the process of agrobacterium-mediated plant transformation to generate transgenic lines, the leaf discs showed a delay in shoot regeneration from callus. Also, the number of shoots regenerated was extremely low compared to the wild type and vector control.

Putative lines were molecularly confirmed using genomic DNA and RNA. From genomic DNA PCR and RT PCR for overexpression *StSDG4* in *N. benthamiana*, line numbers 10, 11, 12, and 13 were confirmed and were subjected to qRT PCR for checking the overexpression levels. Out of the four lines with confirmed overexpression, lines number 10 and 11 were selected for the regeneration assay. For overexpression StATX1 in *N.benthamiana*, line numbers 3, 4, 5, 6, 7, 8, 9 and 11 were confirmed using genomic DNA and RT PCR and were further similarly subjected to qRT PCR. Out of these, line numbers 4, 6, 8, and 9 show overexpression compared to the wild type. From which line number 6 and 9 which showed higher were selected for regeneration assay.

To confirm the role of *StSDG4* and *StATX1* in regeneration, a regeneration assay was performed using overexpression lines of these genes that were generated initially in *N.benthamiana*. While performing the assay, it was observed that there is a huge delay of two weeks in callus regeneration in *StSDG4* and one week of delay in callus regeneration in *StATX1* compared to the wild type and vector control line. For shoot regeneration, it was observed that, even after two months of regeneration assay, there is no observed shoot formation from overexpression *StSDG4* and *StATX1*. But, the callus of the *StSDG4* overexpression line shows a pale yellow colour, which indicates a reduced probability of getting shoot regeneration. On the other hand, the callus of *StATX1* remains green in color, which is an indication of a higher probability of shoot regeneration.

To understand the underlying mechanism behind this delay in shoot regeneration, we have selected regeneration markers *WUS, WOX5, WOX11, LBD16, LBD18, PLT3, CUC1* and *STM*(Liu, Jie, et al. 2018, Wei, Xi, et al. 2020) . It is established that markers such as *WOX11, LBD16, LBD18* and *PLT3*(Wei, Xi, et al. 2020) are involved in callus regeneration. Similarly, markers such as *WUS, STM* and *CUC1*(Wei, Xi, et al. 2020) have been shown to be involved in shoot regeneration. *WOX5* is involved in root regeneration as well(Wei, Xi, et al. 2020). We plan to check the quantitative levels of the expression of these regeneration markers in leaf discs that were harvested in intervals of seven days during the regeneration assay. Since we observed callus regeneration (even though after a delay) but no shoot regeneration, we expect to see a deviation in the expression levels of these shoot regeneration markers.

Similarly, to explore the conserved role of *StSDG4* and *StATX1* in the regeneration of A.thaliana, overexpression lines of these two genes are being generated in *A.thaliana* using agrobacterium-mediated plant transformation. It was observed that the transformation efficiency was low for these genes compared to the vector and its promoter. Since the T1 generation is heterozygous, we have grown the transformants and collected the seeds, and they are being processed for genotyping.

To confirm that *SDG4* and *ATX1* itself plays a role in regeneration , we have acquired T3/T4 generation seeds of *AtSDG4* and *AtATX1* knockout lines. Since the acquired lines were putative homozygous, it was subjected to genotyping to reconfirm whether they were heterozygous or homozygous. It was found that all the acquired

and germinated *AtSDG4* knockout line seeds are heterozygous. But for *AtATX1* knockout lines, in genotyping, it was found that it has a similar banding pattern as wild type. So, further investigations proceeded only on *AtSDG4* knockout lines.

Since, for proceeding to regeneration assay, we need homozygous knockout lines. The *AtSDG4* knockouts, which were heterozygous, were grown, and seeds were collected, processed, and plated. During the genotyping of a few lines, it was observed that they are heterozygous. Further seeds are being planted.

It is nearly confirmed that *StSDG4* and *StATX1* play a role in regeneration from previous studies from the lab. The conservation of this gene in the Solanaceae family is confirmed from the experimental result, which was done in *N. benthamiana*. It was observed that regeneration capacity was affected. For understanding the underlying mechanism, regeneration marker levels are being checked now in 7-day interval harvested leaf discs (explant of regeneration). After understanding those deviations, we can look at the hormonal gene markers associated with the respective markers to see whether the hormone biosynthesis, transport, and signalling, or the response is affected for regeneration to take place. Finally, to find the interactors of *SDG4* and *ATX1* in callus and shoot regeneration, immunoprecipitation assay can be done, and further down the line, confirmation can be done with Y2H and BiFC.

Similarly, to show the conservation of these genes in *A. thaliana,* pure homozygous lines for doing assay are being generated. Both overexpression and knockouts are heterozygous, which needs to be grown again to get homozygous such that a regeneration assay can be carried out. If a similar trend is observed in the results of the regeneration assay, then it can be confirmed that these genes play a crucial role in regeneration all over plants. This will bring us one step closer to finding the underlying mechanism and the conserved role of the genes of interest in regeneration combined with the results in *N. benthamiana*.

Salient Findings

- Overexpression of *StSDG4* and *StATX1* (trithorax members), and vector control lines were generated in *N. benthamiana*
- In *de novo* shoot regeneration assays performed, it was observed that compared to controls in the leaf disc of overexpression line of *StSDG4*, callus formation was delayed by 1-2 weeks as well as shoot formation was not observed even after 2 months of regeneration assay. It is also observed that compared to controls, in the *StATX1* overexpression line; the leaf disc doesn't delay in callus formation but shoot formation was observed only after one and half months of regeneration assay in *N. benthamiana.*
- *WUSCHEL* gene expression was noticed to be high in these overexpression lines in *N. benthamiana* compared to controls for leaf discs subjected to regeneration assay suggesting that it may not be directly involved in affecting shoot regeneration process.
- In *de novo* shoot regeneration assays performed in promoter lines of these trithorax members in *N. benthamiana,* GUS activity was visualized in callus of leaf discs suggesting that these *trithorax* genes are likely involved in cell de-differentiation and early steps of regeneration.
- T1 generation of overexpression lines were generated for *StSDG4* and *StATX1* in *A. thaliana.*
- T3 generation of knockout lines were also generated for *AtSDG4* and *AtATX1* in *A. thaliana*.
- In *A. thaliana,* T1 generation of *StSDG4* overexpression as well as *StATX1* overexpression lines showed less proliferated plants with weak stem and retarded height compared with vector control lines.

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Supplementary Data

Constructs	Date of transformation	No. of lines molecularly confirmed	Regeneration Assay
Wild type			✓
pBI121(vector control)	E1-06/06/2024 E2-19/10/2024 E3- 22/12/2024	7	✓
35S::FLAG StSDG4_pBI121	E1-06/06/2024 E2-19/10/2024 E3- 22/12/2024	4	✓
35S::FLAG StATX1_pBI121	E1-06/06/2024 E2-19/10/2024 E3- 22/12/2024	5	✓
pStSDG4	E1-06/06/2024 E2-22/12/2024	8	✓
pStATX1	E1-06/06/2024 E2-22/12/2024	1	✓

Figure S1: Update regarding de novo shoot regeneration of overexpression lines of *StSDG4* and *StATX1* in *N. benthamiana* .

Constructs	Date of transformation	Transformants (T1 generation)	T2 generation	Molecular Confirmation	Regeneration Assay
pBI121(vector control)	E1-26/07/2024 E2-02/09/2024 E3- 17/11/2024	✓	✓	✓	
35S::FLAG StSDG4_pBI121	E1-26/07/2024 E2-02/09/2024 E3- 17/11/2024	✓	✓	✓	
35S::FLAG StATX1_pBI121	E1-26/07/2024 E2-02/09/2024 E3- 17/11/2024	✓	✓	✓	
pStSDG4	E1-06/08/2024	✓	✓		
pStATX1	E1-06/06/2024	✓	✓		

Figure S2: Update regarding de novo shoot regeneration of overexpression lines of *StSDG4* and *StATX1* in *A. thaliana* .

Constructs	T3 generation	Molecular Confirmation	T4 generation	Molecular Confirmation	Regeneration Assay
AtSDG4 Knockout	✓	✓	✓	✓	
AtATX1 Knockout	✓	✓	✓	✓	

Figure S3: Update regarding de novo shoot regeneration of knockout lines of *AtSDG4* and *AtATX1* in *A. thaliana* .