Results......
Cloning, Overexpression and Antisense expression of AtSirB in Arabidopsis thaliana
Results:

*Arabidopsis thaliana* sirohydrochlorin ferrochelatase (*AtSirB*) (Accession Number-NM_103902) has 678 bp of coding region (Fig-1A) for 225 amino acids. Out of 225 amino acids, 46 amino acids at N terminal end act as transit peptide that target *AtSirB* protein into chloroplast. Histidine at position 155 acts as metal chelation site or as general base for the removal of two protons from sirohydrochlorin. Cystein at 198th position may act to house Fe-S cluster (Fig.1A-B).

Dendrogram

A dendrogram was drawn by comparing the amino acid sequences of dicot and monocot plants using phylip (PHYLOgeny inference package) program (Fig. 2). Dendogram of *SirB* protein reveal a high degree of homology between *Arabidopsis thaliana*, *Ricinus communis* and *Populus trichocarpa*. From the lineage of this group *SirB* of several monocot i.e.*Oryza sativa*, *Sorghum bicolor* and *Zea mays* are evolved.

Hydropathic plot

Hydrophilicity plot for *AtSirB* protein was prepared using Macvector 6.0 programs (Kyte and Dolittle). According to the hydrophilicity plot, the *AtSirB* protein was rich in hydrophilic amino acids at its N-terminal and C-terminal regions. However, small stretches of hydrophobic amino acids were present in between the hydrophilic amino acid sequences throughout the whole *AtSirB* protein sequence (Fig. 3A). The hydrophobic patches probably help the protein to loosely bind to the plastidic membrane.

Hopp and Woods Antigenecity Plot

These plots show hydrophilicity instead of hydrophobicity. A more hydrophilic region is a better place for an antigen to bind. Thus an antigenecity plot helps to determine where on a protein a monoclonal antibody would bind well. The X-axis represents the individual amino acids in the protein sequence and the Y axis represents the hydrophilicity. The *AtSirB* protein seems to have even distributions of hydrophilic and hydrophobic regions although there are several hydrophilic peaks where antibodies could bind are present. There are 2 relatively high peaks at amino acids 58 and 93 that could be probable antigenic region in *AtSirB* protein (Fig. 3B).
Fig 1. (A) cDNA sequence of AtSirB showing showing cleavage site of the transit peptide that targets the protein to chloroplast. (B) Amino acid sequences of SirB protein. Out of 225 amino acids of SirB, the N-terminal 46 amino acids act as chloroplastic transit sequence. Histidine at 155th position may act as metal chelation site or as general base for the removal of two protons from sirohydrochlorin. Cystein at 198th position may act to house Fe-S cluster.
Fig 2. Dendrogram of *Arabidopsis thaliana* Sirohydrochlorin ferrochelatase (SirB) protein. Amino acid sequences of SirB protein from different dicot and monocot plants were aligned and the phylogenetic tree was obtained by clustal X software.
Fig 3. (A) Kyte-Doolittle hydropathy plot showing hydropathic residue present in AtSirB. Hydropathy plot shows that AtSirB having no transmembrane domain. (B) Hoop-woods hydrophilicity plot was designed for predicting potentially antigenic regions of polypeptides. Values greater than 0 are hydrophilic and thus likely to be exposed on the surface of a folded protein.
**Light-dependent AtSirB expression**

Seven-day-old etiolated seedlings were exposed to light (80 μmoles photons m⁻² s⁻¹) for 3h, 6h, 12h, and 24h and their total RNA was isolated from etiolated and light-exposed seedlings. RT-PCR analysis of AtSirB was carried out as described before. The AtSirB mRNA was found very low amount in etiolated seedlings that were increased after 3h to 24h of light exposure (Fig. 4). Actin was used as an internal control.

**Tissue specific expression of AtSirB**

*Arabidopsis thaliana* plants were grown in cool-white fluorescent light (100μmoles photon m⁻² s⁻¹) at 22°C in 14h L and 10h D photo-period and their total RNA was isolated from different tissues (roots, shoots, leaf, floral bud, flower and seed pod). Organ specific expression of the AtSirB transcript level was checked by RT-PCR as described in “Materials and Methods”. Three μg of total RNA extracted from different samples i.e., roots, shoots, leaf, floral bud, flower and seed pod were used for cDNA synthesis followed by RT-PCR. The cDNA was prepared from the total RNA and the RT-PCR analysis was done using AtSirB gene specific primers that yielded a fragment of 678 bp. The amplification reactions consisted of 45 sec at 94°C for denaturation, 30 sec at 55°C for annealing and 1 min at 72°C for extension. To ensure linearity of the reaction, the minimum number of cycles needed to visualize the transcript was first determined (it was found to be 27 cycle). When the conditions for RT-PCR linearity were established, runs were performed and repeated thrice, using independent samples. The level of the AtSirB transcript was much higher in leaves and floral buds than those of roots, stems, and flowers and seed pod (Fig. 5). Actin was shown as an internal control.

**Cloning of Sirohydrochlorin ferrochelatase (AtSirB) from Arabidopsis thaliana**

For the characterization of AtSirB gene and its function, its cDNA was amplified from *Arabidopsis thaliana* by polymerase chain reaction (PCR) using gene specific forward (SirB F) and reverse (SirB R) primers (table-5). *Arabidopsis* cDNA from mature leaves was used as template for PCR. PCR was performed using high fidelity Taq DNA polymerase for AtSirB amplification. PCR temperatures were set as denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec and extension at 72°C
Fig 4. Light dependant expression of AtSirB in Arabidopsis thaliana. Six-day-old etiolated A. thaliana seedlings were transferred to light (100μmoles photons m⁻² s⁻¹) for different time periods (0h, 3h, 6h, 12h and 24h). Total RNA was isolated from each sample and 3μg of total RNA was taken for cDNA synthesis and gene expression was analysed by PCR using AtSirB specific primer. Actin was taken as endogenous control.
Fig 5. Tissue specific expression of AtSirB. 
A. thaliana plants were grown in cool-white fluorescent light (100μmoles photon m⁻² s⁻¹) at 22°C in 14h L and 10h D photo-period and total RNA were isolated from different tissues (roots, shoots, leaf, floral bud, flower and seed pod). 3 μg of RNA was taken for RT PCR analysis. AtSirB gene specific primers were used to check their expression. Actin was used as internal control.
for 60 sec for each cycle and was continued for 30 cycles. PCR yielded a fragment of 678 bp (Fig. 6A). PCR fragment was cloned in pGEMT-easy vector by AT ligation using T4 DNA ligase. pGEMT easy-SirB recombinant vector was transformed into DH5α competent cells. Colony PCR was done to select positive colonies (Fig. 7A). Confirmation of transformed colonies was done by restriction digestion of the pGEMT –easy-SirB plasmid with EcoRI which gives a fall out of 678bp (Fig. 7C). Mature part of the SirB was cloned using forward primer (mature AtSirB) from the cleavage site of the transit peptide after amino acid threonine, positioned at no. 46, and the reverse primer of AtSirB (Table 5). The transit peptide cleavage site was obtained by the ChloroP 1.1 prediction program. PCR was carried out using cDNA of Arabidopsis as template. A fragment of 540bp was amplified (Fig. 6B), cloned into pGEMT –easy vector and transformed into DH5α competent cell. Positive colonies were screened with colony PCR (Fig. 7B) and confirmed after restriction digestion with BamHI and SacI that yielded a fall out of 540bp (Fig. 7D).

### Over-expression of mature AtSirB in E. coli

Mature AtSirB cloning into pET- 30a protein expression vector was done as shown in Fig. 8. pGEMT-easy vector containing the mature AtSirB cDNA was digested with BamHI and SacI and the digested fragment was ligated to pET 30a expression vector. pET 30a-Sirb recombinant vector was transformed into BL21 (DE3) competent cell. Positive colonies were selected by colony PCR (Fig. 9A). For the confirmation of ligation, the Pet 30a expression vector containing the mature AtSirB was digested with BamHI and SacI which yielded a fragment of 540 bp (Fig. 9B). E.coli BL 21 (DE3) cells containing the plasmid pET 30a-mature AtSirB were induced to express SirB protein using 0.3 mM IPTG for overnight at 16°C. Low temperature incubation was given to get the maximum amount of protein into soluble fraction. Supernatant and pellet fraction of sonicated E.coli cells overexpressing mature part of SirB were loaded on 12.5% SDS PAGE. Fig. 10A shows the polypeptide profile of pellet and supernatant fractions of un-induced and IPTG- induced SirB. The heterologously expressed protein had molecular weight of 23.1 KDa that includes the 3.3 KDa His tag.

Recombinant SirB protein was purified from the supernatant fractions of the sonicated E.coli cells over-expressing mature part of SirB via batch purification with
Fig 6: PCR Amplification of AtSirB. At SirB (A) Full length and (B) mature (without transit peptide sequence) were PCR amplified from cDNA using gene-specific forward and reverse primers for (A) and mature forward and gene specific reverse primer for (B) of *Arabidopsis thaliana*. 
Fig 7: Cloning of AtSirB into pGEMT-easy vector. PCR amplified product was ligated to pGEMT easy vector. Colony PCR was done to check the bacterial colony containing recombinant pGEMT-easy by using gene specific primers. Recombinant pGEMT-easy vector containing full length At SirB was restriction digested with EcoR I that gave two fragments of 1) 3 kb, pGEMT-easy size and 2) 678 bp full length AtSirB (A & C). pGEMT easy clone containing the Mature AtSirB was digested with BamHI and Sac I produced a fragment size of mature AtSirB of 540 bp and 3 kb of pGEMT easy (B & D).
Fig 8. Cloning Strategy for Sir B into expression vector

Amplification of full length AtSirB and Mature AtSirB

Full length AtSirB ligated to pGEMT-Easy

Mature AtSirB ligated to pGEMT-Easy

Mature AtSirB fragment is ligated to pET 30a
Fig 9. Cloning of mature *At SirB* in pET 30a. The *AtsirB* mature fragment obtained by BamHI and SacI digestion from Fig. 3 was ligated to pET 30a vector and the resultant colonies (A) were analysed by PCR using *AtSirB* mature forward and gene-specific reverse primers. (B) Positive colonies were grown and their plasmids were digested by BamHI and SacI that yielded 540 bp *AtSirB* mature fragment.
Ni-NTA beads. Supernatant fraction was incubated with Ni-NTA beads and washed five times with the washing buffer and eluted by the elution buffer containing imidazole according to manufacturer’s instruction. Several eluted fractions were collected and the purity was checked by running 12.5% SDS PAGE. The purified SirB protein had a molecular weight of 23.1 KDa (Fig. 10B).

To positively identify the over-expressed purified protein it was probed with Anti-His tag antibody. The immunoblot clearly identified the over-expressed SirB protein in induced supernatant (Fig. 10C).

Production of Polyclonal antibody against \textit{At SirB}

Polyclonal antibodies were raised against purified SirB Protein in New Zealand rabbit as described in “Materials & Methods” and pictorially shown in Fig-11A. The titre of AtSirB antibodies was checked by Western blotting. Immunoblot were performed using different dilution of antibodies i.e., 1:2500, 1:5000 and 1:10000 (Fig. 11B). An intense signal was observed even at 1:10000 dilutions, whereas no signal was observed with pre-immune serum that demonstrated the specificity of AtSirB antibodies towards AtSirB recombinant protein.

Raising Transgenic Plants of \textit{Arabidopsis thaliana} having \textit{AtSirB} in Sense and Antisense Orientations

For the functional characterization of sirohydrochlorin ferrochelatase (\textit{AtSirB}) a transgenic approach was used. \textit{AtSirB} cDNA was cloned in pCAMBIA 1304 binary vector in sense and antisense orientations to genetically transform \textit{Arabidopsis} plants via \textit{Agrobacterium}- mediated transformation.

Cloning of \textit{AtSirB} in Sense and Antisense Orientations in the Binary Vector

pCAMBIA 1304 plant transformation vector was used for \textit{Agrobacterium}-mediated transformation of modified pCAMBIA 1304 plant transformation vector containing kanamycin (npt) gene and CaMV 35S -Ω- enhancer cassette. Cloning of \textit{AtSirB} cDNA in pCAMBIA 1304 was done by taking out the \textit{AtSirB} fragment form recombinant pGEMT-easy vector (pGEMT-easy-SirB) by digesting with the EcoRI restriction enzymes and ligating it with pCAMBIA 1304 plant transformation vector
Fig 10. *AtSirB* expression in *E.coli*. BL 21 (DE3) Cells containing the recombinant pET 30a-mature AtSirB were induced with 0.3mM IPTG at 16°C overnight. SDS PAGE (12.5%) showing the polypeptide profile of pellet, supernatant and purified recombinant protein of 23.1 kDa SirB (A & B). Recombinant protein was further confirmed by immunoblot using anti-His rabbit antibodies (C) as described in “Materials and Methods”.
**Fig 11. Production of polyclonal antibody in rabbit.** (A) Schematic diagram summarizing the steps involved in raising polyclonal antibody against purified recombinant SirB protein. (B) Western blot showing titre and specificity of the antibody against *the recombinant At SirB protein* (lane 1, pre-immune serum; lane 2, 1:2500 dilution; lane 3, 1:5000 and lane 4, 1:10000 dilutions.)
(Fig-12A-B). Recombinant pCAMBIA 1304-\textit{AtSirB} was transformed into DH5 \alpha competent cell. Positive colonies were screened by colony PCR using \textit{AtSirB} gene specific primers (Fig. 12C). As it was a non-directional cloning, both sense and antisense constructs were obtained after transformation. Positive colonies were used for plasmid isolation and after its digestion with \textit{Xba I} yielded a fragment of 678 bp in sense construct and no fallout in antisense construct (Fig. 12D). Orientation check for cloning of \textit{AtSirB} in pCAMBIA sense construct was done by PCR using 35S internal forward primer and gene specific reverse primer. The antisense construct was confirmed using 35S internal forward primer and gene specific forward primer (Fig. 12E-F). Orientation of \textit{AtSirB} with respect to 35S primer is shown in fig. 13. Recombinant pCAMBIA 1304 plasmid was transformed into \textit{Agrobacterium tumefacience} GV 1301 as described in “Materials and methods”. Recombinant colonies were selected on YEM media plates containing 50 \( \mu \)g ml\(^{-1}\) kanamycin and 25 \( \mu \)g ml\(^{-1}\) rifampicin and confirmed by PCR using \textit{AtSirB} gene specific primers (Fig. 13A-C).

**Plants transformation by \textit{AtSirB}**

\textit{Arabidopsis thaliana} plants were transformed with positive colony of recombinant \textit{Agrobacterium tumefacience} by floral dip method as described in “Materials and Methods”. The seeds were harvested after one month from each transformed plant and kept separately. Seeds harvested from each plant were selected in the presence of 30\( \mu \)g ml\(^{-1}\) kanamycin in 0.5X MS medium. Kanamycin resistant plants (T1) were grown in \textit{Arabidopsis} growth room maintained at 22\(^\circ\)C and 100 \( \mu \)moles photons m\(^{-2}\) s\(^{-1}\) to generate seeds. For next generation (T2) the resulting seeds were grown in kanamycin plates and were selected for kanamycin resistance.

**Confirmation of \textit{AtSirB} Transgenic plants**

**PCR analysis**

Kanamycin resistant \textit{AtSirB} over-expression (\textit{AtSirBx}) and antisense (\textit{antiSirB}) (T1) lines were taken and genomic DNA was isolated. Using the kanamycin gene specific forward and reverse primers (Table-5), the genomic DNA from 7 individual lines of T1 generation was amplified by PCR. The PCR condition for (\textit{npt}) amplification was
Fig 12. Cloning of *AtSirB* to pCAMBIA 1304 vector having 35 S –Ω-poly A promoter cassette and *npt* for kanamycin selection. (A) EcoR1 digestion of pGEMT-easy having *AtSirB* and pCAMBIA 1304 vector having CaMV 35 S –Ω-poly A cassette and *npt* gene. Digestion resulted in excision of *AtSirB* gene from pGEMT-easy vector. (B) Ligation of *AtSirB* with linearized modified pCAMBIA 1304 vector. (C) Colony PCR for checking positive colony in DH5α *E. coli* cell. (D) Digestion check with Xba I restriction enzyme of sense clone yielded a fallout of 678 bp (lanes 2 & 3). Antisense clones when digested with XbaI did not yield any fragment (lanes 1 & 4). (E) PCR amplification with 35S internal forward and gene-specific forward primers for *AtSirB* anti-sense clone conformation. (F) PCR amplification with 35S internal forward and gene-specific reverse primers for *AtSirB* sense clone conformation.
Fig 13. Colony PCR confirmation of *AtSirB* Sense (A) and *AtSirB* Antisense (B) construct in *Agrobacterium tumefacience*. (C) Schematic representation of pCAMBIA 1304. Recombinant pGEMT-easy vector having omega enhancer was digested with EcoRI. *AtSirB* fall out was ligated to EcoRI digested pCAMBIA 1304 modified plant transformation vector in sense and antisense orientation that were used to raise over-expression and antisense plants of *Arabidopsis thaliana*. 
denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 60 sec for each cycle and continued for 30 cycles. PCR yielded a fragment of 800 bp from transformants that contained npt kanamycin resistant gene (Fig. 14A). These individual transgenic lines were grown to harvest seeds. Seeds collected from the above plants were again grown in kanamycin plates to select T2 transgenic lines. The seedling resistant to kanamycin were selected and their genomic DNA was isolated for the PCR analysis. AtSirBx and antiAtSirB were confirmed by PCR using 35S internal forward primer and gene specific reverse primer and 35S internal forward primer gene specific forward primer respectively. A fragment of 878bp (678bp AtSirB+200bp 35s sequence) was amplified in all the selected transgenic lines (Fig. 14B-D). pCAMBIA recombinant plasmid containing the AtSirB cDNA was used as positive control and the WT plants genomic DNA as negative control. Transgenic seeds were grown for T3 generation to obtain homozygous transgenic plant (Fig. 15A-B).

GUS histochemical Assay

Some of the transgenic lines positive for AtSirB cDNA along with WT plants were screened by GUS histochemical assay. Only the transformed lines were stained blue whereas the untransformed wild type plants did not acquire the stain (Fig. 15C).

Southern blot Analysis of AtSirB transgenic plants

The stable integration of T-DNA cassette containing AtSirB cDNA into the Arabidopsis thaliana host genome was checked by Southern blot analysis. Genomic DNA from one sense and one antisense line was isolated and digested with EcoRI restriction enzyme and subjected to Southern blot analysis using radio-labelled probe as described in materials and methods. As expected from the restriction digestion pattern of genomic DNA of transformed Arabidopsis plants by EcoRI, a fragment of 678bp (size of the AtSirB cDNA) was observed in transformed sense and antisense plants (Fig. 16). This band was not observed in WT plants. A second band (of size ~7 kb) observed in WT and transgenic plants due to the endogenous AtSirB gene of the host genome.

The number of integration of the T-DNA cassette into the host genome was checked by Southern blot analysis. The genomic DNA extracted from transformed (AtSirBx1
Fig 14. Confirmation of *AtSirB* transgenic (Sense and antisense) plants. (A) Genomic DNA of WT and T1 generation *AtSirB* sense and antisense plants were used as template and PCR of *npt* gene having resistance to kanamycin. A fragment of 0.8 kb corresponding to *npt* was amplified that confirmed the integration of the T-DNA cassette with the host *Arabidopsis* genome. Modified pCAMBIA 1304 vector containing the *AtSirB* gene was taken as positive control. (B) Using 35 S internal forward primer and gene specific reverse primer PCR reaction was performed with genomic DNA isolated form different transgenic lines of T 2 generation plants that yielded a fragment size of 0.868kb integrated in a sense orientation (1s -8s). (C) PCR reaction using 35 S internal forward primer and gene specific forward primer was done with genomic DNA isolated from antisense line A1, A2 and A3 of T2 generation plants that yielded a fragment size of 0.860 kb.(D) For selection homozygous line plants were grown for up to T3 generation, Genomic DNA was isolated and PCR was carried out by using npt gene specific primer. showing a fallout of 0.8Kb.
Fig 15. Confirmation of *AtSirB* transgenic plants: (A) Genomic DNA of the WT and *AtSirB* transgenic homozygous T3 generation (overexpressor selected lines S1, S2 and S3) used as template and PCR was performed by using 35S internal Forward primer and gene specific reverse primers. A fragment of 0.878 kb was amplified. (B) Genomic DNA of the WT and *AtSirB* transgenic T3 generation (Antisense plants selected lines A1, A2 and A3) used as template and PCR was done by using 35S internal Forward primer and gene specific forward primers. A fragment of 0.878 kb was amplified. (C) The WT and T3 generation *AtSirB* plants (Sen1, Sen 2 and Anti 1, Anti 2) were stained with X-Gluc for the GUS histochemical assay as described in the material and methods. All the *AtSirB* plants showed GUS Staining.
Fig 16. Southern blot analysis of WT and AtSirB transgenic plants. Genomic DNA (20μg) from untransformed WT and PCR confirmed AtSirB transgenic plants were digested with EcoR I and transferred onto nylon membrane. The blot was probed with radiolabeled AtSirB cDNA. The AtSirB cDNA fragment used for prob preparation was obtained by PCR with a pair of gene specific Primers. A fragment size of 678bp was observed in transformed SirB transgenic plants and that was not found in untransformed WT plants.
and *antiSirB*1 lines) as well as non-transformed WT plants was digested by *Sph I* or *Spe I* restriction enzyme that specifically cuts the T-DNA of cassette introduced into host genome. Southern blot analysis of *Spe I*-digested genomic DNA probed with radio-labelled *AtSirB* cDNA revealed a band of size approximately 1.3kb in *AtSirB x* plant and around 1.8kb in antisense plant. The other restriction enzyme *Sph I* that cuts the *npt* gene in the T-DNA cassette was used to digest the genomic DNA of WT and *AtSirB* transgenic plants. The digest was probed with radio-labelled *AtSirB* cDNA and revealed a band of size approximately 1.8Kb in *AtSirB x1* plants and a band of 2.5 kb size in antisense plant. A second band pertaining to endogenous *AtSirB* was observed as a 7kb band in all WT and transgenic plants. This confirms the single integration of the trans-gene to the host genome (Fig. 17).

**Gene expression of *AtSirB* in WT and transgenic plants**

To check the gene expression of *AtSirB* in WT and transgenic plants, semi-quantitative RT PCR, real-time PCR and Northern analysis were performed. RNA (3µg) extracted from WT and *AtSirB* transgenic plants were taken for first strand cDNA synthesis. Using the cDNA as template, semi-quantitative PCR was done by using *AtSirB* gene specific forward and reverse primers (Fig. 18A). RT PCR reveals that as compared to WT, in *AtSirB x* lines 1 and 2 *AtSirB* RNA expression increased by 100% whereas in antisense lines its expression decreased by 60%.

**Real-Time PCR analysis of WT and *AtSirB* transgenic plants**

*AtSirB* expression was confirmed by quantitative real-time PCR with RNA extracted from 21-d-old WT and transgenic plants. The message abundance of sense and antisense lines is compared relative to that of WT. The results clearly showed that as compared to WT, *AtSirB* gene expression was increased almost 100% in *AtSirB x* lines and reduced by 70% in antisense lines (Fig-23A-B-C). Actin expression was used as an internal control (Fig. 19 A-B) (Fig. 20A-C).

**Northern blot analysis of WT and *AtSirB* transgenic plants**

To further confirm the extent of *AtSirB* RNA over-expression and silencing in sense and antisense plants, RNA was isolated from the WT, Sense lines (sense 1, sense 2)
Fig. 17. Southern blot analysis continued. Genomic DNA (20μg) from untransformed WT and AtSirB transgenic plants (sense and antisense lines) were digested with Sph I, a cutter of npt gene and Spe I, a cutter of T-DNA. Agarose Gel was run and transferred onto nylon membrane and probed with radiolabeled AtSirB cDNA.

Genomic DNA (20μg) from untransformed WT and AtSirB transgenic plants (sense and antisense lines) were digested with Sph I, a cutter of npt gene and Spe I, a cutter of T-DNA. Agarose Gel was run and transferred onto nylon membrane and probed with radiolabeled AtSirB cDNA.
Fig 18. RT PCR analysis of *AtSirB* transgenic plants. WT and *AtSirB* transgenic plants (*AtSirBx1*, *AtSirBx2*, and *antiSirB*1, *antiSirB*2). Total RNA was isolated from 3 to 4 weeks old plants grown under cool white fluorescent +incandescent light (100 µMoles photon m⁻²s⁻¹) in 14 h L and 10 h D photoperiod at 22 °C. First strand synthesis was done by using reverse transcriptase. (A) PCR of *AtSirB* was done by using gene specific primer. (B) PCR was done for actin (as endogenous control) to show equal loading. (C) Strand Specific RT PCR was used to check the antisense RNA expression in antisense *Arabidopsis thaliana* plants.
Dissociation curve of Actin as an endogenous control

Amplification plot of Actin as an endogenous control

Fig 19. Real time PCR standarization of actin. Out of total RNA isolated from plants 3 μg was taken for quantitative RT PCR analysis for actin using appropriate primers. (A) Dissociation curve for checking primer dimer. (B) Amplification of actin as endogenous control.
Fig 20. Relative expression of AtSirB was analysed using real time RT PCR. Out of total RNA isolated from plants 3 μg was taken for quantitative RT PCR analysis. (A) dissociation curve for AtSirB for standardization of primer concentrations, (B) amplification of SirB RNA with respect to number of cycles, (C) relative expression of AtSirB RNA in WT and SirB transgenic plants taking WT as reference. Sen1, Sen 2 and Anti1, Anti2 are AtSirBx and antiSirB lines respectively.
Results

and antisense lines (Anti 1, 2). Northern blot analysis was performed using the *AtSirB* cDNA as probe. Northern blot analysis revealed that as compared to WT, in *AtSirBx* lines 1 and 2 *AtSirB* RNA expression increased by 100% whereas in antisense lines its expression decreased by 65% (Fig. 21).

**Anti-sense mRNA Expression in Antisense *AtSirB* plants**

Instead of using Oligo (dT) or random hexamers to prime first-strand cDNA synthesis, gene specific primer was used. To detect antisense mRNA, the forward primer was used for reverse transcription from RNA isolated from *antiAtSirB* transgenic lines. The forward primer binds only to the antisense mRNA. Thus, there will be no first-strand cDNA synthesis from the forward primer if there is no antisense mRNA. The first-strand reactions then served as targets for PCR together using the forward and reverse primers for *AtSirB*. The result demonstrates that *AtSirB* gene product was amplified when PCR was performed using first-strand cDNA prepared from gene specific forward primer only (Fig. 18C). A cDNA prepared using total RNA from wild-type plants was used as template for another set of PCR that served as negative control.

**Western blot analysis of the WT and *AtSirB* transgenic plants**

To ensure if transgenic plants result in an expected change in AtSirB protein expression, Western blot analysis was conducted. Total protein was isolated from 3 weeks old plants of *AtSirBx* and *antiSirB* lines as described in “Materials & Methods”. Equal loading of the protein (25μg) was checked by running 12.5% SDS-PAGE (Fig. 22A). Polyclonal antibody raised against *Arabidopsis AtSirB* protein was used to immunodetect the AtSirB protein in different *AtSirB* transgenic plants. Fig. 22B showed that *AtSirB* protein expression was higher in Over-expression line and lower in antisense transgenic plant. This confirmed the over-expression of *AtSirB* in *AtSirBx* plants and reduced expression in *antiSirB* plants.

**Morphology of *AtSirB* overexpressor (*AtSirBx*) and antisense (*antiSirB*) transgenic plants**

The *AtSirB* transgenic plants showed a typical alteration in their morphology. *AtSirBx* plants were bigger in size and greener in colour in comparison to that of WT. Most
Fig 21. Northern blot analysis of \textit{AtSirB}. Northern blot analysis of \textit{AtSirB} mRNA in WT and \textit{AtSirB} transgenic plants (over expression lines S1 and S2 and antisense lines A1 and A2). Total RNA was isolated from 3- to 4-week-old plants grown under cool white fluorescent (100 μmoles photon m$^{-2}$ s$^{-1}$) in 14 h L and 10 h D photo-period at 22°C. The SirB full length DNA fragment was used for probe preparation obtained by PCR with gene-specific primers. 10 μg of total RNA was loaded in each lane and resolved on 1.2 % denaturing formaldehyde Agarose gel. Ethidium bromide (EtBr) stained gel showing equal loading of RNA is shown below.
Fig 22. Western blot Analysis of *AtSirB* plants. Total protein profile (12.5 % SDS-page) of leaves extracted from 3-4 week old WT and *AtSirB* transgenic (Over-expression line *AtSirBx1*, *AtSirBx2* and antisense lines *antiSirB1*, *antiSirB2*) grown at 22°C under light intensity of 100 mmoles photons m⁻² s⁻¹ with 14 h L and 10h D photoperiod. 40μg of protein was loaded in each lane and SDS PAGE was run to check the equal loading (A). Protein samples from the gel were transferred onto nitrocellulose membrane and immunoblot analysis of SirB protein (19.8 kDa) (B). was done as described in material and method.
Fig 23. Phenotypes of the *AtSirB* Transgenic plants. Phenotypes of *AtSirB* transgenic plants grown under cool-white-fluorescent light (100 μmoles photon m⁻² s⁻¹) in 14 h L and 10 h D photoperiod at 22°C. Morphology of 3-week-old WT and *AtSirB* transgenic (Sen 1, Sen2 and Anti 1, Anti 2) plants grown in MS medium. Antisense(Anti 3) seedlings were lethal and did not survive.
Fig 24. WT and \textit{AtSirB} transgenic plants grown in soil. Morphology of 5- to 6-week-old WT and AtSirB transgenic (\textit{AtSirB}x 1 and \textit{antiSirB}1) grown in agropit soil.
Fig 25. Measurement of morphological parameters of WT and *AtSirB* transgenic plants. WT and *AtSirb* (Sense line 1,2 and antisense line 1,2) plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 3 weeks in MS medium. (A) Root morphology of WT and *AtSirB* transgenic plants. (B) Root length (C) Number of lateral roots of WT and *AtSirB* transgenic plants. Each data point is an average of five replicates. The error bar represents standard deviation (SD).
Fig 26. Measurement of Physical parameter of WT and \textit{AtSirB} plants continue. WT and \textit{AtSirb} (Sense 1 and antisense1) plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 20 days in MS medium. Rosette diameter (A), Fresh weight (B) and Dry weight (C) of WT and \textit{AtSirB} transgenic plants. Each data point is an average of five replicates. The error bar represent standard deviation(SD).
antiAtSirB plants were very pale and could not survive. A few antisense lines survived. However they were smaller than WT (Fig. 23-24). AtSirB transgenic root morphology changed especially in overexressor. As compared to that of WT, number of lateral roots per plant was higher in AtSirBx plants although root length was almost equal (Fig. 25A). In antisense plants root length was longer than that of WT. However, number of lateral roots of antisense plants was same as that of WT (Fig. 25B-C). The floral diameter, fresh weight and dry weight of AtSirBx were more than that of WT whereas in antisense plants these parameters were almost equal to or smaller than that of WT (Fig. 26A-C).

AtSirB genetic manipulation and nitrogen assimilation

Protein Contents

WT and AtSirB (Sense 1 and antisense1) plants were grown at 22°C in 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m⁻² s⁻¹) for 3 weeks in MS medium and their proteins were extracted and quantified as described in “Materials & Methods”. In comparison to WT, in AtSirBx plants protein contents increased by 44%, while in antisense plants the same declined by 14% (Fig. 27A).

Gene Expression of Nitrite reductase and Nitrate reductase in AtSirB transgenic plants

To understand if the changes in the protein contents of plants were triggered by gene expression of NiR and NR, two main nitrogen assimilating enzymes the message abundance of nia2 (At1g37130), coding for nitrate reductase and NiR (At2g15620) coding for nitrite reductase were studied by semi-quantitive RT PCR. The reaction was stopped after 27 cycles when they were still in exponential phase. The gene expression of both NiR, NR increased in AtSirBx plants. Interestingly, as compared to that of WT, the message abundance of antiSirB plants also substantially increased (Fig. 28).
Fig 27. Protein and N₂ content of WT and *AtSirB* transgenic plants.

(A) WT and *AtSirB* (Sense 1 and antisense1) plants were grown at 22°C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 3 weeks in MS medium and their proteins were extracted and quantified as described in “Materials & Methods”. Each data point is an average of five replicates. The error bar represent standard deviation (SD).

(B) WT and *AtSirB* (Sense 1 and antisense1) plants were grown at 22°C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 3 weeks in MS medium and their nitrogen contents were quantified by CHNS analyzer using a L-cystein standard as described in “Materials & Methods”.
Fig 28. Gene expression for enzyme involved in Nitrogen metabolism. WT and AtSirB transgenic plants (over expression line AtSirBx1, 2 and antisense lines antiSirB1, 2). Total RNA was isolated from 3 to 4 weeks old plants grown under cool white fluorescent light (100 μMoles photon m⁻²s⁻¹) in 14 h L and 10 h D photoperiod at 22 °C. First strand synthesis was done by using reverse transcriptase. PCR of nitrate reductase (Nia2) and nitrite reductase (NiR) was done by using gene specific primer of respective gene. Actin was taken as an internal control.
Protein abundance of Nitrite reductase (NiR) in *AtSirB* transgenic plants

To understand if gene expression correlates to protein expression, protein abundance of NiR and SiR was analysed by Western blot. In agreement with that of gene expression, *AtSirB* lines had increased protein abundance of NiR substantially over that of WT. However, in antisense lines their protein abundance also increased similar to that of over-expressors, and the same was substantially above their gene expression level (Fig. 29).

**Nitrite reductase activity**

Nitrite reductase is the crucial chloroplastic enzyme that requires siroheme as a prosthetic group. Its activity was measured as disappearance of $\text{NO}_2^-$ per $\mu$g of protein. Four week old WT and *AtSirB* transgenic plants (over-expression and antisense lines) grown at $22^0 \text{C}$ in agropit soil under 14h L/10h D photoperiod in cool-white-fluorescent light ($100\mu$moles photon $\text{m}^{-2} \text{s}^{-1}$) were taken for estimation of NiR activity. As compared to that of WT, due to increased siroheme availability, NiR activity increased by 30% in *AtSirB* plants whereas in antisense plants the same declined by 50% (Fig. 30). In *AtSirB* plants, increased activity of NiR efficiently consumes its substrate $\text{NO}_2^-$, whose over-accumulation could be toxic to the plant.

**Nitrate reductase activity**

$\text{NO}_2^-$ is generated by NR that reduces $\text{NO}_3^-$. To ascertain if NiR could modulate NR, its activity was estimated in 4-week-old WT and *AtSirB* transgenic plants (over-expression and antisense line) grown at $22^0 \text{C}$ under 14h L/10h D photoperiod in cool-white-fluorescent light ($100\mu$moles photon $\text{m}^{-2} \text{s}^{-1}$). NR activity in *AtSirB* over-expresssor plants increased by 39% over that of WT. Conversely, in antisense plants its activity declined by 20% (Fig. 31).

**Nitrogen content in *AtSirB* transgenic plants**

This significant change in the activity of two crucial enzymes involved in N metabolism and protein contents led us to quantify total N content in *AtSirB* transgenic (Overexpressor and antisense lines) plants and that of WT plants grown at $22^0 \text{C}$ under 14h L/10h D photoperiod in cool-white-fluorescent light ($100\mu$moles photon $\text{m}^{-2} \text{s}^{-1}$) for 4 weeks. The Nitrogen content was analyzed by Euro- E.A.
Fig 29. Immunoblot analysis of Nitrite reductase enzyme involved in Nitrogen metabolism. Total protein was isolated from 3-4 week old WT and AtSirB transgenic plants (S1, S2 over expression and A1, A2 antisense lines) grown at 22°C under 14h L /10h D photoperiod in cool-white-fluorescent light (100μmoles photon m⁻² s⁻¹ ) as described in “Materials & Methods”. (A) 25μg of protein was loaded in each lane and SDS-PAGE (12.5% ) was run to check the equal loading of protein. (B) Protein samples were transferred onto nitrocellulose membrane and immunoblot analysis was done as described in “Material & Methods”.
Fig 30. Nitrite reductase activity in WT and AtSirB transgenic plants. Whole shoots of Photo-periodically (14h L and 10h D) grown 4- to 5 week old WT and AtSirB transgenic plants planted in agropit soil under cool-white fluorescent light (100 μmoles photons m⁻² s⁻¹) were used for measuring Nitrite reductase activity as described in “Material & Method”. Nitrite reductase activity was measured as utilization of nitrite in NiR reaction mixture.
Fig 31. Nitrate reductase activity in WT and AtSirB transgenic plants. Whole shoots of Photoperiodically (14h L and 10h D) grown 4- to- 5 week old WT and AtSirB transgenic plants planted in agropit soil under cool-white fluorescent light (100 μmoles photons m⁻² s⁻¹) were used for measuring Nitrate reductase activity as described in "Material & Method". For optimal NR induction 3mM of KNO₃ was added to agropit soil for 24 hour to induce NR. The activity was measured as formation of nitrite in NR reaction mixture. (In- induced).
elemental analyzer as described in “Materials & Methods”. N₂ content increased 31% in AtSirBx plants and a declined by 15% in antiSirB plants in comparison with that of WT (Fig. 27B). This is in agreement with that of protein contents.

**Pigment Contents of WT, AtSirBx and antiSirB Plants**

**Chl contents**

WT and AtSirB transgenic plants were grown in MS plate for three weeks (Fig. 23). Their total Chl, Chl a, Chl b and carotenoids contents were measured as described in “Material & Methods”. AtSirBx had higher amounts of total Chl (46%), Chl a (45%), Chl b (47%) and carotenoids (28%), than that of WT (Fig-32A-E). In antiSirB plants, as compared to that of WT, total Chl, chl a, chl b and carotenoids contents declined by 30%, 32.9%, 24% and 33% respectively.

**Chlorophyll biosynthetic intermediates**

To study the Chl biosynthetic potential of WT, AtSirBx and antiSirB plants, the contents of Pchlide, an intermediate of chlorophyll biosynthesis pathway, was estimated by spectrofluorometry (Hukmani et al., 1992). Under steady state illumination (100μmoles photons m⁻² s⁻¹), as compared to that of WT, the amounts of Pchlide increased by 53% in AtSirBx and decreased by 37% in antiSirB plants (Fig. 33).

**Protein abundance of chlorophyll biosynthetic enzymes (Western blot analysis)**

Chl contents increased in AtSirBx plants and declined in antiSirB plants. To study if changes in Chl contents of transgenic plants were modulated by enzymes involved in Chl biosynthesis, the protein abundance of a few tetrapyrrole biosynthetic enzymes was analyzed by Western blot (Fig. 34).

**Glutamyl t-RNA Synthase (GluRS):** As compared to WT, the steady state level of GluRS increased in AtSirBx plants and declined in antiSirB plants.

**Uroporphyrinogen decarboxylase (UROD):** UROD level increased in AtSirBx lines, whereas it declined in antiSirB lines.
Fig 32. Pigment content of WT and AtSirB Transgenic (AtSirBx1 and antiSirB1) plants. WT, AtSirBx and antiSirB plants were grown at 22°C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for three weeks in MS medium and their (A) Chl, (B) carotenoids, (C) Chla, (D) Chlb and (E) Chla/b were measured as described in “Material & Method”. Each data point is an average of five replicates. The error bar represent standard deviation (SD).
Fig 33. Measurement of Protochlorophyllide and Proto IX in WT and AtSirB transgenic plants. WT and AtSirb (Sense 1 and antisense 1) plants were grown at 22°C under 14 h L and 10 h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for three weeks in MS medium and their chlorophyll biosynthetic intermediate (A) Protochlorophyllide and (B) Proto IX were measured as described in "Materials and Methods". Each data point is an average of five replicates. The error bar represents standard deviation (SD).
Fig 34: Western blot analysis of enzyme involved in chlorophyll biosynthesis pathway. (A) Protein profile of plastids isolated from 4-5 week old WT and AtSirB transgenic plants (AtSirBx1, 2 and antiSirB1, 2) grown at 22°C under 14h L/10h D photoperiod in cool-white-fluorescent light (100μmoles photon m⁻² s⁻¹). 35 μg of protein was loaded in each lane and SDS-PAGE (12.5%) was run to check the equal loading of protein. (B) Protein samples were transferred onto nitrocellulose membrane and immunoblot analysis was done as described in “Material & Methods”.
Coproporphyrinogen oxidase (CPO): This enzyme catalyzes the oxidative decarboxylation of propionate side chains on ring A and B to yield Protoporphyrinogen IX. The steady state protein abundance of CPO was increased in AtSirBx lines while in antiSirB lines it partially declined.

Magnesium-chelatase subunit (ChlD): Similarly there was an increase in protein expression of ChlD in AtSirBx plants whereas the same declined in antiSirB line than that of WT plants.

Protochlorophyllide oxidoreductase C (PORC): There was no change in the expression of PORC in AtSirBx, WT and antiSirB lines.

Pulse amplitude modulated (PAM) chlorophyll a fluorescence measurements

To understand if increased Chl and protein contents of plants results in increased photosynthetic capacity, Chl fluorescence was monitored as a signature of photosynthesis (Govinjee, 1995) in WT and AtSirB transgenic plants.

Chlorophyll a fluorescence was measured in WT and AtSirb (Sense 1 and antisense) lines plants grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m2 s-1) for three weeks in MS medium (Fig-23). Various Chl a fluorescence parameters i.e., Fo, Fm, Fv/Fm, electron transport rate (ETR), quantum yield of PSII (QLPSII), photochemical quenching (qP) and non-photochemical quenching (qN) were studied as described in “Materials & Methods”.

Fo: Fo was measured in the dark adapted leaves as initial minimum fluorescence. In AtSirB transgenic (overexpressor as well as antisense) plants, there was only small change in Fo values (Fig. 35A).

Fm: Fm was measured as maximal fluorescence during the first saturation pulse after dark adaption. As compared to that of WT, the Fm in AtSirBx plants increased by 58%, and almost remained same in antiSirB plants (Fig. 35B).
Fig 35: Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and AtSirB plants. WT and AtSirB transgenic plants photoperiodically (14h L and 10h D) grown in 100 μmoles photons m⁻² s⁻¹ light intensity for 3 weeks were used for studying various parameter of Chlorophyll a fluorescence measurement including Fo(A), Fm (B) and Fv/Fm (C) as described in “Materials & Methods”.
Fig 36: Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and *AtSirB* plants. WT and *AtSirB* transgenic plants photoperiodically (14h L and 10h D) grown in 100 μmoles photons m⁻² s⁻¹ light intensity for 3 weeks were used for studying various parameter of Chlorophyll a fluorescence measurement including (A) electron transport rate (ETR), (B) Photochemical quenching (qP) and (C) nonphotochemical quenching (qN) as described in "Materials & Methods".
Fv/Fm: This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers by dark-adapted leaves. In *AtSirBx* plants, as compared to that of WT, the Fv/Fm ratio increased by 22%, whereas in *antiSirB* plants the same decreased marginally (Fig. 35C).

**Electron transport rate (ETR):** The ETR (µmoles electron m⁻² s⁻¹) increased in response to photosynthetic active radiation (PAR) (µmoles photons m⁻² s⁻¹) (Fig- 29). As compared to WT, the ETR was higher in *AtSirBx* plants both in limiting and saturating light intensities. In *antiSirB* plants the ETR substantially declined at saturating light intensities. However, in limiting light intensities (up to 60 µmoles photons m⁻² s⁻¹) the ETR was almost similar to that of WT (Fig. 36A).

**Photochemical quenching (qP):** The qP represents photochemical quenching and is a measure of the fraction of still open PSII reaction centers. It decreased in response to increased photosynthetic active radiation (PAR) (µmoles photons m⁻² s⁻¹). In *AtSirBx* plants qP was higher than that of WT plants in different light intensities. In *antiSirB* plants qP was lower than that of WT plants (Fig. 36B).

**Non-Photochemical quenching (qN):** The qN is non-photochemical quenching and is a measure of heat dissipation and a combined total for the combination of photoprotective mechanisms, state 1 and state 2 transition quenching, and photo-inhibition and photo-damage. It increased in response to increased light intensity both in WT and transgenic plants. As compared to WT, the qN was higher in *antiSirB* plants and lower in *AtSirBx* plants (Fig. 36C).

**AtSirB Over-expresssor Tolerate Nitrogen Starvation**

To investigate the Role of *AtSirB* in Nitrogen metabolism *AtSirB* transgenic plants were grown in different nitrogen deficiency conditions by reducing the NO₃⁻ level of the growth medium. Initially WT and transgenic plants were grown in normal MS medium for 15 days and were subsequently transferred to N deficient media in agar plates containing Hoagland medium adjusted to different N levels by reducing NO₃⁻ concentrations i.e., 0.1N, 0.25N and 0.50N of the normal concentrations. After 7 days of growth in N-deficient medium there was visible difference in *AtSirB* transgenic plants in comparison to that WT.
Plant phenotype

Due to N deficiency the phenotype of WT plants looked pale-green and in extreme N starvation (10% of control), they almost blanched. Under identical growth conditions the *AtSirBx* plants looked greener than WT. However, in *antiSirB* plants the N starvation caused severe phenotypical changes including bleaching even at 0.5N (Fig. 37).

Pigment contents

As compared to that of WT, total Chl, Chl a, Chl b and carotenoids contents were higher in *AtSirBx* and lower in *antiSirB* plants at all N level. The Chl contents of over-expressors were 47%, 49% and 86% higher than that of WT in 0.5N, 0.25N and 0.1N levels respectively. Similarly carotenoids contents increased in *AtSirBx* plants by 48%, 49% and 63% than that of WT. In antisense plants Chl decreased by 27%, 28% and 30% as compared to that of WT at 0.5N, 0.25N and 0.1N respectively. Similarly carotenoids contents decreased in *antiSirB* plants by 28%, 29% and 39% than that of WT. However, Chlorophyll a/b ratio did not change due to N starvation in WT and transgenic plants (Fig. 38, 39, 40).

Protein contents

As compared to that of WT, total protein contents were higher in *AtSirBx* and lower in *antiSirB* plants. Under normal (1N) N level, protein contents of overexpressor were 37% higher and in under-expressors the same were lower by 11% than that of WT. The protein contents of over-expressors were 38%, 39% and 96% higher than that of WT in 0.5N, 0.25N and 0.1N levels respectively. In antisense plants protein contents declined by 14%, 25% and 32% as compared to that of WT in 0.5N, 0.25N and 0.1N respectively (Fig. 41).

Nitrite reductase activity

To understand the mechanism of tolerance of *AtSirBx* plants to N starvation, activities of enzymes involved in N assimilation were studied in WT and transgenic plants grown in normal and N deficient growth medium. The siroheme-containing NiR mediates 6 electron reduction of NO$_2^-$ to NH$_3$. As compared to that of WT, its activity at optimum N level (1N) increased by 29% in *AtSirBx* plants. The same declined by...
Fig 37. Phenotypes of WT and *AtSirB* transgenic (sense and antisense) plants grown in normal (1.0N) and nitrogen starvation growth conditions. WT and *AtSirB* transgenic plants [Sens1 (*AtSirBx1*) and Anti1(*antiSirB1*)] were grown under normal MS medium for 15 days and subsequently transferred for 7 days to different concentration of Nitrate (0.1N, 0.25N, 0.5N, and 1.0N). Nitrogen concentration for 1.0N was 5mM. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 µmoles photons m⁻² s⁻¹)
Fig 38. Total chlorophyll of WT and $AtSirB$ transgenic (sense and antisense) plants grown in normal (1N) and nitrogen starvation growth conditions. WT and $AtSirB$ transgenic plants [Sens1 ($AtSirB$x1) and Anti1($antiSirB1$)] were grown under normal MS medium for 15 days and subsequently transferred for 7 days to different concentrations of nitrate (0.1N, 0.25N, 0.5N, and 1.0N). Nitrogen concentration for 1.0N was 5 mM. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m$^{-2}$ s$^{-1}$). Total chlorophyll was extracted and estimated as described in “Materials and Methods”. Each data point is an average of five replicates. The error bar represent standard deviation (SD).
Fig 39. Total carotenoid of WT and AtSirB transgenic (sense and antisense) plants grown in normal (1N) and nitrogen starvation growth conditions. WT and AtSirB transgenic plants [Sens1 (AtSirBx1) and Anti1(antiSirB1)] were grown in normal MS medium for 15 days and subsequently transferred for 7 days to different concentrations of nitrate (0.1N, 0.25N, 0.5N, and 1.0N). Nitrogen concentration for 1.0N was 5 mM. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). Total carotenoid was extracted and estimated as described in “Materials and Methods”. Each data point is an average of five replicates. The error bar represent standard deviation (SD).
Fig 40. Chlorophyll a/b ratio of WT and *AtSirB* transgenic (sense and antisense) plants grown in normal (1.0N) and nitrogen starvation growth conditions. WT and *AtSirB* transgenic plants [Sens1 (*AtSirBx1*) and Anti1(*antiSirB1*)] were grown under normal MS medium for 15 days and subsequently transferred for 7 days to different concentrations of nitrate (0.1N, 0.25N, 0.5N, and 1.0N). Nitrogen concentration for 1.0N was 5 mM. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). Chla, chlb was extracted and chl a/b ratio was estimated as described in “Materials and Methods”. Each data point is an average of five replicates. The error bar represent standard deviation (SD).
Fig 41. Total Protein of WT and AtSirB transgenic [Sens1 (AtSirBx1) and Anti1(antiSirB1)] plants grown in normal (1.0N) and nitrogen starvation growth conditions. WT and AtSirB transgenic plants [Sens1 (AtSirBx1) and Anti1(antiSirB1)] were grown under normal MS medium for 15 days and subsequently transferred for 7 days to different concentrations of nitrate (0.1N, 0.25N, 0.5N, and 1.0N). Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). Total Protein was extracted and estimated as described in “Materials and Methods”. Each data point is an average of five replicates. The error bar represent standard deviation (SD).
Fig 42. Nitrite reductase activity of WT and AtSirB transgenic (sense and antisense) plants grown in normal (1.0N) and nitrogen starvation growth conditions. WT and AtSirB transgenic plants [Sens1 (AtSirBx1) and Anti1(antiSirB1)] were grown under normal MS medium for 15 days and subsequently transferred for 5 days to different concentrations of nitrate (0.1N, 0.25N, 0.5N, and 1.0N). Nitrogen concentration for 100% was 5 mM. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). Nitrite reductase activity assay was performed as described in “Materials and Methods”.
38% in *antiSirB* plants. In N deficient conditions, as compared to that optimal N level, the extent of enhancement of NiR activity increased in over-expressors and decreased in under-expressers. The NiR activities of *AtSirBx* plants were 31%, 36% and 60% higher than that of WT in 0.5N, 0.25N and 0.1N levels respectively. As compared to that of WT, in *antiSirB* plants the NiR activity declined by 45%, 53% and 56%, in 0.5N, 0.25N and 0.1N respectively (Fig. 42).

**Nitrate reductase activity**

To understand if the change in the NiR activity modulates the NR, the latter was assayed in WT and *AtSirB* transgenic plants grown under normal and N deficient media. As compared to that of WT, its activity in optimum N level (1N) increased by 26% in *AtSirBx* plants. Conversely, the same declined by 14% in *antiSirB* plants. The NR activities of *AtSirBx* plants were 29%, 35% and 38% higher than that of WT in 0.5N, 0.25N and 0.1N correspondingly. As compared to that of WT, antisense plants grown in N deficient media had reduced (16-20%) NR activity (Fig. 43).

**Gene expression of NR and NiR**

To ascertain, if reduced enzymatic activities of NR and NiR observed during N-starvation is due to reduced gene expression, their message abundance was monitored by semi-quantitative RT-PCR in WT, *AtSirBx* and *antiSirB* plants grown in normal and N-deficient growth media. As compared to WT, in optimal growth media, NR and NiR gene expression increased in *AtSirBx* plants. However, in antisense plants their expression was not reduced in optimal growth media. In N-starvation conditions both NR and NiR expression severely declined in WT and disappeared in antisense plants. Under identical conditions, their gene expression was only partially declined in *AtSirBx* plants (Fig. 44).

**Pulse amplitude modulated (PAM) chlorophyll a fluorescence measurements in WT and transgenic plants grown in optimal and N-deficient media**

WT and transgenic plants were grown in normal MS medium for 15 days and were subsequently transferred to agar plates having Hoagland medium. The N level of the Hoagland medium was adjusted to 0.5N, 0.25N and 0.1N of the normal
Fig 43. Nitrate reductase activity assay of WT and AtSirB transgenic (sense and antisense) plants grown in normal (1.0N) and nitrogen starvation growth conditions. WT and AtSirB transgenic plants [Sens1 (AtSirBx1) and Anti1(antiSirB1)] were grown under normal MS medium for 15 days and subsequently transferred for 5 days to different concentrations of nitrate (0.1N, 0.25N, 0.5N, and 1.0N). Nitrogen concentration for 100% was 5 mM. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). Nitrate reductase activity assay was performed as described in “Materials and Methods”.
Nitrate reductase (Nia2)

Nitrite reductase (NiR)

Actin

Fig 44. Relative gene expression of Nitrate reductase (Nia2) and Nitrite reductase (NiR) in WT and AtSirB transgenic plants grown in optimal (1.0N) and limiting concentration (0.1N and 0.25N) of nitrate. WT and AtSirB transgenic plants were grown for 15 days in normal MS medium were transferred into optimal (1.0N) and limiting N level (0.1N and 0.25N). After 7 days of growth total RNA was isolated and RT-PCR was performed by using gene specific primers of Nia2 and NiR as described in “Materials and Methods”. Actin was taken as internal control.
concentrations by reducing NO$_3^-$ concentrations. After 7 days of growth in N deficient medium various fluorescence parameters i.e., $F_0$, $F_m$, $F_v/F_m$, electron transport rate (ETR), photochemical quenching (qP) and non-photochemical quenching (qN) were studied as described in “Materials & Methods”.

$F_0$: It is the dark adapted initial minimum fluorescence. The $F_0$ was measured in WT and AtSirB transgenic (Sense and antisense) plants grown in optimal and different N starvation level. The $F_0$ partially increased in AtSirBx plants over that of WT, grown in all N levels, mostly due to their higher Chl contents. However, under extreme N-deficiency (0.1N) the $F_0$ of antiSirB plants substantially increased (Fig. 45).

$F_m$: Maximal fluorescence was measured during the first saturation pulse after dark adaption. The $F_m$ was measured in WT and AtSirB transgenic (Sense and antisense) plants grown under different concentration of N. In sense lines, the $F_m$ value increased over that of WT, in plants grown in optimal (1N) and N-starvation conditions (0.5N, 0.25N, 0.1N). Conversely, in antiSirB plants the $F_m$ declined (Fig. 46).

$F_v/F_m$: This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers in dark-adapted samples. The $F_v/F_m$ ratio was measured in WT and AtSirB transgenic (Sense and antisense) plants grown under optimal and N-deficiency conditions. In AtSirBx plants the $F_v/F_m$ ratio increased over that of WT plants grown in all N levels. Conversely in antisense plants, the same declined. However, the decrease of $F_v/F_m$ was high in antiSirB plants grown in extreme N starvation (0.1N) (Fig. 47).

Electron transport rate (ETR): The ETR ($\mu$moles electrons $m^{-2} s^{-1}$) increased in response to photosynthetic active radiation (PAR) ($\mu$moles photons $m^{-2} s^{-1}$) (Fig. 48,49). However, the increase in ETR of AtSirBx plants was observed in low as well as high light intensities. This was consistently observed in AtSirBx plants grown in optimal and N-deficient (0.5N, 0.25N and 0.1N) medium. Increased slope of ETR at limiting light intensities suggest better energy capture by light-harvesting complex and the higher light-saturated rate implies an increase in the components of electron transport chain in sense plants. As compared to that of WT, the extent of increase of light-saturated ETR was higher in AtSirBx plants in N-deficient media than that of optimal growth conditions. In antiSirB plants, the ETR decreased in low as well as
Fig 45. Pulse amplitude modulated (PAM) Chl a fluorescence measurements for WT and \textit{AtSirB} transgenic plants [Sens1 (\textit{AtSirB}x1) and Ant1 (\textit{antiSirB}1)] grown in optimal (1.0N) and Nitrogen starvation (0.5N, 0.25N, 0.1N). Photo-periodically (14h L and 10h D) (100 μmoles photons m$^{-2}$ s$^{-1}$) grown 15-d-old WT and \textit{AtSirB} transgenic plants (sense and antisense) in normal MS medium were transferred to nitrogen deficient Hoagland media (0.5N, 0.25N, 0.1N). After 7 days of growth in nitrogen deficient medium plants were taken for measurements of PAM Chl a fluorescence. The F0 was measured in 20 min-dark-adapted leaves. The data points are average of 5 replicates and error bars represent SD.
Fig 46. The Fm of Chl a fluorescence measurements for WT and AtSirB transgenic plants [Sens1 (AtSirBx1) and Anti1 (antiSirB1)] grown in optimal (1.0N) and Nitrogen starvation (0.5N, 0.25N, 0.1N). Photo-periodically (14h L and 10h D) (100 μmoles photons m\(^{-2}\) s\(^{-1}\)) grown 15-d-old WT and AtSirB transgenic plants (sense and antisense) grown in MS-agar media were transferred to nitrogen deficient Hoagland media (0.5N, 0.25N, 0.1N). After 7 days of growth in nitrogen deficient medium plants were taken for measurements of PAM Chl a fluorescence. The Fm was measured in 20 min-dark-adapted leaves. The data points are average of 5 replicates and error bars represent SD.
Fig 48. Pulse amplitude modulated (PAM) Chl a fluorescence measurements for WT and AtSirB transgenic plants grown in optimal and nitrogen starvation MS medium (0.5N). Photo-periodically (14h L and 10h D) (100 μmoles photons m⁻² s⁻¹) grown 15-d-old WT and AtSirB transgenic plants (sense and antisense) in normal MS medium were transferred to nitrogen deficient MS medium (1.0N and 0.5N). After 7 days of growth in nitrate deficient medium plants were taken for measurements of PAM Chl a fluorescence to calculate electron transport rate (ETR) as described in "Materials & Methods".
Fig 49. Pulse amplitude modulated (PAM) Chl a fluorescence measurements for WT and *AtSirB* transgenic plants grown in nitrogen starvation MS medium (0.25N and 0.1N). Photo-periodically (14h L and 10h D) (100 μmoles photons m⁻² s⁻¹) grown 15-d-old WT and *AtSirB* transgenic plants (sense and antisense) in normal MS medium were transferred to nitrogen deficient MS medium (0.25 and 0.1N). After 7 days of growth in nitrate deficient medium plants were taken for measurements of PAM Chl a fluorescence to calculate electron transport rate (ETR) as described in “Materials & Methods”.
Fig 50. Pulse amplitude modulated (PAM) Chl a fluorescence measurements for WT and *AtSirB* transgenic plants grown in optimal (1.0N) and nitrogen starvation medium (0.5N, 0.25N and 0.1N). Photo-periodically (14h L and 10h D) (100 μmoles photons m⁻² s⁻¹) grown 15-d-old WT and *AtSirB* transgenic plants (sense and antisense) in normal MS medium were transferred to optimal (1.0N) nitrogen deficient hoagland medium (0.5N, 0.25N, 0.1N). After 7 days of growth in nitrate deficient medium plants were taken for measurements of PAM Chl a fluorescence to calculate photochemical quenching (qP) as described in “Materials & Methods”.
Fig 51. Pulse amplitude modulated (PAM) Chl a fluorescence measurements for WT and AtSirB transgenic plants grown in optimal (1.0N) and nitrogen starvation medium (0.5N, 0.25N and 0.1N). Photo-periodically (14h L and 10h D) (100 μmoles photons m⁻² s⁻¹) grown 15-d-old WT and AtSirB transgenic plants (sense and antisense) in normal MS medium were transferred to optimal (1.0N) and nitrogen deficient hoagland medium (0.5N, 0.25N, 0.1N). After 7 days of growth in nitrate deficient medium plants were taken for measurements of PAM Chl a fluorescence to calculate non-photochemical quenching (qN) as described in “Materials & Methods”.
high light intensities in all N level demonstrating a decrease in energy capture and its utilization.

**Photochemical quenching (qP):** It is the quenching parameter that represents coefficient of photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. The qP decreased in response to photosynthetic active radiations (PAR) (μmoles photons m⁻² s⁻¹). In $AtSirBx$ plants the qP was higher than that of WT while in $antiSirB$ plants the same was lower in all different concentrations of N (Fig. 50).

**Non-Photochemical quenching (qN):** The qN is is a measure of heat dissipation and a combined total for the combination of photo-protective mechanisms, state 1 and state 2 transition quenching, and photo-inhibition and photo-damage. The qN was measured in WT and $AtSirB$ transgenic plants grown under different concentration of N. The qN increased in response to higher light intensities. In $AtSirBx$ plants the qN was lower than that of WT, while in $antiSirB$ plants the same was higher when grown in N-limiting or N-sufficient (1N) media (Fig. 51).

**Alteration of carbohydrate metabolism in WT and $AtSirB$ plants**

Nitrogen deficiency results in accumulation of sugars and starch in leaves. WT and transgenic plants were grown in normal MS medium for 15 days and were subsequently transferred for 7 days in agar plates having Hoagland medium adjusted to 0.25N, 0.1N of the normal concentrations by reducing NO₃⁻ concentrations. After seven days of growth in nitrogen starvation media, WT and $AtSirB$ transgenic plants were stained with iodine to visualize the differences in the distribution of starch as described in “Materials and Methods”. In optimal N concentration no starch accumulation was observed in WT, sense and antisense plants. In low nitrate concentrations (0.25N) blue colour developed due to carbohydrate accumulation in antisense plants. Under extreme N-starvation (0.1N), carbohydrate accumulated in the leaves of WT and antisense plants. However, the $AtSirBx$ plants did not accumulate carbohydrates when grown in 0.1N growth medium suggesting that they could tolerate extreme N-starvation (Fig. 52).
Fig 52. Localization of Starch in WT and AtSirB transgenic plants [Sens1 (AtSirBx1) and Anti1(antiSirB1)] grown in (A) optimal (1N) and N starvation [(B) 0.25N and (C) 0.1N] media. WT and AtSirB transgenic plants were grown under normal MS medium for 15 days and subsequently transferred to different concentration of Nitrate (0.25N and 0.1N of normal MS concentration) for 5 days were taken and iodine staining was done to check the translocation of starch as described in “Material s&Methods”.
**AtSirB Over-expressors Overcome Sulphur Deficiency**

To investigate the role of *AtSirB* in Sulphur metabolism, WT and *AtSirB* transgenic plants were grown in sulphur starvation conditions by reducing the SO$_4^{-}$ level of the growth medium as described in “Materials and Methods”. Initially, WT and transgenic plants were grown in normal MS medium for 15 days and were subsequently transferred to agar plates containing Hoagland media having minimal sulphur concentration (0.1S). After 7 days of growth in S-deficient medium there was visible difference in *AtSirB* transgenic plants in comparison to that WT.

**Plant phenotype**

The phenotype of WT plants looked pale-green after seven days of growth in S starvation (0.1S). Under identical growth conditions the *AtSirB* plants looked greener than WT. However, in *antiSirB* plants the S starvation caused severe phenotypical changes and the plants became severely blanched (Fig. 53).

**Gene Expression of sulphite reductase (SiR)**

As siroheme is a prosthetic group of *SiR* (At5g04590) its expression was studied in optimally grown (1S) WT, *AtSirB* and *antiSirB* plants. As compared to that of WT, *SiR* expression substantially increased in *AtSirB* plants. Interestingly, in antisense plants *SiR* message abundance also increased, although to a reduced extent (Fig. 54).

**Protein abundance of sulphite reductase (SiR)**

To understand if gene expression correlates with that of protein expression, protein abundance of SiR was analysed by Western blot. In agreement with that of gene expression, *AtSirB* lines had increased protein abundance of *SiR* substantially over that of WT. However, in *antiSirB* lines their abundance also increased similar to that of over-expressors (Fig. 55).

**Pigment contents**

As shown in figure-56, plants looked pale due to S starvation. To estimate the change in pigment contents, WT and *AtSirB* transgenic plants grown in optimal (1S) and minimal (0.1S) sulphur growth media were analyzed. Chl a, Chl b and carotenoids contents were measured as described in “Material & Methods”. Total Chl contents
Fig 53. Phenotypes of WT and \textit{AtSirB} transgenic plants due to sulfur starvation. WT and \textit{AtSirB} (Sense 1 and antisense 1 lines) plants were grown at 22°C under 14 h L And 10 h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 15 days in optimal sulphur media (1S) and subsequently transferred into minimal sulfur (0.1S) media for seven days.
Fig 54. Gene expression of sulfite reductase (SiR) of WT and *AtSirB* transgenic plants. Total RNA was isolated from 3-week-old plants grown in optimal sulphur media (1S) and cool-white-fluorescent light (100 μmoles photon m⁻²s⁻¹) (14 h L and 10 h D) at 22°C. First strand synthesis was done by using reverse transcriptase. PCR of *SiR* was done using gene specific primers. Actin was taken as an internal control.
Fig 55. Immunoblot analysis of Sulfite reductase (SiR) of WT and AtSirB transgenic plants. Total protein was isolated from 3-week-old plants grown in optimal sulphur media (1S) and cool- white-fluorescent light (100 μmoles photon m$^{-2}$s$^{-1}$) (14 h L and 10 h D) at 22°C. (A) 25μg of protein was analysed by SDS-PAGE (12.5%) to check the equal loading of proteins. (B) Protein samples were transferred onto nitrocellulose membrane and immunoblot analysis was done as described in “Material & Methods”.
**Fig 56.** Total chlorophyll of WT and *AtSirB* transgenic [Sens1 (*AtSirBx1*) and Anti1(*antiSirB1*)] plants grown in optimal (1S) and sulphur starvation (0.1S) growth media. WT and *AtSirB* transgenic plants were grown in optimal sulphur MS medium (1S) for 15 days and subsequently transferred for 7 days to sulphur starvation (0.1S) medium. Plants were grown at 22°C under 14 h L ⁄ 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). (A) Total Chl and (B) carotenoid was extracted and estimated as described in “Materials and Methods”. Each data point is an average of five replicates and the error bars represent SD.
were higher in \textit{AtSirBx} than that of WT plants grown in optimal S media (1S). In response to S starvation (0.1S) Chl contents declined and that of carotenoids increased in both WT and transgenic plants. However, the Chl content of \textit{AtSirBx} plants was much higher than that of WT in minimal S medium. In \textit{antiSirB} plants the decline of Chl content in S-starvation conditions was severe i.e., 72%. Conversely the increase in carotenoids contents was highest (150%) in S-starved antisense plants. As revealed from fig. 57, in response to S deficiency, the Chl a/b ratio increased in WT and sense plants and decreased in antisense plants.

**Protein content**

As compared to that of WT, total protein contents were higher in \textit{AtSirBx} and lower in \textit{antiSirB} plants. In optimal growth media (1S), total protein content of \textit{AtSirBx} plants was 38% higher than that of WT. Conversely, protein content was reduced (29%) in antisense plants. In response to S-starvation (0.1S), the protein content of over-expressors was 45% higher than that of WT. The protein content declined by 50% in antisense plants grown in S-deficient medium (Fig. 58).

**Pulse amplitude modulated (PAM) chlorophyll \textit{a} fluorescence measurements in WT and \textit{AtSirB} transgenic plants grown in optimal (1.0 S) and minimal (0.1 S) concentrations of sulphur**

WT and transgenic plants were grown in normal MS medium for 15 days and were subsequently transferred to agar plates having Hoagland medium. The S concentration of the Hoagland medium was adjusted to 1.0S or 0.1S by reducing \(\text{SO}_4^{2-}\) and replacing it with \(\text{Cl}^-\). After 7 days of growth in S-deficient medium various fluorescence parameters i.e., \(F_0\), \(F_m\), \(F_v/F_m\), electron transport rate (ETR), photochemical quenching (qN) and non-photochemical quenching (qN) were studied as described in “Materials & Methods”.

**\(F_0\):** It is the initial minimum fluorescence in dark adapted plants. The \(F_0\) was measured in WT and \textit{AtSirB} transgenic (Sense and antisense lines) plants grown in normal MS medium for 15 days and subsequently transferred into sulphur starvation (0.1S) media. In optimal growth media (1S), the \(F_0\) almost remained unchanged in
Fig 57. Chl a, Chl b and Chl a/Chl b ratio of WT and AtSirB transgenic [(Sens1 (AtSirBx1) and Anti1(antiSirB1)) plants grown in optimal (1S) and sulphur starvation (0.1S) growth media. WT and AtSirB transgenic plants were grown under optimum S (1S) MS media for 15 days and subsequently transferred for 7 days to S starvation (0.1S) media. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). (A) Chl a, (B) Chlb and (C) Chl a/b ratio were estimated as described in “Materials and Methods”. Each data point is an average of five replicates and the error bar represents SD.
Fig 58. Total protein of WT and AtSirB transgenic [Sens1 (AtSirBx1) and Anti1(antiSirB1)] plants grown in optimal (1S) and sulphur-starvation (0.1S) growth media. WT and AtSirB transgenic plants were grown under optimum S (1S) MS media for 15 days and subsequently transferred for 7 days to S starvation (0.1S) media. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). Total Protein was extracted and estimated as described in “Materials and Methods”. Each data point is an average of five replicates. The error bar represents SD.
WT, AtSirBx and antiSirB plants. However, in S-deficiency (0.1N) the F$_0$ of sense and antisense plants increased by 21-23% (Fig. 59).

**Fm**: Maximal fluorescence was measured during the first saturation pulse after dark adaption. The Fm was measured in WT and AtSirB transgenic (Sense and antisense) plants grown in optimal and S-starvation media. In AtSirBx lines, the Fm value was higher than that of WT and antiSirB plants grown in optimal and sulphur deficient media (Fig. 60).

**Fv/Fm**: This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers in dark-adapted samples. The Fv/Fm ratio was measured in WT and AtSirB transgenic (Sense and antisense) plants grown in optimal and S-deficiency conditions. In AtSirBx plants the Fv/Fm ratio was higher than that of WT plants grown in optimal and S starvation media. Conversely, under identical conditions in antiSirB plants, the Fv/Fm ratio declined (Fig. 61).

**Electron transport rate (ETR)**: The ETR ($\mu$moles electrons m$^{-2}$ s$^{-1}$) increased in response to photosynthetic active radiation (PAR) ($\mu$moles photons m$^{-2}$ s$^{-1}$) (Fig. 62). However, the increase in ETR of AtSirBx plants was observed in low as well as high light intensities. In S starvation media increase of ETR in AtSirBx plants over that of WT was highly pronounced both in limiting and saturating light intensities. Increased slope of ETR at limiting light intensities suggest the better energy capture by light-harvesting complex and the higher light-saturated rate implies an increase in the amounts of electron transport components in sense plants. In antiSirB plants, the ETR decreased in high light intensities in S-deficient media.

**Photochemical quenching (qP)**: It is the quenching parameter that represents coefficient of photochemical quenching. It is a measure of the fraction of still open PSII reaction centers. The qP decreased in response to photosynthetic active radiations ((PAR) ($\mu$moles photons m$^{-2}$ s$^{-1}$). In AtSirBx plants the qP was higher than that of WT while in antiSirB plants the same was lower in optimal and S starvation growth conditions (Fig. 63).

**Non-Photochemical quenching (qN)**: The qN is a measure of heat dissipation and a combined total for the combination of photo-protective mechanisms, state 1 and state 2 transition quenching, and photo-inhibition and photo-damage. The qN was
Fig 59. Pulse amplitude modulated (PAM) Chl a fluorescence measurements for WT and *AtSirB* transgenic (sense and antisense) plants grown in optimal (1S) and sulphur starvation (0.1S) media. WT and *AtSirB* transgenic plants were grown under optimum S (1S) MS media for 15 days and subsequently transferred for 7 days to S starvation (0.1S) media. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). The minimal Fo fluorescence was measured in 20 min-dark-adapted leaves. The data points are average of 5 replicates and error bar represents SD.
Fig 61. Fv/Fm ratios of Chl a fluorescence of WT and AtSirB transgenic plants grown in optimal (1S) and sulphur starvation (0.1S) media. WT and AtSirB transgenic plants were grown under optimum S (1S) MS media for 15 days and subsequently transferred for 7 days to S starvation (0.1S) media. Plants were grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). The Fv/Fm ratio was calculated as in “Materials and Methods”. The data points are average of 5 replicates and error bar represents SD.
Fig 62. Electron transport rate (ETR) estimated by Chl a fluorescence measurements of WT and AtSirB transgenic plants grown in optimal (1S) and Sulphur starvation medium (0.1S). WT and AtSirB transgenic plants were grown under optimum S (1S) MS media for 15 days and subsequently transferred for 7 days to S starvation (0.1S) media. Plants were grown at 22°C under 14 h L/10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). The ETR was calculated as in “Materials and Methods”. The data points are average of 5 replicates and error bar represents SD.
Fig 63. Photochemical quenching (qP) was monitored by PAM Chl a fluorescence measurements of WT and AtSirB transgenic plants grown in optimal (1S) and Sulphur starvation medium (0.1S). WT and AtSirB transgenic plants were grown under optimum S (1S) MS media for 15 days and subsequently transferred for 7 days to S starvation (0.1S) media. Plants were grown at 22°C under 14 h L/10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). The qP was measured as in “Materials and Methods”. The data points are average of 5 replicates and error bar represents SD.
Fig 64. Non-photochemical quenching (qN) was monitored by PAM Chl a fluorescence measurements of WT and *AtSirB* transgenic plants grown in optimal (1S) and Sulphur starvation medium (0.1S). WT and *AtSirB* transgenic plants were grown under optimum S (1S) MS media for 15 days and subsequently transferred for 7 days to S starvation (0.1S) media. Plants were grown at 22°C under 14 h L/10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹). The qN was measured as in “Materials and Methods”. The data points are average of 5 replicates and error bar represents SD.
Fig 65. Immunoblot analysis of Rieske Fe-S center protein. (A) SDS-PAGE protein profile of thylakoid membranes isolated from 3 week old WT and AtSirB transgenic plants (S1, S2 over expression and A1, A2 antisense lines) grown at 22°C under 14h L/10h D photoperiod in cool-white-fluorescent light (100 μmoles photon m⁻² s⁻¹). 25 μg of protein was analysed by SDS-PAGE (12.5%) to check equal loading of proteins. (B) Protein samples were transferred onto nitrocellulose membrane and immunoblot analysis was done as described in “Material & Methods”.
measured in WT and \textit{AtSirB} transgenic plants grown under different concentration of S. The qN increased in response to higher light intensities. In \textit{AtSirBx} plants the qN was lower than that of WT, while in \textit{antiSirB} plants the same was higher when grown in S-limiting or S-sufficient media (Fig. 64).

\textbf{Expression of Rieske protein having 2Fe-2S center}

Siroheme is a cofactor of sulphite reductase responsible for sulphur metabolism. Sulfur assimilation is a prerequisite for the synthesis of Fe-S clusters. Rieske protein binds to high potential 2Fe-2S moiety and is present in cytochrome b/f complex of chloroplasts. Therefore, the abundance of Rieske-Fe-S protein was monitored by using antibodies raised against chloroplastic Rieske protein. As compared to that of WT, Rieske protein abundance increased substantially in \textit{AtSirBx} plants and declined in \textit{antiSirB} plants (Fig. 65).
Cloning, Overexpression and Antisense expression of AtDvr in Arabidopsis thaliana
Modulation of Monovinyl and Divinyl Protochlorophyllide by Genetic Manipulation of Divinyl Reductase

Arabidopsis thaliana Divinyl reductase (AtDvr) (Accession Number- NM_121871) has 1254 bp of coding region for 417 amino acids. Out of 417 amino acids, 49 amino acids at N terminal end act as transit peptide that target AtDvr protein into chloroplast (Fig. 66A-B).

Dendrogram

A dendrogram was drawn by comparing the amino acid sequences of dicot and monocot plants using phylip (PHYLOGeny inference package) program (Fig. 67). Dendogram of DVR proteins reveal a high degree of homology between monocots, i.e., Sorghum bicolor, Zea mays and Oryza sativa. Most dicot plants i.e., Arabidopsis thaliana, Populus trichocarpa, Vitis vinifera formed a separate phylogenetic group.

Hydropathic plot

A hydropathy plot or Kyte and Doolittle Plot show the hydrophobicity of a protein along the Y axis. The amino acids are shown by their numbers in the protein sequence in the X axis. In the present study, the Kyte and Doolittle plot is used to determine whether the protein is a transmembrane protein. Peaks in Kyte-Doolittle hydropathy plot around 2 indicate strong hydrophobic regions making the protein a strong candidate for a transmembrane protein (Kyte and Doolittle, 1982). Hydrophobicity plot for AtDvr shows that there is one one peak score around 1.6 that starts from amino acid 331 to amino acid 353. This confirms the spanning of Dvr protein to membrane by this transmembrane domain (Fig. 68A).

Hopp and Woods Antigenecity Plot

These plots show hydrophilicity instead of hydrophobicity. A more hydrophilic region is a better place for an antigen to bind. Thus an antigenecity plot helps to determine where on a protein a monoclonal antibody would bind well. The X-axis represents the individual amino acids in the protein sequence and the Y axis represents the hydrophilicity. The protein seems to have even distributions of hydrophilic and hydrophobic regions although there are several hydrophilic peaks where antibodies could bind. There are relatively 2 high peaks at approximately amino acids 78 and 125 (Fig.68B).
Fig 66. (A) cDNA sequence of Arabidopsis thaliana divinyl reductase (*Atdivr*) having 1254 bp. Out of 1254 bp 147 bp code for chloroplast targeting peptide. (B) Amino acid sequence of Dvr protein containing 417 amino acids out of which 49 amino acids at N-terminal act as a transit peptide.
Fig 67. Dendrogram of Divinyl reductase (Dvr) of higher plants. Amino acid sequences of Dvr protein from different dicot and monocot plants were aligned and the phylogenetic tree was obtained by phylip software.
Fig 68. (A) **Kyte-Doolittle** hydropathy plot showing hydrophilic residue present in Divinyl reductase. Amino acid residue position at 331 to 353 in square are representing transmembrane domain. (B) **Hoop-woods** hydrophilicity plot Hopp-Woods scale was designed for predicting potentially antigenic regions of polypeptides. Values greater than 0 are hydrophilic and thus likely to be exposed on the surface of a folded protein.
Cloning of *Divinyl Reductase (AtDvr)* from *Arabidopsis thaliana*

For the characterization of *AtDvr* gene and its function, its cDNA was amplified from *Arabidopsis thaliana* by polymerase chain reaction (PCR) using gene specific forward (Dvr F) and reverse (Dvr R) primers (table-5). *Arabidopsis* cDNA from mature leaves was used as template for PCR. PCR was performed using high fidelity Taq DNA polymerase for Dvr amplification. PCR temperatures were set up at denaturation at 94°C for 45 sec, annealing at 56°C for 30 sec and extension at 72°C for 1 min 15 sec for each cycle and was continued for 30 cycles. PCR yielded a fragment of 1254 bp (Fig. 69A). PCR fragment was cloned in pGEMT- easy vector by AT ligation using T4 DNA ligase. pGEMT easy-Dvr recombinant vector was transformed into DH5α competent cells. Colony PCR was done to select positive colonies (Fig. 71A). Confirmation of transformed colonies was done by restriction digestion of the pGEMT−easy-Dvr plasmid with EcoR I which gives a fall out of 1254bp (Fig. 71C). The transit peptide was predicted by the chloroP program that determined the transit peptide cleavage cite after amino acid Serine, positioned at no. 49. Mature part of the *AtDvr* was cloned using mature forward primer (mature Dvr F) from the cleavage site of the transit peptide and the reverse primer of *AtDvr* (Dvr R) having cDNA of *Arabidopsis* as template (Table 5). A fragment of 1107bp was amplified (Fig. 69B), cloned into pGEMT−easy vector and transformed into DH5α competent cell (Fig. 70). Positive colonies were screened with colony PCR (Fig. 71B) and confirmed after restriction digestion with BamHI and Sall that yielded a fall out of 1107bp (Fig. 71D).

Overexpression of mature *AtDvr* in *E. coli*

Mature *AtDvr* was cloned into pMAL c2x protein expression vector as shown in fig. 72C. pGEMT-easy vector containing the mature *AtDvr* cDNA was digested with BamHI and Sall and the digested fragment was ligated to pMAL c2x protein expression vector. pMAL c2x -Dvr recombinant vector was transformed into BL21 (DE3) competent cells. Positive colonies were selected by colony PCR (Fig. 72A). To confirm ligation, the pMAL c2x expression vector containing the mature *AtDvr* was digested with BamHI and Sall which yielded a fragment of 1107 bp (Fig 72B). Ecoli BL 21 (DE3) cells containing the plasmid pMAL c2x -mature *AtDvr* was induced to express Dvr protein using 0.3 mM IPTG for 16 h at 16°C. This induction protocol yielded the maximum amount of protein into soluble fraction. Proteins of
Fig 69. PCR Amplification of Atdvr. Atdvr (A) Full length and (B) mature (without transit peptide sequence) were PCR amplified from cDNA using gene-specific forward and reverse primers for full length and mature forward and gene specific reverse primer for mature Atdvr cDNA of Arabidopsis thaliana.
Fig 70. Cloning Strategy For \textit{AtDvr} in pMAL-c2x protein expression vector.
Fig 71. Cloning of *AtDvr* into pGEMT-easy vector. PCR amplified products were ligated to pGEMT easy vector. Colony PCR was done to check the bacterial colony containing recombinant pGEMT-easy- precursor (A) and mature-*AtDvr* (B). Recombinant pGEMT- easy vector containing *Atdvr* was digested with EcoRI produced a fragment size of 1.254 kb and 3 kb of pGEMT easy (C). Recombinant pGEMT- easy vector containing mature *Atdvr* was digested with BamHI and SalI produced a fragment size of mature *Atdvr* of 1.107kb and 3 kb of pGEMT easy (D).
**Fig 72. Cloning of mature AtDvr in pMAL-c2x.** The *AtDvr* mature fragment obtained by BamHI and SalI digestion from Fig. 3 was ligated to pMAL-c2X vector and the resultant colonies (A) were analyzed by PCR using *AtDvr* mature forward and gene-specific reverse primers. (B) Positive colonies were grown and their plasmids were digested by BamHI and SalI that yielded 1107bp *AtDvr* mature fragment. (C) Schematic representation of cloning in pMAL c2x vector.
supernatant and pellet fractions of sonicated Ecoli cells over-expressing mature part of *AtDvr* were separated by 12.5% SDS-PAGE that shows the polypeptide profile of pellet and supernatant fractions of un-induced and IPTG-induced Dvr protein. The heterologously expressed protein had molecular weight of 82.9 KDa that includes the 42.5 KDa Maltose binding protein (MBP) (Fig. 73A).

Recombinant Dvr protein was purified from the supernatant fractions of the sonicated E. coli cells over-expressing mature part of Dvr via batch purification with Amylose resin beads. Supernatant fraction was incubated with amylase resin and washed five times with the washing buffer and eluted by the elution buffer containing Maltose according to manufacturer’s instruction. Several eluted fractions were collected and checked the purity by running 12.5% SDS PAGE. The Purified Dvr-MBP fusion protein had a molecular weight of 82.9 KDa (Fig-73B). It was subsequently cut by factor X to yield the recombinant Dvr protein.

**Production of polyclonal antibody against AtDvr protein**

Polyclonal antibodies were raised against purified Dvr Protein in New Zealand rabbit as described in “Materials & Methods” and pictorially shown Fig. 74A. The titre of AtDvr antibodies was checked by western blot. Immunoblots were performed using different dilution of antibodies i.e., 1:1000, 1:5000 and 1:10000 (Fig-74B). An intense signal was observed even at 1:10000 dilutions, whereas no signal was observed with pre-immune serum that demonstrated the specificity of AtDvr antibodies towards AtDvr recombinant protein.

**Raising Transgenic Plants of *Arabidopsis thaliana* having *AtDvr* in Sense and Antisense Orientations**

For the functional characterization of *Divinyl reductase (AtDvr)* a transgenic approach was used. *AtDvr* cDNA was cloned in pCAMBIA 1304 binary vector in sense and antisense orientations to genetically transform and *Arabidopsis* plants via *Agrobacterium*-mediated transformation.

**Clonining of *AtDvr* in Sense and Antisense Orientations in the Binary Vector**

pCAMBIA 1304 plant transformation vector was used for *Agrobacterium*-mediated transformation of modified pCAMBIA 1304 plant transformation vector containing
**Fig 73. Atdvr expression in E.coli.** BL 21 (DE3) Cells having the recombinant pMAL- c2x-mature AtDvr were induced with 0.3mM IPTG at 16°C overnight. SDS PAGE (12.5%) showing the polypeptide profile of (A) pellet, supernatant and (B) purified recombinant 82.5 kD MBP-Dvr fusion protein.
**Fig 74. Production of polyclonal antibody in rabbit.** (A) Schematic diagram summarizing the steps involved in raising polyclonal antibody against purified recombinant dvr protein. (B) Western blot showing titer and specificity of the antibody against the recombinant MBP-AtDvr fusion protein (lane 1, pre-immune serum; lane 2, 1:1000 dilution; lane 3, 1:5000 and lane 4, 1:10000 dilutions).
kanamycin \((\text{npt})\) gene and CaMV 35S -\(\Omega\)-enhancer cassette. Cloning of \(\text{AtDvr}\) cDNA in pCambia 1304 was done by taking out the \(\text{AtDvr}\) fragment from recombinant pGEMT-easy vector (pGEMT-easy-\(\text{Dvr}\)) by digesting with the \(\text{EcoRI}\) restriction enzymes and ligating it with pCambia 1304 plant transformation vector (Fig.75A-B). Recombinant pCambia 1304-\(\text{AtDvr}\) was transformed into DH5\(\alpha\) competent cell. Positive colonies were screened by colony PCR using \(\text{AtDvr}\) gene specific primers (Fig. 75C). As it was a non-directional cloning, both sense and antisense constructs were obtained after transformation. Positive colonies were used for plasmid isolation and after its digestion with XbaI yielded a fragment of 1254 bp in sense construct and no fallout in antisense construct (Fig-75E). Orientation of \(\text{AtDvr}\) with respect to 35S primer is shown in fig-76C. Recombinant pCambia 1304 plasmid was transformed into \textit{Agrobacterium tumefacience} GV 1301 as described in “Materials and methods”. Recombinant colonies were selected on YEM media plates containing 50 \(\mu\)g ml\(^{-1}\) kanamycin and 25 \(\mu\)g ml\(^{-1}\) rifampicin and confirmed by PCR using 35S internal forward and gene specific reverse and forward primers (Fig. 76A-B).

**Plants transformation by \(\text{AtDvr}\)**

\textit{Arabidopsis thaliana} plants were transformed with positive colony of recombinant \textit{Agrobacterium tumefacience} by floral dip method as in “Materials and Methods”. The seeds were harvested after one month. After harvesting, seeds were selected in the presence of 30\(\mu\)g ml\(^{-1}\) kanamycin in 0.5X MS medium. Kanamycin resistant plants (T1) were grown in \textit{Arabidopsis} growth room maintained at 22\(^\circ\)C and 100 \(\mu\)moles photons m\(^{-2}\) s\(^{-1}\) to generate seeds. For next generation (T2) the resulting seeds were grown in kanamycin plates and were selected for kanamycin resistance.

**Confirmation of \(\text{AtDvr}\) Transgenic plants**

**PCR analysis**

Kanamycin resistant \(\text{AtDvr}\) over-expression and antisense (T1) lines were taken and genomic DNA was isolated. Using the kanamycin gene specific forward and reverse primers (Table-5), the genomic DNA from 14 individual lines of T1 generation was amplified by PCR. The PCR condition for \((\text{npt})\) amplification was denaturation at
Fig 75. Cloning of AtDvr to pCAMBIA 1304 vector having 35 S – Ω-poly A promoter cassette and npt for kanamycin selection. (A) EcoR1 digestion of pGEMT-easy having AtDvr and pCAMBIA 1304 vector having CaMV 35 S – Ω-poly A cassette and npt gene. Digestion resulted in excision of Atdvr gene from pGEMT-easy vector. (B) Ligation of Atdvr with linearized modified pCAMBIA 1304 vector. (C) Colony PCR for Checking positive colony in DH5α E. coli cell. (D) Digestion check with EcoR1 restriction enzyme yielded a fallout of 1.254kb. (E) Sense and antisense orientation was checked by XbaI restriction digestion yielded a fragment of 1.254 in sense clone and no fallout in antisense clone.
Fig 76. Sense (A) and Antisense (B) confirmation of pCAMBIA+AtDvr in agrobacterium (GV 1304) by colony PCR. (C) Schematic representation of pCAMBIA 1304. Recombinant pGEMT-easy vector having omega enhancer was digested with EcoRI. AtDvr fall out was ligated to EcoRI digested pCAMBIA 1304 modified plant transformation vector in sense and antisense orientation that were used to raise overexpression and antisense plants of Arabidopsis thaliana.
Results

94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 60 sec for each cycle and continued for 30 cycles. PCR yielded a fragment of 800 bp from transformant that contained npt kanamycin resistant gene (Fig. 77A). These individual transgenic lines were grown to harvest seeds. Seeds collected from the above plants were again grown in kanamycin plates to select T2 transgenic lines. The seedling resistant to kanamycin were selected and their genomic DNA was isolated for the PCR analysis. *AtDvr* over expression and antisense lines were confirmed by PCR using 35S internal forward primer and gene specific reverse primer and 35S internal forward primer and gene specific forward primer respectively. A fragment of 1454bp (1254bp *AtDvr* +200bp 35s sequence) was amplified in all the selected transgenic lines (Fig. 77B-C). WT plants genomic DNA was used as negative control. Transgenic seeds were grown for T3 generation to obtain homozygous transgenic plant.

**Southern blot analysis of *AtDvr* transgenic plants**

The stable integration of T-DNA cassette containing *AtDvr* cDNA into the *Arabidopsis thaliana* host genome was checked by Southern blot analysis. Genomic DNA from two sense and two antisense lines were isolated and digested with *EcoRI* restriction enzyme and subjected to Southern blot analysis using radio-labelled probe as described in “Materials and Methods”. As expected from the restriction digestion pattern of genomic DNA of transformed *Arabidopsis* plants by *EcoRI*, a fragment of 1.254kb (size of the *AtDvr* cDNA) was observed in transformed sense and antisense plants (Fig. 78A). This band was not observed in WT plants. There was a second band (size of ~8 kb) observed in WT and transgenic plants due to the endogenous *AtDvr* gene of the host genome.

**GUS histochemical Assay**

Some of the transgenic lines positive for *AtDvr* cDNA along with WT plants were screened by GUS histochemical assay. Only the transformed lines were stained blue whereas the untransformed wild type plants did not acquire stain (Fig. 78B).

**Gene expression of *AtDvr* in WT and transgenic plants**

To check the gene expression of *AtDvr* in WT and transgenic plants, semi-quantitative RT PCR and Northern analyses were performed. RNA (3μg) extracted from WT and *AtDvr* transgenic plants were taken for first strand cDNA synthesis. Using the cDNA
Fig 77. Confirmation of *Atdv* transgenic (Sense and antisense) plants. (A) Genomic DNA of WT and and T1 generation *Atdv* sense and antisense *Arabidopsis* plants were used as template and PCR of *npt* gene having resistance to kanamycinA fragment of 0.8 kb corresponding to *npt* was amplified that confirmed the integration of the T-DNA cassette with the host genome. (B) Using 35 S internal forward primer and gene specific reverse primer PCR reaction was performed with genomic DNA isolated form different transgenic lines of T 2 generation plants that yielded a fragment size of 1.454 kb integrated in a sense orientation (S1 – S9). (C) PCR reaction using 35 S internal forward primer and gene specific forward primer was done with genomic DNA isolated from antisense line A1, A2 and A3 of T2 generation plants that yielded a fragment size of 1.454 kb.
Fig 78. (A) Southern blot analysis of WT and AtDvr transgenic plants. Genomic DNA (20μg) from untransformed WT and PCR confirmed AtDvr transgenic plants were digested with EcoRI, separated on 1% agarose and transferred onto nylon membrane. The blot was probed with radiolabeled AtDvr cDNA.The AtDvr cDNA fragment used for prob preparation was obtained by PCR with a pair of gene specific Primers. A fragment size of 1254bp was observed in transformed AtDvr transgenic plants and that was not found in untransformed WT plants.(B) GUS histochemical assay. The WT and T3 generation AtDvr plants (S1, S2 and A1, A2 ) were stained with X-Gluc for the GUS histochemical assay as described in the material and methods. All the AtDvr plants showed GUS Staining.
Fig 79. RT PCR analysis of AtDvr sense and antisense transgenic plants. Total RNA of WT and AtDvr transgenic plants (AtDvr1x 1, 2 and antiDvr 1, 2) was isolated from 3 to 4 weeks old plants grown under cool white fluorescent light (100 μMoles photon m-2 s-1) in 14 h L and 10 h D photoperiod at 22°C. (A) First strand synthesis was done by Using reverse transcriptase. PCR was done for actin (as endogenous control) to show equal loading. PCR of AtDvr was done by using gene specific forward and reverse primers. (B) Strand specific RT PCR. RT was performed using the gene specific forward primer to check the antisense RNA expression the PCR was done using gene specific forward and reverse primers.
as template, semi-quantitative PCR was done by using \textit{AtDvr} gene specific forward and reverse primers (Fig. 79A). RT PCR analysis reveals that as compared to WT, transgenic \textit{AtDvrx} lines 1 and 2 had increased (35\%) \textit{AtDvr} gene expression whereas in antisense lines its expression decreased by 60\%.

**Northern blot analysis of WT and \textit{AtDvr} transgenic plants**

To further confirm the extent of \textit{AtDvr} RNA over-expression and silencing in sense and antisense plants, RNA was isolated from the WT, Sense lines (sense 1, sense 2) and antisense lines (Anti 1, 2). Northern blot analysis was performed using the \textit{AtDvr} cDNA as probe. Northern blot analysis revealed that as compared to WT, in \textit{AtDvrx} lines 1 and 2 \textit{AtDvr} RNA expression increased by 55\% whereas in antisense lines its expression decreased by 65\% (Fig. 80).

**Anti-sense mRNA expression in \textit{antiDvr} plants**

Instead of using Oligo (dT) or random hexamers to prime first-strand cDNA synthesis, gene specific primer was used. To detect antisense mRNA, the forward primer was used for reverse transcription from RNA isolated from \textit{antiAtDvr} transgenic lines. The forward primer binds only to the antisense mRNA. Thus, there will be no first-strand cDNA synthesis from the forward primer if there is no antisense mRNA. The first-strand reactions then served as targets for PCR together using the forward and reverse primers for \textit{AtDvr}. The result demonstrates that \textit{AtDvr} gene product was amplified when PCR was performed using first-strand cDNA prepared from gene specific forward primer only (Fig. 79B). A cDNA prepared using total RNA from wild-type plants were used as template for another set of PCR that served as negative control.

**Western blot analysis of the \textit{AtDvr} transgenic plants**

To ensure if transgenic plants had the expected change in \textit{AtDvr} protein expression, western blot analysis was conducted. Total protein was isolated from 4-week-old plants of \textit{AtDvr} over-expression and silenced lines as described in “Materials & Methods”. Equal loading of the protein (25\mu g) was checked by running 12.5\% SDS-PAGE (Fig. 81A). Polyclonal antibody raised against \textit{Arabidopsis} Dvr protein was used to immunodetect the \textit{AtDvr} protein in transgenic plants. Fig. 81B showed that \textit{AtDvr} protein expression was higher in over-expression lines 1 and 2. In antisense
Fig 80. Northern blot analysis of *AtDvr*. (A) Northern blot analysis of *AtDvr* mRNA in WT and *Atdvr* transgenic plants (*AtDvr* x 1, 2 and *antiDvr* 1, 2). Total RNA was isolated from 3- to 4-week-old plants grown under cool white fluorescent (100 μmoles photon m⁻² s⁻¹) in 14 h L and 10 h D photo-period at 22°C. The *AtDvr* full length DNA fragment was used for probe preparation obtained by PCR with gene-specific primers. 10 μg of total RNA was loaded in each lane and resolved on 1.2 % denaturing formaldehyde agarose gel. (B) Ethidium bromide (EtBr) stained gel having equal loading of RNA is shown below.
**Fig 81. Western blot Analysis of *AtDvr* plants.** Total protein profile (12.5 % SDS-page) of leaves extracted from 3-4 week old WT and *Atdvr* transgenic (Over-expression line S1 and S2 and antisense line A1 and A2) grown at 22°C under light intensity of 100 mmoles photons m⁻² s⁻¹ with 14 h L and 10h D photoperiod. 35 μg of protein was loaded in each lane and SDS page was run to check the equal loading. Protein samples from the gel were transferred onto nitrocellulose membrane and immunoblot analysis of dvr protein (40.5 kDa) was done as described in material and method.
line 1, the AtDvr protein abundance was significantly reduced. This confirms Dvr was overexpressed in sense lines and silenced in one of the anti-sense lines.

**Morphology of of AtDvr overexpressor (AtDvrx) and Antisense (antiDvr) transgenic plants**

In comparison with WT plants AtDvrx plants were not significantly different from that of WT. Among morphological parameters i.e., plants height, rosette diameter, root length, and fresh weight the latter was partially higher in AtDvrx plants (Fig. 82A-B). However, as compared to that of WT, in AtDvr antisense plants the plant height, rosette diameter, root length, and fresh weight were reduced by 60%, 27%, 31%, 53% respectively. The AtDvr antisense plants were pale in phenotype in comparison to that of WT plants. Several anti-sense lines perished before flowering (Fig. 83A-D).

**Pigment and Protein Contents of WT, AtDvrx and antiDvr Plants**

*Chlorophyll and carotenoid contents*

To know the pigment contents, WT and AtDvr transgenic plants were grown in MS plate for four weeks and their Chl and Chl a/b and carotenoids contents were studied. In AtDvrx plants the Chl and carotenoids contents were higher than that of WT. In antisense plants, the Chl contents decreased and carotenoids contents increased. The Chl a/b ratio increased by 32% in sense plants and 155% in antisense plants (Fig. 84A-E).

*Protein contents:* Total protein contents of AtDvrx plants were higher (15%) than that of WT. In antisense plants the same declined by 29% (Fig. 85).

**Pulse amplitude modulated (PAM) chlorophyll a fluorescence measurements**

To understand if increased Chl and protein contents of plants results in increased photosynthetic capacity, Chl fluorescence was monitored as a signature of photosynthesis in WT and AtDvr transgenic plants.

Chlorophyll a fluorescence was measured in WT and AtDvr (Sense 1 and antisense lines) plants grown at 22°C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for four-week in agropit soil (Fig. 82B).
Fig 82. Phenotypes of the *AtDvr* Transgenic plants. Phenotypes of *AtDvr* transgenic plants grown under cool-white-fluorescent light (100 μmoles photon m⁻² s⁻¹) in 14 h L and 10 h D photoperiod at 22°C. (A) Morphology of 4-week-old WT and *AtDvrX* and *antiDvr* Arabidopsis plants grown in MS medium. (B) Morphology of 4- to 5-week-old WT and *AtDvrX* and *antiDvr* plants grown in agropit soil.
Fig 83. Measurement of morphological parameters of WT and \textit{AtDvr} transgenic plants. WT and \textit{AtDvr} (Sense line 1 and antisense line1) plants were grown at 21\textdegree C under 14 h L / 10h D photoperiod in cool-white-fluorescent light (100 \, \textmu m moles photons m\textsuperscript{-2} s\textsuperscript{-1}) for 4 week in MS medium. (A) Plants height. (B) Root length. (C) Rossette diameter. (D) fresh weight. (E) Dry weight were measured. Each data point is an average of five replicates. The error bar represents standard error (SE).
Fig 84. Pigment content of WT and AtDvrx1 and antiDvr plants. WT and AtDvr (Sense lines 1 and antisense lines 1) plants were grown at 21°C in 14 h L and 10 h D photoperiod of cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 3-to-4 weeks in MS medium and their (A) Chl (B) carotenoids (C) Chl a (D) Chl b (E) Chl a/b ratio were measured as described in "Materials & Methods". Each data point is an average of five replicates. The error bar represent standard deviation (SD).
Fig 85. Total protein content of WT and AtDvrx and antiDv r plants. WT, AtDvrx and antiDvr plants were grown at 22°C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 4-weeks in MS medium. Total protein was extracted and estimated as described in “Materials & Methods”. Each data point is an average of five replicates. The error bar represent standard deviation (SD).
Various Chl a fluorescence parameters i.e., Fo, Fm, Fv/Fm, electron transport rate (ETR), quantum yield of PSII (ΦPSII), photochemical quenching (qP) and non-photochemical quenching (qN) were studied as described in “Materials & Methods”.

**Fo:** Fo was measured in the dark adapted leaves as initial minimum fluorescence. In *AtDvr* transgenic (overexpressor as well as antisense) plants there was no significant change in Fo values (Fig. 86A).

**Fm:** The Fm was measured as maximal fluorescence during the first saturation pulse after dark adaption. In *AtDvr* transgenic plants Fm remained almost unchanged (Fig. 86B).

**Fv/Fm:** This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers by dark-adapted leaves. In *AtDvr* transgenic plants there was no significant difference of Fv/Fm ratio as compared to that of WT. (Fig. 86C).

**Electron transport rate (ETR):** The ETR (μmoles electron m⁻² s⁻¹) increased in response to photosynthetic active radiation (PAR) (μmoles photons m⁻² s⁻¹). As compared to WT, the ETR was higher in *AtDvrx* plants at saturating light intensities. In *antiDvr* plants the ETR substantially declined at limiting (50 μmoles photons m⁻² s⁻¹) as well as saturating light intensities (Fig. 87A).

**Photochemical quenching (qP):** The qP represents photochemical quenching and is a measure of the fraction of still open PSII reaction centers. It decreased in response to increased photosynthetic active radiation (PAR) (μmoles photons m⁻² s⁻¹). In *AtDvrx* plants qP was higher than that of WT plants in different light intensities. In *antiDvr* plants it was lower than that of WT (Fig. 87B).

**Non-Photochemical quenching (qN):** The qN is non-photochemical quenching and is a measure of heat dissipation and a combined total for the combination of photoprotective mechanisms, state 1 and state 2 transition quenching, and photo-inhibition and photo-damage. The qN increased in response to increased light intensity both in WT and transgenic plants. As compared to WT, the qN was higher in *antiDvr* plants and lower in *AtDvrx* plants (Fig. 87C).
Fig 86. Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and *AtDvr* transgenic plants. WT and *AtDvr* transgenic (*AtDvr* and *antiDvr*) plants photoperiodically (14h L and 10h D) grown in 100 μmoles photons m⁻² s⁻¹ light intensity for 4 weeks in agropit soil were used for studying various parameter of Chlorophyll a fluorescence measurement including (A) Fo, (B) Fm and (C) Fv/Fm as described in "Materials & Methods".
Fig 87. Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and AtDvr transgenic plants. WT and AtDvr transgenic plants photoperiodically (14h L and 10h D) grown in 100 μmoles photons m⁻² s⁻¹ light intensity for 4-week in agropit soil were used for studying various parameter of Chlorophyll a fluorescence measurement including (A) electron transport rate(ETR), (B) Photochemical quenching (qP) and (C) non-photochemical quenching (qN) as described in “Materials & Methods”.
Protochlorophyllide contents: Since the amount of chlorophyll in \textit{AtDvr} transgenic plants were higher and that of antisense plants lower than that of WT, their Pchlide, an import Chl biosynthesis intermediate was extracted from leaves harvested during light period of 4-week-old green plants. As compared to WT, in \textit{AtDvrx} plants the Pchlide contents increased over that of WT and in antisense plants the same declined to a larger extent (Fig. 89A)

Monovinyl and Divinyl Pchlide

The Pchlide and other Chl biosynthetic intermediates were transferred from Hear fraction to ether and their fluorescence emission (E440) and excitation (F625) spectra were recorded at 77K as in “Materials and Methods”. Fig. 88. A-B, shows the 77K fluorescence emission (E440) (A) and excitation (F625) (B) spectra of ether extract of hexane extracted acetone residue solvent mixture (HEAR) of WT, \textit{AtDvrx} and \textit{antiDvr} plants. The emission spectra reveal a fluorescence emission peak at 625 nm due to Pchlide. To determine the ratio of monovinyl Pchlide/divinyl Pchlide, their 77K fluorescence excitation spectra were monitored. The excitation spectra of all samples were normalized to 1.0 at 443 nm. At 77K 90% of the Pchlide pool occurs in the pentacoordinated state, while the remaining 10% occurs in the hexacoordinated state. Soret excitation electronic transitions of the pentacoordinated MV and DV Pchlide are very clearly split into shorter wavelength By (0-0) and longer wavelength Bx (0-0) components. Although pentacoordinated MV Pchlide (E437F625) and pentacoordinated DV Pchlide (E443F625) possess very similar fluorescence emission maxima at 624 and 625nm respectively, they exhibit different By (0-0) Soret excitation maxima at 437 and 443nm respectively. The corresponding Bx (0-0) transitions of pentacoordinated MV and DV Pchlide exhibit excitation maxima at 443 and 451nm respectively. As a consequence of these observations and because of the aforementioned Soret excitation overlap between the pentacoordinated MV Pchlide Bx(0-0) and the DV By (0-0) transitions at 443nm the following strategy was adopted. It was decided to discriminate between the MV and DV Pchlide via the MV Pchlide excitation maximum at 437 nm and via the DV Pchlide excitation at 451nm, respectively. Using appropriate equations (Tripathy and Rebeiz, 1985 and Hukmani and Tripathy, 1992), the ratio of MV Pchlide/DV Pchlide was calculated.
Fig 88. Accumulation of DV and MV protochlorophyllide in WT and AtDvr transgenic plants. WT, AtDvr (line 1) and antiAtDvr (line 1) transgenic plants grown at 21°C under 14 h L and 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 3-to-4 weeks in MS medium were grind in 90% Ammonical acetone in dark. Pigments were transferred to ether by exreaction of HEAR (hexane extracted acetone residue solvent mixture) preparation. MV and DV protochlorophyllides were quantified by 77K spectrofluorometry as described in “Material & Methods”. The excitation spectra were normalized at 443 nm. The excitation spectra recorded at excitation and emission slit widths of 4nm:. (A) Emission spectra and (B) Excitation spectra.
Fig 89. Accumulation of Divinyl (DV) and monovinyl (MV) protochlorophyllide in WT and AtDvr transgenic plants. WT, AtDvr (line 1) and antiAtDvr (line 1) transgenic plants grown at 21°C under 14 h L And 10h D photoperiod in cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 3-to-4 weeks in MS medium were grind in 90% Ammonical acetone in dark. Pigments were transferred to ether by exreaction of HEAR (hexane extracted acetone residue solvent mixture) preparation. MV and DV protochlorophyllides were quantified by 77K spectrofluorometry as described in “Material & Methods”.
In the WT plants the MV Pchlide and DV Pchlide were 8% and 92% respectively. In sense plants due to overexpression of Dvr the proportion of DV Pchlide decreased to 85% and consequently that of MV Pchlide increased to 15%. In antisense plants due to reduced Dvr reaction the relative proportion of DV Pchlide increased to 96% and consequently that of MV Pchlide decreased to 4%. This demonstrates that genetic manipulation of \textit{AtDvr} may not drastically alter the MV or DV pool of other Chl biosynthesis intermediates (Fig. 89B-C).
Transformation of
Brassica juncea
with
Divinyl reductase (Dvr)
Cloning of \textit{DVR} in pCAMBIA 1304 (hygromycin) for transformation of \textit{Brassica juncea} cv. varuna

Cloning of \textit{AtDvr} in Sense and Antisense Orientations in the Binary Vector

pCAMBIA 1304 plant transformation vector was used for \textit{Agrobacterium}-mediated transformation of modified pCAMBIA 1304 plant transformation vector containing hygromycin (\textit{hpt}) gene and CaMV 35S -\Omega- enhancer cassette. Cloning of \textit{AtDvr} cDNA in pCAMBIA 1304 was done by taking out the \textit{AtDvr} fragment from recombinant pGEMT-easy vector (pGEMT-easy-Dvr) by digesting with the EcoRI restriction enzymes and ligating it with pCAMBIA 1304 plant transformation vector. Recombinant pCAMBIA 1304-\textit{AtDvr} was transformed into DH5\(\alpha\) competent cell. Positive colonies were screened by colony PCR using \textit{AtDvr} gene specific primers (Fig. 90A). Positive colonies were used for plasmid isolation and after its digestion with EcoRI yielded a fragment of 1254 bp in sense construct. Recombinant pCAMBIA 1304 plasmid was transformed into \textit{Agrobacterium tumefaciense} GV 1301 as described in "Materials and Methods". Recombinant colonies were selected on YEM media plates containing 50 \(\mu\)g ml\(^{-1}\) kanamycin and 25 \(\mu\)g ml\(^{-1}\) rifampicin and confirmed by PCR using \textit{AtDvr} 35S internal forward and gene specific primers (Fig. 90B-C).

Transformation of \textit{Brassica juncea} plants

\textit{Brassica juncea} plants were transformed by \textit{AtDvr} via plant tissue culture methods. Eight to 10 d after sowing, the hypocotyls of brassica seedling were cut in 7 mm segments. About 15 hypocotyl explants were floated in a 9 cm Petri dish on 10 mL of infection medium A2 (Table 9). To each Petri dish, 20\(\mu\)L of a late log Agrobacterium culture having final observance 0.2 to 0.3 was added. These plates were incubated at low light intensity (25 \(\mu\)moles photons m\(^{-2}\) s\(^{-1}\)). After 24 hour the hypocotyl segments were washed with A3 medium containing 250 mg/L augmentin, patted dry on filter paper, and placed on selection medium A4 containing hygromycin. The Petri dishes (2.2 cm high and 14 cm in diameter) were sealed with parafilm and kept in light (80 \(\mu\)moles m\(^{-2}\) s\(^{-1}\)) in 16 h L/8 h D photoperiods at 28°C. Three to 6 weeks after selection, calli with small shoots were formed (Fig. 91A). Entire calli with the shoots were removed from the hypocotyl explants and transferred to medium A4 (without selection) (Table-9) for root generation (Fig. 91B). The shoots grew out and were
Fig 90. Cloning of DVR into pCAMBIA 1304 plants transformation vector having hpt gene for Hygromycin resistance. (A) Clony PCR for AtDvr cloned in pCAMBIA 1304 having hpt gene for hygromycin resistance. (B) Digestion check for AtDvr in pCAMBIA 1304 by EcoRI restriction enzyme that yielded 1.254 kb AtDvr cDNA. (C) confirmation of AtDVR+pCAMBIA sense construct in transformed in GV 1301 competent cell using 35S internal forward and gene specific reverse primers.
Fig 91. Generation of DVR overexpressing Brassica Juncea cv varuna (DvrBx) plants. (A) Brassica hypocotyls in shoot induction media with Hygromycin. (B) Brassica plants in root induction media. (C) Mature Brassica transgenic plants in green house. (D) T2 generation plants of Dvr brassica overexpressor (DvrBx) plants grown for 3 month in Green house.
isolated. Healthy shoots (2 cm or larger) were transferred directly to vermiculite soil wrapped in poly-bag. After few days transformed plants were transferred to the greenhouse (Fig. 91C). Plants were grown upto T2 generation for analysis (Fig. 91D).

**Confirmation of AtDvr Transgenic plants in Brassica**

**PCR analysis**

Hygromycin resistant *AtDvr Brassica juncea* over-expression lines were taken and genomic DNA was isolated. Using the hygromycin gene (*hpt*) specific forward and reverse primers (Table-5), the genomic DNA from 6 individual lines of T1 generation was amplified by PCR. The PCR condition for *hpt* amplification was denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 60 sec for each cycle and continued for 30 cycles. PCR yielded a fragment of 1.0kb in transformants that contained *hpt* hygromycin resistant gene (Fig. 92A). These individual transgenic lines were grown to harvest seeds. Seeds collected from the above plants were again grown in hygromycin plates to select T2 transgenic lines. The seedlings resistant to hygromycin were selected and their genomic DNA was isolated for the PCR analysis. *AtDvr* over expression lines (*DvrBx*) were confirmed by PCR using 35S internal forward primer and gene specific reverse primer. A fragment of 1454bp (1254bp *AtDvr*+200bp 35s sequence) was amplified in all the selected transgenic lines. PCR was also performed to by using *AtDvr* gene specific primer that yielded a fragment of 1254 bp that is corresponding to *AtDvr* cDNA (Fig. 92B-C).

**Southern blot Analysis of AtDvr transgenic plants**

The stable integration of T-DNA cassette containing *AtDvr* cDNA into the *Brassica juncea* was checked by Southern blot analysis. Genomic DNA from two sense lines of *Brassica* overexposer plants grown for 3 month in green house were isolated and digested with *EcoRI* restriction enzyme and subjected to Southern blot analysis using radio-labelled probe as described in “Materials and Methods”. As expected from the restriction digestion pattern of genomic DNA of transformed *Brassica* plants by *EcoRI*, a fragment of 1.254kb (size of the *AtDvr* cDNA) was observed in transformed plants (Fig. 93). The same band was not observed in WT plants.
Fig 92. Confirmation of Advr Brassica transgenic (DvrBx) plants. (A) Genomic DNA of WT and DvrBx Brassica plants were used as template and PCR of hpt gene having resistance to hygromycin. A fragment of 1.0 kb corresponding to hpt was amplified that confirmed the integration of the T-DNA cassette in the host genome. (B) Using 35 S internal forward primer and gene specific reverse primer PCR reaction was performed with genomic DNA isolated form different transgenic lines of T 2 generation plants that yielded a fragment size of 1.456 kb integrated in a sense orientation (S1 –S9). (C) PCR reaction using AtDvr gene specific primer that yielded a fragment of 1.254kb corresponding to Arabidopsis Dvr cDNA.
Fig 93. Southern blot analysis of WT and *Brassica* transgenic plants: Genomic DNA was isolated from WT and *DvrBx* plants. Genomic DNA was restriction digested with *EcoRI* restriction enzyme and southern blot analysis was done using *AtDvr* cDNA as a probe as described in “Materials & Methods”.
Gene expression of $AtDvr$ in WT and transgenic plants

To check the gene expression of $AtDvr$ in WT and transgenic plants, semi-quantitative RT PCR was performed. RNA (3μg) extracted from WT and $AtDvr$ Brassica transgenic plants were taken for first strand cDNA synthesis. Using the cDNA as template, semi-quantitative RT PCR was done by using $AtDvr$ gene specific forward and reverse primers. RT PCR analysis reveals that $AtDvr$ expression in Brassica transformants and its absence from the WT (Fig. 94).

Chlorophyll and carotenoid contents

To know the pigment contents, WT and $DvrBx$ transgenic Brassica plants were grown in soil for 2 months and their Chl and carotenoids contents were estimated. In $DvrBx$ plants the Chl and carotenoids contents were higher than that of WT (Fig. 95A-B).

Protein contents

Total protein contents of $DvrBx$ plants increased by 20% over that of WT (Fig. 96).

Pulse amplitude modulated (PAM) chlorophyll a fluorescence measurements

To understand if increased Chl and protein contents of plants results in increased photosynthetic capacity, Chl fluorescence was monitored as a signature of photosynthesis (Govinjee) in WT and $Dvr$ Brassica transgenic plants.

Chlorophyll a fluorescence was measured in WT and $DvrBx$ plants grown at 28°C in green house for 3 month in vermiculite soil (Fig. 91D). Various Chl a fluorescence parameters i.e., Fo, Fm, Fv/Fm, electron transport rate (ETR), quantum yield of PSII (ØPSII), photochemical quenching (qP) and non-photochemical quenching (qN) were studied as described in “Materials & Methods”.

Fo: Fo was measured in the dark adapted leaves as initial minimum fluorescence. In $DvrBx$ plants there was no significant change in Fo values (Fig. 97A).

Fm: The Fm was measured as maximal fluorescence during the first saturation pulse after dark adaption. In $DvrBx$ plants Fm remained almost unchanged (Fig. 97B).
Fig 94. RT PCR confirmation of *Brassica DvrBx* plants. Total RNA was isolated from WT and DvrBx Brassica plants (T2 generation). First strand synthesis was done by using reverse transcriptase. PCR of *Atdvr* was done by using gene specific primer for Arabidopsis *Dvr* cDNA.
Fig 95. Pigment contents of WT and DvrBx Brassica plants. WT and DvrBx plants were grown at 28°C in 14 h L and 10 h D photoperiod of cool-white-fluorescent light (100 μmoles photons m⁻² s⁻¹) for 2 month in soil and their Chl and carotenoids contents were measured as described in "Materials & Methods". Each data point as an average of five replicates. The error bar represent standard deviation (SD). (A) Total Chl (B) Carotenoids
Fig 96. Total protein content content of WT and Dvr Brassica overexpressor (DvrBx) plants. WT and AtDvrBx plants were grown at 28°C in 14 h L and 10h D photoperiod of cool-white-fluorescent light (200 μmoles photons m⁻² s⁻¹) for 2 month in soil and their total protein was extracted and estimated as described in “Materials & Methods”. Each data point as an average of five replicates. The error bar represent standard deviation (SD).
**Fv/Fm**: This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers by dark-adapted leaves. In *DvrBx* plant there was no significant difference of Fv/Fm ratio as compared to that of WT. (Fig. 97C).

**Electron transport rate (ETR)**: The ETR (µmoles electron m\(^{-2}\) s\(^{-1}\)) increased in response to photosynthetic active radiation (PAR) (µmoles photons m\(^{-2}\) s\(^{-1}\)). As compared to WT, the ETR was higher in *DvrBx* plants at saturating light intensities (Fig. 98A).

**Photochemical quenching (qP)**: The qP represents photochemical quenching and is a measure of the fraction of still open PSII reaction centers. It decreased in response to increased photosynthetic active radiation (PAR) (µmoles photons m\(^{-2}\) s\(^{-1}\)). In *DvrBx* plants qP was higher than that of WT plants in different light intensities. (Fig. 98B).

**Non-Photochemical quenching (qN)**: The qN is non-photochemical quenching and is a measure of heat dissipation and a combined total for the combination of photo-protective mechanisms, state 1 and state 2 transition quenching, and photo-inhibition and photo-damage. The qN increased in response to increased light intensity both in WT and transgenic plants. As compared to WT, the qN was lower in *DvrBx* plants (Fig. 98C).

**Protochlorophyllide contents**: Since the amounts of chlorophyll in *DvrBx* plants were higher than that of WT, their Pchlide, an important Chl biosynthesis intermediate was extracted from leaves harvested during light period of 3-month-old green plants. As compared to WT, in *DvrBx* plants the Pchlide contents partially increased over that of WT (Fig. 100A).

**Monovinyl and divinyl Pchlide**

The Pchlide and other Chl biosynthetic intermediates were transferred from Hear fraction to ether and their fluorescence emission (E440) and excitation (F625) spectra were recorded at 77K as in “Materials and Methods”. Figure 4 shows the 77K fluorescence emission (E440) (A) and excitation (F625) spectra of ether extract of hexane extracted acetone residue solvent mixture (HEAR) of WT, AtDvrx and antiAtDvr plants. The emission spectra reveal a fluorescence emission peak at 625 nm due to Pchlide. To determine the ratio of monovinyl Pchlide/divinyl Pchlide, their
Fig 97. Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and \textit{DvrBx} plants. WT and \textit{AtDvrBx} transgenic plants grown photo-periodically (14h L/10h D) in 200 \textmu moles photons m$^{-2}$ s$^{-1}$ light intensity for 2 month in soil were used for studying various parameter of Chlorophyll a fluorescence measurement i.e., (A) Fo, (B) Fm and (C) Fv/Fm as described in "Materials & Methods".
Fig 98. Pulse amplitude modulated (PAM) Chlorophyll a fluorescence measurements of WT and DvrBx plants. WT and AtDvrBx transgenic plants grown photo-periodically (14h L/10h D) in 200 μmoles photons m⁻² s⁻¹ light intensity for 2 month in soil were used for studying various parameter of Chlorophyll a fluorescence measurement i.e., ETR, qP and qN as described in “Materials & Methods”.
Fig 99. Acumulation of monovinyl and divinyl protochlorophyllide in WT and DvrBx plants. WT and DvrBx transgenic Brassica plants were grown at 28°C under 14 h L/10h D photoperiod in cool-white-fluorescent light (200 μmoles photons m⁻² s⁻¹). Their leaves were ground in 90% ammonical acetone in dark and HEAR was prepared and their Pchlide were quantified by spectro-fluorometry as described in “Material & Methods”. (A) Emission spectra at E440 nm of WT and DvrBx plants. (B) Excitation spectra at F625 nm of WT and DvrBx plants. The excitation spectra were normalized at 443 nm. The excitation spectra recorded at excitation and emission slit widths of 4nm.
Results

77K fluorescence excitation spectra were monitored. The excitation spectra of all samples were normalized to 1.0 at 443 nm. At 77K 90% of the Pchlide pool occurs in the pentacoordinated state, while the remaining 10% occurs in the hexacoordinated state. Soret excitation electronic transitions of the pentacoordinated MV and DV Pchlide are very clearly split into shorter wavelength By (0-0) and longer wavelength Bx (0-0) components. Although pentacoordinated MV Pchlide (E437F625) and pentacoordinated DV Pchlide (E443F625) possess very similar fluorescence emission maxima at 624 and 625 nm respectively, they exhibit different By (0-0) Soret excitation maxima at 437 and 443 nm respectively. The corresponding Bx (0-0) transitions of pentacoordinated MV and DV Pchlide exhibit excitation maxima at 443 and 451 nm respectively. As a consequence of these observations and because of the aforementioned Soret excitation overlap between the pentacoordinated MV Pchlide Bx(0-0) and the DV By (0-0) transitions at 443 nm the following strategy was adopted. It was decided to discriminate between the MV and DV Pchlide via the MV Pchlide excitation maximum at 437 nm and via the DV Pchlide excitation at 451 nm, respectively. Using appropriate equations (Tripathy and Rebeiz, 1985 and Hukmani and Tripathy, 1992), the ratio of MV Pchlide/DV Pchlide was calculated.

In the etiolated WT plants the relative amounts of MV Pchlide and DV Pchlide were 80% and 20% respectively. In sense plants due to overexpression of Dvr the proportion of DV Pchlide decreased to 10% and consequently that of MV Pchlide increased to 90% (Fig. 101).

To compare the relative amounts of MV Pchlide abd DV Pchlide of etiolated plants with that of green plants, the Pchlide was extracted from green leaves and their MV and DV Pchlide contents were estimated. In contrast to etiolated tissue, in green plants, the relative amount of MV Pchlide and DV Pchlide were 10% and 90% respectively. In DvrBx green plants due to overexpression of Dvr the proportion of DV Pchlide decreased to 80% and consequently that of MV Pchlide increased to 20% (Fig. 100B-C).

To understand ALA is the substate of tetrapyrroles. To induce excess Pchlide synthesis leaf discs were treated with ALA in dark for 3 h. Subsequently, its Pchlide was fractionated to HEAR and transferred to ether. The 77K fluorescence excitation spectra (F625) reveal that the relative amounts of MV Pchlide and DV Pchlide in WT
Fig 100. Accumulation of monovinyl and divinyl protochlorophyllide in WT and DvrBx plants. WT and DvrBx transgenic Brassica plants were grown at 28°C under 14 h L/10h D photoperiod in cool-white-fluorescent light (200 μmoles photons m−2 s−1). Their leaves were ground in 90% ammonical acetone in dark and HEAR was prepared and their (A) Pchlide contents and the ratio of (B) divinyl and (C) monovinyl protochlorophyllide were quantified by spectro-fluorometry as described in “Material & Methods”.
Fig 101. Accumulation of DV and MV protochlorophyllide in etiolated WT and DvrBx transgenic plants. WT and AtDvrBx plants were grown at 28°C in dark for 7 days in MS medium were ground in 90% Ammonical acetone in dark and HEAR (hexane extracted acetone residues) preparation was done. MV and DV protochlorophyllide quantification was done by spectrofluorometry as described in “Material & Methods”. The excitation spectra were normalized at 443 nm. The excitation spectra recorded at excitation and emission slit widths of 4nm.
Fig 102. Fluorescence excitation spectra in ether at 77K of WT and DvrBx plants. Leaf disk of 3-4 month old WT and DvrBx plants were used. Leaf disks were incubated with ALA for 3 hour in dark. After incubation leaf disk in water (con) and leaf disk with ALA were grounded in ammonical acetone and hear was prepared. Excitation spectra at 77K were recorded (A) leaf disk without ALA (B) leaf disks with ALA. The excitation spectra were normalized at 443 nm. The excitation spectra recorded at excitation and emission slit widths of 4nm.
Fig 103. Distribution of MV and DV Pchlide in WT and DvrBx leaf discs incubated in dark without (Con) or with ALA.
Brassica were 10% and 90% respectively. In Brassica over-expressssor plants, the proportion of DV Pchlide decreased to 75% and consequently that of MV Pchlide increased to 25% (Fig. 102A-B, 103A-B).