Methyl CpG Binding Domain proteins (MBDs) are known to bind to methylated DNA but the molecular mechanism by which these proteins act in the cellular environment to silence a gene is still unclear. In the present study, the role of these proteins in the process of gene silencing has been studied.

**Role of Methyl CpG Binding Domain (MBD) proteins in RNA directed DNA methylation**

To investigate the role of AtMBDs in gene silencing in *Arabidopsis thaliana*, initially a hypothesis was formulated. The hypothesis was that if a particular AtMBD protein is crucial for gene silencing pathway, knocking down of that AtMBD would hinder the silencing of any reporter gene by its RNAi construct. The reporter gene used in the current study is *uidA* gene which encodes β-glucorunidase enzyme. To validate this hypothesis, an experiment involving two consecutive transformations was designed. In the first transformation, *uidA* (*gus*) gene along with antisense/RNAi construct of gene coding for one of the AtMBDs was introduced into *Arabidopsis* plants. These transgenic lines were subsequently transformed with a construct designed for silencing the pre-existing *gus* gene. According to the hypothesis if the particular AtMBD would be essential for gene silencing pathway, then there would be no silencing of the *uidA* reporter gene by RNAi method.
Figure 1. Flow chart depicting hypothesis for analyzing the role of AtMBD proteins in gene silencing: If a particular AtMBD protein has a role in gene silencing, then knocking down of its gene would hamper silencing of a reporter gene by RNAi. To validate this hypothesis, two consecutive transformations were performed; in the first transformation, *uidA* expressing AtMBD knockdown transgenic plants were generated. In the second transformation, these transgenics were transformed with a construct for RNAi of the *gus* gene. No silencing of *gus* reporter gene would be observed in AtMBD knockdown lines by *gus*-RNAi, if the particular AtMBD had a role in gene silencing.

Cloning and generation of AtMBD11 knockdown Arabidopsis transgenic plants

The *AtMBD11* gene was cloned in antisense orientation in pCAMBIA2301, where it is under the control of 35S promoter (Fig. 2). The vector backbone had *nptII* selectable marker and *gus* reporter genes. This vector was used for *Agrobacterium*-mediated stable transformation. Floral dip method of transformation was used for the generation of transgenic Arabidopsis plants. The screening of transgenic plants was performed on MS medium containing kanamycin (100 µg/ml). Transgenic nature of plants that grew on selection medium was further verified by performing PCR for detection of *nptII* gene using genomic DNA as a template. The PCR result clearly showed amplification of a 554 bp band only in case of transgenic plants and none of this size in untransformed plant (Fig. 3). The PCR-positive plants were subsequently analyzed by GUS histochemical assay for the expression of *gus* gene. The transgenic
plants turned blue when incubated in GUS buffer for overnight whereas the untransformed wild type plants did not show GUS activity (Fig. 4).

**Figure 2. Schematic diagram of the construct for antisense suppression of AtMBD11 gene:** AtMBD11 was cloned in antisense orientation downstream to 35S promoter in pCAMBIA2301. The vector backbone also had gus reporter gene and nptII selectable marker gene.

**Figure 3. PCR verification of AtMBD11-AS transgenic lines:** The transgenic nature of AtMBD11 antisense plants was verified by PCR amplification of nptII gene. A 554 bp amplified fragment was clearly visible in the transgenic lines whereas absent in the wild type plant. Wild type genomic DNA was used as negative control whereas the binary vector harboring the AtMBD11 was used as a positive control. Lanes 1 to 10 represent different transgenic lines which were used for PCR analysis. Lane 1 contains 50 bp DNA ladder marker. WT, wild type.

The transcript accumulation of AtMBD11 gene was analyzed in the knockdown lines using quantitative Real Time PCR (qRT-PCR). The qRT-PCR results clearly indicated the down-regulation of AtMBD11 transcript in the knockdown lines by 3 to 5 folds (Fig. 5). Morphological analysis of AtMBD11 knockdown transgenic plants displayed serrated leaves in T1 generation (Fig. 6). However this phenotype could not be observed in next generation.
Results

AtMBD11-AS lines

WT  1  2  3  4  5  6

Figure 4. GUS histochemical assay in transgenic plants expressing AtMBD11 in antisense and gus gene in sense orientation: The expression of gus reporter gene was analyzed by GUS histochemical assay. Leaves from WT and AtMBD11 antisense plants were incubated in GUS reaction buffer for overnight at 37°C. After that the leaves were washed with acetone. The transgenic plants (lanes 1 to 6) displayed blue color accumulation whereas no blue color was observed in the wild type (WT) plants.

Figure 5. qRT-PCR analysis of AtMBD11 gene in WT and AtMBD11-AS transgenic plants: The relative expression of AtMBD11 gene in different transgenic lines was determined using WT as a control. The fold change is shown on Y-axis. The X-axis represents different transgenic lines. The error bars indicate standard deviation from the mean value. Three biological replicates were employed in the analysis.
Results

Figure 6. Morphological comparison between the leaves of WT and AtMBD11 knockdown plants: The wild type untransformed plant showed normal phenotype (left) while AtMBD11 knockdown lines showed serrated leaves (right) in T₁ generation. WT, wild type.

Cloning and generation of AtMBD10 knockdown transgenic plants

The AtMBD10 gene was cloned in antisense orientation in the binary vector backbone pCAMBIA2301, where it was driven by a constitutive 35S promoter (Fig. 7). The construct was then moved to Agrobacterium. Floral dip method of transformation was used for the generation of transgenic Arabidopsis plants. The screening of transgenic plants was carried out using MS medium containing kanamycin (100 µg/ml). Transgenic plants that grew on selection media were further verified by performing PCR analysis of nptII gene specific primers and using genomic DNA as a template. An amplified product of 554 bp was observed in case of transgenic plants as compared to the untransformed plant where the band was found to be absent (Fig. 8).

Figure 7. Schematic diagram of AtMBD10-AS: AtMBD10 was cloned in antisense orientation downstream to 35S promoter in pCAMBIA2301. The vector backbone also had gus reporter gene and kanamycin selectable marker.
Results

**Figure 8.** PCR verification of AtMBD10-AS transgenic lines: The transgenic nature of AtMBD10 antisense plants was verified using PCR for nptII gene. A 554 bp fragment was obtained in the transgenic lines whereas it was found to be absent in the wild type plants. The first lane contains 50 bp DNA ladder marker; WT genomic DNA was used as a negative control and the binary vector harboring the gene was used as a positive control. Lanes 1 to 9 represent different transgenic lines. WT, wild type.

The expression of gus reporter gene was further analyzed by GUS histochemical assay. When GUS histochemical assay was performed, leaves of transgenic plants turned blue in presence of its substrate x-gluc (5-bromo-4-chloro-3-indolyl glucuronide), whereas the blue color did not appear in case of wild type plants (Fig. 9). Analysis of the expression of AtMBD10 gene in the knockdown lines was performed by qRT-PCR of AtMBD10 gene. qRT-PCR analysis clearly showed down-regulation of AtMBD10 transcript by >5 fold in various transgenic lines in comparison to their wild type counterpart (Fig. 10). The knockdown lines of AtMBD10 gene showed multiple flowers/siliques at a single node whereas this phenotype was absent in the wild type plant (Fig. 11).

**Figure 9.** GUS histochemical assay of AtMBD10-AS transgenic plants: The expression of gus reporter gene was verified by GUS histochemical assay in the transgenic lines. Leaves from untransformed and transgenic plants were incubated in GUS buffer for overnight at 37°C and were then washed with acetone. The transgenic plants (1 to 6) displayed blue color whereas the leaves from wild type (WT) plants did not turn blue when GUS histochemical assay was performed.
Results

Figure 10. qRT-PCR analysis of transgenic plants harboring AtMBD10-AS construct: The expression of AtMBD10 gene in knockdown lines was analyzed by qRT-PCR. The X-axis represents different transgenic lines while the Y-axis represents the relative fold change in the transcript level of AtMBD10 gene.

Figure 11. Morphological analysis of nodes of AtMBD10-AS transgenic plants: The transgenic plant harboring AtMBD10 antisense gene displayed more than one flower/silique at a single node (right), whereas the untransformed plant showed normal phenotype (left).

Cloning of AtMBD6 gene and generation of AtMBD6-RNAi transgenic plants

The AtMBD6 gene was amplified from genomic DNA. To generate an RNAi construct of AtMBD6, the first exon and the first intron of AtMBD6 gene were cloned in sense orientation in BamHI and XbaI sites in pCAMBIA2301 vector. Subsequently,
the first exon was cloned in antisense orientation at XbaI and SacI restriction sites in the same vector. The final construct that was generated had first exon of the gene in sense and antisense orientation spaced by its first intron (Fig. 12)

![Diagram](image)

**Figure 12. Schematic diagram of pCAMBIA2301-AtMBD6-RNAi vector:** The first exon of AtMBD6 was cloned in sense and antisense orientation separated by its intron downstream to 35S promoter in pCAMBIA2301. The vector backbone also contained gus reporter gene and nptII selectable marker.

The AtMBD6-RNAi construct was transformed to Agrobacterium and the transformed Agrobacteria were further used for floral dip method to generate transgenic plants. The seeds from the plants employed for floral dip were harvested and grown on MS medium containing kanamycin (100 µg/ml). Plants that grew on the selection medium were selected for their functional characterization. The transgenic nature of the plants was verified using PCR analysis. PCR was performed using nptII gene-specific primers. Genomic DNA isolated from the transgenic plants was used as a template. The result clearly showed amplification of a 554 bp fragment in case of transgenic plants (Fig. 13). Genomic DNA obtained from untransformed plants was used as a negative control.

The expression of gus reporter gene was analyzed by GUS histochemical assay. Leaf tissues from the transgenic plants were incubated in GUS buffer containing x-gluc at 37°C for overnight. The transgenic leaves turned blue whereas the untransformed wild type plants did not show any GUS activity (Fig. 14).
Results

Figure 13. PCR verification of AtMBD6-RNAi transgenic lines: The transgenic nature of AtMBD6-RNAi plants was confirmed using PCR analysis with nptII specific gene primers; A 554 bp fragment was obtained from the transgenic lines whereas it was found to be absent in the wild type plant. Genomic DNA isolated from wild type plant was used as a negative control whereas the vector harboring this gene was used as a positive control. The first lane contains 50 bp DNA marker; lanes 1 to 10 represent different transgenic lines which were used for PCR amplification. WT, wild type; +ve; positive control.

Figure 14. GUS histochemical assay of AtMBD6-RNAi transgenic plants: The expression of gus reporter gene was analyzed by GUS histochemical assay. Leaves obtained from transgenic plants (1 to 6) turned blue whereas those from wild type (WT) plants did not turn blue when GUS histochemical assay was performed.

The transcript accumulation of AtMBD6 gene was determined using qRT-PCR. The expression analysis showed that there is a significant down-regulation of the transcript in the transgenic RNAi lines as compared to the wild type (Fig. 15). Expression of AtMBD6 gene in the transgenic lines declined to 20-25% of its expression level in wild type plants. Morphological comparisons of the transgenic plants with RNAi lines for AtMBD6 showed abnormal phenotype. Many AtMBD6-RNAi transgenic plants showed profuse root system and multiple flower/siliques at a single node. The wild type plants grown under the same condition did not show such phenotypic abnormalities (Fig. 16).
**Results**

**Figure 15.** qRT-PCR analysis of transgenic plants showing silencing of *AtMBD6*:

The expression of *AtMBD6* gene in knockdown lines was analyzed by qRT-PCR. The X-axis represents different transgenic lines while the Y-axis represents the relative fold change in transcript level. WT, wild type.

**Figure 16.** Phenotypic variation in *AtMBD6*-RNAi transgenic lines:

The transgenic *AtMBD6*-RNAi plants showed profuse root system as compared to the untransformed wild type plants. The transgenic plants also had more than one flower/silique at a single node. The wild type plants did not show any such alteration.
Generation of transgenic Arabidopsis plants transformed with gus gene (uidA)

To study the silencing of gus reporter gene, control transgenic plants were generated using the binary vector pCAMBIA2301. Arabidopsis plants were transformed using floral dip method. Transgenic plants were screened on MS medium containing kanamycin and selected plants were used for GUS histochemical assay (Fig. 17).

![GUS histochemical assay of plants transformed with uidA gene](image)

**Figure 17. GUS histochemical assay of plants transformed with uidA gene:** The expression of gus reporter gene was analyzed by GUS histochemical assay. Leaves obtained from transgenic plants (1 to 6) displayed blue color after the assay whereas leaves from untransformed wild plant did not show any GUS activity.

Construction of a vector for RNAi of gus gene and development of double transgenic plants

In order to study the role of AtMBDs in gene silencing, analysis of the expression of gus reporter gene was employed as a tool. A construct GUS-RNAi was designed to silence the pre-existing gus gene by RNAi. A clone of gus gene in pBSK vector in antisense orientation along with the catalase intron was available in the laboratory. The gus gene along with the intron were excised from this construct and the excised fragment was then cloned in the binary vector pBI121 at XbaI and SacI sites. This led to the construction of GUS-RNAi cassette. This cassette was then moved to pCAMBIA1301 vector along with 35S promoter and NOS terminator (Fig. 18A). Confirmation of the cloning was carried out using restriction digestion analysis (Fig. 18B). The confirmed recombinant clone was then used to transform Agrobacteria.
Figure 18. Cloning of GUS-RNAi: (A) The gus gene was cloned in sense and antisense orientation flanking the catalase intron leading to the final GUS-RNAi construct in pCAMBIA1301 binary vector. (B) Restriction digestion pattern of the construct: 4.9 kb, 5 kb and 2.8 kb fragments were released when digested with EcoRI + HindIII, BstXI + HindIII and BstEII + HindIII, respectively.

Subsequently, different gus-expressing AtMBDs knockdown transgenic plants were transformed with GUS-RNAi to generate double transgenic Arabidopsis plants. A control transgenic plant having pCAMBIA2301 was also used for the second transformation. Floral dip method was used to generate the double transgenics.

Silencing of gus reporter gene in double transgenic lines

The double transgenic plants were screened on MS media containing kanamycin (100 µg/ml) and hygromycin B (15 µg/ml). The expression of gus reporter gene was detected by GUS histochemical assay. The double transgenic control plants with normal expression of AtMBD genes showed the silencing of gus reporter gene. This suggested that the GUS-RNAi construct was working properly. The double transgenic
Results

lines with down-regulated AtMBD6 and AtMBD10 did not show silencing of gus reporter gene (Fig. 19). These results indicated that normal expression of AtMBD6 and AtMBD10 genes was essential for RNAi mediated gene silencing. Due to the absence of these proteins in their respective knockdown lines, the silencing of gus reporter gene was not achieved. This phenomenon was not observed in AtMBD11 knockdown lines (Fig. 19).

<table>
<thead>
<tr>
<th>First transformation</th>
<th>Second transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCAMBIA2301</td>
<td>GUSRNAi</td>
</tr>
<tr>
<td>pCAMBIA-MBD6-RNAi</td>
<td>GUSRNAi</td>
</tr>
<tr>
<td>pCAMBIA-MBD10AS</td>
<td>GUSRNAi</td>
</tr>
<tr>
<td>pCAMBIA-MBD11AS</td>
<td>GUSRNAi</td>
</tr>
</tbody>
</table>

Figure 19. GUS histochemical assay of double transgenic plants: The pre-existing gus gene was silenced in plants with normal or knock down expression of AtMBD11, whereas AtMBD6 and AtMBD10 knockdown transgenic plants did not show the silencing of gus reporter gene.

Identification of genes involved in RNA directed DNA Methylation

To identify the MBD protein that is involved in RNA mediated gene silencing in Arabidopsis, T-DNA insertion mutant lines of atmbd4, atmbd5, atmbd6, atmbd8,
Results

*atmbd9*, *atmbd11* and *atmbd13* genes were screened. All the mutant plants mentioned above were transformed with *gus* expressing vector pCAMBIA1301 (Fig. 20). The first transformation was carried out by floral dip using pCAMBIA1301, which contain a *gus* reporter gene downstream to 35S promoter and a hygromycin resistance gene as a selection marker. Seeds were harvested after floral dip and transgenic plants were screened on MS media containing hygromycin B (15 µg/ml).

**Figure 20. Schematic diagram of pCAMBIA1301:** The vector contain hygromycin B selectable marker and *gus* reporter gene under the control of 35S promoter.

The transgenic nature of these plants was checked by PCR analysis. PCR reaction was performed using *hptII* gene specific primers. Genomic DNA isolated from untransformed and transformed plants was used as a template. The presence of *hptII* gene specific band on agarose gel confirmed the transgenic nature of these plants. Such band was found to be absent in the untransformed plants (Fig. 21).

The PCR-positive transgenic plants were further analyzed for GUS activity (Fig. 22). Leaf tissue from the untransformed and transformed mutant plants were incubated in GUS buffer at 37°C for overnight. The incubation was followed by washing of the leaves with acetone to remove pigments. The transformed plants showed GUS staining and developed blue color while the untransformed plants did not show the blue color.
**Figure 21. PCR verification of transgenic nature of plants in different mutant backgrounds:** The mutant plants transformed with pCAMBIA1301 were checked by PCR analysis using *hptII* gene specific primers. Left panel shows the genotype of the mutant. Middle panel of the figure shows transgene used for PCR amplification. Right most panel shows the gel picture of PCR amplified product in the specific genotype. Lane M, 50bp marker; lane 1-5 represent different transgenic lines; WT, wild type.

<table>
<thead>
<tr>
<th>Genotype/mutant</th>
<th>Vector used (transgene used for PCR)</th>
<th>PCR analysis of transgene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>pCAMBIA2301 (<em>nptII</em>)</td>
<td>M +ve WT 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd4</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>M WT +ve 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd5</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>WT M +ve 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd6</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>M WT +ve 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd8</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>M +ve WT 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd9</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>M +ve 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd11.1</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>M WT +ve 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd11.2</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>M WT +ve 1 2 3 4 5</td>
</tr>
<tr>
<td><em>atmbd13</em></td>
<td>pCAMBIA1301 (<em>hptII</em>)</td>
<td>M WT +ve 1 2 3 4 5</td>
</tr>
</tbody>
</table>
### Figure 22. GUS histochemical assay of transgenic plants in different mutant backgrounds:

The expression of *gus* gene was checked in different genetic background. Left panel show the genotype of the mutant. Middle panel of the figure shows vector used for transformation. Right most panel shows the GUS histochemical assay in the specific genotype. The first lane showed untransformed mutant plant (UT), lanes 1 to 3 are for the transformed plants.

<table>
<thead>
<tr>
<th>Genotype/mutant</th>
<th>Vector used</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>pCAMBIA2301</td>
</tr>
<tr>
<td><em>atmbd4</em></td>
<td>pCAMBIA1301</td>
</tr>
<tr>
<td><em>atmbd5</em></td>
<td>pCAMBIA1301</td>
</tr>
<tr>
<td><em>atmbd6</em></td>
<td>pCAMBIA1301</td>
</tr>
<tr>
<td><em>atmbd8</em></td>
<td>pCAMBIA1301</td>
</tr>
<tr>
<td><em>atmbd9</em></td>
<td>pCAMBIA1301</td>
</tr>
<tr>
<td><em>atmbd11.1</em></td>
<td>pCAMBIA1301</td>
</tr>
<tr>
<td><em>atmbd11.2</em></td>
<td>pCAMBIA1301</td>
</tr>
<tr>
<td><em>atmbd13</em></td>
<td>pCAMBIA1301</td>
</tr>
</tbody>
</table>
Results

Silencing of gus gene using GUS-RNAi in mutant background

The gus-expressing mutant plants were used to silence gus gene using RNAi construct designed to target the gus transcript. The construct was transiently expressed in the gus-expressing mutant plants by agroinfiltration. The silencing effect due to GUS-RNAi infiltration was analyzed by GUS histochemical assay. The GUS histochemical assay showed the silencing of gus reporter gene in the control gus expressing plant where no attempt to alter the expression of any AtMBD gene was made. The silencing of gus gene was also found in the mutant backgrounds including atmbd4, atmbd6, atmbd8, atmbd11.1 and atmbd11.2 mutants. In case of three mutants atmbd5, atmbd9 and atmbd13 the silencing was not there and expression of gus gene was observed (Fig. 23). These observations indicate that normal expression of AtMBD5, AtMBD9 and AtMBD13 genes is required for RNAi-mediated gene silencing.

Silencing of gus gene using microRNA-mediated silencing in mutant background

The mutant plants expressing gus gene were further attempted for the silencing of gus gene silenced using an artificial microRNA vector, designed to silence the gus gene (amiRNA-gus). Arabidopsis leaves of mutant background, expressing gus gene were transformed with this vector in a transient manner. The silencing of gus gene was analyzed by GUS histochemical assay. The assay results confirmed the silencing of gus reporter gene in the control. However in the two mutants atmbd6 and atmbd13 silencing of gus reporter gene was not observed (Fig. 24). In the control plant and in other mutants backgrounds silencing of gus reporter gene was clearly seen (Fig. 24).
Results

Figure 23. Silencing of *gus* gene in mutant background using *gus* gene specific RNAi: The silencing of *gus* expression in the mutants was attempted by transient transformation using GUS-RNAi construct. Left panel shows the transgenic plant in different mutant background. Middle panel of the figure shows vector used for agroinfiltration. Right most panel shows the GUS histochemical assay in the specific mutant backgrounds. In *atmbd5*, *atmbd9* and *atmbd13* mutants no silencing of the *gus* gene was observed.

<table>
<thead>
<tr>
<th>Stable transformation (Genotype)</th>
<th>GUS histochemical assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCambia2301 (Wild type)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd4</em>)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd5</em>)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd6</em>)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd8</em>)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd9</em>)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd11.1</em>)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd11.2</em>)</td>
<td></td>
</tr>
<tr>
<td>pCambia1301 (<em>atmbd13</em>)</td>
<td></td>
</tr>
</tbody>
</table>
Results

Figure 24. Silencing of *gus* gene in mutant backgrounds using amiRNAGUS vector: The silencing of *gus* expression was attempted in different by transiently expressing a microRNA targeting *gus* gene. The *gus* expression in different mutant background is shown in the figure. In the *atmbd6* and *atmbd13* mutants no silencing of *gus* reporter gene by miRNA construct was observed. Left panel shows the vector used for different mutants. Middle panel of the figure shows vector used for transient expression by argroinfiltration. Right most panel shows the GUS histochemical assay in the different mutants.
Functional characterization of \textit{AtMBD4}

**Generation of \textit{AtMBD4} over-expressing transgenic \textit{Arabidopsis} plants**

In order to amplify \textit{AtMBD4} gene, cDNA library from 10-day-old \textit{Arabidopsis} seedlings was prepared. PCR reaction was performed using \textit{AtMBD4} gene specific primers and a 561 bp PCR product was amplified. This PCR product was cloned in pUC19 cloning vector. The cloning of \textit{AtMBD4} in pUC19 was verified by restriction digestion analysis and sequencing of cloned product using T3 and T7 primers. The \textit{AtMBD4} gene was then moved to pBI121 binary vector, where the expression of the gene was governed by a constitutive 35S promoter. The confirmation of this cloning was achieved by restriction digestion analysis (Fig. 25). Once confirmed, the construct was used for \textit{Agrobacterium}-mediated stable transformation of \textit{Arabidopsis} plants to generate transgenic lines.

**Figure 25. Cloning of \textit{AtMBD4} in binary vector:** (A) Schematic diagram of the construct, where \textit{AtMBD4} gene was cloned downstream to 35S promoter in pBI121 binary vector. (B) The construct pBI121-\textit{MBD4} when digested with \textit{Hind} III, \textit{Eco} RI and \textit{Xba} I + \textit{Sac} I released a 1150 bp, 409 bp and 561 bp fragment, respectively. The first and fifth lanes represent 1 kb DNA ladder marker and 50 bp DNA ladder marker respectively.
Transgenic plants over-expressing *AtMBD4* were generated by floral dip method using *Agrobacterium* transformed with pBI121-*AtMBD4* construct. Seeds were harvested and screened on MS medium containing kanamycin (100 µg/ml) and cefotaxime (250 µg/ml). Plants which grew on the selection medium were verified by PCR using nptII gene specific primers. Genomic DNA was used as template for this analysis (Fig. 26). Segregation analysis of the *AtMBD4* transgene was performed in T1 plants by growing *Arabidopsis* seeds on MS medium containing kanamycin (100 µg/ml). The transgenic lines that showed 3:1 segregation ratio were used for further molecular characterization.

**Figure 26. PCR verification of *AtMBD4* over-expressing transgenic plants:** The transgenic nature of *AtMBD4* over-expressing plants was analyzed using PCR analysis of nptII gene. A 554 bp fragment was obtained in the transgenic lines whereas it was found to be absent in the wild type. Wild type genomic DNA was used as a negative control. The binary vector harboring this gene was used as a positive control. Lane 1 to 10 represent the different PCR positive transgenic lines. M; 50 bp DNA ladder marker, +ve, positive control, WT, wild type.

**Characterization of *AtMBD4* over-expressing transgenic plants**

The expression level of *AtMBD4* gene was determined by qRT-PCR, using *AtMBD4*-specific primers. *AtACTIN12* was used as an internal control in this analysis. A significant increase in the transcript level of *AtMBD4* gene was observed in transgenic plants, as compared with the wild type, although to varied levels. Transgenic lines 1 to 4 showed over-expression of this gene by 8 to 14 fold, whereas the fifth transgenic line showed increase in its transcript accumulation only by ~3 fold (Fig. 27).
**Results**

**Figure 27. qRT-PCR of AtMBD4-OE transgenic plants:** The expression of AtMBD4 gene in transgenic lines was analyzed by qRT-PCR. Relative expression of AtMBD4 gene in the transgenic lines was determined against its expression values in wild type plant. The X-axis represents different transgenic lines while the Y-axis represents the relative fold change in transcript level. The error bar indicates the standard deviation from the mean value.

**Figure 28. Analysis of methylation status of AtMBD4-OE plants:** The methylation status of repeats and transposons was analyzed by methylation sensitive PCR. Genomic DNA were digested with HpaII enzyme. A decreased intensity of PCR products in the over-expression lines were detected as compared to the wild type. This indicates hypomethylation in the over-expression lines. *AtGAP-C* (glyceral dehyde 3 phosphate dehydrogenase C) gene was used as an internal control which did not show any change in the methylation pattern.
Results

Over-expression of AtMBD4 shows hypomethylation of genomic DNA

The methylation status of genomic DNA was determined by Methylation Sensitive PCR (MSP) of some known loci where methylation is prominent. The centromeric 180 bp repeats and 18S rRNA genes are usually highly methylated in the wild type Arabidopsis. The transposons AtSN1 and Ta3 were found to be highly methylated in the wild type. MSP was performed using same amount of HpaII digested genomic DNA and gene specific primers. The amplified products from the transgenic plants were found to be less intense as compared to the wild type (Fig. 28). This indicates that AtMBD4 over-expressing transgenic plants have reduced cytosine methylation in these loci.

Characterization of atmbd4 mutant

The atmbd4 mutant has a T-DNA insertion within the coding region of the AtMBD4 gene (Fig. 29A). The mutant plant was verified by northern blotting using AtMBD4 specific probe. In case of wild type plant the transcript was clearly detected whereas in the mutant, no AtMBD4 specific transcript was observed (Fig. 30). Morphological analysis of atmbd4 mutant plants showed less root growth with more branching (Fig. 29.B) and late flowering phenotype as compared to the wild type plants (Fig. 31).
**Figure 31. Late flowering phenotype of atmbd4 mutant:** Seeds of wild type and mutant plants were grown in soil and these plants were looked for any phenotypic change in their development related to flowering. The wild type plants showed flowering after 30(±3) days of germination whereas the mutant showed flowering after 35(±3) days of germination.

**Figure 29.** (A) Schematic diagram of T-DNA insertion in the second exon of atmbd4 mutant (B) Phenotype of the mutant: These mutants showed altered phenotype in root development. The mutant plants produced more root branches with less root length as compared to the wild type. Wild type and mutant seedling grown for 15 days vertically in MS medium were used for phenotypic analysis.
Results

Figure 30. Northern analysis of *atmbd4* mutant: Northern hybridization of RNA samples from wild type *Arabidopsis* and *atmbd4* mutant plants was performed using *AtMBD4* gene specific probe. The northern blot analysis clearly showed the absence of *AtMBD4*-specific transcript in the mutant plant whereas it was found to be very prominent in the wild type plant.

Molecular basis of late flowering phenotype in *atmbd4* mutant

The late flowering phenotype can be explained by ectopic expression of *FWA* (Flowering WAgeningen) gene. This gene has an inverted repeat in its transcriptional initiation site, thus silenced in wild type plant. Ectopic expression of *FWA* gene produces a dominant late flowering phenotype. Semi quantitative RT-PCR was performed to detect the transcript of *FWA* gene in wild and mutant plants. These results clearly showed that *FWA* gene was silenced in wild type plant whereas ectopic expression was observed in the mutant (Fig. 33).

Figure 33. Analysis of expression of *FWA* gene: The expression of *FWA* gene was analyzed by semi-quantitative RT-PCR. The PCR results show the ectopic expression of *FWA* gene in the mutant whereas no such expression was observed in the wild type plant. The constitutive gene *GAP-C* (glyceraldehyde-3-phosphate dehydrogenase c) was used as a positive control for PCR amplification.
**Results**

**Transcriptome analysis of atmbd4 mutant**

To understand the downstream mechanism of its action, transcriptome of *atmbd4* mutant and wild type plants were studied by performing microarray experiment. Affymatrix ATH1 gene chip was used in this analysis and 15 days old seedlings of wild type and mutant *Arabidopsis* plants were used to isolate RNA. The quality and quantity was verified using Bioanalyzer and Nanodrop spectrophotometer. The preparation of cRNA and hybridization were carried out as per manufacturer’s instructions. In the mutant, expression of 329 genes was found to be altered in comparison to wild type plant (fold change ≥ 2 at p value 0.05). Out of the genes 242 genes were up-regulated while 87 genes were down-regulated in the mutant plant (Fig. 34). The differentially expressed genes were further annotated according to TAIR9 guidelines and grouped in different functional categories, such as genes involved in phosphate starvation, response to stimulus (treatment of chemicals, hormones and nutrients etc.), response to stress and metabolic processes (Fig. 36).

![Bar chart showing expression of genes in wild type and mutant plants](image)

**Figure 34. Transcriptome analysis of atmbd4 mutant seedlings:** Expression of 329 genes was modulated in the mutant plants in comparison to wild type. It was found that 242 genes were up-regulated whereas 87 genes were down-regulated in the mutant.
Figure 35. Validation of microarray data by qRT-PCR analysis: qRT-PCR analysis of selected genes was performed to validate microarray data. Genes which showed a high level of differential expression in the mutant were selected for this analysis. Similar expression pattern was observed in both microarray and qRT-PCR. The X-axis represents different genes whereas the Y-axis represents relative expression of mutant with reference to wild type. Blue and red colors represent the expression value in microarray and qRT-PCR respectively.

The microarray results were again verified by performing qRT-PCR analysis of some selected genes. The expression pattern observed in microarray was found to be similar with that of qRT PCR results (Fig. 35).
Figure 36. Functional categorization of differentially expressed genes in *atmbd4* mutant: (A). up-regulated genes (B) down-regulated genes
Figure 37. Expression of genes involved in metabolism in atmbd4 mutant. Transcript levels in atmbd4 mutant relative to the levels in wild type were displayed on a log$_2$ scale using the MapMan software (Thimm et al. 2004). Gene transcripts that do not change by more than a threshold value (log$_2$ more than ±1) are shown in white; genes that showed an increase and a decrease in expression are shown by an increasingly intense red and green coloration, respectively, as shown in the color bar.

The microarray result was further analyzed using MapMan software (Thimm et al. 2004), which mapped the up/down-regulated genes in different pathways, giving a false color according to the log$_2$ values. This allows a clear resolution and understanding of comparative cellular response in the mutant. The mutant showed altered gene expression that affected many cellular responses. An increase was seen in expression of genes related to flavonoid biosynthesis, nitrogen metabolism, peroxidase, ethylene response and cell wall biosynthesis; whereas a significant decrease in expression of genes related to wax biosynthesis was observed (Fig. 37).
AtMBD4 is involved in phosphate starvation

The root phenotype of atmbd4 mutant mimics the phenotype in plants affected with phosphate starvation. There was a significant up-regulation of genes involved in phosphate starvation. The genes which showed a significant up-regulation in the mutant code for phosphate transporters (AtPHT1 and AtPHT2), phosphate induced 1 (PHI-1), MYB90, WRKY18, SPX1, SPX3 and RNS1. Plants that face phosphate starvation usually show up-regulation in flavonoid biosynthetic genes. The mutant also showed up-regulation of genes involved in anthocyanin biosynthetic genes encoding flavonoid-3-O-glucosyltransferase, AACT1, DIHYDROFLAVONOL-4-REDUCTASE and leucoanthocyanidin dioxygenase enzymes. To validate the expression data, anthocyanins were estimated in wild type and mutant plants. It was found that atmbd4 mutant produced more anthocyanin as compared to the wild type plants (Fig. 38). Two anthocyanins (pelargonidine and pg-soph-5-glu) contents were found to be induced significantly.

The microarray result of atmbd4 mutant was compared with some other microarray data available in the public domain under the screen name E-GEOD-16722. When the differentially regulated genes of atmbd4 were compared with that of phosphate starved shoot, 103 common genes were found to be up-regulated in both. However when compared with the microarray data of the root tissue affected with phosphate starvation only 32 genes were found to be in common (Fig. 39). When these common genes were analyzed it was found that out of 103 genes in shoot majority of genes are annotated as responsive to phosphate starvation. Many genes involved in response to different stimuli and different stresses were also found to be commonly up-regulated. The common up-regulated genes in atmbd4 and root affected with phosphate starvation are annotated as responsive to different stimuli. Many genes known to have role in phosphate starvation were found in the common in both sets, such as PHI-1 (phosphate induced 1), PHT (phosphate transporter 1), SPX1 (SPX domain gene 1), SPX3 (SPX domain gene 3), RNS1 (ribonuclease 1), ATPT2 (Arabidopsis thaliana phosphate transporter 2) and APT1 (Arabidopsis phosphate transporter 1).
**Results**

**Figure 38.** Secondary metabolism in *atmbd4* mutant plants: (A) Genes encoding flavonoids biosynthesis enzymes like 3-O-glucosyltransferase, AACT1, dihydroflavonol-4-reductase and leucoanthocyanidin dioxygenase were up-regulated in *atmbd4* mutant.

(B) Biochemical estimation of anthocyanin concentration in *atmbd4* mutant: Total anthocyanins were extracted in ethanol containing 1% v/v HCL from leaf tissue of wild type and *atmbd4* mutant plants. The optical density was measured in spectrophotometer in different wave length corresponding to respective anthocyanin. The X-axis represents different anthocyanins molecules while the Y-axis represents the optical density obtained from per gram of tissue extract resuspended in 1 ml 1% v/v HCL-ethanol.

**Figure 39.** Comparison of *atmbd4* microarray data with the publicly available microarray data of phosphate starved roots and shoots tissues of *Arabidopsis*. Comparison of differentially expressed genes of phosphate deficient root and *atmbd4* mutant seedling resulted in identification of 32 commonly up-regulated genes whereas comparison of phosphate deficient shoot and *atmbd4* mutant seedling data led to identification of 103 commonly up-regulated genes.
Results

Analysis of the expressions of genes involved in oxidative stress

The \textit{atmbd4} mutant showed up-regulation of genes involved in oxidative stress. Genes like peroxidases and glutathione-S-transferases were up-regulated in the mutant. There are some other genes involved in this process such as gene encoding FE-superoxide dismutase, thioredoxin-dependent peroxidase 2 and peroxiredoxin which were also up-regulated in the mutant (Fig. 40).

Up-regulation of heat stress-related genes in \textit{atmbd4} mutant

Microarray analysis also showed that there was a significant up-regulation in the genes involved in heat stress in the \textit{atmbd4} mutant. Seven heat shock proteins encoding genes (\textit{Hsp90.1}, \textit{Hsp26.5}, \textit{Hsp17.4}, \textit{Hsp17.6}, \textit{Hsp17.8}, \textit{Hsp17.6A} and \textit{Hsp23.5}) showed up-regulation in the mutant. Interestingly, two genes (\textit{COR15A} and \textit{COR15B}) involved in cold stress were down-regulated (Fig. 40).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mapman.png}
\caption{Genes involved in various biotic and abiotic stresses mapped by MapMan: The heat shock protein genes up-regulated in the mutant whereas the cold stress response protein encoding genes were down-regulated. The redox reaction related genes were also up-regulated in the mutant. Each small square represents a gene, red color represents up-regulation and green color represent down-regulation.}
\end{figure}
**Yeast two hybrid screening for AtMBD4 interacting proteins**

To identify the interacting partner of AtMBD4, yeast-two-hybrid assay was performed. The bait gene *AtMBD4* was cloned in fusion with GAL4-DNA binding domain in the yeast expression vector pGBKT7 (Fig. 41). Yeast strain Y187 was transformed with this vector. The expression of gene encoding the fusion protein GAL4BD-*AtMBD4* was under the control of a constitutive promoter of gene encoding alcohol dehydrogenase1.

The prey library was created in yeast strain *AH109* using the vector pGADT7-rec where the gene encoding expressed proteins were cloned in fusion with gene encoding GAL4 activation domain. The two strains containing the bait and prey were used for mating followed by screening on nitrogen basal medium with drop out -AHLT. The colonies that grew on the selection medium were analyzed for α-galactosidase activity. The colonies which turned blue in presence of X-α-gal (5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside) were selected. Initially, 33 colonies were selected which grew on –histidine medium and showed α-galactosidase activity. Isolation of the prey plasmids and sequencing led to the identification of some putative proteins that showed interaction with AtMBD4. One such selected putative protein i.e. AtUBC36, a ubiquitin conjugating enzyme was selected for further verification (Fig. 42).
Results

Figure 41. Cloning of \textit{AtMBD4} in pGBKT7 vector: (A) \textit{AtMBD4} was cloned in fusion with gene encoding GAL4 DNA binding domain. The vector backbone contains \textit{Trp1} selectable marker gene.

B. Restriction digestion of pGBKT7-\textit{AtMBD4}: Restriction digestion analysis of the clone produced expected size fragments of 598 bp, 416 bp and 2454 bp when digested with \textit{NcoI}+\textit{PstI}, \textit{EcoRI} and \textit{BstXI}, respectively.

Figure 42. Yeast two hybrid screening: Screening of yeast two hybrid library led to the identified \textit{AtUBC36} (a ubiquitin conjugating enzyme) as an interacting partner of \textit{AtMBD4}. Yeast transformed with pGBKT7-p53 and pGADT7rec-SV40 was used as a positive control whereas yeast transformed with pGBKT7Lam and pGADT7rec-SV40 was used as a negative control.
Results

Florescence Resonance Energy Transfer (FRET) and co-localization of AtMBD4 and AtUBC36

The interaction between AtMBD4 and AtUBC36 which was identified in yeast two-hybrid was further verified *in-vivo* by Florescence Resonance Energy Transfer (FRET). Both the bait and prey were cloned in pSITE1CA and pSITE3CA vectors (Fig. 43). These constructs were co-bombarded into onion epidermal cells. The onion peels were then analyzed using a confocal microscope.

![Figure 43: Cloning of AtMBD4 and AtUBC36 in pSITE vectors](image)

**Figure 43. Cloning of AtMBD4 and AtUBC36 in pSITE vectors:** Schematic diagram of cloning of AtMBD4 and AtUBC36 in pSITE1C and pSITE3C vectors respectively. The gene encoding AtMBD4 was cloned in pSITE1CA in fusion with 3’ of CFP; AtUBC36 was cloned in fusion with 3’ of YFP in pSITE3CA.

![Figure 44: Co-localization of AtMBD4-CFP and AtUBC36 in onion epidermal cell by confocal microscopy](image)

**Figure 44. Co-localization of AtMBD4-CFP and AtUBC36 in onion epidermal cell by confocal microscopy:** Subcellular localization of AtMBD4 and AtUBC36 as Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) fusion proteins respectively. Open reading frame (ORF) of the genes were fused with the fluorescent protein coding region at the 3’-end and was visualized after onion epidermal cells bombardment. Cyan and Yellow are pseudocolors representing fluorescence of CFP and YFP respectively.
The CFP and YFP fused AtMBD4 and AtUBC36 proteins when visualized under confocal microscope showed co-localization of these proteins in the nucleus. The protein AtUBC36 was also present in the cell membrane where the partner was absent. FRET was performed in the nucleus and membrane in three different cells. The FRET efficiency in the nucleus was found to be 16.33% whereas no FRET was detected in the membrane.

**Figure 45. Interactions of AtMBD4 and AtUBC36:** The protein-protein interaction was studied by fluorescence resonance energy transfer (FRET) analysis using a confocal microscope. Both the genes were cloned in fusion with YFP and CFP were co-bombarded in onion epidermal cells. Protein–protein interaction was studied by FRET-acceptor bleaching protocol. FRET efficiency for AtMBD4 and AtUBC36 interaction is shown in histogram below.
Functional characterization of \textit{AtMBD6}

Generation of \textit{AtMBD6} over-expressing \textit{Arabidopsis} transgenic plants

The gene encoding AtMBD6 was PCR amplified from genomic DNA and cloned in pUC19 cloning vector. The cloning of \textit{AtMBD6} in pUC19 was verified by restriction digestion and sequencing. This fragment was then moved to binary vector pCAMBIA2301 in \textit{BamHI} and \textit{SacI} sites, where the gene is governed by \textit{35S} promoter. The confirmation of the cloning was done by restriction digestion. Restriction digestion analysis of the construct released 1156 bp cloned fragment when digested with \textit{BamHI} and \textit{SacI} (Fig. 46).

\textbf{Figure 46. Cloning of \textit{AtMBD6} in sense orientation:}

(A) Schematic diagram of the vector for expression of \textit{AtMBD6}: \textit{AtMBD6} gene was cloned in \textit{BamHI} and \textit{SacI} sites downstream to \textit{35S} promoter to generate pCAMBIA2301-\textit{AtMBD6}. The vector backbone contains \textit{nptII} selectable marker gene and \textit{uidA} reporter gene.

(B) Restriction digestion analysis of pCAMBIA2301-\textit{AtMBD6}: The construct pCAMBIA2301-\textit{AtMBD6} released 1165 bp fragment when digested with \textit{BamHI} + \textit{SacI}. The first lane represents the \textit{\lambda} \textit{HindIII} DNA marker and the fifth lane represents 50 bp DNA ladder.
Characterization of *AtMBD6* over-expressing transgenic plants

Transgenic plants over expressing *AtMBD6* were generated by floral dip method. Seeds were screened on MS medium containing kanamycin and cefotaxime. The transgenic nature of the plants was verified by PCR analysis using the genomic DNA as template and primers specific to *nptII* gene. PCR result clearly showed amplification 554 bp fragment of *nptII* gene from transgenic plants whereas the fragment was absent in the DNA from wild type plant (Fig. 47A). The expression of *gus* reporter gene was performed by GUS histochemical assay. The leaf tissues from wild type and transgenic plants were incubated in GUS buffer for overnight at 37°C and then washed with acetone to remove the pigments. The histochemical result clearly showed the expression of *gus* reporter gene in *AtMBD6* over-expression lines (Fig 47B).

**Figure 47.** A. PCR verification of *AtMBD6* over expressing (*AtMBD6-OE*) transgenic lines: The transgenic nature of *AtMBD6* over-expression plants was verified using PCR for *nptII* gene. A 554 bp fragment was obtained with the DNA from transgenic lines whereas it was absent when DNA from wild type plant was used. The first lane contains 50 bp marker, WT genomic DNA was used as a negative control and the vector was used as a positive control. WT, wild type; +ve; positive

B. GUS histochemical assay of *AtMBD6-OE* transgenic plant: The expression of *gus* reporter gene was verified by GUS histochemical assay. Leaves from WT and transgenic plants were incubated in GUS assay buffer for overnight at 37°C. After that the leaves were washed with acetone. The leaves of transgenic plants (lanes MBD6 S1 to MBD6 S9) accumulated blue color whereas the wild type (WT) plants did not turn blue. WT, wild type.
The expression level of \textit{AtMBD6} transcript was determined by qRT-PCR analysis, using \textit{AtMBD6} specific primers and \textit{AtACTIN12} was taken as an internal control. The qRT PCR analysis was performed using two biological replicates and three technical replicates. The transcript level of \textit{AtMBD6} was higher in case of transgenic plants as compared to the wild type as shown in the graph (Fig. 48A). Plants over-expressing \textit{AtMBD6} showed reduced length and poorly developed root system and small size as compared to the wild type plants grown in same condition (Fig. 48B).

**Figure 48 A.** The analysis of \textit{AtMBD6-OE} transgenic plants by qRT-PCR: The expression of \textit{AtMBD6} gene in over expressing transgenic lines was analyzed by qRT-PCR using primers specific to \textit{AtMBD6}. The X-axis represents different transgenic lines while the Y-axis represents the relative fold change in transcript level. Error bars indicate standard deviation from mean value.

**B. Morphological analysis of \textit{AtMBD6-OE} transgenic plant:** The \textit{AtMBD6-OE} transgenic plant produced very poor root system compared to the wild type plant, grown in same condition.

**Characterization of \textit{atmbd6} mutant**

The \textit{atmbd6} mutant has a T-DNA insertion within the coding region of the \textit{AtMBD6} gene (Fig. 49A). The \textit{atmbd6} mutant plants showed late flowering in long day condition and more number of rosette leaves as compared to the wild type plant (Fig.
Results

49). The wild type plant bolted 24±3 days after germination whereas in case of mutant the bolting was seen after 32±3 days after germination. The expression of AtMBD6 gene was analyzed in the mutant plant by northern hybridization. Total RNA from wild type and mutant plants were isolated and transferred to membrane. Radiolabeled probe, specific to AtMBD6 was prepared and hybridized. In case of wild type plant, the transcript of AtMBD6 was clearly detected, whereas in the mutant the transcript specific to AtMBD6 was not seen, suggesting the lack of of AtMBD6 gene expression in the mutant (Fig. 50).

![Figure 49](image_url)

**Figure 49.** A. Schematic diagram of T-DNA insertion: The T-DNA is inserted in the second exon of atmbd6 mutant. B. morphological analysis in atmbd6 mutant: The atmbd6 mutant showed late flowering as compared to wild type, grown in same condition. The number of rosette leaves was also more in the mutant as compared to the wild type. The wild type plant showed flowering 24 (±3) days after germination whereas the atmbd6 mutant showed flowering in 32 (±3) days after germination (shown in histogram C). The number of rosette leaves in the mutant was found to be more than that of wild type plants (shown in histogram D).
Results

**Figure 50. Northern analysis of atmbd6 mutant:** RNA from wild type and atmbd6 mutant were used for northern analysis. Hybridization of AtMBD6-specific probe showed presence of transcript in wild type plants whereas it was absent in the mutant. WT; wild type, rRNA; ribosomal RNA.

**Transcriptome analysis of atmbd6 mutant**

To understand the mechanism of action of AtMBD6 regulation, microarray was performed using affymetrix ATH1 gene chip. Seedlings of wild type and atmbd6 mutant plants grown for 15 days were used for this analysis. RNA samples from the wild type and mutant were isolated in triplicates and the quality was verified using Bioanalyzer. RNA with OD_{260/280} ratio ≥2 and OD_{260/230} ratio ≥2 was used for the microarray analysis. The preparation of cRNA and hybridization were carried out as described in material and method. The mutant showed altered expression of 717 genes (fold change ≥2 at p value 0.05) out of which 521 genes were up-regulated while 196 genes were down-regulated (Fig. 51). The differentially expressed genes were annotated according to TAIR9. They belong to different functional categories such as nuclear factors, metabolic enzymes, transporters and structural proteins (Fig. 53). The microarray results were again verified by quantitative RT-PCR analysis for some of the selected genes. The expression of these genes was similar to the expression pattern observed in microarray analysis (Fig. 52).
**Results**

**Figure 51:** Transcriptome analysis of *atmbd6* mutant seedling: The microarray analysis identified 717 differentially expressed genes in *atmbd6* mutant, out of them 521 genes showed up-regulation and 196 were down-regulated in the mutant as compared to wild type seedling.

**Figure 52.** Validation of microarray data using qRT-PCR analysis: The qRT-PCR was performed to validate the microarray data. The differentially expressed genes which showed a high log$_2$ value of fold change were selected for the validation. Similar expression pattern was observed in microarray and qRT-PCR analysis. The X-axis represents different genes whereas the Y-axis represents the fold change in transcript level. Blue and red colors represent the expression value in microarray and qRT PCR, respectively.
Figure 53. Functional categorization of differentially expressed genes: The functional categorization of differentially expressed genes in *atmbd6* mutant. A. Functional categorization of up-regulated genes in *atmbd6* mutant. B. Functional categorization of down-regulated genes in the mutant.
The microarray result was analyzed using MapMan software (Thimm et al. 2004), which mapped the up/down-regulated genes in different pathways, giving a false color according to the log₂ values. The mutant showed altered gene expression that affected many cellular processes like light reactions of photosynthesis, ethylene response, cell wall biogenesis, anthocyanin biosynthesis, phenylpropanoids synthesis and RNA metabolic process (Fig. 54).

**Figure 54. Metabolic changes in atmbd6 mutant:** Transcript levels in atmbd6 mutant relative to the level in wild type were displayed on a log₂ scale using the MapMan software. Gene transcripts that do not change by more than a threshold value are shown in white, genes that were increased and decreased significantly are shown by an increasingly intense red and green coloration, respectively, as shown in the color bar. The differentially expressed genes are involved in light reaction, cell wall biosynthesis, lipid metabolism, anthocyanin biosynthesis and phenylpropanoid biosynthesis. log₂ value of more than ±1 was used as threshold.
Decreased transcript level of light reaction-associated genes

Microarray analysis identified that there was a significant decrease in the transcription level of genes involved in light reaction of photosynthesis (Fig. 55). The structural proteins D1, Photo system-II K (PSII-K) and Photo systemII-I (PSII-I) proteins were highly down-regulated. Gene encoding ATPase subunits, present in chloroplast inner membrane such as subunit-A, subunit-H, subunit-F, ATPaseIII along with NADH dehydrogenase were down-regulated. The gene encoding oxygen evolving enhancer3 was also down-regulated. Interestingly, majority of the down-regulated genes are encoded by the chloroplast genome including the large subunit of RUBISCO. This suggests that AtMBD6 may be involved in nuclear-chloroplast anterograde signaling.

Figure 55. Expression of light reaction genes in atmbd6 mutant: The genes involved in light reaction are down-regulated in the mutant. Interestingly, the genes were encoded by the chloroplast genome. Each small square represents a gene, green and red color for down and up-regulated genes respectively.
Results

Up-regulation of phenylpropanoid, lignin and lignans-related genes in \textit{atmbd6} mutant

The secondary metabolism is highly affected in \textit{atmbd6} mutant as indicated by increase in level of expression of genes involved in phenylpropanoid, lignin and lignans biosynthesis pathways (Fig. 56). The enzymes involved in phenylpropanoid biosynthesis especially those activated by elicitor like gene, coumarate-CoA ligase and caffeoyl-CoA O-methyltransferase were up-regulated. Some other proteins predicted to be involved in this pathway like \textit{mitogen-activated protein kinase 3}, \textit{phytoalexin deficient 3}, \textit{WRKY 33}, \textit{MAP kinase kinase 9} and \textit{CYP71A13} were also up-regulated in the mutant. The up-regulation of these genes provides resistance against bacterial pathogen. Interestingly, \textit{atago4} mutant plant also shows resistance against bacterial pathogen. Both AtAGO4 and AtMBD6 are localized in the nucleolus, and are involved in miRNA mediated gene silencing. This suggests that these two genes may be part of a single complex and regulate the expression of these genes.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure56.png}
\caption{Modulation of secondary metabolism in \textit{atmbd6} mutant: The genes involved in anthocyanin biosynthesis and flavonoid biosynthesis were up-regulated in the mutant. Phenylpropanoid and glucosinolates biosynthesis genes are also up-regulated. Each small square represents a gene; red and green color for up and down-regulated genes respectively.}
\end{figure}
Results

Ethylene-mediated signaling

Microarray data indicated up-regulation of genes involved in ethylene mediated signaling and responses, including \textit{ACC oxidase1}, \textit{MAP KINASE 9}, \textit{ERF6} and \textit{ERF1}. Some of the nuclear factors, involved in ethylene response like \textit{MYB-44}, \textit{MYB-77}, \textit{MYB-51}, \textit{MYB-73} and \textit{MYB-2} were also getting up-regulated in the mutant.

Up-regulation of cell wall biogenesis genes

The structural components of cell wall proteins and structural carbohydrates were also up-regulated in the mutant. There was a significant increase in the transcript level of genes encoding enzymes involved in synthesis and modification of cell wall pectins. However there was decrease in transcript levels of gene encoding cell wall degradating enzymes, like pectate lyase in the mutant. The mutant also showed an increase in expression of genes encoding enzymes related to mannose and xylose biosynthetic genes.

Yeast two hybrid screening for AtMBD6 interacting proteins

The screening of protein-protein interaction was carried out by yeast two hybrid using AtMBD6 as bait. The ORF of \textit{AtMBD6} gene was cloned in pGBK7 in fusion with GAL4-DNA binding domain (Fig. 57). A library was prepared using 10 days old seedling and mating was performed. Screening of the positive clones was performed in N2 base medium containing dropout –AHLT. The positive colonies that grew on selection medium were verified by $\alpha$-galactosidase activity. Plasmids from the positive colonies were isolated and sequenced. The sequencing results identified some putative interacting partners of AtMBD6. Two putative partners which were found are AtNTF2 (nuclear transport factor2) and AtRPS2C (a 40S ribosomal protein) (Fig. 58).
Results

Figure 57. Cloning of $AtMBD6$ in pGBKT7 vector: A. schematic diagram of cloning of $AtMBD6$ gene in pGBKT7 vector. The gene was cloned in fusion with GAL4 DNA binding domain. The vector backbone also contain Trp1 nutritional selectable marker.

B. Restriction digestion analysis of pGBKT7-$AtMBD6$: the cloning confirmation was performed by restriction digestion analysis. When the clone was digested with EcoRI+$PstI$ and $BstXI+HindIII$ produces 678 bp and 1632 bp fragments respectively.
Results

Figure 58. Screening of yeast two hybrid: The two hybrid screening identified AtNTF2 and AtRPS2C; the bait and prey grew on –AHLT medium and were positive to alpha galactosidase activity. Yeast transformed with pGBK7-p53 and pGAD7rec-SV40 were used as a positive control whereas yeast transformed with pGBK7Lam and pGAD7rec-SV40 was used as a negative control.

Florescence Resonance Energy Transfer (FRET) and co-localization of AtMBD6, AtNTF2 and AtRPS2C

The putative proteins which were identified in yeast two hybrid analyses were further verified in-vivo by Florescence Resonance Energy Transfer (FRET). Both the bait and prey were cloned in pSITE1CA and pSITE3CA vectors respectively (Fig. 59) and transiently expressed in onion peels. The onion peels were then analyzed using a confocal microscope (Leica).
Results

**Figure 59. Cloning of bait and prey in pSITE vectors:** AtMBD6 was cloned in pSITE1CA vector in fusion with 3’ of CFP. AtRPS2C and AtNTF2 were cloned in pSITE3CA in fusion with 3’ of YFP.

The bait protein AtMBD6 was localized in the nucleus whereas AtRPS2C was found in both nucleus and cytoplasm (Fig. 60). AtMBD6 and RPS2C interaction was verified by FRET. The efficiency of energy transfer between CFP and YFP was found to be 13.69%, which suggested it to be a strong interaction (Fig. 61).

AtNTF2 was found in both nucleus and plasma membrane (Fig. 62). The interaction of AtMBD6 and AtNTF2 was verified by FRET. The efficiency of FRET was found to be 14.67%, which suggested these two proteins interacted with each other in vivo (Fig. 63).
Figure 60. Co-localization of AtMBD6 and AtRPS2C in onion cell using confocal microscope: Subcellular localization of CFP-AtMBD6 and YFP-AtRPS2C fusion proteins was performed in onion epidermal cells. Open reading frame (ORF) of the genes were fused with the fluorescent protein coding region at the 3’-end and were visualized after onion epidermal cell bombardment. Cyan and Yellow are pseudo colors representing fluorescence of CFP and YFP respectively.

Figure 61. Fluorescence Resonance Energy Transfer (FRET) analysis using confocal microscope: The protein-protein interaction was carried out by Fluorescence Resonance Energy Transfer (FRET) analysis. Protein–protein interaction was studied by FRET-acceptor bleaching protocol. FRET efficiency for AtMBD6 and AtRPS2C was found to be 13.69% as shown in histogram below.
Results

Figure 6. Colocalization of AtMBD6 and AtNTF2 in onion cell using confocal microscope: Subcellular localization of AtMBD6 and AtNTF2 was detected in onion peels as Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) respectively. Open reading frame (ORF) of the genes were fused with the fluorescent protein coding region at the 3’-end and was visualized after onion epidermal cells bombardment. Cyan and Yellow are pseudocolors representing fluorescence of CFP and YFP respectively.

Figure 63. Interaction of AtMBD6 and AtNTF2 using confocal microscope: The protein–protein interaction was carried out by fluorescence resonance energy transfer (FRET) analysis. Both the genes were cloned in fusion with YFP and CFP were co-bombarded in onion epidermal cells. Protein–protein interaction was studied by FRET-acceptor bleaching protocol FRET efficiency for AtMBD6 and AtNTF2 was found to be 14.67% as shown in histogram below.
Results

Functional characterization of *AtMBD10*

**Generation of transgenic plants over-expressing *AtMBD10* gene**

The gene encoding *AtMBD10* was over-expressed in *Arabidopsis*. A clone of the gene was available in the laboratory, in which it was under the control of 35S promoter in binary vector pCAMBIA2301 (Fig. 64). The construct was used for *Agrobacterium* mediated stable transformation of *Arabidopsis* to generate transgenic plants.

![Figure 64. Schematic diagram of the vector for over expression of *AtMBD10*: The ORF encoding *AtMBD10* gene was cloned downstream of 35S promoter in pCAMBIA2301 binary vector. The vector backbone also contains kanamycin (*nptII*) resistance gene and *uidA* reporter gene.](image)

**Characterization of *AtMBD10* over-expressing transgenic lines**

Transgenic plants over expressing *AtMBD10* (*AtMBD10*-OE) were generated by floral dip. Seeds were harvested and screened on MS medium containing kanamycin (100 µg/ml) and cefotaxime (250 µg/ml). Plants which grew on the selection medium were verified by PCR using *nptII* gene specific primers. Genomic DNA from untransformed and transformed plants was used as a template in this analysis. PCR analysis clearly indicate the presence of *nptII* gene in the transformed plants whereas it was absent in the untransformed wild type plant (Fig. 65).

The expression of *uidA* (*gus*) reporter gene was analyzed by GUS histochemical assay. When GUS histochemical assay was performed in transgenic plants they turned blue in presence of its substrate x-gluc, whereas the wild plant lacked the GUS activity (Fig. 66).
**Results**

**Figure 65. PCR verification of AtMBD10-OE transgenic plants:** The transgenic nature of AtMBD10 over-expressing plants was verified using PCR of nptII gene. A 554 bp fragment was obtained in the transgenic lines whereas it was absent in the wild type plant. The first lane contain 50 bp marker; WT genomic DNA was used as a negative control; the vector DNA was used as a positive control and lane 10S1 to 10S5 are independent transgenic lines. WT, Wild type: +ve, positive

**Figure 66. GUS histochemical assay of AtMBD10-OE transgenic plants:** The expression of gus reporter gene was analyzed by GUS histochemical assay. Leaves from untransformed and transgenic plants were incubated in GUS assay buffer for overnight at 37°C and were bleached with acetone. The transgenic plants (lane 2 to 6) displayed blue color whereas the leaves from wild type (WT) plants were negative to GUS histochemical assay. WT, wild type

The expression level of AtMBD10 transcript was determined by quantitative RT-PCR, using gene specific primers and AtACTIN12 as an internal control. The expression of AtMBD10 gene in transgenic plants was found to be higher than the wild type plant. The expression level of AtMBD10 gene in the transgenic plant varies from 3 to 10 folds as compared to the untransformed plant (Fig. 67).

<table>
<thead>
<tr>
<th>M</th>
<th>WT</th>
<th>+ve</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>nptII</td>
<td>554 bp</td>
<td></td>
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WT

10S 1

10S 2

10S 3

10S 4

10S 5
**Results**

**Figure 6.** qRT-PCR of *AtMBD10-OE* transgenic lines: The expression of *AtMBD10* gene in transgenic lines was analyzed by qRT-PCR. Graph picture clearly indicate the increased transcript level of *AtMBD10* in the transgenic lines. The X-axis represents different transgenic lines while the Y-axis represents the relative fold change in transcript level. Wild represents the untransformed plant; 10S1 to 10S5 represent different transgenic lines. The error bars indicate standard deviation from the mean value.

**Construction of an artificial microRNA-based vector to silence *AtMBD10* gene**

To silence *AtMBD10* gene, an artificial microRNA (amiRNA) specific to *AtMBD10* was cloned in binary vector pCAMBIA2301 using backbone of an endogenous microRNA AthmiRNA164C. Cloning of 21 nucleotides from 3’ UTR of *AtMBD10* gene was performed in sense and antisense orientation spaced by *miRNA164C* loop. The amiRNA was driven by 35S promoter. The cloning of amiRNA-*AtMBD10* was confirmed by restriction digestion (Fig. 68).

**Figure 68. Schematic diagram of the construct amiRNA-*AtMBD10*:** 21 nucleotides from 3’ UTR region of *AtMBD10* was cloned in sense and antisense orientation separated by the loop of microRNA164C downstream to 35S promoter.

Transgenic plants harboring artificial microRNA, designed to silence *AtMBD10* gene were generated by floral dip method. The transgenic nature of the plants was verified using PCR of *nptII* gene (Fig. 69) and GUS histochemical assay (Fig. 70). The transcript level of *AtMBD10* was detected by qRT-PCR analysis which indicated
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down-regulation of the gene in transgenic lines. The qRT-PCR analysis of transgenic lines showed down-regulation of AtMBD10 as compared to the wild type (Fig. 71).

Figure 69. PCR verification of amiRNA-AtMBD10 transgenic lines: The transgenic nature of amiRNA-AtMBD10 plants was verified using PCR of nptII gene. A 554 bp fragment was obtained in the transgenic lines whereas it was absent in the wild type plant. The first lane contains 50 bp DNA marker; lanes 1 to 7 depict different transgenic lines. The gene amplification of 554 bp fragment was not observed in wild type plant. WT, wild type; M, marker

Figure 70. GUS histochemical assay of amiRNA-AtMBD10 RNA expressing transgenic plants: The expression of gus reporter gene was verified by GUS histochemical assay in the transgenic lines. Leaves from untransformed and transgenic plants were used for GUS histochemical assay. The transgenic plants (1 to 10) displayed blue color whereas the leaves from wild type (WT) plants did not turn blue when GUS histochemical was performed. WT, wild type
Figure 71. Quantitative RT-PCR of transgenic plants harboring amiRNA-AtMBD10: The expression of AtMBD10 gene in amiRNA-AtMBD10 lines was analyzed by qRT-PCR. The X-axis represents different transgenic lines while the Y-axis represents the relative fold change in transcript level. The error bars indicate standard deviation from the mean value. WT, wild type.

Yeast two hybrid screening for AtMBD10-interacting proteins

The yeast two hybrid screening was performed using full length AtMBD10 gene as bait. Initially, 18 colonies were found to grow on -AHLT medium containing 2 mM 3AT and they turned to blue in α-gal containing medium. Sequencing of prey plasmid identified a clone containing AtPAP14 that grew on -histidine medium and showed +ve α-galactosidase activity (Fig 72).

Figure 72. Yeast two hybrid screening: Screening of yeast two hybrid library identified AtPAP14 (purple acid phosphatase14) as an interacting partner of AtMBD10. Yeast transformed with pGBK7-p53 and pGADT7rec-SV40 was used as positive control whereas yeast transformed with pGBK7Lam and pGADT7rec-SV40 was used as negative control. L, T, H and A represent leucine, trpophan, histidine and adenine hemisuplate respectively.
Results

Sub cellular localization and Florescence Resonance Energy Transfer (FRET) analysis

The putative interacting partner which was identified in yeast two-hybrid was further demonstrated in-vivo by Florescence resonance energy transfer (FRET). Both the bait and prey were cloned in pSITE1CA and pSITE3CA vectors respectively (Fig. 73). The CFP-AtMBD10 and YFP-AtPAP14 fused constructs were bombarded into onion peels. The onion peels were then analyzed using a confocal microscope.

The Purple Acid Phosphatase 14 (PAP14) gene produces two transcripts. The small splicing variant was found to interact with AtMBD10 in yeast two-hybrid. The interaction between AtMBD10 and AtPAP14 was detected by FRET where these proteins were fused with CFP and YFP respectively. The FRET efficiency was found to be 14.46%, suggesting a strong interaction between them (Fig. 74 and Fig. 75).

Figure 73. Cloning of bait and prey in pSITE vectors: Schematic diagram showing cloning of AtMBD10 in pSITE1CA vector in fusion with 3’ of CFP. AtPAP14 was cloned in pSITE3CA in fusion with 3’ of YFP.
Figure 74. Colocalization of AtMBD10 and AtPAP14 in onion cell by confocal microscopy: Subcellular localization of AtMBD10 and AtPAP14 was performed using CFP and YFP fusion proteins respectively: Open reading frame (ORF) of the genes were fused with the fluorescent protein coding region at the 3’-end and were visualized after onion epidermal cell bombardment. Cyan and Yellow are pseudocolors representing fluorescence of CFP and YFP respectively.

Figure 75. Interaction of AtMBD10 and AtPAP14 using confocal microscopy: The protein-protein interaction was analyzed by fluorescence resonance energy transfer (FRET) analysis. The fusion constructs were co-bombarded into onion epidermal cells. Protein–protein interaction was studied by FRET-acceptor bleaching protocol. FRET efficiency for AtMBD10 and AtPAP14 is shown in histogram below.
Characterization of AtMBD11

Generation of transgenic plants over-expressing AtMBD11

A clone of the gene encoding AtMBD11 was available in the laboratory in the binary vector pCambia2301, where the coding sequence was placed in between 35S promoter and NOS terminator (Fig. 76). Transgenic plants over expressing AtMBD11 were generated by floral dip. Seeds were screened in MS medium containing kanamycin and cefotaxime. The transgenic nature of the plants was verified by PCR analysis using the genomic DNA as template and primers specific to nptII gene. PCR result clearly showed amplification 554 bp fragment of nptII gene from transgenic plants whereas the fragment was absent in the DNA from wild type plant (Fig. 77A). The expression of gus reporter gene was analyzed by GUS histochemical assay. The leaf tissues from the transgenic plants were then incubated in GUS assay buffer containing x-gluc at 37°C for overnight. The transgenic plant showed GUS staining whereas the untransformed plants did not show GUS activity (Fig. 77B).

Figure 76. Schematic diagram of cloning of AtMBD11 in pCambia2301: The gene was cloned in BamHI and SacI sites downstream to 35S promoter. The vector backbone contain kanamycin selectable marker and gus reporter gene.
Results

Figure 7. (A) PCR verification of AtMBD11-OE transgenic lines: The transgenic nature of AtMBD11-OE lines was confirmed using PCR analysis with nptII specific gene primers; A 554 bp fragment was obtained from the transgenic lines whereas it was found to be absent in the wild type plant. Genomic DNA isolated from wild type plant was used as a negative control whereas the vector harboring this gene was used as a positive control. The first lane contains 50 bp DNA marker; lanes 1 to 8 represent different transgenic lines which were used for PCR amplification. WT, wild type; +ve, positive control.

(B). GUS histochemical assay of transgenic plant over-expressing AtMBD11: The expression of gus reporter gene was verified by GUS histochemical assay. Leaves from WT and transgenic plants were incubated in GUS assay buffer for overnight at 37°C. After that the leaves were washed with acetone. The transgenic plants (lanes 2 to 8) displayed blue color whereas the wild type (WT) plants did not turn blue.

The expression level of AtMBD11 transcript was determined by quantitative RT-PCR, using AtMBD11 specific primers and AtACTIN12 was taken as an internal control. The transcript level of AtMBD11 was higher in case of transgenic plants as compared to the wild type (Fig. 7B).
Results

**Figure 78. The expression analysis of AtMBD11-OE transgenic plants by q-RT-PCR:** The expression of AtMBD11 gene in over-expression lines was analyzed by qRT-PCR using AtMBD11 gene specific primers. The X-axis represents different transgenic lines while the Y-axis represents the relative fold change in transcript level. The error bars indicate standard deviation from the mean value.

**Microarray analysis of atmbd11 mutant**

Microarray analysis of atmbd11 mutant was performed using Affymetrix ATH1 gene chip. The expression of a large number of genes was affected in the mutant showing fold change $\geq 2$ at p value 0.05. The mutant showed altered expression of 1738 genes, out of which 520 genes were up-regulated and 1218 genes were down-regulated (Fig. 79). The microarray result was verified by quantitative RT-PCR analysis of some selected genes. The expression analysis of qRT-PCR was found to be similar with that of microarray data (Fig. 80). The differentially expressed genes were annotated according to TAIR9. These genes belong to different functional categories such as nuclear factors, transporters, lipid metabolism, carbohydrate metabolism and cellular N2 compound biosynthesis (Fig. 81).
Figure 79. **Microarray analysis of *atmbd11* mutant**: The microarray result identified 1738 differentially expressed genes, out of which 520 genes were up-regulated in the mutant whereas 1218 genes were down-regulated.

Figure 80. **Verification of microarray data**: qRT-PCR was performed to validate microarray data. Genes which showed a high level of differential expression in the mutant were selected for this analysis. Similar expression pattern was observed in both microarray and qRT PCR. The X-axis represents different genes whereas the Y-axis represents the fold change in transcript level. Blue and red colors represent the expression value in microarray and qRT PCR data.
Figure 81. Functional categorization of differentially expressed genes in *atmbd11* mutant: A. Up-regulated genes. B. Down-regulated genes.
The microarray result was mapped on MapMan software (Thimm et al. 2004), which mapped the up/down-regulated genes in different pathways, giving a false color according to the log₂ values. The mutant showed altered gene expression that affected cellular processes like post translational modification, protein degradation, protein targeting, lipid metabolism and carbohydrate metabolism (Fig. 82).

Figure 82. Expression of genes involved in metabolism in atmbd11 mutant: Transcript levels in atmbd11 mutant, relative to the level in wild type were displayed on a log₂ scale using the MapMan software (Thimm et al. 2004). Gene transcripts that do not change by more than a threshold value are shown in white; genes that showed an increase and a decrease in expression are shown by an increasingly intense red and green coloration respectively, as shown in the color bar.

Nuclear factors

The microarray result showed that 214 transcription factors show altered expression in the mutant, out of which 90 genes were up-regulated whereas 124 genes were down-regulated. Among the transcription factors are members of MYB, AP2-EREBP, bHLH, HB, WRKY, C2H2 gene family.
Results

Post-translational modification

There are a large number of genes involved in posttranslational modification of proteins which showed altered expression in the mutant. The expression analysis indicate that 91 such genes were differentially regulated out of which 74 were down-regulated whereas 17 were up-regulated. Most of these proteins have kinase activity (40.6%) and transferase activity (25.6%).

Protein degradation

Genes involved in the protein degradation pathway also showed altered expression in the mutant. Out of 154 genes which were differentially regulated 109 genes were down-regulated. The up-regulated gene are belongs to ubiquitin proteosome pathway and ubiquitin E3 RING pathway. Most of the up-regulated genes are C3HC4-type RING finger protein.

Protein targeting

Microarray result identified some transport factors which were down-regulated in the mutant. Genes involved in protein targeting to nucleus, especially importin-α, importin-β and NTF2 like genes were highly down-regulated. Genes involved in protein targeting to endoplasmic reticulum and Golgi complex were also down-regulated in the mutant. This suggests that the protein shorting is highly affected in the mutant.

Abiotic stress

The mutant shows altered expression of genes involved in abiotic stress like heat stress, drought stress and salt stress. Heat responsive genes like heat shock proteins were up-regulated in the mutant. The highly up-regulated HSPs were HSP83, HSP17.3, HSP26, HSP17, HSP101, HSP70 and HSP23. The genes involved in salt stress and drought stress especially dehydration responsive proteins and proteins which show early response to dehydration were down-regulated.
Chlorophyll biosynthesis and light reaction

There was down-regulation of genes involved in tetrapyrrole synthesis. Estimation of pigments also showed that there was a decrease in chlorophyll content in the mutant (Fig. 83). The mutant also showed decrease in light reaction associated gene, most of which are encoded by the chloroplast genome.

Figure 83. Tetrapyrrole biosynthesis in atmbd11 mutant: A. The genes involved in tetrapyrrole biosynthesis are down-regulated in the mutant. Each small square represent a gene; red and green color indicate up and down-regulated genes respectively. B. Estimation of chlorophyll from wild and mutant suggests decrease pigment content in the mutant.
Results

Lipid metabolism

The mutant also showed an alteration in lipid metabolism. The genes involved in triacyl glycerol synthesis were up-regulated. These induced genes encoding olosin 1 and olosin 2. There was down-regulation in genes coding for fatty acid desaturases (FA desaturase 1, FA desaturase 2, FA desaturase 3, FA desaturase 6 and FA desaturase 7) and FA synthesis and elongation.

Carbohydrate metabolism

The carbohydrate metabolism is highly affected in the mutant. There was a significant down-regulation in the genes encoding enzymes of glycolysis, transporters of carbohydrate intermediates and Calvin cycle (Fig. 84). The transporters responsible for metabolite transport present in chloroplast membrane, like phosphate translocator, GPT antiport, PPT2, AAC1 and APE1 were also down-regulated. Among the enzymes of glycolysis pyruvate kinase, hexokinase 1, UDP-glucose pyrophosphorylase, glyceraldehydes 3 phosphate dehydrogenase, UDP-glucose pyrophosphorylase, pyruvate kinase pyruvate decarboxylase and diphosphate-fructose-6-phosphate 1-phosphotransferase were down-regulated.
Figure 8. Carbohydrate metabolism in atmbd11 mutant: (A) Down-regulation of transporters present in chloroplast membrane and starch degradation enzymes. (B) Genes involved in TCA cycle were down-regulated in atmbd11 mutant. The genes involved in electron transport system are up-regulated in the mutant.
Results

Yeast two hybrid screening for AtMBD11-interacting proteins

The analysis of protein-protein interaction was screened by yeast two hybrid assay using AtMBD11 as bait. The ORF of \textit{AtMBD11} was cloned in pGBKT7 vector in fusion with GAL4-DNA binding domain. A library was prepared using 10 days old seedling and mating was performed. The colonies that grew in –His medium were verified using \( \alpha \)-galactosidase activity (Fig. 85). Plasmids from the positive colonies were isolated and sequenced. The sequencing results identified one clone that grew in –His medium. Sequencing of the prey vector identified the gene as \textipa{At1g03250}, which contain R3H domain was predicted to bind with ssRNA.

\textbf{Figure 85. Yeast two hybrid screening:} Screening of yeast two hybrid library identified \textipa{At1g03250} (a nuclear protein containing RNA binding domain) as an interacting partner of AtMBD11. Yeast transformed with pGBKT7-p53 and pGADT7rec-SV40 was used as positive control whereas yeast transformed with pGBKT7Lam and pGADT7rec-SV40 was used as negative control.