5.1 Regulation of seed maturation in A-ZIP53 expressing *Arabidopsis thaliana*

Gene expression is regulated by contributing action of regulatory elements including cis regulatory viz., enhancer, silencer, promoter, and trans regulatory elements such as transcription factor. Transcription factors are the major contributing players in transcription machinery which regulate all biological processes including growth, development, stress, pathogen defense, light signaling, seed and flower development (Jakoby et al. 2002). The model plant *Arabidopsis thaliana* is well-studied and explored to understand the process of seed development and maturation (Santos-Mendoza et al., 2008). The seed maturation (MAT) is fine-tuned processes that occur only in angiosperms. This process collectively occurs in different tissues of seed that ultimately contributes to seed quality (Vicente-Carbajosa and Carbonero 2005). High-throughput genome and transcriptome technologies help to identify genes that are involved in seed maturation (Soeda et al. 2005; Sreenivasulu and Wobus 2013). Some of the genes reported to play prominent role during this process are seed storage protein genes (SSP), ABI3, FUS3, and LEC1 (Parcy et al. 1997; Braybrook et al. 2006; Santos-Mendoza et al. 2008). Mutation in these genes results in severe seed phenotype with reduced content of seed storage proteins (To et al. 2006). In addition to seed phenotype, other pleiotropic effects also appeared in mutants including accumulation of chlorophyll in dry seeds, desiccation intolerance, defected cotyledonary identity, and others (Meinke 1992; Keith et al. 1994; Meinke et al. 1994; Parcy et al. 1994; Stone et al. 2001; Kroj et al. 2003). These genes alone or in combination can initiate or maintain maturation phase. Regulation of these genes are under the direct control of several regulatory elements including B–ZIP class of TFs (Santos-Mendoza et al. 2008).

In the previous chapter, we have elaborated on designing of dominant negative (A-ZIP53) protein and its derivatives that preferentially heterodimerizes with B-ZIP53 and its dimerizing partners i.e., B-ZIP10 and B-ZIP25 involved in seed maturation. In this chapter, we aimed to understand the phenomenon of seed maturation by inhibiting the binding of B-ZIP53 and its heterodimerizing TFs to their DNA binding sites in the promoter of genes regulated. A-ZIP53 expressing transgenic *Arabidopsis* lines have been developed which showed retarded seed phenotype. *In vitro* studies showed the A-ZIP53 interaction with wild type B-ZIPs (B-ZIP10, B-ZIP25, and B-ZIP53) and inhibit their DNA binding to the target DNA. In order to evaluate the function of A-ZIP53, *in vivo* transgenic *Arabidopsis* expressing A-ZIP53 was produced. A-ZIP53 showed specificity by targeting B-ZIP53 and its
heterodimerizing partners that were confirmed with down-regulation of genes responsible for seed maturation and seed-specific storage proteins. In conclusion, our designed DN protein can be used to regulate the function of closely related B-ZIPs which is difficult to attain by other loss of function mutation strategies like CRISPR/Cas, zinc finger nuclease, and TALEN.

5.2 Construct preparation and transformation

For transient transfections and *Agrobacterium* mediated transformation, A-ZIP53 was cloned as NdeI and EcoRI double digested fragment into pRI101 AN vector under the control of CaMV35S promoter. For cloning, vector was double digested with NdeI-EcoRI and A-ZIP53 coding DNA was introduced as NdeI-EcoRI fragment using standard molecular biology techniques.

**Figure 43** Multiple cloning site of pRI101AN plant transformation vector in which gene of A-ZIP53 was inserted.

Presence of insert was confirmed by PCR using cloned plasmid as template that was further confirmed by double digestion of cloned plasmid (Figure 44).

**Figure 44** Cloning and conformation of A-ZIP53 insert into plant expression vector (A) Double digestion of pRI101 AN (NdeI-EcoRI) (B) PCR with gene specific primers confirmed the presence of A-ZIP53 insert (C) Confirmation of insert using double digestion of construct with the NdeI-EcoRI restriction enzymes.
5.3 A-ZIP53 ectopic expression causes abnormal phenotype in *Arabidopsis*

We have examined the consequences of A-ZIP53 expression on the plant growth and development. In earlier studies, the efficacy of the designed dominant negative protein against target B-ZIP transcription factors (TFs) both have been shown *invitro* and *invivo* (Olive et al. Rishi et al. 2004; Gerdes et al. 2006). Now we have generated transgenic *Arabidopsis* lines (Pro35S:A-ZIP53) that expressed A-ZIP53 under the control of CaMV35S constitutive promoter (Figure 45A and 45B). A-ZIP53 transcript presence in the plants was confirmed by PCR (Figure 45C). A-ZIP53 expression causes altered phenotype including retarded growth, dwarfism, and late flowering compared to the mutant of B-ZIP53, B-ZIP25, B-ZIP10, and the wild type *Arabidopsis* (Figure 45D). Fifteen different transgenic lines in the T-1 generation were analyzed, which showed the differential and retarded growth pattern. Comparative expression of the A-ZIP53 was done by the qRT-PCR in two different transgenic lines. The transgenic line with a more severe and dwarf phenotype has higher expression of the A-ZIP53 transcript (Figure 45E). It shows the designed DN protein A-ZIP53 is effective and functional in plants suggesting that it may act by inhibiting the DNA binding of target B-ZIP transcription factors, that are structurally and functionally similar (Alonso et al. 2009; Dietrich et al. 2011; Hartmann et al. 2015).

5.4 A-ZIP53 expression inhibits the DNA binding of B-ZIP53, B-ZIP10, and B-ZIP25 in the transient transfection using By-2 cell line protoplast

Earlier, ChIP-seq analysis has shown that B-ZIP53 is directly involved in the activation of seed-specific genes (Alonso et al. 2009). We studied the inhibition of the DNA binding activity of B-ZIP53 using A-ZIP53 in transient transfection studies. Figure 46 shows the inhibition of B-ZIP53 mediated reporter gene activity by the A-ZIP53. We examined whether A-ZIP53 expression in protoplasts of By-2 cell line could inhibit the target TFs (B-ZIP53, B-ZIP10, and B-ZIP25) specific reporter activity. For that purpose, the A-ZIP53 construct was co-transformed with the B-ZIP53, B-ZIP10, and B-ZIP25 under the control of constitutive promoter in the By-2 cell line protoplasts. To determine the effect of the A-ZIP53, it is necessary to use a reporter plasmid that reflects the condition of the natural promoter. Earlier, the network of class C/S1 B-ZIP has been reported and confirmed through the protoplast transient transfections using the GUS/NAN reporter assay (Jakoby et al. 2002; Weltmeier et al. 2006). To determine the DNA binding of B-ZIP TFs to promoter, the reporter plasmid (2S2: GUS) (Alonso et al. 2009), that has a unique binding site for the target
B-ZIP transcription factor was co-transformed with the effector plasmid of B-ZIP53 that resulted in the increased level of reporter activity. The GUS expression decreased in a dose-dependent manner by co-transfection with the A-ZIP53 (CaMV35S:A-ZIP53) (Figure 46A). Further, the efficacy of the A-ZIP53 protein against the B-ZIP10 and B-ZIP25 DNA binding is shown in Figure 46B. GUS/NAN reporter activities mediated by binding of B-ZIP53, B-ZIP10, and B-ZIP25 is demonstrated in the presence of the A-ZIP53, indicating that A-ZIP53 inhibited the DNA binding of target B-ZIP TFs.

![Figure 45](image)

**Figure 45** Constitutive expression of A-ZIP53 in wild type *Arabidopsis thaliana*  
(A) Schematic of Pro35S: A-ZIP53 construct as described in Materials and Methods. (B) Selection of A-ZIP53 expressing transgenic *Arabidopsis* on kanamycin containing selection media. (C) Confirmation of transgenics using PCR. (D) Comparison between the phenotypic variation and abnormal growth pattern of 12 weeks old transgenic *Arabidopsis*, wild type, and insertion mutants of B-ZIP10, B-ZIP25, and B-ZIP53 in the T-1 generation. (E) Differential expression of A-ZIP53 in transgenic lines.
A-ZIP53 inhibits the reporter gene activity of B-ZIP53, B-ZIP10, B-ZIP25, and their heterodimers in transient transfection experiments using Arabidopsis protoplast. Plasmid coding for B-ZIP53 can transactivate the GUS reporter gene under the control of the 2S2 promoter that contain the G-box binding site. NAN plasmid was used as an internal control. A) Suspension of the By-2 tobacco plant cell line and isolated protoplast. B) Transient expression of A-ZIP53 inhibits the normalized GUS reporter activity in a dose-dependent manner. Protoplasts were co-transfected with plasmids coding for A-ZIP53. Column 2-5 depict reporter activity at B-ZIP53: A-ZIP53 molar ratio of 1:1, 1:2, 1:3, and 1:5, respectively. C) B-ZIP10 and B-ZIP25 overexpression can transactivate the GUS reporter gene, suggesting that these B-ZIPs can bind to the G-Box containing promoter in vivo. Reporter activity was inhibited in the presence of 3 molar excess of A-ZIP53 plasmid suggesting that A-ZIP53 can compete with G-Box DNA sequence for B-ZIP53 binding. Enhanced GUS/NAN activity was observed when protoplasts were co-transfected with 9 µg each of B-ZIP53, B-ZIP10, and B-ZIP25. A-ZIP53 inhibits the reporter gene activity at 3 molar excess concentrations.
5.5 RNA-seq of transgenic *Arabidopsis* revealed that A-ZIP53 inhibits the expression of seed-specific genes

RNA samples from immature siliques of three independent transgenic lines were sequenced using Illumina Next seq 500 system, which generated 20,420,244 reads (Reads with unknown nucleotide “N” larger than 5%), adaptor sequence, ambiguous reads (reads with more than 10% quality threshold (QV) <20 phred score) were removed and rest of reads were processed using trimmomatic-0.35. The high quality reads (QV >20) were used for the reference based mapping using Tophat v2.1.1. with default parameters.

![Venn diagram showing differentially expressed downregulated transcripts](image)

**Figure 47** Venn diagram showing differentially expressed downregulated transcripts (Appendix 3) (log₂ fold ≥ 2; P-value ≤ 0.001) between transgenics and wild type *Arabidopsis*.

5.6 Analysis of differential expression of genes in immature siliques and seeds

There are total of 37,865 genes in the *Arabidopsis thaliana*. Out of which 27,411 are protein coding. The differential gene expression was carried out using cut diff v1.3.0. Fold change values less than zero were downregulated (Figure 48). P value threshold of 0.05 was used to filter significant results (Appendix 3). These genes were further categorized on the basis of their statistical significance (which can be either “yes” or “no”) depending on
whether p value is less than 0.05 and the FDR (False discovery rate 0.05 after Benjamin-Hockberg correction for multiple-testing) for their significant expression.

Figure 48 Functional classification of assembled transcripts based on the Agri GO analysis. The results are categorized into the three main GO categories: biological processes, cellular components, and molecular functions. The x axis represents the GO terms while Y axis (left side) shows the % of genes while Y axis (right side) shows the number of genes belonging to the GO terms.

5.7 Temporal expression pattern of seed specific genes in A-ZIP53 transgenic Arabidopsis and their validation using transcriptome analysis

Genes and pathways involved in seed maturation have been extensively studied. The storage proteins are present in different seed compartments and provide nutrition during germination. The temporal expression pattern of several genes responsible for seed maturation and development were checked using gene-investigator. RNA-seq data was analyzed to check the expression of genes responsible for seed maturation in the A-ZIP53 expressing transgenic plants. The transcript profiling revealed the absence or lower expression of genes involved in seed maturation. Genes responsible for seed maturation are
cruciferin (CRU) (At4g28520), asparagine synthase 1 (ASN1) (At3g47340), cruciferina (Sun et al. 2010) (At5g44120), hydroxysteroid dehydrogenase 1 (HSD1) (At5g50600), seed storage albumin (2S2) (At4g27150), proline dehydrogenase (ProDH) (At5g38710), and the late embryogenesis accumulating 76 (LEA76) (At3g15670), which are involved in different stages of seed development and maturation, and are targets of B-ZIP53 and its heterodimerizing B-ZIP partner (Figure 48) (Weltmeier et al. 2006; Alonso et al. 2009).

5.8 In silico promoter analysis of target genes

The promoter region of putative genes identified from the transcriptome data were analyzed, which revealed the presence of G-box (GTCAGTCAGGCCACGTGGCAGCT). G-box is a proximal binding site for B-ZIP transcription factor especially, B-ZIP53, B-ZIP10, and B-ZIP25 that are involved in the seed development and maturation (Lara et al. 2003; Alonso et al. 2009). Protein binding microarray (Weirauch et al. 2014), ChIP, and the overexpression data (Weltmeier et al. 2006) (Table-19) showed the putative binding site for target B-ZIP TFs present in the promoter region of genes. However, DAP-seq showed the C-box as a probable binding site for B-ZIP53. DNA binding sites preferences may depends on its heterodimerizing partners (Weltmeier et al. 2006; Alonso et al. 2009; O’Malley et al. 2016). Heterotypic interaction between B-ZIP TFs may be responsible for differential expression of target genes.

**Table 19** Probable DNA binding site of B-ZIP transcription factor

<table>
<thead>
<tr>
<th>S.No.</th>
<th>B-ZIP</th>
<th>Gene</th>
<th>Binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>B-ZIP10</td>
<td>CRU</td>
<td><img src="" alt="binding_site_1.png" /></td>
</tr>
<tr>
<td>2.</td>
<td>B-ZIP25</td>
<td>CRU</td>
<td><img src="" alt="binding_site_2.png" /></td>
</tr>
<tr>
<td>3.</td>
<td>B-ZIP39</td>
<td>CRU</td>
<td><img src="" alt="binding_site_3.png" /></td>
</tr>
<tr>
<td>4.</td>
<td>B-ZIP10, B-ZIP25</td>
<td>CRU</td>
<td><img src="" alt="binding_site_4.png" /></td>
</tr>
<tr>
<td>5.</td>
<td>B-ZIP10, B-ZIP25</td>
<td>CRU</td>
<td><img src="" alt="binding_site_5.png" /></td>
</tr>
<tr>
<td>6.</td>
<td>B-ZIP10, B-ZIP25</td>
<td>LEA</td>
<td><img src="" alt="binding_site_6.png" /></td>
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<tr>
<td>7.</td>
<td>B-ZIP10, B-ZIP25</td>
<td>LEA</td>
<td><img src="" alt="binding_site_7.png" /></td>
</tr>
<tr>
<td>8.</td>
<td>B-ZIP25</td>
<td>LEA</td>
<td><img src="" alt="binding_site_8.png" /></td>
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<tr>
<td>9.</td>
<td>B-ZIP39</td>
<td>LEA</td>
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</tr>
<tr>
<td>10.</td>
<td>B-ZIP10, B-ZIP25</td>
<td>ASN</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>B-ZIP10, B-ZIP25</td>
<td>ASN</td>
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</tr>
<tr>
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<td>ASN</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>B-ZIP39</td>
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</tr>
<tr>
<td>14.</td>
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<td>HSD</td>
<td></td>
</tr>
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<td>B-ZIP25</td>
<td>HSD</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>B-ZIP39</td>
<td>HSD</td>
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</tr>
<tr>
<td>17.</td>
<td>B-ZIP10, B-ZIP25</td>
<td>ProDH</td>
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</tr>
<tr>
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<td>ProDH</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>B-ZIP25</td>
<td>CRA</td>
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<td>B-ZIP25</td>
<td>CRA</td>
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<tr>
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<td>B-ZIP25</td>
<td>CRA</td>
<td></td>
</tr>
<tr>
<td>23.</td>
<td>B-ZIP53</td>
<td>CRU</td>
<td></td>
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</tbody>
</table>

### 5.9 Expression of target genes during plant development

Figure 49 shows the transcription profiling of seed-specific genes during different development stages of *Arabidopsis*. To study expression of target genes, gene-investigator database search was performed. The continuous expression of the B-ZIP53 and B-ZIP25 observed throughout developmental stages of the plant starting from seedling to seed maturation while B-ZIP10 expression is higher during the seed maturation. The target genes including 2S2, LEA76, ASN1, CRA1, and CRU have higher expression in the later phase of seed maturation. Expression of the ProDH which is a direct target of B-ZIP53 has an optimal expression throughout the development of plant (Weltmeier et al. 2006). B-ZIP39 and B-ZIP72 that were chosen to show the specificity of A-ZIP53. The proteins B-ZIP39 (ABI-5) (a non-target protein of A-ZIP53) has higher expression in the later stage of seed maturation compared to the other target protein B-ZIP72 (Bensmihen et al. 2005; Le et al. 2010;
Belmonte et al. 2013). The expression of SHB-1 a target gene of B-ZIP39 has lower expression in the later stage of seed maturation in Arabidopsis (Bensmihen et al. 2005; Cheng et al. 2014). It may be suggested that the regulation of genes is a combinatorial effect of B-ZIP TFs.

![Figure 49](image)

Geneinvestigator has revealed the expression of B-ZIP53 (Red), B-ZIP10 (Blue–first panel), B-ZIP25 (Light green), target genes (2S2: Orange, CRU3: Violet, LEA76: Yellow, ProDH: Brown, ASN1: Sky Blue, CRA1: Gray), Non heterodimerizing partner (B-ZIP72: Blue-second panel, B-ZIP39:Green), and Non-target gene (SHB1: Orange-second panel) in the different development stage of Arabidopsis are also shown.

5.10 Altered growth parameters in different transgenic lines

To gain insight into the impact of A-ZIP53 on the expression of target genes of B-ZIP53, B-ZIP10, and B-ZIP25 (Weltmeier et al. 2006; Weirauch et al. 2014), transgenic Arabidopsis were subjected to the expression analysis. Two different A-ZIP53 expressing transgenic lines were analyzed for the expression of seven target genes (2S2, CRU, LEA76, ProDH, ASN1, CRA1, and HSD1), and the non-target gene (SHB-1)(Alonso et al. 2009; Cheng et al. 2014; Weirauch et al. 2014)using qRT-PCR. Leaves from the T-1 generation while immature siliques and seeds from the T-2 generation were subjected to the expression analysis using qRT-PCR (Figure 50B and 50D). The T-1 and T-2 generation of transgensics have higher expression of B-ZIP53 compared to the wild type plant. This could be a plant response to compensate for the B-ZIP53 loss of function due to heterodimerization with the
A-ZIP53. The expression of target genes of B-ZIP53, B-ZIP10, and B-ZIP25 including CRU, ASN1, CRA, and HSD1 were downregulated in both T-1 and T-2 generations (Figure 50B, 50D). Transcript expression of the seed storage albumin (2S2) and the late embryogenesis accumulating 76 (LEA76) was not observed in the T-1 generation (Figure 49B) while both genes were downregulated in the T-2 generation (Figure 50D). Transgenic has higher expression of ProDH in the T-1 generation compared to the T-2 generations (Figure 50B and 50D) (Weltmeier et al. 2006).

Figure 50 Phenotypic variation in the growth of transgenic compare to wild type (A) T-1 generation: four week old transgenic and (B) qRT-PCR revealed the expression of B-ZIP53, B-ZIP39, target (CRU, ProDH, ASN1, CRA, and HSD1) of B-ZIP53 and non-target gene (SHB-1) from the leaves of four weeks old transgenic. (C) T-2 generation: Phenotypic variation in the six week old plants (D) Expression of target genes (2S2, LEA76, CRU, ProDH, ASN1, CRA, and HSD1) and non-target gene (SHB-1) in the immature siliques and seeds of Arabidopsis (E) Differences in the silique size of wild-type and transgenic (F) Seed size variation between wild type and transgenic.

The specificity of the A-ZIP53 was checked against the expression of SHB-1, a target gene of ABI-5 using qRT-PCR. The transgenic has higher expression of SHB-1 compared to
the wild type *Arabidopsis*. It shows that A-ZIP53 is specific and did not alter the expression of non-target genes. Together, above data suggests that the A-ZIP53 can specifically regulate the redundant behavior of B-ZIP TFs by forming heterotypic interactions with closely related B-ZIPs. Seeds from most of the transgenic lines failed to germinate except of the lower A-ZIP53 expressing transgenic lines. The phenotypes were analyzed in the T-4 generation and siliques and immature seeds were subjected to the qRT-PCR (Figure 51). The expression of seed-specific genes were lower compared to the wild type plants and had similar expression pattern as in T-1 and T-2 generation.

![Figure 51](image)

**Figure 51** Phenotypic alteration and expression analysis of target genes of B-ZIP53 and its dimerizing partners in the T-4 generation using qRT-PCR. (A and B) The growth of the four and eight week old wild type and transgenic. (C, D, and E) Eight weeks old insertion mutants of B-ZIP10, B-ZIP25, and B-ZIP53. (F) Differences in the Rosette diameter of three weeks old transgenic, mutants (B-ZIP53, B-ZIP10, and B-ZIP25) and WT under standard condition. Error bar represents ± mean and SD of 8-10 individual plants. (G) Expression analysis of target genes of involved in the seed maturation from the immature silique and seeds of transgenic. Error bar represent ±S.D. of three technical replicates.
5.11 A-ZIP53 expression causes abnormal reproductive phase parameters in the transgenic

To investigate the effect of A-ZIP53 on the reproductive phase of plants, flowers of the A-ZIP53 expressing transgenic plants (T-4 generation) were analyzed against the mutants of B-ZIP53, B-ZIP10, B-ZIP25, and the wild type Arabidopsis (Figure 52A). Under standard growth conditions, delayed flowering was observed in A-ZIP53 expressing transgenic plants relative to the wild type and mutants of B-ZIP53, B-ZIP10, and B-ZIP25. Beside from the flowering time, other phenotypes were also studied including flower size, silique, and mature seeds. Transgenic has significantly small sized flowers compare to the wild type and the mutant of B-ZIP53, B-ZIP10, and B-ZIP25. The siliques has shorter length compared to the wild type and mutants (Figure 53A & 53B) while the number of siliques per 0.5 gm of weight are more compared to the wild type (Figure 53C). Additionally, seeds were stunted and small compared to the wild type and mutants (Figure 54A and 54B).

Figure 52 (A) Differences between length and size of flowers of mutants (B–ZIP10, B-ZIP25, and B-ZIP53) and transgenic of six weeks old plant (B) Significant differences between height and width of transgenic flowers and mutants compared to control were observed (± mean and S.D. of three independent biological replicates n= 8-12).
**Results**

**Figure 53** (A) Silique size of transgenic was smaller compared to mutants and wild type. (B) Height and width of siliques were compared. Error bar represents ±mean and SD of siliques (n = 8-12). (C) Number of siliques per 0.5 gm of silique weight were more in transgenics compared to wild type.

**Figure 54** Differences in the seed size of transgenic, WT and mutants (B-ZIP53, B-ZIP10, and B-ZIP25) (A) Representative pictures of WT, mutants and transgenic seeds (B) Seed of transgenic are smaller size compared to mutants and WT.
Variation in developing phase of transgenic lines were observed compared to the wild type and mutant of B-ZIP53 before and after anthesis. Compared to the wild type, transgenic flower has significant reduction in the length and width of flowers. These results suggested that unlike wild type, A-ZIP53 expression has major effect on the reproductive phase of the Arabidopsis. The effect of A-ZIP53 is continuous from the developing flower to the mature seed. The mature seeds of transgenic plant are retarded and smaller compare to the wild type.

Figure 55 Comparison between developing stages of the transgenic and wild type Arabidopsis
5.12 A-ZIP53 expressing transgenics has retarded seed phenotype

Seeds of the A-ZIP53 expressing transgenics were subjected to sectioning followed by staining with the toludine blue. Seeds of the late cotyledonary stage from the different siliques are smaller and abnormal compared to the wild type (Figure 56). It confirms the effect of the A-ZIP53 on the seed maturation in *Arabidopsis*.

![Figure 56](image)

**Figure 56** Light microscope of the late cotyledonary stage after thin sectioning and staining with the toluidine blue. A, wild type B, C, and D Transgenics

5.13 Germination assay using transgenic seeds

The germination efficiency of transgenic lines were checked. Seeds of most of transformed plants failed to germinate. Seeds (n=50) of the four independent line of the T-2 generation were subjected to the germination on the plain M.S. media, all seeds failed to germinate (Figure 57). It signifies that the A-ZIP53 by inhibiting B-ZIP53 and its heterodimerizing partners interfere with the developmental and reproductive growth of *Arabidopsis*. Transgenic lines that have higher expression of A-ZIP53 showed strongest phenotype with severe seed morphology (Figure 56B).

![Figure 57](image)

**Figure 57** Seeds of the A-ZIP53 expressing losses their viability on the half strength M.S. media. (A) Wild type (B, C, D) Transgenic.
5.14 Western blotting

Total protein was isolated from the immature siliques and seeds of the A-ZIP53 expressing transgenics. Protein extract was run on the SDS PAGE and subjected to Western blotting. The Western blot confirms the presence of the A-ZIP53 protein in cell extract of transgenic Arabidopsis (Figure 58).

![Western blotting of A-ZIP53 expressing transgenic Arabidopsis](image)

**Figure 58** Western blotting of A-ZIP53 expressing transgenic Arabidopsis (A) Ponceau stain of Blot, 1- Pure protein, 2- transgenic, 3- transgenic (B) Western blot of A-ZIP53 from independent lines.

5.15 Putative heterotypic interaction of A-ZIP53 in Arabidopsis

Our finding showed that A-ZIP53 can form the heterotypic interaction with the B-ZIP53, B-ZIP10, and B-ZIP25 in vitro (Figure 32 and 33) and in vivo (Figure 42). In order to know other heterodimerizing partners of A-ZIP53, whole protein extract from immature silique and seeds were subjected to the immunoprecipitation followed by the mass-spectrometry (IP-nano LC-MS). A schematic representation of IP-MS has been shown in the figure59. For the IP-MS seed and siliques of A-ZIP53 expressing transgenic lines were chosen to find out the other putative target B-ZIPs, which are involved in the seed development and maturation and heterodimerizing partners of the AZIP53. Total protein was immunoprecipitated using the primary antibody against T-7 tagged A-ZIP53 and separated on the 15% SDS PAGE (Figure 60). Based on molecular weight, proteins were selectively excised from the gel, trypsinized and analyzed by the nano-LC-MS/MS (Figure 61). The MS result in the identification of B-ZIP TFs that may be new heterodimerizing partners of the B-ZIP53 or its partner B-ZIPs. However, the excessive Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) interfered with the MS-based protein identification.
proteins that were identified in more than one sample with at least one proteotypic peptide were considered as a high confidence candidate. Eight B-ZIP TFs (B-ZIP14, B-ZIP17, B-ZIP19, B-ZIP23, B-ZIP29, B-ZIP33, B-ZIP34, and B-ZIP69) were obtained which may indicate them to be interacting partners of the B-ZIP53. Putative heterotypic interactions between B-ZIP53/A-ZIP53 with wild type B-ZIPs are given in appendix-I and appendix-II.

Figure 59: Schematic representation of IP-MS for the identification of heterodimeric partners of B-ZIP53.

Figure 60: Immunoprecipitation of A-ZIP53 from transgenic *Arabidopsis* (A) After IP: i, ii, iii (col-o leaves, transgenic leaves, transgenic siliques), first washing: iv, v, vi (col-o leaves, transgenic leaves, transgenic siliques), water: vii, viii, ix (col-O leaves, transgenic leaves, transgenic siliques) (B) IP (i-pureprotein, ii-transgenic leaves, iii-transgenic silique).
**Figure 61** Schematic representation of IP-MS for the identification of heterodimeric partners of B-ZIP53.
RESULTS

Here we describe a transgenic *Arabidopsis*, expressing a novel designed dominant negative protein A-ZIP3 under the control of the CaMV35S constitutive promoter. A-ZIP53 is shown to form specific heterotypic interaction with target B-ZIP transcription factors i.e., B-ZIP53, B-ZIP10, and B-ZIP25 which are involved in the seed maturation in *Arabidopsis*. In an earlier study, the functional significance of the target B-ZIP TF i.e., B-ZIP53 was shown by the gain of function approach which resulted in the retarded growth and delayed bolting compared to wild type plants, but showed higher expression of seed-specific genes (Alonso et al. 2009). However, genome scale transcriptome data failed to define B-ZIPs that are responsible for the seed maturation suggesting the combinatorial effect of B-ZIP TFs. Earlier it was shown that seed maturation is caused by the synergistic effect of B-ZIP53, B-ZIP10, and B-ZIP25 TFs (Alonso et al. 2009) although knockdown lines of these three B-ZIPs still set viable seeds that suggest functional redundancy of B-ZIP TFs. This prompted us to design a protein inhibitor that target B-ZIP53 and its heterodimerizing partners in the hope that knock out activity of A-ZIP53 will leads to the stronger phenotype, which is not possible through other loss of function technologies.

A-ZIP53 consists of the dimerization domain of B-ZIP53 and a designed poly glutamic acid rich acidic extension that replaced the basic region of wild type B-ZIP53. *Arabidopsis* expressing A-ZIP53 partially mimics the phenotype of B-ZIP53 overexpressing lines with a dwarf and delayed bolting phenotype (Alonso et al. 2009). However, transgenic plants showed a gradient of growth pattern due to the differential expression of the A-ZIP53 (Figure 45). Transient transfection assay using By-2 cell line protoplast shows that A-ZIP53 specifically inhibits the reporter activity mediated by B-ZIP53, B-ZIP10, and B-ZIP25 and their putative heterodimers that have earlier showed using *Arabidopsis* protoplast. The B-ZIP53 is redundant in its function and its expression is not limited to the seed. Earlier, it was shown that B-ZIP53 can heterodimerize with the B-ZIP1 and could partially complement its function. The intervention of heterodimerization and DNA binding activity of the B-ZIP53 TF leads to the stress as it is also involved during the regulation of hypo-osmolarity responses (Weiltmeier et al., 2006) and other stresses (Dietrich et al. 2011; Hartmann et al. 2015). Although, several heterodimeric partners of the B-ZIP53 have been defined but a detailed understanding of all its putative partners during seed maturation is lacking, which play
coordinated role during the seed maturation and stress (Alonso et al. 2009; Hartmann et al. 2015).

Immature siliques and seeds of the A-ZIP53 expressing Arabidopsis were subjected to the m-RNA profiling. Genes responsible for the seed development and maturation, are reported to be targets of B-ZIP53 and its heterodimerizing partners that are down regulated compared to the wild type plants (Figure 49). Genes including cruciferin, asparagine synthase, cruciferina, hydroxysteroid dehydrogenase, seed storage albumin, proline dehydrogenase, and late embryogenesis accumulating76 that have binding sites for B-ZIP53 and its dimerizing partners are found to be down-regulated in A-ZIP53 transgenic plants, as confirmed by the qRT- PCR. The lower expression of genes could be a reason for the abnormal and retarded seed phenotype (Figure 54).

**A-ZIP53 expression causes delayed growth and retarded seed phenotype**

B-ZIP53 over expression causes higher expression of genes responsible for the seed development and maturation (Alonso et al. 2009). To nullify the effect of the B-ZIP53 and its heterodimerizing partners, we overexpressed the A-ZIP53. Phenotypic examination of the A-ZIP53 expressing Arabidopsis revealed the contrasting features of various growth pattern (Figure 50). The transgenic lines have lesser rosette number, small height, small sized flower, and siliques. This indicates the pleiotropic nature of B-ZIP53 TF and also showed that a change in the DNA binding could lead to the severe impact on the plant morphology. Previous studies showed that B-ZIP53 is also involved in the salt stress (Hartmann et al. 2015). DNA binding inhibition of the B-ZIP53 transcription factor leads to the abnormal growth in A-ZIP53 transgenic plant. Germination assay confirmed the viability loss of the transgenic seed.

**Additional dimerizing partners of the A-ZIP53**

To find out the additional partner of B-ZIP53 and its heterodimerizing partners total protein of immature siliques and seeds of A-ZIP53 expressing Arabidopsis were subjected to the immunoprecipitation followed by mass spectrometry. The mass spectrometry helps to find additional B-ZIP TFs that may be involved during seed development and maturation process. These results validated the DNA binding of B-ZIP transcription factors as a target for studying seed development and maturation phenomenon in plants.