1. Introduction

Genomic DNA serves as an information carrier that specifies the protein coding mRNA and provides the regulatory sequence for the enactment of gene expression. The regulation of gene expression is a contributing action of several regulatory elements including cis-regulatory elements viz., enhancer, silencer, promoter, and trans-regulatory elements such as transcription factors. TFs are the major contributing part of transcription machinery in plants that regulate all biological processes. These DNA binding proteins interact with the other components of the transcriptional regulation machinery by allowing or restricting the RNA polymerase access to the promoter of the gene.

Basic leucine zipper (B-ZIP) are the dimeric eukaryotic class of bipartite transcription factors (TFs) that have an N-terminal DNA binding domain while the other half is a C-terminal dimerization domain. The dimerization domain in a human B-ZIP is typically composed of six to seven heptad \((g, a, b, c, d, e, f)\) repeat of amino acids which are responsible for the stability and specificity whereas in plants they are 8 heptads or longer. B-ZIP TFs can bind to their cognate site as a dimer either as homo- or heterodimer. The dimeric interactions between interacting B-ZIPs are dynamic, which can be homotypic or heterotypic. The profound biochemical and biophysical studies have defined the different heterodimerization partners of B-ZIP TFs in the *Arabidopsis thaliana* (Jakoby et al. 2002; Lara et al. 2003; Deppmann et al. 2004; Weltmeier et al. 2006; Alonso et al. 2009; Weltmeier et al. 2009). The dimeric interactions are imperative for B-ZIP TFs functions (Dietrich et al. 2011). In the dimeric interaction, each monomer can recognize a distinct half-consensus DNA binding site destined to the different biological function (Amoutzias et al. 2008; Mann et al. 2013).

In *Arabidopsis thaliana*, B-ZIPs are involved in various biological processes including the growth, development, stress, pathogen defense, light signaling, flower development, seed development, and maturation (Jakoby et al. 2002). The seed maturation is a fine-tuned process, which is regulated by the expression of Maturation-associated (MAT) genes. This process collectively takes place in different parts of the seed, which ultimately contributes to the viability and quality of seed (Vicente-Carbajosa and Carbonero 2005). Maturation phase can be collectively divided into different phases in which the early and mid-phase are dominated by the action of ABA and in the late phase synthesis of the Late embryogenesis abundant (LEA) protein take place due to the lower expression of the ABA
(Figure 1). The synthesized LEA protein helps in the desiccation tolerance during the dehydration. In earlier studies, high-throughput genome and transcriptome technique have helped to identify genes which are involved in the seed maturation (Keith et al. 1994; Meinke et al. 1994; Parcy et al. 1994; Meinke et al. 1998; Boyes et al. 2001; Stone et al. 2001; Kroj et al. 2003; Bensmihen et al. 2005; Le et al. 2010; Belmonte et al. 2013; Sreenivasulu and Wobus 2013). Genes which play a prominent role in this process are seed storage protein genes (SSP), ABI3, FUS3, and leafy cotyledon1 (LEC1) (Parcy et al. 1994; Jakoby et al. 2002; Braybrook et al. 2006; Santos-Mendoza et al. 2008). Mutation in these genes results in the severely affected seed phenotype with the reduced content of seed storage proteins (To et al. 2006). Beside seed phenotype, mutants displays other pleiotropic effects like the accumulation of chlorophyll in dry seeds, desiccation intolerance, defected cotyledonary identity, etc. (Keith et al. 1994; Meinke et al. 1994; Parcy et al. 1994; Meinke et al. 1998; Boyes et al. 2001; Stone et al. 2001; Kroj et al. 2003; Bensmihen et al. 2005). These genes alone or in combination can initiate or maintain the maturation phase. (To et al. 2006). The promoter of MAT (Maturation associated) genes are marked by several cis-regulatory elements including ACGT elements, RY (CATGCA), AACA, and CTTT motifs (Vicente-Carbajosa and Carbonero 2005). These regulatory elements are the probable binding sites of several TFs including the B-ZIP TFs.

![ENDOSPERM DEVELOPMENT](image)

**Figure 1** Different stages of plant development presented from early seed development, later maturation, and finally germination.

During seed maturation, various B-ZIPs like ABI-5, B-ZIP67 (Belmonte et al. 2013), B-ZIP15, B-ZIP72 (Le et al. 2010), and the B-ZIP53 are expressed. B-ZIP53 is reported as a major regulator of MAT genes (Alonso et al. 2009). To investigate the function of the B-ZIP53, the gain of function approach in the *Arabidopsis* has been used. For example, the gain
of function of the B-ZIP53 leads to the activation of MAT and LEA by forming the heterodimer complex with the B-ZIP10 and B-ZIP25 proteins (Alonso et al. 2009). In contrast, B-ZIP53 also regulates the expression of genes involved in the dark-induced starvation (Dietrich et al. 2011) and the salt-induced stress where it partially overlaps loss of function of B-ZIP1 (Hartmann et al. 2015). However, knockdown lines of B-ZIP53 still set viable seeds which suggests that some other B-ZIPs may partially or completely substitute the function of B-ZIP53 (Alonso et al. 2009).

Extensive biochemical and biophysical studies have established rules that dictate the homo- and heterodimerization of B-ZIP TFs. Studies involving Drosophila, Human, and Arabidopsis have revealed that many features that are critical in specifying dimerization properties of B-ZIPs are conserved across the kingdoms (Fassler et al. 2002; Vinson et al. 2002; Deppmann et al. 2004). Previous studies, on the basis of their dimerization potential have placed Arabidopsis B-ZIP proteins in 14 families. Phylogenetic analysis showed that none of the B-ZIPs in Arabidopsis is homologous to human B-ZIP proteins but have similar repetition of amino acids in heptads that regulate dimerization specificity (Deppmann et al. 2004). For example in both the cases, dimerization specificity of the B-ZIP leucine zipper is defined by charged amino acids in a, g, and e positions whereas their stability is governed mostly by hydrophobic amino acids in a and d positions (Figure 2) (Vinson et al. 2002; Deppmann et al. 2004). In vitro studies have confirmed that compared to homodimer, heterodimers are more proficient and impart a synergistic effect on the gene expression (Vinson et al. 1993).

Based on its dimerizing properties, B-ZIP53 was placed in the Group H with complex heterotypic properties (Deppmann et al. 2004). In Arabidopsis, numbers of studies have reported the heterodimerizing network of group C/S1 B-ZIP proteins involved in the seed
development and maturation (Jakoby et al. 2002; Ehlert et al. 2006; Alonso et al. 2009; Dietrich et al. 2011; Hartmann et al. 2015). In a previous study, the B-ZIP53 protein was shown to play a central role in regulating MAT genes (Alonso et al. 2009). B-ZIP53 has enhanced expression during seed maturation that later localizes to the embryo and endosperm (Alonso et al. 2009). It binds to the G-box (ACGTG) containing 2S2 promoter with the partner B-ZIPs i.e., B-ZIP10 and BZIP25 as well as to the C-box (ACGTC) (Alonso et al. 2009; O'Malley et al. 2016). Knock-out lines of B-ZIP53, B-ZIP10, and B-ZIP25 have no significant differences in the phenotype and seed development. It may be stated that in the absence or sub-optimal expression of the B-ZIP53 protein other B-ZIP TFs like B-ZIP10, B-ZIP25, or some hitherto unknown protein(s) may regulate seed-specific gene expression. B-ZIP53 is reported as a member of the complex group of B-ZIP TF that has >8 heptads in its leucine zipper region. B-ZIP53 can also partially overlap the function of the B-ZIP1 during salt stress (Hartmann et al. 2015) and low osmolarity (Dietrich et al. 2011). The understanding and regulation of the dimerization behavior of target B-ZIPs can be a major step to unravel the function of B-ZIP TFs. However, structural determinants that mediate heterodimerization are not well-understood and there is a lack of in vitro biochemical and biophysical studies that address the dimerizing potential of these three or other groups of plant TFs.

The function of any gene can be addressed by gain or loss of function mutation approaches. Loss of function mutation can be achieved using the site-specific recombinase (Gaj et al. 2016), engineered endonuclease such as Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) system(Cong et al. 2013), Zinc finger endonuclease (ZFN) (Zhang et al. 2010), Transcription activator-like effector nucleases (TALEN) (Chen and Gao 2013), or by si-RNA mediated interference (Gaj et al. 2016). However, these techniques do not address the problem of the redundant and pleiotropic behavior of TFs. To unravel the biological function of a B-ZIP TFs, it is useful to use proteins that inhibit the DNA binding and function of the individual B-ZIP protein. These types of proteins are called “dominant negative”. The term ‘dominant’ refers to their genetic dominance. The term ‘negative’ describes the inhibition of the function of cellular proteins. In its simplest form, a dominant negative could be a truncated B-ZIP protein, which would heterodimerize with an endogenous wild type protein, producing an inactive heterodimer. However, the binding of DNA stabilizes the B-ZIP structure and this complicates the designing of biologically active
dominant negative proteins. One can address this problem by designing dominant negatives based on two different strategies.

Figure 3 Diagrammatic representation of the DNA binding ability of A-ZIP and B-ZIP motif containing DNA binding protein (Krylov et al. 1994).

The first strategy is to tweak the leucine zipper motif. This can be done by changing amino acids in e and g positions of a heptad. Another way is based on the concept that an amino acid sequence could mimic the properties of DNA. Knowing that the B-ZIP basic region could form an alpha helix when bound to the DNA, a protein sequence was designed such that it could mimic the DNA. In these dominant negatives termed A-ZIPs, the designed protein sequence replaces the DNA-binding region. This sequence forms a very stable heterodimer with the wild type B-ZIP protein and prevents the B-ZIP complex to bind to DNA. The heterodimer complex between target wild type B-ZIPs and designed dominant negative may be 100 fold more stable compared to homodimer or heterodimer of B-ZIP proteins (Figure 3) (Krylov et al. 1994). In the present study, the novel designed dominant negative protein A-ZIP53 is used to regulate the dimeric interaction of B-ZIP53 and its dimerizing partners involved during the seed maturation (Alonso et al. 2009). Designed dominant negative protein can interact with the B-ZIP53 as well as with its heterodimerizing partners i.e., B-ZIP10 and B-ZIP25. By targeting the triad, we expect to study the direct effect of the target B-ZIP TFs and indirect effect of their target genes. Considering the above observations, following objectives were undertaken:

1. To rationally design and characterize a dominant negative protein A-ZIP53 that can simultaneously inhibit the DNA binding of B-ZIP53, B-ZIP10, and B-ZIP25 by heterodimerizing with them.
2. To design derivatives of A-ZIP53 each with different homo- and heterodimerizing properties.

3. To study seed development and maturation in the A-ZIP53 expressing transgenic *Arabidopsis*.

4. Transcriptome profiling of differentially expressed genes in the A-ZIP53 expressing transgenics and validation of the gene expression responsible for the seed development and maturation.

5. Identification of different heterodimerizing partners of B-ZIP53 in transgenic *Arabidopsis* by biochemical and mass spectrometry approaches.