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A Pivotal Role of the Basic Leucine Zipper Transcription Factor bZIP53 in the Regulation of *Arabidopsis* Seed Maturation Gene Expression Based on Heterodimerization and Protein Complex Formation [W]

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Transcription of *Arabidopsis thaliana* seed maturation (MAT) genes is controlled by members of several transcription factor families, such as basic leucine zippers (bZIPs), B3s, MYBs, and DOFs. In this work, we identify *Arabidopsis* bZIP53 as a novel transcriptional regulator of MAT genes. *bZIP53* expression in developing seeds precedes and overlaps that of its target genes. Gain- and loss-of-function approaches indicate a correlation between the amount of bZIP53 protein and MAT gene expression. Specific in vivo and in vitro binding of bZIP53 protein to a G-box element in the albumin *2S2* promoter is demonstrated. Importantly, heterodimerization with bZIP10 or bZIP25, previously described bZIP regulators of MAT gene expression, significantly enhances DNA binding activity and produces a synergistic increase in target gene activation. Full-level target gene activation is strongly correlated with the ratio of the correspondent bZIP heterodimerization partners. Whereas bZIP53 does not interact with ABI3, a crucial transcriptional regulator in *Arabidopsis* seeds, ternary complex formation between the bZIP heterodimers and ABI3 increases the expression of MAT genes in planta. We therefore propose that heterodimers containing bZIP53 participate in enhanceosome formation to produce a dramatic increase in MAT gene transcription.

INTRODUCTION

As an outstanding adaptation of terrestrial plants, seed formation favors dispersal of species and allows the interruption of the life cycle and its resumption once optimal growth conditions are newly established (for review, see Vicente-Carbajosa and Carbonero, 2005; Weber et al., 2005; Santos-Mendoza et al., 2008). Seeds are formed after a double fertilization event, triggering the development of a complex organ, which comprises the embryo, the endosperm, and the seed coat derived from the integuments and other surrounding layers of maternal origin. Seed development can be divided into three phases: first, embryogenesis is characterized by cell division and differentiation until embryo morphology is established. Second, the maturation phase is dominated by storage compound accumulation, growth arrest, and acquisition of desiccation tolerance. Third, the embryo can enter into a dormancy state that is broken upon germination. With respect to seed morphology, physiology, and gene regulation, considerable variations occur among species. *Arabidopsis thaliana* has been developed as a well-established model system for dicot seed development, and several similarities and differences with monocot model systems have been described (Vicente-Carbajosa and Carbonero, 2005; Santos-Mendoza et al., 2008).

Important programs of gene expression related to the metabolic changes that occur during seed maturation are highly coordinated and tightly regulated (Gutierrez et al., 2007). An understanding of gene expression control in the seed was tackled from early studies in plant molecular biology, with maize (*Zea mays*) Opaque2 (O2) representing a hallmark as one of the first plant transcription factor (TF) genes cloned and characterized (Hartings et al., 1989; Schmidt et al., 1990). Similarly, orthologous genes from wheat (*Triticum aestivum*) (SPA) and barley (*Hordeum vulgare*) (BLZ2) play the same roles as O2 in their corresponding species (Albani et al., 1997; Oñate et al., 1999). In dicot species, key TFs have been characterized that control gene expression programs during seed maturation.

The class of maturation genes (MAT) expressed during seed maturation typically includes seed storage protein (SSP) genes, such as albumin and cruciferin genes, which are induced in early or mid-maturation phase. The late embryogenesis abundant (LEA) genes are induced at later stages of maturation and include genes proposed to be involved in acquisition of desiccation tolerance (for review, see Tunnacliffe and Wise, 2007). MAT promoter analyses have revealed several conserved

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cis-regulatory elements with functional relevance in the control of gene expression during seed maturation. Among them, G-box-related ACGT elements, RY (CATGCA), AACA, and CTTT motifs are the best described examples (for review, see Vicente-Carbajosa and Carbonero, 2005). The corresponding associated TFs belong to the basic leucine zipper (bZIP), B3, MYB, and DOF TF families, respectively. Cooperation of these regulatory units in the control of gene expression appears to be an evolutionarily conserved pattern that can be traced back to the origins of the Spermaphyta (Vicente-Carbajosa and Carbonero, 2005; Schallau et al., 2008).

TFs of the bZIP class, related to cereal O2-type TFs, have been identified in *Arabidopsis* (Lara et al., 2003), namely, bZIP10 and bZIP25, which have been classified into group C of the *Arabidopsis* bZIP TF family (Jakoby et al., 2002). Expression during seed development, specific binding to G-box-like ACGT elements of the albumin and cruciferin promoters, and in vivo regulation of these target genes have been demonstrated (Lara et al., 2003). A peculiarity exists in that none of the four genes in the bZIP group C in *Arabidopsis* shows seed-specific expression, in contrast with characterized members of the cereal O2-type TFs. This suggests that in *Arabidopsis*, combinatorial interactions and expression levels of different TFs may be of major relevance in the induction of seed-specific gene expression patterns.

ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON2 (LEC2) have been implicated in seed development and belong to the B3 family of TFs (Parcy et al., 1994; Kroj et al., 2003; Braybrook et al., 2006; Santos-Mendoza et al., 2008). Mutations in any of these genes result in pleiotropic phenotypes affecting SSP accumulation and acquisition of desiccation tolerance. Recent genetic analysis demonstrated that these B3-type TFs are controlled by a complex self-regulating network including LEC1, LEC2, FUS3, and ABI3 (To et al., 2006). The corresponding genes clearly differ in expression and function despite being partially redundant. Although binding of ABI3, FUS3, and LEC2 to the RY element, mediated by the B3 DNA binding domain, has been shown in vitro and in yeast systems (Kroj et al., 2003; Mönke et al., 2004; Braybrook et al., 2006), RY motifs are not sufficient to confer seed-specific expression patterns to a target promoter (Ezcurra et al., 2000; Nakashima et al., 2006). Cooperation with neighboring *cis*-elements and the cognate TFs has been demonstrated to be important to establish seed-specific transcriptional patterns. bZIP10 and bZIP25 physically interact with ABI3, which in turn enhances in vitro DNA binding of the bZIP proteins to SSP promoters as well as their in vivo activation capacity (Lara et al., 2003). Therefore, cooperation of transcriptional regulators by protein–protein interactions provides an efficient mechanism to control gene expression in seeds and explains some of the molecular mechanisms underlying SSP gene expression. However, our knowledge on the protein partners and their interplay remains limited.

In general, bZIP TFs bind DNA as homo- or heterodimers (Ellenberger et al., 1992). Recently, we demonstrated that group C bZIPs, such as bZIP10 and bZIP25, preferentially interact with group S1 bZIPs, resulting in a set of specific heterodimers designated the C/S1 network of bZIP TFs (Ehlert et al., 2006). In particular, members of the C/S1 network have been implicated in energy homeostasis (Baena-Gonzalez et al., 2007; Usadel et al., 2008), amino acid metabolism (Weltmeier et al., 2006; Hanson et al., 2008), stress response (Kaminaka et al., 2006), and sink-specific gene expression (Rook et al., 1998; Weltmeier et al., 2009). Importantly, these heterodimers were shown to convey synergistic activation properties to target genes, suggesting that heterodimerization serves as an efficient mechanism of signal integration (Weltmeier et al., 2006). With respect to the regulation of the seed gene *Em*, the importance of bZIP heterodimers and the ABI3 othologous regulator VP1 has already been proposed in rice (*Oryza sativa*) (Nantel and Quatrano, 1996).

In this work, we aimed to identify S1 bZIP members that could be involved in seed gene regulation as important potential partners of previously described group C bZIPs. We define the S1 TF bZIP53 as a key regulator of MAT gene transcription. bZIP53 enhances MAT gene expression by specific heterodimerization with bZIP10 or bZIP25. Furthermore, these bZIP heterodimers interact with ABI3, which further increases MAT gene activation. Therefore, we propose that bZIP53 plays a pivotal and crucial role in quantitative control of MAT gene transcription levels by cooperation with several TFs forming enhanceosome-like protein complexes.

RESULTS

bZIP53 Is Expressed in Seed Tissues during Maturation

We have previously shown that *Arabidopsis* S1 bZIP proteins support strong heterodimerization with members of the group C bZIPs using two-hybrid systems in yeast and protoplasts and bimolecular fluorescence complementation (BiFC) techniques (Ehlert et al., 2006; Weltmeier et al., 2006). Since two group C members, bZIP10 and bZIP25, are known activators of SSP genes (Lara et al., 2003), we surveyed the expression patterns of S1 members during seed development to identify possible partners for C-S1 interactions in this process. According to the mRNA profiles of S1 bZIPs during silique and seed development derived from public AtGenExpress microarray data (http://www.genomforschung.uni-bielefeld.de/GF-research/AtGenExpress-SeedsSiliques.html), *bZIP53* is the S1 member with the highest expression in mid and late maturation phases. Induction of *bZIP53* precedes and overlaps the activation of typical SSP and LEA genes, supporting the hypothesis that bZIP53 might be involved in MAT gene regulation.

Confirmation of the *bZIP53* temporal pattern of expression and its localization within the seed were obtained by in situ hybridization studies (Figure 1A). *bZIP53* mRNAs produced a strong signal in the embryo cotyledons during late maturation. In addition, we performed histochemical localization of β-glucuronidase (GUS) activity in seeds of *Arabidopsis* transgenic plants expressing the *GUS* reporter gene under control of the *bZIP53* gene promoter (ProbZIP53:GUS). The results presented here were confirmed in three independent transgenic lines, all of which showed the same profiles of GUS expression. As seen in Figure 1B, GUS staining in the developing embryo was observed from the torpedo to the green cotyledon stages during seed development (Bowman, 1994). Altogether, these results demonstrate that *bZIP53* expression increases during seed development and localizes to the embryo and endosperm during the maturation phase.

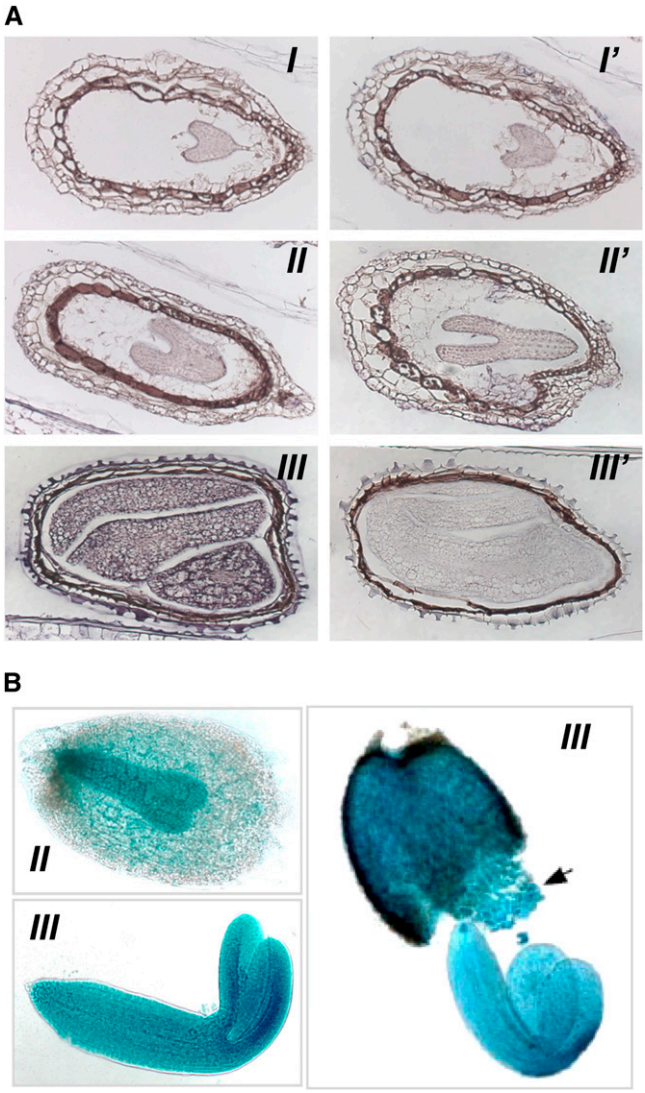

Figure 1. Expression of *bZIP53* in Seeds.

(A) In situ mRNA hybridization for *bZIP53* at different stages of seed development. Longitudinal sections of siliques with seeds at heart (I and I'), early torpedo (II and II'), and green cotyledon (III and III') stages of development are shown. Sections were hybridized with a *bZIP53* antisense (I, II, and III) or a sense probe (I', II', and III').

(B) Histochemical analysis of Columbia (Col-0) seeds harboring a *bZIP53* promoter fused to GUS (ProbZIP53:GUS). GUS staining of late torpedo stage (II) and green cotyledon stage (III) embryo development are shown. The arrow indicates the developing endosperm; to facilitate viewing, the seed has been pressed to push out the embryo.

Ectopic Expression of *bZIP53* Results in Abnormal Plant Growth and Expression of Seed-Specific Genes in Leaf Tissue

Because of its particularly high levels of expression during seed development, bZIP53 could be expected to participate in seed gene regulation. To address this question, we followed a gain-of-function approach and obtained transgenic *Arabidopsis* plants with ectopic expression of *bZIP53* driven by the cauliflower mosaic virus 35S promoter (Pro35S:bZIP53) (Weltmeier et al., 2006). As seen in Figure 2A, strong overexpression of *bZIP53* resulted in phenotypic alterations, including dwarfism and delayed bolting compared with the wild-type plants. To test whether these modifications were associated to altered patterns of seed gene expression, mRNA isolated from wild-type and Pro35S:bZIP53 plants was subjected to quantitative RT-PCR (qRT-PCR) to check for *bZIP53*, MAT, and LEA gene expression. Figure 2B shows data of wild-type and two independent Pro35S:bZIP53 transgenic lines with increased levels of *bZIP53* mRNA as well as induced expression of seed MAT and LEA genes, indicating that bZIP53 is able to activate seed-specific expression in *Arabidopsis* leaves. Accumulation of SSP transcripts in leaves, as exemplified by the cruciferin (*CRU3*) and albumin (*2S2*) genes, was also obtained by overexpression of HA-tagged bZIP53 (see Supplemental Figures 1A and 1B online). The presence of the transgene-encoded protein was confirmed by

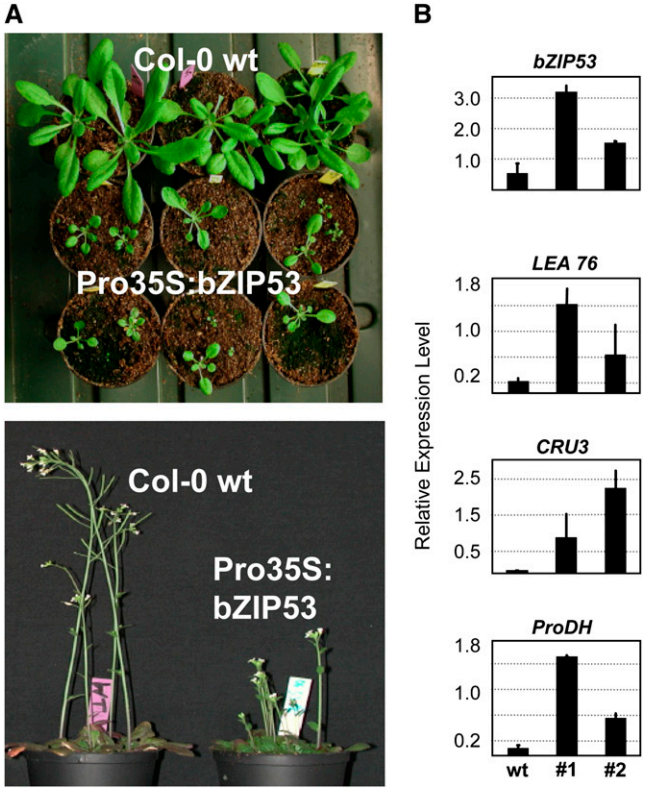

Figure 2. Plants with Constitutive Expression of *bZIP53* Have Growth Defects and Increased Levels of Seed Maturation Transcripts.

(A) Plants overexpressing *bZIP53* (Pro35S:bZIP53) have a stunted and late flowering phenotype compared with Col wild-type (Col-0 wt) plants.

(B) mRNA samples from 2-week-old wild-type and two Pro35S:bZIP53 lines (#1 and #2) were analyzed by qRT-PCR to quantify the transcript levels of *bZIP53*, *LEA76*, *CRU3*, and *ProDH*. Expression levels are given relative to a *UBIQUITIN* gene for normalization. Given are mean values and standard deviation of two to three replicates.

immunoblot analysis. Further examples of seed genes misexpressed in leaves are legumin-like *CRA1* (Wang et al., 2007) and the 11-β-hydroxysteroid dehydrogenase gene (*HSD1*) (Li et al., 2007; see Supplemental Figure 1C online). In addition, these plants also show enhanced expression of the proline dehydrogenase (*ProDH*) gene, as previously reported (Weltmeier et al., 2006), or the *Asparagine Synthetase1* (*ASN1*) gene, both typical genes involved in amino acid metabolism (Lam et al., 2003) (Figure 2B; see Supplemental Figure 1C online). Altogether, these data support the assumption that overexpression of bZIP53 in leaves triggers the misexpression of a substantial fraction of seed-specific genes.

The Albumin *2S2* Gene Is a Direct Target of bZIP53

To elucidate whether the SSP promoters were directly targeted by bZIP53, we performed chromatin immunoprecipitation (ChIP) from leaves of plants expressing ectopical bZIP53 tagged at the N terminus with a 3xHA epitope. These plants have been previously described and display the same phenotype as the untagged Pro35S:bZIP53 plants described in Figure 2A (Weltmeier et al., 2006).

Figure 3A shows the results of qPCR analyses for the *2S2* promoter on chromatin samples isolated from wild-type and Pro35S:HA-bZIP53 plants. The relative enrichment of the *2S2* gene promoter in Pro35S:HA-bZIP53 immunoprecipitated samples supports direct binding of a protein complex that includes bZIP53.

Heterodimerization of bZIP53 with Group C bZIP10 or bZIP25 Promotes Strong Activation of Seed-Specific Genes

Activation of seed-specific genes in leaves of Pro35S:bZIP53 plants as well as ChIP analyses suggest that bZIP53 is directly involved in this regulation. We studied the activation properties of bZIP53 in *Arabidopsis* leaves transformed by particle bombardment (Lara et al., 2003). Figures 3B and 3C show an example of *2S2* promoter constructs driving the expression of a GUS reporter that is cotransformed with effector plasmids for bZIP53, bZIP10, and bZIP25. Individually, none of these bZIPs is able to produce a significant increase in the basal activity of the reporter. Previous studies showed no significant heterodimerization for bZIP10 and bZIP25, whereas strong heterodimerization of group C bZIPs and bZIP53 was reported (Ehlert et al., 2006). Accordingly, when bZIP53 was cotransformed with bZIP10 or bZIP25, a dramatic increase in the reporter activity was observed. These results were confirmed in transiently transformed leaf mesophyll protoplasts using two different seed-specific reporter constructs, driven by the *2S2* or the *CRU3* promoter (see Supplemental Figure 2 online). Similarly, immunoblot analysis confirmed expression of the bZIP genes (see Supplemental Figure 3A online) in transient assays in protoplasts. Unexpectedly, we observed that coexpression of bZIP10 and bZIP53 leads to enhanced protein levels, suggesting that heterodimer formation might stabilize the bZIP proteins from degradation. This data emphasize the importance of heterodimer formation between bZIP53 and group C bZIPs in the observed enhancement of gene activation.

Activation of the *2S2* Seed Storage Protein Gene by bZIP53 and Its Heterodimers Relies on the G-Box Promoter Element

The in vivo regulation of bZIP53 and its heterodimers was further investigated on the *2S2* promoter. A modified version of the native promoter was used, in which the G-box element was mutated to a sequence that prevents bZIP DNA binding (Figure 3B; Lara et al., 2003). Different from the behavior of the native promoter, the activation mediated by the bZIP TFs is completely abolished in this mutant (Figure 3C), indicating that the regulation of the *2S2* promoter by these bZIP proteins requires an intact G-box motif.

bZIP53 Binds in Vitro to the G-Box Present in the *2S2* Promoter, and Heterodimerization with bZIP10 or bZIP25 Increases Its Binding Activity

To establish if bZIP53 was able to bind to seed-specific gene promoters, we performed in vitro DNA binding experiments with an epitope-tagged bZIP53 (T7-bZIP53) protein. A native-sequence oligonucleotide (wild type) containing a functional bZIP (G-box) binding site (Ezcurra et al., 2000) derived from the *2S2* promoter and a mutated version were used (Figure 3B). Double-stranded oligonucleotides were covalently attached to 96-well ELISA plates before doing the binding assays as described in Methods. Figure 3D shows a positive correlation between the amount of T7-bZIP53 detected with the epitope-specific antibody and the amount of T7-bZIP53 protein extract added to the wells, indicating T7-bZIP53 binding to the wild-type oligonucleotide in a concentration-dependent manner. To analyze the specificity of this binding, competition experiments were performed with increasing amounts of the wild-type or mutated oligonucleotides added to the binding reactions. As shown in Figure 3D, the wild-type, but not the mutated oligonucleotide, was able to reduce the amount of T7-bZIP53 bound to the plate, thus confirming the specificity of the binding.

We have shown in previous work and in this study that bZIP53 heterodimerization with bZIP10 or bZIP25 increases its transactivation properties (Ehlert et al., 2006; Weltmeier et al., 2006). To determine if the heterodimerization was also affecting the binding capabilities of these proteins, we incubated increasing amounts of T7-bZIP53 in the wild-type oligo-coated wells in the presence or absence of fixed amounts of bZIP10. Figure 3E illustrates the effect of heterodimerization between bZIP53 and bZIP10 and shows that the binding activity for the concentration series of bZIP53 is higher in the presence (than in the absence) of bZIP10. Likewise, the same behavior is observed in the reciprocal experiment for a bZIP10 series, in support of an increased binding activity of the heterodimers.

ABI3 Does Not Interact with bZIP53 Alone, but Heterodimers with bZIP10 or bZIP25 Form a Ternary Complex in Yeast and in Planta

ABI3 is an important regulator of gene expression in seeds of *Arabidopsis* (Giraudat et al., 1992; Parcy et al., 1994) and is able to interact with bZIP10 or bZIP25 to increase their activation capacity on the *2S2* promoter (Lara et al., 2003). We used a yeast

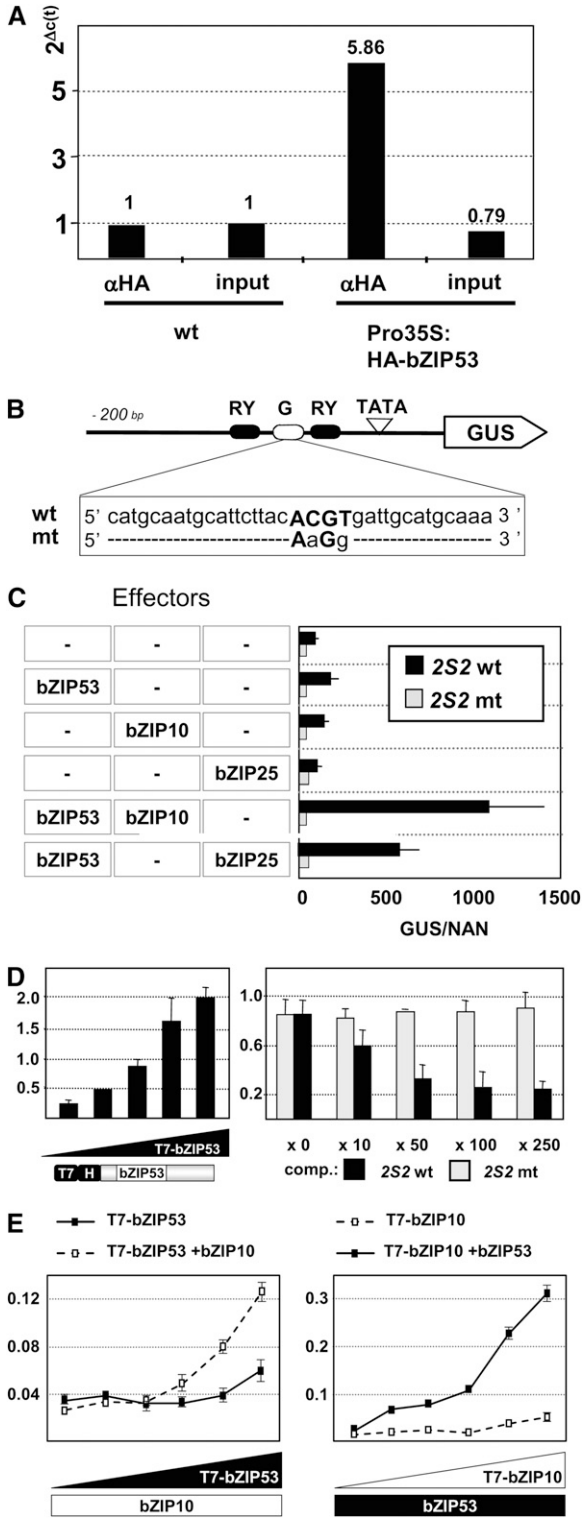

Figure 3. In Vivo and in Vitro Binding of the bZIP53 Protein to the Albumin *2S2* Promoter and Interaction with bZIP10 and bZIP25.

(A) Chromatin extracts from wild-type plants and plants overexpressing a HA-tagged bZIP53 protein (Pro35S:HA-bZIP53) were subjected to qRT-PCR analysis with *2S2* promoter-specific primers before (input) and after immunoprecipitation with an anti-HA antibody (α-HA). Ct values for Pro35S:HA-bZIP53 samples were subtracted from the Ct values of the equivalent wild type, and the differentials are shown on top of the right bars in the graph. A value of 1 was assigned to the Col-0 samples. For normalization, an actin (*ACT7*) gene was used (see Methods).

(B) Schematic view of the *2S2* promoter fused to GUS used as reporter in transient expression analysis. Depicted are RY (black) and G-box (white) elements. The sequence of the wild type and mutated G-box (mt) is shown.

(C) *Arabidopsis* leaves were transformed with the reporter constructs containing sequences described in **(B)** and effector constructs containing specific *bZIP* genes under control of the Pro35S in cobombardment experiments as described by Lara et al. (2003). Three microliters of control plasmid Pro*35S*:NAN was included in all the experiments to normalize GUS expression values for differences in bombardment efficiencies (Kirby and Kavanagh, 2002). The *x* axis values are expressed as GUS activity relative to NAN. Given are mean values and standard deviation of three independent experiments.

(D) In vitro binding of bZIP53 to the G-box sequence from the *2S2* promoter. A biotinylated oligonucleotide containing the G-box sequence was bound to streptavidin-coated wells and incubated with increasing amounts of a T7-tagged bZIP53 protein (1 to 1:90). Nonbound proteins were removed from the reaction wells, and the amount of T7-bZIP53 protein was quantified by immunodetection with an anti-T7 antibody (α-T7; left panel). The binding specificity of bZIP53 to the *2S2* G-box was analyzed in competition experiments where increasing amounts of unlabeled oligonucleotides (as indicated) containing a wild-type (*2S2 wt*; black bars) or a mutated 2S2 *mt* version (gray bars) were incubated with a fixed amount of the T7-bZIP53 protein and the biotinylated oligonucleotide containing the wild-type G-box sequence (right panel).

(E) Effect of heterodimerization on bZIP53 and bZIP10 binding affinity. A biotinylated oligonucleotide containing the G-box sequence was bound to streptavidin-coated wells and incubated with increasing amounts (1 to 1:30) of a T7-bZIP53 protein in the absence (filled squares) or presence of a fixed amount of bZIP10 protein (open squares). Nonbound proteins were removed from the reaction wells, and the amount of T7-bZIP53 protein was quantified by immunodetection with an α-T7 antibody (left panel). The reciprocal experiment was performed with a T7-bZIP10 protein and a nontagged bZIP53 protein (right panel). Given are mean values and standard deviation of three repetitions.

two-hybrid system to determine if bZIP53 was also able to interact with ABI3. We expressed the ABI3 and bZIP53 proteins as fusions to the GAL4 binding (BD) or activation (AD) domains, respectively, and introduced them into yeast strains containing *LacZ* or *HIS* reporter genes under the control of GAL4 binding sites. Different combinations of ABI3 and bZIP53 were tested for possible interactions in this system, alongside ABI5, another bZIP TF that has been implicated in seed gene expression serves here as a negative control (Finkelstein and Lynch, 2000). As seen in Figure 4A, no interaction could be detected between bZIP53 and ABI3 or between bZIP53 and ABI5 in any of the experimental systems used. As previously reported, positive interactions between bZIP53 and bZIP10 or bZIP25 confirmed their capacity to heterodimerize. To further analyze the possibility that these heterodimers could still interact with ABI3, three-hybrid system experiments were devised. Yeast cells expressing BD-bZIP53 were cotransformed with either AD-bZIP10 or AD-bZIP25 in the presence or absence of ABI3, and β-galactosidase reporter

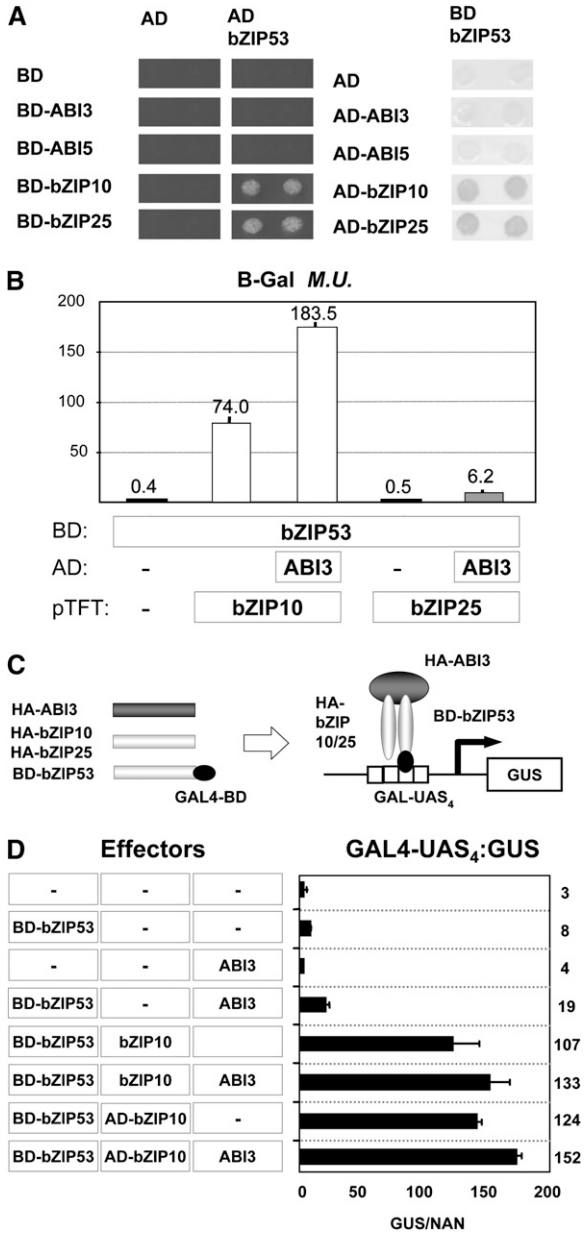

Figure 4. Interaction of bZIP53, bZIP10, and bZIP25 Homo- or Heterodimers with ABI3.

(A) Protein interactions in yeast two- and three-hybrid systems. ABI3, ABI5, bZIP10, and bZIP25 proteins were fused to the GAL4 DNA BD or AD and introduced separately into a yeast strain containing the AD-bZIP53 (HF7c) or BD-bZIP53 (SFY526) protein, respectively. Activation of the reporter genes HIS3 (growth in a His-depleted medium; left panel) and LacZ (blue colored colonies; right panel) indicates positive protein–protein interactions.

(B) Yeast strains (SFY526) expressing different combinations of BD-bZIP53, AD-ABI3, and bZIP10 or bZIP25 were assayed for β-galactosidase activity. The latter were provided in the three-hybrid vector pTFT (Egea-Cortines et al., 1999). Average values (Miller units) and standard errors from six replicates and two independent experiments are shown.

(C) Schematic overview of a three-hybrid assay in *Arabidopsis* protoplasts. Constructs are shown on the left, and a model of reporter gene activation is shown on the right.

(D) Activation of the GAL4-UAS_4:GUS reporter after cotransfection with the constructs indicated in **(C)**. The *x* axis values are expressed as GUS activity relative to NAN (Ehlert et al., 2006). Average values and standard errors from four transfections are shown. Numbers along the *y* axis represent fold induction values relative to nontransfected control cells. The experiments were repeated three times with similar results.

activities were quantified (see Supplemental Figure 4 online). As previously observed, the interaction between BD-bZIP53 and either AD-bZIP10 or AD-bZIP25 was confirmed by an increase of the reporter activity. However, when a construct designed to express ABI3 was cotransformed into the strains, a further increase of the β-galactosidase activity was observed, in support of a trimeric interaction. As expected for a negative control, the bZIP factor ABI5, which interacts with ABI3 (Nakamura et al., 2001) but does not heterodimerize with AtbZIP53, did not show activation indicative of ternary complex formation.

Reciprocal three-hybrid analysis where the noninteracting proteins bZIP53 and ABI3 were fused to the Gal4BD and Gal4AD, respectively, showed also an increase of reporter activity when assayed in the presence of bZIP10 or bZIP25 (Figure 4), in support of the proposed trimeric interaction. Altogether, these results indicate that ABI3 can be brought into the heterodimer complex, probably by its interaction with bZIP10 and/or bZIP25. Importantly, bZIP53 does not interfere with the interaction between ABI3 and bZIP10 or bZIP25.

Ternary complex formation in plant cells was supported by a similar three-hybrid approach developed in *Arabidopsis* protoplasts. Figure 4C shows a schematic overview of the experimental setup and the structure of the expressed proteins used to transactivate a reporter construct containing the *GUS* gene under the control of a minimal promoter bearing a tetramer of the GAL4 upstream activating sequence (GAL4-UAS_4:GUS) (Ehlert et al., 2006). As shown in Figure 4D, comparable results to the yeast system were achieved. Transactivation of the reporter with BD-bZIP53 alone results in low levels of GUS activity, whereas cotransformation with either HA-bZIP10 or AD-bZIP10 displayed enhanced activity indicative of heterodimer formation as previously observed in the yeast two-hybrid system. Expression of the corresponding proteins was assayed by immunoblot analysis with HA-tag and BD-specific antibodies (see Supplemental Figure 3B online). Equally, in both systems, the supplementary inclusion of HA-ABI3 resulted in an additional increase of reporter activity, in support of its capacity to interact with the heterodimers in plant cells.

BiFC (Walter et al., 2004) was used as an additional experimental system to verify the detected ternary interactions in plant cells. bZIP53 and ABI3 proteins fused to different domains of yellow fluorescent protein (YFP) were coexpressed in onion cells in distinct combinations, rendering no reconstruction of YFP activity. However, when a third construct expressing bZIP10 protein was cotransfected, YFP fluorescence was observed in support of a tethering function of this protein to promote a ternary complex formation (Figure 5).

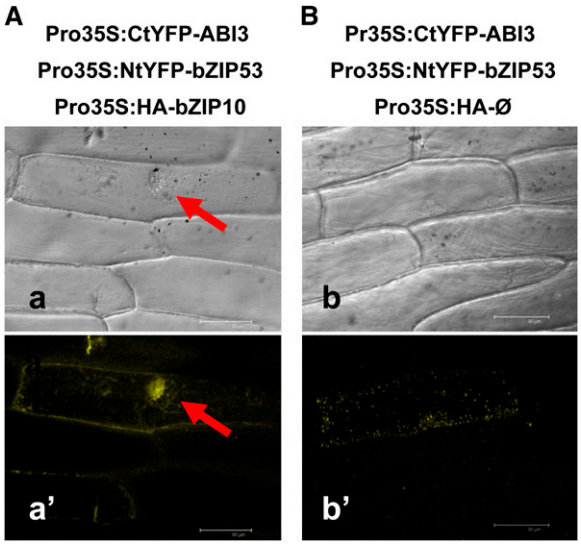

Figure 5. bZIP53, bZIP10, and ABI3 Protein–Protein Interaction Studied by BiFC (Walter et al., 2004).

Onion epidermis cells have been transiently transformed by particle bombardment. Fusion proteins of the C-terminal part of YFP (CtYFP) and ABI3 and the N-terminal part of YFP (NtYFP) and bZIP53 have been coexpressed with HA-tagged bZIP10 (HA-bZIP10) (a and a') or an empty vector control (b and b'). Bright-field images (top panels) and epifluorescence images taken by confocal microscopy (bottom panels) are shown.

Heterodimer Formation and Interaction with ABI3 Regulate the Activation Properties of bZIP53 on SSP Gene Expression in Plant Cells

To study the functional implications of the interaction between ABI3 and bZIP heterodimers in plant cells, we performed transient expression analysis in *Arabidopsis* protoplasts using two different SSP promoters, corresponding to the *2S2* and *CRU3* genes. Figure 6A shows that the use of bZIP effectors individually or in combination with ABI3 resulted in limited activation capacity on both promoters. Combination of bZIP53 and bZIP10 led to an important increase in the activity, in accordance with previous results. Moreover, the inclusion of ABI3 in this combination produced an additional increase in the activation of both promoters.

This reporter gene activation cannot be observed when using an effector construct expressing bZIP10 with two Pro exchange mutations (bZIP10pp); these mutations disrupt the zipper dimerization surface and therefore abolish the interaction with bZIP53 (Weltmeier et al., 2006). Expression of bZIP10pp was found to be comparable to the wild-type protein as confirmed by immunoblot analysis (Figure 6B). Accordingly, disruption of the bZIP heterodimer using bZIP10pp abolishes this induced gene activation both in the presence or absence of ABI3. These results indicate a functional interaction between bZIP53/bZIP10 heterodimer and ABI3 and confirm a positive effect of ABI3 on heterodimer-mediated transcription.

To further investigate this effect, a comprehensive analysis of ABI3 action on the heterodimer was performed. In Figure 6C, different ratios of bZIP53 and bZIP10 were used to transactivate the *2S2* promoter in *Arabidopsis* protoplasts, either in the presence or in the absence of ABI3. In all cases, the activation effect of the heterodimer was enhanced in the presence of ABI3 by a similar increment. However, maximum promoter activity was greatly determined by the ratio of the heterodimerization partners. A 1:1 ratio, which enables optimal heterodimerization, results in maximum promoter activation.

bZIP53 Loss-of-Function Plants Display Reduced Expression of SSP Genes

Results from the analysis of ectopic expression of *bZIP53* suggested a regulatory role in SSP gene expression. We used a complementary loss-of-function strategy making use of a *bzip53* T-DNA insertion mutant (Weltmeier et al., 2006) to corroborate the functional relevance of bZIP53 for SSP expression. We quantified SSP gene expression in *bzip53* plants during seed development. Siliques of wild-type and *bzip53* plants were collected at different stages of seed development, and total RNA was isolated and used in qRT-PCR experiments. Figure 7A shows that levels of *bZIP53* mRNA were greatly reduced throughout all the stages of silique development in the *bzip53* plants. However, residual levels of *bZIP53* were detected, probably due to the leaky nature of the *bzip53* mutation since the T-DNA is inserted in the promoter region (Weltmeier et al., 2006). Observed differences in the timing of RNA accumulation between *CRU3*, *2S2*, and *LEA76* genes were in accordance with those previously described for MAT and LEA genes (Parcy et al., 1994). Comparisons between wild-type and *bzip53* plants show significantly reduced levels of SSP transcript in the latter. Although the timing of mRNA accumulation for these genes in the *bzip53* samples was not significantly altered, the mRNA levels were reduced throughout the maturation stages and no peaks of RNA accumulation were detected, in contrast with the wild-type samples (Figure 7A).

As seen above, impaired bZIP53 activity leads to decreased SSP gene expression in the seed, probably by limiting the activation capacity of the regulatory complex involving bZIP10/bZIP25 and ABI3. To test this hypothesis, we performed transactivation experiments using a *2S2* promoter driving a GUS reporter in different plant backgrounds. Figure 7B shows results of particle bombardment experiments on *Arabidopsis* leaves from wild-type, Pro35S:bZIP53, and *bzip53* plants. As previously reported (Lara et al., 2003), the use of bZIP10 and ABI3 as effectors in this system resulted in the activation of the *2S2* promoter. This activation was significantly enhanced in Pro35S:bZIP53 plants and reduced in the *bzip53* knockdown mutant line, consistent with the participation of bZIP53 in this regulatory complex. The same results were obtained for bZIP25 in combination with ABI3 (see Supplemental Figure 5 online).

DISCUSSION

Seed gene expression relies on specific TFs acting in a combinatorial fashion. Both in monocot and dicot species, group C

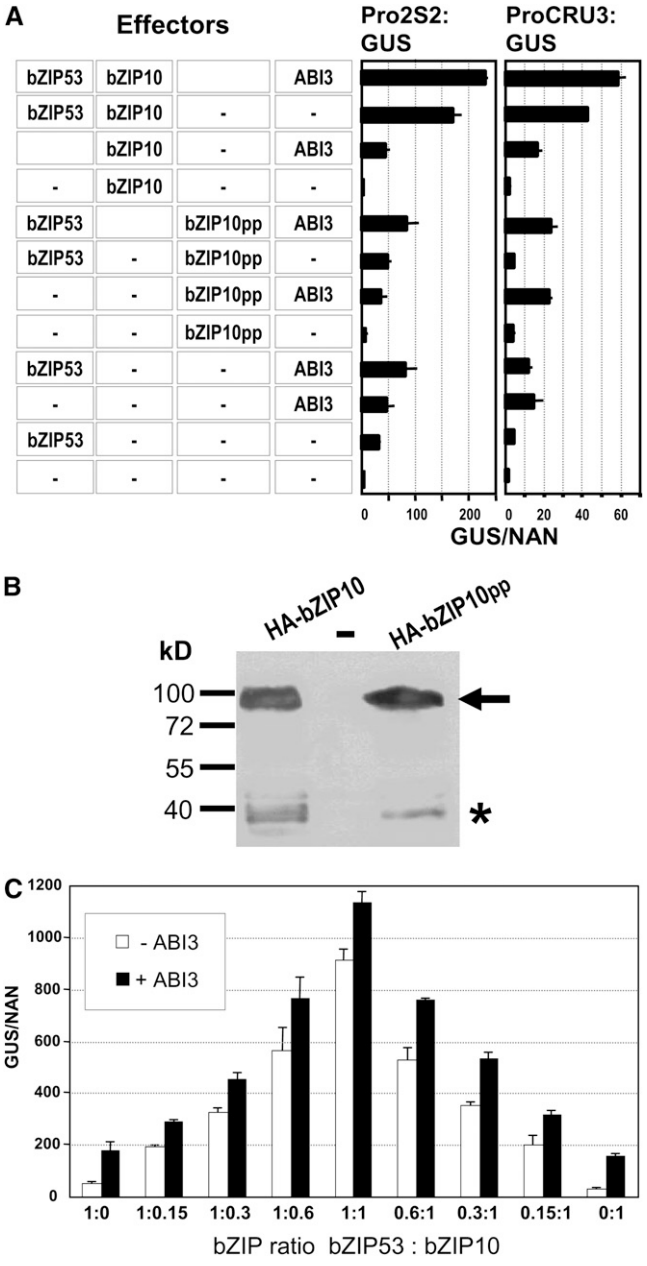

Figure 6. Effect of ABI3 on the Transcriptional Activation Mediated by the bZIP53/bZIP10 Heterodimers.

(A) GUS reporter activity under the control of the *2S2* or *CRU3* promoter was measured in transiently transformed *Arabidopsis* protoplasts after cotransfection of the effector constructs indicated. bZIP10, bZIP53, and ABI3 are expressed under the control of Pro35S. For immunodetection, 3xHA-epitope-tagged derivatives were used (see Supplemental Figure 3 online). The importance of bZIP heterodimerization was demonstrated by including bZIP10pp, which is impaired in zipper-mediated dimerization (Weltmeier et al., 2006). Given are mean values and standard deviation of four independent transfections.

(B) Immunoblot analysis of transiently transformed protoplasts confirms that expression of HA-bZIP10 is comparable to HA-bZIP10pp-untransformed control protoplasts. HA epitope-tagged proteins were detected using an α-HA antiserum. The arrow indicates HA-bZIP10 and bZIP10pp proteins, and the asterisk shows degradation products.

(C) Transient expression using a GUS reporter gene under the control of the *2S2* promoter. Combinations of bZIP53 and bZIP10 at different ratios (indicated are ratios of input DNA) in the presence (black bars) or absence (white bars) of ABI3 were used as effectors. The *x* axis values are expressed as GUS activity relative to NAN as described by Ehlert et al. (2006). Average values and standard errors from four transfections are shown.

bZIP factors related to maize O2 have been shown to be important players in this regulation. However, expression of the corresponding *Arabidopsis* bZIP genes is not seed specific. The recently described functional interaction between group C and S1 bZIPs (Ehlert et al., 2006) prompted us to investigate the possible participation of S1 members in seed gene regulation. In this study, we identified bZIP53 as the major S1 member expressed during seed maturation. Evidence of its participation in seed gene regulation was obtained by different approaches, including the analysis of plants with bZIP53 gain and loss of function. Moreover, functional studies show that bZIP53 plays a key role in the strong activation of seed-specific genes, directly involved in a seed regulatory protein complex encompassing the group C bZIP10 and bZIP25 and the B3 seed regulator ABI3.

bZIP53 Constitutes a Transcriptional Regulator of Seed Maturation Genes

Analyses of transcriptomic data of S1 bZIPs from repository databases show an increase in expression of *bZIP53* (and to a lesser extent for *bZIP1*) during intermediate and late stages of seed development. This suggests a prominent role of bZIP53 in regulating seed gene expression in conjunction with the group C members. We further confirmed the precise pattern of expression of *bZIP53* in seed tissues by mRNA in situ hybridization and promoter reporter fusions in transgenic *Arabidopsis* plants.

Gain-of-function approaches in transgenic plants and *Arabidopsis* leaf protoplasts ectopically expressing *bZIP53* demonstrate that bZIP53 is able to activate several seed MAT and LEA genes, such as *2S2*, *CRU3*, *CRA1*, *HSD1*, and *LEA76*. However, as observed before, the presence of MAT RNA does not necessarily result in the detection of the corresponding proteins. Additional cellular requirements and tissue-specific protein degradation determine high-level accumulation of MAT proteins (Gruis et al., 2004)

Complementing the gain-of-function results, reduced expression of *bZIP53* in a knockdown line that harbors a T-DNA insertion in the promoter leads to strong reduction in the expression of the corresponding target genes. However, these plants still set viable seeds, which might be due to a residual amount of bZIP53 protein. The lack of complete knockout lines in mutant collections might be explained by an essential function of this gene. Alternatively, other genes among the 75 bZIP TFs identified in *Arabidopsis* (Jakoby et al., 2002) may partly substitute for bZIP53 function. Within the group S1, which harbors the closest homologs of bZIP53, only *bZIP1* is intermediately induced during seed maturation with timing comparable to

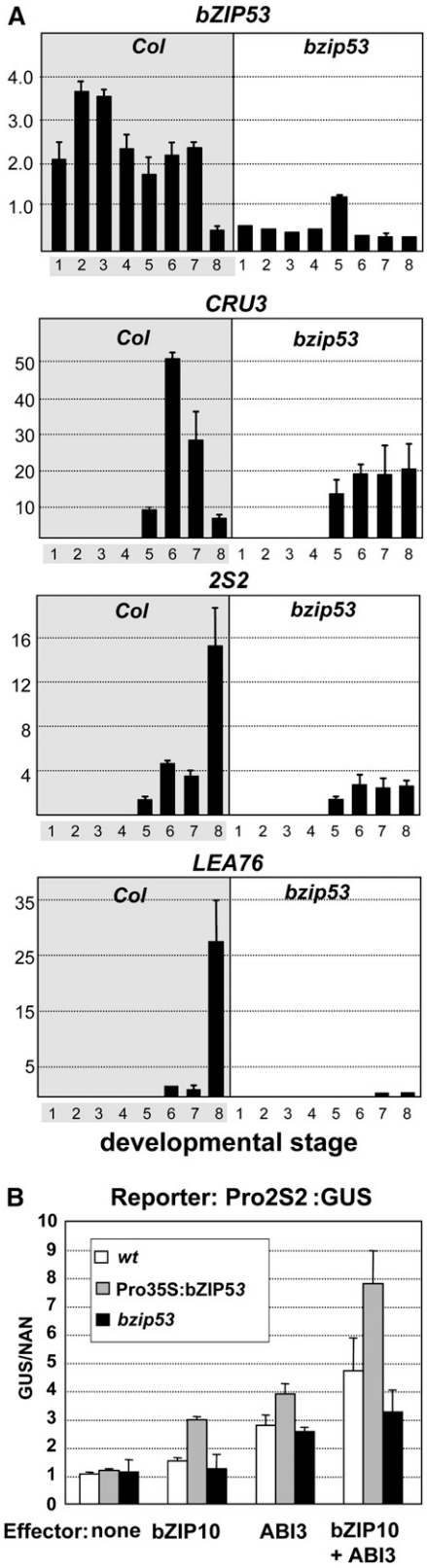

Figure 7. Effect of a bZIP53 T-DNA Insertion Mutant on Seed-Specific Gene Regulation.

(A) mRNA levels detected of the seed maturation genes indicated by qRT-PCR in Col wild-type and *bzip53* siliques at different stages of development. Lane 1, 0 d after flowering (DAF); lane 2, 2 DAF; lane 3, 4 DAF; lane 4, 6 DAF; lane 5, 9 DAF; lane 6, 12 DAF; lane 7, 15 DAF; lane 8, ≥18 DAF. Data are normalized using *UBIQUITIN* expression values. Average values and standard errors from at least two technical replicates are shown.

(B) Transient expression by microparticle bombardment of *Arabidopsis* leaves from the wild type (white bars) and plants with increased (*Pro*35S*: bZIP53*; gray bars) or decreased (*bzip53*; black bars) expression of *bZIP53.* Effector constructs containing bZIP10 or ABI3 under the control of a 35S promoter (Pro35S) and a *GUS* reporter gene under the control of the *2S2* promoter were used. The *x* axis values are expressed as GUS activity relative to NAN (Kirby and Kavanagh, 2002). Average values and standard errors from four replicates and two different experiments are shown.

bZIP53. However, ectopic overexpression of At *bZIP1* does not lead to activation of MAT genes, indicating a lack of functional redundancy of these genes in MAT gene regulation (Weltmeier et al., 2009).

High-level ectopic expression of *bZIP53* results also in a dwarf growth phenotype in vegetative phases. Based on these observations, it can be assumed that regulation of SSP genes reflects only a subset of bZIP53 targets. In previous work, we and others demonstrated that bZIP53 is also involved in regulating hypoosmolarity responses (Satoh et al., 2004; Weltmeier et al., 2006). In particular, the promoter of the *ProDH* gene, which is involved in regulating amino acid metabolism, has been defined as an in vivo target of bZIP53. Furthermore, a second amino acid metabolism gene, *ASN1*, was shown to be regulated by bZIP53 (this study) and other members of the C/S1 network (Baena-Gonzalez et al., 2007; Hanson et al., 2008). Amino acid synthesis, transport, and SSP synthesis are linked processes that have to be coordinated in vegetative tissues as well as in the seed. In accordance, ectopic overexpression of *ASN1* enhances the SSP content and nitrogen status of the seed (Lam et al., 2003). Further studies are necessary to test whether bZIP53 is coordinating other pathways in addition to SSP synthesis, such as amino acid metabolism during seed and vegetative development.

bZIP53 expression is not restricted to the seed and can be observed in vegetative tissues, particularly under certain stress conditions. Interestingly, salt treatment dramatically induces *bZIP53* transcription in roots (Weltmeier et al., 2009). In the context of the seed, it is worth mentioning that besides storage compound accumulation, dehydration occurs during late stages of the maturation phase. Accordingly, stress-related programs of gene expression are also important in the establishment of desiccation tolerance. We have determined that bZIP53 can also control the expression of the seed-specific gene *LEA76*. Although our knowledge of LEA76 function is limited, LEA proteins are known to accumulate in seed and vegetative plant tissues following environmental stress (Xu et al., 1996) and also in desiccation-tolerant bacteria and invertebrates (Bies-Etheve et al., 2008; Hundertmark and Hincha, 2008). The hypothesis whether bZIP53 is involved in establishing stress tolerance in vegetative tissues requires further testing.

Specific Heterodimerization of bZIP53 and Group C bZIPs Executes an Efficient Mechanism to Enhance Transcription of Seed Maturation Genes

MAT gene regulation is one of the early investigated areas in plant transcriptional control. The importance of G-box *cis*-elements in MAT gene promoters has been determined both in monocot and dicot species (Vicente-Carbajosa and Carbonero, 2005; Santos-Mendoza et al., 2008), and its regulation by seed-specific O2-type bZIPs has been firmly established in cereals. By contrast, genome scale transcriptomic data in *Arabidopsis* do not reveal a seed-specific candidate among all bZIPs, indicating that in this species (and probably in nonendospermic seeds from dicot plants), combinatorial effects are likely of major importance in the regulation of seed gene expression. In this framework, the group C bZIPs related to O2, bZIP10, and bZIP25 are able to interact with ABI3 and have been proposed as *Arabidopsis* counterparts of the monocot bZIP seed regulator (Schmidt et al., 1990; Lara et al., 2003). The corresponding genes, which are not seed specific, are constitutively expressed at low levels in other tissues, and bZIP10 has been implicated in plant stress responses to pathogen attack in vegetative tissues (Kaminaka et al., 2006). Previous work has defined a specific heterodimerization network of group C members with group S1 bZIPs, such as bZIP53 (Ehlert et al., 2006). The expression of this gene increases during seed maturation, and it strongly heterodimerizes with bZIP10 and bZIP25 as demonstrated in yeast and plant protoplasts (Ehlert et al., 2006), as well as by BiFC. Additionally, in vitro binding studies show that they can bind DNA as heterodimers (Weltmeier et al., 2006).

Efficient methods for in vivo DNA binding studies in *Arabidopsis* seeds have not been established. However, here, we demonstrated binding of bZIP53 to the 2S2 promoter using leaf-derived chromatin from Pro35S:HA-bZIP53 plants, which in turn show vegetative transcription of the *2S2* gene. Although this approach does not unequivocally confirm participation of bZIP53 in regulation of MAT genes, it provides circumstantial evidence in line with other results presented in this study. Mutational approaches confirm the *2S2* G-box as the in vitro binding site and also as the relevant in vivo *cis*-regulatory element. At high protein concentration, bZIP53 displays interaction with G-box elements in vitro, but titration experiments demonstrate that binding of bZIP53 in the presence of bZIP10 or bZIP25, presumably by heterodimer formation, is significantly enhanced. The in vivo relevance of heterodimer formation is further supported by expression of bZIP mutants, which are impaired in zipper-mediated dimerization, and consequently target gene activation. Detailed titration experiments in protoplasts also demonstrate that the level of target gene expression is determined by the ratio of the bZIP heterodimerization partners. The 1:1 ratio of bZIP53 and bZIP10 leads to the highest target gene activity. Altogether, these data provide conclusive evidence that bZIP heterodimers show enhanced binding to and gene activation through G-box-like *cis*-elements. Importantly, heterodimerization provides an efficient mechanism by which bZIP53 drives MAT gene expression.

Possible mechanisms of heterodimer-enhanced transactivation have been described elsewhere (Weltmeier et al., 2006), including (1) increased affinity for binding target sites, (2) stabilization of dimer structures (as effective DNA binding forms) via

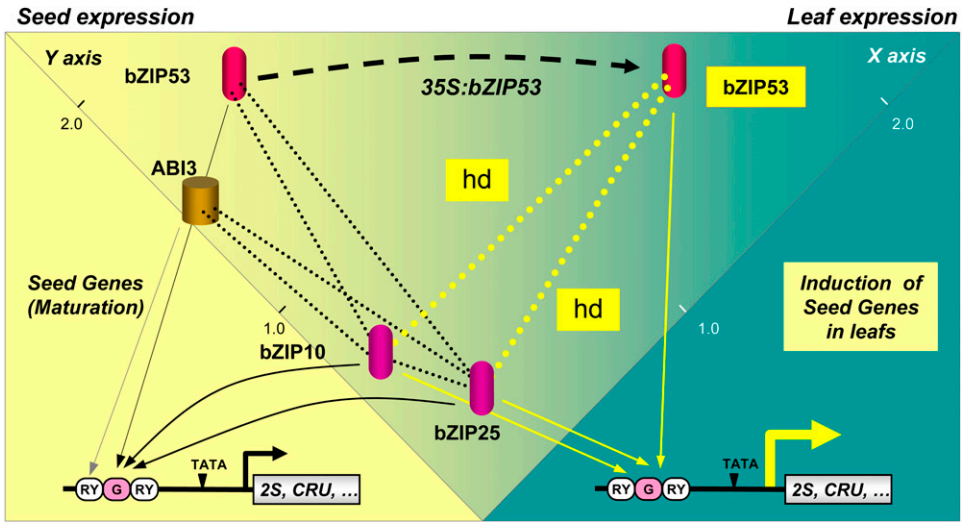

Figure 8. Model for bZIP53 Regulation of Seed Maturation Gene Expression.

Yellow and blue backgrounds represent expression in the seed and leaf, respectively. Positions of the indicated TFs were represented in a coordinate system according to relative expression values in seeds (*y* axis) and leaves (*x* axis) (data derived from AtGenExpress). Structure of a typical SSP promoter-like albumin (*2S2*) or cruciferin (*CRU3*) is depicted, indicating regulatory elements in their promoters (RY, RY-box; G, G-box; TATA, TATA-box). Continuous and dotted lines indicate DNA–protein interactions and protein–protein interactions, respectively. A displacement arrow (open line) indicates overexpression of bZIP53 in Pro35S:bZIP53 plants. bZIP53 increased expression in leaves favors heterodimer formation (hd) with group C bZIP10 and bZIP25 and triggers induction of seed gene expression in this organ.

equilibrium displacement in dimer-monomer concentrations, and (3) heterodimer interactions with other protein factors that increase activation. Here, we showed that the second and third possibilities must be operating in vivo in the regulation of MAT genes. In support of this is the increased activation observed in protoplast systems for the heterodimers, which is independent of their DNA binding when used as GAL4-BD protein fusions (Figure 4E), and their interaction and enhanced gene activation when assayed with ABI3 (Figure 6). In addition, heterodimer formation seems to protect bZIP proteins from degradation (see Supplemental Figure 3 online) and therefore provides an additional mechanism that contributes to the observed synergistic effect on transcription. Although not investigated in this study, the first possibility might also explain how diversification in target binding sites can lead to variations in the expression of different SSP genes (Conceicao Ada and Krebbers, 1994), depending on the binding properties of the heterodimers prevailing temporally and spatially in the seed. In conclusion, the concurrence of several regulatory mechanisms must be operating on the underlying bZIP network.

bZIP53, Group C bZIPs, and ABI3 Physically and Functionally Interact in the Control of Seed Maturation Genes

Current models of regulation of MAT gene expression in the seed entail the participation of bZIP and B3 interacting proteins, particularly bZIP10 and 25 and ABI3. We have demonstrated that bZIP53 is a positive factor in this regulation and that its interaction with group C bZIPs results in enhanced activation of the heterodimers. Although yeast and protoplast two-hybrid data indicate that bZIP53 on its own is not able to interact with ABI3, heterodimerizations should not preclude the group C bZIP-ABI3 interactions. Three-hybrid and BiFC analyses confirmed that bZIP heterodimerization of bZIP10 or bZIP25 with bZIP53 does not interfere with ABI3 interaction and supports the existence of a ternary complex. ABI3 has been shown to bind to the RY element (Mönke et al., 2004), and a functional interplay between G-box and RY-element in MAT gene regulation has been previously described (Ezcurra et al., 2000; Lara et al., 2003). In this report, we clearly demonstrate functional interplay between bZIP heterodimers and ABI3, presumably by ternary complex formation.

By altering the bZIP expression levels in leaves, we can still see functionality of the bZIP heterodimers despite the absence of the ABI3 protein (Parcy et al., 1994), though their mediated activation occurs at a significantly lower level. Further experiments in bZIP53 overexpressing and knockdown plants show that although bZIP10 and/or ABI3 can enhance MAT gene activation to a certain extent, full-level expression clearly depends on the presence of bZIP53 (see Figure 7B). In this respect, nonseed model systems, such as protoplasts and bombarded leaves, might not fully reflect the native situation in seeds. Nevertheless, all results obtained in these systems are in agreement with the intrinsic reduction of MAT gene expression observed in seeds of mutant *bzip53* plants. On the other hand, similar experiments performed in cereal systems that allowed the postulation of regulatory models have been confirmed in null mutants and transgenic plants (Norre et al., 2002). In summary, the output in MAT gene expression depends on all partners, ABI3 and bZIPs, and is quantitatively triggered by the amount of bZIP heterodimers.

Complex formation between the bZIP TF ABI5 and ABI3 have been recognized in LEA but not in MAT gene regulation (Bensmihen et al., 2002). Our findings suggest that the same principle based on ABI3 protein interactions operates on MAT expression, but inclusion of different bZIP partners defines distinct subsets of target genes. Therefore, this study provides further insight into the synergistic action of TFs and formation of enhanceosome-like protein-DNA complexes, which control seed gene transcription.

A Model of Seed Maturation Gene Expression Triggered by bZIP53 Heterodimerization and Protein Complex Formation

Based on previous findings and data from this study, the working model in Figure 8 is proposed. The function of the previously described group C bZIP factors bZIP10 and bZIP25 in seed maturation strongly depends on bZIP53 as a heterodimerization partner. Spatial and developmentally controlled expression of *bZIP53* is precisely timed, leading to increased amounts in the seed from mid maturation. Subsequent accumulation of bZIP53 would trigger heterodimer formation with bZIP10 or bZIP25 as prerequisite for efficient promoter binding and target SSP gene activation. ABI3 protein, which also accumulates during seed maturation, interacts with the bZIP heterodimer via bZIP10 or bZIP25 and provides an additional increment in promoter activation. Hence, SSP gene regulation is driven by the interplay of (at least) two maturation-dependent transcriptional regulators, namely, bZIP53 and ABI3. The crucial role of ABI3 in this process was largely uncovered through the characterization of *abi3* loss-of-function mutants (Parcy et al., 1994). This factor has a prominent role in seed maturation and germination gene expression programs. In this respect, its participation in a ternary complex with C/S bZIPs might partly explain many of numerous pleotropic effects observed in *abi3* mutants. Interestingly, transcriptomic data suggest that *bZIP53* and *bZIP10* expression levels are reduced in mutant *abi3-4* developing seeds (Carrera et al., 2008).

In vegetative tissues, where bZIP10 and bZIP25 are constitutively present at low levels, the ectopic high-level expression of ABI3 can lead to the activation of SSP genes (Parcy et al., 1994; Parcy and Giraudat, 1997). Likewise, ectopic high-level expression of bZIP53 can substitute for the presence of ABI3, triggering SSP gene expression even more effectively than this factor. In summary, we propose that bZIP53 acts as a key regulator, which drives a subset of MAT genes mainly by the formation of heterodimers with a high activation capacity. Although further research is needed to elucidate distinct interacting partners and target genes both in vegetative and seed tissues, bZIP53 might represent an important target for manipulating seed storage compound accumulation and establishment of desiccation tolerance.

METHODS

Plant Materials and Transformation

For plant and protoplast transformation, particle bombardment, and ChIP experiments, *Arabidopsis thaliana* ecotype Col-0 was grown on soil or

Murashige and Skoog plates under controlled environmental conditions at 16-h-light/8-h-dark cycles. To improve germination uniformity, seeds were pretreated at 4°C for 2 to 4 d. Floral dip transformations were performed using the *Agrobacterium tumefaciens* strain GV3101 (Weigel and Glazebrook, 2002). Pro35S:bZIP53, Pro35S:HA-bZIP53, ProbZIP53:GUS, and *bzip53* (NASC ID: N569883) plants were described by Weltmeier et al. (2006).

In Situ Hybridization and Histochemistry

In situ hybridization was performed according to Ferrandiz et al. (2000). Forward 5′-TTGTCCAATGCAACCCAATCA-3′ and reverse 5′-ACAAGACTAGAGGACTGAGGCT-3′ *bZIP53*-specific oligonucleotides were used to amplify a 200-bp fragment from the 3′ untranslated region. The fragment was inserted into pGEM-T easy (Promega), and the sense and antisense probes were generated as specified by Lara et al. (2003). The construction of ProbZIP53:GUS plants has been described by Weltmeier et al. (2006). GUS staining was performed according to Stangeland and Salehian (2002) with some modifications. The siliques were incubated in GUS assay buffer overnight at room temperature under vacuum and transferred to an ethanol/acetic acid (1:1) solution for 4 h (young seeds) or 12 h (mature seeds). Clearing was performed by incubating tissues in Hoyer's light medium (100 g chloral hydrate in 60 mL water) for 12 h (young seeds) or 24 h (mature seeds) at room temperature. Samples were analyzed with a Zeiss Axiophot microscope.

qRT-PCR

RNA was isolated from seedlings and siliques as described by Oñate-Sánchez and Vicente-Carbajosa (2008). cDNA synthesis was performed according to Oñate-Sánchez and Singh (2002), and between 8 and 16 ng were used as a template for qRT-PCR together with forward and reverse oligonucleotides (0.5 μM each) in 1× Power SYBR Green PCR Master mix (Applied Biosystems). Cycling conditions (ABI Prism 7300; Applied Biosystems) were as follows: 10 min at 95°C, 50 cycles of 15 s at 95°C, and 60 s at 60°C, linked to a default dissociation stage program to detect nonspecific amplification. Primers are provided in Supplemental Table 1 online. At least two technical and two biological replicates were analyzed in all the experiments

Transient Expression Assays and Constructs

Protoplast isolation, transformation, construction of effector plasmids, and immunoblot analysis were performed according to Ehlert et al. (2006) and Weltmeier et al. (2006). For reporter gene assays, 9 μg reporter and 14 μg of each effector were used if not stated otherwise. Three micrograms of Pro35S:NAN plasmid was added for normalization (Kirby and Kavanagh, 2002). For three-hybrid analysis, 9 μg of each reporter, BD-, AD-, and HA-plasmid were applied. The overall amount of DNA was set to 40 μg by adding pUC19 plasmid DNA. Particle bombardment was performed according to Lara et al. (2003). Data presented for the different assayed constructs are derived from three independent experiments, including six plates with 10 leaves per plate. Pro2S2:GUS reporter vector is a pUC19-derived plasmid containing the GUS reporter gene under the control of the *2S2* promoter and fused to the 3′-terminator of the nopaline synthase gene (3′-nos) (Lara et al., 2003). Effector plasmids for the expression of bZIP53, bZIP10, bZIP25, and ABI3 were generated by cloning the corresponding coding sequence under the control of the Pro35S and a 3′-nos terminator (Lara et al., 2003) in the pJIT60 plasmid (Guerineau, 1995). Three micrograms of Pro35S:NAN plasmid was added for normalization. GUS and NAN enzyme assays were performed according to Kirby and Kavanagh (2002). Immunoblot analysis was performed according to Weltmeier et al. (2006) using α-HA and α-BD antisera (SantaCruz).

ChIP

ChIP was performed as described by Weltmeier et al. (2006) using an HA-specific antibody (SantaCruz). *2S2* primers were used to amplify a 306-bp fragment of the *2S2* promoter (5′-GACCGGTGACCTGCGTGTA-3′ and 5′-GACTTGCATGGAGTTCACGTG-3′). The difference between the resulting C(t) values of wild-type and Pro35S:HA-bZIP53 overexpressor was calculated and normalized with the input controls of these samples that were analyzed with the same primers. For normalization, PCR was performed with unspecific actin (*ACT7*) promoter primers (5′-CGTTTCGCTTTCCTTAGTGTTAGCT-3′ and 5′-AGCGAACGGATCTAGAGACTCACCTTG-3′).

ELISA-Based Protein-DNA Binding Assays

The cDNAs encoding bZIP10, bZIP25, bZIP53, and ABI3 proteins were cloned into the expression vectors pET23a (Novagen) to generate T7-epitope tagged proteins (Lara et al., 2003). Expression in *Escherichia coli* and preparation of protein extracts were performed as previously described (Lara et al., 2003). Biotinylated complementary oligonucleotides derived from the *At2S2* promoter containing a G-box sequence (5′-CATGCAATGCATTCTTAC**ACGT**GATTGCATGCAAA-3′ and 5′-TTTGCATGCAATC**ACGT**GTAAGAATGCATTGCATG-3′) or a mutated version (5′-CATGCAATGCATTCTTAC**AaGg**GATTGCATGCAAA-3′ and 5′-TTTGCATGCAATCC**CtTg**TAAGAATGCATTGCATG-3′) were annealed in TES buffer (10 mM Tris, pH 8, 1 mM EDTA, and 300 mM NaCl). The appropriate double-stranded DNA (2 pmoles; target) in 60 μL of TBS-T buffer (20 mM Tris, pH 7.4, 180 mM NaCl, and 0.1% Tween 20) was added to each well of a streptavidin-coated plate (Nunc Inmobilizer) and incubated 1 h at 37°C, and nonbound targets were removed by washing three times with TBS-T. Wells were blocked by incubating 30 min at room temperature and gentle shaking (150 rpm) with 60 μL of 1% blocking agent (Roche) in TBS-T and washed three times with TBS-T. Twenty microliters of the protein extracts were mixed with 40 μL of binding buffer (4 mM HEPES, pH 7.5, 100 mM KCl, 0.2% BSA, 8% glycerol, and 5 mM DTT) in microfuge tubes and incubated 15 min on ice. The mixes were added to the wells of the plate, the binding reactions incubated for 1 h at room temperature with gentle shaking, and well washed three times with TBS-T. For epitope detection, we added 60 μL of a 1:5000 dilution of the antibody (T7 tag horseradish peroxidase conjugate; Novagen) in TBS-T to each well. Following incubation for 1 h at room temperature at 150 rpm, the wells were four times washed with TBS-T. The antibody peroxidase-conjugated reaction was performed according to manufacturer's instructions, and the activity was measured at 492 nm using an ELISA plate reader (Tecan) with filter setting at 650 for reference.

Yeast Two- and Three-Hybrid Analyses

Yeast two-hybrid analyses were done as described previously (Lara et al., 2003). BD and AD constructs were generated using appropriate restriction enzymes and cloning the indicated open reading frames into the pGBT9 and pGAD424 plasmids (Clontech), respectively, as follows: *Eco*RI/*Sal*I sites for the BD-bZIP53, AD-bZIP53, BD-ABI5, and AD-ABI5 constructs and *Bam*HI/*Sal*I sites for the BD-ABI3 and AD-ABI3 constructs. BD-bZIP10, AD-bZIP10, BD-bZIP25, and AD-bZIP25 were described by Lara et al. (2003). To analyze ternary complex formation in yeast, we used BD and AD constructs from the two-hybrid assays and expressed a third protein whose coding sequences were inserted into the *Eco*RI/*Sal*I sites of the pTFT1 plasmid (Egea-Cortines et al., 1999).

BiFC

Experimental procedures were according to Moreno-Risueno et al. (2007). In general, at least two independent assays were performed that

included four independent plates bombarded for every individual construct or the different combinations. Comparisons were based on transformation efficiencies estimated to Pro35S:green fluorescent protein as a reference. Vectors used have been described by Weltmeier et al. (2006).

Accession Numbers

Arabidopsis Genome Initiative identifiers for the genes mentioned in this article are as follows: b*ZIP53* (At3g62420), b*ZIP10* (At4g02640), b*ZIP25* (At3g54620), b*ZIP1* (At5g49450), *2S2* (At4g27150), *CRU3* (At4g28520), *LEA76* (At3g15670), *ProDH* (At5g38710), *UBI* (At5g25760), *ACT7* (At5g09810), *ABI3* (At3g24650), *ABI5* (At2g36270), *ASN1* (At3g47340), *CRA1* (At5g44120), and At *HSD1* (At5g50600).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Analysis of Putative bZIP53 Target Genes in Plants Constitutively Expressing HA-bZIP53 or bZIP53.

Suplemental Figure 2. Transient Expression of bZIP53 in Combination with bZIP10 or bZIP25 Synergistically Enhances Transactivation of *CRU3* and *2S2* Promoters.

Supplemental Figure 3. Analysis of Effector Proteins Transiently Expressed in *Arabidopsis* Protoplasts.

Supplemental Figure 4. Ternary Protein Interaction Studied in a Yeast Three-Hybrid System.

Supplemental Figure 5. Transient Expression by Microparticle Bombardment of *Arabidopsis* Leaves from Columbia Wild Type and Plants with Increased (Pro35S:bZIP53) or Decreased (*bzip53*) Expression of *bZIP53*.

Supplemental Table 1. Primers Used in This Study.

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