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Combinatorial control of *Arabidopsis* proline dehydrogenase transcription by specific heterodimerisation of bZIP transcription factors

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Proline metabolism has been implicated in plant responses to abiotic stresses. The *Arabidopsis thaliana* proline dehydrogenase (ProDH) is catalysing the first step in proline degradation. Transcriptional activation of *ProDH* by hypo-osmolarity is mediated by an ACTCAT *cis* element, a typical binding site of basic leucine zipper (bZIP) transcription factors. In this study, we demonstrate by gain-of-function and loss-of-function approaches, as well as chromatin immunoprecipitation (ChIP), that *ProDH* is a direct target gene of the group-S bZIP factor AtbZIP53. Dimerisation studies making use of yeast and *Arabidopsis* protoplast-based two-hybrid systems, as well as bimolecular fluorescence complementation (BiFC) reveal that AtbZIP53 does not preferentially form dimers with group-S bZIPs but strongly interacts with members of group-C. In particular, a synergistic interplay of AtbZIP53 and group-C AtbZIP10 was demonstrated by colocalisation studies, strong enhancement of ACTCAT-mediated transcription as well as complementation studies in *atbzip53 atbzip10* T-DNA insertion lines. Heterodimer mediated activation of transcription has been found to operate independent of the DNA-binding properties and is described as a crucial mechanism to modulate transcription factor activity and function.

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Introduction

Osmotic stress, caused by drought, high salinity and cold is a key determinant of plant growth and productivity (Hoekstra *et al*, 2001). In response to environmental conditions causing osmotic stress, many higher plants accumulate protective compounds, like the compatible osmolyte L-proline (L-Pro) (Delauney *et al*, 1993; Liu and Zhu, 1997; Parvanova *et al*, 2004). This compound has been proposed to participate in the protection of plasma membrane integrity (Mansour, 1998) and to act as a scavenger of radicals (Smirnoff and Cumbes, 1989). In addition, L-Pro is discussed to be a source of reducing power (Walton and Boldingh, 1991) and to serve as a transient storage of carbon and nitrogen (Peng *et al*, 1996).

The L-Pro homeostasis is tightly regulated by its synthesis (Delauney *et al*, 1993), degradation (Verbrüggen *et al*, 1996) and transport (Rentsch *et al*, 1996). L-Pro is synthesised in the cytosol from glutamine or ornithin precursors (Figure 1A). In osmotically stressed tissues, L-Pro mainly results from the glutamine pathway by the activity of the NADPH using enzymes Δ^1-pyrroline-5-carboxylate-synthetase (P5CS) and P5C reductase (P5CR) (Delauney *et al*, 1993; Roosens *et al*, 1999). L-Pro accumulation correlates with osmotic stress tolerance, however, a stringent causal relationship is not yet firmly established (Nanjo *et al*, 1999). Furthermore, accumulation of L-Pro is toxic and thus L-Pro and its metabolites have been discussed to function as signalling molecules in stress-induced cell death (Hellmann *et al*, 2000; Nanjo *et al*, 2003; Deuschle *et al*, 2004).

Induced by rehydration, L-Pro is degraded by a pathway localised in the mitochondria, which makes use of the enzymes proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) (Peng *et al*, 1996; Verbrüggen *et al*, 1996; Nakashima *et al*, 1998; Yoshiba *et al*, 1999). ProDH catalyses the rate-limiting step in-Pro degradation. Transgenic *Arabidopsis* plants expressing a *ProDH* antisense construct display high L-Pro levels and enhanced tolerance to freezing conditions and high salinity (Nanjo *et al*, 1999).

In *Arabidopsis thaliana*, the *ProDH* gene (At3g30775) is induced in response to hypo-osmotic conditions occurring during rehydration after stress recovery (Kiyosue *et al*, 1996). *ProDH* transcription is also enhanced by high levels of L-Pro and reduced by dehydration (Peng *et al*, 1996; Verbrüggen *et al*, 1996). Detailed dissection of the 1.4-kb *ProDH* promoter revealed an ACTCAT motif which is sufficient for induction of the *ProDH* gene by L-Pro or hypo-osmolarity, respectively (Nakashima *et al*, 1998; Satoh *et al*, 2002). Microarray analysis confirmed that among 121 rehydration-inducible genes, 48% harbour the ACTCAT motif in their promoters (Oono *et al*, 2003). These findings indicate that this motif is of general relevance with respect to gene regulation during recovery after osmotic stress. Therefore, ACTCAT-mediated transcription might serve as a fruitful model system for

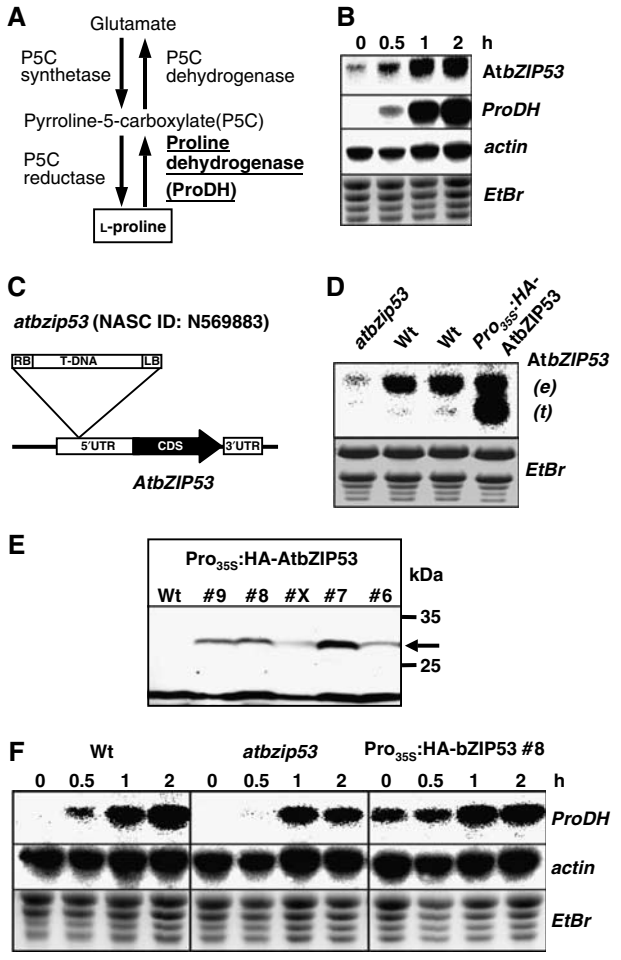


Figure 1 The bZIP transcription factor AtbZIP53 regulates *ProDH* transcription in the hypo-osmolarity response. (**A**) Schematic overview of the metabolic pathways involved in L-proline (L-Pro) synthesis and degradation in plant cells. P5CS: Δ^1-pyrroline-5-carboxylate-synthetase; P5CR: reductase; ProDH: L-Pro dehydrogenase; P5CDH: P5C dehydrogenase (Peng *et al*, 1996; Verbrüggen *et al*, 1996; Nakashima *et al*, 1998; Yoshiba *et al*, 1999). (**B**) Gene induction by hypo-osmotic stress treatment. *Arabidopsis* plants, 14-day-old, were transferred from MS medium (58 mM sucrose) to desalted water (Satoh *et al*, 2004). RNA of control (0 h) and stress-treated plants (time points as indicated) was analysed by Northern hybridisation. Depicted are hybridisation experiments with *ProDH*- and *AtbZIP53*-specific probes, respectively. (**C**) Schematic illustration of the T-DNA insertion mutant *atbzip53*. The insertion was mapped inside the 5′utr 430 bp upstream of the ATG. (**D**) Northern analysis of wild-type (Wt), AtZIP53 T-DNA insertion lines (*atbzip53*), and AtbZIP53-overexpressing plants (Pro_{35S}:HA-AtbZIP53). Endogenous (e) and transgene encoded (t) *AtbZIP53* RNAs show slightly different mobility due to the length of their 5′ leaders. (**E**) Western Analysis of wild-type (Wt) and several independent transformants of Pro_{35S}:HA-AtbZIP53. The transgene-encoded proteins are detected by a HA-specific antibody (arrow). (**F**) Induction of *ProDH* transcription in response to hypo-osmolarity treatment. RNA was isolated after the time points indicated and *ProDH* transcript level of Wt, *atbzip53*, and Pro_{35S}:HA-AtbZIP53 plants are compared. Equal loading was verified by ethidium bromide (EtBr) staining and hybridisation with an actin-specific probe. All experiments have been replicated at least three times with similar results.

studying transcriptional control of osmotic stress responses, which is of importance for both, basic science as well as genetic engineering of crop plants.

The ACTCAT-motif is related to the GCN4-binding site (ATGA(C/G)TCAT) (Ellenberger *et al*, 1992) recognised by members of the basic leucine zipper (bZIP) transcription factor family (Landschulz *et al*, 1988). In the model plant *Arabidopsis thaliana,* 75 bZIP genes have been identified and classified into 10 groups (Jakoby *et al*, 2002). *In vitro* DNA-binding studies and transfection experiments performed in transiently transformed protoplasts revealed that distinct members of *Arabidopsis* group-S bZIP proteins are involved in ACTCAT-mediated transcription (Satoh *et al*, 2004). Although 17 group-S bZIPs have been identified, only a subgroup consisting of five highly related members (AtbZIP1, AtbZIP2, AtbZIP11/ATB2, AtbZIP44 and AtbZIP53) seem to be involved in *ProDH* regulation (Satoh *et al*, 2004) and will be referred to as group-S1 bZIPs.

bZIP proteins are phylogenetically widely distributed transcriptional regulators, characterised by a basic DNA-binding domain (b) (Landschulz *et al*, 1988). Although DNA binding of bZIP monomers has been described, they normally interact with DNA as dimers (Ellenberger *et al*, 1992; Metallo and Schepartz, 1997; Cranz *et al*, 2004). Dimerisation is mediated by the so-called leucine zipper domain, a heptad repeat of leucine or other bulky hydrophobic amino acids creating an amphipathic helix (Landschulz *et al*, 1988; Baxevanis and Vinson, 1993). The formation of bZIP homo- or heterodimers offers a huge combinatorial flexibility to regulatory transcription systems. By heterodimerisation, DNA-binding specificity and affinity, transactivation properties and ultimately, cell physiology might be altered (Naar *et al*, 2001). For instance, in the animal system, heterodimers of the bZIP proteins Jun and Fos have been shown to recognise the AP1 motif, which is not targeted by Jun homodimers (Kouzarides and Ziff, 1988). As bZIP proteins do not heterodimerise promiscuously but specifically (Newman and Keating, 2003), an important function for gene regulation is anticipated. *In planta*, bZIP heterodimerisation has been shown for closely related bZIPs, for example, the GBFs (Schindler *et al*, 1992). In tobacco (Strathmann *et al*, 2001) and parsley (Rügner *et al*, 2001), specific heterodimerisation was found between members of two groups of bZIPs that are related to the *Arabidopsis* groups S and C. Interestingly, heterodimerisation between these groups is strongly preferred in comparison to homotypic dimerisation.

In this work, we demonstrate by means of transgenic and ChIP approaches that *ProDH* is a direct target gene of the group-S1 bZIP transcription factor AtbZIP53. Using yeast two-hybrid, BiFC and interaction assays in protoplasts, we show that AtbZIP53 does not function as a homodimer, but preferentially heterodimerises with group-C bZIP transcription factors. AtbZIP53 and the group-C heterodimerisation partner AtbZIP10 were found to be colocalised and to control ACTCAT-mediated *ProDH* transcription in a synergistic manner. We describe bZIP heterodimerisation as an essential mechanism to modulate transactivation properties of transcriptional regulators *in planta*. Furthermore, we provide *in vivo* evidence that *ProDH* is not regulated by a single bZIP but by a complex heterodimerisation network of group-S1/C bZIP factors.

Results

AtbZIP53 directly participates in the hypo-osmolarity-induced transcription of the ProDH gene

Using *in vitro* DNA-binding studies and transfection experiments in *Arabidopsis* protoplasts, Satoh *et al* (2004) demon-

strated that the group-S1 bZIP transcription factors bind to the ACTCAT *cis* element located in the *ProDH* promoter. However, expression data of the correspondent *bZIP* genes, obtained from the RARGE database (http://rarge.gsc.riken.jp) or by Satoh *et al* (2004), suggest that AtbZIP53 constitutes as the main regulator of hypo-osmolarity-induced *ProDH* transcription. Whereas *AtbZIP53* is strongly induced by hypo-osmolarity treatment (Figure 1B), *AtbZIP11* or *AtbZIP44* are not. We therefore focussed on studying the function of AtbZIP53 in the regulation of *ProDH* transcription. Expression of an HA-tagged *AtbZIP53* gene driven by the 35S promoter (Pro_{35S}:HA-AtbZIP53), as monitored by Northern and Western analyses (Figure 1D and E), resulted in a constitutive activation of the *ProDH* gene (Figure 1F). Comparable results were obtained with several transgenic lines expressing an untagged construct indicating that this response is not due to the HA-tag (data not shown). Complementarily, a partial loss-of-function mutant of *AtbZIP53* (*atbzip53*) (Figure 1C and D) displayed a significantly reduced and delayed induction of *ProDH* transcription after hypo-osmolarity treatment (approximately by 40%) (Figure 1F). Owing to redundancy, AtbZIP53 and other group-S1 bZIP transcription factors (Jakoby *et al*, 2002) might show an overlapping function which results in the residual activation of *ProDH* expression in the *AtbZIP53* plant.

To verify direct binding of AtbZIP53 to the *ProDH* promoter *in vivo*, ChIP was performed applying chromatin obtained from HA-AtbZIP53-expressing plants (Pro_{35S}:HA-AtbZIP53) using an HA-tag-specific antibody. A significant enrichment of *ProDH* promoter fragments was detected by PCR amplification, using *ProDH* promoter-specific primers (Figure 2). Control primers, unrelated to the AtbZIP53 target gene did not result in an enrichment following immunoprecipitation (Figure 2). In summary, the *in vitro* and *in vivo* evidence support the hypothesis that the *ProDH* promoter is a direct target of the AtbZIP53 transcription factor.

AtbZIP53 heterodimerises with group-C bZIP transcription factors in yeast and plant cells

bZIP factors usually bind DNA as homo- or heterodimers, consequently, dimer formation allows the establishment of complex regulatory networks based on combinatorial interactions. It is tempting to speculate that heterodimers of AtbZIP53 formed with the related group-S1 bZIP factors are regulating *ProDH* transcription. However, when applying a yeast two-hybrid approach, no AtbZIP53 homodimers or AtbZIP53 containing heterodimers were formed with any of the group-S1 bZIP proteins or other less related group-S bZIP factors (Figure 3A). A quantitative assay for protein–protein interactions confirmed the weak heterodimerisation capacity

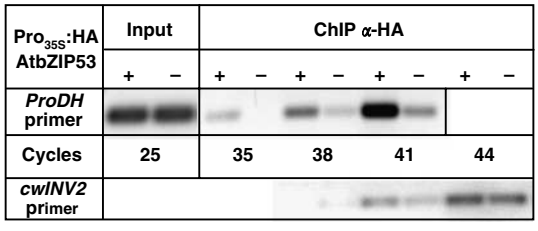


Figure 2 *In vivo* binding of AtbZIP53 to the *ProDH* promoter. ChIP analysis of chromatin obtained from Pro_{35S}:HA-AtbZIP53 (+) and Wt (−) plants using a HA-specific antibody for immunoprecipitation. *ProDH* primers were used to amplify a 191 bp fragment of the *ProDH* promoter. Given are the PCR products obtained after 35, 38, or 41 cycles, respectively. As input control, PCR amplicons are shown which have been obtained after 25 cycles with material before immunoprecipitation. The specificity was verified by using primers amplifying the promoter of a nontarget gene (cwINV2, At3g52600). Given are amplicons obtained after 38, 41 or 44 cycles, respectively. The PCR products were stained by ethidium bromide. The experiments have been replicated three times with similar results.

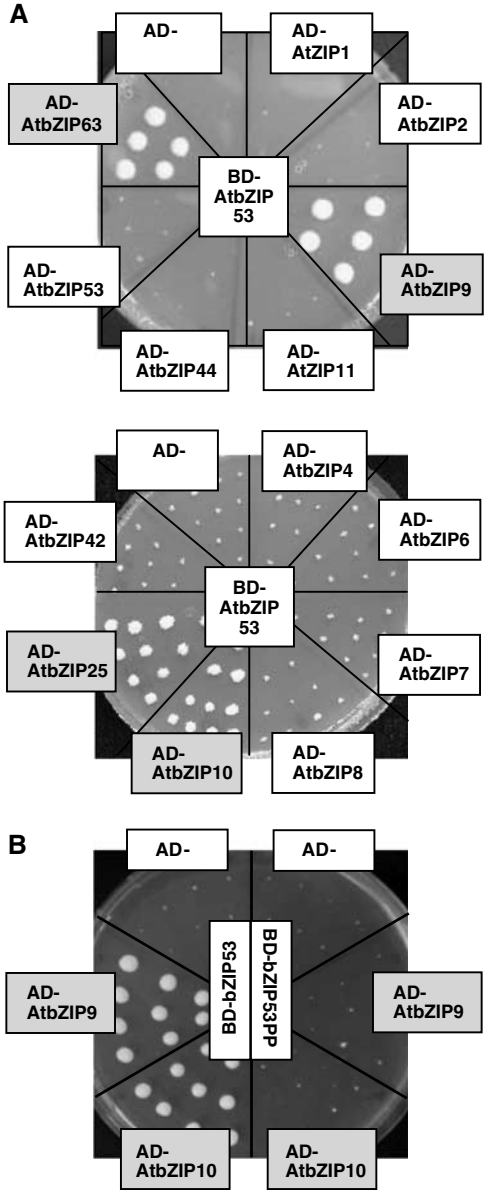


Figure 3 Yeast two-hybrid analysis studying heterodimerisation of AtbZIP53 with group-S and -C bZIP factors. (**A**) Interaction of BD-AtbZIP53 and the AD-bZIP fusion proteins indicated lead to prototrophic growth of the yeast strain MAV203 (ProQuest®, Invitrogen) on medium lacking uracil. Group-C bZIP (AtbZIP63, AtbZIP9, AtbZIP25, AtbZIP10) and group-S bZIP proteins (AtbZIP53, AtbZIP2, AtbZIP44, AtbZIP11, AtbZIP42, AtbZIP7, AtbZIP6, AtbZIP4, AtbZIP8) are labelled by a grey or white background, respectively. (**B**) Interaction of AtbZIP53 and group-C bZIP proteins is mediated by the ZIP domain. ZIP-specific exchanges of the amino acids $leucine_{51}$ and $alanine_{72}$ by Pro ($AtbZIP53_{PP}$) disrupt the α-helical structure and results in loss of interaction (right) in comparison to the wild-type AtbZIP53 (left).

of AtbZIP53 with other group-S1 members (data not shown). We further analysed the potential of AtbZIP53 to interact with other bZIP factors. As described for tobacco (Strathmann *et al*, 2001) and parsley (Rügner *et al*, 2001), group-S1-like bZIP proteins specifically heterodimerise with group-C-like bZIPs. Strong heterodimerisation of AtbZIP53 was detected with all *Arabidopsis* group-C bZIP proteins, namely AtbZIP9, AtbZIP10, AtbZIP25 and AtbZIP63 (Figure 3A). As no heterodimerisation with members of the bZIP groups A (e.g. AtbZIP39) and D (e.g. AtbZIP22) was found, this heterodimerisation is assumed to be specific. In order to analyse whether the protein–protein interaction is mediated by the leucine zipper domain, two conserved residues (leucine$_{51}$ and alanine$_{72}$) were changed to prolines which should disrupt the α-helical structure of the ZIP domain (AtbZIP53$_{PP}$). As shown in Figure 3B, no interaction between AtbZIP53$_{PP}$ and group-C members, such as AtbZIP10 and AtbZIP9 was observed.

To verify bZIP heterodimerisation *in planta*, BiFC assays were performed in *Agrobacterium*-infiltrated tobacco (*Nicotiana benthamiana*) leaves (Walter *et al*, 2004) using the strongly interacting AtbZIP53 and AtbZIP10 as a representative example. Both bZIP factors localise to the nucleus when expressed as YFP fusion proteins (Figure 4A and B). When fused to the N- and C-terminal YFP fragment and coexpressed in tobacco leaves, a strong BiFC signal was detected in the nuclear compartment of the transformed cells (Figure 4C), confirming the interaction of AtbZIP53 and AtbZIP10 *in planta*. Control experiments in which C-YFP-AtbZIP53 and unfused N-YFP protein are expressed do not show any fluorescence (Figure 4D). In order to demon strate the specificity of BiFC system, the C-YFP-AtbZIP53$_{PP}$ mutation was coexpressed with N-YFP-AtbZIP10. Specific mutation of the ZIP domain (see Figure 3B) interferes with heterodimerisation and in consequence does not result in positive BiFC signals (Figure 4E). In order to confirm expression of the BiFC constructs, Western analyses have been performed (data not shown). Finally, homodimerisation of AtbZIP53 and AtbZIP10 was analysed in this system, but no or only significantly weaker homotypic interactions could be detected (data not shown). In summary, these experiments provide conclusive evidence for the formation of group-S1/C heterodimers *in planta*.

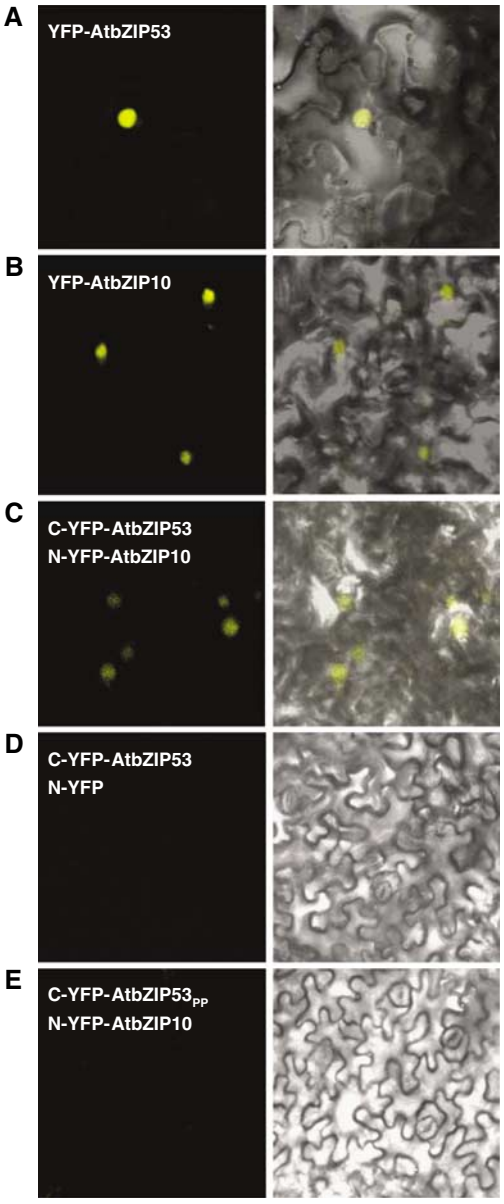


Figure 4 BiFC analysis of AtbZIP53/AtbZIP10 heterodimerisation *in planta*. Localisation of YFP-AtbZIP53 (**A**) and YFP-AtbZIP10 (**B**) fusion proteins in *Nicotiana benthamiana* leaf epidermal cells after *Agrobacterium*-mediated transient transformation. (**C**) Fluorescence observed by confocal microscopy which results from complementation of the C-terminal (C-YFP) and N-terminal (N-YFP) part of the YFP protein fused to AtbZIP53 and AtbZIP10, respectively. (**D**) Negative control making use of C-YFP-AtbZIP53 and an unfused N-YFP protein. (**E**) Coexpression of the C-YFP-AtbZIP53pp mutant (see Figure 3B) and N-YFP-AtbZIP10. YFP-Epifluorescence (left) and bright field overlay images (right) are depicted.

Heterodimerisation with group-C bZIPs enhances the transactivation properties of AtbZIP53 in planta

In order to quantify bZIP heterodimerisation *in planta*, a two-hybrid approach was established in *Arabidopsis* protoplasts as described by Ehlert *et al* (2006). AtbZIP53 and the related group-S1 and group-C bZIP factors were fused to the GAL4 DNA-binding domain (BD) (Giniger *et al*, 1985) and expressed under control of the 35S promoter. Cotransfection with a GUS reporter, which is driven by GAL4 upstream activating sequence (GAL-UAS$_4$:GUS), allows quantification of bZIP activation properties independent of their DNA binding (Figure 5A). Whereas group-S1 members AtbZIP11, AtbZIP44 and AtbZIP2 showed significant activation capacity; AtbZIP53, AtbZIP1 and the group-C members displayed almost no intrinsic activation (Figure 5B).

In order to study heterodimerisation of AtbZIP53, BD-AtbZIP53 was individually coexpressed with GAL4 activation domain (AD) fusions of the bZIP heterodimerisation partners (Figure 5C). Similar to the results obtained in yeast, almost no AtbZIP53 homodimerisation and only weak interaction with the group-S1 members AtbZIP2, AtbZIP11 and AtbZIP44 was observed (Figure 5D). A strong heterodimerisation of AtbZIP53 with the group-C members AtbZIP10, AtbZIP25 and AtbZIP9 was detected. In contrast, interaction between AtbZIP53 and group-C AtbZIP63 was less pronounced.

Heterodimerisation might modulate the transactivation capacity of bZIP transcription factors. We therefore co-expressed BD-AtbZIP53 with bZIP partners which have not been fused to the AD domain (Figure 5D). Strong reporter

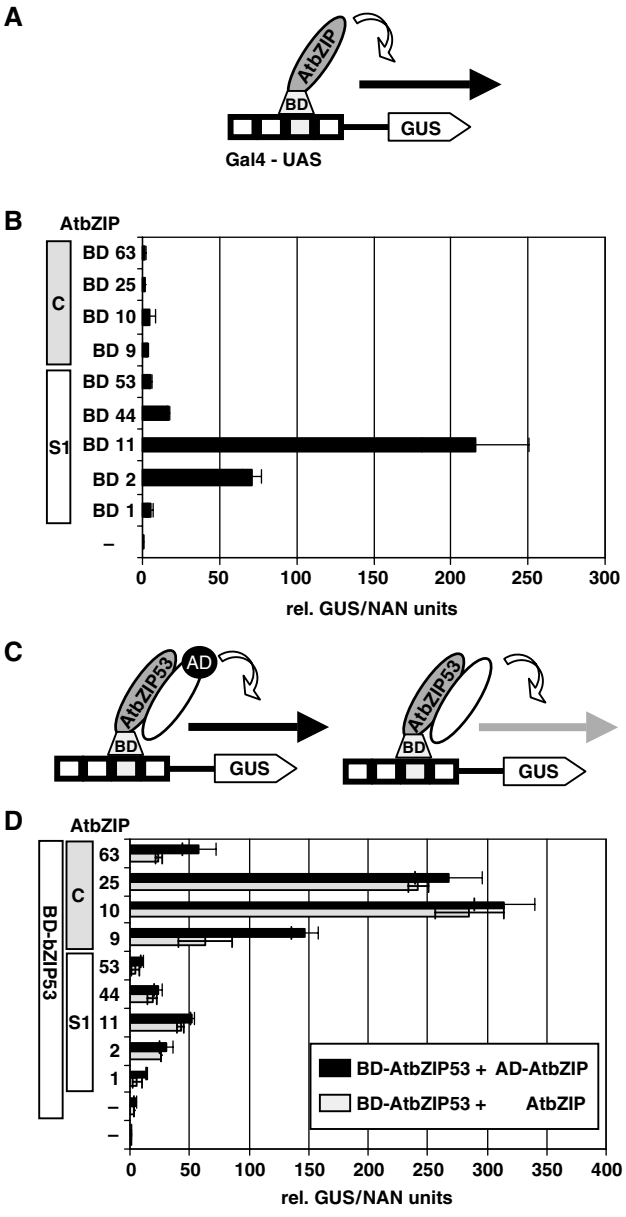


Figure 5 Analysis of AtbZIP53 heterodimerisation in transiently transformed *Arabidopsis* protoplasts. Experimental set-up (**A**) to analyse bZIP activation properties. The activation of a *GUS* reporter driven by multimeric GAL4-UAS DNA-binding sites (GAL-UAS_4:GUS) was measured after expression of GAL4-BD fusions of group-S1 and C bZIP proteins (BD-AtbZIP) (**B**). Experimental set-up (**C**) to analyse AtbZIP53 heterodimerisation. Activation of a GAL-UAS_4:GUS reporter was measured after coexpression of BD-AtbZIP53 and bZIP proteins fused to an AD domain of GAL4 (AD, black bars) or an HA-tag (grey bars) (**D**). In single transfection experiments, 9 μg of reporter and 28 μg of effector plasmid DNA were applied. In cotransfections, 14 μg of each AtbZIP effector plasmid were used. Variations in transformation efficiency are normalised by using 3 μg of a Pro_{35S}:NAN plasmid construct per transfection (Kirby and Kavanagh, 2002). Given are mean values and s.d.'s of four independent transfections as relative GUS/NAN units.

gene activation was obtained for AtbZIP53/AtbZIP10 and AtbZIP53/AtbZIP25 heterodimers. As neither AtbZIP53 nor group-C bZIPs exhibit strong activation on their own (Figure 5B), these data indicate that specific heterodimerisation may act as a key mechanism to modulate transactivation properties *in planta*. This mechanism will be referred to as heterodimer-induced transactivation (HIT).

In order to analyse the HIT mechanism, the following control has been included into the experimental set-up. Fusing a BD domain to the N-terminus of the bZIP proteins might mask endogenous activation properties of the bZIP proteins analysed in Figure 5B. We therefore coexpressed BD-AtbZIP53 with the other BD-bZIP proteins which also lead to the synergistic reporter gene activation in the case of AtbZIP53/AtbZIP10 and AtbZIP53/AtbZIP25, respectively (data not shown).

The HIT mechanism is not restricted to AtbZIP53/AtbZIP10 and AtbZIP53/AtbZIP25 heterodimers. A complete analysis of all group-S1/C interactions, according to the set-up described in Figure 5C, revealed that the HIT mechanism is a typical feature of distinct heterodimers (Supplementary Table S1). It is particularly observed between AtbZIP25, AtbZIP10 and most group-S1 members. Moreover, HIT is also observed inside group-C, as demonstrated for AtbZIP10/AtbZIP63. These data suggest that HIT is of general importance for the function of the group-C and S bZIP factors.

Group-S1 and C bZIPs bind specifically the ACTCAT-regulatory element present in the ProDH promoter

ProDH promoter analysis revealed an ACTCAT motif to be necessary and sufficient for hypo-osmolarity-induced gene activation (Satoh *et al*, 2002). To assess the significance of AtbZIP53 homo- or heterodimer participation in ACTCAT-mediated transcriptional control, we transiently expressed AD-bZIP proteins in *Arabidopsis* protoplasts in conjunction with a reporter vector containing this regulatory element. In this experimental system, activation of a $(ACTCAT)_2$:GUS reporter should solely rely on the binding properties of the bZIP proteins and be independent of their intrinsic activation capacity. The results in Figure 6 show that all bZIP proteins tested are able to interact with this element, and demonstrate that AtbZIP53 binds more effectively the ACTCAT motif than group-C bZIPs. The specificity of this interaction was studied by introducing mutations into the ACTCAT element. The use of a $(ACTtta)_2$:GUS reporter resulted in a loss of activation for all bZIPs, confirming that the ACTCAT motif is a specific binding site for these proteins *in planta* (Figure 6).

AtbZIP53-containing heterodimers bind the ProDH promoter ACTCAT-cis-element and regulate ACTCAT-mediated transcription

The results of the heterodimerisation assays prompted us to analyse whether *ProDH* is regulated by AtbZIP53-containing heterodimers. We therefore coexpressed AtbZIP53 in combination with AD-bZIP fusions of group-S1 and C bZIPs (Figure 7). As we observed for the GAL-UAS supported transcription, synergistic enhancement of the ACTCAT-mediated transcription was obtained by coexpression of AtbZIP53 and its group-C heterodimerisation partners AtbZIP9, AtbZIP10, AtbZIP25 and AtbZIP63, respectively. In order to analyse the endogenous activation properties, the bZIP proteins were expressed without the AD domain (Figure 7). Equal expression of the proteins was monitored by Western blot using an HA-tag-specific antibody (data not shown). Whereas expression of single bZIP proteins resulted in weak reporter activation, a significant synergistic activation was obtained with all AtbZIP53/group-C hetero-

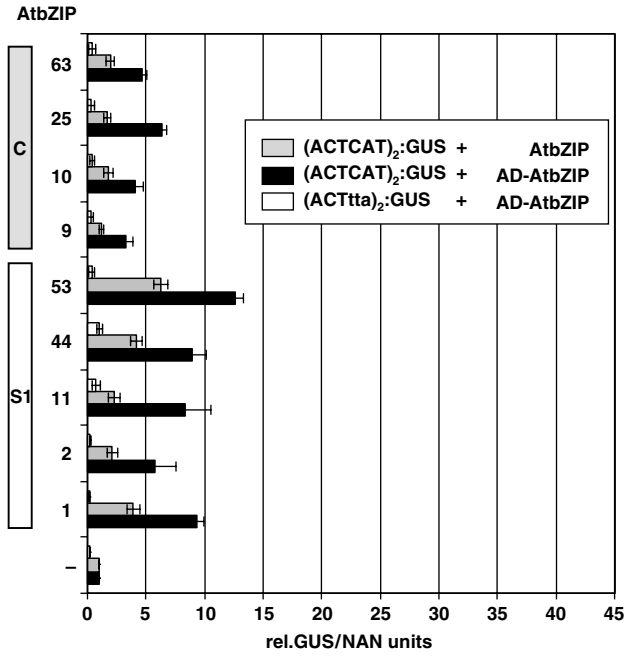


Figure 6 ACTCAT-specific DNA binding of group-S1 and C bZIP factors. *Arabidopsis* protoplasts were transiently transformed with a GUS reporter construct driven by the ACTCAT motif ($(ACTCAT)_2$:GUS) (black and grey bars) or a mutated version ($(ACTttg)_2$:GUS) (white bars). Activation of the reporters was measured after expression of AtbZIP proteins (grey and white bars) or AD-AtbZIP proteins (black bars). In single transfection experiments, 9 μg of reporter and 28 μg of effector plasmid DNA was applied. Variations in transformation efficiency are normalised by using 3 μg of a Pro_{35S}:NAN plasmid construct per transfection (Kirby and Kavanagh, 2002). Given are mean values and s.d.'s of four independent transfections as relative GUS/NAN units.

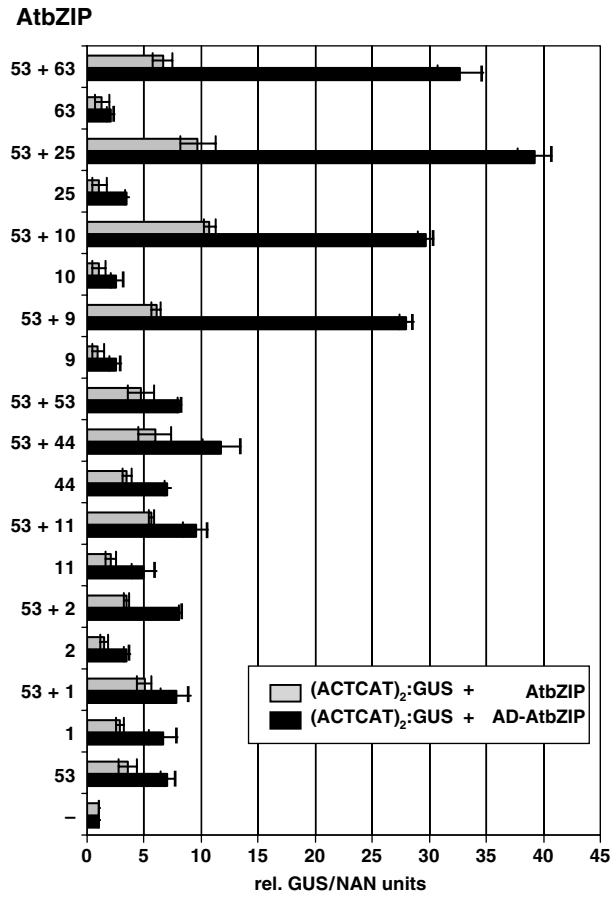


Figure 7 The ACTCAT-mediated transcription is regulated by AtbZIP53 heterodimers. Regulation of the $(ACTCAT)_2$:GUS reporter by heterodimers was analysed by coexpression of AtbZIP53 and group-S1 and C bZIP (grey bars) or AD-AtbZIP proteins (black bars). In single transfection experiments, 9 μg of reporter and 28 μg of effector plasmid DNA was applied. In cotransfections, 14 μg of each bZIP effector plasmid were used. Variations in transformation efficiency are normalised by using 3 μg of a Pro_{35S}:NAN plasmid construct per transfection experiment (Kirby and Kavanagh, 2002). Given are mean values and s.d.'s of four independent transfections as relative GUS/NAN units.

dimers. Based on these data, a number of heterodimers seem to fulfil a partly redundant function in ACTCAT-mediated transcription.

AtbZIP53 and AtbZIP10 cooperate in regulating hypo-osmolarity induced ProDH transcription

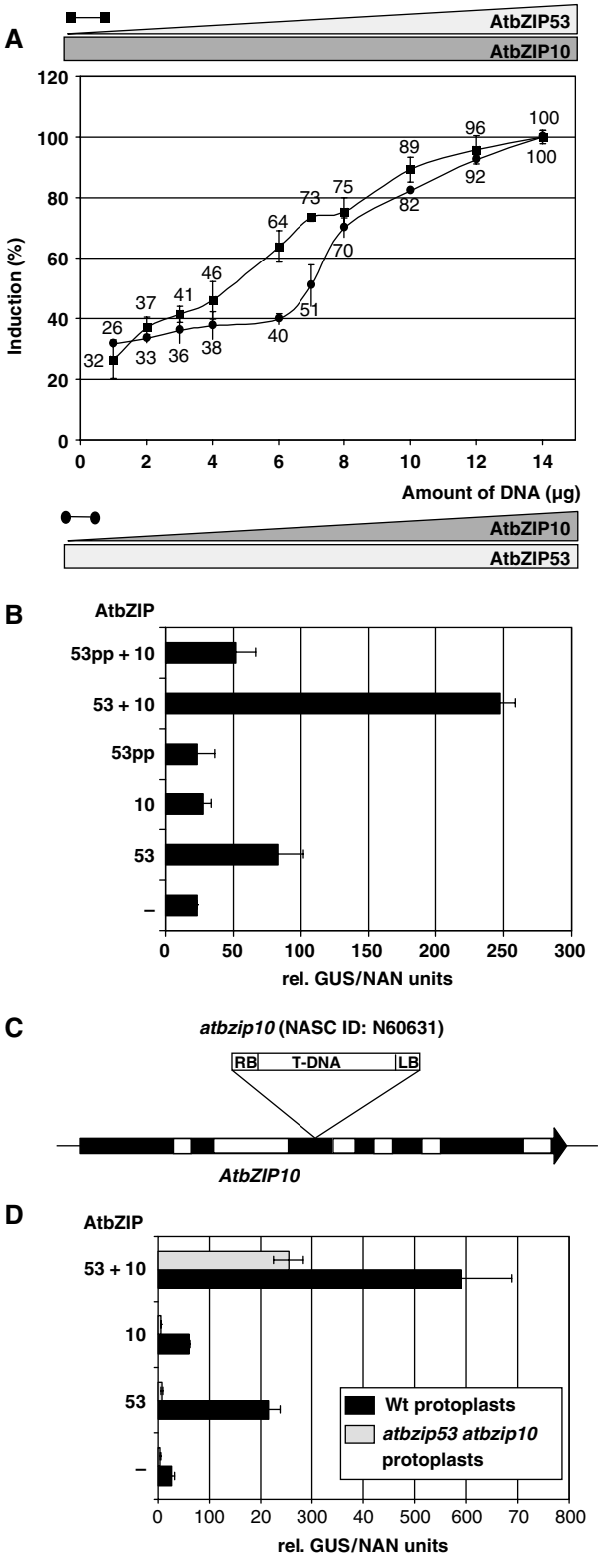
The data described above suggest that a complex network of heterodimers is involved in regulation of *ProDH*. To get further insight in this regulatory mechanism, we focused on AtbZIP53 and AtbZIP10, the two partners that show the highest degree of ACTCAT-mediated reporter gene activation. By means of ChIP analysis we could demonstrate that *ProDH* is a direct target of both, AtbZIP53 (Figure 2) as well as AtbZIP10 (Supplementary Figure S1). Furthermore, electrophoretic mobility shift assays (EMSA) confirmed that heterodimers can be formed on the ACTCAT motif *in vitro* (Supplementary Figure S2). A detailed experimental series was initiated in which the ratio of bZIP effector plasmids was altered. Figure 8A shows that the synergistic effect of AtbZIP53 and AtbZIP10 clearly depends on the ratio of the two heterodimerisation partners. Furthermore, the synergistic effect depends on the ZIP-mediated heterodimerisation function. Disruption of the α-helical structure in the $AtbZIP53_{PP}$ construct impairs activation of the reporter by AtbZIP53/AtbZIP10 (Figure 8B).

To verify the impact of heterodimerisation on reporter gene expression, similar experiments as described above were performed in protoplasts obtained from *atbzip53 atbzip10* plants. For efficient reporter gene expression, both AtbZIP53 and AtbZIP10 are required (Figure 8D). All together, these data strongly suggest that AtbZIP53/AtbZIP10 heterodimers are regulating ACTCAT-mediated *ProDH* transcription.

Colocalisation of the bZIP partners in the same tissue is a prerequisite for heterodimerisation. Whereas transcription of *AtbZIP53* is strongly induced by hypo-osmolarity (Figure 1B), only slight induction can be obtained for *AtbZIP10* (Supplementary Figure S3). Analysis of AtbZIP53 and AtbZIP10 promoter GUS reporter lines ($Pro_{AtbZIP53}$:GUS and $Pro_{AtbZIP10}$:GUS) revealed a partial overlap of expression patterns primarily in the meristematic tissues of the *Arabidopsis* plantlet. Strong enhancement of GUS activity after transfer from MS media to distilled water was found for both, $Pro_{AtbZIP53}$:GUS and $Pro_{AtbZIP10}$:GUS reporter lines (Figure 9). In contrast, $Pro_{AtbZIP25}$:GUS lines do not show this induction, indicating that this bZIP transcription factor is not involved in this response. Coexpression of *AtbZIP53* and *AtbZIP10* was also observed in parts of the flower and

seeds (data not shown) in which a Pro_{ProDH}:GUS construct is strongly expressed (Nakashima *et al*, 1998).

In conclusion, these data demonstrate that AtbZIP53 and AtbZIP10 colocalise and interact with high affinity. Moreover, protein availability, which is regulated by environmental and developmental cues, restricts heterodimer formation and ultimately controls target gene transcription (Figure 10).



Discussion

The amino acid L-Pro is essential for basic metabolism as well as for plant development. Moreover, under stress conditions, it has been proposed to serve as a protective compound or as a signalling molecule (Delauney *et al*, 1993; Liu and Zhu, 1997). Consequently, final effects derived from its availability depend on L-Pro homeostasis, a process tightly regulated by synthesis, degradation and transport. In this work we focus on the transcriptional control of the *ProDH* gene, encoding the rate-limiting enzyme of L-Pro degradation and found that it is directly regulated by the transcription factor AtbZIP53. We disclosed that AtbZIP53 action is modulated by other bZIP heterodimerising partners that integrate a fine regulatory network supported by combinatorial interactions.

The ProDH promoter ACTCAT motif is a direct target of AtbZIP53 transcription factors in vivo

Previously reported *in vitro* DNA-binding studies (Satoh *et al*, 2004), alongside with the different experimental results presented in this work, including overexpression in transgenic plants, ChIP analysis, and activation of ACTCAT reporter constructs in protoplasts, provide conclusive evidence that

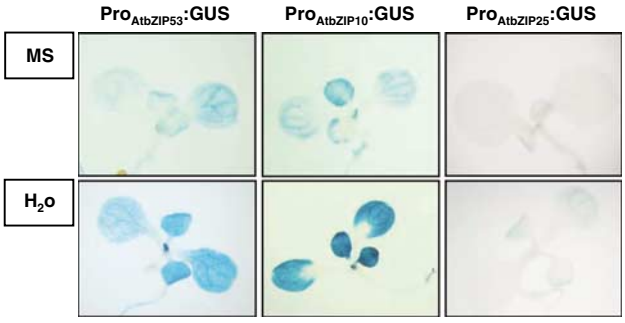


Figure 9 Coexpression studies of *AtbZIP53* and *AtbZIP10*. Activation of $Pro_{AtbZIP53}$:GUS (left), $Pro_{AtbZIP10}$:GUS (middle) and $Pro_{AtbZIP25}$:GUS (right) lines in 14 day-old plantlets grown on MS media (upper panel) and after transfer to distilled water (hypo-osmolarity treatment) for 12 h (lower panel).

Figure 8 The ACTCAT-mediated transcription in *Arabidopsis* protoplasts depends on AtbZIP53/AtbZIP10 heterodimers. (**A**) Reporter activation depends on the ratio of AtbZIP53 and AtbZIP10. Activation of $(ACTCAT)_2$:GUS reporter was measured using 14 µg of AtbZIP10 and increasing amounts of AtbZIP53 encoding plasmid (squares) or 14 µg of AtbZIP53 and increasing amounts of AtbZIP10 encoding plasmid (circles), respectively. Given are reporter gene induction values in %. Reporter gene activation after applying 14 µg of each effector is defined as 100%. (**B**) Activation of the $(ACTCAT)_2$:GUS reporter by AtbZIP53/AtbZIP10 depends on a ZIP-mediated heterodimerisation. ZIP-specific exchanges of the amino acids $leucine_{51}$ and $alanine_{72}$ by proline ($AtbZIP53_{PP}$) disrupts the α-helical ZIP structure, bZIP heterodimerisation (see Figure 3B) and reporter gene activation. (**C**) Graphical description of the T-DNA insertion mutant *atbzip10*. Exons are depicted as black bars, introns as white bars. The insertion was mapped inside the third exon. (**D**) Activation of $(ACTCAT)_2$:GUS reporter by AtbZIP53, AtbZIP10 or both was compared in protoplasts derived from wild-type (black bars) and *atbzip53 atbzip10* double mutant plants (grey bars), respectively. For each bZIP encoding construct, 14 µg of DNA have been used. Given are relative GUS/NAN units.

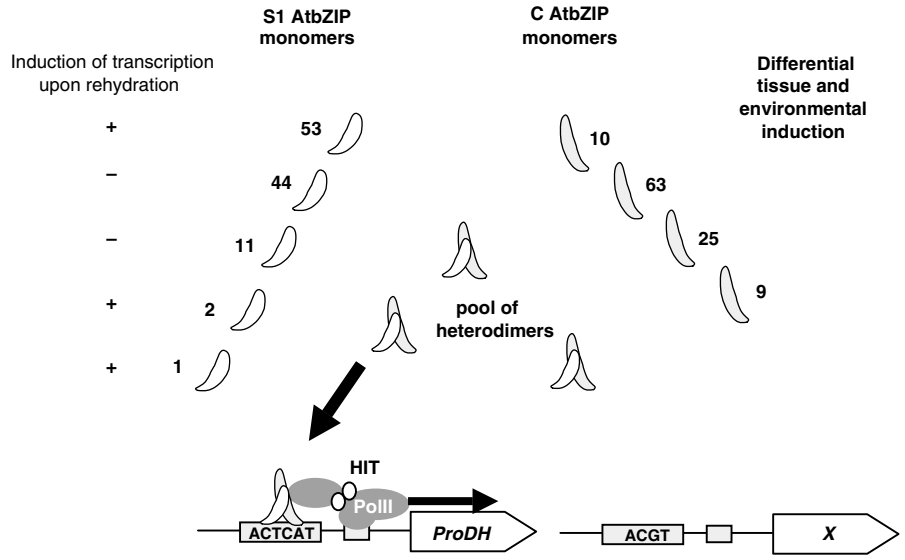


Figure 10 Model describing the combinatorial control of *ProDH* transcription by the network of group-S1/C heterodimers. (1) Developmental and tissue-specific cues define expression of A*tbZIP* genes and consequently, the cellular amount of transcription factors. In particular, AtbZIP53, AtbZIP1, and AtbZIP2 are transcriptionally induced by environmental signals, for example, rehydration after osmotic stress (hypo-osmolarity). As a consequence, colocalisation defines which heterodimers can be formed. (2) Specific group-S1 and C heterodimers are formed with high affinity before DNA binding. Therefore, a pool of specific heterodimers can be postulated to exist in the nucleus. (3) Heterodimers select target genes, such as *ProDH*. Moreover, group-S1/C heterodimers exhibit enhanced transactivation capacity (heterodimer-induced transactivation, HIT), which is reflected by efficient recruitment of transcriptional cofactors and subunits of the polymerase II-dependent transcription initiation complex (PolII). We therefore postulate that bZIP heterodimerisation acts as a crucial mechanism for signal integration on the level of transcription.

the *ProDH* gene is a direct target of the bZIP transcription factor AtbZIP53. Loss-of-function approaches show, however, that although AtbZIP53 clearly mediates hypo-osmolarity-induced *ProDH* transcription, it is not the only factor involved in this process, as the elimination of AtbZIP53 activity does not result in complete lack of ProDH activation. In this respect, additional data obtained in transient expression assays in *Arabidopsis* protoplasts suggest that the closely related group-S1 bZIP proteins might also participate in *ProDH* regulation (Satoh *et al*, 2004). In conclusion, a subset of five group-S1 bZIP factors including AtbZIP53 seems to have overlapping, partly redundant functions. As single and double knockout lines (FW, KD and WDL, unpublished results) only partly reduce *ProDH* target gene expression, multiple ko lines are needed to assess their function.

HIT—a central mechanism to modulate activation properties of bZIP transcription factors in plants

By means of yeast two-hybrid analysis, interaction studies in protoplasts and BiFC, we could establish that AtbZIP53 homodimerisation and heterodimerisation with other group-S members is not favoured, but instead AtbZIP53 preferentially interacts with group-C bZIP proteins. A similar heterodimerisation behaviour has already been described for homologous proteins from tobacco (Strathmann *et al*, 2001) and parsley (Rügner *et al*, 2001). We have also determined that this interaction relies on the ZIP structure, as it could be abolished by introducing amino-acid exchanges that disrupt the α-helical structure of the ZIP domain. Moreover, BiFC assays confirmed the occurrence of this preferred heterodimerisation *in planta*, as revealed by fluorescence due to complementation of two unfunctional parts of the YFP protein fused to AtbZIP53 and AtbZIP10. The cellular localisation of YFP fusions of AtbZIP53 and AtbZIP10 could also be assigned to the nucleus, in support of a role in transcription.

While the BiFC method provides information about the cellular localisation of an interaction, it does not give quantitative information about affinity of the protein partners. To address this question, we reproduced the principle of the yeast two-hybrid system to *Arabidopsis* protoplasts. Basically, results obtained in the yeast system could be confirmed *in planta*. Moreover, this assay allows discriminating weak interactions inside the group-S1 from strong interactions between groups S1 and C.

In the model plant *Arabidopsis thaliana*, 75 bZIP genes have been identified (Jakoby *et al*, 2002) and heterodimerisation has been experimentally shown for closely related bZIP proteins, like the GBFs (Schindler *et al*, 1992) or Hy5/HyH (Holm *et al*, 2002). Based on data obtained for dimerisation specificity of human bZIP proteins, a model for prediction of the *Arabidopsis* bZIP dimerisation has been proposed (Deppmann *et al*, 2004). However, group-S1 and group-C interactions, which have been confirmed experimentally, have not been predicted. Dimerisation of human bZIPs has been studied experimentally by means of protein-CHIP techniques (Newman and Keating, 2003); however, bioinformatic studies using these experimental data conclude that no easy rules can be inferred for predictions (Fong *et al*, 2004). Hence, further experimental studies are needed to assess the complex heterodimerisation patterns in *Arabidopsis*. In general, group-S1 and -C have been found to form specific heterodimers (Ehlert *et al*, 2006). As group-S1 and -C share an unusual long ZIP domain harbouring eight repeats, we assume that the observed high-affinity interaction is due to the intrinsic structural properties of this ZIP domain.

In vitro DNA-binding experiments show that group-S1 bZIP proteins can bind DNA without a group-C partner. This finding could be explained by monomeric DNA binding which has been described before (Metallo and Schepartz, 1997; Cranz *et al*, 2004). Alternatively, structural analyses of bZIP proteins have demonstrated that by DNA binding the conformation of the basic domain renders into a coiled structure which is linear with the ZIP domain (Pu and Struhl, 1991). Hence, DNA–bZIP protein complex formation might alter dimerisation properties. Our experimental system makes use of the GAL4 DNA-binding domain and hence, measured protein–protein interactions are independent of DNA-binding activities. We therefore conclude that under *in vivo* conditions, group-S1 and C ZIP domains show a strong affinity without need to be associated to its target promoter DNA. It is therefore tempting to speculate that pools of group-S1/C heterodimers are formed in the nucleus, which has implications on transcriptional control of target genes (Figure 10).

The functional impact of bZIP heterodimerisation *in vivo* has rarely been addressed *in planta*. A recent publication deals with the rice bZIP factor LIP19/OBF1 in regulation of low-temperature-induced genes (Shimizu *et al*, 2005). In this case, heterodimerisation is postulated to modulate DNA-binding specificity. This mechanism is related to the well-described prototype in animals in which Fos homodimers cannot bind DNA but by heterodimerisation with Jun, another bZIP protein, the AP-1-binding site is recognised (Kouzarides and Ziff, 1988). Depending on the recruitment of bZIP heterodimerisation partners, members of the small MAF proteins can activate or repress transcription of their cognate target genes (Igarashi *et al*, 1994; Dlakic *et al*, 2001). MAF proteins are characterised by an extended DNA-binding domain and therefore guide their heterodimerisation partners to promoter binding sites which are not recognised by the bZIP on its own. In contrast to these examples, specific heterodimers of AtbZIP53 lead to strong transactivation, although neither of the single transcription factors displays significant activation potential by itself. As we have observed a comparable synergistic enhancement of transcription in a system based on ACTCAT binding and a GAL-UAS binding, this mechanism is independent of protein–DNA complex formation. These data indicate that formation of defined heterodimers generates new properties, which are relevant for gene activation under *in vivo* conditions. bZIP heterodimerisation might lead to the interaction with cofactors which cannot be recruited by homodimers. Alternatively, heterodimerisation might result in structural changes that might alter protein-interaction surfaces and recruitment properties. In animal systems, Jun/Fos heterodimers have been found to promote interaction with general transcription factors (GTFs) like TFIIE-34, TFIIE-30 and TFIIF-74 *in vitro* (Martin *et al*, 1996). Here, we provide conclusive evidence for the impact of a HIT mechanism *in vivo*. The isolation of protein-interaction partners of the group-S1/C heterodimers will give future insight into transcriptional control. In a comprehensive study, all group-C and -S1 interactions have been analysed, indicating that HIT is not restricted to AtbZIP53/AtbZIP10 heterodimers but is a general feature of several group-S1/C heterodimers. We therefore postulate that HIT is a general and important mechanism for fine tuning of transcription in plants.

The C/S1 network of bZIP transcription factors—signal integration on the level of transcription

A fine-tuned mechanism can be anticipated by the disclosed participation of different bZIP factors in the regulation of common target genes. In particular, here we have studied the important contribution of distinct heterodimer combinations, whose concerted action will be ultimately dependent on common localisation, protein expression levels and functional capacity to interact.

Northern analysis described in this work and web-based expression resources demonstrate that members of the C/S1 network are transcriptionally regulated by various developmental and environmental cues. As L-Pro homeostasis has to be tightly regulated, the C/S1 network might serve to adjust gene transcription in response to a variety of endogenous and exogenous signals. In particular, *AtbZIP53*, *AtbZIP1*, and *AtbZIP2* have been shown to be activated by hypo-osmolarity (RARGE database: http://rarge.gsc.riken.jp) and promoter:GUS assays reveal that *AtbZIP53* and *AtbZIP10* are upregulated by hypo-osmolarity. As heterodimerisation has been shown *in vivo* in the nucleus independent of DNA binding to target sequences, we assume that depending on the concentration of particular bZIP proteins, pools of homo- or heterodimers can be formed. These dimers compete for promoter target sites such as the ACTCAT motif in order to activate or repress transcription (Figure 10).

In the case of AtbZIP53 and AtbZIP10, it has been shown that the formation of heterodimers depends on the concentration of both partners. In addition, the amount of functionally active bZIP protein in the nucleus is regulated by various means. For instance, post-transcriptional mechanisms, like sucrose-induced repression of translation (SIRT) (Wiese *et al*, 2004) have been described for bZIP11/ATB2 and other S1-like bZIP genes. Nuclear translocation of AtbZIP10 has been shown to be controlled (KH, unpublished results) by the protein-interaction partner LSD1 (Dietrich *et al*, 1997). In summary, availability of bZIP heterodimerisation partners is regulated in a highly complex manner by transcriptional and post-transcriptional mechanisms.

The biological function of the members of the C/S1 network is not well established. Recent findings provide evidence that AtbZIP10 is involved in oxidative stress response and LSD1-mediated programmed cell death (KH, unpublished results). Accordingly, proline metabolism has been postulated to play a crucial role in cell death signalling (Deuschle *et al*, 2004) and very recently, a function in pathogen-induced hypersensitive response has been demonstrated (Fabro *et al*, 2004). We therefore assume that the C/S1 network is not only involved in recovery after osmotic stress but also represents a general regulatory circuit in protection against oxidative stress. Owing to the well-characterised promoter elements, the growing knowledge on *trans* acting factors and the physiological role of proline in cell-death, *ProDH* will develop as a valuable marker gene for oxidative stress responses.

Group-C bZIP factors are highly related to well-characterised bZIP proteins involved in seed-storage protein synthesis, such as O2 in maize (Schmidt *et al*, 1992). In *Arabidopsis*, AtbZIP10 and AtbZIP25 have been implicated in regulation of seed-storage protein genes (Lara *et al*, 2003). *ProDH* transcription is also highly controlled during seed development (Nakashima *et al*, 1998). Hence, we assume that besides gene regulation in oxidative stress response, the

C/S1 network is involved in developmentally regulated gene control.

A characteristic feature of the C/S1 network is overlapping functional redundancy. Single ko in group-S1 or -C could not completely abolish *ProDH* gene activation. Heterodimerisation of group-S1 and -C bZIP factors provide an efficient mechanism to integrate signals and to adjust target gene transcription. A functional study of the bZIP proteins, the identification of target genes and a detailed expression and heterodimerisation analysis is needed to further elucidate the impact of the C/S1 network on gene regulation in higher plants.

Materials and methods

Plant material, plant treatment, plant transformation

For protein isolation, protoplast preparation and ChIP, *A. thaliana* ecotype Columbia (Col-O) was grown on soil under controlled environmental conditions at 16 h light/8 h dark cycles. To improve germination uniformity, plants were pretreated at 4°C for 2–4 days. Hypo-osmolarity treatment was performed according to Satoh *et al* (2002). Floral dip transformations were performed by using *Agrobacterium tumefaciens* strains GVG3101 and wild-type *A. thaliana* (ecotype Col-0) (Weigel and Glazebrook, 2002).

Identification of T-DNA mutants

Seeds of the T-DNA insertion mutants *atbzip10* (NASC ID: N606031) and *atbzip53* (NASC ID: N569883) were obtained from the Nottingham *Arabidopsis* Stock Centre. Homozygous mutants were identified by PCR as described in (http://signal.salk.edu/tdnaprimers.2.html). *AtbZIP53*-specific primers were 5′-CTGCAGCTTAG GACAGCTCATCACCA-3′ (AtbZIP53pGUSf) and 5′-GTCGACCTCGTT GACTTTTTGACTTC-3′ (AtbZIP53pGUSr), *AtbZIP10*-specific primers were 5′-TCTTCTCCATTGACGATTTCTC-3′ (AtbZIP10for) and 5′-TGATCTTCCGAGAAGCATCGG-3′ (AtbZIP10rev), the T-DNA-specific primer was 5′-TGGTTCACGTAGTGGGCCATCG-3′ (Lba1). Homozygous *atbzip10* and *atbzip53* T-DNA insertion mutants were crossed and in the f2-generation double mutants were identified by PCR.

Molecular biological techniques

Standard DNA techniques have been described in Sambrook *et al* (1989). Plant RNA was isolated using TRIZOL Reagent (Life Technologies, Rockville, MD, USA), Northern analysis has been described in Heinekamp *et al* (2002). Hybridisation probes were produced by PCR amplification from cDNA using specifically designed primers. *ProDH*: 5′-TGGTCGGTCTTTGTCAGCAT-3′ and 5′-ATGGCAACCCGTCTTCTCCG-3′; *AtbZIP53*: 5′-CATACAAAGACAC GAACAAATTGAA-3′ and 5′-GTGCTTGAGATGGTTGAAGAAATTA-3′; *actin8*: 5′-GCTGGATTCGCTGGAGATGA-3′ and 5′-AGGTCTCCATCT CTTGCTCG-3′, respectively. A hybridisation probe for AtbZIP10 was obtained by digestion of pDONR201-AtbZIP10 with *Bsp*1407I. Western analysis was described in Heinekamp *et al* (2002) using a polyclonal HA-antibody (Santa Cruz, Santa Cruz, CA, USA). Vector constructions are described in detail in Supplementary methods S1. ChIP was performed according to Johnson *et al* (2001) with modifications described in Supplementary methods S1.

Yeast two-hybrid analysis

ProQuest® Y2H vectors pDEST22 and pDEST32 containing the different AtbZIP genes were transformed into the yeast strain MaV203 (Invitrogen, Karlsruhe, Germany) according to the protocol of Dohmen *et al* (1991). Handling of yeast cultures, plate growth assays and quantitative β-galactosidase assays were performed as described in the Clontech Yeast Protocols Handbook (1996).

Protoplast transformation and GUS assays and BIFC

Protoplast isolation and transformation was performed as described by Ehlert *et al* (2006). BiFC was performed in 6-week-old *Nicotiana benthamiana* plants after *Agrobacterium*-mediated transient transformation according to Walter *et al* (2004). For analysis of the BiFC signal, a confocal microscope (Leica TCS SP2 AOBS) was applied.

Electrophoretic mobility shift assays

EMSAs were performed as described by Lara *et al* (2003). The oligonucleotides PRODHS (5′-ATTCATCATCC ACTCAT CCT-3′) and PRODHAS (5′-AGATATGAAGG ATGAGT GGA-3′) were used as dsDNA probe.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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