Interactions of Class I Homeodomain Leucine Zipper Proteins

Ole Herud

Degree project in biology, Bachelor of science, 2008
Examensarbete i biologi 30 hp till kandidatexamen, 2008
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Introduction

Plants react like all organisms to endo- or exogenous stimuli by the adjustment of their proteome. The expression could be modified on different steps on the way between gene and functional protein or even later on by often reversible modification of existing proteins. Proteins could survive long times and can travel between cells, therefore the proteome does not necessarily resemble the transcriptome. The existence of one mRNA is neither necessary nor sufficient for the expression of the corresponding gene. A good example are maternal proteins or mRNAs allowing the survival of embryos missing essential genes as suggested in several cases (e.g. Steinborn et al., 2002).

The abundance of functional proteins depends on and is controlled by several steps: transcription, capping, splicing, polyadenylation, localisation and cleaving. The first step involves transcription factors (TFs), they bind to binding sites in the promoters of their target genes and regulate the transcription positively or negatively – alone, antagonistically or cooperatively.

Many different families of TFs are known and nearly all of them could be found in all taxa. Encouraged through this fact and the importance of homeodomain (HD) TFs like ANTENNAPEDEA in the development of Drosophila melanogaster, Mattsson et al., (1992) searched for similar proteins in Arabidopsis thaliana. They found genes in which together with the HD, another well known motif is present: a leucine zipper (LZ) (Fig. 1). The LZ is a helical structure in which (mostly) leucine residues are arranged in heptamer repeat cycles. These residues create an amphipathic protein-protein interaction motif, while the HD is responsible for RNA- or DNA-protein interactions. In addition to the DNA binding ability the HD could also contain localisation and transport signals (Bolduc et al., 2008). Because the homeodomain is – in respect to the leucine zipper – similarly localised to the DNA binding region in the b-Zip family this TF family was named homeodomain leucine-zipper Family (HD-Zip) and a similar dimerisation model was suggested.

For HD-Zip class I proteins no in vivo interaction data are published but the classes II and III have been extensively investigated.

HD-Zip class II members form multimers through disulfide bridges (Ariel et al., 2007), this could resemble the necessity of additional stable interactions in this class. Likewise the HD-Zip class III differs structurally from the other HD-Zip proteins. In between the HD and the LZ an additional four amino acid loop is present. A similar shift would prevent interactions between HD-Zip class I proteins. The HD-Zip class I proteins could therefore not interact in an identical manner (Chan et al., 1998). The class IV, members contain one loop in the LZ (Ariel et al., 2007) suggesting an interruption of the leucine zipper motif.

![Figure 1: Schematic representation of HD-Zip transcription factor (Ariel et al., 2007)](image1)

Based on sequence homologies the HD-Zip genes have been divided in 4 classes (HD-Zip class I – IV). The LZ and its localisation to the HD differ between the various classes, hence no global dimerisation model could be found.

Chan et al. (1998) proposed binding of DNA by the HD-Zip proteins as both homodimers or heterodimers with other members of their class, both being already demonstrated in vitro by Johannesson et al. (2001). Other distinct classes of HD TFs in Arabidopsis were also reported to work as dimers, or even as higher order complexes (Noyes et al., 2008).

![Figure 2: Phylogenetic relationships among investigated HD-Zip class I proteins, Aligned with ClustalX2.03 (Larkin et al., 2007) and drawn with TreeView (Page, 1996); numbers at the branches give Bootstrap 10000 values](image2)
ABA signalling through ABI4 (Rook et al., 2006). Because this signalling did not change ATHB13 mRNA abundance, the protein is most likely regulated by interaction with other proteins like phosphatases acting on any of the 11 putative phosphorylation sites reported by PhosPhAt (Swarbreck et al., 2008).

Because of the implication of these genes in the same pathways, the published interaction between AtHB6 and ABI1 and the abundance of putative phosphorylation sites, interactions of different HD-Zip class I proteins with the PP2Cs ABI1 and ABSCISIC ACID INSENSITIVE 2 (ABI2) were investigated.

Two different approaches were used; first, the direct interaction among different HD-Zip proteins, ABI1 and ABI2 in a wild-type background. Secondly, the putative dephosphorylation effect of PP2Cs on HD-Zips by using the gain-of-function mutant abi1-1, the single knock-out abi1-2 and the triple knock-out including HOMOLOGY TO ABI1 (HAB 1) knock-out (abi1-2/abi2-2/hab1 (Table 1)).

Here the dimerisation properties of 13 HD-Zip class I proteins were investigated in vivo by a Bimolecular Fluorescence Complementation (BiFC) assay. This assay uses the intrinsic property of different domains of the reporter protein yellow-fluorescence-protein (YFP) to regain their activity when the major chains come in close contact. The reassembling of the YFP takes place when two fragments, covering the N-terminal 155 (YFP\textsuperscript{N}) and the 86 C-terminal (YFP\textsuperscript{C}) amino acids get in close contact (Walter et al., 2004).

Material and Methods

For this BiFC assay a protocol provided by Kristina Rizzardi and based on an approach by Walter et al. (2004), was used. Because the protocol has been modified within the time of this study the used plants and RNA silencing suppressors are indicated under the results.

Experimental overview

Table 1: ABA signalling impaired mutants used in the study

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Effected Gen</th>
<th>Mutation Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>abi1-1</td>
<td>gain-of-function of ABI1</td>
<td>Reduce the ABA response by constitutive phosphorylation of its targets</td>
<td>Gosti et al., 1999</td>
</tr>
<tr>
<td>abi1-2</td>
<td>loss-of-function of ABI1</td>
<td>Enhanced response to ABA</td>
<td>Saez et al., 2006</td>
</tr>
<tr>
<td>abi1-2 / abi2-2 / hab1</td>
<td>loss-of-function of ABI1, ABI2 and HAB1</td>
<td>Enhanced response to ABA and reduced water loss under drought conditions</td>
<td>Saez et al., 2006</td>
</tr>
</tbody>
</table>

The open reading frame (ORF), excluding the stop codon, of 13 different HD-Zip class I genes (Table 2) have been amplified by polymerase chain reactions. Besides them, the TF DREB2A and the phosphatases ABI1 and ABI2 have been cloned.

Template cDNA was obtained from different tissues or vectors bearing the cDNA of interest. The primers were designed with attB overhangs, to allow the further use of the Gateway® system and for cloning the genes into the kanamycin resistant expression vectors pUC-SPYNE\textsuperscript{G} and pUC-SPYCE\textsuperscript{G} (Figure 3). These expression vectors have been developed by Walter et al. (2004) on the basic of the pUC19 vector. They harbour the strong constitutive 35S promoter of the cauliflower mosaic virus, attR sites and the terminator of the Nos gene (NosT). In the pUC-SPYNE\textsuperscript{G} the ORF is fused to a spacer, a c-Myc tag and the 155 N-terminal YFP amino acids. In the pUC-SPYCE\textsuperscript{G} vector the ORF is fused to the same spacer, an HA-tag and the 86 C-terminal amino acids of YFP (Figure 3).

All the inserts were sequenced once into this vectors, to ensure that a mistake-free PCR product have then been transformed into Escherichia coli. The plasmid was transformed into Agrobacterium tumefaciens strain C58C1 which harbour the helper plasmid pHCh32 which confers tetracyclin resistance (Hellens et al., 2000).

Table 2: Investigated proteins

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<td>ATHB53</td>
<td>ABI1</td>
<td>ABI2</td>
<td>DREB2A</td>
</tr>
</tbody>
</table>

Figure 3: Representation of the vectors pUC-SPYNE\textsuperscript{G} and pUC-SPYCE\textsuperscript{G}, Walter et al., 2004
The *A. tumefaciens* cells have been used for transient leaf transformation of “4- to 8-week-old” wildtype and mutant *A. thaliana* Columbia ecotype plants (Table 1) or “3- to 6-week-old” wildtype *Nicotiana benthamiana* plants.

All *A. thaliana* plants were grown under short-day conditions to increase the number of leaves while *N. benthamiana* was grown under long day conditions.

To increase the expression levels of our genes of interest, two different viral suppressors of plant mRNA silencing have been used: the potato A potyvirus helper component proteinase (Hc_Pro) and the tomato bushy stunt virus p19 protein.

For agro-infiltration, *A. tumefaciens* cultures with an optical density between 0.7 and 1 at a wavelength of 600 nm (OD$_{600}$) have been used. *A. tumefaciens* harbouring mRNA silencing suppressors were grown to OD$_{600}$ = 1, those harbouring pUC-SPYNE$^\alpha$ or pUC-SPYCE$^\alpha$ with or without the ORFs of the investigated genes have been used at an OD$_{600}$ of 0.7.

All pictures shown have been made with the confocal laser microscope TCS SP DM IRBE with a RSP500 filter. Additionally an epifluorescence microscope with a GFP filter has been used for fast screening.

### Results and Discussion

#### Growth Conditions

Growth conditions for the *N. benthamiana* plants were changed after comparing our plants with those growing at the Sveriges lantbruksuniversitet (SLU). It was noticed that plants, grown in the SLU FISON growth chambers, looked greener and developed bigger leaves (not measured). Therefore, from that moment, plants were grown in bigger pots in FISON growth chambers (hereafter referred as NB plants) instead of the medium-sized pots previously used (NM plants).

<table>
<thead>
<tr>
<th>Plant</th>
<th>l</th>
<th>t</th>
<th>Plant</th>
<th>l</th>
<th>t</th>
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<tr>
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<td>7.0</td>
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<td>2</td>
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<td></td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 3: Size of the two biggest leaves of 6 plants in cm; measuring accuracy 0.5 cm, l: longitudinal leaf length, t: transversal leaf length

The size of the leaves was different in both growth conditions. Average size of the two biggest leaves of NB plants was, after 4 weeks 10 x 9 cm (longitudinal (l) x transversal (t)) while even after 9 weeks the two biggest leaves of NM plants were on average just 7 (l) x 6 (t) cm big (Table 3).

The likelihood that NM and NB plants differ is higher than 99.9%. For the longitudinal length ANOVA revealed $P < 0.001$ ($F_{1,10} = 35.60$) for the transversal length $P < 0.0001$ ($F_{1,10} = 46.463$).

No detailed data about the leaf size at other points of time are available. It seems possible that the change in the growth is somehow related to a change in transformation efficiency. Comparison of the autofluorescence showed a decrease in the faster grown NB plants compared to NM plants. In the following pictures the kind of plants used is indicated.
Control Experiments
Three control fluorescence analyses have been done, 2 positive controls and one negative control.

- Negative control: the fluorescence profile of untransformed leaves has been analysed. Fluorescence excitation noticed here is caused by intrinsic substances (Figure 4). Fluorescence signals in a leaf sample that did not differ clearly from signals obtained in this negative control were not be assumed as a positive result.

A reduction of autofluorescence (AF) in the faster and bigger grown N. benthamiana plants compared to the first ones is observable. Autofluorescent substances are mainly aromatic and several are implicated in plant defence and adaptation to stress. A higher level of stress is mostly accompanied by a slower growth rate. Hence fast growth rates complemented by small stress related AF seems likely. One explanation for this stress could be the low humidity in the growth rooms of 37 % and the high air circulation, leading to a reduced temperature at evaporating surfaces.

- The second control experiment done was transformations with a GFP construct under the 35S promoter and fused to a signal targeting to the endoplasmic reticulum (er::GFP). This has been done to get an insight in the transformation efficiency (transformation control). Plants have been transformed with the construct alone or together with p19\(^{\text{pBIN61}}\). Signals in the cytoplasm (Figure 5) and in cytoplasmic bodies (not shown) have been noticed.

- The second positive control done was transformations with the b-Zip transcription factor bZIP63 cloned into the pUC-SPYNE\(^{\text{G}}\) and pUC-SPYCE\(^{\text{G}}\) vectors. Due to the already reported homodimerisation of this protein the functionality of the BiFC assay with these particular vectors has been demonstrated by Walter \textit{et al.} (2004).

Figure 4: Fluorescence analysis of untransformed N. benthamiana plants; A (NB): YFP-signal in stomata; B (NM): YFP signal in central localised bodies and probable on the epidermal surface; TM: transmission pictures, show the focal plane for orientation in the tissue; YFP: fluorescence, detected in the bandwidth of 514 - 540 nm; AF: signal in the bandwidth of 652 - 800 nm; OL: inverted multiplied overlays of all detected bandwidths for co-localisations

Figure 5: NB plants transfected with er::GFP (cotransfections with p19\(^{\text{pBIN61}}\)) after 40 h

YFP fluorescence signals are detectable in nearly all cells. Stomata (Fig. 4 A) and especially thick cell walls are fluorescent, prohibiting the differentiation between positive signals and AF in these regions. In the case of chloroplasts, comparison between YFP signals and AF signals allows to exclude them as YFP signal (not shown). AF signal in the bandwidth of 652 - 800 nm is generally low. This was expected because the manufacturer proclaims that the use of RSP500 beam splitter only allows detection between 500 and 600 nm.

Further complication in the detection of interactions
is due to unknown bodies notable in several cells (Figure 4 B). They emit fluorescence in the same wavelength than the YFP signal and are observable both in A. thaliana and (mainly) medium-size N. benthamiana plants even when those have not been agroinfiltrated. In most cases where these bodies have been noticed in untransformed plants, obvious non specific signals could be detected in the environment of the affected cells. These signals were e.g. located on the surface. In later analyses it has been tried to eliminate these false positive signals by analysing the environment.

A very faint YFP signal was shown in the bZIP63 positive control, this was located in small bodies near the cell wall (Figure 6). Sequencing of the two constructs revealed they both carried the C-terminal part of YFP and could thereby not complement each other – the signals are wrong positives. Besides this the sequence differed in some irrelevant details, the peptide starts 2 amino acids before the canonical start methionine and contains 4 silent mutations.

**Interaction tests**

Fluorescence signals in the cytoplasm indicate 6 different dimerisations: AtHB5 with AtHB6, AtHB7 and DREB2A (Figure 7) as well as AtHB7 and AtHB12 homodimers and AtHB7 / AtHB12 heterodimers (of 45 possible interactions tested). The table 4 summarises the positive results. Because all noticed interaction partners are implicated in similar pathways an interaction seems possible.

**Table 4:** positive interactions; Yes: interaction in cytoplasm, No: no interaction in cytoplasm noticed, NA: not analysed, triple: abi1-2, abi2-2, hab1 triple knock-out.

<table>
<thead>
<tr>
<th>Background \ Interaction</th>
<th>A. thaliana wildtype / N. benthamiana</th>
<th>Triple</th>
</tr>
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<tbody>
<tr>
<td>AtHB5/AtHB6</td>
<td>Yes</td>
<td>NA</td>
</tr>
<tr>
<td>AtHB5/AtHB7</td>
<td>Yes</td>
<td>NA</td>
</tr>
<tr>
<td>AtHB5/DREB2A</td>
<td>Yes</td>
<td>NA</td>
</tr>
<tr>
<td>AtHB7/AtHB7</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>AtHB7/AtHB12</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>AtHB12/AtHB12</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
In AtHB7 and AtHB12 there are respectively 5 and 4 possible phosphorylation sites as reported by PhosPhAt (Swarbreck et al., 2008). They could be directly affected by the knocked-out PP2Cs. Actually, other HD-Zip, AtHB6, has been already reported as a direct target of ABI1 (Himmelbach et al., 2002). But also an indirect effect is possible. The different PP2Cs could affect different phosphorylation sites of the HD-Zip class I proteins, different proteins or they could modify other genes antagonistically.

No interactions have been observed between HD-Zip genes and the PP2Cs ABI1 and ABI2, but it is possible that such interactions could not be detected.
because of methodology constrains. To detect an interaction both partners have to interact at least 60 seconds (Hu et al., 2002) it is possible that dephosphorylation reactions, as a rapid cell response to stress, are too fast to be detected. All interactions noticed have been localised in the cytoplasm. While YFP signals in small bodies have been ignored because they have been noticed in untransformed plants, no nucleus specific interaction was observable. Different approaches could explain this: in the case of HD-Zip class II Ariel et al. (2007) suggested inactivation through multimerisation, our results suggest either an unspecific localisation or even an exclusion out of the nucleus (Figure 8 B). If the observed HD-Zip class II dimers bind DNA they could not do this in the noticed localisation. HD-Zip proteins could in contrast be excluded of the nucleus by dimerisation like it was suggested for HD-Zip class I proteins.

A different explanation is the binding of RNA additional to DNA. knotted-1 and several Drosophila melanogaster HD proteins are able to bind their own RNA, allowing them to autoregulate themselves (Bolduc et al., 2008). A similar mechanism seems also possible for HD-Zip class I genes.

Reassembled YFP has a half-life of more than 24 h (Hu et al., 2002), it is possible that the interaction partners are transported to different compartments within this time, indicating localisation at places they are not active, such as the cytoplasm.

The mode of action could be revealed by ChIP experiments, the effect of the stability of reassembled YFP could be excluded by antibody studies.

The interaction experiments done with AtHB7 and AtHB12 in wildtype and PP2C knock-out background show that HD-Zip interactions at least in some cases depend on additional factors. To study these factors analysis in A. thaliana under the natural promoter would be necessary. Interactions may only occur in some tissues or specific conditions, even when the proteins are co-expressed. This cell dependent influence was not examined by the assay.

But like always negative results do not proof no interaction. Especially on interaction studies in mutants background only preliminary data be on hand because the experiments have not been repeated. Also in the case of positive results caution is necessary because interactions of proteins under the strong 35S promoter do not necessarily resemble normal behaviour.

The 35S promoter could lead to an overexpression, allowing interactions which would not occur in the wildtype. A strong indication for this effect were the often noticed small inclusion bodies in many plants (Table 5). Plants tend to destroy proteins of overexpressed genes by transporting them to degradation bodies. Here the environment is strongly unphysiological, this changes and mostly inactivates proteins. In this study such bodies have been ignored, but the bodies suggest that HD-Zip proteins could interact with most others HD-Zip proteins and with the PP2C ABI2 and the TF DREB2A when the concentration is high enough. On the other hand these bodies could also contain autofluorescent substances so the florescent signals obtained have not been related to HD-Zip proteins. In the case of the bZIP63 control, positive signals have to result due to autofluorescence, because only the c-terminal part of YFP was present.

To study in vivo interactions under physiological conditions it would be necessary to clone the genes under the natural promoters and fuse them with an easy-to-notice interaction detector like a split version of β-galactosidase. This would allow a total overview about interaction in the whole plant. ChIP analyses in progress will also allow conclusions about the modes of action.

### Table 5: YFP signals in cytoplasmic bodies in interaction studies between the horizontal and the vertical interaction partner;

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<tbody>
<tr>
<td>AthB5</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes*</td>
<td>+</td>
<td>NA</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
<td>AthB6</td>
<td>No</td>
<td>Yes*</td>
<td>Yes</td>
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</table>

Yes: positive signals in cytoplasmic bodies obtained, +: YFP signal in cytoplasm, No: no signal, NA: not analysed
Acknowledgements

I would like to thank my supervisor Ana Elisa Valdés for practical and theoretical help with all experiments. Peter Engström, Henrik Johansson and Elin Övernas for helpful discussions about the HD-Zip genes. I’m also grateful to the whole Lab for the kind welcome, nice atmosphere and great help with the experiments, especially to Kristina Rizzardi for her help with the BiFC assay.

I’m also thankful to Annelie Carlsecker, George Lomonossoff, Tadeusz Wroblewski and Eugene Savenkov for plants, plasmids, seeds and technical help and to Peter Bozhkov for providing the epifluorescent microscope.

Literature


