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STRUCTURE-FUNCTION ANALYSES OF THE ARABIDOPSIS E3 LIGASE ARI12 AND THE POTENTIAL SUBSTRATE NCBP

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Abstract

Ubiquitin ligases E3 are key protein regulators mainly known for ubiquitylating proteins targeted for degradation by the 26S proteasome. However, depending on the ubiquitylation site and number of attached ubiquitin moieties the fate of the substrate can be different. The class of RBR ubiquitin ligases E3 consist of two RING (really interesting novel gene) domains (RING1 and 2) separated by a in between RING domain (IBR). E3 ligases are known to bind both the substrate and the ubiquitin conjugating E2 enzyme which delivers the activated ubiquitin for ligation. ARI12 is a member of the RBR ubiquitin ligases E3 of *Arabidopsis thaliana*. Previous studies have shown that ARI12 interacts with nCBP (novel cap binding protein), a member of eIF4E (eukaryote initiation factor 4E) that play key roles in mRNA cap-binding, mRNA nucleus export and translation initiation. In *ari12* mutants the abundance of nCBP is higher suggesting that ARI12 regulates nCBP's stability. The aim of my project was the identification of those ARI12 domains which interact with nCBP and another member of the eIF4E family. Therefore I cloned truncated versions of the ARI12 gene in Yeast Two Hybrid vectors and performed protein interaction assays. Using both growth assays and beta-galactosidase quantifications I could show that ARI12 interacts specifically with nCBP and not with eIF4E. While the N-terminal-RING1 construct interacts with nCBP, the RING2-C-terminal construct did not. However, both interactions were enhanced in combination with the IBR domain suggesting that the RING1 and the IBR alone are sufficient to mediate interaction with nCBP. These findings are in contrast to that of the human homologs of ARI12 and nCBP, HHARI and 4EHP, which only interact with the RING1 domain.
To determine the biological role of the ARI12 and nCBP interaction I generated overexpression constructs and transformed them into wildtype and mutants of *ARI12* and *nCBP*. The characterization of these lines is beyond this diploma thesis

Kurzfassung

Ubiquitin Ligasen E3 sind wichtige Proteinregulatoren. Diese Ligasen ubiquitinylieren zum größten Teil Proteine, die für das 26S Proteasom bestimmt sind und dort abgebaut werden. Je nach Ubiquitinylierungsstelle und Anzahl der transferierten Ubiquitinmoleküleinheiten entscheidet sich das Schicksal der Substrate. Die Klasse der Ubiquitin Ligasen E3 besteht aus 2 RING (really interesting novel gene) Domänen (RING1 und 2), die durch eine IBR (in between RING) Domäne unterbrochen wird. E3 Ligasen binden am Substrat und am Ubiquitin konjugierenden Enzym E2, welches aktiviertes Ubiquitin für die Ligation bereitstellt. ARI12 gehört zur Familie der RBR Ubiquitin Ligasen E3 der Modellpflanze *Arabidopsis thaliana*. Frühere Studien zeigten, dass ARI12 mit nCBP (novel cap binding protein) sowie einem Mitglied der Familie eIF4E (eukaryote initiation factor 4E), das eine zentrale Rolle im Binden des Caps von mRNA, mRNA Export aus dem Zellkern und in der Translationsinitiation einnimmt, interagiert. In *ari12* Mutanten wurde nCBP im größeren Ausmaß vorgefunden, woraus man schließen kann, dass ARI12 die Stabilität von nCBP reguliert. Ziel meines Projektes ist die Identifizierung jener ARI12 Domänen, die mit nCBP und einem anderen Mitglied der Familie eIF4E interagieren. Zu diesem Zweck klonierte ich Teilfragmente des Gens ARI12 in Yeast Two Hybrid Vektoren und führte damit Protein-Interaktionsanalysen durch. Sowohl durch Wachstumsassays als auch unter Anwendung von Beta-Galactosidase Quantifizierungen konnte ich zeigen, dass ARI12 spezifisch mit nCBP, aber nicht mit eIF4E interagiert. Während der N-terminale Konstrukt RING1 mit nCBP Wechselwirkung zeigte, war keine Proteininteraktion bei RING2-C-Terminus mit nCBP zu beobachten. Jedoch konnte die IBR Domäne in Kombination mit RING1 oder RING2 beide Interaktionen jeweils verstärken, was darauf hinweist, dass RING1 und IBR alleine für die Interaktion mit nCBP ausreichen. Diese Resultate decken sich nicht mit jenen der homologen Gene im

Menschen von ARI12 und nCBP, nämlich HHARI und 4EHP, welche nur über die RING1 Domäne miteinander interagieren.

Um die biologische Funktion von ARI12 und nCBP zu untersuchen, konstruierte ich Überexpressionskonstrukte und transformierte diese in Wildtyppflanzen und Mutanten von ARI12 und nCBP. Die Charakterisierung dieser Linien geht allerdings über den Rahmen dieser Diplomarbeit hinaus.

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1 Introduction

1.1 The ubiquitin 26S proteasome pathway

26S proteasome mediated proteolysis in eukaryotes has its function in coordinating regulatory proteins and enzymes. Target proteins for degradation are tagged in the ubiquitylation process and processed by the proteasome in a system termed UPS (ubiquitin-proteasome system). 26S proteasomes only exist in eukaryotes, but archaea and bacteria possess 20S proteasomes that have less complex subunit compositions. (Naujokat et al. 2002).

The ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2 and the ubiquitin ligase E3 are the key enzymes in the ubiquitylation process. E3 recruits ubiquitin that is thioester-linked to an E2, attaches it to the substrate and thus is thought to facilitate the ubiquitylation (Freemont et al. 1991; Freemont 1993; Lorick et al. 1999; Hatakeyama et al. 2001). Ubiquitylated target proteins, especially those with substrate Lys 48 residue linked polyubiquitin chains, are mainly degraded in the 26S proteasome. Deubiquitylating enzymes (DUBs) release both ubiquitin molecules and target intact (before proteolysis) or ubiquitin alone (after proteolysis of the target in the proteasome) (Figure 1.1). However, the fate of the substrate depends on the number of the ubiquitin molecules attached (mono-, multi- or polyubiquitylation) and the location of their attachment (Vierstra 2009).
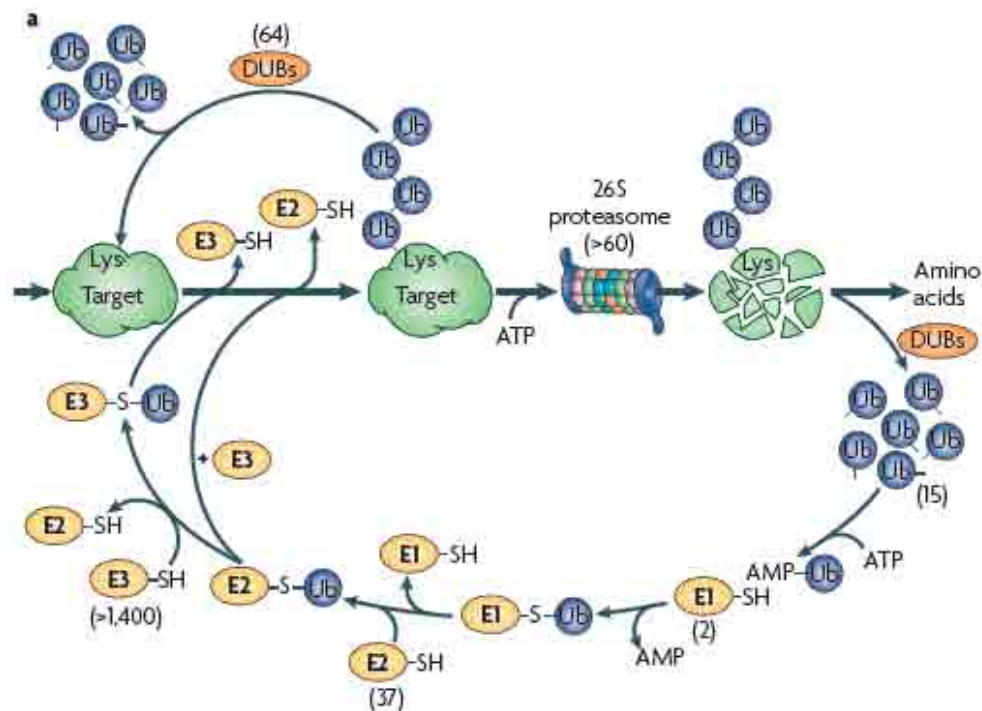
Figure 1.1. The 26S ubiquitin proteasome system

Ubiquitin is attached to the Cys residue of E1 (ubiquitin-activating enzyme) in an ATP-dependent step. The thioester bond activates the ubiquitin moiety that is transferred to an E2 (ubiquitin-conjugating enzyme) by trans-esterification. E2 is often the ubiquitin donor, while E3 (ubiquitin ligase) identifies the target and catalyses the ubiquitylation. The fate of the target proteins is determined by the number and manner of attached ubiquitin molecules. Deubiquitylating enzymes (DUBs) release the ubiquitin moieties from the protein and recycle them (Vierstra 2009).

Monoubiquitylation is a non-proteasomal signal: the ligation of a single ubiquitin molecule to the target is a transport signal in the endocytic pathway. Adaptor proteins deliver ubiquitylated cargo to the vesicle budding machinery (Schnell et al. 2003; Umebayashi

2003). Furthermore monoubiquitylated histones (one sort of histone modification) are involved in DNA damage response (Hofmann 2009).

In contrast, attaching polyubiquitin chains to Lys 48 residue on the substrate targets the ubiquitylated protein to the 26S proteasome pathway and its degradation (Freemont 2000; Jackson et al. 2000; Joazeiro et al. 2000; Thrower et al. 2000). Target proteins ubiquitylated with a polyubiquitin chain at the Lys 63 residue are either activated, or involved in stress response or DNA damage repair (Hoege et al. 2002; Shi et al. 2003; Zhou et al. 2004) (Figure 1.2).
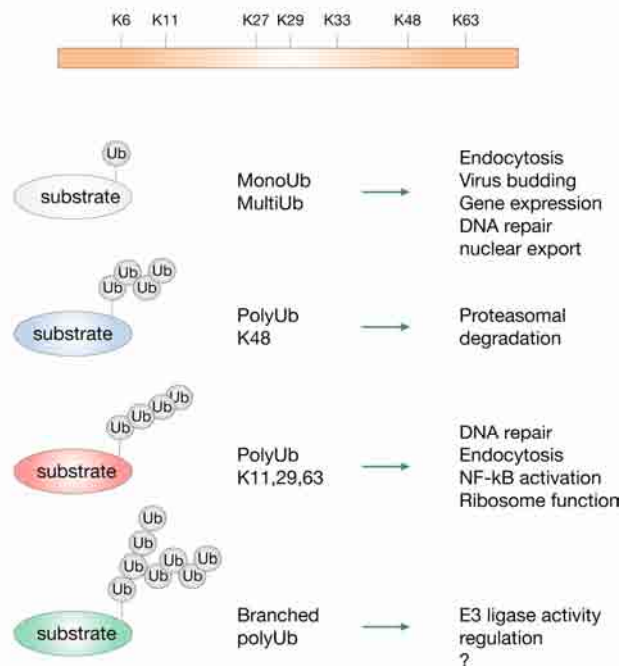
Figure 1.2. Fates of target proteins attached with ubiquitin

Depending on the number of attached ubiquitin molecules and binding location the fate of the target proteins is determined. Question marks indicate unknown functions. Monoubiquitylation of the target can be a signal in the endocytic pathway, while monoubiquitylated histones are involved in DNA repair. Polyubiquitylation at the Lys 48 residue leads to proteasomal degradation of the substrate (Woelk et al. 2007).

It has been reported that ubiquitin-26S proteasome system (UPS) proteins make up 6% of the *Arabidopsis thaliana* proteome. *Arabidopsis thaliana* is used as a model plant in the field of molecular biology, since its genome is fully sequenced. The UPS of *Arabidopsis* targets regulators of hormone signalling, chromatin structure and transcription and is involved in plant morphogenesis, selfincompatibility, responses to environmental challenges and pathogens (Vierstra 2009).

1.2 Classes of ubiquitin ligases E3

Ubiquitin ligases E3 determine the specificity of the UPS system, since their specific recognition modules are responsible for the identification and interaction with their substrates. Currently four main types of ubiquitin ligases E3 are known, classified by their mechanisms and composition: HECT, RING, U-box and cullin-RING ubiquitin ligases (CRLs). CRLs again can be divided into four subtypes that all share common modules: a cullin, RING-box 1 (RBX1 binding to E2-ubiquitin) and a variable target recognition domain (Figure 1.3.).

HECT, RING and U-box ligases are single polypeptides, whereas the cullin-RING ligases (CRLs) consist of different subunits that form an enzyme complex. HECT E3 ligases form

a thioester intermediate at a Cys with ubiquitin before ubiquitylation. RING or U-box proteins have a structurally related RING or U-box motif that interacts with the E2-ubiquitin intermediate and allosterically promotes attachment of ubiquitin to the substrate (Vierstra 2009).
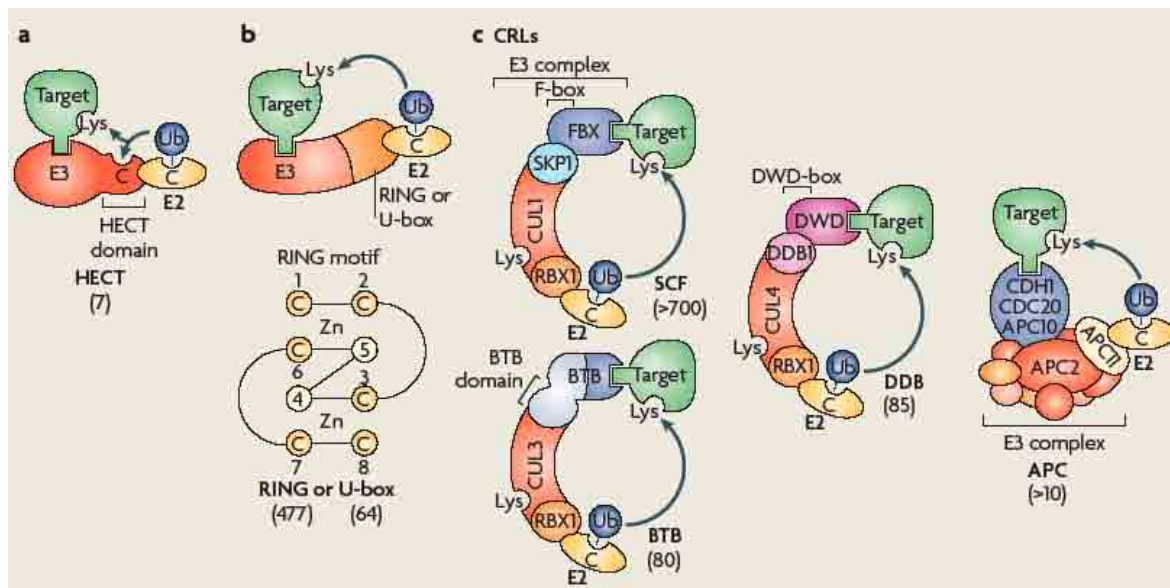
Figure 1.3. Types and organization of ubiquitin ligase E3

In this figure (a) represents the HECT domain type, (b) the RING or U-box type and (c) the cullin-RING type in enzyme complexes of E3 ligases. (a) Ubiquitin makes a thioester bond with a Cys in the HECT domain of HECT-type ligases before ubiquitylation. RING or U-box ligases (b) are single polypeptides with a RING or U-box motif which binds the E2-ubiquitin intermediate and promotes ubiquitin attachment to the target allosterically. The cross-braced RING motif structure (position 4 and 5 can be either Cys or His) has an octet of Cys or His residues chelating two zincs, whereas the U-box stabilizes the E2-ubiquitin binding pocket by electrostatic interactions. (c) Cullin-RING ligase E3s (CRLs) are multisubunit complexes that generally contain a cullin, an E2-ubiquitin binding RING-box 1 (RBX1) and a variable target recognition module. The F-box of S phase kinase-associated protein 1 (SKP1)-cullin 1(CUL1)-F-box(SCF) E3s determines the specificity for the target and is connected to CUL1 via the bridging protein SKP1; the bric-a-brac-tramtack-broad complex (BTB) E3s have BTB proteins with a dual function that recognize targets and interact with CUL3 as well. The DNA damage-binding (DDB) E3s use WD40 domain-containing DWD proteins for target recognition and bind CUL4 via a DDB1 protein. The anaphase-promoting complex E3s (APC) consist of 11 or more subunits, including relatives of cullins (APC2) and RBX1 (APC11) and interchangeable recognition subunits are cell division cycle protein 20 (CDC20), CDC30-homology 1 (CDH1) and APC10. Yellow colours represent factors with RING motifs. The ubiquitin-binding Cys in E2s and HECT E3s is abbreviated with C. Lys at the target indicates the ubiquitin-attachment site and Lys in cullins shows Lys modified by related to ubiquitin protein 1 (RUB1). The number of predicted *Arabidopsis thaliana* genes encoding the target recognition module of the corresponding E3 type is printed in parenthesis (Vierstra 2009).

1.3   The class of RBR-proteins

The large class of ring between ring fingers (RBR) proteins are characterized by three ring finger-type domains, separated by loops: the RING1 (binds two zinc ions, folds into a classical cross-brace ring finger), the RING2 (binds only one zinc ion) and the IBR domain. The RBR domain appears to mediate protein-protein interaction. The sequence of the RBR domain is characterized by a cluster of eight Cys and His residues that bind metal ions.

RBR proteins are divided into 15 subfamilies, abbreviated by one alphabet (A-I, P, S, T, U, X, Z). Subfamily A (Ariadne-like proteins) again can be further subdivided into around 10 clusters (A0-A9). Some RBR protein subfamilies are limited to specific taxonomic groups: Subfamily S proteins are only found in viruses, subfamily T consists of the TRIAD3

proteins and subfamily Z proteins only exist in protozoa. Subfamily members C, I, P, U or X can be only observed in animals. Subfamily E and F are specific for fungi, G and H for plants. A large number of RBR proteins can be observed in higher eukaryotes, but also some occur in unicellular eukaryotes or viruses. However, archaeal or eubacterial genomes do not contain genes coding for RBR.

Two RBR proteins were found in the yeast *Saccharomyces cerevisiae*, 6 in *Drosophila melanogaster*, 10 in *Caenorhabditis elegans*, around 23 in zebrafish (*Danio rerio)* and about 15 in humans. Approximately 40 RBR proteins can be found in *Arabidopsis thaliana*, among them 16 ARI genes. Although these ARIs share conserved sequence, they have distinct gene structures with the number of exons ranging from one (*ARI3*) to 15 (*ARI5*, *ARI7* and *ARI18*).

Due to alternate splicing, spliced isoforms of RBR are widespread and some shortened isoforms might control the cellular localization and function of the corresponding larger isoform.

RBR typically consists of 200 consecutive amino acids and the ring domains usually contain: 60 residues (RING1), 50 residues (IBR) and 40 residues (RING2). Cys/his positions are more conserved in RING1 and the IBR, whereas substitutions of Cys/His with non-cysteine and non-histidine residues are only observed in the RING2 domain. While most RBR proteins are functional without RING2, parkin and some other depend on RING2 for ubiquitin ligase E3 activity.

RING1 in the human UbcM4-interaction protein 4 is very similar to a classical ring-finger domain. There is no structural information available for IBR. HHARI 3D structure reveals that RING2 folds into a novel structure binding a single zinc ion in human Ariadne protein.

The function of additional segments to and their cooperation with RBR is not elucidated, but hydrophobic segments suggest functions in and at membranes. Subfamily D member dorfin was unable to bind ubiquitylated substrates without the carboxy-terminal (contains hydrophobic segments).

Apart from expected combination of the RBR region with domains and motifs known from the ubiquitylation pathway the association with helicase domains (DXDc and HELICc), nucleic-acid-binding domains (KH and RRM) and protein-binding domains (RWD, Armadillo/HEAT repeats and ankyrin segments) would show functions in the gene expression regulation. Ubiquitylation and RNA metabolism generally appears to have functional links.

The function of most RBR family members is still unknown, but a subset is reported to have ubiquitin ligase E3 activity. Analysed RBR proteins have a wide range of different functions: regulatory, signalling functions, control of protein quality. XAP3 and rat TBCK1 were discovered with regulatory domains of a protein kinase. Ariadne-like ubiquitin ligase E3 PAUL was found in a complex with a muscle-specific kinase.

Mutations in the gene encoding parkin cause some types of parkinsonism and are also susceptible to intracellular pathogens (*Salmonella typhi*, *S. parathyphi* and *Mycobacterium leprae* and cancer). Several other RBR proteins are involved in human neurodegenerative diseases, susceptibility to infections, and cancer.

Ubiquitin ligase E3 activity is reported for 15 RBR proteins. They interact with E2s and catalyse the ubiquitylation of targets. For most RBR proteins (with some exceptions binding in the non-RBR region), the RING domains are essential for recruiting specific E2s and binding substrates. RBR proteins are considered single-molecule ubiquitin ligases E3.

Parkin and parkin-like cytoplasmic protein (PARC) interact with components of the SCF-like ubiquitin ligase E3 complex. Parkin and dorfin protect dopaminergic neurons from consequences of mitochondrial damage and are involved in endoplasmic reticulum-associated degradation pathway (ERAD). Toll receptors TLR4 and TLR9, plasma membrane receptors, are substrates for the RBR protein TRIAD3A (controls pro-

inflammatory responses in Toll pathway). Parkin might regulate signalling from epidermal growth factor receptor (EGFR) through the ability to bind EGFR and the EGFR pathway substrate 15 (EPS15) before ubiquitylating EPS15.

At least 3 RBR proteins are involved in the regulation of the cell cycle and apoptosis: PARC, p53RFP and ZIN. PARC is a negative regulator of the tumour suppressor protein p53. On the contrary, p53RFP (of subfamily C) targets an inhibitor of cell cycle progression (p21^{WAF1}) for degradation. ZIN, a zinc finger protein, supports the degradation of an inhibitor of NF-κB activation (RIP), while overexpression of p53RFP or RIP induces apoptosis.

Some RBR proteins interfere with virus replication. Protein Vif, important for the assembly of HIV-1 particles and the stability of the reverse transcription complex, induces the translocation of ZIN to the nucleus. RBR protein heme-oxidized IRP2 ubiquitin ligase (HOIL-1) enhances the ability of X protein of hepatitis B virus to activate X-responsive promoters.

Shuttling is frequent with RBR proteins. They can be recruited by their interaction partners to particular subcellular compartments, as RBR protein ZIN mentioned before. For example, RBCK1 is translocated to the nucleus by its splicing variant RBCK2. In *Drosophila* interaction of the RBR protein ARI-2 with a classical nuclear transport receptor, karyopherin has been shown. Some RBR members may be self-regulating in shuttling.

Parkin ubiquitylates Ran-binding protein 2 (RanBP2), a component of the nuclear pore complex and related to the small ubiquitin-related modifier (SUMO). Ligase E3 family SUMO-1 binds and enhances parkin's nuclear translocation and auto-ubiquitylation.

RBR proteins of the Ariadne subfamily are probably involved in translational regulation, as human HHARI ubiquitylates the eukaryotic mRNA cap-binding protein 4EHP, which alters the binding efficiency of 4EHP to mRNA caps. Moreover, 4EHP ubiquitylation may be a signal leading to intracellular compartmentalization of specific mRNAs.

RBR proteins are subject to posttranslational regulation (and their E3 activity is inhibited). Parkin's stability is controlled by the RING finger domain ligase E3 FLRF/Nrdp1. Auto-ubiquitylation inhibits E3 activity in members of Ariadne, androgen receptor-associated protein 54 (ARA54), RNF144, dorfin, parkin and TRIAD3 subfamilies. Chaperone-like protein 14-3-3η suppresses Parkin's E3 activity through binding. The chaperone Hsp70 inhibits parkin's E3 activity by forming a complex with unfolded Pael-R (substrate of parkin) and parkin, whereas the carboxy terminus of the Hsc-70-interacting protein (CHIP) induces the dissociation of Hsp70 and enhances parkin's activity and the degradation of Pael-R. Parkin is also inhibited when interacting with Bcl-2-associated athanogene 5 (BAG5) and Hsp70.

Phosphorylation negatively regulates parkin, as reduction of phosphorylation increases activity. Furthermore, parkin is controlled by reversible nitric-oxide (NO) modifications.

RBR proteins and their interaction partners are involved in all major cellular events: transcription and RNA metabolism, translation, subcellular tethering, regulation of posttranslational modification and protein stability, cellular and stress signalling, cell-cycle control, and the course of microbial infection. Aside from ligase E3 function, RBR might serve as activation domains, work as tethering modules and interact with cytoskeletal components (Eisenhaber et al. 2007).

1.4 The ARIADNE family of RBR-proteins

ARIADNE proteins are characterized by an N-terminal acid-rich cluster, an N-terminal leucine-rich region, the RBR motif typical for RBR proteins (RING1, IBR and RING2 domains), a coiled-coil domain and an additional leucine-rich region at the C-terminus (Mladek et al. 2003). ARI proteins function as E2-dependent E3 ligases that interact with the E2s (ubiquitin conjugating enzymes) UbcD10, UbcM4, UbcH7 and UbcH8 in fruit flies,

mice and humans (Martinez-Noel et al. 1999; Moynihan et al. 1999; Aguilera et al. 2000; Ardley et al. 2001).

ARIADNE RING-finger domains consist of a conserved Cys-rich region with a $C\text{-}X_2\text{-}C\text{-}X_{9\text{-}39}\text{-}C\text{-}X_{1\text{-}3}\text{-}H\text{-}X_{2\text{-}3}\text{-}C/H\text{-}X_2\text{-}C\text{-}X_{4\text{-}48}\text{-}C\text{-}X_2\text{-}C$ signature coordinating two Zn^{2+} ligands. These zinc fingers were shown to have substrate binding characteristics. Cys/His positions in RING1 and IBR are strongly conserved (Borden et al. 1996; AGI 2000; Mladek et al. 2003; Eisenhaber et al. 2007).

There are 4 ARIADNE members in human, 5 in mice and 3 in *Drosophila melanogaster* (Eisenhaber et al. 2007). However, the biological function of human ARIADNE member HHARI is still unknown (Tan et al. 2000). Human HHARI interacts with the E2s through the RING1 domain and therefore is involved in the ubiquitylation process, either as a part of the E3 or as a protein that facilitates the process. In co-immunoprecipitation studies interaction of HHARI with UbcH7 could be detected. The distance between RING1 and IBR is essential for UbcH7 binding possibly due to protein folding (Ardley et al. 2001; Tan et al. 2003). *ariadne-1* and *ariadne-2* in *Drosophila melanogaster* were one of the first ARIADNE genes identified. As novel RING-finger proteins they were reported to be required for neural development in *Drosophila* (Aguilera et al. 2000). ARI-1 in *Caenorhabditis elegans* regulates pharyngeal development with UBC-18 (Qiu et al. 2006). Ariadne-like ubiquitin ligase rbrA in *Dictyostelium* is required for development and disruption of rbrA leads to cells unable to form intact slugs producing fruiting bodies. rbrA shows strong sequence similarity to the Ariadne family (34% identity and 54% to human HHARI) (Whitney et al. 2006).

The biological role of ARIADNE proteins in plants is still unknown; since knockout plants did not show a specific phenotype under standard growth conditions (Mladek et al., unpublished data).

16 ARIADNE (ARI) genes among 40 RBR proteins in *Arabidopsis thaliana* were identified by homology and motif searches.

ARIADNE proteins in Arabidopsis are divided into 3 subgroups according to their sequence and amino acid similarity, as well as to the phylogenetic analysis. Within the groups the similarity is higher (less than 44% sequence and 33% amino acid similarity between groups). Two groups are lacking in yeast, invertebrates and vertebrates including mammals and could be exclusive to plants. Subgroup A consists of *AtARI1/2/3/4*, subgroup B of *AtARI5/6/7/8/9/10/11/12* and C with *AtRI13/14/15/16*. Group A includes the closest homologs to the yeast and animal ARI proteins. In group B ARI proteins of barley, rice and *C reinhardtii* can be found. However, there are no other plant ARIs belonging to group C (Mladek et al. 2003).

Some ARI proteins in *Arabidopsis thaliana* (At) have a complete RING1-IBR-RING2 domain: AtARI4/5/7/8/9/10/11. All AtARIs except AtARI12 have the conserved IBR signature $C\text{-}X_{4\text{-}5}\text{-}C\text{-}X_{15\text{-}24}\text{-}C\text{-}X\text{-}C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ (C6HC). RING2 of AtARI1/2/3 have a Leu instead of the central His, whereas AtARI12 lacks of the central $C\text{-}X_{1\text{-}3}\text{-}H$ residues in RING2. Subgroup C that consists of AtARI13/14/15/16 has two members (AtARI14/16) that have lost the first two Cys residues in RING1.

AtARI8 has shown ubiquitin ligase activity with E2s UBC8, UBC10 and UBC11 in ubiquitylation assays in *vitro* (Kraft et al. 2005; Stone et al. 2005).
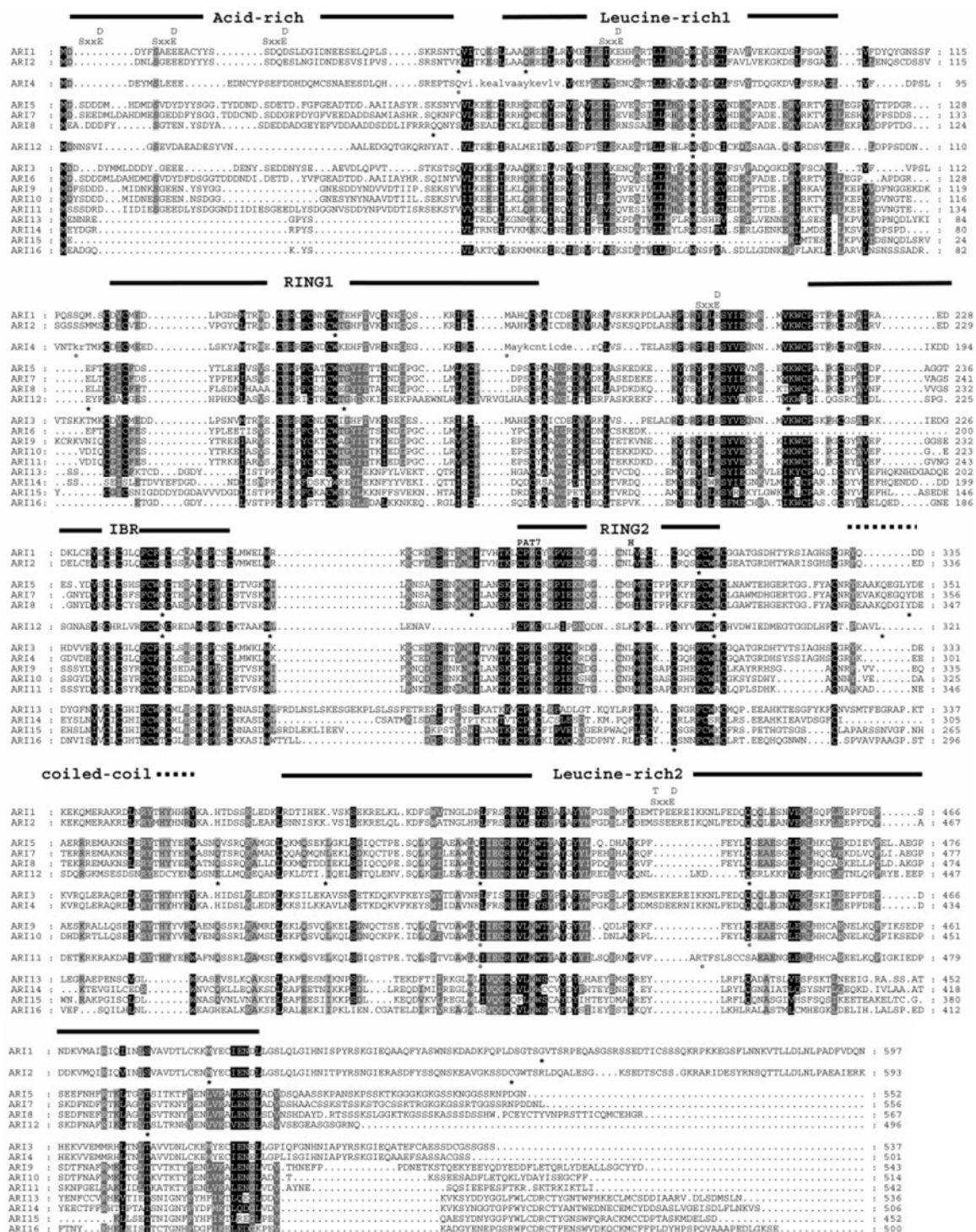
Figure 1.4. Protein alignment of the Arabidopsis ARIADNE family

Black shading indicates 80%, dark grey 70% and light grey shading 60% amino acid similarity between the members of the Arabidopsis ARIADNE family. Gaps are marked with periods; stars below the sequence indicate confirmed intron positions and circles represent postulated intron positions. The conserved acid-rich, Leu-rich1 and 2, RING1-IBR-RING2 and the coiled-coil regions are printed on top of the alignments with solid and dotted lines, respectively. The CKII phosphorylation signatures, the nuclear localization signal (PAT7) and the conserved His (H) of RING2 are also indicated above the alignment. The small letters in the AtARI4 protein sequence mark the putative amino acids of the putative first and third intron. Compared to other AtARIs, AtARI12 lacks of the central C-X1-3-H residues in RING2 (Mladek et al. 2003).

AtARI12 is transcribed in organ-specific manner, i.e. in the roots of AtARI12 according to real time PCR expression analysis (Mladek et al. 2003). Elevated expression under UVB

and cold stress can be observed according to e-Northern microarray data (Toufighi et al. 2005; Nigam et al. 2008). Expression of AtARI12 peaks and is highly induced 2 h after exposing AtARI12 to UVB stress, indicating a role of ARI12 in these abiotic stresses. Indeed *ari12* mutants exposed to elevated UVB radiation and cold develop better than wildtype plants (Mladek et al. 2008). This suggests that ARI12 is a negative regulator of UVB and cold stress response.

The highest expression levels can be observed in the dry seed, at flower stage 9 and in the shoot apex (inflorescence and transition) according to Arabidopsis eFP browser microarray data (http://bbc.botany.utoronto.ca).

ARIADNE RING-finger proteins have a C-X_2-C-X_{9-39}-C-X_{1-3}-H-X_{2-3}-C/H-X_2-C-X_{4-48}-C-X_2-C signature coordinating two zinc ion ligands, while AtARI12 is lacking the central C-X_{1-3}-H residues of RING2. The alignment of the Arabidopsis ARIADNE family depicts conserved regions and the difference of AtARI12 in RING2 to other AtARIs (Figure 1.4). Conserved regions are shown with shades in black (90% similarity), dark grey (80%) and light grey (70%) (Mladek et al. 2003).

In my studies the gene of interest was *Arabidopsis thaliana ARI12* of the ARIADNE gene family, a RING finger protein that interacts with its potential substrate nCBP via the RBR domain (Mladek 2005; Nigam et al. 2008).

1.5 nCBP is a member of eIF4E initiation factors

eIF4E belongs to the group of eukaryotic initiation factors that initiate the translation of mRNAs. In general, eIF4E binds to the 5'-end cap of the mRNA, recruits ribosomes to the mRNA, and binds to eIF4G. eIF4E and eIF4G together form the eIF4F complex that is supposed to be one of the most frequently targeted proteins for translational regulation. The ternary complex (TC), which consists of transport factor eIF2-GTP and Met-tRNA$_i^{Met}$, associates with the 40S ribosomal subunit, generating the 43S pre-initiation complex. This binding is promoted by eIF3 and eIF1A. eIF3 consists of at least 11 non-identical subunits in mammals and 12 non-identical subunits in *Arabidopsis thaliana*: eIF3a, eIF3b, eIF3c, eIF3d, eIF3e, eIF3f, eIF3g, eIF3h, eIF3i, eIF3j, eIF3k, eIF3l. The 43S complex binds to the mRNA-eIF4F construct with the help of RNA helicase eIF4A, forming the 48S initiation complex. The mRNA is scanned for the initiation start codon AUG, GTP is hydrolysed by eIF2, which triggers the release of factors from the 48S complex and allows the binding of this complex to the large 60S ribosomal subunit, generating the 80S ribosome needed for translation elongation and protein synthesis. (Gingras et al. 1999; Browning et al. 2001; Burks et al. 2001; Klann et al. 2004; Pestova et al. 2007; Rhoads et al. 2007; Vardy et al. 2007)

3 different eIF4Es exist in *Arabidopsis thaliana*: eIF4E, eIF(iso)4E and nCBP (novel cap binding protein). The cap-binding complex eIF4F formed by eIF4E and eIF4G has 50% amino acid similarity to eIF(iso)4F that consists of eIF(iso)4E and a large subunit eIF(iso)4G. Cap-binding protein eIF(iso)4E and thus the protein complex eIF(iso)4F, as well as nCBP are limited to plants (Browning 2004; Rhoads et al. 2007).

nCBP is categorized as a Class II member of eIF4Es. Class II members include the *Drosophila* homolog d4EHP and the human homolog 4EHP to nCBP. Although it is not shown for nCBP homologous eIF(iso)4E, a highly-conserved cap-binding protein is reported to have mRNA export function in the nucleus (Culjkovic et al. 2007) and works as an initiation factor for protein translation in the cytoplasm. Chromatography studies revealed that recombinant *A. thaliana* nCBP's affinity to m7GTP is 5- to 20-fold higher than eIF(iso)4E's and differs significantly in sequence. In *Arabidopsis*, nCBP's aromatic residues substitute for two conserved tryptophan residues (Trp 49 and Trp 65). eIF4E mutants lacking these amino acids failed to bind to the cap, whereas the distantly related protein nCBP was capable to (Ruud et al. 1998).

1.6 Interaction partners of eIF4E with roles in cap-binding and cap-dependent initiation of protein synthesis in eukaryotes

Class I eIF4E in the nucleus seems to be regulated by homeodomain proteins (in Drosophila and vertebrates) with conserved eIF4E binding motifs that inhibit mRNA export or regulate the cap-binding (Culjkovic et al. 2007; Goodfellow et al. 2008): One of them is the vertebrate PML (promyelocytic leukaemia protein) that binds eIF4E-1 via a RING domain. In humans, the majority of nuclear eIF4E colocalises with PML nuclear bodies upon stress (i.e. virus infections, interferon treatment) (Lai et al. 2000). Furthermore, the vertebrate proline rich homeodomain protein (PRH) inhibits eIF4E-1's mRNA export and oncogenic transformation functions. Mutant forms of PRH cannot enter the nucleus and failed to export mRNA. In contrast, some homeodomain proteins stimulate eIF4E's function on export and/or translational initiation. Cyclin D1 mRNA export is enhanced by vertebrate HOXA9. Other homeodomain proteins regulating eIF4E include Engrailed in worms, Bicoid in Drosophila (eIF4E-8), human Emx2 and Hox11 etc. (Culjkovic et al. 2007).
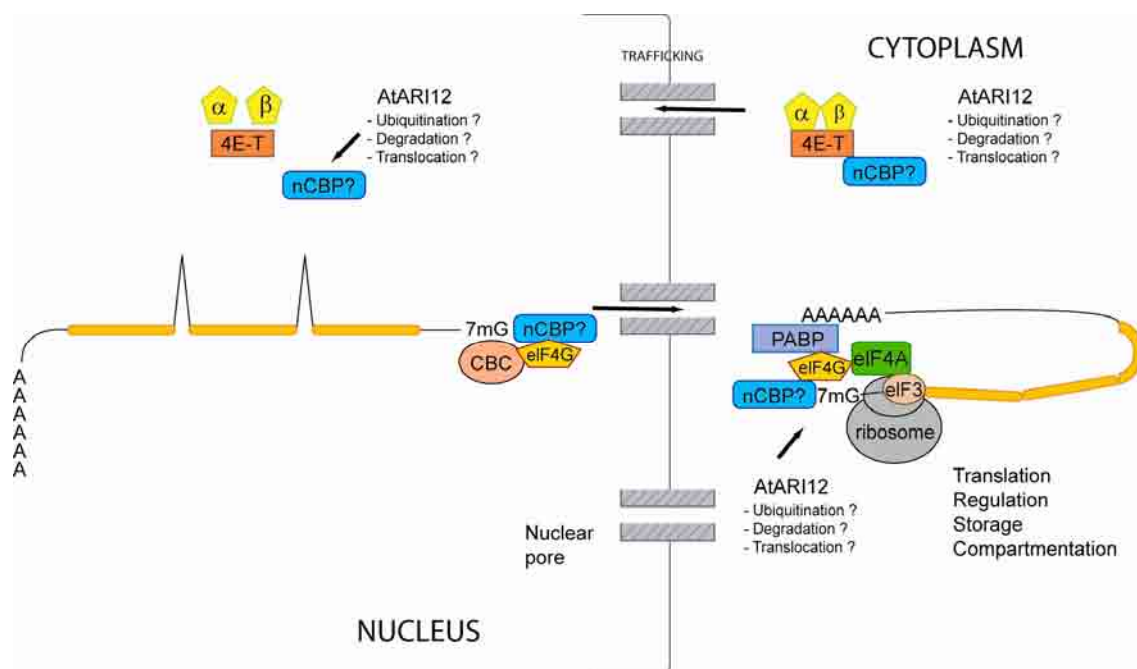
Figure 1.5. Hypothesis of the function of ARI12 and nCBP in the cytoplasm and the nucleus in *Arabidopsis thaliana*

Possible functions of AtARI12 and its substrate nCBP are depicted in this scheme. nCBP might be imported into the nucleus by eIF4E-binding protein 4E-T (only reported for class I eIF4E and not in plants so far) and an α/β importin complex, where the importin complex dissociates and nCBP can bind to the cap of eukaryotic mRNA (indicated with 7mG). The nuclear cap binding complex (CBC) and eIF4G (eukaryotic initiation factor 4G) might associate with nCBP in the nucleus. Bound mRNA can be transported to the cytoplasm, where the CBC complex dissociates and translation initiation begins in the initiation complex including PABP, eIF4G, eIF4A, nCBP, eIF3 and the ribosome. PABP indicates the poly A-binding protein that binds to the poly-A tail of the target mRNA. The stability of the initiation complex can be regulated by phosphorylation. The capped mRNA could be stored or compartmented. Ubiquitylation of nCBP by AtARI12 in the cytoplasm might regulate the abundance and/or localization of the initiation factor (Mladek 2005). Question marks indicate that the interaction of AtARI12 with nCBP is not fully understood.

During translational inactivity in yeast, eIF4E could be detected in stress granules or mRNA granules called EGP-bodies (eIF4E-, eIF4G- ad Pab1-containing bodies arising under glucose starvation) suggesting eIF4E's role in mRNA regulation and compartmentalization (Hoyle et al. 2007; Gaillard et al. 2008; Weber et al. 2008). Pab1 is multifunctional and the major poly(A)-binding protein in yeast. The protein mediates

cellular functions associated with the 3'-poly(A)-tail of messenger RNAs (Brune et al. 2005). In yeast, eIF4E also colocalises with eIF4E-binding protein 4E-T in processing bodies (p-bodies), which are thought to be sites of mRNA decay. Experiments in human HeLa lines also show inhibitory effect of 4E-T (Ferraiuolo et al. 2005). It is currently unknown if 4E-T interacts with other eIF4E family members than class I that are not represented in plants (Rhoads et al. 2007). In yeast, 4E-T and the α/β importin complex shuttle eIF4E from the cytoplasm into the nucleus and there eIF4E binds to the 5'-cap of mRNA for exporting this mRNA to the cytoplasm (Dostie et al. 2000). *Arabidopsis* nCBP was localised in the nucleus and in the cytoplasm supporting the hypothesis that it might function as initiation factor 4E and is imported with a transport-protein like 4E-T in yeast (Figure 1.5). However, to date 4E-T cannot be found in plants that would support this working hypothesis.

4E-BPs (4E binding proteins) that bind and inhibit eIF4E members, have only been found in some protists and could not be observed in plants, nematodes, or most fungi. Regarding eIF4G and 4E-BP interaction in mammals, mammalian eIF4E-2 does not interact with eIF4G but does interact with 4E-BPs, whereas for mammalian eIF4E-3 it is vice versa (interacts with eIF4G but not with 4E-BPs) (Joshi et al. 2004).

Expression of eIF4E-1 is important for ovary and embryo development and *Drosophila* mutants lacking eIF4E-1 die (Hernandez et al. 2004).

Given the observation that eIF4Es are versatile and expressed in different tissues and developmental stages (Hernandez et al. 2005), previous eIF4E studies can only give an idea of nCBP's function in plants or *Arabidopsis. Drosophila* eIF4E-8/d4EHP was reported to inhibit translation (Cho et al. 2005).

BTF3, the β subunit of the heterodimeric nascent polypeptide-associated complex (NAC) (Wiedmann et al. 1994), interacts with eIF(iso)4E in Arabidopsis, but the biological context of the interaction is still unknown, since BTF3 is reported to have dual functions in different cellular compartments (Freire 2005).

In addition, classical eIF4E and eIF(iso)4E have potyviral resistance capability in plants by changing amino acids, preventing binding of the virus' VPg that is necessary for infection (Nicaise et al. 2003; Gao et al. 2004; Kang et al. 2005; Ruffel et al. 2005; Rhoads et al. 2007; Yeam et al. 2007; Charron et al. 2008).

Arabidopsis eIF4E and eIF(iso)4E mRNA expression can be found in all tissues, except in some root cells (http://bbc.botany.utoronto.ca/).

It has been shown that HHARI (human homolog to AtARI12) interacts with 4EHP (eIF4E homolog protein) and ubiquitylates it (Tan et al. 2003). 4EHP is a member of eIF4E that is inhibiting the translation of specific mRNAs in *Drosophila* (Cho et al. 2005). Five eIF4E genes have been found in *Arabidopsis thaliana* (http://browning.cm.utexas.edu): nCBP is the closest homolog to it (Rhoads 2009).

2 Aim of the study

2.1 Defining the protein-protein interaction domain of AtARI12 for interactions with members of the eIF4E family

Previous analyses have identified interactions of a subset of AtARI proteins with the mRNA cap binding protein and member of the eIF4F family of translation initiation factors, nCBP (Mladek 2005). Since the abundance of nCBP was higher in mutants of *AtARI2*, *AtARI12* and *AtARI15,* a subset of AtARI proteins might be involved in regulating the stability of this translation initiation factor.

To define the domain(s) responsible and the specificity of the AtARI12/nCBP interaction extended Y2H analyses were performed with the classical eIF4E and truncated versions of the AtARI12 protein. The interactions were quantified by beta-galactosidase assays.

2.2 Establishing transgenic Arabidopsis plants overexpressing tagged versions of ARI12 and nCBP

To date nearly nothing is known on the function of the *ARI12* and *nCBP* genes. Expression and first phenotypic analyses of *ari12* and *ncbp* mutants indicated that these proteins have opposing effects upon UVB stress. To determine if a C-terminal tag of about 183 amino acids is interfering with the function of ARI12 and nCBP I cloned full length cDNAs into the pSPYCE-35S vector. This vector comprises the C-terminal part of the YFP protein which is sufficient to bind to anti-GFP antibodies and thus can be used in future immunolocalisation and –pull down studies. To determine if this constructs are functional and/or result in measurable overexpression phenotypes they were transformed in wild type, *ari12-1* and *ncbp* mutants.

3 Material

Restriction enzymes

- BamHI (10 U/µL, Fermentas MBI, #ER0051)

- EcoRI (10 U/µL, Fermentas MBI, #ER0271)

- HindIII (10 U/µL, Fermentas MBI, #ER0501)

- NcoI (10 U/µL, Fermentas MBI, #ER0571)

- PstI (10 U/µL, Fermentas MBI, #ER0611)

- XhoI (10 U/µL, Fermentas MBI, #ER0691)

- XmaI (5 U/µL, Promega, R649A)

Other enzymes

- Antarctic phosphatase (5 U/µl, NEB, #M0289S)

- RNase A (10 mg/mL) (Roth, 7156.1)

- Shrimp Alkaline Phosphatase (1 U/µL) (SAP) (Roche, 11758250001)

- T4 DNA ligase (5 Weiss U/µl, Fermentas MBI, #EL0331)

- T4 Polynucleotide Kinase (PNK) (10U/µL, Fermentas MBI, #EK0031)

- T4 Polynucleotide Kinase buffer 10x (Fermentas MBI)

- TAQ DNA polymerase (5 U/µl, home-made)

- TAQ DNA polymerase (recombinant) (5 U/µl, Fermentas MBI, #EP0401)

Kits

- DNA Extraction Kit (Fermentas MBI, #K0513)
    - o Silica Powder Suspension
    - o Binding Buffer
    - o Concentrated Washing Buffer
    - o TBE Conversion Buffer

- DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare)
    - o Terminator reaction mix

- NucleoBond® Xtra Midi (Macherey-Nagel, 740410.10)
    - o NucleoBond Xtra Midi Columns with inserted NucleoBond Xtra Column Filters
    - o Buffers (RES, LYS, NEU, EQU, WASH, ELU)
    - o RNase A (Sigma)

- Phusion Site-Directed Mutagenesis Kit (Finnzymes, F-541)
    - o Phusion Hot Start DNA Polymerase
    - o 5x Phusion HF Buffer
    - o dNTP mix, Quick T4 DNA Ligase (NEB)
    - o 2x Quick Ligation Buffer (NEB)
    - o Control Plasmid with Control Primer Mix

- Phusion® Flash High-Fidelity PCR Master Mix (Finnzymes, F-548)

- Quant-iT Protein Assay (Invitrogen, Q33211)

- TOPO TA Cloning Kit For Sequencing (Invitrogen, 45-0071)
  - Salt solution (1.2 M NaCl, 0.06 M MgCl$_2$)
  - pCR®4-TOPO® vector
  - One Shot® Chemically Competent E. coli
  - S.O.C. Medium

Primers

- Primers for the ARI12 deletion constructs

Table 3.1. Truncated ARI12 constructs and their sequences of used PCR primers to amplify them. Underlined sequences indicate BamHI GGATCC or EcoRI GAATTC restriction sites.

Construct	Size	Primer	Primer sequence
ARI12-RING1	522 bp	01 Fwd	GGA TCC ATA TGG ATA ATA ATT CTG TAA T
		02 Rev	GGA TCC CTG GAC AAG AAG CGT GGA GA
ARI12-RING1-IBR	759 bp	01 Fwd	GGA TCC ATA TGG ATA ATA ATT CTG TAA T
		03 Rev	GGA TTC ACA AGG CAC CGC ATT TTC TA
ARI12-IBR-RING2-C	861 bp	04 Fwd	GAA TTC ATG CAT CCT ATC CAG GGG TCT
		05 Rev	GAA TTC TTG ATT ACG GCC TGA ACC A
ARI12-RING2-C	693 bp	06 Fwd	GAA TTC ATG GTG CCT TGT CCC AAG TGT
		05 Rev	GAA TTC TTG ATT ACG GCC TGA ACC A
ARI12-C	549 bp	07 Fwd	GAA TTC ATG CAT GTC GAC TGG ATT GAA GG
		05 Rev	GAA TTC TTG ATT ACG GCC TGA ACC A

- Phusion PCR primers for the ARI12 deletion constructs

Table 3.2. Truncated pGADT7-ARI12 constructs and their sequences of used Phusion PCR primers are shown. PCR products were ligated with Finnzymes T4 ligase to form a circular plasmid. Primer 10 includes a BamHI site (underlined) that is already located on the pGADT7 vector.

pGADT7 construct	Size	Primer	
ARI12-RING1	8522 bp	10 Fwd	CAT CGA TAC GGG ATC CAT CGA GCT
		11 Rev	CTG GAC AAG AAG CGT GGA GAC CAA
ARI12-RING1-IBR	8759 bp	10 Fwd	CAT CGA TAC GGG ATC CAT CGA GCT
		12 Rev	ACA AGG CAC CGC ATT TTC TAG TAG
ARI12-IBR-RING2-C	8861 bp	13 Fwd	CAT CCT ATC CAG GGG TCT AGA TGC
		14 Rev	ACT GGC CTC CAT GGC CAT ATG AGC
ARI12-RING2-C	8693 bp	15 Fwd	GTG CCT TGT CCC AAG TGT AAG CTA
		14 Rev	ACT GGC CTC CAT GGC CAT ATG AGC
ARI12-C	8549 bp	16 Fwd	CAT GTC GAC TGG ATT GAA GAC ATG

		14 Rev	ACT GGC CTC CAT GGC CAT ATG AGC

- Primers for sequencing the pSPYCE-35S constructs

The sequencing reaction was performed with the primers in the table below:

Table 3.3. Primers used for sequencing pSPYCE-35S constructs: pSPYCE-35S-ARI12 (14078 bp), pSPYCE-35S-nCBP (13274 bp)

Construct	Direction	Primer name	Primer sequence
pSPYCE-35S-ARI12	Forward	ariUpm	AGCCTAACGCGGAACCACTA
pSPYCE-35S-ARI12	Reverse	ariR9R	TTAAGTGAGAAAGCAGAAGTGTGG
pSPYCE-35S-nCBP	Forward	5g18110_F2	CTCGCTTCTGGGAGGATC
pSPYCE-35S-nCBP	Reverse	5g18110_R2	GATCTGTTGGACTAGGCAAG

Table 3.4. Primer used for sequencing of amplified and ligated pGADT7-ARI12 constructs (ARI12 deletion constructs). All pGADT7-ARI12 constructs were sequenced with the pGAD_Fw primer; pGADT7-ARI12-RING1 was additionally sequenced with the pGAD_Rev primer.

Construct	Direction	Primer name	Primer sequence
pGADT7-ARI12 constructs	Forward	pGAD_Fw	TACCACTACAATGGATG
pGADT7-ARI12-RING1	Reverse	pGAD_Rev	GTTGAAGTGAACTTGCGGGG

Buffers and solutions

- Blocking solution

  5% low fat dry milk powder in 50 mL TBS-T Buffer

- Buffer B 10x (Promega, R002A)

- Buffer O 10x (orange) (Fermentas MBI, #BO5)

  Tris-HCl (50 mM) (pH 7.5 at 37°C), $MgCl_2$ (10 mM), NaCl (100 mM), BSA (0.1 mg/mL)

- Buffer R 10x (red) (Fermentas MBI, #BR5)

  Tris-HCl (100 mM) (pH 8.5 at 37°C), $MgCl_2$ (100 mM), KCl (1 M), BSA (1 mg/mL)

- Buffer Tango 10x (yellow) (Fermentas MBI, #BY5)

  Tris-acetate (330 mM) (pH 7.9 at 37°C), Mg-acetate (100 mM), K-acetate (660 mM), BSA (1 mg/mL)

- Buffer Z

  Na_2HPO_4 (16.1 g/L) (Roth, T877.1), NaH_2PO_4 (5.5 g/L) (Roth, 2370.2), $MgSO_4 * 7 H_2O$ (0.246g/L) (Riedel-de Haën, 13142)

- Coomassie Blue staining solution

  0.25% (w/v) Coomassie brilliant blue R250 (Roth, 3862.1), 45% (v/v) methanol (Roth, P717.1), 10% (v/v) acetic acid (Roth, 3738.5)

- Dephosphorylation buffer 10x (NEB)

- GTE buffer

  D(+)-Glucose anhydrous (50 mM) (Fluka, 49140), EDTA (10 mM) (Roth, X986.2), Tris-HCl pH 8.0 (25 mM) (Roth, 5429.2 and 4625.2) in H_2O

- KCM buffer 5x

KCl (0.5 M) (Roth, 6781.1)

CaCl$_2$ (0.15 M) (Roth, 5239.1)

MgCl$_2$ (0.15 M) (Fluka, 63065)

- Ligation buffer A 10x (home-made)

pH 7.8 at 25°C; Tris-HCl (400 mM), MgCl$_2$ (100 mM), DTT (100 mM), ATP (5 mM), EDTA (1 mM), rATP (5 mM)

- PCR buffer 10x (home-made)

Tris-HCl (100 mM) (Roth, 5429.2 and 4625.2), KCl (500 mM) (Roth, 6781.1), MgCl$_2$ (15 mM) (Fermentas MBI), 1.5% Triton X-100 (Sigma-Aldrich, X100), H$_2$O

- T4 DNA ligase buffer 10x (Fermentas MBI)

- TAE buffer 1x

Made from TAE buffer 50x stock that was prepared as follows: 242 g Tris base (Roth, 5429.2) is added to 750 mL and 57.1 mL acetate acid glacial (Roth, 3738.5) and 100 mL of EDTA pH 8.0 (0.5 M) (Roth, X986.2) is added. The solution is adjusted to 1L.

- TAQ DNA polymerase buffer with KCl 10x (Fermentas MBI)

- TBS-T buffer 1x

Tris-HCl pH 7.6 (20 mM) (Roth, 5429.2 and 4625.2), NaCl (137 mM) (Roth, 3957.2), 0.1% Tween 20 Tween 20 (Sigma, P-7949)

- TE buffer pH 7.5 10x

Tris-HCl (0.1 M) (Roth, 5429.2), EDTA (10 mM) (Roth, X986.2)

- TE buffer pH 8.0 1x

Tris-HCl (10 mM) (Roth, 5429.2), EDTA (1 mM) (Roth, X986.2)

- Transfer buffer pH 8.3 for Western blot

Tris (500 mM) (Roth, 5429.2), boric acid (500 mM) (Roth, 6943.1)

- Tris-glycine SDS-polyacrylamide gel running buffer 5x without SDS

72 g glycine (Roth, 3908.3), 15.1 g Tris base (Roth, 5429.2) in 1 L H$_2$O

- Tris-HCl pH 6.8 (1 M) (Roth, 5429.2 and 4625.2)

- Tris-HCl pH 6.8 (1.5 M) (Roth, 5429.2 and 4625.2)

- Tris-HCl pH 8.8 (1.5 M) (Roth, 5429.2 and 4625.2)

- TSB-Buffer

50 mL YT liquid media, 100 µL MgSO$_4$ (25 mM) (Riedel-de Haën, 13142), 40 µL MgCl$_2$ (100 mM) (Fluka, 63065), 860 µL H$_2$O, 2.5 mL DMSO (Sigma, D4540-500ML), 4.75 g PEG 4000 (Fluka, 81240)

Media

- MS 1.0 plates with BASTA selection

Murashige and Skoog Basal Salt Mixture (4.3 g/L) (Sigma, M5524), sucrose (10 g/L), agar (10 g/L) (Fluka, 05039), titrated to pH 5.6-5.8 with several drops of KOH (1 M) (Merck) and adjusted to the volume. After media autoclaving, filter-sterilized

BASTA (AgrEvo, 1637404) selection was added to a final concentration of 10 mg/L.

- MS 2.5 plates

  Murashige and Skoog Basal Salt Mixture (4.3 g/L) (Sigma, M5524), sucrose (25 g/L), agar (10 g/L) (Fluka, 05039), titrated to pH 5.6-5.8 with several drops of KOH (1 M) (Merck) and adjusted to the volume

- SD liquid media –Leu –Trp, –Leu –Trp –His and –Leu –Trp –His (3-AT (3 mM))

  see SD plates without adding agar

- SD plates –Leu –Trp and –Leu –Trp –His

  Minimal SD Base (Clontech, Cat# 630411) and amino acid is mixed according to the manufacturer's procedures: either -Leu/-Trp DO Supplement (Clontech, 630417) or –Leu –Trp –His -Leu/-Trp/-His DO Supplement (Clontech, 630419). Then agar (15g/L) (Fluka, 05039) was added. The media was adjusted to pH 5.8 (with KOH (1M) (Merck))

- SD plates –Leu –Trp –His (with 3 mM 3-AT)

  for aminotriazol plates filter sterilized 3-Amino-1,2,4-triazole (1 M) (Aldrich, A81609) was added to SD –Leu –Trp –His plate media, respectively to a final concentration of 3 mM

- YPD liquid media (composition see YPD plates without agar)

- YPD plates

  Casamino acids (20 g/L) (Roth, AE41.1), yeast extract (10g/L) (Roth, 2363.2), agar (18g/L) (Fluka, 05039), titrated to pH 5.8

  Liquid glucose (50%) (Fluka, 49140) was added to a final concentration of 2% prior to plating out

- YT liquid media

  composition see YT plates, but without agar

  see "antibiotic selection" for working concentrations of specific selections

- YT plates

  Yeast extract (10 g/L) (Roth, 2363.2), casamino acids (10 g/L) (Roth, AE41.1), agar (15 g/L) (Fluka, 05039), NaCl (5 g/L) (Roth, 3957.2) and H_2O up to desired volume

  see "antibiotic selection" for working concentrations of specific selections

- Antibiotic selection for YT plates and YT liquid media

  Rifampicin, final concentration 100 mg/L (Sigma, R3501)

  Gentamycin, final concentration 50 mg/L (Roth, 0233.2)

  Kanamycin, final concentration 50 mg/L (Roth, T832.4)

  Ampicillin, final concentration 100 mg/L (Roth, K029.1)

Table 3.5 Antibiotic selection used for competent cells

Organism	Selection, final conc.
Agrobacterium tumefaciens GV3101 (pMP90)	rifampicin, 100 mg/L gentamycin, 50 mg/L

E. coli DH5α	no selection
Saccharomyces cerevisiae PJ69-4a	no selection

Table 3.6 Antibiotic selection used for the corresponding construct

Construct	Organism	for	Selection, final conc.
TOPO-ARI12	E. coli	Miniprep	kanamycin, 50 mg/L or ampicillin, 100 mg/L
pGADT7-ARI12 deletion constructs	E. coli	Miniprep	ampicillin, 100 mg/L
pSPYCE-35S constructs	E. coli	Miniprep, Midiprep	kanamycin, 50 mg/L
pSPYCE-35S constructs	Agrobacterium tumefaciens	Miniprep, Transformation	rifampicin, 100 mg/L gentamycin, 50 mg/L kanamycin, 50 mg/L

Antibodies

- Primary antibody: c-myc-epitope (1 µg/mL) (epitope 9E10, Roche). The primary antibody was diluted in blocking solution with 0.02% NaN_3.

- Primary antibody: HA-epitope (1 µg/mL) (epitope 12CA5, purified by the group of Dr. Lukas Mach, DAGZ, BOKU Wien)

- Secondary antibody: Immunopure Goat Anti-Mouse-IgG HRP (0.8 mg/mL) (Pierce)

Chemicals

- Agarose gel

  Agarose (Eurogentec, EP-0010-05) in TAE buffer 1x

  0.8% gel: 0.8 g agarose in 100 mL TAE buffer 1x

  1 % gel: 1 g agarose in 100 mL TAE buffer 1x

- 10% ammoniumperoxodisulfate (APS) (Roth, 9592.2)

- $CHCl_3$, chloroform (Roth, T901.1)

- dATP (2 mM) (Solis Biodyne)

  100 mM solution in TE buffer

  (100 mM Tris pH 7.8, 1 mM EDTA)

- Destaining solution

  25% ethanol (AustriaAlco), 10% acetic acid (Roth, 3738.5)

- DMSO, Dimethyl sulfoxide, minimum 99.5% GC (Sigma, D4540-500ML)

- dNTP mix (2 mM) (Solis Biodyne)

dATP (0.5 mM) (Solis Biodyne), dCTP (0.5 mM) (Solis Biodyne), dGTP (0.5 mM) (Solis Biodyne), mM dTTP (0.5 mM) (Solis Biodyne)

- 70% ethanol

  made from ethanol 99% (AustriaAlco) and H_2O

- 95% ethanol

  made from ethanol 99% (AustriaAlco) and H_2O

- 50 % glucose (Fluka, 49140)

- 86% glycerol (Roth, 4043.3)

- Herring testes carrier DNA (10 mg/mL) (Clontech, S0177)

- Isopropanol (2-Propanol) (Roth, 6752.5)

- KOAc pH 4.8 solution (3 M/5 M)

  Potassium acetate (3 M) (Sigma, P-5708), titrated to pH 4.8 with acetic acid glacial (Roth, 3738.5)

- LiAc 10x

  Lithium acetate (1 M) (Roth, 5447.1), adjusted to pH 7.5 with diluted acetic acid (Roth, 3738.5)

- LiAc/PEG4000

  50% PEG4000 (Fluka, 81240), TE 10x pH 7.5, LiAc 10x

  LiAc/PEG4000 mixture contains TE 10x pH 7.5, LiAc 10x and 50% PEG4000 in a 1:1:8 ratio

- Liquid N_2

- 1% low melting agarose

  1 g low melting agarose (Biozym, 850080) in 100 mL distilled H_2O

- 5% (v/v) 2-mercaptoethanol (Roth, 4227.1)

- Methanol (Roth, P717.1)

- $MgCl_2$ (25 mM) (Fermentas MBI)

- $MgSO_4$ * 7 H_2O (Riedel-de Haën, 13142)

- Na_2CO_3 stop solution (1 M) (Roth, A135.2)

- NaOH (0.2 M)/1% SDS

  NaOH (0.4 M) (Roth, 9356.2), 2% sodium lauryl sulphate (Roth, 4360.2) in a 1:1 ratio

- ONPG (o-Nitrophenyl-B-D-Galactopyranoside)

  4 mg/mL ONPG (ONPG 2-Nitrophenyl beta-D-galactopyranoside (Aldrich, N21502) in buffer Z (freshly prepared or stored in -20°C freezer)

- Phenol-chloroform-isoamyl alcohol (24:24:1)

  Phenol saturated solution (Amresco, 0945-400ML), CHCl3 chloroform (Roth, T901.1), isoamyl alcohol (Roth, T870.1)

- Pre-stained Protein Molecular Weight Marker (Fermentas MBI, #SM0431)

- rATP (10 mM)

- Roti-Lumin (Roth, P078.1): chemiluminescent substrate for Horseradish Peroxidase-Labeled Reporter Molecule

- 40% Rotiphorese® gel 40 (29:1) acrylamid (Roth, A515.1)

- SDS (Roth, 4360.2)

- SDS-PAGE loading dye 4x (including DTT)

  SDS-PAGE loading dye 1x contains Tris-HCl pH 6.8 (50 mM) (Roth, 5429.2 and 4625.2), DTT (100 mM) (Invitrogen, Y00147), 2% SDS (Roth, 4360.2), 0.1% bromophenol blue (Fluka, 18030), 10% glycerol (Roth, 4043.3)

- Silwet L-77

- NaAc pH 4.8 (3 M) (Roth, 6773.2)

- NaAc pH 5.2 (3 M) (Roth, 6773.2)

- Sterilization solution

  5% hypochlorite (Roth, 9062.1), Tween-20 (1 drop/5 mL) (Sigma, P-7949)

- 5% sucrose (Wiener Zucker)

- 99% TEMED p.a. (Roth, 2367.1)

Plasmids and constructs

- pGADT7 (Clontech)

- pGBKT7 (Clontech)

- pSPYCE-35S

- TOPO-ARI12

- pUC-SPYCE-nCBP

- pGADT7-ARI12

- pGBKT7-nCBP

- pGBKT7-eIF4E

E. coli cells

- DH5α *Escherichia coli* (Chung et al. 1993)

Yeast cells

- PJ69-4a *Saccharomyces cerevisiae*

- pGADT7-ARI12 x BK-eIF4E in PJ69-4a yeast cells

- pGADT7-ARI12 x BK-nCBP in PJ69-4a yeast cells

- pGADT7-ARI12 x BK in PJ69-4a yeast cells

Agrobacteria cells

- GV3101 (pMP90) *Agrobacterium tumefaciens*

Plant lines

- Columbia wildtype *Arabidopsis thaliana*

- Landsberg wildtype *Arabidopsis thaliana*

- *ncbp* knockout mutant (SALK 131503, SALK institute, http://www.signal.salk.edu; ColWT background; Alonso et al., 2003) *Arabidopsis thaliana* (Figure 3.1)

- *ari12-1* knockout mutant (ET7329, homozygous, CSLH Gene/Enhancer trap collection http://genetrap.cshl.org; LerWT background; Sundaresan et al., 1995) *Arabidopsis thaliana* (Figure 3.1)

Other Materials

- Roti-PVDF membrane (Roth, T830.1)

AtARI12

ari12-1 (Ler)

nCBP

ncbp (Col)
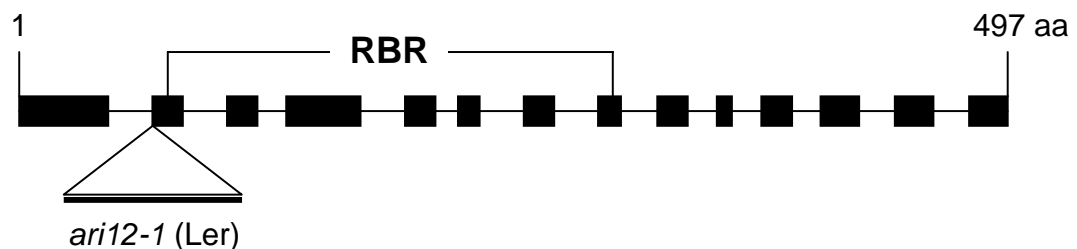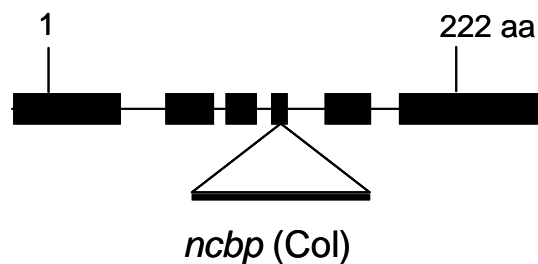
Figure 3.1. T-DNA insertion of ari12-1 (ET7329) mutant line and T-DNA insertion of nCBP SALK (131503) mutant line were used for transformation (*Arabidopsis thaliana*).

4   Methods

4.1   General procedures

4.1.1   Plasmid Miniprep from *E. coli* cells

The original protocol is termed Quickprep II for colony screening by Manuel Simon.

On the first day *E. coli* colonies from the plate were inoculated into 5 mL YT medium per sample. Ampicillin from stock was added to a final concentration of 100 mg/L (in case of kanamycin the working concentration was 50 mg/L). Then the samples were shaken at 37⁰C and 180 rpm overnight.

Stock cultures were made from the overnight culture on the second day (see "Preparation of *E.coli* stock cultures" in 4.1.3).

Plasmid isolation

2 mL of the overnight culture was transferred to an Eppendorf tube. The sample was centrifuged for 3 min. Then the supernatant was removed. 200 µL GTE buffer was added and the pellet was fully suspended in the solution. 400 µL fresh NaOH/1% (0.2 M) SDS was added and inverted 3 times for lysis. Incubation time thereafter was 5 min. 300 µL NaAc pH 4.8 (3 M) was added and the sample was shaken hardly. It was incubated on ice for 10 min and centrifuged again for 15 min. Approximately 800 µL supernatant was transferred to a new tube while avoiding cell residues. DNA was precipitated with 600 µL isopropanol during 10 min of incubation at room temperature. After a 30 min centrifugation step the supernatant was removed and the pellet was washed with 70% ethanol. The next centrifugation step took 15 min. The supernatant was removed and the DNA was dried by the Eppendorf concentrator for 5 min. Finally, the isolated DNA was suspended in 40 µL 1x TE pH 8.0 and 2 µL RNAse A (10 mg/mL). For thorough suspension the sample was shaken for 10 min and incubated at 37°C (RNA digestion). If not stated otherwise in the paragraph, the centrifugation steps were performed at 13200 rpm and 4⁰C.

Restriction enzymes were added to the sample after the plasmid DNA isolation to confirm successful cloning. Incubation time was set to 2 h at 37°C if not mentioned otherwise in the manufacturers' protocol. The sample and the undigested DNA isolate (as a control) were loaded on a 0.8% agarose gel and run in an electrophoresis chamber at 100V for 60 min.

4.1.2   Plasmid Midiprep from *E. coli* cells

E. coli clones containing the confirmed pSPYCE-35S-ARI12 or the pSPYCE-35S-nCBP constructs were grown overnight in 100 mL YT liquid media with kanamycin (50 mg/L) selection and plasmid DNA was isolated by Nucleobond Xtra Midi kit (Macherey and Nagel) according to the manufacturer's protocol.

4.1.3   Stock cultures

Preparation of *E.coli* stock cultures

Stocks were made by mixing 1 mL of an overnight culture with 400 µL 87% glycerol. The stocks were frozen by liquid nitrogen and stored at -80°C.

Preparation of *Agrobacteria* stock cultures

Stocks were made by mixing 500 µL of a 2 days culture with 500 µL 87% glycerol. The stocks were frozen by liquid nitrogen and stored at -80⁰C.

Preparation of yeast stock cultures

Stocks were made by mixing 1 mL of a 2 days culture with 400 µL 87% glycerol. The stocks were frozen by liquid nitrogen and stored at -80°C

4.1.4   Restriction digests with conventional restriction enzymes

1 µg of DNA was mixed with 2 µL of the recommended buffer 10x and 0.5 µL-1.0 µL restriction enzyme. Water was added up to 20 µL. The sample was gently mixed and spinned down. If not stated otherwise, the incubation time was 2 h at 37°C.

4.1.5   Gel purification

The desired DNA fragment was excised from a gel and purified with the extraction kit (Fermentas MBI, #0513) according to the protocol. The DNA was eluted with 20 µL H$_2$O.

4.1.6   cDNA synthesis

cDNA was already available as TOPO-ARI12 for ARI12 and pUC-SPYCE-nCBP for nCBP. cDNA synthesis of ARI12 and nCBP was done by Dr. Juan-Antonio Torres-Acosta and Nina Chumak, respectively.

4.1.7   PCR reactions

Table 4.1: Summary of all conventional PCR conditions used

Template	Enzyme	Primer	Product size	Programme	PCR machine
Plasmid TOPO-ARI12	TAQ (Fermentas MBI)	01 Fwd 02 Rev	522 bp	SSLP55	HYBAID Omn–E PCR machine
Plasmid TOPO-ARI12	TAQ (Fermentas MBI)	01 Fwd 03 Rev	759 bp	SSLP55	HYBAID Omn–E PCR machine
Plasmid TOPO-ARI12	TAQ (Fermentas MBI)	04 Fwd 05 Rev	861 bp	SSLP55	HYBAID Omn–E PCR machine
Plasmid TOPO-ARI12	TAQ (Fermentas MBI)	06 Fwd 05 Rev	693 bp	SSLP55	HYBAID Omn–E PCR machine
Plasmid TOPO-ARI12	TAQ (Fermentas MBI)	07 Fwd 05 Rev	549 bp	SSLP55	HYBAID Omn–E PCR machine
Plasmid pGADT7-ARI12-RING1 (Colony PCR)	TAQ (Fermentas MBI)	10 Fwd pGAD_ Rev	522 bp	SSLP55	Eppendorf Mastercycler

4.1.7.1 PCR reaction with TAQ polymerase (Fermentas MBI)

A PCR reaction was performed to amplify the truncated ARI12 constructs

TAQ DNA polymerase buffer with KCl 10x (Fermentas MBI)	2 µL
dNTP mix (2 mM) (Solis Biodyne)	2 µL
MgCl$_2$ (25 mM) (Fermentas MBI)	1.6 µL
Primer Fwd (20 µM)	0.25 µL
Primer Rev (20 µM)	0.25 µL
TAQ DNA polymerase (recombinant) (5 U/µl, Fermentas MBI, #EP0401)	0.8 µL

TOPO-ARI12 (2 ng/µL)	1 µL
H$_2$O	12.1 µL
TOTAL	20 µL

PCR reactions were performed with the programme SSLP 55°: 95°/3 min – (55°/30 s – 72°/1 min – 94°/30 s) x 35 on a HYBAID Omn–E PCR machine. Bands of expected size (table 4.1) could be detected on an agarose gel.

The obtained PCR fragments were cloned into the pCR4-TOPO vector (Invitrogen, TOPO kit, 3956 bp) as described in section 4.1.8. This step was only successful for the RING2-C constructs. Therefore the final cloning step from TOPO to the Clontech pGADT7 vector for Y2H assays was attempted with this construct only.

4.1.7.2 Colony PCR protocol

One *E. coli* colony was mixed in 10 µL H$_2$O with a toothpick. The suspension was cooked for 5 min at 100°C on a heat block and centrifuged for 5 min at full speed at room temperature. 1 µL from it was added to the following PCR reaction

TAQ DNA polymerase (recombinant) (5 U/µl, Fermentas MBI, #EP0401)	0.8 µL
TAQ DNA polymerase buffer with KCl 10x (Fermentas MBI)	2.0 µL
Primer 10 Fwd (20 µM)	0.25 µL
Primer pGAD_Rev (20 µM)	0.25 µL
dNTP mix (2 mM) (Solis Biodyne)	2.0 µL
MgCl$_2$ (25 mM) (Fermentas MBI)	1.6 µL
E. coli cell with pGADT7-ARI12-RING1	1.0 µL
H$_2$O	12.1 µL
TOTAL	20 µL

Eppendorf Mastercycler: Programme SSLP55 94°/3m – (55°C/15s – 72°/1m – 94°/15s) x 35. Bands of expected size (table 4.1) could be detected on an agarose gel.

4.1.8 TOPO TA-Cloning

Adding poly A tail when proof reading TAQ is used

PCR fragment (see section 4.1.7.1.)	8 µL
PCR buffer 10x (home-made)	1 µL
dATP (2 mM) (Solis Biodyne)	1 µL
TAQ DNA polymerase (5 U/µl, home-made)	0.5 µL
TOTAL	10.5 µL

The PCR fragment was incubated for 10 min at 72°C in an Eppendorf Mastercycler.

TA Cloning reaction

PCR fragment with poly A	4 µL
Salt solution (1.2 M NaCl, 0.06 M MgCl$_2$) (Invitrogen, TOPO Kit)	1 µL
H$_2$O	0.5 µL

pCR®4-TOPO® vector (10 mM) (Invitrogen, TOPO Kit)	5 µL
TOTAL	10.5 µL

The TA Cloning reaction was incubated for 5 min at room temperature and put on ice immediately thereafter.

Transformation into competent TOP10 cells

2 µL cloning reaction was transferred to 30 µL of competent TOP10 cells each. The tubes were mixed gently and incubated on ice for 30 min. The samples were put to heat-shock at 42°C for 30 s. The cells were put on ice and 250 µL room temperature S.O.C. medium (Invitrogen, TOPO Kit) was added. The cells were shaken for 1 h at 200 rpm in the 37°C room and plated out on YT agar media (50 mg/L kanamycin). The plates were incubated overnight at 37ºC

4.1.9   Making competent DH5α cells after Chung and Miller

Competent DH5α *E. coli* cells were inoculated in 5 mL YT media without antibiotics. This culture was shaken over night at 220 rpm and 37°C. 100 µL of this overnight culture was inoculated in 50 mL YT media. The bacteria were shaken at 37°C, 220 rpm until OD600 reached 0.3-0.6. The cell suspension was transferred to a Falcon tube and centrifuged in the Christ Minifuge (Rotor 816) at 2500 rpm and 4°C for 10 minutes. The supernatant was discarded. The pellet was carefully resuspended in 5 mL 4°C cold TSB-Buffer and incubated for 10 minutes on a shaker on ice. Eppendorf tubes were pre-cooled on ice. The competent cells were aliquoted in those tubes (100 µL/tube). The tubes were frozen in nitrogen and stored at -80°C until use. Before use the competent cells were thawn on ice.

4.1.10 Transformation into DH5α cells

20 µL KCM 5x buffer and 60 µL H$_2$O were added to samples A and B. Furthermore a negative control C and a transformation control D were prepared. 50 µL competent DH5α *E. coli* cells from -80ºC stocks per sample were thawed on ice. Then the mixes A, B, C and D were added to one tube of competent DH5α cells each and incubated on ice for 30 min. A heat shock was performed for 90 seconds at 42ºC. 800 µL YT liquid media without selection was added. The tubes were incubated at 37ºC at 180 rpm for 50 min.

The cells were plated out on YT agar plates with antibiotic selection in the working concentration in volumes of 10, 100, 250 µL and the remaining culture. These were incubated overnight at 37ºC.

4.1.11 DNA Sequencing

The BIG DYE programme on a HYBAID Omn–E thermocycler (96°/30 s – 55°/15 s – 60°/4 min) x 25 was chosen for the sequencing mix:

Terminator Reaction Mix	
(GE Healthcare, DYEnamic ET Terminator Cycle Sequencing Kit)	4.0 µL
Template 250 ng DNA	x µL
20 µM Primer (6 pmol)	0.3 µL
H$_2$O	15.7 – x µL
TOTAL	20 µL

After completion of the PCR reaction 50 µL 95% ethanol and 2 µL NaAc pH 5.2 (3 M) were mixed in a tube and the PCR products were added to it. The tube was mixed again

and vortexed. The sample was incubated for 15 min on ice for precipitation and centrifuged at full speed for 30 min. The supernatant was removed and 500 μL 70% ethanol was added. The sample was centrifuged again at full speed for 15 min and the supernatant was removed. The pellet was dried for 5 min in the concentrator and the fully dry DNA was sent to sequencing analysis.

4.1.12 DNA purification by ethanol precipitation

2.5x 95% ethanol of the sample volume was mixed gently with 1/10 NaAc pH 5.2 (3 M) of the sample volume. Then the sample was added to this solution and mixed again. After 20 min incubation on ice, the sample was centrifuged for 30 min at full speed and 4ºC. The DNA was washed with 500 μL 70% ethanol once and dried in the Eppendorf concentrator for 5 min. The sample was suspended in 10 μL H_2O and stored at 4ºC after thorough mixing.

4.2   Y2H cloning

Creating pGADT7-ARI12 Y2H constructs with the Finnzymes Phusion Mutagenesis Kit and the Finnzymes Phusion® Flash High-Fidelity PCR Master Mix

Primer construction for Finnzymes Phusion PCR

See results section 5.1

Phosphorylation of the primers

Primers were phosphorylated as follows for the recirculation of the plasmid after the Finnzymes Phusion PCR reaction:

300 pmol oligonucleotide primer	3 μL
T4 Polynucleotide Kinase (PNK) (10U/μL, Fermentas MBI, #EK0031)	1 μL
rATP (10 mM)	5 μL
H_2O	36 μL
T4 Polynucleotide Kinase buffer 10x (Fermentas MBI)	5 μL
TOTAL	50 μL

The primers were incubated at 37ºC for 30 min with the T4 polynucleotide kinase, followed by a heat-inactivation step at 65ºC.

PCR with Finnzymes Phusion® Flash High-Fidelity PCR Master Mix

Finnzymes PCR Phusion Flash with pGADT7-ARI12-RING1, pGADT7-ARI12-RING1-IBR, pGADT7-ARI12-IBR-RING2, pGADT7-ARI12-RING2, pGADT7-ARI12-C

The pGADT7-ARI12 construct served as a template for the PCR reaction.

Phusion® Flash High-Fidelity PCR Master Mix (Finnzymes, F-548)	10 μL
Primer Fwd (final conc. 0.5 μM)	1.7 μL
Primer Rev (final conc. 0.5 μM)	1.7 μL
350 pg Template DNA	2.2 μL
H_2O up to 20 μL	4.4 μL
TOTAL	20 μL

See above for details on the primer combinations.

The Phusion Flash PCR reactions were performed in the Eppendorf Mastercycler

Programme: 98°/10 s – (98°/1 s - 71°/5 s - 72°/2 min 10 s) x 30 - 72°/1 min – 4° hold

Table 4.2. Summary of Phusion PCR reaction

Template	Enzyme	Primer	Product size	Programme	PCR machine
Plasmid pGADT7-ARI12	Finnzymes Phusion Flash PCR Master MIX	10 Fwd 11 Rev	8522 bp	98°/10 s – (98°/1 s - 71°/5 s - 72°/2 min 10 s) x 30 - 72°/1 min – 4°	Eppendorf Mastercycler
Plasmid pGADT7-ARI12	Finnzymes Phusion Flash PCR Master MIX	10 Fwd 12 Rev	8759 bp	98°/10 s – (98°/1 s - 71°/5 s - 72°/2 min 10 s) x 30 - 72°/1 min – 4°	Eppendorf Mastercycler
Plasmid pGADT7-ARI12	Finnzymes Phusion Flash PCR Master MIX	13 Fwd 14 Rev	8861 bp	98°/10 s – (98°/1 s - 71°/5 s - 72°/2 min 10 s) x 30 - 72°/1 min – 4°	Eppendorf Mastercycler
Plasmid pGADT7-ARI12	Finnzymes Phusion Flash PCR Master MIX	15 Fwd 14 Rev	8693 bp	98°/10 s – (98°/1 s - 71°/5 s - 72°/2 min 10 s) x 30 - 72°/1 min – 4°	Eppendorf Mastercycler
Plasmid pGADT7-ARI12	Finnzymes Phusion Flash PCR Master MIX	16 Fwd 14 Rev	8549 bp	98°/10 s – (98°/1 s - 71°/5 s - 72°/2 min 10 s) x 30 - 72°/1 min – 4°	Eppendorf Mastercycler

Ligation of the Phusion PCR products

25 ng of the PCR products were ligated. The plasmid circulized and was transformed into competent E. coli cells. The ligation was performed as duplicates.

25 ng Phusion PCR product	4.5 µL
H_2O	0.5 µL
Quick Ligation Buffer 2x (NEB, Finnzymes F-541 Kit)	5.0 µL
Quick T4 DNA Ligase (NEB, Finnzymes F-541 Kit)	0.5 µL
TOTAL	10.5 µL

Transformation of ligated Phusion PCR products into competent DH5α E. coli

For details on the transformation protocol see section 4.1.10. The transformed *E. coli* were plated out on YT plates with 100 µg/µL ampicillin.

Confirming pGADT7-ARI12 deletion constructs

Minipreps as described in section 4.1.1 were performed for the Phusion PCR constructs. The constructs were digested with NcoI and BamHI (pGADT7-ARI12-RING1-IBR) or with NcoI and EcoRI (pGADT7-ARI12-IBR-RING2-C, pGADT7-ARI12-RING2-C and pGADT7-ARI12-C). For pGADT7-ARI12-RING1, a colony PCR was performed in order to check the size of the insert.

Digestion of the RING1-IBR miniprep

DNA miniprep pGADT7-ARI12-RING1-IBR	1 µL
NcoI (10 U/µL, Fermentas MBI, #ER0571)	0.5 µL
BamHI (10 U/µL, Fermentas MBI, #ER0051)	0.5 µL
Buffer Tango 10x (yellow) (Fermentas MBI, #BY5)	4 µL
H_2O	14 µL
TOTAL	20 µL

Digestion of IBR-RING2-C, RING2-C and C miniprep

DNA miniprep pGADT7-ARI12-IBR-RING2-C or pGADT7-ARI12-RING2-C or pGADT7-ARI12-C	1 µL
NcoI (10 U/µL, Fermentas MBI, #ER0571)	0.5 µL
EcoRI (10 U/µL, Fermentas MBI, #ER0271)	0.5 µL
Buffer Tango 10x (yellow) (Fermentas MBI, #BY5)	4 µL
H_2O	14 µL
TOTAL	20 µL

The samples were incubated for 2.5 h at 37ºC. 1 µL digested and undigested DNA each was loaded on a 0.8% agarose gel and run in an electrophoresis chamber for 60 min at 100V.

To verify the inserts of the pGAD-ARI12 constructs, all were sequenced with the pGAD_Fw primer (see table 3.6 – in materials section). Additionally, pGADT7-ARI12-RING1 was sequenced with the reverse pGAD_Rev primer (Table 3.6).

4.3   Cloning of ARI12 sequence (CDS, 1470 bp) into pSPYCE-35S

The genes of interest ARI12 (CDS, 1470 bp) was cloned from TOPO-ARI12 into the MCS of the overexpression pSPYCE-35S vector. ARI12 was directly cloned into the pSPYCE-35S vector after restriction of TOPO-ARI12 by the restriction enzyme XhoI (the restriction site is located in the MCS of TOPO) without gel purification.

Restriction digest of pSPYCE-35S with XhoI

2 µg pSPYCE-35S	27.5 µL
XhoI (10 U/µL, Fermentas MBI, #ER0691)	1.7 µL
Buffer R 10x (red) (Fermentas MBI, #BR5)	5 µL
H_2O	15.8 µL
TOTAL	50 µL

Restriction digest of TOPO-ARI12

2 µg TOPO-ARI12	8.6 µL	
XhoI (10 U/µL, Fermentas MBI, #ER0691)	2 µL	
NcoI (10 U/µL, Fermentas MBI, #ER0571)	0.5 µL	
Buffer Tango 10x (yellow) (Fermentas MBI, #BY5)	6 µL	(end conc. 2x)
H_2O	12.9 µL	
TOTAL	30 µL	

NcoI cuts the TOPO backbone to reduce background.

Dephosphorylation step

1.35 µg pSPYCE-35S (XhoI restricted)	48 µL
Shrimp Alkaline Phosphatase (SAP)	
(1 U/µL, Roche, 11758250001)	3 µL
TOTAL	51 µL

The sample was incubated for one h at 37⁰C and the alkaline phosphatase was inactivated for 15 min at 65⁰C on a heat block thereafter.

Ligation control pSPYCE-35S XhoI

Kinase was added in the following mix:

A) Ligation of dephosphorylated vector

26 ng pSPYCE-35S, SAP (XhoI restricted)	1 µL
Ligation buffer A 10x (home-made)	2 µL
rATP (10 mM)	2 µL
T4 Polynucleotide Kinase (PNK) (10 U/µL, Fermentas MBI, #EK0031)	0.5 µL
H_2O	14.5 µL
TOTAL	20 µL

B) Background of undigested vector in the ligase buffer

52 ng pSPYCE-35S, SAP (XhoI restricted)	2 µL
Ligation buffer A 10x (home-made)	2 µL
rATP (10 mM)	2 µL
H_2O	14 µL
TOTAL	20 µL

The samples A and B were incubated for one h at 37⁰C and the kinase inactivated by 10 min at 65⁰C. 0.6 µL of Fermentas MBI T4 ligase was added and the sample was incubated at 22⁰C for one h. 20 µL KCM buffer 5x and 60 µL H_2O were added to samples A and B.

A) Ligation ARI12 in pSPYCE-35S

Vector:insert ratio 1:5

52 ng pSPYCE-35S, SAP (XhoI restricted)	2 µL
31 ng ARI12 (CDS, XhoI restricted)	1 µL
T4 DNA ligase buffer 10x (Fermentas MBI)	2 µL
T4 DNA ligase (5 Weiss U/µl, Fermentas MBI, #EL0331)	0.6 µL
H_2O	14.4 µL
TOTAL	20 µL

B) Background control for dephosphorylated and digested vector

52 ng pSPYCE-35S, SAP (XhoI restricted)	2 µL
T4 DNA ligase buffer 10x (Fermentas MBI)	2 µL
T4 DNA ligase (5 Weiss U/µl, Fermentas MBI, #EL0331)	0.6 µL
H$_2$O	15.4 µL
TOTAL	20 µL

The samples were incubated for 1h at 22°C with the T4 ligase. Then 60 µL H$_2$O and 20 µL KCM buffer 5x was added to A and B each. Furthermore a negative control C and a transformation control D were prepared.

C) Background of undigested vector

52 ng pSPYCE-35S, SAP (XhoI restricted)	2 µL
KCM buffer 5x	20 µL
H$_2$O	78 µL
TOTAL	100 µL

D) Undigested vector only (transformation control)

36 ng pSPYCE-35S	0.5 µL
KCM buffer 5x	20 µL
H$_2$O	79.5 µL
TOTAL	100 µL

The further transformation step was performed with all samples (A, B, C, D) as described in section 4.1.10 using YT agar plates with 50 µg/µL kanamycin. These were incubated overnight at 37°C.

Pre-screening for pSPYCE-35S-ARI12

Cloning of ARI12 into the pSPYCE-35S vector was performed without purification of ARI12 after restriction digest from TOPO-ARI12. This resulted in a high background of TOPO vector containing *E. coli* during the cloning process making a pre-screening necessary. Plasmid DNA of colonies which only grew on kanamycin and not on ampicillin agar plates were potential positive pSPYCE-35S-ARI12 clones and confirmed by plasmid DNA (miniprep protocol section 4.1.1, antibiotic selection kanamycin 50 µg/µL) and restriction digest (concentrations below in 4.5). TOPO provides AMP and KAN resistance, whereas pSPYCE-35S only codes for KAN resistance. pSPYCE-35S-ARI12 candidate minipreps were digested with XhoI (10 U/µL, Fermentas MBI, #ER0691) and Buffer R 10x (red) (Fermentas MBI, #BR5).

4.4 Transfer of nCBP coding sequence from pUC-SPYCE-nCBP into pSPYCE-35S

The HindIII restriction site is not located in the multiple cloning site (MCS), but the sequences of pUC-SPYCE and pSPYCE-35S vectors are identical between HindIII and XmaI (35S cassette and MCS). Thus these sites are suitable for the transfer of nCBP into the binary pSPYCE-35S vector.

pUC-SPYCE-nCBP was digested with HindIII and XmaI and excised from an agarose gel. The 35S promoter and nCBP containing fragment was isolated with the DNA Extraction Kit (Fermentas MBI, #K0513) and ligated into binary pSPYCE-35S vector for the

generation of transgenic plants which stably overexpress under the 35S promoter nCBP fused to the C-terminal half of the YFP protein.

Restriction digest of binary pSPYCE-35S vector with XmaI, HindIII and PstI

2 µg pSPYCE-35S	27.5 µL
XmaI (5 U/µL, Promega, R649A)	0.5 µL
Buffer B 10x (Promega, R002A)	5 µL
H_2O	17 µL
TOTAL	50 µL

Restriction digest of pUC-SPYCE-nCBP

3.2 µg pUC-SPYCE-nCBP	5.5 µL
XmaI (5 U/µL, Promega, R649A)	0.5 µL
Buffer B 10x (Promega, R002A)	3 µL
H_2O	21 µL
TOTAL	30 µL

Both XmaI reactions were incubated at 37ºC for 3 h. To check for successful linearization 3µl of the restriction digest reactions were separated on a 0.8% agarose gel. Before the HindIII digest the XmaI linearized pUC-SPYCE-nCBP and pSPYCE-35S were purified by ethanol precipitation and dissolved in 10 µl H_2O (section 4.1.12).

pSPYCE-35S XmaI	10 µL
HindIII (10 U/µL, Fermentas MBI, #ER0501)	0.5 µL
Buffer R 10x (red) (Fermentas MBI, #BR5)	2 µL
H_2O	7.5 µL
TOTAL	20 µL

The samples were incubated with HindIII for 3 h at 37ºC. Afterwards PstI (incubation time: 3 h) was added to XmaI and HindIII digested pSPYCE-35S without additional buffer or water. PstI restriction reduced background, since incompletely cut pSPYCE-35S vectors are less prone to religate when further digested with PstI.

The cDNA of nCBP with 35S promoter and additional flanking regions of the pUC-SPYCE vector that had a size of 1570 bp was excised from the gel and gel purification was performed (see 4.1.5).

A) Ligation nCBP in pSPYCE-35S

Vector:insert ratio 1:10

50 ng pSPYCE-35S (HindIII, XmaI, PstI)	0.8 µL
67.6 ng nCBP	2.6 µL
T4 DNA ligase buffer 10x (Fermentas MBI)	2 µL
T4 DNA ligase (5 Weiss U/µl, Fermentas MBI, #EL0331)	0.6 µL
H_2O	14 µL
TOTAL	20 µL

B) Background with ligase buffer

50 ng pSPYCE-35S (HindIII, XmaI, PstI)	0.8 µL
T4 DNA ligase buffer 10x (Fermentas MBI)	2 µL
T4 DNA ligase (5 Weiss U/µl, Fermentas MBI, #EL0331)	0.6 µL
H$_2$O	16.6 µL
TOTAL	20 µL

The samples were incubated for 1h at 22⁰C with the T4 ligase. Then 60 µL H$_2$O and 20 µL KCM buffer 5x was added to A and B each.

C) Transformation control

72 ng pSPYCE-35S	1 µL
KCM buffer 5x	20 µL
H$_2$O	79 µL
TOTAL	100 µL

The further transformation step is as in section 4.1.10. Here, YT agar plates with 50 µg/µL kanamycin were used. These were incubated overnight at 37⁰C.

4.5   Verifying the cloning of pSPYCE-35S-nCBP and pSPYCE-35S-ARI12

To identify the clones that harbor the nCBP or ARI12 cDNAs, diagnostic restriction digest were done with XhoI for ARI12 and double digest with PstI and EcoRI for nCBP.

pSPYCE-35S-ARI12 candidates with insert are expected to release a fragment with the size of 1470 bp. The vector residue should be approximately 12600 bp. For pSPYCE-35S empty vector a 14084 bp size linearized DNA is expected.

pSPYCE-35S-nCBP candidates were expected to have fragment sizes of 1105 bp, 1109 bp, 4480 bp and 6600 bp (EcoRI and PstI double digest) or 2x 6600 bp (PstI only) or 1105 bp + 12095 bp (EcoRI only), respectively.

Minipreps (DNA plasmid isolation) of pSPYCE-35S-nCBP and pSPYCE-35S-ARI12 candidates were performed as described in section 4.1.1.

ARI12

Digestion with XhoI

pSPYCE-35S-ARI12 candidates	3 µL
XhoI (10 U/µL, Fermentas MBI, #ER0691)	0.3 µL
Buffer R 10x (red) (Fermentas MBI, #BR5)	2 µL
H$_2$O	14.7 µL
TOTAL	20 µL

nCBP

Digestion with PstI and EcoRI (double digest)

pSPYCE-35S-nCBP candidates	2 µL

PstI (10 U/µL, Fermentas MBI, #ER0611)	0.5 µL
EcoRI (10 U/µL, Fermentas MBI, #ER0271)	0.5 µL
Buffer O 10x (orange) (Fermentas MBI, #BO5)	2 µL
H$_2$O	15 µL
TOTAL	20 µL

The samples were incubated at 37⁰C for 2 h.

Digestion of positive colony 34 with PstI or EcoRI

pSPYCE-35S-nCBP candidates	2 µL	2 µL
PstI (10 U/µL, Fermentas MBI, #ER0611)	0.5 µL	-
EcoRI (10 U/µL, Fermentas MBI, #ER0271)	-	0.5 µL
Buffer O 10x (orange) (Fermentas MBI, #BO5)	2 µL	2 µL
H$_2$O	15.5 µL	15.5 µL
TOTAL	20 µL	20 µL

The sample was incubated at 37⁰C for 2 h.

The sizes of the DNA fragments were verified on a 0.8% agarose gel: nCBP CDS with the 35S cassette 1609 bp (HindIII and XmaI) and ARI12 1470 bp (XhoI) were confirmed to be inside. *E. coli* clones containing the confirmed pSPYCE-35S-ARI12 or the pSPYCE-35S-nCBP constructs were isolated (kanamycin selection, 50mg/L) by Nucleobond Xtra Midi kit (4.1.2).

4.6 Yeast Transformation

pGADT7 and pGBKT7 constructs were transformed into yeast by the following protocol:

Preparation of competent yeast cells

On the first day, competent cells of the yeast strain PJ69-4a from YPD plate were inoculated in 25 mL YPD media (with 2% Glucose). The culture was incubated at 30⁰C and 180 rpm overnight.

OD600 of the culture was measured on the next day, which should be around 1-1.5. 100 mL YPD (with 2% Glucose) was added with such an amount of overnight culture to reach OD600 0.2-0.3. This culture was shaken for 3 h at 180 rpm. Then the culture was centrifuged in several 50 mL Falcon tubes for 10 min at 5000 rpm and 4⁰C. The supernatant was removed immediately after centrifugation and the pellet was suspended in 30 mL sterile water. The culture with water was centrifuged and the pellet was resuspended in 1 mL of a 1x TE/LiAc/H$_2$O mixture. This mixture was prepared by mixing 1 mL 10x TE pH 7.5, 1 mL 10x LiAc and 8 mL H$_2$O freshly.

Transformation of competent yeast cells

100 ng of each plasmid was transferred to a new tube. 0.1 mg herring testes carrier DNA per sample and construct combination was added to the tubes and mixed. 100 µL of yeast competent cells (in TE/LiAc/H$_2$O) was transferred to each tube and mixed well. 600 µL of sterile fresh PEG/LiAc (1mL TE 10x pH 7.5/1 mL LiAc/8 mL PEG) solution was added to each tube and mixed by hand. The samples were incubated for 30 min at 180-200 rpm and 30⁰C thereafter. 70 µL DMSO was added and the solution was mixed by gentle inversion. The samples were heat-shocked at 42°C for 20 min. The tubes were mixed every 2 min. The cells were immediately chilled on ice for 20 min after the heat shock.

The cells were centrifuged for 2 min at 4000 rpm, the supernatant was removed and the cells were resuspended in 80 µL 1x TE pH 7.5. 20 µL transformed cell suspension was spread on SD –Trp -Leu plates, 1 plate per construct combination. The plates were incubated at 30°C until colonies were grown and clearly visible (2 to 3 days).

Transformed constructs in the PJ69-4a yeast strain

Sample	Constructs
1	pGADT7-ARI12-RING1 x BK-eIF4E
2	pGADT7-ARI12-RING1 x BK-nCBP
3	pGADT7-ARI12-RING1 x BK
4	pGADT7-ARI12-RING1-IBR x BK-eIF4E
5	pGADT7-ARI12-RING1-IBR x BK-nCBP
6	pGADT7-ARI12-RING1-IBR x BK
7	pGADT7-ARI12-IBR-RING2-C x BK-eIF4E
8	pGADT7-ARI12-IBR-RING2-C x BK-nCBP
9	pGADT7-ARI12-IBR-RING2-C x BK
10	pGADT7-ARI12-RING2-C x BK-eIF4E
11	pGADT7-ARI12-RING2-C x BK-nCBP
12	pGADT7-ARI12-RING2-C x BK
13	pGADT7-ARI12-C x BK-eIF4E
14	pGADT7-ARI12-C x BK-nCBP
15	pGADT7-ARI12-C x BK
19	AD x BK

Following transformed constructs were already available

16	pGADT7-ARI12 full length x BK-eIF4E
17	pGADT7-ARI12 full length x BK-nCBP
18	pGADT7-ARI12 full length x BK

4.7   Yeast Two Hybrid growth assay

3 colonies per construct were inoculated into 4 mL of SD –Leu –Trp liquid medium and incubated at 30°C and 180 rpm for two days. 200 µL from these overnight yeast cultures were diluted by adding 800 µL sterile water and the optical density was measured at OD600. For every measurement a blank was taken with SD medium (200 µL SD in 800 µL sterile water). The volume of yeast culture necessary for the proceeding step was calculated with the formula $V = (0.25 \times 1 \text{ mL})/OD600$. The calculated volume was transferred to a tube and centrifuged for 3-4 min at 4000 rpm and room temperature. The supernatant was discarded and the pellet was suspended in 1 mL sterile water (1st concentration). The culture was diluted 1:10 and 1:100. 12 µL of every concentration and construct was spotted onto a SD –Leu –Trp, a SD –Leu –Trp –His and a SD –Leu –Trp –His 3 mM aminotriazol media plate. After all spots had dried, the plates were incubated at 30°C over 2 nights.

4.8   Beta-galactosidase assay

The original source of the methods described here are from Guarente (Guarente 1983). Transformed yeast colonies were inoculated in 5 mL liquid SD –Leu –Trp selection medium and incubated at 30°C and 180 rpm over 2 nights. If available, 3 colonies of each construct were grown. On the third day 1 mL overnight culture was transferred into 4 mL fresh SD –Leu –Trp medium and incubated for 4h at 30°C and 180 rpm. OD600 of the overnight culture was measured in 1:1 dilution. In case of weak growth the measurement was made with 1 mL undiluted culture.

After 4 h of incubation 1.5 mL culture was transferred to a 1.5 mL Eppendorf tube. The tubes were centrifuged for 5 min at 14000 rpm and 4°C. The supernatant was discarded and the pellet was washed once with 1.5 mL Buffer Z. Another centrifugation step for 5

min at 14000 rpm and 4°C was performed. The pellet was resuspended in 500 µL Buffer Z. A blank sample was also prepared by adding 500 µL Buffer Z to a new tube. 25 µL chloroform was added to each sample and the cells were vortexed for 15 seconds for lysis. They were pre-incubated for 10 min at 30°C and 100 µL ONPG was added per sample thereafter. After another incubation step with the substrate for 20 min shaken gently at 30°C, the reaction was stopped with 250 µL 1M Na_2CO_3. The tubes were centrifuged for 5 min at 10000 rpm and the supernatant was transferred to cuvettes for OD420 measurement without taking up any chloroform.

The OD_{420} was measured and LacZ activity was calculated by the formula:

beta-galactosidase units = 1000 x OD_{420} / (t x V x OD_{600})

OD_{420} … OD420 of the reaction solution

t … elapsed time (in min) of incubation; is omitted because all samples are incubated for the same time period

V … 0.1 mL x concentration factor (here 1.5/0.5 mL = 3)

OD_{600} … OD600 of 1 mL overnight culture

4.9 Western blot

SDS Page

12.5% SDS gels were used because protein sizes are between 20 and 75 kDa. Four 12.5% gels with 10 slots each were made. The mix for the SDS separating gel was pipetted into BIORAD glass plates. An isopropanol film was added to avoid air bubbles. After 20-30 min the gel solidified and remaining isopropanol was flushed with sterile water. The SDS stacking gel was mixed and added afterwards. The combs were inserted. After 20 min the gel solidified and was stored at 4°C until the next day.

Table 4.3. Protocol for making a 12.5% SDS separating gel and a SDS stacking gel

Ingredients

SDS separating gel

	12.50%			
	2 gels		4 gels	
	14 mL		28 mL	
dH$_2$O	5.84	mL	11.68	mL
40% AA (29:1)	4.38	mL	8.76	mL
1.5M Tris-HCl pH 8.8	3.5	mL	7	mL
10% SDS	0.14	mL	0.28	mL
10% APS	0.14	mL	0.28	mL
TEMED	0.0084	mL	0.0168	mL

SDS stacking gel

	2 gels		4 gels	
	7 mL			
dH2O	5.18	mL	10.36	mL
40% AA (29:1)	0.91	mL	1.82	mL
1.5M Tris-HCl pH 6.8	0.875	mL	1.75	mL
10% SDS	0.07	mL	0.14	mL
10% APS	0.07	mL	0.14	mL

| TEMED | 0.007 | mL | 0.014 | mL |

Protein isolation of yeast cultures for Western blots

The "Rapid Yeast Protein Prep for SDS PAGE and Western" protocol by Horvath and Riezman, Yeast, 1994 from the Gottschling Lab has been modified and was performed for the Y2H Western blotting. A quantification step could be introduced by modifying the protocol. Less 2-mercaptoethanol was added. Instead of mixing bromophenol blue to the buffer, loading dye was added before the loading step.

Original concentrations	Original volumes	**Modified protocol**
Sample Buffer:	10 mL	**2 mL**
0.06 M Tris-HCl pH 6.8	0.6 mL 1M Tris-HCl pH 6.8	**0.12 mL 1 M Tris-HCl pH 6.8**
10% (v/v) glycerol	2 mL 50% glycerol	**0.233 mL 86% glycerol**
2% (w/v) SDS	2 mL 10% SDS	**0.4 mL 10% SDS**
5% (v/v) 2-mercaptoethanol	0.5 mL 2-mercaptoethanol	**0.05 mL 2-mercaptoethanol (2.5%, final concentration. 1.75 mM for QuBit)**
0.0025% (w/v) bromophenol blue	0.1 mL Sat. bromophenol blue	**No bromophenol added for quantification with Invitrogen QuBit**
	4.9 mL H2O	**1.197 mL H$_2$O**

Yeasts carrying the Yeast Two Hybrid constructs were incubated over two nights at 30ºC and 180 rpm. The OD600 of the culture was measured in intervals. When a culture reached 0.5-1.0 at OD600, the cells of this sample were harvested, centrifuged at 13000 rpm for 3 min at room temperature. After the supernatant was removed, the cells were frozen with liquid nitrogen and stored at -80ºC.

The cells were taken from the stocks on the day of blotting. The pellets were washed once with 500 ⌠L H2O and collected again by centrifugation. The cells were resuspended in 100 µL sample buffer: see above in modified protocol for the composition. The proteins were denatured for 5 min at 95ºC. A centrifugation step for 5 min at 14000 rpm followed. 15 µg of every sample was loaded onto a gel after adding 5 µL 4x loading dye (with DTT) to every sample. The cells were normalised according to a quantification done with the Quant-iT Protein Assay (Invitrogen, Q33211) kit.

The quantification was performed according to the manufacturers' instructions. 1 µL Quant-iT Reagent (Invitrogen, Quant-iT Protein Assay Kit) and 199 µL Quant-iT Buffer (Invitrogen, Quant-iT Protein Assay Kit) per sample were mixed. 199 µL of this mix was added to 1 µL protein sample for measurement. To establish a standard curve 190 µL of this mix was used for each 10 µL BSA standard. The samples and standards were incubated for 15 min at room temperature and then measured in the QuBit fluorometer (Invitrogen).

Loading the SDS gel and electrophoresis

5 mL 4x loading Dye was added to 15 µg sample. Before loading, the samples were denatured for another 3 min at 95ºC and stored on ice. Running buffer 5x was diluted to 1x and 1g SDS/L was added.

The gels were taken from 4ºC. The electrophoresis chamber was filled up with 1x running buffer. 3 µL pre-staining protein molecular weight marker (Fermentas MBI #SM0431) was loaded to the first slot of every gel. 15 µg protein sample each was loaded from left to the right. Electrophoresis was performed at 40 mA and for 1h 10 min (Mini-Protean-electrophoresis, BIORAD).

Electro-Blotting

Transfer buffer (for Western blot) was made by diluting a 10x western transfer buffer stock to a 1x buffer. The membranes were activated by gently shaking them in methanol. Tank

blotting was carried out at constant 30V overnight at 4ºC (Trans-Blot Cell, BIORAD) using transfer buffer pH 8.3 (for Western blot; 500 mM Tris, 500 mM boric acid). The transfer direction is from the cathode (negative pole) to the anode (positive pole).

To check whether blotting was successful, the gels were shaken in coomassie blue dye for about 2 h and destained with a destaining solution again for 2 h. Protein bands were lacking on the gels, which proves that the blotting was successful.

Immunodetection

On the next day, the membranes were taken from the blotting apparatus and gently shaken in blocking solution for an h. The blocking solution was discarded and the membranes were washed with 1x TBS-T buffer briefly. Antibodies were diluted in TBS-T buffer. 2 mL of primary antibodies with a dilution of 1:1000 for MSα c-Myc antibodies or 2 mL HA antibodies in 1:100 dilution were applied to a parafilm attached in a square petri dish (per membrane and antibody combination). The membranes with the blotted protein side facing the parafilm were laid upon the antibodies and incubated for 1 h on a rotary shaker. Washing steps with enough TBS-T buffer covering the membranes were performed: 1 x 15min and 2 x 5 min. The secondary antibody anti-mouse-HRP (Pierce) in 1:10000 dilution (freshly made from 0.8 mg/mL stock) was applied to all membranes, 2 mL each. The samples were incubated for another h and washings steps were repeated (1 x 15 min, 2 x 5 min). 2 mL Chemoluminescence substrate Roti Lumin (Roth, P078.1) was made (mix of Lumin 1 with Lumin 2 in 1:1 ratio), added to the membranes in a foil and incubated for 2 min. Then the substrate was discarded and the membrane was welded in foil. An x-ray film was exposed to chemoluminescence light for 3 min, 10 min, 30 min and 60 min, respectively, and developed. Since the Western Blot gave very week signals the Western Blot analysis was repeated with a new batch of antibodies and 30 µg protein. Exposure time was extended to 90 min.

4.10 Overexpression of ARI12 and nCBP in *Arabidopsis thaliana*

4.10.1 Generation of transgenic plants overexpressing ARI12 and nCBP tagged with the C-terminal YFP

a) Sterilization and plating of the seeds

Sterilization protocol

The seeds were put into an Eppendorf tube and sterilized with approximately 1.2 mL sterilization solution for 3 min in the tubes and centrifuged briefly. Then the solution was removed and the seeds were washed twice with sterile water. 10 drops 1% low melting agarose were added to each tube.

20 seeds per batch of Col WT, Ler WT, *ari12-1* (Ler WT), *ncbp* SALK (Col) were plated on a MS 2.5 agar plate. The plates were put on 4ºC for 48 h for synchronisation of the germination (stratification). Then the plates were transferred to the RUMED at 22ºC. The lines were moved onto soil after 15 or 9 days respectively after germination. 2 batches of plants were grown. A batch consists of 8 pots per ecotype and 4 plants per pot.

b) *Agrobacteria* transformation

Preparation of electrocompetent *Agrobacterium tumefaciens* cells

6 mL YT liquid medium containing the appropriate antibiotics were inoculated with a single colony and incubated at 140 rpm overnight at 28 °C. The next day, 500 mL of YT liquid selection medium was inoculated with 5 mL of the overnight culture and grown to an OD600 of 0.6-0.8. From this stage on every step was done on ice and with cold solutions: cells were transferred into 50 mL Falcon tubes, chilled on ice for 15 minutes and pelleted

at 4000 *g* for 15 minutes at 4 °C. The supernatant was discarded and the cells were resuspended in 250 mL sterile H_2O. The centrifugation step was repeated three times, and the cells were resuspended step by step in 25 mL sterile H_2O, 2 mL sterile H_2O, and 2 mL sterile 10% glycerol. Finally, aliquots of 100 µL were frozen in liquid N_2 and stored at 80 °C.

Electroporation

Following constructs were transformed into agrobacterium and plated out on YT media plates with the antibiotics in the material section:

1.) pSPYCE-35S-nCBP-31

2.) pSPYCE-35S-ARI12-308

3.) Control for plant transformation: pSPYCE-35S empty

4.) Negative control: no DNA

4 clean electroporation cuvettes (1 mm, Equibio, yellow cap) were put into the UV cross linker for sterilization, which was set to 6000 kJ. The cuvettes were then cooled on ice. 100 ng DNA of each construct was added to 50 µl competent agrobacteria that were previously thawn on ice. Electroporation was performed with the "AGR" programme (2.45 kV, 25 µF and 200 ohm, 1 pulse) on the BIORAD MicroPulser Electroporation System. The time constant should be between 4.4 – 4.8 ms. After electroporation 700 µL YT medium was added to the electrophorated agrobacteria in the cuvette and the mixture was transferred back to the reaction tube. The tube was subsequently shaken at 30°C for 50 min at 180 rpm. The cells were plated with the following volumes on YT plates with the appropriate antibiotics (see Material – table 3.6): 50 µL, 100 µL, 200 µL and incubated for two days at 30°C.

To confirm the transformation of the constructs 5 colonies of each transformation (nCBP, ARI12, control) were inoculated in 5 mL YT medium with antibiotic selection and incubated over two nights at 30°C.

c) Plasmid miniprep from *Agrobacteria*

The original protocol is taken from Li X., Stahl R. and Brown G.G. (Li et al. 1995).

2 mL of overnight culture was transferred to a tube and centrifuged at 5000 rpm for 5 min at 4ºC. The supernatant was removed and 200 µL GTE buffer was added. The pellet was well suspended in the GTE buffer and incubated for 5 min at room temperature. 400 µL fresh-made 0.2 M NaOH/1% SDS was added and the samples were incubated on ice for 15 min. 300 µL 3 M K/5 M acetate pH 4.8 solution (made of 3 M CH_3COOK und 2M CH_3COOH, titrated to pH 4.8 with additional acetate) was added afterwards. After rigorous shaking the mix was incubated for 30 min on ice. The samples were centrifuged for 15 min at 13000 rpm and 4ºC. The supernatant was transferred to a new tube while avoiding taking up the cell residues. A 500 µL phenol-chloroform-isoamyl alcohol 24:24:1 mixture was added. The tube was manually inverted for 1 min and another centrifugation step of 5 min at 13000 rpm and 4ºC followed. The supernatant was transferred to a tube with 1200 µL isopropanol. The tube was inverted and centrifuged for 30 min at 13000 rpm and 4°C. The supernatant was discarded and the DNA was washed with 500 µL 70% ethanol once. The sample was centrifuged for 10 min at 13000 rpm and 4°C again. The supernatant was removed with a pipette and the pellet was dried with the Eppendorf concentrator for 5 min. The DNA pellet was suspended in 20 µL TE pH 8.0 and 2 µL RNase A (10 µg/mL). After 10 min of shaking 10 µl of the plasmid prep was used for restriction digestion. To confirm the presence of the correct constructs, isolated plasmid DNA from agrobacteria was digested with 5 U EcoRI or EcoRI and PstI (each 5 U, see below). Plasmid DNA of

the pSPYCE-35S constructs isolated from *E. coli* with the Nucleobond Xtra Midi Kit (see section 4.1.2) were taken as controls.

Restriction digest of pSPYCE-35S-ARI12 (DNA from agrobacteria)

DNA from agrobacteria (pSPYCE-35S-ARI12 candidate)	10 µL
PstI (10 U/µL, Fermentas MBI, #ER0611)	0.5 µL
Buffer O 10x (orange) (Fermentas MBI, #BO5)	2 µL
H$_2$O	7.5 µL
TOTAL	20 µL

Restriction digest of pSPYCE-35S-ARI12 (DNA from *E. coli*)

300 ng DNA from *E.coli* (pSPYCE-35S-ARI12)	x µL
PstI (10 U/µL, Fermentas MBI, #ER0611)	0.5 µL
Buffer O 10x (orange) (Fermentas MBI, #BO5)	2 µL
H$_2$O	up to 20 µL
TOTAL	20 µL

Restriction digest of pSPYCE-35S-nCBP (DNA from agrobacteria)

DNA from agrobacteria (pSPYCE-35S-nCBP candidate)	10 µL
PstI (10 U/µL, Fermentas MBI, #ER0611)	0.5 µL
EcoRI (10 U/µL, Fermentas MBI, #ER0271)	0.5 µL
Buffer O 10x (orange) 10x (Fermentas MBI, #BO5)	2 µL
H$_2$O	7 µL
TOTAL	20 µL

Restriction digest of pSPYCE-35S-nCBP (DNA from *E. coli*)

300 ng DNA from *E.coli* (pSPYCE-35S-nCBP)	x µL
PstI (10 U/µL, Fermentas MBI, #ER0611)	0.5 µL
EcoRI (10 U/µL, Fermentas MBI, #ER0271)	0.5 µL
Buffer O 10x (orange) 10x (Fermentas MBI, #BO5)	2 µL
H$_2$O	up to 20 µL
TOTAL	20 µL

Samples were incubated for 2.5 h. 10 µL of digested and undigested DNA (from agrobacteria) as well as 20 µL digested plasmid DNA isolated from *E. coli* (as a control) were loaded on a 0.8% agarose gel and run in an electrophoresis chamber for 45 min at 100V.

d) Plant transformation

Confirmed agrobacteria cells containing the 35S plasmid DNA were inoculated in 5 mL YT liquid media with antibiotics selection (see material) over 2 nights at 30ºC. 1 mL was taken from this overnight culture and inoculated in another 4 mL YT liquid media with antibiotics selection. After 2 nights of incubation at 30ºC 2 mL overnight culture was inoculated in 3

mL YT liquid media with antibiotics selection. Finally, 2 mL from this overnight culture was inoculated in 500 mL YT liquid media with antibiotics selection and incubated for 3 days at 30⁰C in a 1L flask on a shaker (180 rpm). In general, inoculating the first overnight culture in 500 mL YT liquid media is sufficient. However, several overnight cultures were needed in this experiment only to maintain the workflow of other analyses.

The density and thus growth phase of the last overnight Agrobacteria culture was determined by measuring the OD at 600 nm. The optimum for plant transformation is between 1 and 2. The OD600 was slightly above 2 in my transformation experiment. The agrobacteria were harvested by centrifugation at 6000 rpm for 15 min in a Sorvall centrifuge at 22⁰C. The supernatant was removed and new agrobacteria were added for centrifugation in a second harvest. After the supernatant was removed the agrobacterium pellet was resuspended in 100 mL 5 % sucrose. The suspension was transferred to a 1000 mL beaker and diluted with 5 % sucrose to OD600 of 0.8. The siliques of approximately 5 week old flowering Arabidopsis plants were removed before transformation. For higher transformation efficiency Silwet (300 µL per 800 µL sucrose media) was added to the Agrobacteria suspension. Up to 20 plants were dipped into the solution for 2 min. The dipped plants were kept for 24 h in the dark under a plastic lid to reduce evaporation and then transferred to the growth chamber for seed production.

e) Selecting transformed T1 plants

Sterilization and preparation of T1 seeds

T1 seeds were harvested from the transformed T0 plants, stored at least for one week at room temperature for drying. Sterilization time was prolonged to 5 min due to high contamination of the seeds.

Plating T1 seeds

The seeds of every transformed pot (4 plants per pot) were plated on MS 1% selection media with 10 mg/L BASTA generously for screening. In order to avoid false positives, seeds should not stick to each other. The plates were put on 4⁰C for 48 h for synchronisation of the germination (stratification). Then the plates were transferred to the growth chamber (RUMED) at 22⁰C under continues light (75 µEinstein/m^{-2}s^{-1}). 9-18 days after germination the transformants which are resistant to BASTA were transferred to soil (shown in table 5.5). Survived plants on soil are shown in table 5.6. From every ecotype and overexpression construct combination transgenic plants are available.

Plating T2 lines

After sterilization of the seeds (4.10.1a) 6 rows (with 20 seeds each in a row) were plated on each MS 1.0 BASTA plates, 40 seeds (2 rows) per construct/ecotype combination.

5 Results

5.1 Cloning five ARI12 deletion constructs for Y2H interactions studies

In Yeast Two Hybrid (Y2H) interaction studies one protein (X) is fused to the GAL4 activation domain (AD), while the other protein (Y) is fused to the GAL4 DNA binding domain (BK). The Y2H constructs are co-transformed into competent yeast cells. Interaction of X and Y activates the promoter and the reporter gene will be expressed (Figure 5.1) in yeast.

Figure 5.1. Schematic presentation of the principle of Y2H interaction studies.

The promoter is activated, if the two hybrid proteins (protein X fused to GAL4 DNA binding domain and protein Y fused to GAL4 activation domain) are interacting. Upon activation the reporter gene (i.e. lacZ) downstream of the promoter will be expressed and can be quantified (i.e. with ONPG for lacZ). In this figure the upstream activating sequence (UAS) is GAL1 which is recognized by fusion proteins with GAL4 (http://www.mblab.gla.ac.uk/~maria/Y2H/Y2H.html).

ARI12 deletion constructs were made in the AD (activation domain) Y2H vector for interaction studies with nCBP in the BK (binding domain) vector. Previous experiments have shown that full length ARI12 interacts with nCBP strongly in this AD-BK combination (Nigam, personal communication).

The cloning of ARI12 deletion constructs (PCR products) into TOPO vector and later to pGADT7 was not successful and asked for a change of strategy. The deletion constructs were created with the Phusion TAQ (Finnzymes) in Phusion PCR reactions. While the Finnzymes Phusion® Site-Directed Mutagenesis Kit for creating pGADT7-ARI12 constructs produced low yield, further PCRs were finally successfully set up with Finnzymes Phusion Flash TAQ. The characteristics of this polymerase is high processivity, an error rate 50-fold lower than that of *Thermus aquaticus* and high annealing temperatures. Hence the Phusion TAQ was the first choice for creating large size (> 8500 bp) ARI12 deletion constructs in the AD vector

Specific full matching, HPLC purified primers were used for the Finnzymes Phusion PCR reaction. Phusion PCR primers with annealing temperatures falling between 65°C and 72°C were designed to meet the requirements. Longer stretches of T or A at the end of the primers were avoided for stability reasons.

ARI12 Y2H constructs AD-RING1, AD-RING1-IBR, AD-IBR-RING2-C, AD-RING2-C and AD-C were generated by Phusion PCR reactions and subsequent ligation of the PCR product with the Quick T4 DNA ligase (NEB, Phusion Site-Directed Mutagenesis Kit, Finnzymes, F-541) (AD-RING1, AD-RING1-IBR, AD-IBR-RING2-C, AD-RING2-C and AD-C, section 4.2). The schematic domain structure of the ARI12 deletion constructs is shown in figure 5.2 The ARI12 domain deletion constructs were cloned and propagated in *E. coli* and co-transformed with BK-eIF4E, BK-nCBP or empty BK vector (negative control) into the yeast pJ69-4a strain. AD-ARI12 (full length ARI12) and empty AD vector as controls were also co-transformed with these BK constructs.
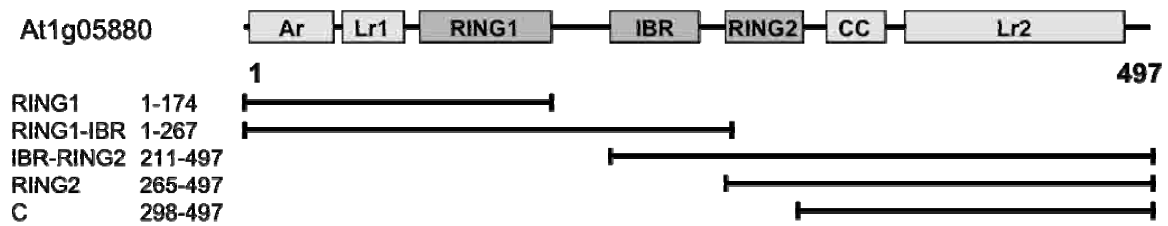
Figure 5.2. Schematic representation of the domain structure of ARI12 (At1g05880) and the deletion constructs used for Yeast Two Hybrid interaction analyses with nCBP and eIF4E.

Ar = Acid-rich, Lr = Leucine-rich, IBR = in between RING, CC = coiled-coil

The PCR product bands of the ARI12 deletion constructs are running at 10000 bp, approximately 1000 bp higher than expected.

5.1.1 Confirming the ARI12-RING1 deletion construct with colony PCR

The presence of the ARI12-RING1 construct was confirmed by a colony PCR. The estimated correct sizes were compared to the PCR product on a 0.8% agarose gel separated by electrophoresis. The result is shown in figure 5.3. The expected size of the construct was 613 bp, while the digest released an insert with the size of about 650 bp.
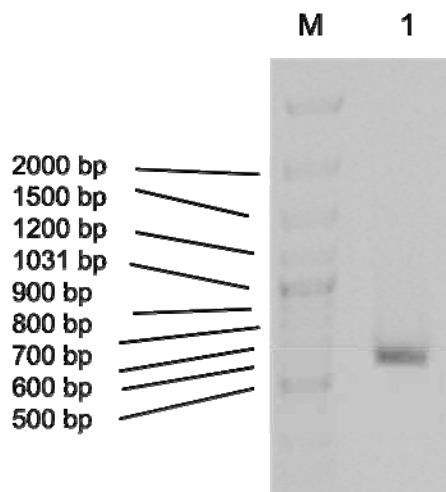

Figure 5.3. Example of colony PCR with pGAD_Fw primers of pGADT7-ARI12-RING1 candidate.

The expected size of the construct was 613 bp and matched to the band on the gel. M = 100 bp marker.

5.1.2 Confirming ARI12-RING1-IBR, ARI12-IBR-RING2-C, ARI12-RING2-C and ARI12-C construct with restriction digests

To confirm the presence of RING1, RING1-IBR, IBR-RING2-C, RING2-C and C constructs, minipreps (plasmid DNA isolation) from *E. coli* cells (grown on YT plates with kanamycin selection) were made. The DNA was digested with BamHI and NcoI (RING1-IBR) or EcoRI and NcoI (IBR-RING2-C, RING2-C and C) and run on a 0.8% agarose gel in the electrophoresis chamber.

Expected and observed band sizes of all ARI12 deletion constructs are listed in table 5.1. The expected size of the released insert were 613 bp (RING1), 759 bp (RING1-IBR), 861 bp (IBR-RING2-C), 693 bp (RING2-C) and 549 bp (C). Except RING1 and RING2-C the observed bands of the ARI12 constructs on an electrophoresis agarose gel were approximately 100 bp higher than expected (not shown). Positive clones were sequenced

and used for further experiments. However, sequencing data matched with the expected DNA sequence.

Table 5.1. Expected and observed band sizes of ARI12 deletion constructs on the agarose gel. The column "positive clones" indicate the number of positive E. coli clones out of the number of tested clones.

ARI12 construct	Confirmation by	Expected size	Observed size	Positive clones
RING1	Colony PCR	613 bp	approx. 650 bp	1 of 1
RING1-IBR	Miniprep	759 bp	approx. 900 bp	6 of 6
IBR-RING2-C	Miniprep	861 bp	approx. 1000 bp	6 of 6
RING2-C	Miniprep	693 bp	approx. 600-700 bp	6 of 8
C	Miniprep	549 bp	approx. 700 bp	6 of 6

5.1.3 DNA sequencing of ARI12 deletion constructs reveals point mutations

According to the sequencing data analysis 4 mutated nucleotides in my ARI12 deletion constructs could be detected. Table 5.2 shows mutated amino acids found and which constructs are affected. Amino acid 20, valine, is replaced by isoleucine, 65 aa serine is replaced by proline, 142 aa glycine is replaced by serine and 332 aa is a silent mutation. At position 332 aa the third nucleotide of aspartic acid thymine is replaced by cytosine, but the triplet is still coding for aspartic acid. The amino acids 20, 65 and 142 are present in the ARI12-RING1 and ARI12-RING1-IBR deletion constructs, the amino acid mutation at 332 aa affects ARI12-IBR-RING2-C, ARI12-RING2-C and ARI12-C. Mutation at 332 aa could also be found in the TOPO-ARI12-RING2-C construct (not shown here). Mutations at the same position for different constructs suggest that the DNA template was already mutated at these four positions.

Table 5.2. Mutated nucleotides in ARI12 constructs were detected at 20 aa, 65 aa, 142 aa and 332 aa. Original amino acid (Mladek et al. 2003) column shows correct coding triplets in Arabidopsis ARI12.

Amino acid	Original amino acid	Mutated amino acid	in construct
20 aa	GTC Val	ATC Ile	RING1, RING1-IBR
65 aa	TCC Ser	CCC Pro	RING1, RING1-IBR
142 aa	GGC Gly	AGC Ser	RING1, RING1-IBR
332 aa	GAT Asp	GAC Asp	IBR-RING2-C, RING2-C, C-Terminus

The Val20Ile mutation is located in the N-terminal acidic-region of ARI12. Both have similar physicochemical characteristics, they are neutral and apolar. Although Ser65 is relatively conserved in other ARI proteins the serine can be replaced by a lysine or threonine (Figure 5.4). Gly142 is located in RING1 and not conserved either among the other ARI proteins where a lysine or glutamic acid is present at this position. In *C. elegans* and *Dictyostelium discoideum* serine is actually the amino acid at this protein position. The fourth mutation of the triplet of amino acid 332 does not cause an amino acid substitution and is a silent synonymous mutation.

My clones do not contain frame shift mutations and the cysteines or histidines essential for the formation of the RING1 domain are not affected. Hence I accepted the mutations and proceeded with the Yeast Two Hybrid analyses with these constructs. We can assume that the mutations were already present in the full length ARI12 template used for PCR creating the constructs.
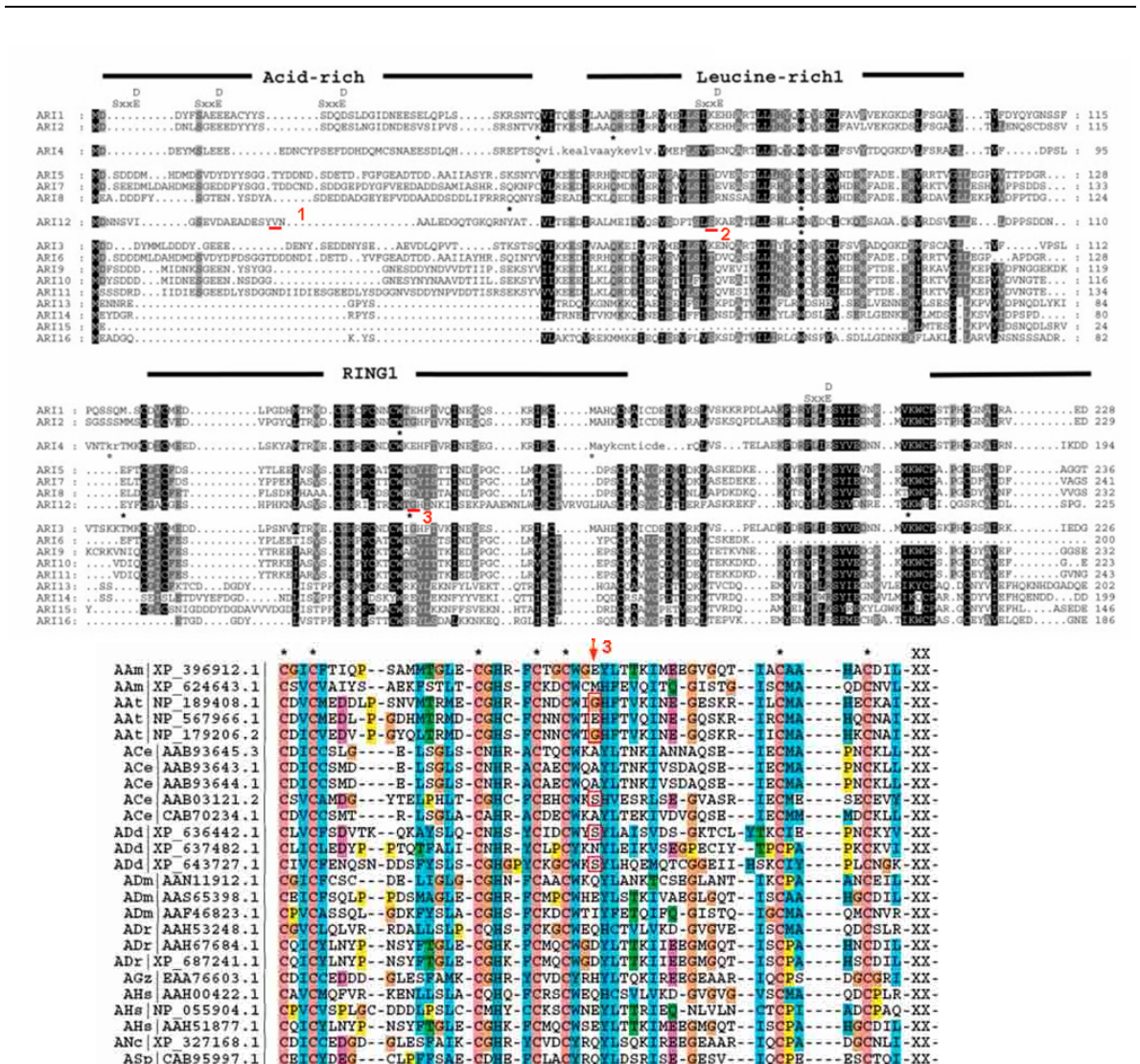
Figure 5.4. Position of the found mutations in the ARI12 alignment (top) and in RING1 of all organisms (bottom).

1 = Val20Ile mutation in the acidic-rich region, 2 = Ser65Pro and 3 = Gly142Ser mutation. Ad 3: Even though glycine is relatively conserved in Arabidopsis thaliana at position 142, serine can be actually found in *C. elegans* and *Dictyostelium discoideum*. The accession number in GenBank is followed by the subfamily and organism on the left of the sequence (bottom). *Am, Apis mellifera; At, Arabidopsis thaliana; Ce, Caenorhabditis elegans; Dd, Dictyostelium discoideum; Dm, Drosophila melanogaster; Dr, Danio rerio; Gz, Gibberella zeae; Hs, Homo sapiens; Nc, Neurospora crassa; Sp, Schizosaccharomyces pombe.* (Mladek et al. 2003; Eisenhaber et al. 2007)

5.2 Y2H growth assays of ARI12 deletion constructs with nCBP and eIF4E

Figure 5.5 shows that all Yeast Two Hybrid co-transformed ARI12 constructs interact with their potential substrate nCBP, as seen on selective SD medium –Leu –Trp –His. However, the negative control AD X BK-nCBP shows comparable growth with RING2-C and C-terminus due to auto-activation of BK-nCBP. As expected, the full length ARI12 constructs show the strongest interaction on 3-AT plates. IBR-RING2 is almost as strong, followed by the RING1 and RING1-IBR, RING2-C, C constructs and the negative control.

RING1, RING1-IBR and IBR-RING2 x eIF4E growth is relatively weak and full length ARI12 fails to show growth on the most selective medium –Leu –Trp –His 3-AT. ARI12 is not interacting at all with eIF4E when comparing it with the negative control.
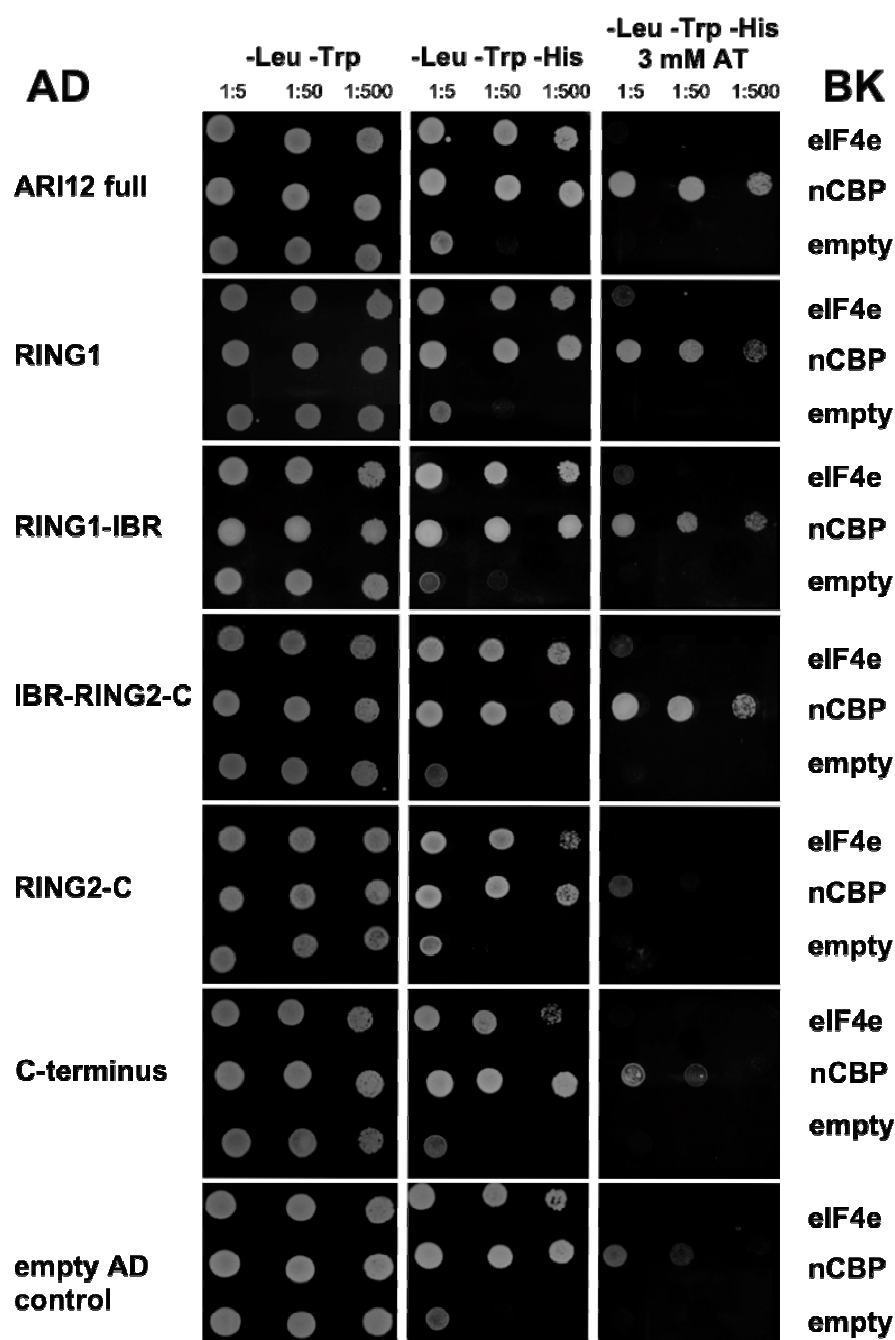
Figure 5.5. Yeast pJ69-4a strain was co-transformed with AD-ARI12 deletion constructs and BK-eIF4E or BK-nCBP, respectively.

AD empty and BK empty vectors serve as negative controls. Growth of all constructs on selective SD medium lacking leucine, tryptophan and histidine (–Leu –Trp –His; second column) show interaction with ARI12's potential substrate nCBP. However, strength of interaction shows difference in the extent of interaction on SD medium supplemented with additional 3 MM 3-Amino-1,2,4-triazole (-Leu –Trp –His 3-AT). Medium lacking leucine and tryptophan (-Leu –Trp) only are positive growth controls for all constructs. The yeast culture was diluted in three concentrations (1:5, 1:50. and 1:500).

5.3 Quantitative evaluation of Y2H interactions with enzymatic beta-galactosidase assays

Beta-galactosidase, encoded by the lacZ gene, is able to cleave beta-D-galactosides into a molecule of glucose and a molecule of galactose facilitating growth on carbon sources

like lactose. In the beta-galactosidase assay the substrate o-nitrophenyl-beta-D-galactopyranoside (ONPG) is used instead of lactose. When the enzyme cleaves ONPG, o-nitrophenol which has a yellow colour is released and absorbs light at OD420. Thus, beta-galactosidase activity can be determined in a photometer at 420 nm that allows numerical quantification of the protein interaction of the Y2H proteins, which is an advantage of the beta-galactosidase assay. However, disadvantages are relatively high standard deviations and results depend on the handling of the user that makes comparisons between users difficult. Only a small number of selected transformants can be assayed in liquid culture assays and ONPG may not be sensitive enough to quantify weak or transient two-hybrid interactions.

OD600 of the overnight culture was measured and after 4 h of incubation, yeast culture was centrifuged twice. The pellet was washed with and resuspended in Buffer Z. The samples were pre-incubated for and the substrate ONPG was added thereafter. After another incubation step with the substrate for 20 min at 30°C, the reaction was stopped with Na_2CO_3. The tubes were centrifuged and the supernatant was measured at OD420 (for detailed instructions see section 4.8). LacZ activity was calculated by the formula: beta-galactosidase units = 1000*OD420 / (t x V x OD600). Since all measurements were taken with the same volume and incubation time, the formula was simplified to: beta-galactosidase units = 1000*OD420 / OD600 in a relative manner. The results in the figure 5.5 and 5.6 were obtained from data of 2 to 4 independent experiments.

Figure 5.6. Interaction of AD RING domains with BK-nCBP.

Negative controls AD empty or BK empty vector are also included. The beta-galactosidase activity of the co-transformed yeast-two-hybrid constructs was determined in total protein extracts. Error bars correspond to standard deviation.

The beta-galactosidase results of the AD-ARI12-RING with BK-nCBP constructs are consistent with the Y2H growth assays and confirm the high auto-activation activity of BK-nCBP (figure 5.6). However, the full length ARI12 as well as the ARI12-RING1, ARI12-RING1-IBR and ARI12-IBR-RING2-C constructs show significant higher beta-galactosidase activities than the negative controls. There is no significant difference between RING1-IBR or IBR-RING2-C and full length ARI12 (p-value > 0.05). Although ARI12-RING2 and ARI12-C have measurable beta-galactosidase activities, they are not significantly higher than the nCBP auto-activation.
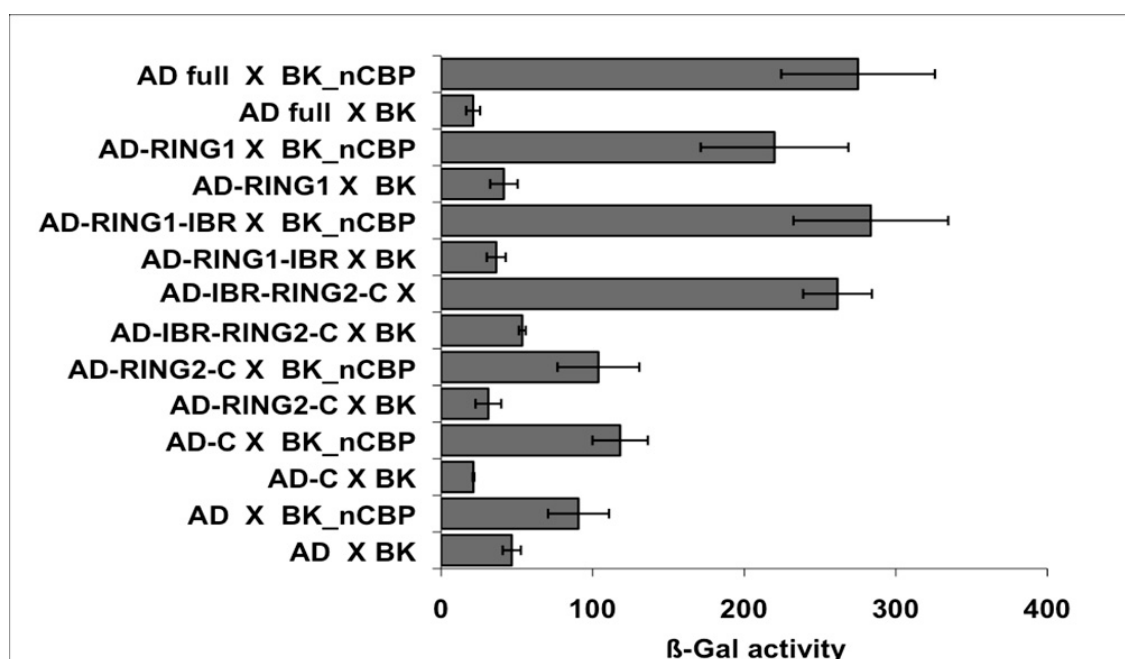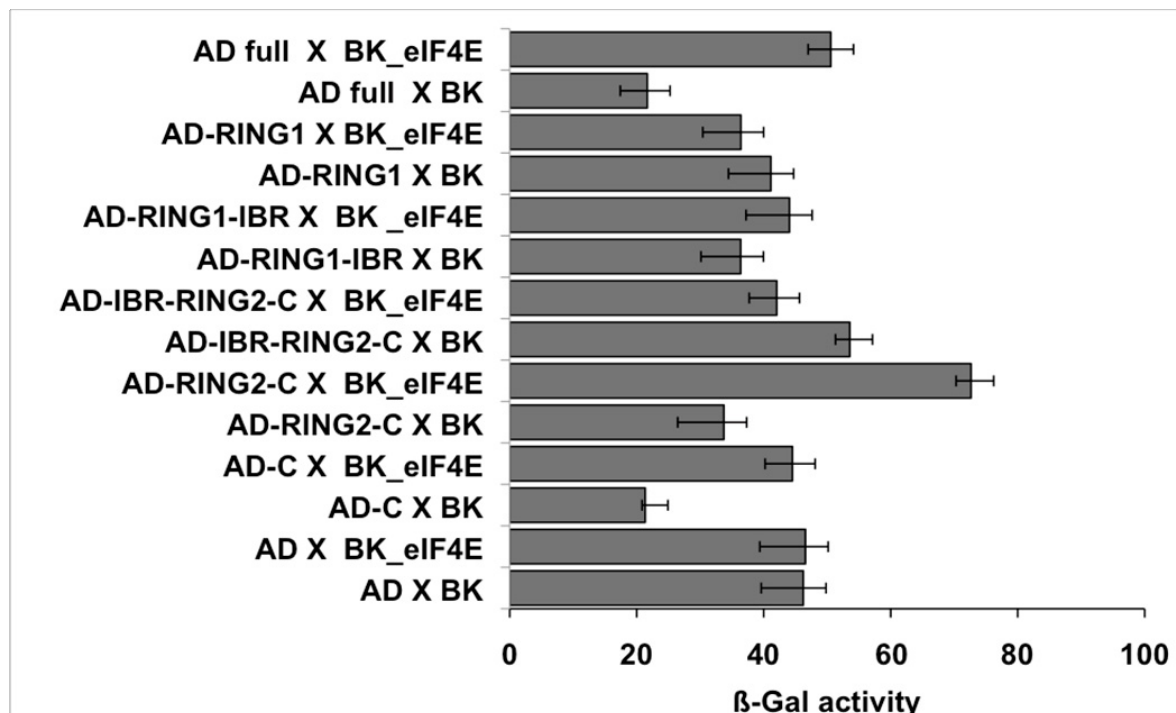
Figure 5.7. Interaction of AD RING domains with BK-eIF4E.

Negative controls AD empty or BK empty vector are also included. The beta-galactosidase activity of the co-transformed yeast-two-hybrid constructs was determined in total protein extracts. Error bars correspond to standard deviation.

The beta-galactosidase activities of the AD-ARI12–RING with BK-eIF4E constructs do not indicate significant interactions (Figure 5.7). Only the interaction between AD-ARI12-RING2-C and BK-eIF4E generates slightly higher than or similar beta-galactosidase activities as the full length ARI12 construct or the negative control. However, the results of the Y2H growth assay show weaker interaction of AD-ARI12-RING2-C than the negative control and comparable growth to AD-ARI12-C alone. Since the beta-galactosidase activities are much weaker than the ARI12-nCBP interaction, it is unlikely that ARI12 interacts with the full length eIF4E.

5.4 Quantification of the Y2H construct expression by Western Blot analyses

To determine if the yeast-two-hybrid clones express at equal levels for the two interaction partners, quantitative Western blot analyses were performed. One advantage of the Matchmaker yeast-two-hybrid constructs is that the interaction partners are fused to either a myc-tag (for BD-nCBP, BD-eIF4E) or a HA-tag (for AD-ARI12 deletion constructs). Although good antibodies for nCBP are available, I have chosen to use antibodies against the two tags since good antibodies were not available for ARI12 or for eIF4E, respectively.

The Western blot of Y2H clones expressing the HA-tagged ARI12 deletion constructs in combination with their potential interactors eIF4E and nCBP are displayed in figure 5.8. While specific bands for RING2-C were not visible, all other constructs show specific bands with expected sizes. At approximately 50 kDa to 90 kDa (stars) many unspecific bands can be seen and this make it difficult to identify the specific ones tagged with HA. For RING1 another unspecific protein band at 45 kDa is visible. The weaker signal below can be considered the specific band with 39 kDa. While the estimated size of 20.3 kDa could be detected for the negative control AD x BK, there are also unspecific bands at 25 and 50 kDa. The constructs ARI12-RING2-C and ARI12-C seems to be loaded with less

proteins since the unspecific 50 kDa band is weakly visible. Thus it might be possible that the specific band is present but below the detection limit. All other constructs show similar protein expression levels on this blot. However, AD-RING2-C X BK-nCBP's weaker interaction or beta-galactosidase assays due to lower expression or weaker Y2H yeast growth with the construct BK-nCBP cannot be ruled out. The results of the Western blot with anti-myc antibodies (for detection of BK vectors) are not shown here due to weak signal.
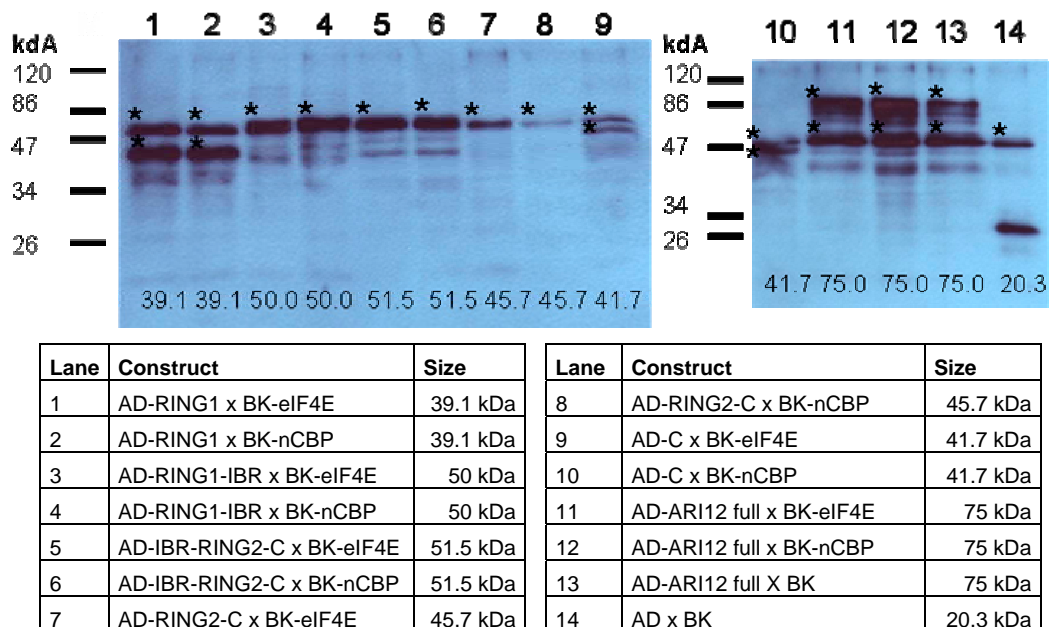

Lane	Construct	Size	Lane	Construct	Size
1	AD-RING1 x BK-eIF4E	39.1 kDa	8	AD-RING2-C x BK-nCBP	45.7 kDa
2	AD-RING1 x BK-nCBP	39.1 kDa	9	AD-C x BK-eIF4E	41.7 kDa
3	AD-RING1-IBR x BK-eIF4E	50 kDa	10	AD-C x BK-nCBP	41.7 kDa
4	AD-RING1-IBR x BK-nCBP	50 kDa	11	AD-ARI12 full x BK-eIF4E	75 kDa
5	AD-IBR-RING2-C x BK-eIF4E	51.5 kDa	12	AD-ARI12 full x BK-nCBP	75 kDa
6	AD-IBR-RING2-C x BK-nCBP	51.5 kDa	13	AD-ARI12 full X BK	75 kDa
7	AD-RING2-C x BK-eIF4E	45.7 kDa	14	AD x BK	20.3 kDa

Figure 5.8. Western blot of the HA-tagged AD-ARI12 constructs with BK-nCBP and BK empty, hybridized to HA-antibody.

30 µg protein was loaded per lane. The film was developed for 90 min. Expected sizes are shown below the bands. Loading is indicated in the table below. Bands of expected protein sizes are detectable (i.e. RING1, lane 1 and 2 at 39 kDa below the unspecific band at 45 kDa) except for AD-RING2-C (lane 7 and 8), which also are lower expressed. Unspecific proteins are abundant (especially from approximately 50 kDa to 90 kDa, stars). Lane 14 shows a nice band for the negative control AD X BK at 20.3 kDa and unspecific bands at 25 and 50 kDa are visible.

5.5 Generation of transgenic *Arabidopsis thaliana* plants overexpressing ARI12 tagged with the C-terminal end of the YFP

I generated tagged overexpression lines which allow hybridization and detection of the proteins with commonly available antibodies in future studies.

Classical genetic approaches use the phenotypes of mutants to determine the function of genes. In *Arabidopsis*, there are mutants available for nearly every gene (SIGNAL-T-DNA – http://signal.salk.edu/). However, the link between phenotype to genotype has to be proven by complementing the mutant with the wild type gene. In addition, overexpression of the gene of interest might give additional information on its function.

During my thesis another member of the lab determined that mutants of *ari12* are more resistant to UVB, indicating that ARI12 is a negative regulator of UVB responses (Lang-Mladek and Neha Nigam, personal communication). Accordingly, overexpression of ARI12 should results in plants that are more sensitive to UVB.

The 35S promoter from the Cauliflower mosaic virus (CaMV) is a very strong constitutive promoter and widely used. 35S is the coefficient of sedimentation of the viral transcript whose expression is naturally driven by this promoter. High gene expression - when

driven by the 35S promoter - can be observed in dicot plants (http://www.patentlens.net/daisy/promoters/242/g1/250.html).

In my study pSPYCE-35S containing the half YFP-tag was the vector of choice, because YFP tagged proteins can be detected with common GFP-antibodies and good ARI12 antibodies are not available in the lab. Furthermore, pSPYCE-35S is a binary vector and can be used for stable plant transformations.
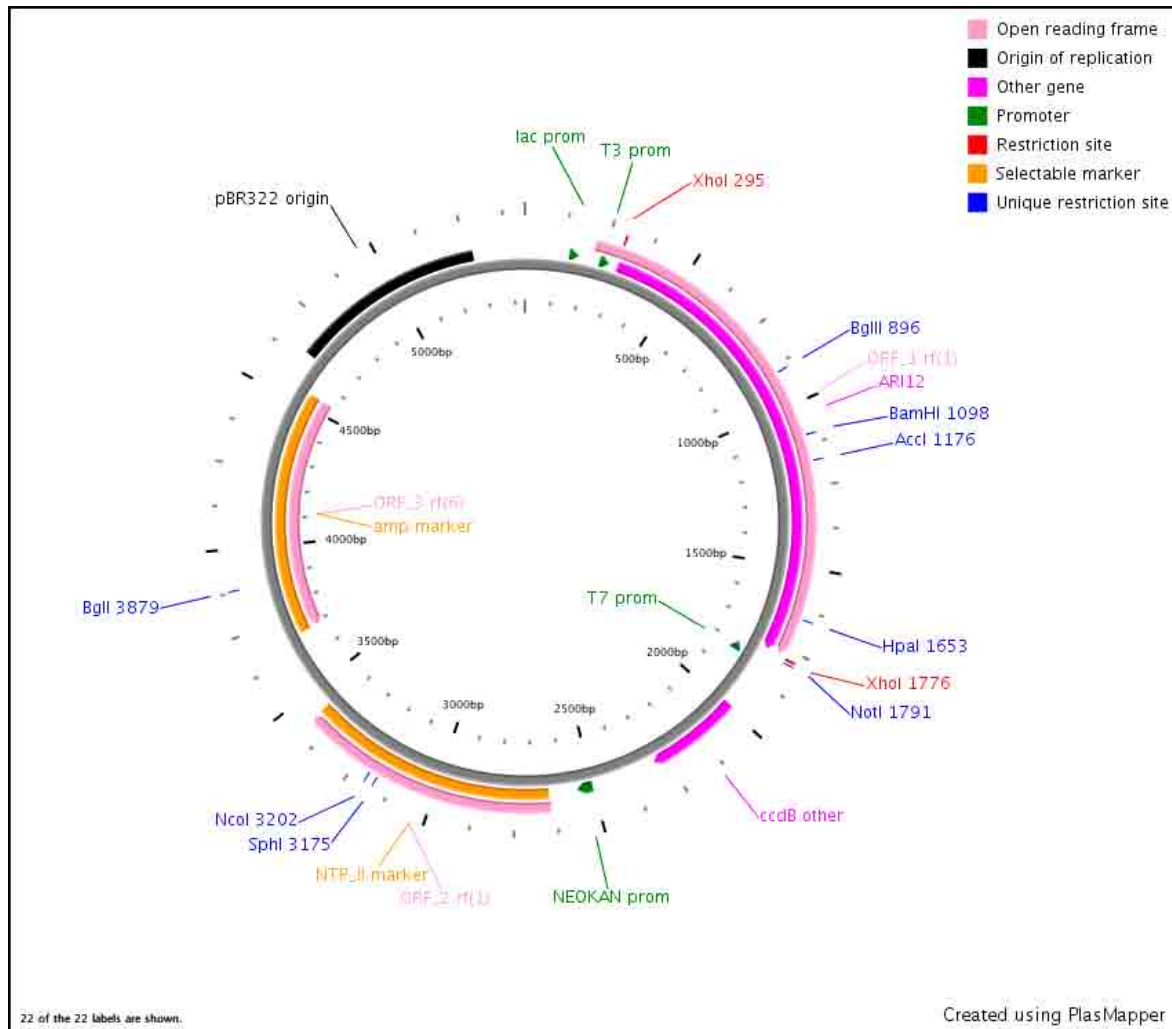

Figure 5.9. Schematic representation of the TOPO-ARI12 construct.

ARI12 is located between the XhoI sites (295bp-1776bp), marked in dark pink. The cDNA of *ARI12* was cloned from this TOPO-ARI12 to the pSPYCE-35S overexpression vector by XhoI restriction (created with PlasMapper http://wishart.biology.ualberta.ca/PlasMapper/).

AtARI12 cDNA was already cloned in the sequencing vector pCR®4-TOPO® (Invitrogen) (Map of TOPO-ARI12 in Figure 5.9.) and transferred to pSPYCE-35S by using XhoI restriction sites. Since the insert was not purified after digestions and only one cloning enzyme was used, several clones contained the TOPO-backbone. To reduce this background two strategies were used. One strategy was to further digest the TOPO backbone with NcoI which did not cut in the insert. The other strategy was to preselect the colonies, because the TOPO vector contains ampR and kanR, while pSPYCE-35 has only kanR. *E. coli* cells growing on kanamycin plates only were chosen for plasmid isolation and restriction digest analyses with XhoI. Only 1 out of 330 tested colonies contained the correct insert. The map of the successful cloned pSPYCE-35S-ARI12 is shown in figure 5.10.
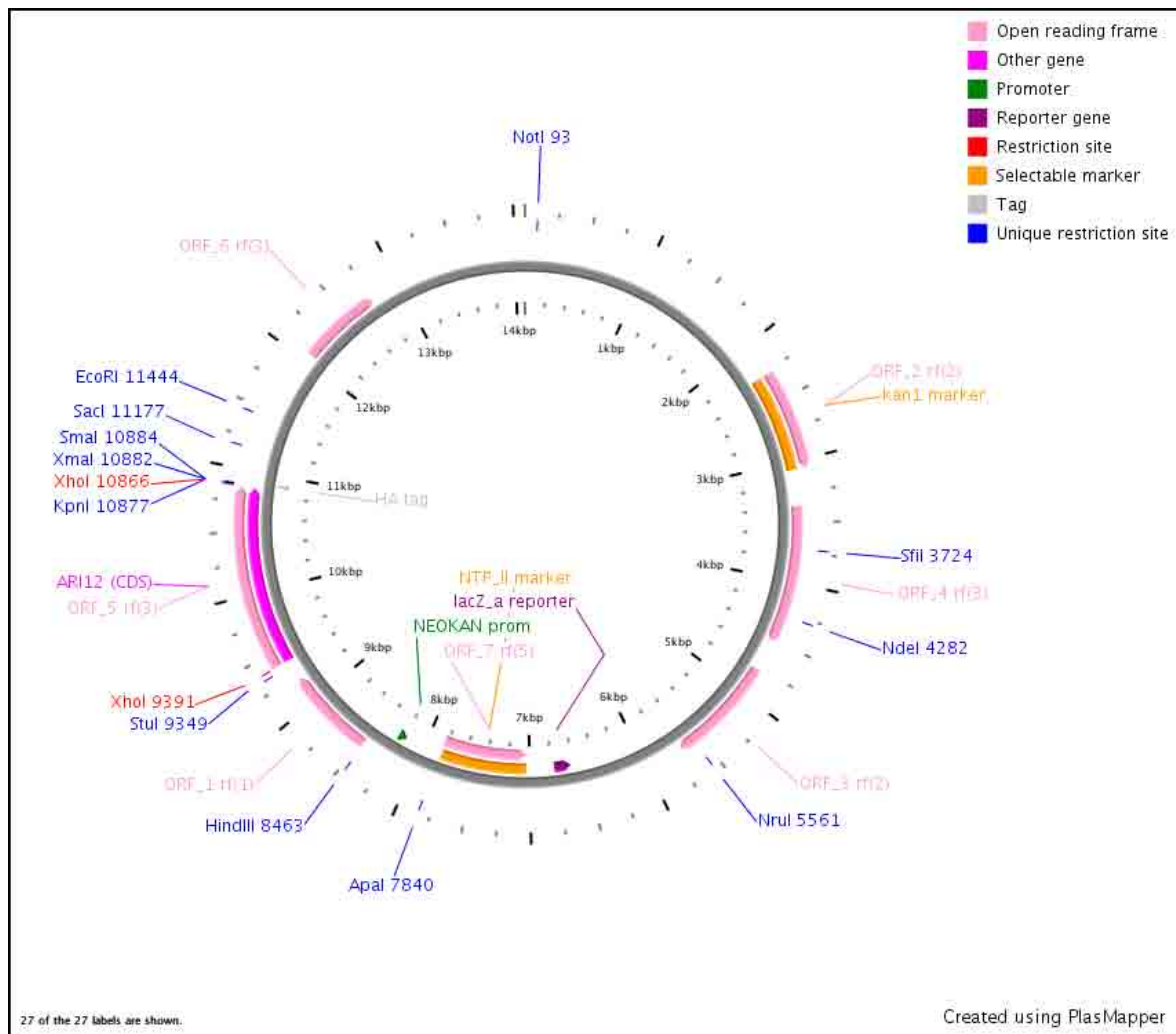
Figure 5.10 Schematic representation of the pSPYCE-35S-ARI12 clone.

The cDNA of ARI12 is located (9391-10866 bp) between the XhoI restriction sites, marked in dark pink (created with PlasMapper http://wishart.biology.ualberta.ca/PlasMapper/).

The sizes of the isolated DNA fragments were verified on a 0.8% agarose gel: ARI12 (1470 bp) was confirmed to be inside the pSPYCE-35S vector in clone number 308 which was used for plant transformation. Figure 5.11 shows confirmed pSPYCE-35S-ARI12 constructs on a 0.8% agarose gel.
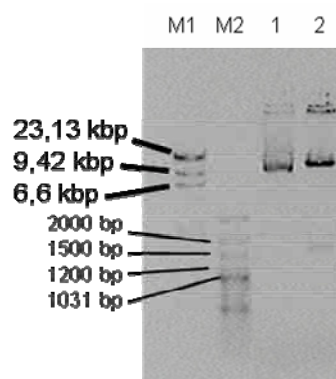
Figure 5.11. Confirmation of the construct pSPYCE-35S-ARI12 by miniprep digested with XhoI.

Lane 1 (undigested) and 2 (XhoI) shows the positive plasmid that was chosen for transformation to Arabidopsis.

Successful transformation of the pSPYCE-35S constructs into *Agrobacteria* was tested with an *Agrobacteria* miniprep. The estimated expected fragment sizes (Table 5.3.) could be detected on an agarose gel after digestion with XhoI (pSPYCE-35S-ARI12).

Table 5.3. Expected and observed DNA sizes from a miniprep isolation of the pSPYCE-35S-ARI12 construct match.

| Construct	Enzyme	Expected band size	Observed band size
pSPYCE-35S-ARI12	XhoI	1705 bp	Ca. 1700 bp

The clone 308 was confirmed by sequencing with the primer ariUpm, ariR9R (ARI12). The sequence was free of mutations and the fusion with YFP was successful.

5.6 Generation of transgenic *Arabidopsis thaliana* plants overexpressing nCBP tagged with the C-terminal end of the YFP

Tagged overexpression lines were also generated for nCBP. Advantages of the fusion to a tagged YFP vector were already mentioned in section 5.5.

Mutants of *nCBP* have shown to be hyper-sensitive to UVB and thus nCBP is considered to be a positive regulator of UVB responses (Lang-Mladek, personal communication). In a working hypothesis, overexpression of nCBP is expected to make a plant more resistant to UVB.

The cDNA of nCBP (666 bp) was already cloned in the pUC-SPYCE vector. From there nCBP with a part of the 35S cassette (1609 bp) was restricted with the enzymes HindIII and XmaI. Then it was separated on a gel, excised from there, purified by DNA extraction and transferred to the target binary vector pSPYCE-35S (Figure 5.12).

The target vector pSPYCE-35S was also cut with HindIII and XmaI without subsequent purification. Then the vector was further restricted with PstI to avoid that the pSPYCE-35S backbone only cut with one restriction enzyme would contaminate the ligation step (by religation) (Figure 5.13). The sequences of the pUC-SPYCE and pSPYCE vectors are identical between HindIII and XmaI (including the 35S promoter cassette and MCS). pUC-SPYCE is a transient vector, whereas the binary pSPYCE-35S is a stable vector and can be used for *Agrobacteria* and plant transformation.

The pSPYCE-35S-nCBP (Map in figure 5.14) clone was verified with the restriction enzymes PstI and EcoRI (pSPYCE-35S-nCBP) on a 0.8% agarose gel. The estimated expected fragment sizes (Table 5.4) could be detected on an agarose gel after digestion with PstI and EcoRI.

Table 5.4. Expected and observed DNA sizes from a miniprep isolation of the pSPYCE-35S-nCBP construct match.

Construct	Enzyme	Expected band size	Observed band size
pSPYCE-35S-nCBP	PstI, EcoRI	1105 bp, 1109 bp, 4480 bp, 6600 bp	Ca. 2x 1100 bp, 4500 bp, 6600 bp
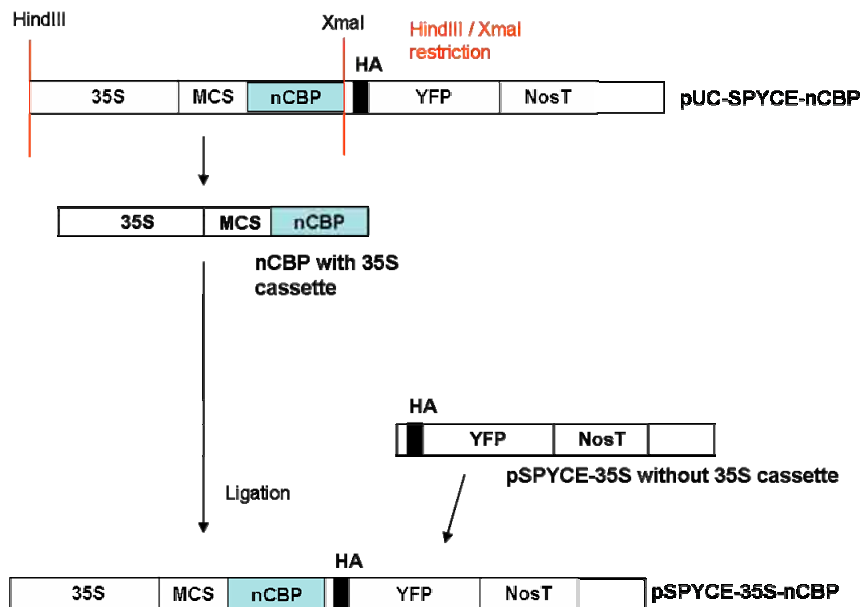
Figure 5.12. Cloning strategy of nCBP from pUC-SPYCE-nCBP into pSPYCE-35S-nCBP.

nCBP with the 35S cassette was restricted with HindIII and XmaI and cloned into the pSPYCE-35S vector.
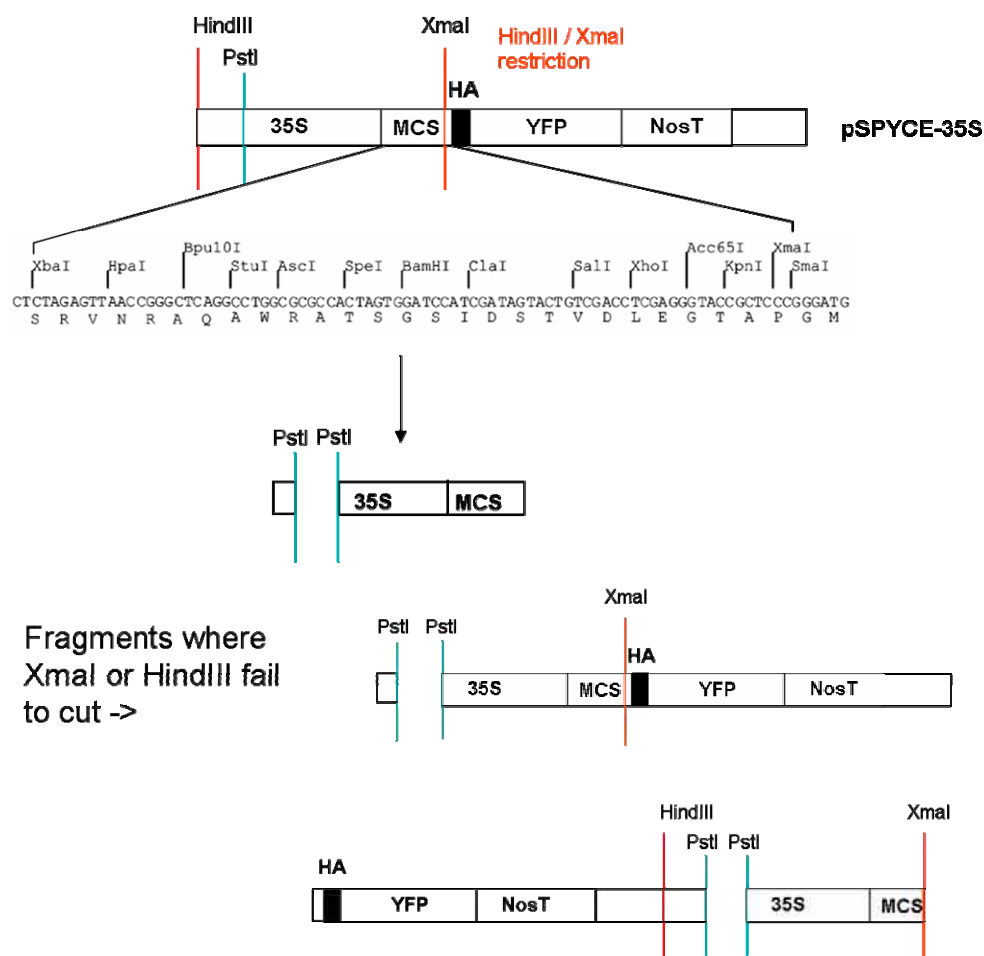
Figure 5.13. Schematic representation of the pSPYCE-35S vector preparation.

To avoid a high background due to incomplete XmaI or HindIII digestion of pSPYCE-35S this acceptor vector was further cut with PstI. PstI cuts incompletely cut pSPYCE-35S fragments and reduces background.
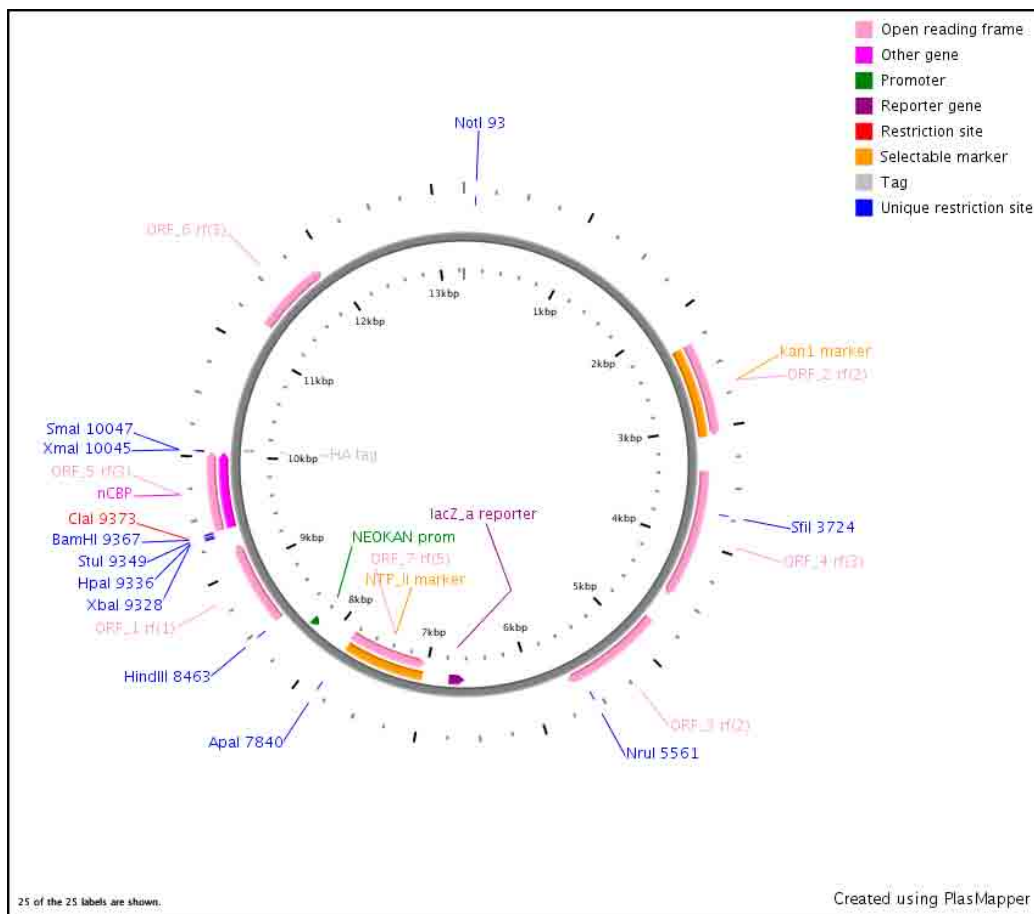
Figure 5.14. nCBP (CDS) was cloned from pUC-SPYCE-35S to pSPYCE-35S with HindIII (8463 bp) and XmaI (10045 bp).

nCBP stretches from 9373 to 10045 bp), marked in dark pink (created with PlasMapper http://wishart.biology.ualberta.ca/PlasMapper/).

pSPYCE-35S-nCBP constructs could be confirmed on a 0.8% agarose gel after digestion with XhoI (pSPYCE-35S-ARI12) and PstI, EcoRI (pSPYCE-35S-nCBP), respectively (Figure 5.15; sizes in table 5.4). Colony number 31 (lane 1 and 2) was chosen for use in plant transformation.
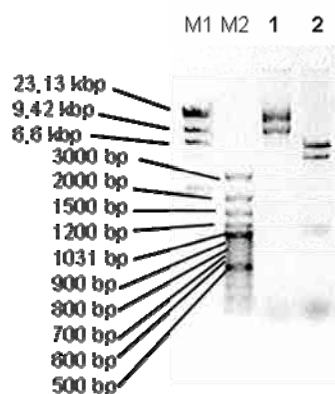
Figure 5.15. Confirmation of the construct pSPYCE-35S-nCBP by miniprep, digested with PstI and EcoRI.

Lane 1 (undigested) and 2 (PstI and EcoRI) show colony candidate nr. 31 chosen for transformation into Arabidopsis plants.

Clone 31 was confirmed by sequencing with the primer 5g18110_F2, 5g18110_R2 (nCBP). According to sequencing data the nCBP gene was successfully cloned into the pSYPCE-35S vector.

Generation of transgenic *Arabidopsis* plants

The overexpression constructs are expected to complement the mutants and might have additional phenotypes once they are transformed into wildtype plants. Therefore plants of the wildtype accession ColWT and LerWT and *ari12-1* and *ncbp* mutants were used to transform the tagged overexpression constructs.

The 35S constructs were transformed into competent GV3101 pMP90 *Agrobacterium* by electroporation. Transformed cells were plated out on YT plates with rifampicin (100 mg/L), gentamycin (50 mg/L) and kanamycin (50 mg/L) selection. Empty pSPYCE-35S vector served as a transformation control. The plates were incubated over two nights at 30°C. To confirm that the *Agrobacteria* contain the desired plasmid an *Agrobacteria* miniprep was performed (Li et al. 1995). pSPYCE-35S-ARI12 was restricted with PstI and pSPYCE-35S-nCBP with PstI and EcoRI.

The plant was dipped into the *Agrobacteria* sucrose solution for 2 min. pSPYCE-35S-ARI12 was transformed into the mutant lines *ari12-1* (Ler background, figure 5.17) and pSPYCE-35S-nCBP into *ncbp* (Col background, figure 5.19). As mentioned above ColWT and LerWT were also transformed (not shown). Empty pSPYCE-35S plasmids served as controls (Figures 5.16 and 5.18).

Table 5.5. Time of transformants plated (T1 generation), germinated and transferred to soil

Mutant lines germination time sheet			
	Plating	**Germination**	**Soil**
BATCH 1	01.08.2008	04.08.2008	19.08.2008
BATCH 2	22.09.2008	25.09.2008	03.09.2008
BATCH 3	21.01.2009	23.01.2009	04.02.2009

Table 5.6. Number of plants transferred from MS 1.0 BASTA plates to soil and total number of transformant plants survived.

Ecotype	Construct	BATCH 1	BATCH 2	BATCH 3	Total on soil	Total survived
Col WT	pSPYCE-35S empty	7	13	33	53	33
nCBP (Col)	pSPYCE-35S empty	5	3	15	23	14
ari12-1 (Ler)	pSPYCE-35S empty	0	3	4	7	4
Ler WT	pSPYCE-35S empty	2	1	3	6	2
Col WT	pSPYCE-35S-nCBP	1	4	3	8	3
nCBP (Col)	pSPYCE-35S-nCBP	2	9	31	42	22
Col WT	pSPYCE-35S-ARI12	8	13	8	29	17
ari12-1 (Ler)	pSPYCE-35S-ARI12	6	9	12	27	16
Ler WT	pSPYCE-35S-ARI12	1	0	21	22	4

The T1 generation of all *Arabidopsis* transformants (list of plants in table 5.5 and 5.6) are stressed, probably due to their selection with the herbicide BASTA and some overgrowth of agrobacterium in the selection plates. Hence accurate phenotyping is impossible. However, construct-specific tendencies could only be observed with *ari12-1* pSPYCE-35S-ARI12 transformants, since they generally tend to develop strong stems (Figure 5.17). The transformation efficiency of Ler plants were very low, although *ari12-1* (Ler) plants had more transformants.
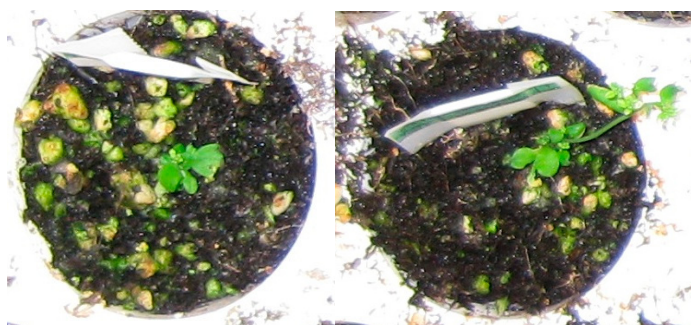
Figure 5.16. Example of *ari12-1* T1 plants transformed with empty pSPYCE-35S, 37 days after germination.

Figure 5.17. Example of *ari12-1* T1 plants transformed with pSPYCE-35S-ARI12, 37 days after germination.

The overexpressing ARI12 T1 plants grew faster and had stronger leaves than their corresponding controls with empty 35S vector.
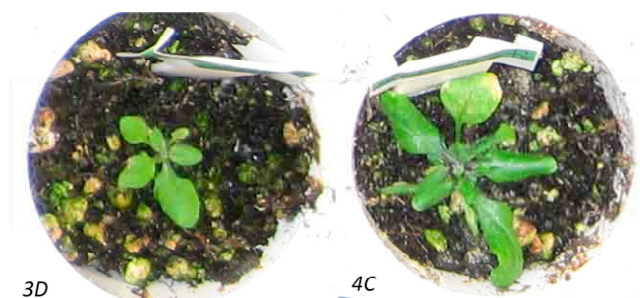

Figure 5.18. Example of *ncbp* T1 plants transformed with empty pSPYCE-35S (control), 37 days after germination.

The overexpressing nCBP T1 plants grow faster and develop stronger leaves than their corresponding controls with empty 35S vector.
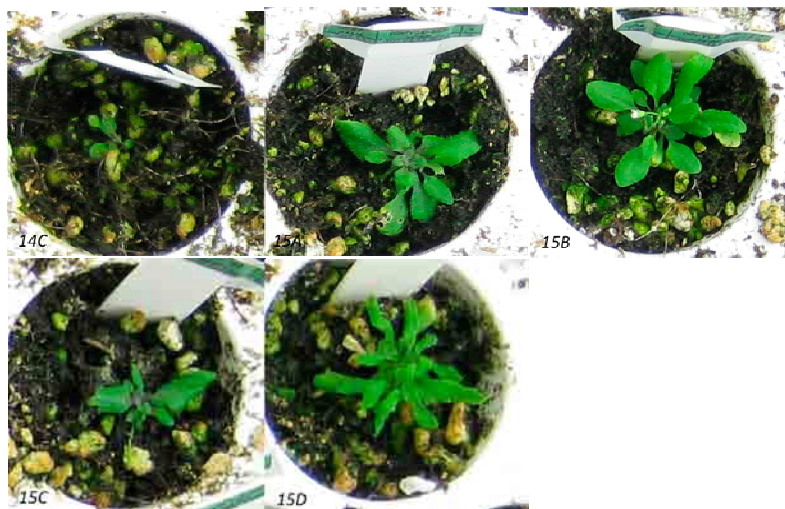

Figure 5.19. Example of *ncbp* T1 plants transformed with pSPYCE-35S-nCBP, 37 days after germination.

The 35S overexpression plant leaves show a variety of phenotypes.

Segregation analysis in the transformants in the T2 generation

It is known that *Agrobacterium* transformation sometimes result in transformants with more copies of the desired gene. A segregation analysis can be carried out to determine the number of inserts by counting positive transformants grown on a plate (with appropriate selection) and comparing the number of resistant plants with the expected number of resistant plants for 1 insert.

Segregation analysis was performed on BASTA (herbicide) plates. Transformants with one, two or three and more inserts could be detected. Assuming one insert is present, the plant should segregate to 1:2:1 (genotype) in T2 and theoretically 25% of the filial generation will be homozygote without an insert, 50% heterozygote with one insert and 25% will be homozygote with 1 insert. The phenotype ratio is 3:1 (3 resistant, 1 sensitive). For 2 inserts the segregation ratio for the phenotype is 15:1 (15 resistant, 1 sensitive) and for 3 inserts it is 63:1 (63 resistant, 1 sensitive). Table 5.7 shows the plants grown for segregation analysis and table 5.8 shows the expected and observed number of transformants of each line and the number of inserts (estimation). With a χ test (p > 0.1) transformants with 1 insert are predicted. Since this project had the aim to produce transgenic plants that overexpress tagged versions of either ARI12 or nCBP transformations, plants with two or multiple insertions might boost the expression and are even desired.

Table 5.7. T2 lines plated out for segregation analysis. The date of plating and the corresponding ecotype and constructs for the code are indicated.

Date/code	Ecotype / Line	Construct
19.03.09		
2C	Col WT	pSPYCE-35S empty
2G	Col WT	pSPYCE-35S empty
4A	*ncbp* (Col)	pSPYCE-35S empty
4B	*ncbp* (Col)	pSPYCE-35S empty
13A	*ncbp* (Col)	pSPYCE-35S-nCBP
19A	Col WT	pSPYCE-35S-ARI12

20.03.09		
19B	Col WT	pSPYCE-35S-ARI12
19C	Col WT	pSPYCE-35S-ARI12
19D	Col WT	pSPYCE-35S-ARI12
21A	*ari12-1* (Ler)	pSPYCE-35S-ARI12
21B	*ari12-1* (Ler)	pSPYCE-35S-ARI12
27A	Ler WT	pSPYCE-35S-ARI12
11A	Col WT	pSPYCE-35S-nCBP
11B	Col WT	pSPYCE-35S-nCBP
23.03.09		
19E	Col WT	pSPYCE-35S-ARI12
20A	Col WT	pSPYCE-35S-ARI12
11C	Col WT	pSPYCE-35S-nCBP
14C	*ncbp* (Col)	pSPYCE-35S-nCBP
15A	*ncbp* (Col)	pSPYCE-35S-nCBP
15B	*ncbp* (Col)	pSPYCE-35S-nCBP
15C	*ncbp* (Col)	pSPYCE-35S-nCBP
15E	*ncbp* (Col)	pSPYCE-35S-nCBP
26.03.09		
20B	Col WT	pSPYCE-35S-ARI12
20D	Col WT	pSPYCE-35S-ARI12
20G	Col WT	pSPYCE-35S-ARI12
20I	Col WT	pSPYCE-35S-ARI12
20J	Col WT	pSPYCE-35S-ARI12
20K	Col WT	pSPYCE-35S-ARI12
22E	ari12-1 (Ler)	pSPYCE-35S-ARI12
22F	ari12-1 (Ler)	pSPYCE-35S-ARI12
23B	ari12-1 (Ler)	pSPYCE-35S-ARI12
23C	ari12-1 (Ler)	pSPYCE-35S-ARI12
27.03.09		
1A	Col WT	pSPYCE-35S empty
1B	Col WT	pSPYCE-35S empty
2H	Col WT	pSPYCE-35S empty
2J	Col WT	pSPYCE-35S empty
3D	*ncbp* (Col)	pSPYCE-35S empty
4C	*ncbp* (Col)	pSPYCE-35S empty
5A	ari12-1 (Ler)	pSPYCE-35S empty
5B	ari12-1 (Ler)	pSPYCE-35S empty

Table 5.8. Segregation analysis presenting expected and observed number of transformants (positive versus negative). A χ test provides the prediction whether there is only 1 insert or more in the plant. In case of 85% (p > 0.1) or more positives, more than 1 insert is assumed. Plants with 2 inserts would produce 15 resistant plants and 1 sensitive in T2 – for 60 plants this would be 54.6 resistant transformants and 5.4 sensitive non-transformants. If all plants on a plate are positive, 3 or more inserts are assumed. Zero equals non-transformants and thus are not overexpressing plants. Summary: no insert – false positive, 1 inserts – positive, 2 inserts – positive, more than 2 inserts – unusual segregation (gene silencing possible)

		Expected with the assumption of 1 insertion		χ test	Observed		
Date/code	Ecotype / Line	Positive	Negative	p value	Positive	Negative	Inserts
19.03.09							
2C	Col WT	38.25	12.75	0.936	38	13	1

2G	Col WT	36	12	0.505	38	10	1
4A	*nCBP* (Col)	45	15	0.000	0	60	0
4B	*nCBP* (Col)	45	15	0.551	43	17	1
13A	*nCBP* (Col)	45	15	1.000	45	15	1
19A	Col WT	45	15	0.000	58	2	2
20.03.09							
19B	Col WT	45	15	0.000	57	3	2
19C	Col WT	45	15	0.551	43	17	1
19D	Col WT	45	15	0.000	58	2	2
21A	*ari12-1* (Ler)	45	15	0.000	60	0	≥3
21B	*ari12-1* (Ler)	45	15	0.233	41	19	1
27A	Ler WT	45	15	0.233	41	19	1
11A	Col WT	45	15	0.001	56	4	2
11B	Col WT	45	15	0.037	52	8	2
23.03.09							
19E	Col WT	45	15	0.551	47	13	1
20A	Col WT	45	15	0.551	47	13	1
11C	Col WT	45	15	0.074	39	21	1
14C	*nCBP* (Col)	45	15	0.233	49	11	1
15A	*nCBP* (Col)	45	15	1.000	45	15	1
15B	*nCBP* (Col)	45	15	0.000	57	3	2
15C	*nCBP* (Col)	45	15	0.007	36	24	1
15E	*nCBP* (Col)	45	15	0.766	46	14	1
26.03.09							
20B	Col WT	45	15	0.000	58	2	≥2
20D	Col WT	45	15	0.000	0	60	0
20G	Col WT	45	15	0.233	41	19	1
20I	Col WT	45	15	0.000	60	0	≥3
20J	Col WT	45	15	0.017	53	7	1 or 2
20K	Col WT	45	15	0.003	55	5	1 or 2
22E	*ari12-1* (Ler)	45	15	0.007	54	6	2
22F	*ari12-1* (Ler)	45	15	1.000	45	15	1
23B	*ari12-1* (Ler)	45	15	0.007	36	24	1
23C	*ari12-1* (Ler)	45	15	0.003	55	5	2
27.03.09							
1A	Col WT	45	15	0.551	43	17	1
1B	Col WT	45	15	0.766	46	14	1
2H	Col WT	45	15	0.017	53	7	1 or 2
2J	Col WT	45	15	0.766	46	14	1
3D	*nCBP* (Col)	45	15	0.037	52	8	1 or 2
4C	*nCBP* (Col)	45	15	0.000	0	60	0
5A	*ari12-1* (Ler)	37.5	12.5	0.253	41	9	1
5B	*ari12-1* (Ler)	45	15	0.233	41	19	1

6 Discussion

6.1 Specificity of the interaction between ARI12 and the eIF4E gene family

The ARI12 protein of Arabidopsis belongs to the ARIADNE subclass of the ring between ring fingers (RBR)-domain containing family of putative E3 ubiquitin protein ligases (Eisenhaber et al. 2007). ARIADNE proteins have been identified in 48 organisms (Eisenhaber et al. 2007). HHARI interaction partners have been only identified for the human member (Figure 6.1) (Eisenhaber et al. 2007).
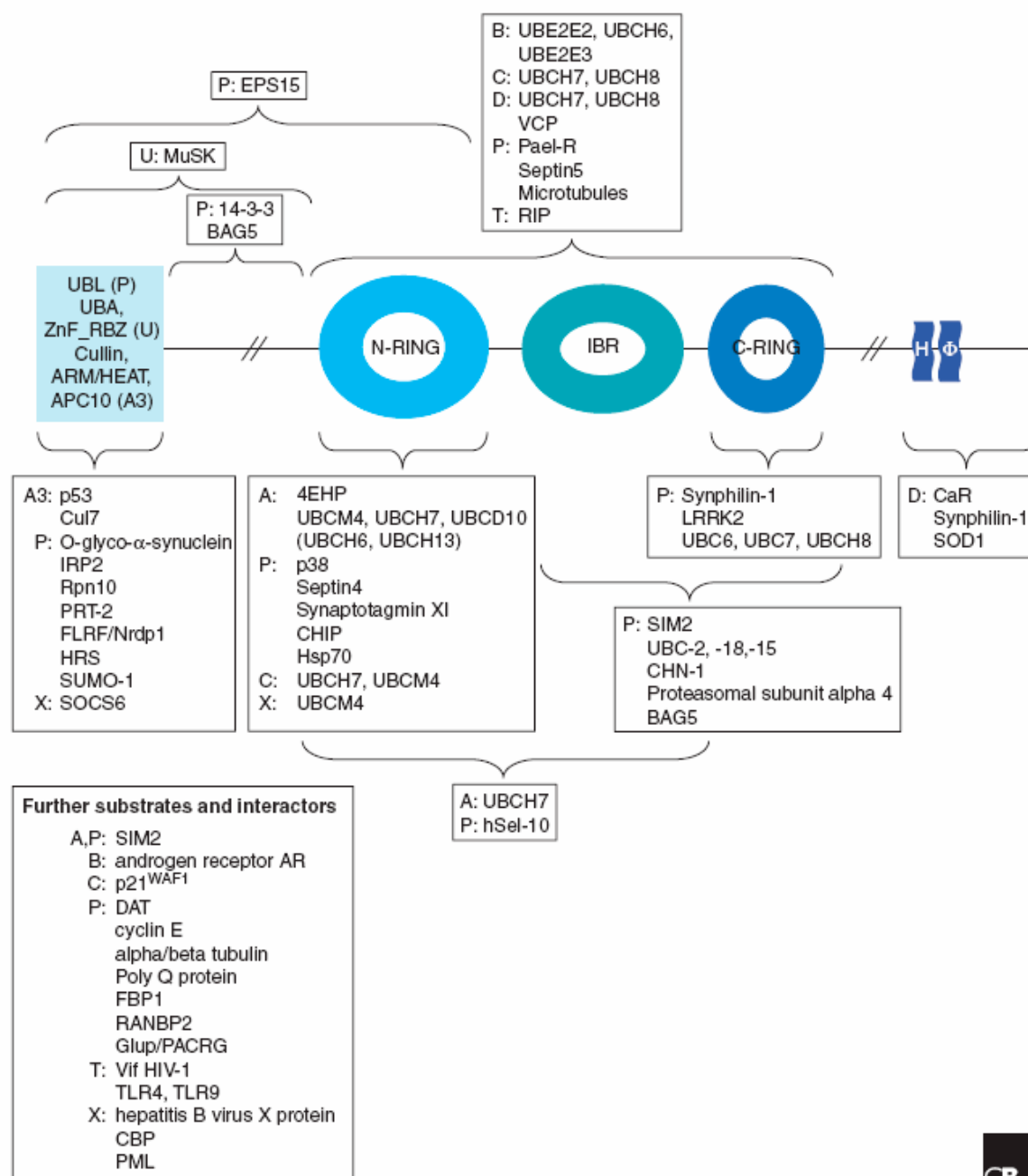
Figure 6.1. Interaction partners of the ring between ring (RBR)-domains.

The substrate 4EHP interacts with the RING1 (here N-RING) of HHARI. To date, one substrate only has been identified for the ARIADNE family member HHARI. 64 interaction partners of RBR proteins are known so far. This scheme depicts the RBR interaction domains with the corresponding interaction partners. A, Ariadne; A3, Parc; B, ARA54; D, dorfin; P, parkin; C, RNF144; X, XAP; U, Paul; T, TRIAD3.

Apart from several ubiquitin conjugating enzymes (UBCs) HHARI interacts with translation initiation factor 4E homologous protein (4EHP/eIF4E-2) (Tan et al. 2003). This protein belongs to the family of translation initiation factors 4E. One of eIF4Es functions is to control the recruitment of mRNAs to the ribosome by binding the 7-methyl-guanosine (7mG)-cap structure of mRNAs. eIF4Es together with the docking protein, eIF4G, and the RNA helicase eIF4A are components of the heterotrimeric eIF4F complex that unwinds the secondary structures of the 5' UTR and recruits the 40S ribosomal subunit to the mRNA (Pestova et al. 2000). Apart from their key role in regulating translation initiation, eIF4Es also function in nucleocytoplasmic transport, stabilization and sequestration of mRNAs into storage compartments in the cytoplasm (Richter et al. 2005).

Multiple eIF4E isoforms exist in higher organisms (Hernandez et al. 2005; Joshi et al. 2005) and they have different interaction specificities to eIF4G and other eIF4E binding proteins (Keiper et al. 2000; Joshi et al. 2004; Hernandez et al. 2005). The Arabidopsis genome codes for three distinct classes of cap-binding proteins, eIF4E-1, -2, -3, eIF(iso)4E and nCBP. eIF4E-1 and eIF(iso)4E assemble into different eIF4F complexes with complementary biological functions (Browning 1996; Rodriguez et al. 1998; Gallie et al. 2001).

That the eIF4E proteins have distinct functions and are differently regulated is further supported by the finding that the interaction between 4EHP/eIF4E-2 and HHARI is specific for 4EHP/eIF4E-2, because the classical eIF4E-1 was unable to co-immunoprecipitate (Tan et al. 2003). This specificity is supported by my results since I could only show a strong interaction of ARI12 with nCBP and not with eIF4E-1 by Yeast Two Hybrid analyses.

These interaction specificities can be partially due to differences in the primary and/or secondary structure of the eIF4E members. For example, from the three important non-polar residues involved in eIF4E/eIF4G interaction, valine-69 and glycine-139 are conserved in all eIF4Es, whereas leucine-131 is exchanged for a glutamate in the subclass of 4EHP/eIF4E-2/nCBP.

The most dramatic differences in the prediction of the secondary structures between the classical and 4EHP/eIF4E-2/nCBPs are the increased loop sizes between secondary structures such as the beta sheet S1, S2 and the beta sheet S4, and helix H2 and beta sheet S7 and S8. These important loops harbor distinctive 7mG-cap binding residues and are close to the eIF4G binding residues.

Thus, in addition to the amino acid identity, the predicted secondary structure variability supports the differences in the interaction with ARI12. However, since ARI12 belongs to a protein family of 14 members, I cannot rule out that other ARI members are interacting with the other eIF4E members. Indeed, my colleague Neha Nigam found evidence for this hypothesis.

6.2 Characterization of the interaction domain of ARI12 with the putative substrate nCBP

To date, about 64 RBR protein interaction partners have been identified and the interaction domains have been characterized (Eisenhaber et al. 2007). Most of the RBR protein interaction partners interact with the RBR domain (Figure 6.1). This holds also true for the ARI subclass of RBR domain proteins. Tan et al. showed that the RING1 finger of the human ARI protein, HHARI, interacts with 4EHP/eIF4E-2 (Tan et al. 2003).

In contrast, my function-structure analyses with the ARI12 deletion constructs identified RING1 and the IBR domain to be responsible for the interaction with nCBP. A similar broadened interaction domain has been characterized for the ARI interaction with UBCH7 (Ardley et al. 2001) and for the interaction of Parkin with hSel (Staropoli et al. 2003).

Although some beta-galactosidase activity could be measured with the ARI12-RING2-C and ARI12-C-terminus deletion constructs, it was not significantly different from the activity of the nCBP alone which showed a strong auto-activation also in the growth assays. These insignificant interactions could also be due to the differences in the protein levels of these clones, since they have been much lower than with the ARI12-RING1, ARI12-RING1-IBR and ARI12-IBR-RING2-C deletion constructs. However, the Western blot did not contain a loading control and thus it is still possible that the gel was unevenly loaded. The latter interpretation is supported by the fact that the unspecific band are also weaker expressed.

It has to be kept in mind that the Yeast Two Hybrid system is a heterologous system and it is known that this protein-protein interaction method is susceptible to false positive and negative results. Thus interaction of ARI12 with nCBP should be supported by other methods such as Bimolecular fluorescence complementation (BiFC), Protein complex immunoprecipitation (Co-IP), colocalization and Fluorescence resonance energy transfer (FRET).

BiFC analysis is based upon fluorescent proteins (GFP, CFP, YFP, PFP, RFP) that are split into halves. These halves are not able to produce fluorescent light on their own. One half is fused to the first interaction partner, while the other half is fused to the second. If interaction takes place, the fluorophore will be reconstituted and fluorescence can be detected via a fluorescence microscope. BiFC allows interaction studies as well as localization of the proteins at the same time.

In Co-IP studies, an antibody targets a known protein that is thought to be a protein complex member. It is possible to pull out the entire complex of the solution and identify the interaction partner of the known protein (unknown members of the complex).

Colocalization works by attaching fluorescent stains to biological samples. If, for example, one protein is stained green and the supposed interaction partner is labelled red, merging the red and green fluorescent channels in the microscope will result in a yellow colour. Spatial dependence and interaction of proteins can be observed.

In FRET analysis, two fluorophore labelled molecules (or two interacting proteins) are mixed together. GFP variants (i.e. YFP) can be attached to the molecules by genetic engineering. Excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon, if both molecules are in proximity. This results in a larger emission peak of the acceptor molecule.

In the future, my constructs can be used for further structure-function analyses to define the domain responsible for the E3 ligase activity.

6.3 Establishing transgenic plants constitutively overexpressing tagged versions of ARI12 and nCBP

Transgenic overexpressing plants will be used for complementation studies on UVB phenotypes. Overexpressing plants are expected to enhance a certain phenotype and can support previous functional studies with mutant lines. Any phenotypical changes of the overexpressing plants will be tested. Proteins and RNA (via cap binding) that form a complex with ARI12 and nCBP can be isolated by means of the tagged 35S constructs and anti-GFP antibodies.

There are constitutive, inducible promoters and tissue-specific promoters. Constitutive promoters override the plants' regulatory system and cannot be switched off by the plant itself. With these promoters expression is possible in all developmental stages of the plant. On the other hand one must consider that constitutive gene expression can be detrimental to the plant and lethal. On the contrary, stress-inducible (chemical or physical; by heat, cold, wounding, pathogens, light) promoters do not have negative effect on plant growth under normal growth conditions. They also allow gene expression in all

developmental stages of the plant by induction with chemicals or other stimuli. However, repressing compounds have to be removed. Sometimes tissue-specific promoters from a recipient plant can be preferable, but using them might result in gene silencing.

The CaMV 35S promoter is a strong promoter that is hardly influenced by environmental conditions and can be efficiently used in all tissues. At the same time, constitutive promoters like 35S are not tissue-specific. However, the 35S promoter allows high expression of genes in dicot plants, whereas it is less effective in monocots, probably due to different regulatory factors. 35S is a viral promoter and plant promoters might be better for regulatory purposes in plants. Other promoters than 35S are more specific and respond to cues in the plant's internal or external environment.

pSPYCE-35S was the vector of choice in my studies, because it is tagged with the C-terminal end of YFP that can be probed with common antibodies and the constitutive 35S promoter drives overexpression efficiently. Furthermore the vector was available in our lab which made the decision for 35S easier.

Overexpression of nCBP and ARI12 do not appear lethal to the plants. More transgenic lines survived than control plants with an empty 35S construct. T1 lines of 35S overexpression transgenic lines varied in phenotype. Due to different copy number and insertion sites of overexpressed nCBP or ARI12 genes in the plant, phenotype variation is common. Segregation analysis with the T2 generation was carried out to determine the number of 35S driven inserts. 2 *ari12-1* plants with more than 1 insert of pSPYCE-35S-ARI12 tend to grow faster and one *nCBP* plant with 2 inserts of pSPYCE-35S-nCBP looked healthier than other *nCBP* plants with 1 insert. There is no indication for that in control plants. However, the sample size is too small to predict any correlations and the phenotype of the T3 generation has to be observed for more accurate measurements.

UVB analysis of mutant lines showed that ARI12 is a negative regulator in UVB response: ARI12 showed elevated expression during UVB exposure (http://bbc.botany.utoronto.ca) and *ari12-1* as well as *ari12-2* mutants performed better than their corresponding wildtype (Ler), while the *nCBP* mutant was more sensitive to UVB (Mladek et al. 2008; Nigam et al. 2009). Therefore we expect overexpression ARI12 Arabidopsis plants to be more sensitive to UVB light and make the hypothesis that overexpressed nCBP plants are more robust under UV stress. However, further experiments with the overexpression plants have to be carried out to support this hypothesis. In T3 the phenotype will also be observed after UVB exposure.

Prep1 in mice was shown to interact with eIF4E homolog cap-binding protein 4EHP and inhibiting translation of *Hoxb4.* Overexpression of *Hoxb4* slowed down embryo development. Considering the fact that 4EHP negatively regulates *Hoxb4,* we can speculate that overexpression of nCBP in plants will have a positive effect on plant growth (Villaescusa et al. 2009).

4EHP and d4EHP specifically bind to certain mRNA by binding to a protein that binds to the 3' UTR of the mRNA. d4EHP in Drosophila simultaneously binds to the 5' mRNA cap of caudal and to BICOID that binds to the 3' UTR region of the caudal mRNA which results in inhibiting translation in the anterior of the embryo. d4EHP also binds to hunchback mRNA (regulator of pattern formation) and the protein complex PUMILIO, NANOS and BRAT in this manner and inhibits translation of hunchback (probably in the central part of the embryo). By regulating Cad and Hb expression, d4EHP plays a key role in establishing an expression (posterior-anterior) gradient in the Drosophila embryo. Mutant of d4EHPs showed patterning defects (Cho et al. 2005; Cho et al. 2006; Vardy et al. 2007), another indication that overexpression of nCBP, as a negative regulator of translation, in Arabidopsis might have positive effects on plant development.

High levels of eIF4E expression could be observed in human cancers (Graff et al. 2003; Bilanges et al. 2007). Playing a crucial role in growth or development, overexpression results in oncogenic transformation and cellular dysregulation in mammals (De Benedetti

et al. 1990; Lazaris-Karatzas et al. 1990; McClusky et al. 2005), it is observed to stimulate growth (McClusky et al. 2005): in foci (De Benedetti et al. 1990), as well forming tumours in nude mice (Lazaris-Karatzas et al. 1990). eIF4E and the human homolog 4EHP levels are thought to be a good indicator for cancer and tumours (Ramaswamy et al. 2003; De Benedetti et al. 2004). eIF4E is thought to export mRNA particles that encode proteins involved in cell proliferation and tumourgenesis (Topisirovic et al. 2003). Supporting this view, only 4E-sensitive mRNAs are upregulated: cyclin D1, ornithine decarboxylase (ODC) and vascular endothelial growth factor (VEGF). They are all involved in growth (Rosenwald et al. 1995; Mamane et al. 2004; Cho et al. 2005).

In fruit flies overexpressed eIF4E in more than one copy lead to cell proliferation, abnormal growth, severe disturbance of the ommatidia lattice pattern, and the presence of extra chaete. Only above a certain threshold phenotypic changes could be observed (Hernandez et al. 2005).

In S. cerevisiae 100 fold overexpression of eIF4E had only a minor effect on growth rates (Lang et al. 1994). Overexpression in Xenopus (Wakiyama et al. 2001) and S. Pombe (Hashemzadeh-Bonehi et al. 2003) modestly increased translation in oocytes or had no effect on cell growth. However, early embryos suffer from defects of eIF4E-1 transgene overexpression (Hernandez et al. 2005).

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8 Abbreviations

µg	microgram
µL	microlitre
µm	micromolar
3-AT	3-Amino-1,2,4-triazole
7mG	7-methyl guanosine
A. thaliana	*Arabidopsis thaliana*
aa	amino acid
AA	acrylamide
AD	activation domain
AGI	*Arabidopsis* Genome Initiative
AMP	ampicillin
approx.	approximately
APS	ammoniumperoxodisulfate
Asp	aspartic acid
ATP	adenosine triphosphate
BASTA	glufosinate ammonium salt
BiFC	bimolecular fluorescence complementation
BK	binding domain
bp	base pair
cDNA	complementary DNA
CDS	coding sequences
Col	Columbia
conc.	concentration
Cys	cysteine
dATP	deoxyadenosine triphosphates
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylendiaminetetraacetic acid
FITC	fluorescein isothiocyanate
Fwd	forward
g	gram
g	gravitational acceleration

GFP	green fluorescent protein
Gly	glycine
GTE	Glucose-Tris-EDTA
h	hours
HA	hemagglutinin
His	histidine
HRP	horseradish peroxidase
IgG	immunoglobulin G
Ile	isoleucine
KAN	kanamycin
kDa	kilo dalton
kJ	kilo joule
L	litre
Ler	Landsberg erecta
Leu	leucine
Lys	lysine
M	molar
MCS	multiple cloning site
Met	methionine
min	minute(s)
mL	millilitre
mM	millimole
mRNA	messenger RNA
ms	millisecond(s)
MS	Murashige Skoog
myc	myelocytomatosis oncogene cellular homolog
NEB	New England Biolabs
ng	nanogram
nm	nanometer
OD	optical density
ONPG	ortho-Nitrophenyl-beta-galactoside
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glucane
pmol	picomol
PNK	polynucleotide kinase
Pro	proline

PVDF	polyvinylidene difluoride
rATP	adenosine triphosphates
Rev	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s	second(s)
S	svedberg
SAP	shrimp alkaline phosphatase
SD	synthetic defined
SDS	sodium dodecyl sulphate
Ser	serine
S.O.C.	Super Optimal broth with Catabolite repression
t	time
TAQ	*Thermophilus aquaticus*
TBS-T	Tris buffered saline with tween-20
TE	Tris and EDTA
TEMED	tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan
Tyr	tyrosine
U	units
UVB	ultraviolet B
V	(1) volt
	(2) volume
Val	valine
WT	wildtype
Y2H	Yeast Two Hybrid
YFP	yellow fluorescent protein
YPD	yeast extract/peptone/dextrose
YT	yeast extract/tryptone