# Characterization of interactions of ARIADNE ubiquitin ligase family members with potential target proteins


by

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# Abstract

ARIADNE (ARI) proteins belong to the ring between ring fingers protein family and are involved in the ubiquitination pathway, where they are responsible for ubiquitin transfer onto substrate proteins, targeting them for degradation by the 26S proteasome. By using the yeast two hybrid system interactions of three members of the ARI family with other ARI proteins and putative substrates as well as ubiquitin were analyzed. Also included in the experiments were UV RESISTANCE LOCUS8 (UVR8) and CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), two components of the UV-B response pathway. Interactions of ARI5 and ARI7 with ARI1, elongation initiation factor 4E (eIF4E) and ubiquitin were detected as well as UV-B dependent interaction of COP1 with UVR8 and homodimerization of COP1.

ARIADNE (ARI) Proteine gehören zur Gruppe der Ring „ring between ring fingers“ Proteinfamilie, die für die Übertragung von Ubiquitin auf Substratproteine verantwortlich sind und somit diese für den Abbau durch das 26S Proteasom markieren. Durch die Verwendung des „Yeast two Hybrid“ Systems wurden Interaktionen von drei Mitgliedern der ARI Familie mit anderen ARI Proteinen und vermeintlichen Substraten, sowie Ubiquitin untersucht. Auch UV RESISTANCE LOCUS8 (UVR8) und CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), zwei Bestandteile des UV-B Signalübertragungsweges wurden bei den Experimenten inkludiert. Es konnten Interaktionen von ARI5 und ARI7 mit ARI1, Elongation Initiation Faktor 4E (eIF4E) und Ubiquitin nachgewiesen werden, ebenso die UV-B abhängige Interaktion zwischen COP1 und UVR8 und die Homodimerisierung von COP1.

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# 1) Introduction

## 1.1) Ring in between ring fingers (RBR) - proteins and the ARIADNE family

Ring in between ring fingers (RBR) - domain carrying proteins form a huge and multifaceted protein family with several hundred members found in eukaryotic organisms and their viruses. The name giving structure is a 200 amino acid long protein segment, which forms three ring finger type domains connected by loops and which are most likely responsible for protein-protein interactions. These rings contain highly conserved cysteine and histidine residues, which are able to bind metal ions, with RING1 binding two zinc ions separated by the in between rings (IBR) domain, supposed to form a ring on its own, from the one zinc ion binding RING2. RBR proteins are involved in various cell processes as regulation of cell cycle, apoptosis and translation as well as degradation of aggregation prone proteins. Some RBR proteins take effect as part of a protein cascade in the ubiquitination pathway, where proteins are targeted with monomeric or polymeric ubiquitin mostly for degradation (Eisenhaber et al. 2007). In the process ubiquitin (UBI), a 76 amino acid peptide, is captured by an ubiquitin activating enzyme (E1) by forming a thioester bond, subsequently passed on to a cysteine residue of an ubiquitin conjugating enzyme (E2) and finally with the help of an ubiquitin protein ligase (E3) transferred onto the target protein, usually condemned to be degraded by the 26S proteasome pathway. Various working mechanisms of different E3 classes exist, either by taking over ubiquitin from E2 before passing it on to the substrate or by merely holding E2 and target protein in place to enable ubiquitin transfer between those two.

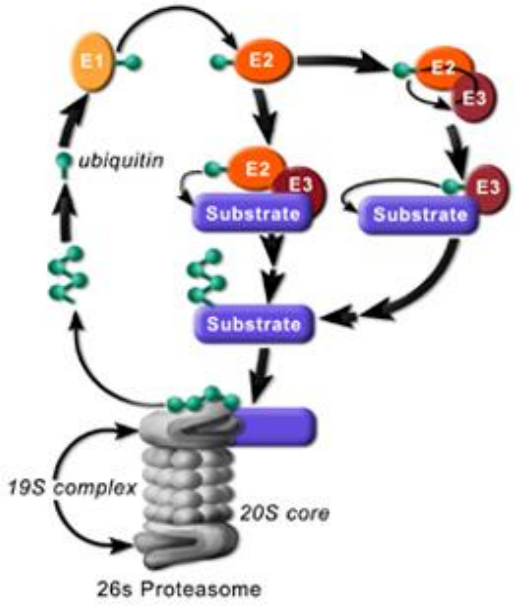


**Figure 1** The ubiquitination pathway leading to target protein degradation by the 26S proteasome: E1 (ubiquitin activating enzyme) binds monomeric ubiquitin and passes it on to E2 (ubiquitin conjugating enzyme) before it is transferred onto the target either with the help of E3 (ubiquitin protein ligase) which holds E2 and target in place during transfer or by E3 taking over ubiquitin itself before marking the substrate. Thus targeted proteins are recognized and degraded by the 26S proteasome , the ubiquitin is monomerized to reenter the pathway (Rubio *2004*).

Ubiquitin protein ligases (E3) are the protein group with the highest diversity of those involved, thus providing specificity for diverse substrates they are directly interacting with (Freemont 2000, Jackson et al. 2000). This is also where RBR proteins come into play. With their characteristic RING structures, which are thought to mediate protein-protein interactions, they fulfil the requirements to bring together activated ubiquitin associated with E2 and substrate-protein. Recent research showed, that RBR E3 themselves form a thioester bond with ubiquitin via a highly conserved cysteine residue of the RING2 structure before transferring it on to substrate proteins (Wenzel and Klevit 2012). One group of RBR E3 ligases are ARIADNE proteins, homologous to human PARKIN, which is involved in Parkinson's disease (Zhang et al. 2000). They were first identified in *Drosophila melanogaster* for interacting with known E2 enzymes (Aguilera et al. 2000). Additionally to the RBR structure ARI proteins characteristically contain an N-terminal acid rich cluster, a potential coiled coil domain as well as a leucine rich region at the C-terminus and a potential phosphorylation site possibly for

de-/activation. ARI and homologues can be found in various organisms; in *Arabidopsis thaliana* 16 possible ARI genes were discovered and divided into three groups by database and phylogenetic analyses. Their high variability might have evolved through genome duplication and retroposition (Mladek, Guger and Hauser 2003). Special interest was drawn to *At*ARI5 and *At*ARI7, highest expressed according to search in the public microarray data collection Bio-Array Resource (BAR; Winter et al. 2007) and *At*ARI12, usually just expressed in roots, but strongly induced in leaves early after UV-B exposure (Lang-Mladek et al. 2012). Possible interaction partners of ARI5 and ARI7 were researched using the yeast two-hybrid system. As 'prey' served already available and the newly cloned ARI proteins (ARI1, ARI2, ARI5, ARI7, ARI8, ARI12, ARI15), their putative substrates and ubiquitin (UBI). Additionally interaction-analyses of ARI12 with two components of the UV-B response pathway were part of the experiments.

## 1.2) Plant translation initiation factors - putative substrates for ARIADNE E3 ligases

As it was shown that the human homologue of *Drosophila* ARIADNE (HHARI) is able to interact with translation initiation factor 4E homologous protein (4EHP) the assumption arose if ARIs might also play an important role in the regulation of protein translation (Tan et al. 2003). In *Arabidopsis thaliana* three translation initiation factors are known, whose distribution is dependent on the tissue and developmental stage: eukaryotic translation initiation factor 4E (eIF4E 1, 2 and 3), its isozyme eIF(iso)4E and novel cap binding protein (nCBP)(Martínez-Silva et al. 2012). Together with eIF4G and eIF4A, eIF4E forms the eIF4F complex, which enables ribosomes to connect with mRNAs. The role of eIF4E is to bind to the RNA cap structure and then recruit the 40S ribosome together with eIF4G, which bridges the connection to eIF3, the 40S ribosome recruiting complex. EIF4A is a helicase, opening the secondary RNA structure and thus providing access for the ribosome to scan the mRNA for startcodons.

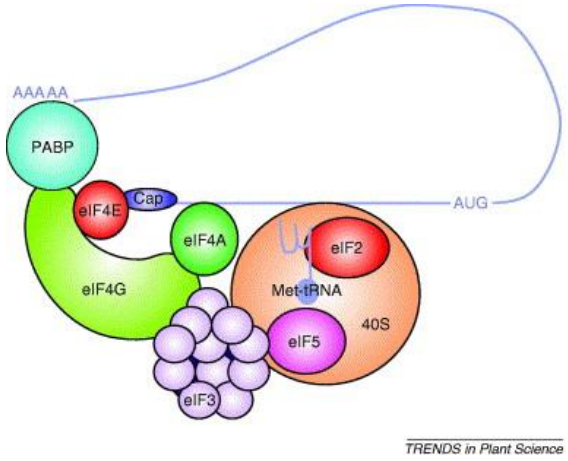


**Figure 2** The eukaryotic translation initiation complex: eIF4E binds to the 7-methylguanosine cap structure of mRNAs and together with eIF4G it forms the mRNA recruiting complex eIF4F. EIF4G interacts with several other initiation factors like eIF4A, a helicase unwinding the secondary RNA structures, poly A binding protein (PABP) and the ribosome 40S subunit recruiting complex (eIF3). eIF2 an 5 are ribosome associated factors (Robaglia and Caranta 2006).

While eIF4E is distributed in all eukaryotes eIF(iso)4E and its corresponding complex partner eIF(iso)4G are unique to plants. The isoform's working mechanism is the same as that of eIF4E but differs depending on the methylation status and length of the secondary structure of the 5'-UTR (Bush et al. 2009). An eIF4E and eIF(iso)4E specific property is the conserved relative position of eight tryptophan residues, in nCBP two of these tryptophans are substituted by other aromatic amino acids. As in eIF4E those residues play an important role for cap binding, not only the sequence but also the binding mechanism of nCBP might differ. To form a RNA-Ribosome mediating complex nCBP is able to interact with eIF(iso)4G, but it is also supposed that it might be responsible for mRNA stabilization for later translation (Ruud et al. 1998). All three have in common, that they bind to the 7-methylguanosine cap structure of mRNA, and therefore are an important element in the selection of mRNAs and regulation of translation (McKendrick, Pain and Morley 1999). As the factors themselves can be regulated in diverse ways it would be of great interest to find such a regulator in ARIADNE proteins, involved in protein degradation. For interaction experiments full length eIF4E, eIF(iso)4E and nCBP as well as deletion mutants of nCBP in its C-tail, N-tail, helix or β-sheet domains, were utilized.

## 1.3) COP1 and UVR8 - two components of the UV-B response pathway

Additionally to a member of the ARIADNE family, ARI12, two proteins involved in UV-B response were included in interaction experiments.

Plants are exposed to ever changing light conditions and have evolved various mechanisms to use light as informational source to adapt their growth and development adequately (Tilbrook et al. 2013). As a part of natural sunlight UV-B (280-315 nm) above 290 nm reaches the earth's surface and can in high doses lead to tissue necrosis, DNA damage and upregulation of stress response genes. Non-damaging doses on the other hand stimulate UV protection mechanisms and have a regulatory effect, affecting plant morphology, development and biochemical composition. Although these low UV-B response processes are not well understood until now, with UV RESISTANCE LOCUS8 (UVR8) and CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) recently two components were identified, which seem to take effect early in this mechanism (Jenkins 2009). UVR8, a UV-B photoreceptor, is a homodimeric protein, highly conserved among plants and expressed constitutively in the whole organism. Low UV-B radiation leads to UVR8 monomerization, by breaking salt bridges, with tryptophan as UV-B chromophore. UVR8 monomers subsequently accumulate in the nucleus where they possibly regulate gene expression by association with chromatin via histones (Tilbrook et al. 2013). For instance it regulates the transcription level of transcription factor ELONGATED HYPOCOTYLE 5 (HY5), which by itself influences the expression of proteins involved in UV protection. Monomerized UVR8 can also interact with COP1, an E3 ligase usually known to repress light signalling in darkness, by targeting positive regulators of photomorphogenic gene expression, for instance HY5, for degradation. When light conditions change to white light COP1 is inactivated and removed from the nucleus, avoiding its destructive effect and enabling normal light development of the plant. But when exposed to low UV-B COP1 reaccumulates in the nucleus and this time becomes a positive regulator of UV response. Its interaction with UVR8 is required to induce UV-B response, also including HY5 expression, maybe by leading degradation of negative regulators of this pathway.

Experiments have shown that COP1 most probably interacts with a UVR8 domain only exposed after monomerization through UV-B exposure.

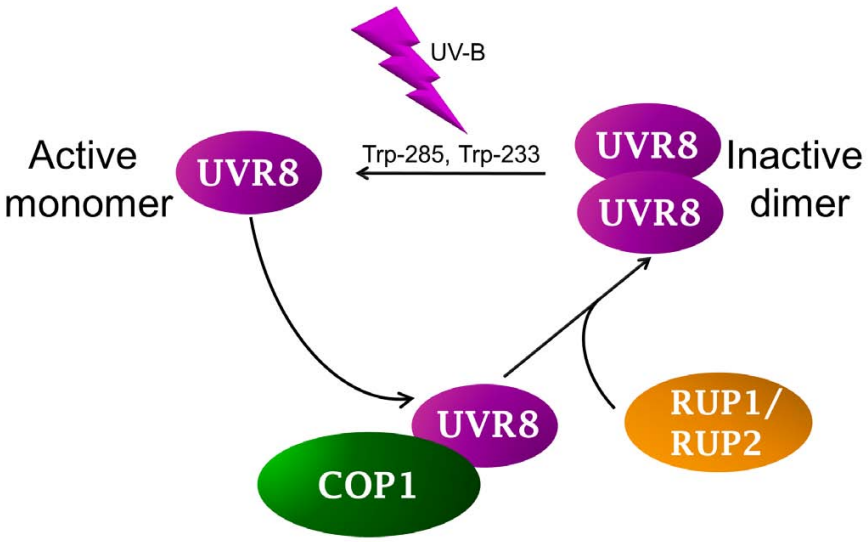


**Figure 3** The inactive UVR8 homodimer is monomerized by UV-B radiation, absorbed by a tryptophane based chromophore. The active monomeric form interacts with COP1, leading to signal transduction in the UV-B response pathway. UVR8 is redimerized after rupture of the UVR8-COP1 association by RUP1 and RUP2 (Tilbrook et al. 2013).

It has still to be resolved if and how UVR8 is involved in COP1's change from negative to positive regulator and which components are additionally involved in UV-B response (Rizzini et al. 2011, Oravecz et al. 2006, Cloix et al. 2012, Brown et al. 2005). The UV-B dependent interaction of UVR8 and COP1 was studied as well as interactions with diverse ARIADNE proteins, possible down-stream UV-B response components.

## 1.4) Protein-protein interaction analyses

The interplay between proteins determines virtually every process in the life of a cell. As ever more genomes are completely sequenced the underlying code would be freely accessible but properties and functions of a great portion of proteins still remain a secret to us. Various methods exist to reveal some of these mysteries (Piehler 2005, Van Criekinge and Beyaert 1999).

On the one hand there are physical, *in vitro* methods like protein affinity chromatography, affinity blotting, co-immunoprecipitation or cross linking (Phizicky and Fields 1995). Based on these 'classical methods' more recent developments are protein array and tandem affinity purification (Piehler 2005). While *in vitro* techniques are always depending on the presence of sufficient amounts of 'bait' proteins, library based methods, like protein probing, phage display and two-hybrid, once cloned are usually readily available (Phizicky and Fields 1995). Because of the usage of transcription factors to be refunctionalized, the classical yeast two-hybrid assays are restricted to interaction analysis in the cell nucleus. Protein fragment complementation assays offer a higher variability of compartments to be chosen as interaction environment and are also successfully applied for membrane proteins. Various systems are known, from complementation of ubiquitin, which subsequently acts as substrate for specific enzymes, or of reporter enzymes, like lactamase or galactosidase, to GFP and YFP, which allow a direct spectroscopic readout. Another spectroscopic mechanism enabling direct observation of protein-protein interactions is FRET. Recent developed methods even allow research on the single molecular level (Piehler 2005).

For my research the yeast two hybrid system was chosen, with GAL4 as the transcription factor to be complemented through interaction of bait and prey proteins. The expression of reporter genes can only occur when GAL4's DNA-binding domain and activation domain are successfully associated, but already weak or transient interactions suffice.

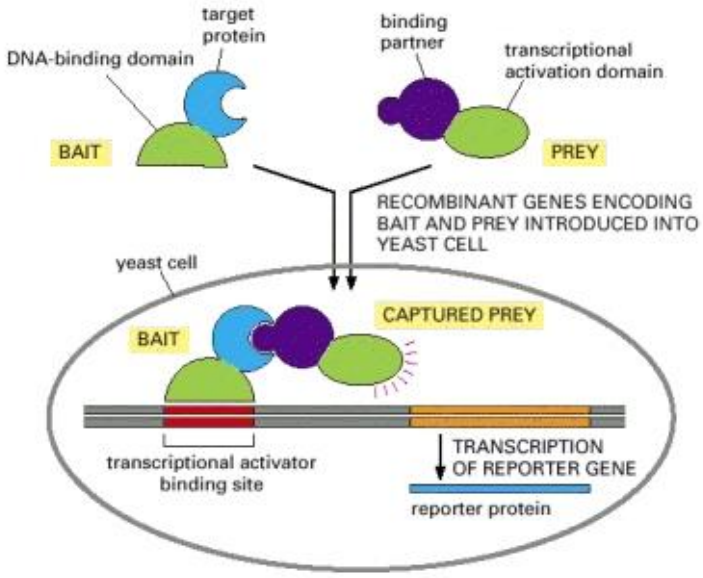


**Figure 4** For yeast two-hybrid experiments a transcription factor is split into DNA-binding domain and transcriptional activation domain which are fused with the target protein and its possible binding partner respectively. Interaction leads to expression of a reporter gene usually permitting growth on a selective medium (Alberts et al. 2002).

One major drawback of this method is the construction of fusion proteins which might change the initial conformation of the proteins of interest and prevent interaction with actual interaction partners. As proteins from plant were used in my study yeast might not be able to produce authentic post-translational modifications. But although yeast two hybrid does not perfectly represent conditions in plant cells it is at least closer to it than *in vitro* techniques. What is always to be considered is that protein combinations researched might never be able to interact because of different localization or expression to various times during cell life (Van Criekinge and Beyaert 1999).

# 2) Aim

Against this background following questions arose:

- if there is some kind of hierarchy in the ARIADNE E3 protein family, which indicates mutual regulation.
- if ARI proteins might have an influence on translation by using translation initiation factors or possible mRNA stabilizing proteins as substrate.
- if my proteins of interest ARI5 and ARI7 do really interact with ubiquitin, which they are thought to take over from E2 enzymes before passing it on to the target proteins.
- if it can be confirmed, that COP1 and UVR8 can only interact upon UV-B radiation, indicating that energy uptake from light alone is sufficient to lead to a structural change.
- if members of the ARIADNE family are able to interact with components of the UV-B response pathway, especially ARI12 also depending on UV-B radiation.

ARI5 and ARI7 were not available in yeast two hybrid shuttle vectors and COP1 only combined with the GAL4 activation domain. Because it was already known that ARI12 associated to the DNA binding domain is autoactivating, combinations of COP1 with ARI12 should also be tested vice versa. This in mind the following objectives for my master thesis were formulated:

- The isolation and cloning of *ARI5*, *ARI7* and *COP1* into both yeast two hybrid shuttle vectors
- Protein-protein interaction analysis of
    - ARI5 and ARI7 with other already available ARI proteins
    - ARI5 and ARI7 with ubiquitin
    - ARI5 and ARI7 with translation initiation factors
    - the UV-B signalling regulators UVR8 and COP1 with ARIs
    - COP1, UVR8 and ARI12 under UV-B influence

# 3) Material

**Enzymes**

Antarctic Phosphatase (NEB #M0289S)

10x Antarctic Phosphatase Reaction buffer (NEB # B0289S)

Blunt Enzyme Mix (NEB #M1201A)

Quickligase (NEB #M2200S)

2x Quickligase buffer (NEB #B2200S)

SLiCE extract prepared by Shengqing Shi according to (Zhang, Werling and Edelmann 2012)

T4 DNA Ligase (Fermentas #EL0011)

10x T4 DNA Ligase buffer

T4 Polynukleotid Kinase (Fermentas #EK0031)

10x T4 PNK buffer

Dream TAQ Polymerase (Thermo Scientific #EP0702)

10x Dream TAQ buffer (Thermo Scientific)

Homemade TAQ Polymerase

10x PCR buffer for Homemade TAQ (20 mM $MgCl_2$)

2x Phusion Flash Mastermix (Finnzymes F-548)

RNase A (ROTH)

*BamH1* Fast Digest (Thermo Scientific #FD0054)

*EcoR1* Fast Digest (Thermo Scientific #FD0274)

*Sac1* Fast Digest (Thermo Scientific #FD1133)

*Sal1* Fast Digest (Thermo Scientific #FD0644)

*Sfi1* Fast Digest (Termo Scientific #FD1824)

*Sma1* Fast Digest (Termo Scientific #FD0663)

*Xho1* Fast Digest (Thermo scientific #FD0694)

10x Fast Digest buffer (Thermo Scientific)

*BamH1* High Fidelity (NEB #R3136S)

*EcoR1* High Fidelity (NEB #R3101S)

*Nco1* High Fidelity (NEB #R3193S)

*Sal1* High Fidelity (NEB #R3138S)

10x NEB buffer 4 (NEB #B7004S)

**Chemicals**

40% Acrylamide-, bis-acrylamide-stock solution 29:1 (AA; ROTIPHORESE ROTH A515.1)

Ammonium persulfate (APS) ≥ 98%, p.a. (ROTH 9592.2)

Chloroform ≥99.8% (ROTH ROTISOLV T901.1)

D-(+)-glucose anhydrous (FLUKA 49140)

Disodium hydrogen phosphate ≥ 78%, $Na_2HPO_4$*2 $H_2O$ (ROTH T877.1)

Dithiothreitol (DTT) ≥ 99%, p.a. (ROTH 6908.2)

EDTA ≥99% p.a. (ROTH 8043.2)

Ethidium bromide (FLUKA 46065)

Glycerol ≥ 86% p.a. (ROTH ROTHIPURAN 4043.3)

Glycine ≥99% (ROTH 3187.4)

Lithium acetate, LiAc ≥99% (ROTH 5447.1)

Magnesium chloride, $MgCl_2*6H_2O$ >98% (FLUKA 63065)

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

2-Mercaptoethanol 90%, p.a. (ROTH 4227.1)

Mineral oil (SIGMA ALDRICH M3516-1L)

2-Nitrophenyl β-D-galactopyranoside (ONPG) ≥99% (ALDRICH N21502-5g)

PEG 4000 (FLUKA 81240)

Potassium chloride ≥99.5% p.a., KCl (ROTH ROTIPURAN 6781.1)

Sodium dodecyl sulphate (SDS) ≥ 99% (ROTH 4360.2)

Sodium carbonate p.a., (ROTH ROTIPURAN A.135.2)

Sodium chloride ≥ 99.5% p.a. (ROTH 3957.2)

Sodium dihydrogen phosphate ≥ 98%, $NaH_2PO_4*2\ H_2O$ (ROTH 2370.2)

TEMED 99%, p.a. (ROTH 2367)

TRIS ≥99.3% (ROTH PUFFERAN AE15.3)

TWEEN-20 polyoxyethylene sorbitan monolaurate (SIGMA P-7949)

**Solvents**

DMSO ≥ 99.5% (GC) (SIGMA D4540)

Ethanol absolute (AustoAlco AAAH-5020-07025-040610)

Isopropanol ≥99.8% (ROTH ROTHIPURAN 6752.5)

Methanol (ROTH P717.1)

**Biochemicals**

100 bp ladder Gene Ruler Plus DNA Ladder (Fermentas #SM0321)

Agarose DNA Grade Electran (VWR Ref 438794L)

Carrier DNA herring testes (Clontech 630439)

Carrier DNA ss salmon testes (SIGMA D9156)

Disodium rATP (Boehringer Mannheim 519979)

λ DNA/HindIII (Fermentas #SM0101)

Milk (fixmilch INSTANT)

Prestained protein molecular weight marker (Fermentas #SM0441)

**Ingredients for media**

Agar-agar, Kobe I (ROTH 5210.2)

Casein hydrolysate (ROTH AE41.2)

DO supplement -leucine/-tryptophan (Clontech 630417)

DO supplement leucine/ -tryptophan/ -histidine (Clontech 630419)

Yeast extract (ROTH 2363.4)

Yeast nitrogen base without amino acids (SIGMA Y0626)

**Buffers and Solutions**

Agarose gels

0.7 - 2 % (w/v) agarose was melted in 1x TAE and ethidium bromide added before pouring the gel
stored wrapped in foil at +4°C

100 bp marker

1x loading dye, 1x TE, 50 ng/µL 100 bp ladder Gene Ruler Plus DNA
aliquots stored at -20°C

Blocking solution

5% milk in TBS-T
prepared freshly

Coomassie Blue staining solution

0.25% Coomassie Blue, 25% ethanol, 10% acetic acid

2 mM dATP

100 mM dNTP – dGTP, dATP, dCTP, dTTP (Solis BioDyne 02-21)

2 mM dNTP Mix

2 mM dATP, 2mM dCTP, 2mM dGTP, 2mM dTTP

1 M, 100 mM DTT

70% Ethanol

40% (w/v) Glucose (autoclaved)

GTE buffer (autoclaved)

50 mM glucose, 25 mM TRIS/HCl pH 8, 10 mM EDTA

1 M KCl (autoclaved)

5x KCM

500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$

stored at +4°C

1 M LiAc (autoclaved)

LiAC/PEG solution

40% (w/v) PEG 4000, 100 mM LiAc, 1x TE

prepared freshly before use from stock solutions

LiAc solution

1x TE, 100 mM LiAc

prepared freshly before use from stock solutions

9x Loading dye

0.09% bromphenol blue, 0.09% xylene cyanol FF, 60% glycerol, 60 mM EDTA

for 1x Loading buffer diluted 1:10 in 87% glycerol

λ-HindIII marker

50 ng/µL λ-HindIII, 1x TE, 1x loading dye

100 mM, 25 mM $MgCl_2$

0.1 M $MgSO_4$ (autoclaved)

1 M $Na_2CO_3$ (not sterile)

1 M $Na_2HPO_4$ (autoclaved)

1 M $NaH_2PO_4$ (autoclaved)

3 M NaOAc pH 4.9 (autoclaved)

0.2 N NaOH/1% SDS

0.4 N NaOH

ONPG

13.3 mM 2-nitrophenyle β-D-galactopyranoside in z-buffer without 2-mercaptoethanol

prepared freshly, to dissolve shaken at least for two hours at room temperature

Panceau S staining solution

0.5% panceau, 1% acetic acid

50% (w/v) PEG 4000 (autoclaved)

100 mM, 10 mM rATP

stored at -20°C

Roti-free stripping buffer (ROTH 0083.1)

Roti-Lumin plus (ROTH 3692.2)

5x Running buffer

1 M glycine, 0.1 M TRIS

for 1x running buffer diluted 1:5 in water and 0.1% (w/v) SDS were added

20, 2 and 0.1% SDS

4 x SDS PAGE loading dye

200 mM TRIS/HCl pH 6.8, 400 mM DTT, 8% SDS, 40% glycerol, 0.01% bromphenol blue

stored at -20°C

1 x SDS-PAGE sample buffer

50 mM TRIS/HCl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol

SLiCE buffer

500 mM TRIS/HCl pH 7.5, 100 mM $MgCl_2$, 10 mM rATP, 10 mM DTT

mixed from stock solutions, stored at -20°C

50 x TAE-buffer (autoclaved)

2 M TRIS, 0.05 M EDTA pH 8, 1 M acetic acid

for use diluted 1:50 in water

10 x TBS-T

1 M TRIS/HCl pH 7.5, 1.37 M NaCl

diluted 1:10 with water, 0.1% TWEEN-20 added

10x TE (autoclaved)

100 mM TRIS/HCl pH 7.5, 10 mM EDTA pH 8

for 1x TE diluted 1:10 in water

10x Transfer buffer

1.92 M glycine, 250 mM TRIS

diluted 1:10 with water before use

1 M TRIS/HCl pH 6.8

1 M TRIS/HCl pH 7.5 (autoclaved)

1.5 M TRIS/HCl pH 8.8

Z-buffer

0.6 M $Na_2HPO_4$, 0.4 M $NaH_2PO_4$, 0.1 M KCl, 0.01 M $MgSO_4$, pH 7

for z-buffer with 2-mercaproethanol additionally 0.35% 2-mercaptoethanol were added

mixed from stock solutions, not autoclaved, store at +4°C

**Kits**

GeneJET Gel Extraction Kit (Thermo Scientific #K0691)

Gene JET Plasmid Midi Prep Kit (Fermentas #K0481)

TOPO TA Cloning kit for Sequencing (Invitrogen K4575-01)

Qubit Protein Assay Kit (Invitrogen Q33211)

**Media**

LB-medium

| | |
|---|---|
| Yeast extract | 5g/L |
| Casein hydrolysate | 10g/L |
| NaCl | 10g/L |
| pH 7-7.4 | |

SD - medium

| | |
|---|---|
| Glucose | 20 g/L |
| Yeast nitrogen base | 6.7 g/L |
| -L/-T or -L/-T/-H DO supplement | 0.64 or 0.62 g/L |
| (Agar agar | 20 g/L) |

All components were weighed in and dissolved in ¾ of final volume. To prepare plates agar agar was optionally added. PH was adjusted to 5.8 with 1N NaOH, the volume filled up and autoclaved. Optionally 3-aminotriazol was added to 1 mM after medium cooled down to 55°C.

TSB – medium

0.95 g of PEG 4000 were dissolved in 10 mL of YT-medium, 100 µL of salt solution for TSB medium (1 M $MgCl_2$, 1M $MgSO_4$) and 0.5 mL DMSO added and filter sterilized.

YPD-medium

| | |
|---|---|
| Yeast extract | 10 g/L |
| Casein hydrolysate | 20 g/L |
| (Agar agar | 20 g/L) |

All components were weighed in and dissolved in ¾ of final volume. Agar agar was added optionally and pH adjusted to 6.5 with diluted HCl. The volume was filled up and autoclaved. 40% glucose was added to a final concentration of 2% right before usage.

YT-medium

| | |
|---|---|
| Yeast extract | 10 g/L |
| Casein hydrolysate | 10 g/L |
| NaCl | 5 g/L |
| (Agar-Agar | 15 g/L) |

All components were weighed in and dissolved in ¾ of final volume. Agar agar was added optionally the volume was filled up and autoclaved. After cooling down to approximately 60°C antibiotics to a final concentration of 50 mg/L were added.

**Antibiotics**

Ampicillin 100 mg/mL (filter sterilized and stored at -20°C)

Kanamycin 100 mg/mL

Streptomycin 100 mg/mL

**Primer**

| | | |
|---|---|---|
| ARI5_AD_F | 5' –CCGGGTGGGCATCGAATGGATTCCGATGATGATATG-3' | SLiCE cloning |
| ARI5_AD_R | 5' -ATCTGCAGCTCGAGCTCAATTGCCATCTGGGTTTC-3' | SLiCE cloning |
| ARI5_BD_F | 5' -ATGGAGGCCGAATTCATGGATTCCGATGATGATATG-3' | SLiCE cloning |
| ARI5_BD_R | 5' -TGCGGCCGCTGCAGGTCAATTGCCATCTGGGTTTC-3' | SLiCE cloning |
| ARI7_AD_F | 5' -CCGGGTGGGCATCGAATGGATTCTGAAGAAGACATG-3' | SLiCE cloning |
| ARI7_AD_R | 5' -ATCTGCAGCTCGAGCTTATAAGTTGTCATCTGGGTTT-3' | SLiCE cloning |
| ARI7_BD_F | 5' -ATGGAGGCCGAATTCATGGATTCTGAAGAAGACATG-3' | SLiCE cloning |
| ARI7_BD_R | 5' -TGCGGCCGCTGCAGGTTATAAGTTGTCATCTGGGTTT-3' | SLiCE cloning |
| ARI7_Sfi_F | 5' -TGGCCATGGAGGCCATGGATTCTGAAGAAGACATG-3' | cloning |
| ARI7_Sfi_R | 5' -TGGCCTCCATGGCCTTATAAGTTGTCATCTGGGTTTC-3' | cloning |
| M13 fwd | 5´-GTAAAACGACGGCCAG-3´ | sequencing (seq) |
| M13 rev | 5´-CAGGAAACAGCTATGAC-3´ | seq |
| pGAD_F | 5' -TACCACTACAATGGATG-3' | seq/colony PCR |
| pGAD_R | 5' -GTTGAAGTGAACTTGCGGGG-3' | seq/colony PCR |

| | | |
|---|---|---|
| pGBK_Eco_rev | 5’ -ATGGAGGCCGAATTC-3’ | PCR |
| pGTB9_F | 5’ -TCATCGGAAGAGAGTAG-3’ | seq/colony PCR |
| pGTB9_R | 5’ -CGTTTTAAAACCTAGAGTCAC-3’ | seq/colony PCR |
| T3 | 5’ -ATTAACCCTCACTAAAGGGA-3‘ | seq |
| T7 | 5´-TAATACGACTCACTATAGGG-3´ | seq |

**cDNA (provided by Christina Lang-Mladek)**

Col wt ctrl uvr8-6, uvr8-6 22.07.09 14:00, uvr8-6 30.10.08 18:00, ctrl 14:00 23.09.08 leaves, col wt 21:00 04.09.08

**Plasmid DNA**

| | |
|---|---|
| pGAD (Clontech) | self-prepared |
| pGAD - ARI1 | self-prepared from stock culture provided by Neha Nigam (3.2.10 pGADT7-ARI1) |
| pGAD - ARI2 | prepared by Juan Antonio Torres-Acosta (#44) |
| pGAD - ARI5 | self-prepared |
| pGAD - ARI7 | self-prepared |
| pGAD - ARI8 | self-prepared from stock culture provided by Neha |
| pGAD - ARI12 | prepared by Antonio (#55) |
| pGAD - ARI12-RING1 | prepared by Hann-Wei Chen |
| pGAD - ARI12-RING1-IBR | prepared by Hann-Wei |
| pGAD - ARI12-IBR-RING2 | prepared by Hann-Wei |
| pGAD - ARI12-RING2-C | prepared by Hann-Wei |
| pGAD - ARI12-C | prepared by Hann-Wei |

| | |
|---|---|
| pGAD - ARI15 | self-prepared from stock culture provided by Neha |
| pGAD - cop1 | provided by Gareth I. Jenkins, University of Glasgow |
| pGAD - eIF4E | prepared by Rafaela Scheidelberger |
| pGAD - eIF(iso)4E | self-prepared from stock culture provided by Neha |
| pGAD - nCBP | prepared by Antonio (#68) |
| pGBK (Clontech) | prepared by Elisabeth Hehenberger (Midiprep) |
| pGBK - ARI1 | self-prepared from stock culture provided by Antonio ( #76) |
| pGBK - ARI2 | prepared by Antonio (#79) |
| pGBK - ARI5 | self-prepared |
| pGBK - ARI8 | self-prepared from stock culture provided by Neha |
| pGBK - ARI15 | prepared by Antonio (#93) |
| pGBK - cop1 | self-prepared |
| pGBK - eIF4E | prepared by Neha |
| pGBK - eIF(iso)4E | prepared by Neha and self-prepared from stock cuture provided by Wolfgang Sommeregger (#4) |
| pGBK - nCBP | prepared by Rafaela |
| pGBK - β-sheet | self-prepared from stock culture provided by Rafaela |
| pGBK - C-tail | self-prepared from stock culture provided by Rafaela |
| pGBK - Helix | prepared by Rafaela |
| pGBK - N-tail | self-prepared from stock culture provided by Rafaela |
| pGBK - $UBI^{K48L}$ | prepared by Conny Habacher (provided by Linda Hicke, University of Texas, Austin) |
| pGBK - uvr8 | provided by Gareth I. Jenkins, University of Glasgow |

**Escherichia coli stock cultures and transformants**

DH10beta cells ($F^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G $\lambda^-$ (Durfee et al. 2008))

Chemical competent DH10beta cells (provided by Shengqing, Katharina Leeb and selfmade)

DH10b cells with pGAD - empty vector (stock culture provided by Neha)

**Yeast stock cultures and transformants**

pJ69-4a (*MAT*a *ura3 his3 leu2 trp1 ade2 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ* ; (James, Halladay and Craig 1996) #4 of Neha)

AD-empty/BD-empty provided by Rafaela

AD-ARI12/BD-empty provided by Rafaela

AD-empty/BD-nCBP provided by Rafaela

AD-ARI12/BD-nCBP provided by Rafaela

AD-empty/BD-ARI1 provided by Neha

**Antibodies**

Rat anti-HA (ROCHE) 1:1000

Rabbit anti-c-myc A14 (Santa Cruz Biotech sc-789) 1:1000 provided by Richard Strasser

HRP goat anti-rat (Jackson) 1:10000 - 1:15000

HRP goat anti-rabbit (Pierce) 1:7500

**Material for Western blot**

Mediphot X-90/RP Medical X-ray film double coated

Roti-PVDF membrane (ROTH T830.1)

# 4) Methods

## 4.1) Cloning

As a first step the genes of interest had to be cloned into pGBKT7 (pGBK) and pGADT7 (pGAD) two shuttle vectors especially constructed for yeast two hybrid assays, where fusion proteins with transcription factor GAL4 DNA-binding (BD) and activation (AD) domain respectively can be constructed. For cloning and plasmid amplification purposes in bacteria they carry a bacterial high-copy-number origin of replication, a multiple cloning site and a resistance for one antibiotic, ampicillin in pGAD and kanamycin in pGBK. For expression in yeast and yeast two hybrid assays they carry a yeast origin of replication and the respective GAL4 domain under the control of the constitutive ADH1 promoter and targeted with a nuclear localization signal. Inside of the open reading frame both vectors posses an epitope tag, pGAD the HA-tag and pGBK the c-Myc tag, to be used for Western blot analyses of the fusion proteins. They also include an auxotrophic selection marker, leucine in pGAD and tryptophan in pGBK. For cloning *ARI5* and *ARI7* a rather new cloning approach, SLiCE cloning, was tested. As *COP1* was only available in pGAD this gene was also cloned into pGBK by restriction/ligation cloning. *UVR8* was used as provided in pGBK. Sequences of *Arabidopsis thaliana* genes were retrieved from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/servlets/Search?action=new_search&type=gene; Sept 09, 2013 on www.arabidopsis.org), sequence information of vectors from Clontech Laborotories, Inc and SnapGene software (from GSL Biotech; available at snapgene.com).

### 4.1.1) SLiCE cloning (Zhang, Werling and Edelmann 2012)

Seamless Ligation Cloning Extract (SLiCE) cloning is a relatively new developed method based on the ability of an extract from the bacteria strain PPY, a modified DH10b strain, to perform *in vitro* homologous recombination, independent of restriction sites and ligases. Appropriate gene insertion sites were found by analyses with EditSeq and MapDraw from DNASTAR, Inc.

**Preparation of vectors**

For SLiCE cloning vectors were linearized by restriction digestion with *BamH1*. The ends were then additionally blunted, because it is described by Zhang et al. (2012) that vectors with compatible overhangs tend to form increased amounts of background colonies with recircularized empty vectors during SLiCE reaction. Purified empty pGBK vector was already available in sufficient amounts; the pGAD plasmid on the other hand had to be freshly prepared.

Preparation of empty pGAD vector - Gene JET Plasmid Midiprep Kit

A single colony from a freshly streaked plate of *E.coli* containing empty pGAD vector was transferred into 5 mL of LB-medium containing 50 µg/mL ampicillin. The culture was incubated at 37°C and 180 rpm on a shaker for eight hours. Then 50 µL of the day culture were transferred into 50 mL of fresh LB-medium (antibiotic was forgotten) and incubated over night at 37°C and 180 rpm. After reaching an $OD_{600}$ of three the culture was split into two 50 mL falcon tubes and centrifuged at 5000 x g at 5°C for 10 minutes. Each pellet was resuspended in 1 mL of Resuspension buffer and the two suspensions were united. 2 mL of Lysis solution were added, the tubes carefully inverted five times and incubated for three minutes at room temperature. 2 mL of Neutralization solution were added and the tubes inverted eight times, precipitation occurred. 0.5 mL of Endotoxin Bindung Reagent were added, the tube inverted eight times and incubated for five minutes at room temperature then centrifuged 20 minutes with 7000 x g. The supernatant was transferred into a fresh 15 mL tube and the same amount of 96% Ethanol was added. Up to 5 mL were loaded onto the

column and centrifuged three min with 2000 x g, the flow through was discarded. This step was repeated until the whole sample was loaded. Washing was performed once with 4 mL Wash Solution 1 and two times with 4 mL Wash Solution 2, each time centrifugation was for two minutes with 3000 x g, flow through was discarded. The column was dried by centrifugation with 3000 x g for five minutes. Then the column was put into a fresh collection tube. The filter was completely coated with 250 µL of Elution buffer, incubated for two minutes at room temperature, then centrifuged five minutes at 3000 x g. Again the column was put into a new collection tube and the elution step was repeated with 150 µL of Elution Buffer. The flow through was transferred into fresh 1.5 mL tubes and the plasmids were stored at -20°C.

Linearization of pGBK and pGAD by restriction digestion

| | |
|---|---|
| 10x buffer 4 | 2 µL |
| *BamH1* high fidelity | 1.5 µL |
| pGBK or pGAD | up to 2 µg |
| double distilled water | filled up to 20 µL |

All components were pipetted into a tube, mixed by snipping, spun down and incubated at 37°C for 15 to 30 minutes. The enzyme was inactivated at 80°C for 30 minutes. To control the success of the digestion an agarose gel was run with 1 µL of the reaction.

Blunting of vectors

2 µL 1 mM dNTPs and 1 µL of Blunt Enzyme were added to 20 µL restriction digestion. The reaction was incubated at room temperature for 15 minutes then inactivated at 70°C for ten minutes. The blunted vectors were stored at -20°C until use.

**Preparation of inserts**

*ARI5* and *ARI7* inserts were amplified from a cDNA mixture generated during UV experiments with *Arabidopsis thaliana*. Gene specific primers, with melting temperatures between 58 and 62°C, were designed using DNASTAR Software and reverse complement (Stothard 2000) with a 15 bp 5' overhang homologous to the plasmid DNA down- and upstream of the *BamH1* restriction site. Special attention had to be paid on in frame positioning of the start codon.

Reverse transcription-PCR

| | | **final concentration** |
|---|---|---|
| 2x PhusionFlash MM | 7.5 µL | 1x |
| Primer Forward 20 µM | 0.38 µL | 0.5 µM |
| Primer reverse 20 µM | 0.38 µL | 0.5 µM |
| double distilled water | filled up to 15 µL | |

First the reagents for the mastermix were combined and vortexed. For every single reaction 2 µL of cDNA were mixed with 13 µL of mastermix and sealed with one drop of mineral oil.

| | | | |
|---|---|---|---|
| Program: CAPS 55°C | 95°C | 3 min | |
| | 35x | 30 sec | 55°C |
| | | 90 sec | 72°C |
| | | 30 sec | 95°C |
| | 20°C | hold | |

Purification of insert DNA - GeneJET Gel Extraction Kit

The PCR reactions were separated on a 0.7% agarose gel and the bands at approximately 1700 bp for both ARIADNE genes were cut out under UV light. For each insert the gel pieces were transferred into a tube and 1 µL of Binding buffer per mg of gel was added. This mixture was incubated on the heating block at 55°C for approximately 12 minutes, with inverting every two minutes, until the gel

was completely dissolved. Up to 800 µL were loaded on the filter column and centrifuged for one minute at 13 000 rpm. The flow through was reloaded and the centrifugation repeated, then the flow through was discarded. If necessary the loading steps were repeated with the rest of the sample. The sample was washed with 700 µL of Wash buffer and centrifugation as before. The column was put into a new collection tube and the filter was dried by centrifugation for one minute at 13 000 rpm. The column was put into a fresh 1.5 mL tube and 20 µL of double distilled water were pipetted directly onto the filter. It was centrifuged for one minute at 13 000 rpm. The elution step was repeated into the same collection tube. The insert DNA was stored at -20°C or quantified. If higher DNA concentrations were needed the volume was decreased by lyophilisation at room temperature.

**SLiCE reaction**

The molar ratios of vector to insert used were between 1:5 and 1:10

| | |
|---|---|
| insert | 50 to 120 ng |
| vector | 50 ng |
| PPY SLiCE extract | 1 µL |
| SLiCE buffer | 1 µL |
| double distilled water | brought to 10 µL |

The reaction was incubated at 37°C for one hour, afterwards it was immediately proceeded with the transformation using 1, 2 or 5 µL.

### 4.1.2) Cloning by restriction/ligation

As it was not possible to clone ARIADNE 7 into pGBK by SLiCE another approach had to be tried out. Additionally *COP1* was cloned into pGBK too. It was taken advantage that both genes were already cloned into pGAD and that the *Sal1* and *Xho1* restriction sites are compatible. Thus the insert DNA could be cut out from pGAD and inserted into pGBK quite straightforward.

**Preparation of pGBK for insertion of *COP1* and *ARI7***

| | |
|---|---|
| 10x Fast Digest buffer | 2 µL |
| *EcoR1* Fast Digest | 1.5 µL |
| *Sal1* Fast Digest | 1.5 µL |
| pGBK | 2 µg |
| double distilled water | filled up to 20 µL |

All components were mixed in a tube by snipping, spun down and incubated at 37°C for 20 minutes. The enzyme was inactivated at 80°C for ten minutes. To control the success of the digestion an agarose gel was run with 1 µL of the reaction.

**Insert preparation from pGAD-ARI7 and pGAD-COP1**

| | |
|---|---|
| 10x Fast Digest buffer | 2 µL |
| *EcoR1* Fast Digest | 1.5 µL |
| *Xho1* Fast Digest | 1.5 µL |
| pGAD-COP1 or pGAD-ARI7 | 1 and 1.5 µg respectively |
| double distilled water | filled up to 20 µL |

All components were mixed in a tube by snipping, spun down and incubated at 37°C for 25 minutes. The enzyme was inactivated at 80°C for five minutes. The digestion reaction was loaded on a 0.7% agarose gel and the fragments at 1700 bp for *ARI7* and at 2000 bp for *COP1* were purified using the GeneJET Gel Extraction Kit.

**Ligation**

The ligation reactions were performed with a molar ratio of vector to insert between 1:1 and 1:5 with Quick Ligase or T4 Ligase.

| | |
|---|---|
| insert | 20 to 50 ng |
| vector | 50 ng |
| 2 x Quick Ligase buffer | 10 µL |
| Quick Ligase | 1 µL |
| double distilled water | brought to 20 µL |

The reaction was incubated for six minutes at room temperature. It was either immediately proceeded with the transformation using 10 µL or the reactions were frozen at -20°C.

As it was suspected that ligation might not be successful because of DNA conformational reasons T4 Ligase preparations were made with and without additional magnesium, which is able to influence conformation (Egli 2002). The molar ratio used was 1:10.

| | |
|---|---|
| vector | 50 ng |
| insert | 120 ng |
| 10 x T4 Ligase buffer | 2 µL |
| T4 Ligase | 2 µL |
| 100 mM $MgCl_2$ | none or 1 µL |
| 100 mM rATP | 0.4 µL |
| double distilled water | brought to 20 µL |

All components were mixed by snipping gently and incubated at 14°C in the water bath over night then it was proceeded with the transformation using 10 µL ligation reaction.

To confirm successful ligation a colony PCR of the pGBK-ARI7 ligation was performed with the primer pairs pGTB9 F/R and ARI7 AD F/pGTB9 R.

### 4.1.3) Cloning of pGBK-ARI7

As it turned out to be quite hard to clone *ARI7* into pGBK two additional approached were tried.

**TOPO-Transformation - TOPO TA Cloning kit for Sequencing**

TOPO TA cloning is a method to insert any PCR amplified DNA fragment into an especially prepared vector. The pCR™4-TOPO® vector was provided linearized, with a single 3‘ thymidine overhang and a covalently bound Topoisomerase I from *vaccinia* virus. When vector and insert DNA with additional 3’ desoxyadenosine overhang are mixed, the inserts 5’ hydroxyl group causes the release of the enzyme and binds covalently to the compatible vector overhang. The plasmids assembled in this way were then transformed into TOP10 competent cells, which generated amounts of insert DNA much larger than obtained with PCR.

Amplification of insert DNA

Insert DNA was amplified by RT-PCR as described above with primers ARI7_Sfi_F/R which hang on *Nco1* restriction sites on both ends of the gene. Those were later used to cut out *ARI7* of TOP10.

Generation of single 3' desoxyadenosine overhang

The Phusion Flash II DNA Polymerase used for RT-PCR does not generate 3' desoxyadenosine overhangs. Thus inserts were first amplified with Phusion Flash and then purified by GeneJET Gel Extraction Kit. To add the A overhang the insert DNA was treated with homemade TAQ Polymerase.

| | |
|---|---|
| Insert DNA | 50 ng |
| 10x Puffer for Homemade TAQ | 1 µL |
| 2 mM dATP | 1 µL |
| Homemade TAQ | 0.5 µL |

All reagents were mixed and incubated at 72°C for ten minutes in the PCR machine. Afterwards it was immediately proceeded with cloning.

Cloning step

| | |
|---|---|
| Insert DNA with A overhang | 4.5 µL (20 ng) |
| Salt Solution | 1 µL |
| pCR™4-TOPO® vector 10 ng/µL | 1 µL |

The reaction was incubated at room temperature for six minutes.

Transformation

For the transformation 2 µL of cloning reaction were mixed with 50 µL TOP10 competent cells and chilled on ice for 12 minutes. The heat shock was performed in the water bath at 42°C for 30 seconds. The cells were chilled on ice briefly, 250 µL SOC medium were added and incubated at 37°C and 200 rpm for one hour. To get a reasonable number of colonies per plate 20 µL and 100 µL

were plated on prewarmed YT plates, containing 50 µg/mL of kanamycin and incubated at 37°C over night.

Grown colonies were screened by colony PCR with primers T3 and T7. After preparation of plasmids by QuickPrep (described later) from positive transformants the insert was cut out by restriction digestion and used for ligation transformation.

Preparation of pGBK for insertion of *ARI7* from TOPO

| | |
|---|---|
| 10x buffer 4 | 2 µL |
| *Nco1* high fidelity | 1.5 µL |
| pGBK | 4 µg |
| double distilled water | filled up to 20 µL |

All components were mixed in a tube by snipping, spun down and incubated at 37°C for 30 minutes. The enzyme was inactivated at 80°C for 20 minutes. To control the success of the digestion an agarose gel was run with 1 µL of the reaction.

Dephosphorylation of the vector

To avoid recircularization of digested vector without insert DNA during the ligation process, it was dephosphorylated by adding 1 µL Antarctic Phosphatase and 2.2 µL 10 x Reaction buffer to 20 µL restriction digestion. The reaction was incubated at 37°C for 15 minutes, inactivated at 72°C for five minutes and stored at -20°C.

Preparation of insert DNA from TOPO

| | |
|---|---|
| 10x buffer 4 | 2 µL |
| *Nco1* high fidelity | 1.5 µL |
| TOPO-ARI7 | 1 µg |
| double distilled water | filled up to 20 µL |

All components were mixed in a tube by snipping, spun down and incubated at 37°C for 25 minutes. For insert purification the whole volume was loaded on a 1.5% agarose gel. Because the TOPO vector contains a *Nco1* restriction site, resulting in a 1400 and a 2600 bp TOPO fragment, ARI7 with 1700 bp had to be cut out carefully. It was further purified by GeneJET Gel Extraction Kit.

For ligation QuickLigase was used as described above. 10 µL of ligation reaction were then utilized for transformation.

**Amplification of the whole vector**

As a last attempt the whole vector containing insert DNA, obtained by restriction/ligation cloning with *EcoR1* and *Sal1*/*Xho1*, was amplified by PCR, recircularized to avoid any background plasmids and then transformed into DH10b.

Amplification of pGBK-ARI7 by PCR

To enable successful ligation in a later step the primers had to be phosphorylated. pGBK-Eco-rev was already available phosphorylated.

| | |
|---|---|
| ARI7_AD_F primer | 300 pmol |
| T4 PNK | 1 µL |
| 10x PNK buffer | 5 µL |
| 10 mM rATP | 5 µL |
| double distilled water | 24 µL |

The reaction was incubated at 37°C for 30 minutes and afterwards inactivated for ten minutes at 70°C. Phosphorylated primer were then used in whole vector PCR.

| | | **final concentration** |
|---|---|---|
| 2x Phusion Flash Mastermix | 10 µL | 1 x |
| primer forward (ARI_AD_F) phosphorylated 6 µM | 1.7 µL | 0.5 µM |
| primer reverse (pGBK-Eco-rev) phosphorylated 6 µM | 1.7 µL | 0.5 µM |
| pGBK-ARI7 | 250 - 350 pg | 12.5 - 17.5 pg/µL |
| double distilled water | brought to 20µL | |

| | | | |
|---|---|---|---|
| Programm: MUTNCBP | 98°C | 10 sec | |
| | 30x | 10 sec | 98°C |
| | | 4 min | 72°C |
| | 72°C | 1 min | |
| | 20°C | hold | |

The whole PCR reactions were loaded on a 0.8% agarose gel and the amplified, now linear vector was purified by GeneJET Gel Extraction Kit and had to be subsequently recircularized.

Phosphorylation and recircularization

To make sure that both ends of the linear vector were successfully phosphorylated this step was repeated with the compatible Quick Ligase buffer.

| | |
|---|---|
| vector | 50 ng |
| 2x Quick Ligase buffer | 10 µL |
| T4 PNK | 1 µL |
| double distilled water | brought to 20 µL |

The reaction was incubated at 30°C for 20 minutes then 1 µL of Quick Ligase was added and incubated at room temperature for 15 minutes. 2 or 5 µL of this reaction were used for transformation with standard or special conditions as described below.

### 4.1.4) Transformation

**Preparation of DH10-Beta (DH10b) chemical competent cells (Chung and Miller 1988)**

DH10b stock culture was streaked out on a YT-plate containing 25 µg/mL streptomycin and incubated over night at 37°C. The next day an overnight culture was prepared: 5 mL of YT-medium containing 25 µg/mL streptomycin were inoculated with a single colony from the plate and incubated over night at 37°C with 180 rpm shaking. On the next day the cells were diluted: in a 250 mL flask 50 mL YT-medium containing 25 µg/mL streptomycin were inoculated with 50 µL overnight culture and incubated at 37°C and 180 rpm until reaching an OD of 0.3 – 0.6, what took about four hours. The suspension was centrifuged at 5°C and 2500 rpm for ten minutes in two falcon tubes. From then on the cells were always kept on ice. The supernatant as discarded and each pellet resuspend in 2.5 mL cold TSB-buffer. The cells were left on ice for ten minutes, then aliquoted (100 µL) in the lamina flow hood, frozen in liquid nitrogen and stored at -80°C.

***Escherichia coli* transformation**

Transformation by heat shock is a quite fast and easy way to insert plasmid DNA into competent cells, which results in a decent amount of successfully transformed bacteria. Therefore the required amount of competent cells was thawed on ice. For each transformation 10 µL 5x KCM were mixed with vector DNA, either prepared by Quickprep (5 to 50 ng) or generated by SLiCE (1 to 5 µL) or ligation reaction (2 to 10 µL) and brought to 50 µL with doubled distilled water. Every time a control transformation with either 5 or 50 ng of pGAD or pGBK vector was carried along. To each

transformation reaction 50 µL competent cells were added with a cut pipette tip and mixed by pipetting up and down several times. Incubation on ice for 30 minutes was followed by a heat shock at 42°C in the water bath for 90 seconds. Then the cells were chilled on ice for two minutes. Afterwards 400 µL of YT-medium were added and the tubes incubated at 37°C with 180 rpm shaking for 50 minutes. Up to 125 µL of cell suspension were plated per plate, containing the corresponding antibiotic (50 µg/mL ampicillin for pGAD and kanamycin for pGBK transformants). The plates were incubated at 37°C over night; the colonies were counted on the next day and screened for desired transformants by colony PCR. Transformation efficiencies of DH10b competent cells were $10^5$ to $10^6$ colony forming units (cfu)/µg pGAD or pGBK DNA/mL transformation broth.

*Escherichia coli* transformation with special conditions (Frangioni and Neel 1993)

Generally this transformation was performed exactly as the one described above except: to the YT-medium (liquid and plates) 0.2% of glucose were added, the incubation before plating of the transformants was performed at 1000 rpm and 25°C for one hour on the heating block. The plates were incubated at room temperature (22°) for seven days.

For colony PCR primer-combinations pGTB9 F/R and ARI7_AD_F/pGTB9 R were used.

For QuickPrep 5 mL YT containing 0.2% glucose were inoculated with a single colony from a plate containing glucose and incubated at room temperature with 250 rpm shaking for 28 hours. The plasmid yield was too low to be sequenced.

Test transformation

By transforming processed vectors into DH10b cells the background of transformants containing empty vector could be estimated. The standard transformation protocol was used with 50 ng of linearized vector. The calculated colony forming units were compared to the transformation efficiency of empty pGAD or pGBK vectors.

**Table 1** Results of test transformations

| **Vector** | **Transformation efficiency [cfu*µg$^{-1}$*mL$^{-1}$]** |
|---|---|
| pGAD empty | $10^5$ to $10^6$ |
| pGADx*BamH1* blunted | $2*10^3$ |
| pGBK empty | $10^5$ to $10^6$ |
| pGBKx*BamH1* blunted | ~$5*10^3$ |
| pGBKx*EcoR1*x*Sal1* | $2*10^3$ |
| pGBKx*Nco1* dephosphorylated | ~$5*10^3$ |

The test transformations showed a decrease of about two orders of magnitudes of growing colonies compared to the empty vector controls, thus plasmids were used without further purification for transformation.

### 4.1.5) Colony PCR

Colony PCR is a fast method to screen transformation plates for clones containing a plasmid with insert DNA with primers hybridizing up- and downstream of the multiple cloning site, for instance pGAD F and R for pGAD transformants and pGTB9 F and R for pGBK transformants. By using insert specific primers the identity of the integrated DNA fragment can be tested. Because of the harsh conditions PCR sometimes didn't work and had to be redone with additional magnesium in the mastermix.

| | | **final concentration** |
|---|---|---|
| 10x DreamTAQ buffer | 2.5 µL | 1x |
| DreamTAQ Polymerase 5 U/µL | 0.1 µL | 0.02 U/µL |
| Forward Primer 20 µM | 0.25 µL | 0.2 µM |
| Reverse Primer 20 µM | 0.25 µL | 0.2 µM |
| 2 mM dNTP | 2.5 µL | 0.2 mM |
| 25 mM $MgCl_2$ | 0.5 or none | + 0.5 mM or + none |
| double distilled water | brought to 24 µL | |

For each sample, water control and empty vector control 24 µL mastermix were prepared in a PCR tube. A single colony was picked with a pipette tip and resuspended into the mastermix and the rest streaked on a YT-plate containing 50 µg/mL of the appropriate antibiotic. As empty vector control colonies from control transformation plates or 1 ng of empty plasmid were used. Reactions were sealed with a drop of mineral oil.

| | | | |
|---|---|---|---|
| PCR-program (yeast colony): | 95°C | 10 min | |
| | 35x | 30 sec | 95°C |
| | | 30 sec | 50°C |
| | | 2 min | 72°C |
| | 72°C | 3 min | |
| | 20°C | hold | |

The PCR reactions were then run on an 1.5 to 2% agarose gel. Transformants containing empty pGAD vector were expected to show bands at 250 bp, those with empty pGBK at 350 bp. *ARI5* and *ARI7* transformants were expected to show bands at about 2000 bp, *COP1* transformants at 2300 bp.

To prepare vectors for yeast transformation, clones with the desired insert were grown and plasmid DNA extracted by QuickPrep.

### 4.1.6) QuickPrep (Manuel Simon)

One colony of the desired transformant was inoculated in 5 mL YT-medium containing 50 µg/mL of the appropriate antibiotic and incubated over night at 37°C with 180 rpm shaking. 2 mL of overnight culture were centrifuged at top speed (13 000 rpm) for four minutes. The supernatant was discarded and the harvesting step repeated in the same tube. The cell pellet was resuspended in 200 µL of GTE buffer, vortexed and then incubated on ice for five minutes. 400 µL of freshly mixed 0.2 N NaOH/1% SDS were added and the tube inverted three times and incubated on ice. After exactly five minutes 300 µL cold 3 M NaOAc were added and the tube "barshaked" three times. The tubes were chilled on ice for ten minutes, and then centrifuged for ten minutes at 5°C with 13 000 rpm. 800 µL supernatant were recovered into a fresh 2 mL tube. If there was some contamination by precipitate the centrifugation step was repeated. 600 µL of isopropanol were added and mixed by inverting five times. The samples were then centrifuged at 5°C and 13 000 rpm for 15 minutes and the supernatant was discarded. The DNA pellet was washed with 500 µL of 70% ethanol by pipetting up and down until the pellet was removed from the tube wall and centrifugation was repeated. The pellet was dried by lyophilisation for about four minutes at room temperature. To dissolve the DNA 40 µL double distilled water were added and the tubes shaken for at least 20 minutes. 2 µL of RNase A (DNAse free) were added to remove contaminating RNA.

### 4.1.7) Sequencing

To finally prove the insert identity and correctness the sample was sequenced before yeast transformation. Therefore 1.2 µg of plasmid were mixed with 30 pmol either forward or reverse primer and filled up to 15 µL with water. Primer pGAD F and R for pGAD transformants and pGTB9 F and R for pGBK transformants were used. Sequencing was performed by Microsynth AG.

### 4.1.8) Glycerine-stock cultures of bacteria and yeast transformants

For long time storage a single bacterial or yeast colony was inoculated in 5 mL liquid medium and incubated over night, bacteria at 37°C and 180 rpm, yeast at 29°C and 200 rpm. 410 µL of ≥ 96% glycerine were mixed with 1 mL of overnight culture by inverting several times, frozen in liquid nitrogen and stored at -80°C.

## 4.2) Yeast two hybrid assays

### 4.2.1) Yeast transformation

PJ69-4a is a yeast strain constructed for yeast two hybrid selections, only $His^+$, $Ade^+$ and β-galactosidase expressing in the presence of functional GAL4 (James, Halladay and Craig 1996). To test autoactivation fusion gene carrying vectors were combined with an empty vector. As samples ARI5 and ARI7 were combined with all available ARIs (1, 2, 8, 12 and its fragments RING1, RING1-IBR, IBR-RING2, RING2-C and C, 15), translation initiation factors (eIF4E, eIF(iso)4E, nCBP and its deletion mutants N-tail, β-sheet, helix and C-tail) and ubiquitin (UBI). COP1 and UVR8 were combined with themselves, and all available ARIs.

PJ69-4a stock culture was streaked on a YPD-plate and incubated at 29°C for three days. For the transformation one colony from the plate was transferred into 25 mL YPD containing 2% glucose and incubated at 29°C and 180 rpm over night. When the culture reached an $OD_{600}$ over one, after 16 to 19 hours, it was diluted in 100 mL YPD containing 2% glucose to an $OD_{600}$ of 0.2 to 0.3 and incubated at 29°C and 180 rpm until the cells had divided at least twice, what took about three to four hours ($OD_{600}$ = 0.4-0.6). The cell suspension was split into two falcon tubes and centrifuged at 5000 rpm and 5°C for ten minutes. Each pellet was washed with 30 mL of prechilled water. The centrifugation step was repeated and the supernatant discarded. Both pellets were resuspended in 500 µL fresh LiAc-solution, and chilled on ice. Plasmid DNA was prepared by mixing both plasmids to

a final amount of up to 1 µg with 100 µg Carrier DNA (denatured by boiling 20 minutes and afterwards immediately cooled on ice). 100 µL competent yeast cells were added to each tube and mixed thoroughly. 600 µL fresh LiAc/PEG were added per reaction, mixed carefully by pipetting up and down several times and incubated at 29°C for 30 minutes with 180 rpm shaking. Then 70 µL of DMSO were also mixed to the suspensions by carefully pipetting up and down. The samples were incubated for 20 minutes at 42°C on the heating block and mixed by snipping the tubes every two to three minutes. Then they were chilled on ice for 20 minutes and centrifuged two minutes with 4000 rpm. The supernatant was removed carefully and the pellet resuspended in 80 µL 1x TE. The whole volume was streaked on a SD -leucine/-tryptophan (SD -L/-T) plate and incubated at 29°C. Colonies usually emerged after two to four days.

### 4.2.2) Spotting assay

Transformants were transferred onto plates additionally lacking histidine (SD -L/-T/-H) and containing 1 mM 3-aminotriazole (SD -L/-T/-H +At), a competitive inhibitor of histidine, where only interaction positives can grow.

Of each transformant one colony was inoculated in 5 mL SD -L/-T medium and incubated over night at 29°C and 200 rpm. As positive control AD-ARI12/BD-nCBP and as negative control AD-empty/BD-empty were included. As far as possible all following steps were performed in a laminar flow hood. The overnight cultures were vortexed vigorously and 200 µL were mixed with 800 µL double distilled water to measure $OD_{600}$. The volume, to proceed with, was calculated by following formula: Volume = 0.25 x 1 mL / $OD_{600}$. This volume was transferred into a fresh tube and centrifuged five minutes with 4000 rpm. The pellet was resuspended in 1 mL double distilled water. 1:10 and 1:100 dilutions were prepared by mixing 100 µL initial suspension or 1:10 dilution with 900 µL double distilled water. Of each suspension 10 µL were dropped on a SD -L/-T, SD -L/-T/-H and SD -L/-T/-H +At plate respectively and incubated at 29°C for two to four days.

To test UV-B dependent interactions plates were incubated lid down in a growth chamber on a 32°C heating block with 3 µmol $m^{-2}$ $s^{-2}$ UV-B with foil (spectral energy irradiance weighted after Flint and Caldwell 2003 was 0.13 W/m² under medium).

### 4.2.3) β-galactosidase-assay (GardnerLab)

In two hybrid transformants the reporter gene β-galactosidase is only expressed when the GAL4 transcription factor is successfully assembled by protein-protein interaction. The activity of β-galactosidase can be assessed by its ability to hydrolyze 2-nitrophenyle-β-D-galactopyranoside into galactose and o-nitrophenol causing a yellow staining, which can be measured photometrically.

A bit of yeast colony was streaked on a new SD -L/-T plate and incubated at 29°C over night (about 20 hours). From each transformant three colonies were tested. The next day 1 mL z-buffer with 2-mercaptoethanol was prepared in a tube for each sample and put into the fridge until the assay was started. A bit of yeast culture was scraped off and resuspend vigorously on a shaker for two minutes, in prepared z-buffer and afterwards chilled on ice. The whole mililiter was used to measure $OD_{600}$ in a fresh cuvette. If it was over one the sample was diluted with z-buffer, if it was below 0.5 more yeast was added. 800 µL of the cell suspension were transferred back into the tube, the rest was discarded. In the fume hood one drop of 0.1% SDS and two drops of chloroform were added with a pasteur pipette. Chloroform is easier to pipette if it is sucked up and down several times before pipetting. The samples were vortexed vigorously for 15 seconds and equilibrated to 30°C for 15 minutes on a heating block. The enzymatic reaction was started by adding 160 µL of ONPG-solution and vortexing for ten seconds. The samples were incubated at 30°C for two hours. To stop the reactions 400 µL of 1 M sodium carbonate were added and the tubes were inverted five times. To pellet cell debris before measuring $OD_{420}$ the samples were centrifuged at full speed for ten minutes. Beta-galactosidase activity was calculated as follows:

$$\text{Miller Unit} = (1000*OD_{420})/(OD_{600}*V*t)$$

t….time in minutes -> 120

V… volume of cell suspension in mL -> 0.8

**UV experiment**

To test UV-dependent interactions, transformants were streaked on fresh SD -L/-T plates and incubated for 20 hours lid down in a growth chamber on a 32°C heating block with 3 µmol $m^{-2}$ $s^{-2}$ UV-B (spectral energy irradiance weighted after (Flint and Caldwell 2003) was 0.13 W/m² under medium). The assay was performed as described above.

### 4.2.4) Expression analysis

SDS-PAGE and Western blotting were performed to confirm successful expression of fusion proteins in yeast transformants. In the process HA- and c-Myc tag were used for immunodetection with corresponding antibodies.

**Harvesting of cells**

Of each plasmid combination of interest one yeast transformant was cultivated over night in 5 mL SD -L/-T at 29°C and 180-200 rpm until reaching an $OD_{600}$ over 0.5. Cells were harvested by centrifuging two times 2 mL in an appropriate tube at 13 000 rpm for three to four minutes. The supernatant was poured off, the cells frozen in liquid nitrogen and stored at -80° until use.

For the *E.coli* sample DH10b cells with empty pGBK vector were grown in 5 mL YT-medium containing 50 mg/mL kanamycin over night at 180 rpm and 37°C. To harvest cells 1, 0.5 and 0.1 mL were

centrifuged respectively for four minutes at 13 000 rpm. The supernatant was discarded, the cells frozen in liquid nitrogen and stored at -80°C.

**SDS-PAGE**

A 12.5% gel was chosen as expected band sizes were between 20 and 90 kDa.

First the separation gel was prepared:

| | |
|---|---|
| double distilled water | 3.75 mL |
| 40% AA | 2.82 mL |
| 1,5 M Tris/HCl pH 8.8 | 2.25 mL |
| 10% SDS | 90 µL |
| APS | 90 µL |
| TEMED | 5.4 µL |

APS and TEMED were added just before pouring the gel. During polymerization the gel was overlaid with isopropanol. After 15 minutes the stacking gel was poured on top of the separation gel.

| | |
|---|---|
| double distilled water | 1.85 mL |
| 40% AA | 325 µL |
| 1 M Tris/HCl pH 6.8 | 313 µL |
| 10% SDS | 25 µL |
| APS | 25 µL |
| TEMED | 2.5 µL |

The comb was inserted immediately and the gel was stored over night in the cold room wrapped in film. Before starting the SDS-PAGE the frozen cells were washed with 500 µL double distilled water,

vortexed vigorously and centrifuged at 13 000 rpm at 5°C for four minutes. The supernatant was discarded and yeast cells resuspended in 100 µL, *E.coli* in 80 µL 1x SDS PAGE sample buffer. The suspensions were incubated for five minutes at 95°C on a heating block and then centrifuged at 5°C and 13 000 rpm for five minutes. The supernatant was transferred into a new tube and used for quantification.

Quantification - Qubit Protein Assay Kit

The working solution was prepared by diluting Qubit reagent 1:200 in Qubit protein buffer. For the samples 199 µL of working solution were mixed with 1 µL undiluted or 1:5 diluted protein extract, for the standards 190 µL working solution were mixed with 10 µL Qubit standard solution. The tubes were vortexed briefly and incubated for 15 minutes at room temperature. First a calibration curve was created with the standards, then the samples were measured and their protein concentrations calculated:

Sample concentration [µg/mL] = QF value [µg/mL] x 200 x dilution factor

Of each sample 30 µg of protein were mixed with 5 µL of 4x SDS-PAGE loading dye and incubated for three minutes at 95°C in the PCR machine. The gel was inserted into the running chamber filled with running buffer and the whole volume of each sample, as well as 3 µL of protein marker were loaded into the slots. The proteins were separated for about 1.5 hours at 25 mA per gel.

**Western Blotting**

Sponges and filter paper pieces were placed in transfer buffer for at least five minutes. The membrane was activated in methanol for one minute and then also put into the transfer buffer. A sandwich of sponge - filter paper - gel - membrane - filter paper - sponge was fixated with a clip and placed in the tank filled with transfer buffer with the membrane towards the anode. The blot was run at constant 20-25 V for 16 hours at 5°C.

**Immunodetection**

To confirm total transfer of proteins from the gel it was shaken in coomassie blue for three hours. The membrane was incubated in Ponceau Solution for five minutes to dye transferred proteins rose. Afterwards the membrane was blocked with 50 mL 5% milk in TBS-T for one hour on the shaker. After washing two times with TBS-T for one minute the membrane was incubated in 1.5 mL primary antibody, either rat anti-HA or rabbit anti-c-Myc A14, each diluted 1:1000 in TBS-T for one hour on the shaker. Washing was performed once for 15 minutes and two times for ten minutes in TBS-T. Then the membrane was incubated in 1.5 mL secondary antibody, either 1:10000 - 1:15000 diluted HRP goat anti-rat or 1:7500 diluted HRP goat anti-rabbit, for one hour on the shaker. 1:10 dilutions of secondary antibodies in TBS-T were stored at 4°C for about one week. The washing steps were performed as before. The membrane was sprayed three times with Roti lumin plus 1 and 2 solutions and wrapped immediately in plastic foil. The membrane was exposed to the x-ray film for 30 seconds to 30 minutes.

**Stripping**

The membrane was covered in Roti-Free and shaken in an incubator at 56°C for 30 minutes. It was washed twice in TBS-T for 20 minutes and blocked with 50 mL 5% milk in TBS-T at 4°C over night.

# 5) Results

## 5.1) SLiCE cloning

By SLiCE cloning *ARI5* could be inserted into both shuttle vectors, *ARI7* only into pGADT7.

SLiCE is a relatively new developed *in vitro* cloning method which enables cloning without the necessity of appropriate restriction sites or the usage of ligases. It is based on the ability of bacterial extracts to recombine DNA fragments which are flanked by at least 15 bp long homologous sequences. The extract can be obtained from RecA deficient strains, like DH10b. For my experiments an especially for SLiCE modified DH10b strain, additionally carrying an optimized λ prophage Red recombination system, called PPY, was used. The mechanisms of the SLiCE mediated recombination are not known but as DH10b itself is $RecA^-$ it has to occur through a RecA independent pathway. Because the Red recombination system is not essential but increases cloning efficiency it is believed that during SLiCE cloning similar mechanisms take place involving a single stand DNA generating exonuclease, the stabilization of these single strand overhangs and the mediation of annealing of complementary DNA strands (Kuzminov 1999). The high fidelity of this method enables the recombination of several DNA fragments one after the other or the insertion of multiple fragments carrying identical homologies into one vector in parallel. One very special feature of SLiCE is the ability to eliminate flanking heterologous sequences up to over hundred base pairs.
For practical implementation the vectors had to be linearized by restriction digestion and were blunted as it is recommended by the authors, but not required. The genes to be inserted were amplified by RT-PCR with primers carrying the 15 bp long 5' overhangs homologous to the chosen recombination sites in the vector sequence up- and downstream of the restriction site. Several flanking heterologous base pairs were left to obtain in frame cloning (Zhang, Werling and Edelmann 2012). Below more details of the pGAD-ARI5 cloning are shown in Figure 5. It was similarly performed for the other vector-insert combinations. Whole sequences of cloned plasmids can be found in the appendix.

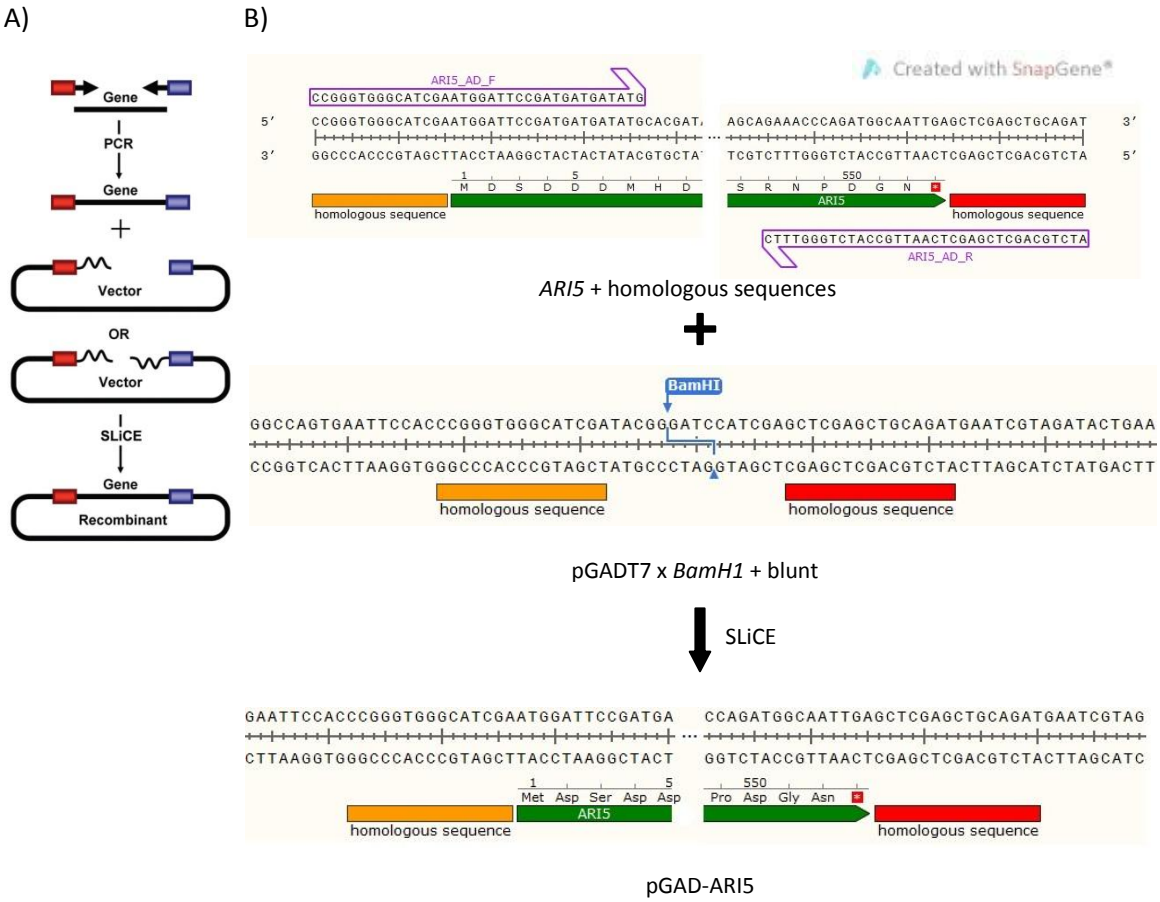


**Figure 5** A) Schematic illustration of SLiCE with simultaneous elimination of flanking heterologous sequences (Zhang, Werling and Edelmann 2012). B) SLiCE cloning of *ARI5* into pGAD: at the top the generation of the homologous 5' overhang at *ARI5* by PCR and therefore used primers ARI5_AD_F and R, in the middle pGAD insertion site with *BamH1* restriction site and flanking heterologous sequence, at the bottom *ARI5* inserted into pGAD between homologous sequences.

After transformation into the *E.coli* DH10b strain, colonies were counted and screened the next day.

**Table 2** Summary of SLiCE cloning results

| Transformant | number of transformations | total amount of utilized empty vector [ng] | obtained colonies | positive colonies |
|---|---|---|---|---|
| pGAD-ARI5 | 1 | 25 | 7 | 1 |
| pGBK-ARI5 | 7 | 190 | 110 | 1 |
| pGAD-ARI7 | 4 | 66 | 13 | 1 |
| pGBK-ARI7 | 11 | 260 | 77 | none |

As for pGBK-ARI7 no positive transformant was found other cloning methods were used.

## 5.2) Cloning of *COP1* into pGBK from pGAD

By sequencing pGAD-COP1 in the forward direction it was found out that *EcoR1* was used for cloning *COP1* into pGAD at the 5' end. The 3' end was not further investigated by sequencing. Test restriction digestions with *EcoR1* alone showed that pGAD-COP1 was just linearized, indicating that a different restriction site was used at the 3' end. *COP1* could be cut out with a combination of *EcoR1* and *Xho1* and was subsequently inserted at the compatible *EcoR1* and *Sal1* sites of pGBK.

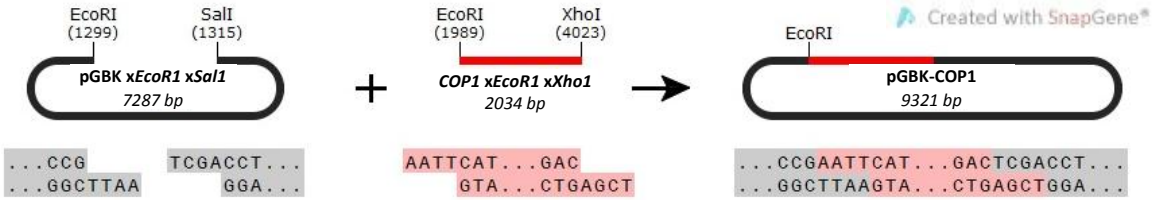


**Figure 6** Cloning of *COP1* into pGBK by cutting out of pGAD and inserting at appropriate restriction sites.

One transformation with 25 ng of linearized vector and vector to insert ratio of 1:1.5 resulted in formation of 1440 colonies (efficiency $1.2*10^5$ cfu*$mL^{-1}\mu g^{-1}$). From ten screened colonies six were positive.

## 5.3) Cloning of pGBK-ARI7

As SLiCE did not work for cloning *ARI7* into pGBK other approaches were chosen. First TOPO TA cloning was tried. Therefore inserts with *Nco1* restriction sites and A overhang were inserted into pCR™4-TOPO® vectors.

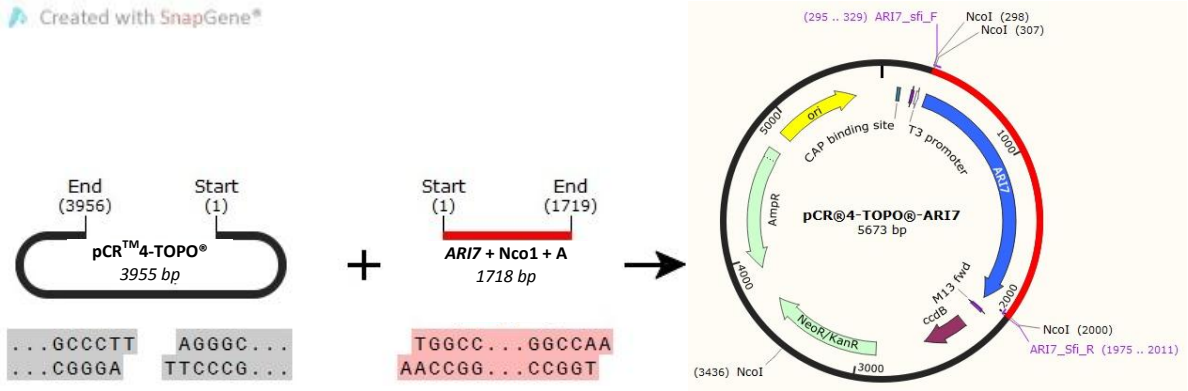


**Figure 7** Cloning of *ARI7* into pCR™4-TOPO® via A/T overhangs. *Nco1* restriction sites are highlighted in the recombinant product

The transformation with 10 ng of linearized vector and a vector to insert ratio of 1:4.7 resulted in the formation of 20 colonies, of which 16 were positive according to colony PCR. The sequence of one colony was checked with M13 F/R primers and one change from asparagine to tyrosine in the second to last codon was considered acceptable.

*ARI7* was cut out with *Nco1* and ligated with dephosphorylated pGBK x *Nco1*. Four transformations resulted in the growth of 332 colonies, of those 115 were tested by colony PCR. Four clones with plasmids carrying an insert were found, but test digestions and PCR revealed none to be *ARI7*. Figure 8 shows two test digestions of one of these clones once with *Nco1* to find out the insert size and once with *BamH1* which should have cut out *ARI7* if it had been inserted the wrong way, due to the usage of just one restriction enzyme. To compare sizes directly, *ARI7* and linearized vector controls were loaded on the gel additionally to the markers.

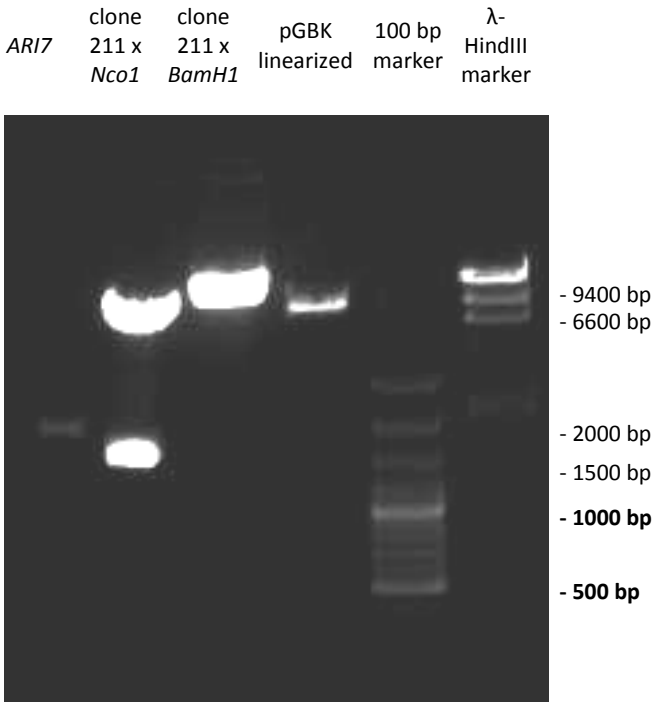


**Figure 8** Test digestions run on a 1.2% agarose gel showed that the plasmid could be linearized with *BamH1*, that indicates that *ARI7* was not inserted in the wrong direction but the insert, cut out with *Nco1*, was smaller than expected.

Test restriction digestions indicated that the insert might be the 1400 bp fragment from the TOPO vector backbone, generated when cutting out *ARI7* from pCR™4-TOPO® and not possible to get completely rid of at the insert purification step.

As *COP1* was successfully cloned from pGAD into pGBK the possibility arose to use the same digestion sites and the already prepared pGBK x *EcoR1* x *Sal1* vector also for *ARI7*.

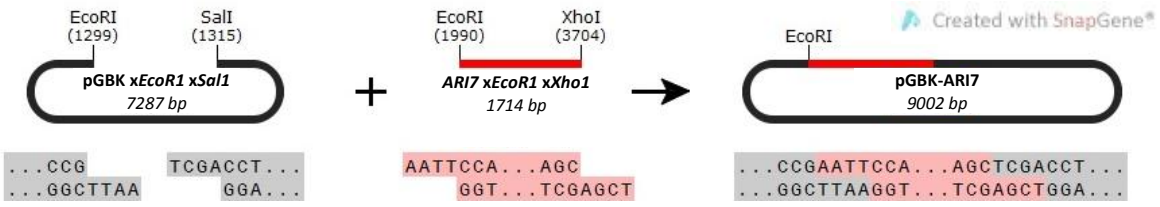


**Figure 9** Schematic illustration of cloning *ARI7* from pGAD into pGBK via compatible restriction sites

Two transformations, with vector to insert ratios of 1:4 and 1:10, resulted in the formation of 1221 colonies, whereof none of the 304 tested by colony PCR contained an insert. By PCR of the ligation preparation with insert unspecific primers pGTB9 F/R and the *ARI7* specific primer combination ARI7_AD F with pGTB9 R the integrity of the ligation process was confirmed.

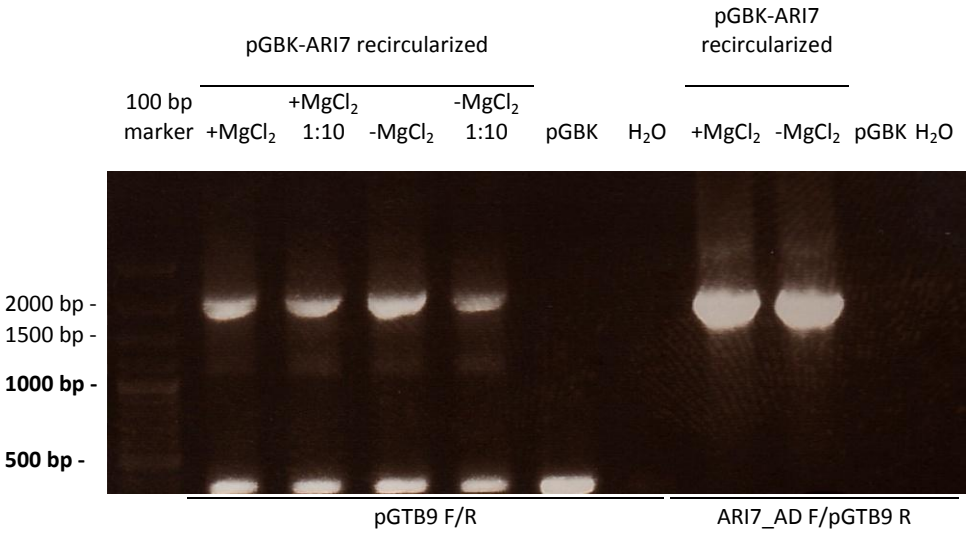


**Figure 10** Success of ligation of pGBK-ARI7 with and without additional $MgCl_2$ was analyzed by PCR (ligation used undiluted or 1:10 diluted) with insert unspecific and *ARI7* specific primer pairs. PCR with pGTB9 F/R shows presence of plasmids with (2000 bp) and without insert (350 bp). PCR with ARI7_AD F primer identifies insert as *ARI7*. PGBK was used as empty vector control and shows the typical 350 bp band with pGTB9 primers but none with *ARI7* specific primers.

PCR confirms successful ligation, independent of additional magnesium, but also shows the presence of empty pGBK vectors. To get purer pGBK-ARI7 vector and thus minimize background colony growing, the successfully ligated vector was amplified by PCR.

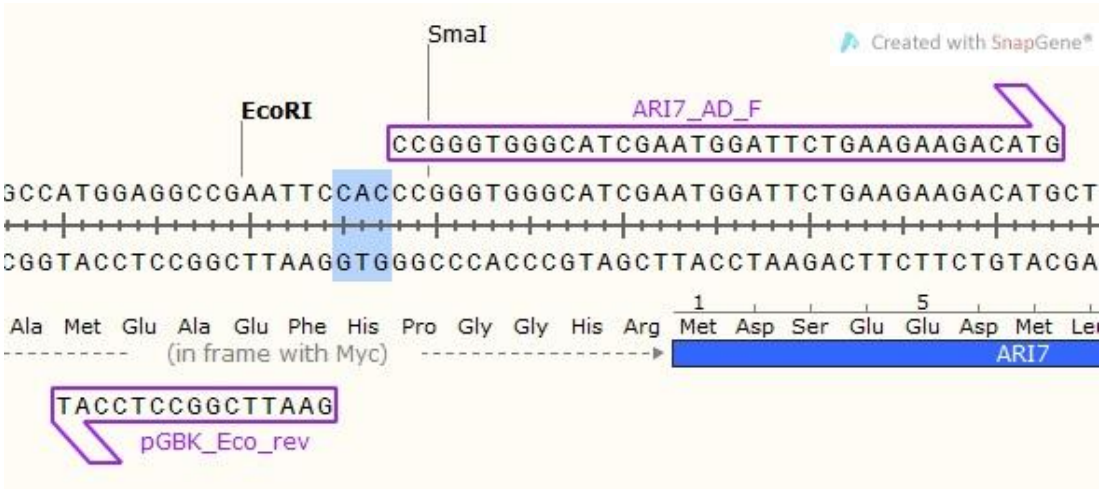


**Figure 11** Illustration of the primer binding sites for amplifying whole pGBK-ARI7 vector. Light blue marked bases are lost after recircularization, but do not result in a frameshift. For test restrictions important sites *EcoR1* and *Sma1*, which is just functional after successful recircularization, are highlighted.

By standard transformation conditions three, by transformation with added glucose and at room temperature 17 colonies grew. Colony PCR revealed diverse patterns of obtained vectors, in the figures below some examples are given.

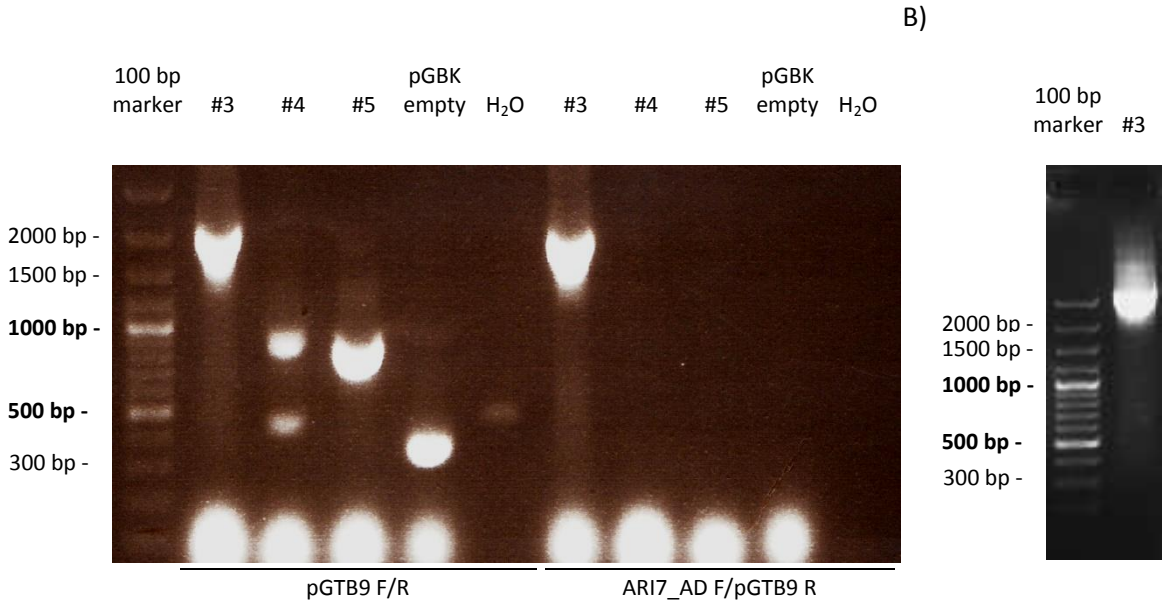


**Figure 12** A) 1.5% agarose gel of colony PCR of clone #3 obtained with standard transformation and clones #4 and #5 with special conditions with insert unspecific and *ARI7* specific primers. Clone three shows 2000 bp bands with both primer pairs indicating presence of pGBK with *ARI7* insert. Clones four and five are negative for *ARI7* but carry a shorter than expected insert. B) Purified plasmid from clone three was reanalyzed by PCR with pGTB9 F/R primers and surprisingly showed a band at 3000 bp on a 1.5% agarose gel.

Clone number four and five carry smaller than expected inserts of about 550 and 450 bp respectively, which do not contain the ARI7_AD F recognition site. Sequencing of purified clone number three revealed an insertion of a transposase into the insert sequence (results of BLAST search shown in appendix). Purified plasmid used for sequencing was subsequently reanalyzed by colony PCR, which showed an increase of insert length of about 1000 bp, indicating that transposase insertion occurred between colony screen and plasmid purification. As clones #15 and 16 showed potential to carry pGBK-ARI7 by colony PCR, plasmids were purified and further tested by restriction digestions.

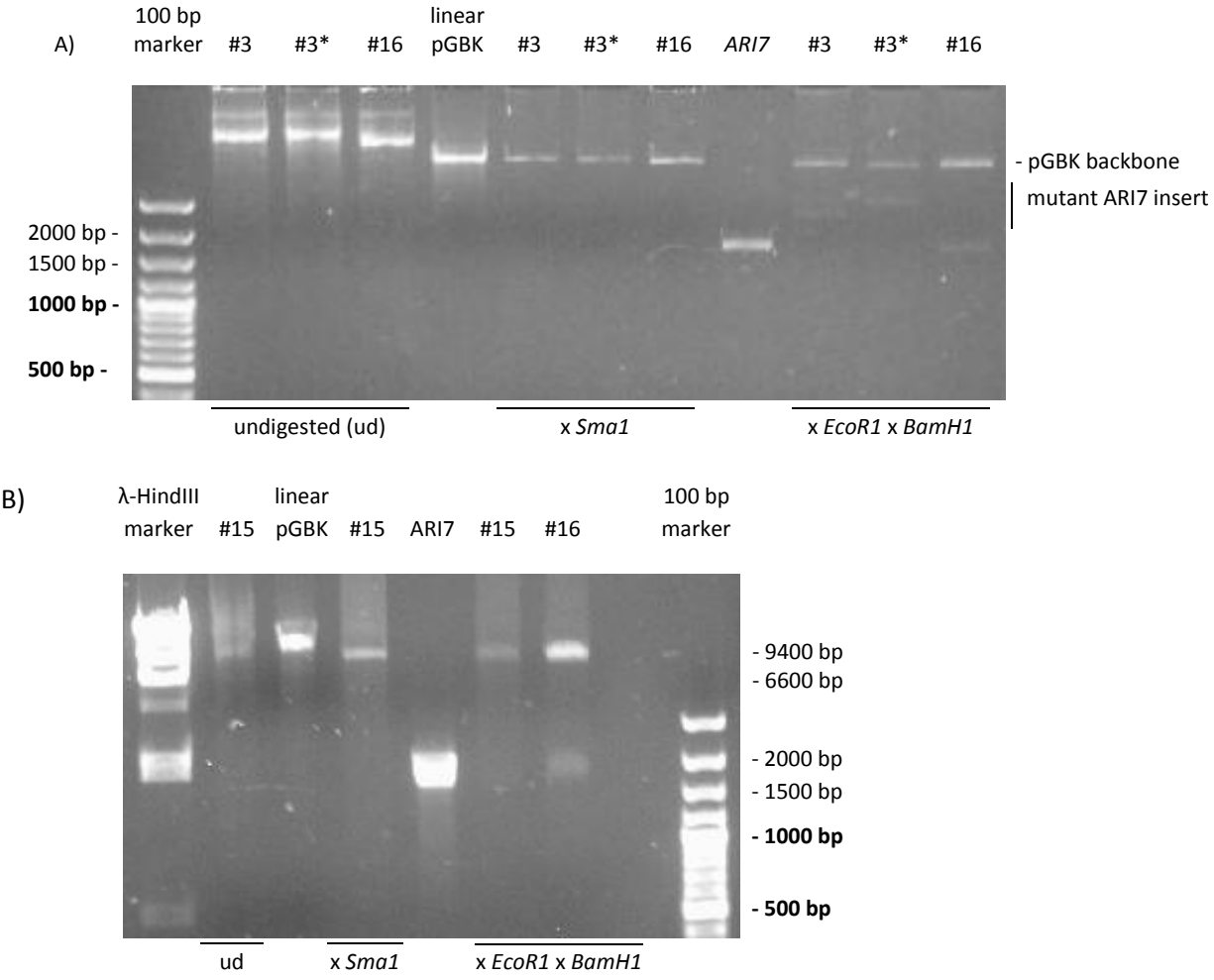


**Figure 13** Test restrictions of clones three, 15 and 16 run on a 1.5% agarose gel show A) and B) successful linearization of all vectors with *Sma1* indicating regained restriction site, destroyed by whole vector PCR. Digestion with *EcoR1* and *BamH1* results in cutting out of insert, which is over the expected size of clone three, not detectable of #15 and of correct size of #16. Clone three was purified from two different cultures grown on plates without (#3*) and with (#3) additional glucose. *ARI7* and linear empty pGBK controls were included on the gels.

Although PCR and restriction digestions suggest both #15 and 16 to carry pGBK-ARI7 not enough plasmid could be generated for successful sequencing, let alone transformation into yeast. Efforts to clone *ARI7* into pGBK were abandoned at this point. As suspicion arose that ARI7 protein was expressed in DH10b cells and thus interfered with colony growing, *E.coli* transformed with empty pGBK vector was included in Western blot analyses.

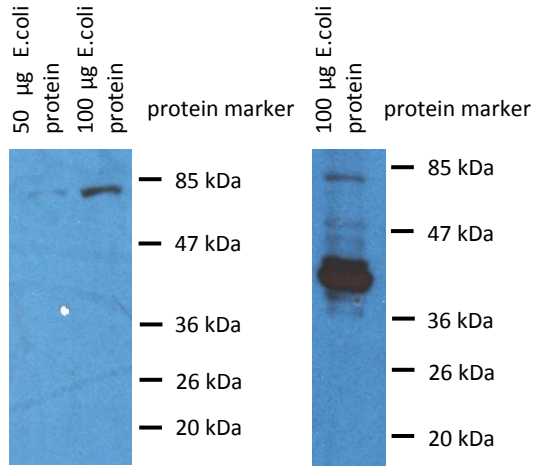


**Figure 14** Western blot of *E.coli* protein preparations of two different harvested volumes from the same liquid culture and immunodetection with 1:1000 rabbit anti-c-Myc antibody and 1:7500 goat anti-rabbit HRP; 20 and 30 min exposure respectively.

Western blot analyses showed a protein expressed in *E.coli* transformed with empty pGBK with a size of about 80 kDa. Accumulation of 40 kDa fragment in the left picture might have resulted from sample refreezing and -thawing, leading to protein degradation. Control of DH10b without plasmid was unfortunately not tested.

**Table 3** Summary of pGBK-ARI7 cloning attempts.

| Method | total amount of utilized empty vector [ng] | total amount of colonies | comment |
|---|---|---|---|
| SLiCE | 260 | 77 | none positive |
| TOPO TA cloning | 10 | 20 | successful; sequencing revealed asparagine to tyrosine mutation in second to last codon |
| TOPO TA cloning and subsequent transfer to pGBK | 100 | 332 | smaller inserts (probably TOPO fragment) |
| restriction/ligation cloning | 50 | 1221 | none positive |
| amplification of whole vector | 43* | 20 | deletion of ca. 650 bp of *ARI7* 5' end and frameshift<br>smaller inserts<br>insertion of transposase<br>low yield |

*whole vector [ng]

## 5.4) Yeast two hybrid results

### 5.4.1) Analyzing autoactivation

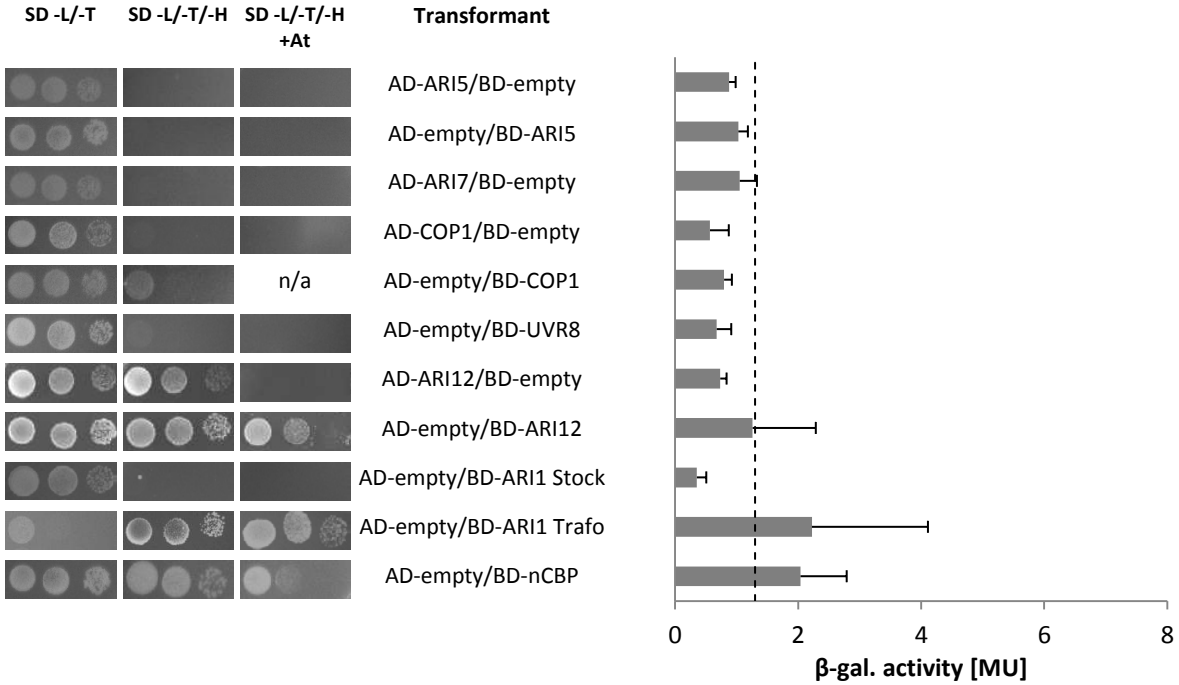


**Figure 15** Spotting and β-galactosidase results for autoactivation experiments. On the left side growth control on SD -L/-T plates and selection for interacting fusion proteins on SD -L/-T/-H and SD -L/-T/-H +At plates with strongest selection pressure with added 3-aminotriazole. On the right side calculated β-galactosidase activity in Miller Units [MU] from three different transformants. The threshold is the highest value reached by AD-empty/BD-empty in diverse experiments.

Autoactivation experiments showed that newly cloned ARI5, ARI7 and COP1 as well as UVR8 are not autoactivating. Controls with ARI12-empty combinations and AD-empty/BD-nCBP were transformed although stock cultures would have been available. As already expected ARI12 and nCBP are autoactivating fused with BD. Astonishingly ARI12 fused to AD showed growing on the SD -L/-T/-H plate although negative according to the β-galactosidase assay. Most surprisingly ARI1 fused to BD showed extremely different results when comparing a stock culture and self-transformed clones. From earlier experiments following fusion proteins were known to be autoactivating: AD-ARI8, AD-nCBP, AD-eIF(iso)4E, BD-ARI2, BD-ARI8, BD-ARI12, and BD-eIF(iso)4E.

### 5.4.2) Analyzing interactions between ARIADNE proteins

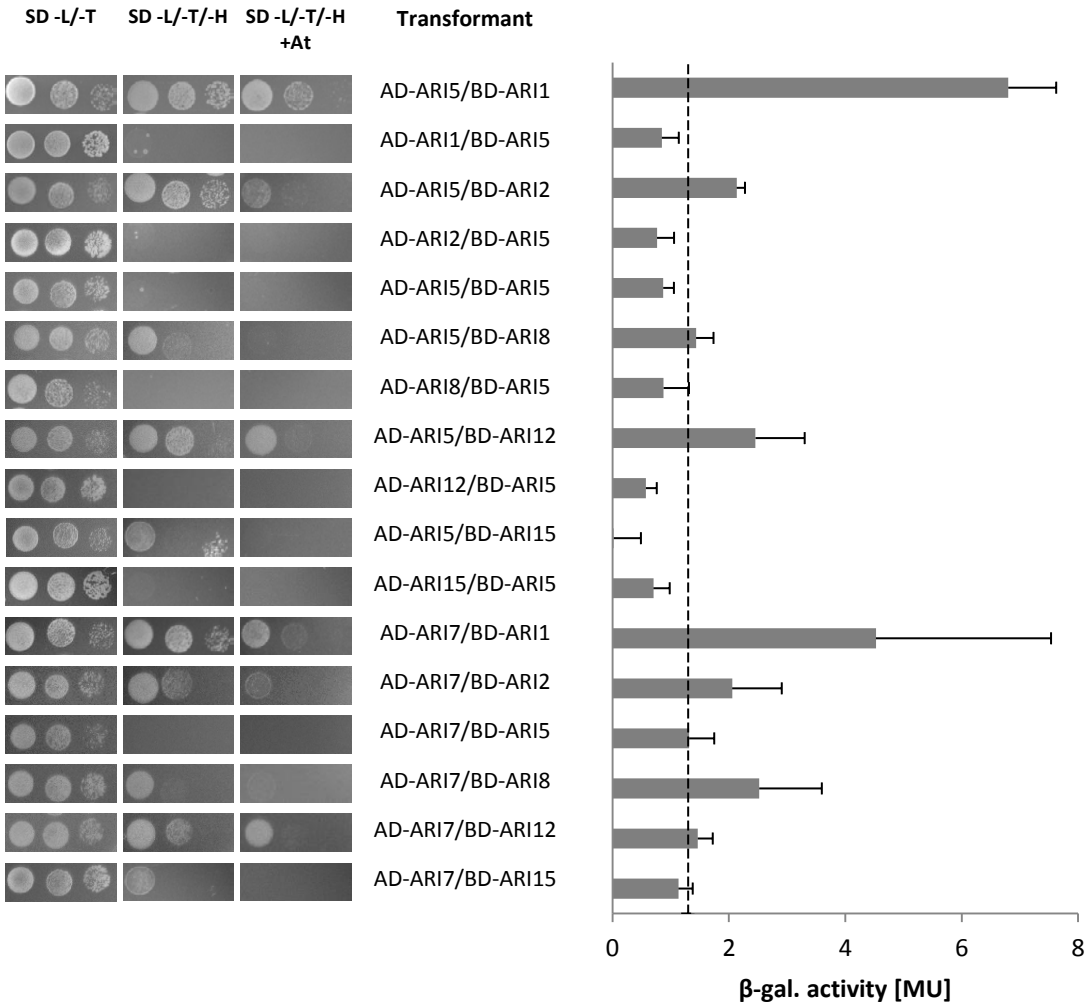


**Figure 16** Spotting and β-galactosidase results for ARI combinations. Arrangement according to **Figure 15**.

ARI5 and ARI7 fused with AD seem to show interaction with ARI1, 2, 8 and 12, but all of those four are autoactivating in BD-position. Nevertheless the β-galactosidase activity of AD-ARI5/BD-ARI1 and AD-ARI7/BD-ARI1 is much higher than in the AD-empty/BD-ARI1 control. None of the combinations show interaction vice versa.

**Table 4** Summary of interactions between ARIADNE proteins

| | **ARI1** | **ARI2** | **ARI5** | **ARI7** | **ARI8** | **ARI12** | **ARI15** |
|---|---|---|---|---|---|---|---|
| **ARI5** | ++ | auto | - | - | auto | auto | - |
| **ARI7** | ++ | auto | - | - | auto | auto | - |

### 5.4.3) Interactions of ARI5 with truncated ARI12

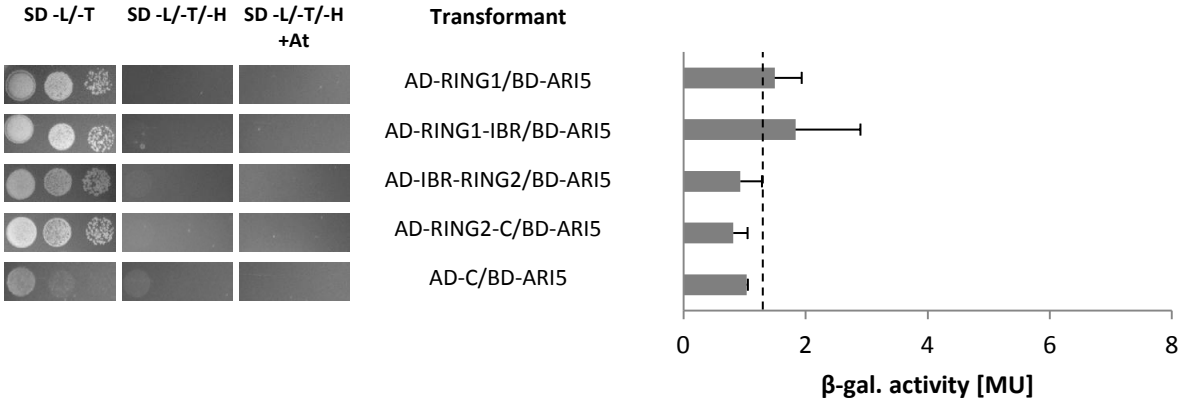


**Figure 17** Spotting and β-galactosidase results for ARI5 combinations with ARI12 fragments. Arrangement according to **Figure 15**.

Although spotting with full length ARI12 already showed no interaction, results for experiments with truncated ARI12 are shown and according to the β-galactosidase assay ARI5 seems to interact weakly with the RING1 domain.

**Table 5** Summary of interactions of ARI5 with ARI12 domains

| | **RING1** | **RING1-IBR** | **IBR-RING2** | **RING2-C** | **C** |
|---|---|---|---|---|---|
| **ARI5** | +/- | +/- | - | - | - |

### 5.4.4) Interactions of ARIADNE 5 and 7 with translation initiation factors and ubiquitin

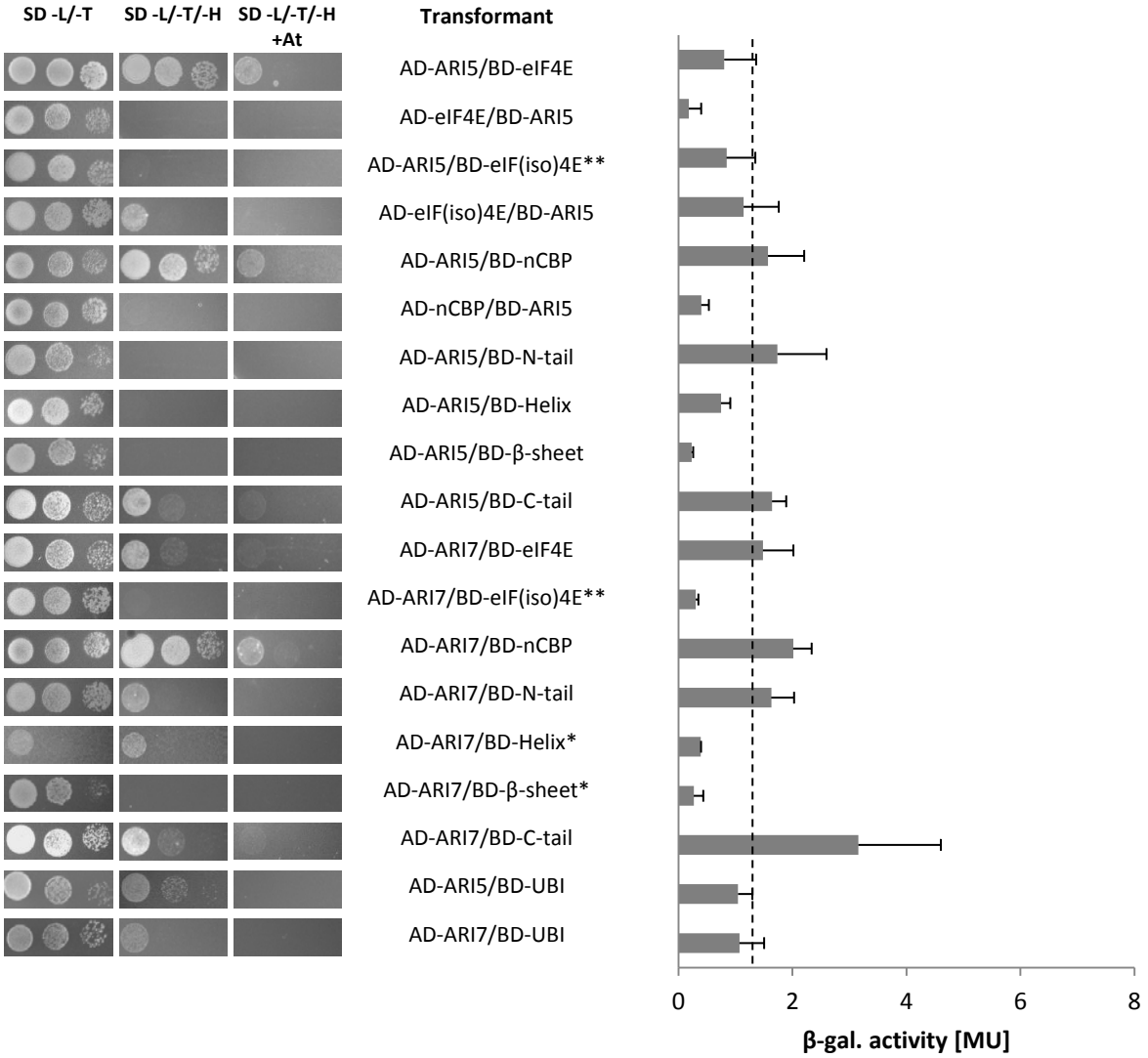


**Figure 18** Spotting and β-galactosidase results of ARI5 and ARI7 with translation initiation factors and ubiquitin. Arrangement according to **Figure 15**.
* Only one transformant was obtained, used three times for β-galactosidase assay.
** Only two transformants were obtained, one used two times for β-galactosidase assay.

BD-nCBP is autoactivating and of its mutants no autoactivation controls were made, so results are unclear. ARI5 and ARI7 both do not interact with eIF(iso)4E, but weakly with eIF4E and UBI (their β-galactosidase activity was clearly over the background in the respective experiment).

**Table 6** Summary of interactions of ARI5 and ARI7 with translation initiation factors and ubiquitin

| | eIF4E | eIF(iso)4E | nCBP | N-tail | Helix | β-sheet | C-tail | UBI |
|---|---|---|---|---|---|---|---|---|
| **ARI5** | + | - | auto | + (?) | - | - | + (?) | + |
| **ARI7** | + | - | auto | + (?) | - | - | ++ (?) | + |

### 5.4.5) Interactions of COP1 and UVR8 with ARIADNE proteins

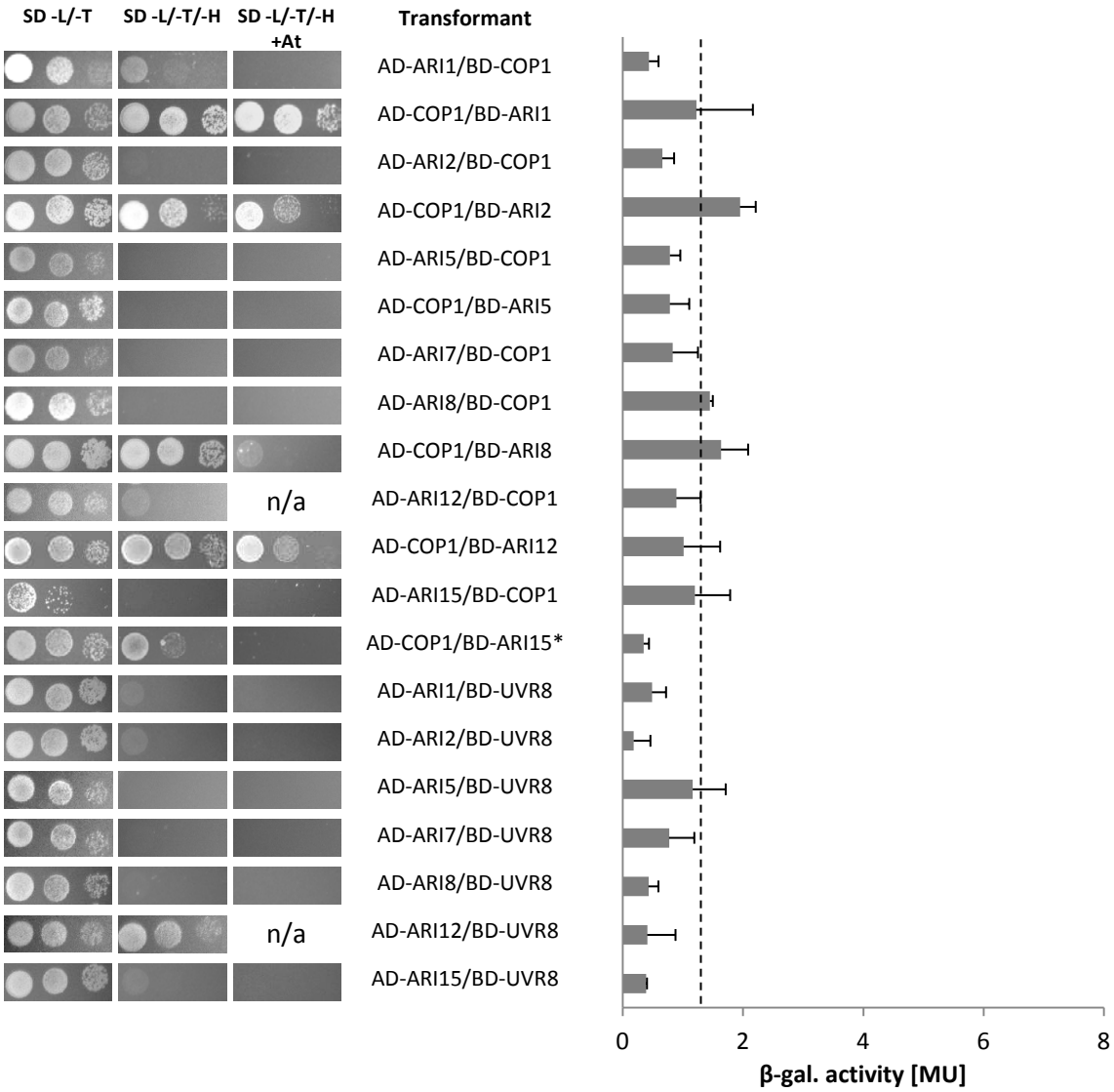


**Figure 19** Spotting and β-galactosidase results of COP1 and UVR8 combinations with ARIs. Arrangement according to **Figure 15**.
* Only one transformant was obtained, used three times for β-galactosidase assay.

COP1 and UVR8 do not interact with any ARI protein. Those seeming to be positive are also autoactivating and their activity is not so much higher than of the according control as for instance AD-ARI5/BD-ARI1.

**Table 7** Summary of interactions of COP1 and UVR8 with members of the ARIADNE family

| | **ARI1** | **ARI2** | **ARI5** | **ARI7** | **ARI8** | **ARI12** | **ARI15** |
|---|---|---|---|---|---|---|---|
| **COP1** | auto | auto | - | - | auto | auto | - |
| **UVR8** | - | - | - | - | - | - | - |

### 5.4.6) Interactions of COP1 and UVR8 dependent on UV radiation

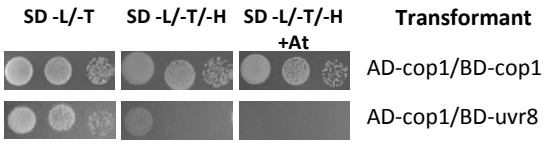


**Figure 20** Spotting results of COP1 with itself and UVR8: On the left side growth control on SD -L/-T plates and selection for interacting fusion proteins on SD -L/-T/-H and SD -L/-T/-H +At plates with strongest selection pressure with added 3-aminotriazole.

Spotting plates under UV did not grow at all.

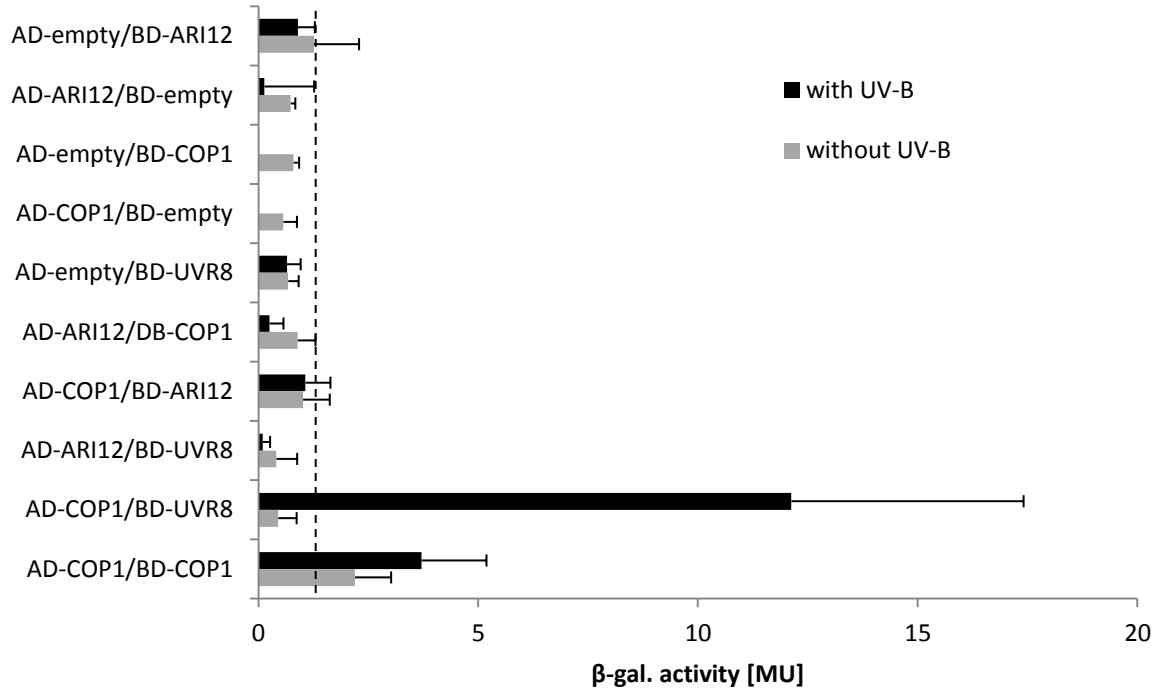


**Figure 21** β-galactosidase activity in Miller Units [MU] for COP1, UVR8 and ARI12 combinations and their respective controls with and without UV calculated from three different transformants. The threshold is the highest value reached by AD-empty/BD-empty. Form AD-ARI12/BD-empty and AD-empty/BD-ARI12 only two transformants grew under UV and could be assayed.

Except AD-empty/BD-ARI12 all controls show no autoactivation. COP1 and UVR8 combinations with ARI12 do interact neither with nor without UV-B radiation. COP1 interacts with itself under both conditions and COP1 does only interact with UVR8 when treated with UV-B.

### 5.4.7) Summary of yeast two hybrid results

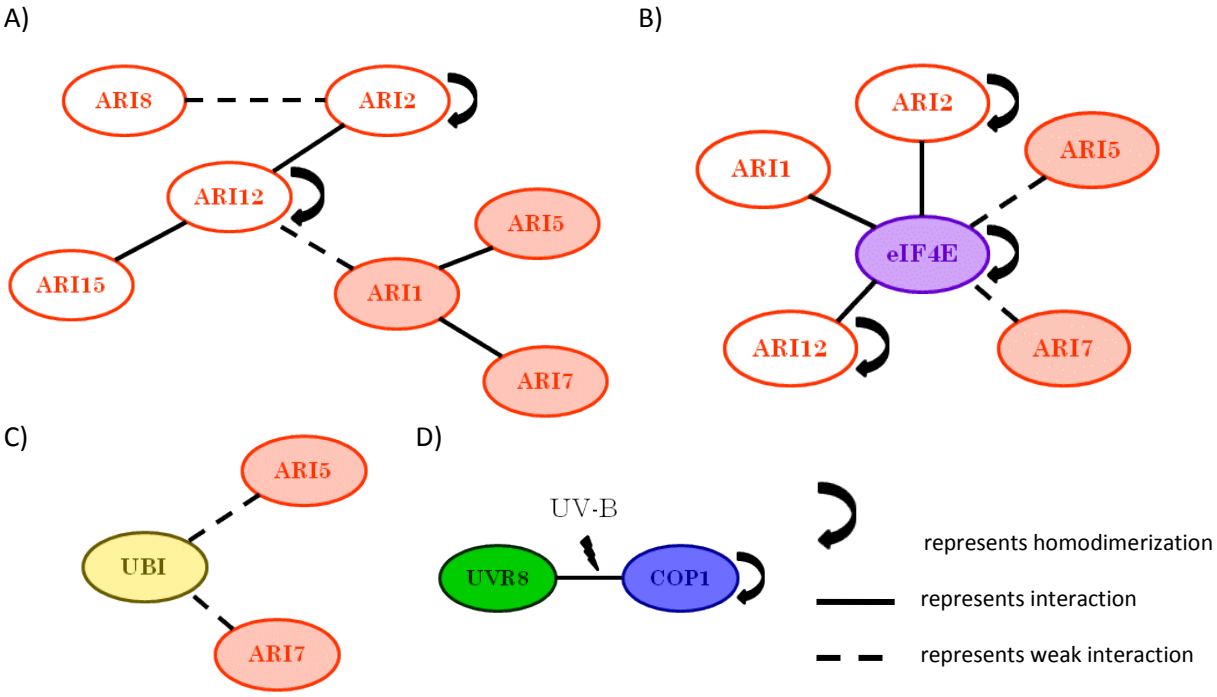


**Figure 22** Illustration of interactions observed by yeast two hybrids assays between A) ARI family members, B) ARI proteins and eIF4E, C) ARI proteins and ubiquitin, D) COP1 and UVR8 depending on UV-B. Data included from earlier research without background colouring.

## 5.5) Results of expression analysis

The fusion protein expression of transformants showing interaction or possible interaction at spotting and β-galactosidase assay was analysed by Western blotting. Immunodetection was first performed with anti-HA antibodies before membranes were stripped and reprobed with anti-c-Myc antibodies. Of each sample 30 µg whole protein extract were loaded. SDS-PAGE and Western blot was repeated for samples not showing any signal, the second time with 100 µg.

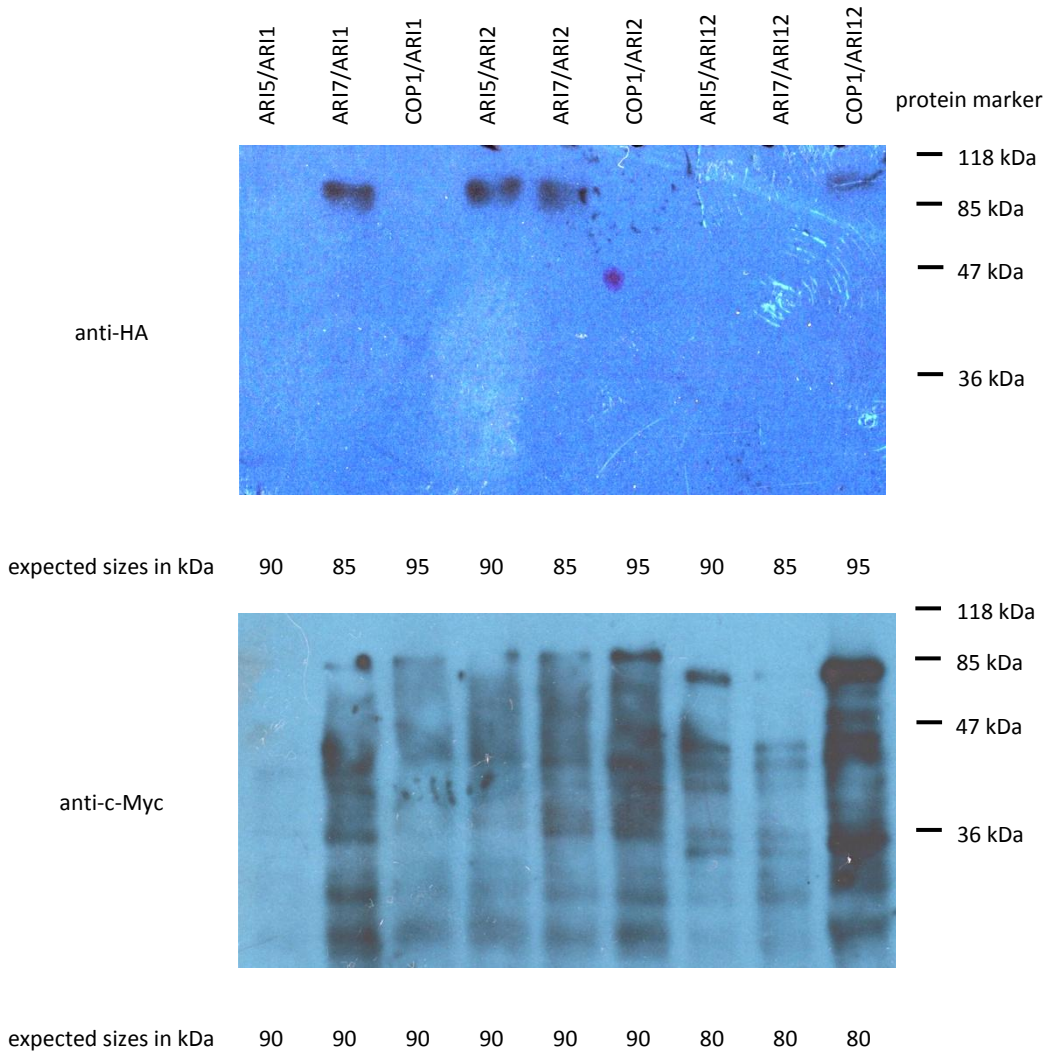


**Figure 23** Western blot 1: At the top 1:1000 rat anti-HA and 1:15000 goat anti-rat HRP; five minutes exposure. At the bottom 1:1000 rabbit anti-c-Myc and 1:10000 goat anti-rabbit HRP resulted in weak signals and was repeated after one washing step with 1:1000 rabbit anti-c-Myc and 1:5000 goat anti-rabbit HRP; exposure 30 seconds.

Those samples giving a signal do correspond to their expected size. Because the immunodetection with anti-c-Myc antibodies was repeated, the background is very high. However highest bands correspond to the expected sizes.

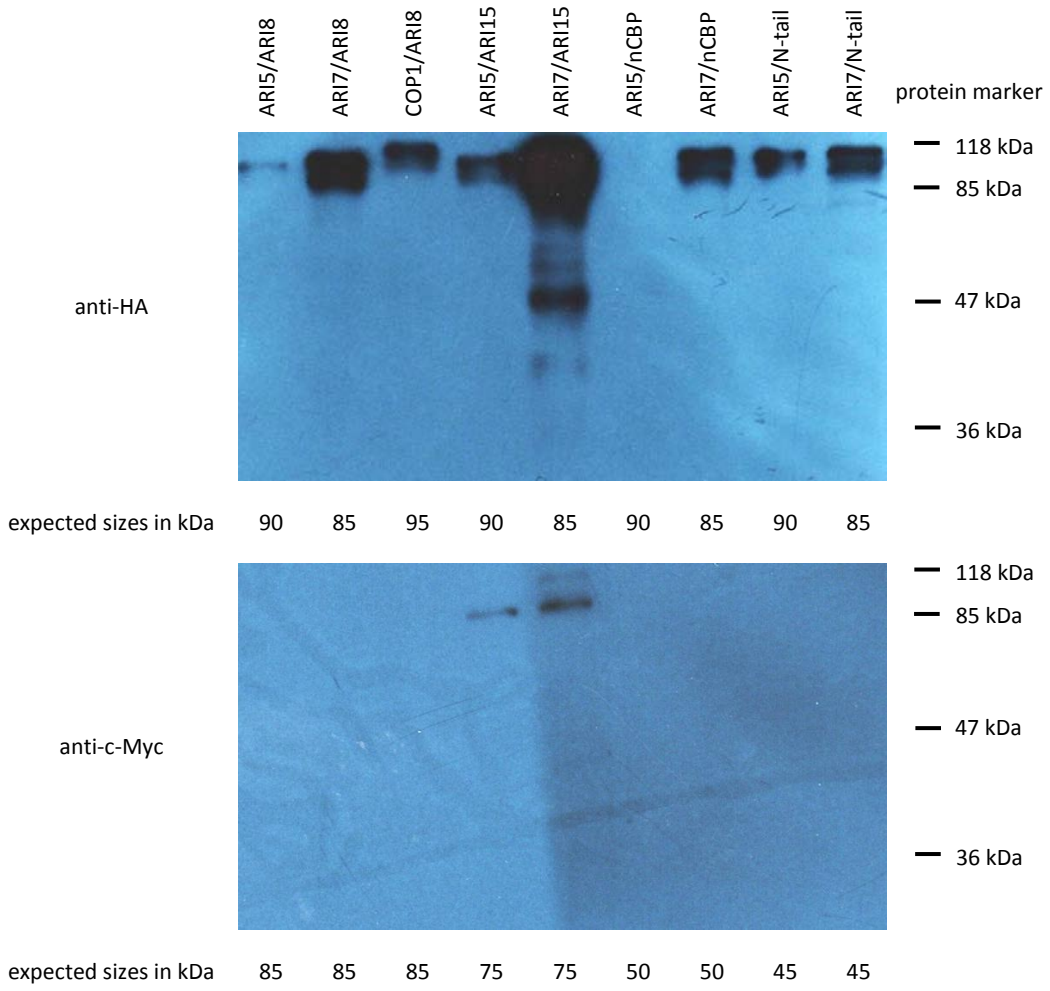


**Figure 24** Western blot 2: At the top 1:1000 rat anti-HA and 1:10000 goat anti-rat HRP; 30 seconds exposure. At the bottom 1:1000 rabbit anti-C-Myc and 1:7500 goat anti-rabbit HRP; exposure 20 min.

Some anti-HA samples do not show exactly the expected sizes in relation to the marker. However comparison between the different protein sizes corresponds to their size differences. Additionally for ARI5 and ARI7 formation of a double band seems to be characteristic. The ARI15 bands detected with anti-c-Myc are higher than expected but on the other hand both have the same size.

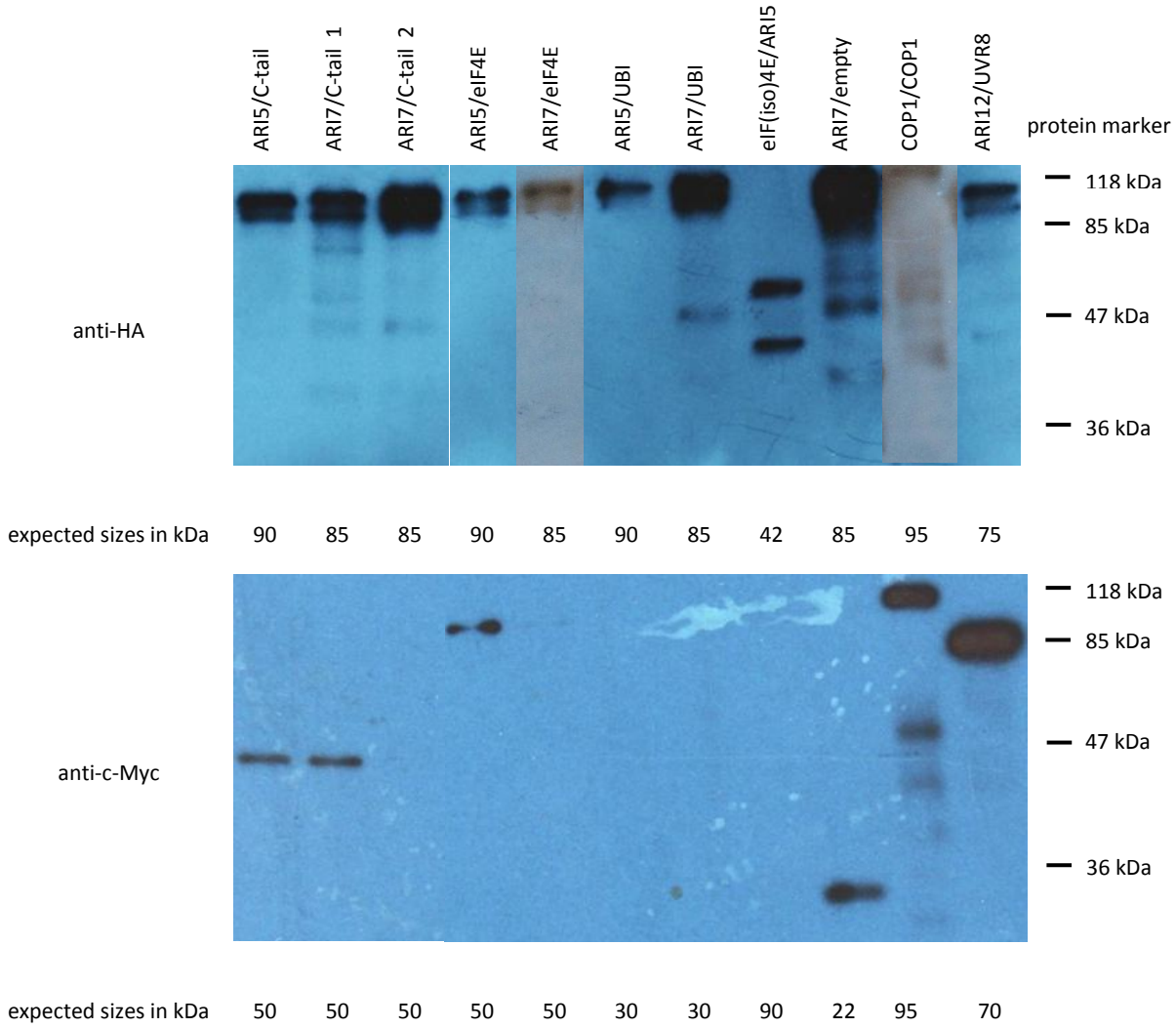


**Figure 25** Western blot 3: At the top 1:1000 rat anti-HA and 1:10000 goat anti-rat HRP; 30 seconds exposure and five minutes for the two extra inserted lanes (grey). At the bottom 1:1000 rabbit anti-C-Myc and 1:7500 goat anti-rabbit HRP; exposure 20 min.

Most of the AD samples show bands of expected sizes. Noticeable are the second band at about 60 kDa of AD-eIF(iso)4E and the higher than expected band of AD-ARI12. Of the analyzed BD-fusions eIF4E has about twice its anticipated size, while the GAL4 BD-domain alone should be just 20 kDa but is about 30 kDa, indicating that the size marker is shifted.

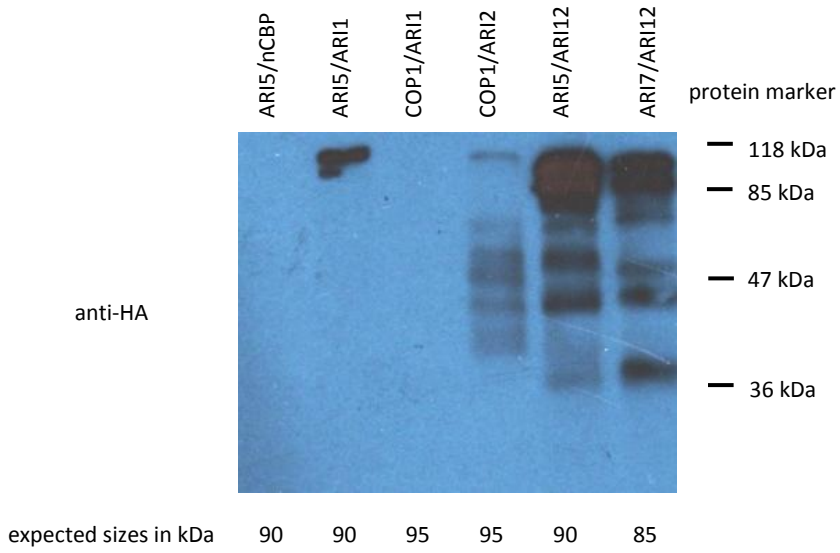


**Figure 26** Western blot 4: 1:1000 rat anti-HA and 1:10000 goat anti-rat HRP; 30 seconds exposure

The AD-fusion protein of AD-ARI5/BD-nCBP and AD-COP1/BD-ARI1 could be displayed extremely brightly on a film after 30 minutes of exposure, not shown here. Samples show expected sizes but also lower bands, probably resulting from re-thawing of protein extracts and hence most probable protein degradation.

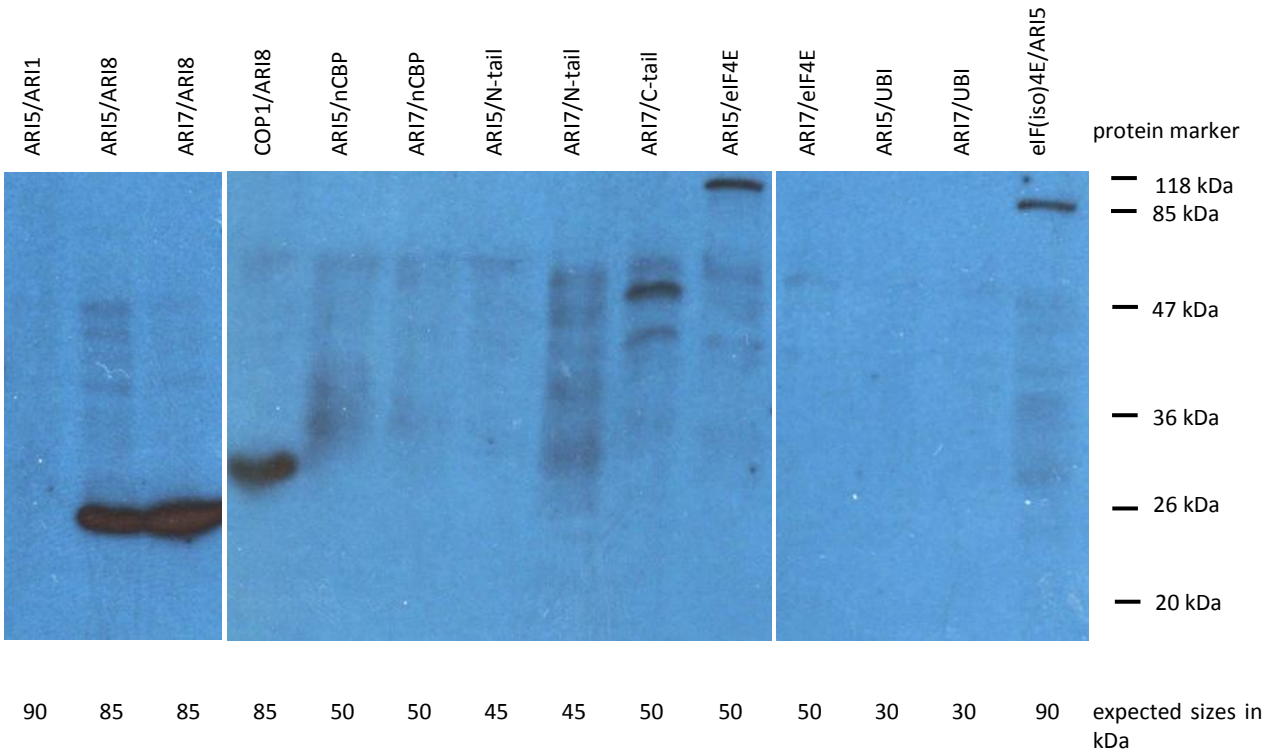


**Figure 27** Western blot 5: 1:1000 rabbit anti-c-Myc and 1:7500 goat anti-rabbit HRP; exposure 30 min.

Most BD-fusions from this Western blot show bad quality and only smears, most probably because of re-thawing of protein extracts. The smear of BD-nCBP starts at the expected 50 kDa. BD-ARI8 shows bands similar to a size expected from the BD vector without fusion. The difference in size on the two gels might be caused by different inner structure of the gels. BD-eIF4E shows bright bands at expected size for both samples but AD-ARI5/BD-eIF4e additionally a band about twice the size. UBI is not to be seen at all, but BD-AIR5 displays the anticipated band.

Only the expression of both BD-UBI fusion proteins and the BD-ARI1 protein combined with AD-ARI5 could not be shown by Western blot. All other proteins were displayed, although some with divergence from expected sizes.

# 6) Discussion

## 6.1) SLiCE

Generally the transformation efficiency with SLiCE was not very high, but as only one correct transformant was needed for subsequent experiments it was nevertheless sufficient. It has to be considered, that very stringent conditions were chosen. With an overhang of just 15 bp the lowest recommended was used, best results achieved by were with 52 bp overhangs. First transformations were always tried with 50 ng of vector and a vector to insert ratio of about 1:5. These parameters were changed to up to 120 ng of vector and a ratio of 1:10. Zhang et al. (2012) described to use up to 200 ng of vector. Another fact to be considered is the big size of the insert and of the whole construct which might have an effect on the transformation efficiency. As a chemical transformation was chosen, efficiency had to be expected to be even lower, as electroporation usually leads to more transformants (Zhang, Werling and Edelmann 2012). Also the amount of SLiCE reaction to be used in the transformation could have been optimized. Only 1, 2 or 5 µL were used and results showed no advantage of one of the volumes, or it was not enough data collected to recognize a pattern. But the SLiCE reaction has a negative effect on transformation efficiency. It would be expected at least as high as the background ($2*10^3$ and $5*10^3$ $cfu*mL^{-1}*\mu g^{-1}$ for pGAD x *BamH1* and pGBK x *BamH1* respectively). Transformation efficiency calculated for SLiCE transformations including all grown colonies is between 400 and 1200 $cfu*mL^{-1}*\mu g^{-1}$and thus considerably lower. What was interesting was the difference between the two vectors. As for pGAD cloning worked out quite nicely, pGBK caused some trouble, as pGBK-ARI5 did just work out after several transformations and pGBK-ARI7 was not found at all, even after some changes in conditions.

SLiCE certainly is, compared to TOPO TA cloning, a very fast and straight forward method, which leaves quite some flexibility considering the insert site. It was newly established in the lab and certainly leaves some room for improvement. Obviously the length of the overhang could be increased, as well as the amount of empty vector DNA used. Background could be reduced by

purifying linearized vectors what would subsequently reduce the amount of colonies to be screened after transformation. Further the transformation conditions could be optimized, as it was not found out which volume of SLiCE reaction works best. Compared to restriction/ligation cloning its advantage is the independence of suitable restriction sites and that no expensive restriction enzymes or ligases are needed, but overall time and effort are quite the same.

## 6.2) Cloning of ARI7 into pGBK

PGBK-ARI7 could not be stably transformed into yeast. First it was thought that efficiency of SLiCE cloning was too low. Then while cloning into the TOPO TA vector worked nicely, the transfer of ARI7 into pGBK did not. Only too small inserts, probably of the TOP10 backbone itself, were taken up. Next a restriction/ligation cloning approach was tried, already successful for cloning *COP1* with the exact same vector. Quite interesting is the comparison of transformation efficiencies of this experiment: pGBK-COP1 resulted in $1.2*10^5$ cfu$*$mL$^{-1}*$µg$^{-1}$, of those about 60% were positive, efficiency of pGBK-ARI7 was $5*10^4$ cfu$*$mL$^{-1}*$µg$^{-1}$, approximately corresponding to the 40% of negative colonies obtained by pGBK-COP1 cloning. As if pGBK-ARI7 transformants just did not grow. As it was proven by PCR that ligation was successful the whole vector was amplified, purified and recircularized to avoid background colonies. On the one hand clones carried plasmids with truncated insert, maybe through damage during purification or DNA-polymerase failure during PCR, on the other hand apparently positive clones were identified, but plasmid could not be successfully recovered. Either because of low yield, not even enough for sequencing could be purified, or because suddenly a transposase was inserted into the gene of interest, a mechanism maybe explainable as stress response to destroy the functionality of the inserted gene (Kovařík et al. 2001). The fusion protein should not be expressed in *E.coli* anyway, because *ADH1* promoter is a yeast promoter but Western blot analyses showed expression of a gene containing the c-Myc tag, unfortunately a control of untransformed DH10b cells was forgotten, leaving the possibility that those bands occurring are not

pGBK specific. But the Western blot results together with the observation that *ARI7* in TOPO vector does not interfere with *E.coli* growth indicates that they might recognize a cryptic promoter sequence in pGBK and that the resulting protein expression is somehow lethal for the transformants.

Of course other possibilities would still have been available, but were not tried because of lack of time. For instance an approach to take advantage of the yeast recombination apparatus would have been quite obvious as this would have been quite similar to SLiCE (Muhlrad, Hunter and Parker 1992). Transformation into a different *E.coli* strain might have been another possibility.

## 6.3) Yeast two hybrid analysis

Transformants of all desired combinations were successfully generated, although of some just one or two colonies were obtained. For analysis at least tree would have been needed. These transformants were especially marked in the results section.

Generally it has to be mentioned that β-galactosidase results were very low, because an approach was chosen where transformants were grown on plates and then scraped off to be resuspended directly in assay buffer. In most protocols liquid cultures have to be grown over night and additionally for some hours on the subsequent day to reach the logarithmic growth phase (Van Criekinge and Beyaert 1999). This was not done with my method, it took less time but cells probably were not in the most advantageous growth phase for reporter gene expression, thus the values achieved were about a factor ten lower than of previous experiments. Variations between single assays were quite huge, this might have been avoided by not just using three different clones per transformant but by also repeating the assay three times. This was not done because of time issues. The background was chosen rather conservative by taking the highest value achieved by AD-empty/BD-empty. This seemed more reasonable than choosing it too low and thus having to cope with a lot of transformants appearing positive, although they were not. For this reason some transformants with interacting proteins dropped below the threshold, so it was especially pointed out in the results

section that in their respective experiment they were positive, for instance AD-ARI5/BD-eIF4E and AD-ARI7/BD-eIF4E. But there were still transformants over the threshold not considered positive, because one of the fusion proteins is autoactivating. Those were: AD-ARI5/BD-ARI2, AD-ARI5/BD-ARI8, AD-ARI5/BD-ARI12, AD-ARI7/BD-ARI2, AD-ARI7/BD-ARI8, AD-ARI7/BD-ARI12, AD-ARI5/BD-nCBP, AD-ARI7/BD-nCBP, AD-COP1/BD-ARI1, AD-COP1/BD-ARI2, AD-ARI8/BD-COP1, AD-COP1/BD-ARI8 and AD-COP1/BD-ARI12. For BD fusions of ARI2 and ARI8 as well as AD fusion of ARI8 autoactivation was not tested and values generated with my β-galactosidase assay were not available for direct comparison. But their autoactivation activity was already known from earlier research. However as the β-galactosidase results of transformants with one of these fusion proteins as interaction partner were mostly just slightly over the background signal, they were considered negative. In contrast the self-made autoactivation test of BD-ARI1 was positive too, but AD-ARI5 and AD-ARI7 combinations with BD-ARI1 gave so much higher values in the β-galactosidase assays, that these combinations were considered positive for interaction. Most strangely the results of a stock culture of AD-empty/BD-ARI1 did not at all indicate autoactivation, this discrepancy cannot be explained.

Interactions of both ARIs of interest with ubiquitin seemed very weak, considering that ARIADNE E3 ligases are thought to directly bind ubiquitin before passing it on to the substrate protein. But this might also be an effect of yeast-own ubiquitin interfering and binding to the recognition site in ARIADNE, thus blocking interaction with BD-UBI.

ARI5 and ARI7 both interacted weakly with eIF4E and thus might be able to influence translation. Considering their task as E3 ligases to cause protein degradation, they might be responsible for decreasing amounts of available cap-binding proteins. Without the help of functional eIF4F complexes ribosomes would not be able to associate with mRNA and as a consequence translation would be inhibited.

If ARI5 and ARI7 can interact with nCBP cannot be clearly said, because autoactivation tests for BD-nCBP were positive, but were not done for its deletion mutants N-tail, Helix, β-sheet and C-tail.

Thus it has to be considered that they might be able to interact with nCBP's core structure. The autoactivation experiments for the mutants have to be done before allowing a complete interpretation of respective results.

It is noticeable that not a single combination with BD-ARI5 showed interaction. That might have been caused by a change in conformation due to the fusion protein formation, one of the major drawbacks of the yeast two hybrid system.

UV-B spotting plates did not grow, most probably because UV-B dose was too high. Additionally of AD-ARI12/BD-empty and AD-empty/BD-ARI12 in each case one transformant did not grow for β-galactosidase assay. Nevertheless it could be shown that COP1 and UVR8 did interact after treatment with UV-B radiation and that COP1 could form homodimers, matching reports of mammalian COP1 to perform autoubiquitination (Dornan et al. 2006). ARI12 did not interact with either COP1 or UVR8. As those two are involved in early signalling in the UV-B response pathway and ARI12 is induced after UV-B exposure they might not directly interact with each other, but COP1 and UVR8 might cause or influence ARI12 expression.

Concerning the problems of growth under UV-B exposure it would have probably been recommendable to further decrease UV-B dose. Success will depend on finding the right proportion between enough UV-B to enable thereof dependent interactions and not too much radiation which apparently causes cell death.

Generally it has to be considered that yeast two hybrid analysis shows interactions that might be possible in plant cells, but do not have to, as protein combinations are forced that might never actually meet in the plant cell. It can just give an idea what is possible and interactions have to confirmed with other experiments directly involving plant cells (Van Criekinge and Beyaert 1999).

## 6.4) Expression analysis

Almost all fusion proteins tested by Western blot could be shown to be expressed, although immunodetection with anti c-Myc antibody could not be optimized. Only BD-UBI was never visible as well as BD-ARI1 of the AD-ARI5/BD-ARI1 combination. ARI5 and ARI7 mostly form double bands, usually suggesting posttranslational modifications to take place. ARI are thought to be autoubiquitinated and the molecular weight of a single ubiquitin is 8.5 kDa. Thus a modification by ubiquitin offers one explanation for the detectable bandshift. Samples often showed multiple bands and smears probably evolving through protease digestion, as protease inhibitors were not used and some protein extracts were re-thawn several times. Of some samples bands did not correspond to expected sizes, that might have been caused by shifting the membrane's position on the film before exposure or when drawing the marker lanes. Especially for smaller fragments it might also have occurred due to inhomogeneous running of the gels. The low bands displayed by BD-ARI8 are hardly explainable. As all three different transformants show quite the same size and after fresh purification of pGBK-ARI8 from a DH10b stock culture no abnormalities in size were seen on an agarose gel (data not shown), it was assumed that there might have occurred a point mutation leading to the formation of a stop codon.

# 7) Conclusion

Finally it can be summarized that with SLiCE a new cloning method could be established in the lab and was successfully applied for cloning pGAD-ARI5, pGAD-ARI7 and pGBK-ARI5. Additionally *COP1* was cloned from pGAD into pGBK. The plasmid construct pGBK-ARI7 could not be generated neither by SLiCE nor by TOPO TA cloning or restriction/ligation cloning. I found evidence that the pGBK vector contains a cryptic promoter that allows the expression of proteins from this vector in *E.coli*.

With yeast two hybrid assays novel interactions of ARI5 as well as ARI7 with ARI1, eIF4E and ubiquitin were detected. Also homodimerization of COP1 and the UV-B dependent interaction of COP1 and UVR8 were confirmed. Expression of the fusion proteins in yeast transformants was demonstrated by Western blotting.

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# Abbreviations

| | |
|---|---|
| (w/v) | weight per volume |
| °C | degrees Celsius |
| 4EHP | translation initiation factor 4E homologous protein |
| 5'-UTR | 5' untranslated region |
| A overhang | adenosine overhang |
| AA | acrylamide-, bis-acrylamide |
| AD | activation domain |
| $Ade^+$ | restored adenine metabolism |
| *ade2* | mutation of phosphoribosylaminoimidazole carboxylase of purine synthesis |
| ADH1 | alcohol dehydrogenase1 |
| APS | ammonium persulfate |
| *ara*D139 | mutation in L-ribulose-phosphate 4-epimerase blocks arabinose metabolism |
| ARI | ARIADNE |
| *At*ARI | ARIADNE from *Arabidopsis thaliana* |
| auto | autoactivating |
| BD | DNA binding domain |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pairs |
| C | C-tail of ARI12 |
| cDNA | complementary DNA |
| cfu | colony forming units |
| Col | Columbia |
| COP1 | CONSTITUTIVELY PHOTMORPHOGENIC1 |
| C-tail | deletion mutant of nCBP at the C-tail |
| ctrl | control |
| dATP | deoxy adenosine triphosphate |

| | |
|---|---|
| dCTP | deoxy cytidine triphosphate |
| dGTP | deoxy guanosine triphosphate |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxy nucleoside triphosphate |
| DO | dropout |
| DTT | dithiotheitol |
| dTTP | deoxy thymidine triphosphate |
| *E.coli* | *Escherichia coli* |
| E1 | ubiquitin activating enzyme |
| E2 | ubiquitin conjugating enzyme |
| E3 | ubiquitin protein ligase |
| EDTA | ethylenediaminetetraacetic acid |
| eIF | eukaryotic initiation factor |
| *end*A1 | mutation of non-specificendonucleaseA1 |
| $F^-$ | does not carry F plasmid |
| F | forward |
| FRET | Förster resonance energy transfer, Fluorescence resonance energy transfer |
| g (mg, µg, ng) | gram(s) (milligram, microgram, nanogram) |
| *GAL2-ADE2* | *ADE2* under control of *GAL2* inserted |
| *gal4Δ* | deletion of GAL4 |
| *gal80 Δ* | deletion of negative regulator of GAL genes, GAL80 |
| *gal*K | mutants cannot metabolize galactose and are resistant to 2-deoxygalactose |
| *gal*U | mutants cannot metabolize galactose |
| GFP | green fluorescent protein |
| GTE | glucose/TRIS/EDTA |
| HA | hemagglutinin |
| Helix | deletion mutant of nCBP at the helix structures |

| | |
|---|---|
| HHARI | human homologue of *Drosophila* ARIADNE |
| $His^+$ | restored histidine metabolism |
| *his3* | mutation of imidazoleglycerol-phosphate dehydratase of histidine biosynthesis |
| HRP | horseradish peroxidse |
| HY5 | ELONGATED HYPOCOTYLE 5 |
| IBR | in between ring fingers |
| IBR-RING2 | IBR and RING2 domain of ARI12 |
| KCM | potassium/calcium/magnesium |
| kDa | kilodalton(s) |
| L (mL, µL) | litre(s) (millilitre, microlitre) |
| -L/-T | -leucine/-tryptophan |
| -L/-T/-H +At | -leucine/-tryptophan/-histidine + 1 mM 3-aminotriazole |
| -L/-T/-H | -leucine/-tryptophan/-histidine |
| LB | lysogeny broth |
| *leu2* | mutation of beta-isopropylmalate dehydrogenase of leucine biosynthesis |
| *LYS2::GAL1-HIS3* | *HIS*3 under control of *GAL1* promoter inserted into *LYS2* site |
| m | metre(s) |
| M (mM, µM) | molar (millimolar, micromolar) |
| mA | milliampere |
| *MAT*a | mating type a |
| *mcrA* | Mutation eliminating restriction of DNA methylated at the sequence $C^mCGG$ |
| *met2*::*GAL7-lacZ* | *LACZ* under control of *GAL7* promoter inserted into *MET2* site |
| min | minute(s) |
| MM | mastermix |
| mRNA | messenger RNA |
| MU | Miller Unit |
| N | normal |

| | |
|---|---|
| n/a | not available |
| nCBP | novel cap binding protein |
| N-tail | deletion mutant of nCBP at the N-tail |
| *nup*G | regulatory gene that allows constitutive expression of deoxyribose synthesis genes |
| $OD_x$ | optical density at wavelength x |
| ONPG | 2-nitrophenyle β-D-galactopyranoside |
| PABP | poly A binding protein |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| pGAD | pGADT7 |
| pGBK | pGBKT7 |
| pH | hydrogen ion concentration |
| PNK | polynucleotid kinase |
| PVDF | polyvinylidene fluoride |
| R | reverse |
| rATP | ribo-adenosine triphosphate |
| RBR | ring in between ring fingers |
| $RecA^-$ | deficient in RecA |
| *rec*A1 | mutation in a gene responsible for general recombination of DNA |
| RING | really interesting new gene |
| RING1 | RING1 domain of ARI12 |
| RING1-IBR | RING1 and IBR domains of ARI12 |
| RING2-C | RING2 domain and C-tail of ARI12 |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| *rps*L | confers resistance to streptomycin |

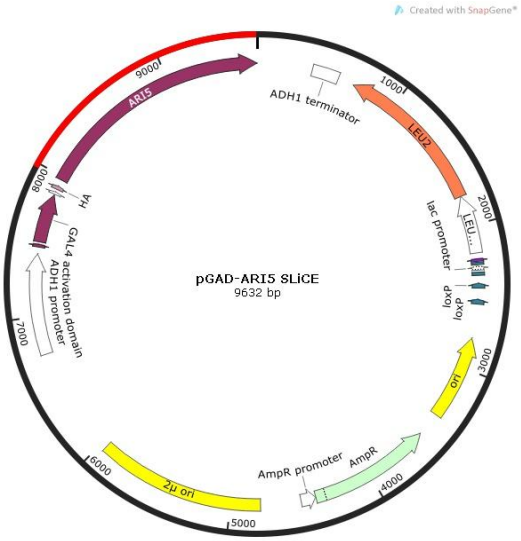
| | |
|---|---|
| RT-PCR | reverse transcription-polymerase chain reaction |
| RUP | repressor of UV-B photomorphogenesis |
| SD | selective dropout |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| sec, s | second(s) |
| seq | sequencing |
| SLiCE | seamless ligation cloning extract |
| SOC | super optimal broth with catabolic repressor |
| ss | single strand |
| T overhang | thymidine overhang |
| t | time |
| TAE | TRIS/EDTA/acetic acid |
| TBS-T | TRIS-buffered saline and TWEEN 20 |
| TE | TRIS/EDTA |
| TEMED | tetramethylethylenediamine |
| TRIS | tris(hydroxymethyl)-aminomethane |
| *trp1* | mutation of phosphoribosylanthranilate isomerase of tryptophan synthesis |
| TSB | tryptic soy broth |
| U | unit(s) |
| UBI | ubiquitin |
| *ura3* | mutation of orotidine-5'-phosphate decarboxylase of pyrimidine metabolism |
| UV | ultraviolet |
| UVR8 | UV RESISTANCE LOCUS8 |
| V | volt(s) |
| V | volume |
| W | watt(s) |

| | |
|---|---|
| wt | wild type |
| x g | units of gravity |
| x | times |
| YFP | yellow fluorescent protein |
| YPD | yeast extract peptone dextrose |
| YT | yeast extract and tryptone |
| β-gal | β-galactosidase |
| β-sheet | deletion mutant of nCBP at the β-sheet structures |
| Δ(*ara leu*) 7697 | deletion of several genes |
| Δ(*mrr-hsd*RMS-*mcr*BC) | mutations in the system of methylation and restriction that allow *E. coli* to recognize DNA as foreign |
| Δ*lac*X74 | deletion of the entire *lac* operon as well as some flanking DNA |
| $\lambda^-$ | lambda lysogen deletion |
| Φ80*lac*ZΔM15 | cell carries the lambdoid prophage ϕ80, carrying lacZ deletion |

# Appendix

**Sequences**

pGAD-ARI5

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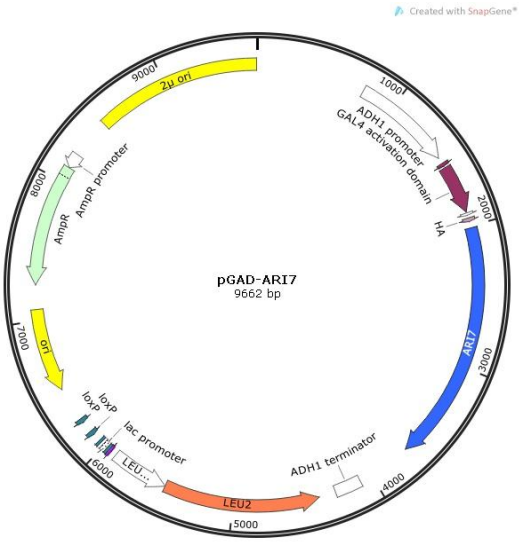


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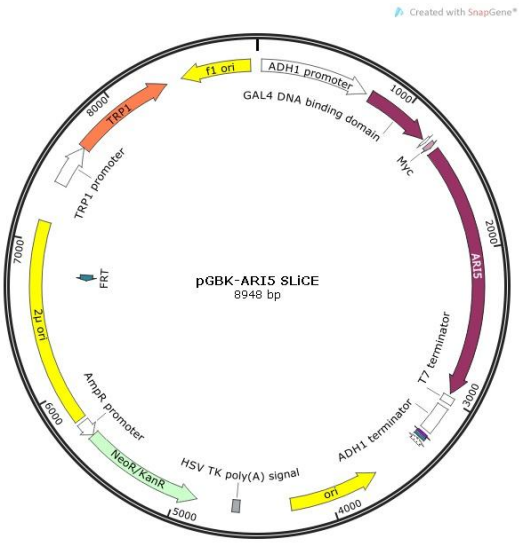


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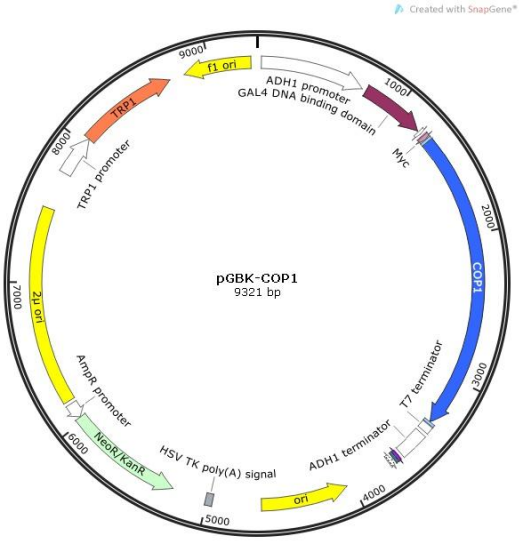


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**BLAST research of the pGBK-ARI7 clone #3 after purification by QuickPrep**

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Database: Nucleotide collection (nt)
           19,363,435 sequences; 49,069,697,512 total letters
Query= 3808995

Length=964



Score     E
Sequences          producing          significant          alignments:
(Bits)  Value

ref|NM_128708.4|    Arabidopsis  thaliana  putative  E3  ubiquitin-p...
800   0.0
gb|GQ167039.1|    Escherichia  coli  strain  EC-917/05  insertion  se...
676   0.0

ALIGNMENTS
>ref|NM_128708.4|  Arabidopsis  thaliana  putative  E3  ubiquitin-protein
ligase ARI7
mRNA, complete cds
Length=2034

 Score =   800 bits (433),  Expect = 0.0
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 Strand=Plus/Minus

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Query  220   ACCTCTTGTTTTGCTACTGCAACCTGTTGATTTAGAGCTTGTTGATTTGCTGCTGCAAGC  279
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Query  280   CGCATGTGAGTCTACATCAGCAAGACCGTTCTCCAGAGCTTTCACCAAATTCTCAAAGTA  339
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Query  340   GTTTTTCGTCACGCTGGTCAAACCAGCTAGTTTTGTCCGGAAATCATTGAAATCTTTTGA  399
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Query  520   CTCAGGTAGGTAGTATCCATATGCATAAGTCCATTTCAAGACTCGCCTACATTCGATGAT  579
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Query  580   CTGAAGCCAAGCT  592
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Sbjct  1269  CTGAAGCCAAGCT  1257
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>gb|GQ167039.1| Escherichia coli strain EC-917/05 insertion sequence IS10 Tnp
(tnp) gene, complete cds; and disrupted outer membrane protein
C (ompC) gene, partial sequence
Length=2627

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 Score =   676 bits (366),  Expect = 0.0
 Identities = 367/368 (99%), Gaps = 0/368 (0%)
 Strand=Plus/Plus

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  473  GAAGCATCAGGGCGATTAGCAGCATGATATCAAAACGCTCTGAGCTGCTCGTTCGGCTAT  532

Query  890  GGCGTAGGCCTAGTCCGTAGGCAGGACTTTTCAAGTCTCGGAAGGTTTCTTCAATCTGCA  949
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  533  GGCGTAGGCCTAGTCCGTAGGCAGGACTTTTCAAGTCTCGGAAGGTTTCTTCAATCTGCA  592

Query  950  TTCNCTTC  957
            ||| ||||
Sbjct  593  TTCGCTTC  600
```

```
  Database: Nucleotide collection (nt)
    Posted date:  Aug 26, 2013  6:26 AM
  Number of letters in database: 49,069,697,512
  Number of sequences in database:  19,363,435

Lambda      K        H
    1.33    0.621     1.12
Gapped
```

```
Lambda      K        H
    1.28    0.460    0.850
Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 0, Extension: 0
Number of Sequences: 19363435
Number of Hits to DB: 0
Number of extensions: 0
Number of successful extensions: 0
Number of sequences better than 10: 8
Number of HSP's better than 10 without gapping: 0
Number of HSP's gapped: 8
Number of HSP's successfully gapped: 8
Length of query: 964
Length of database: 49069697512
Length adjustment: 33
Effective length of query: 931
Effective length of database: 48430704157
Effective search space: 45088985570167
Effective search space used: 45088985570167
A: 0
X1: 13 (25.0 bits)
X2: 32 (59.1 bits)
X3: 54 (99.7 bits)
S1: 13 (25.1 bits)
S2: 23 (43.6 bits)
```