Chapter 2

Introducing the higher order Arabidopsis 14-3-3 mutants

P.J.M. van Kleeff\textsuperscript{1}, N. Jaspert\textsuperscript{2}, S. Rauch\textsuperscript{2}, C. Oecking\textsuperscript{2} and A.H. de Boer\textsuperscript{1}

\textsuperscript{1} Faculty of Earth and Life Sciences, Department of Structural Biology, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, the Netherlands
\textsuperscript{2} Centre for Plant Molecular Biology – Plant Physiology, University of Tübingen, Auf der Morgenstelle 32, 72076 Tübingen, Germany
Abstract
Gene redundancy is a common cause for the absence of observable phenotypic alterations in single mutant plants. Gene redundancy is a phenomenon in which family members of the mutated gene compensate for its loss. In yeast, one protein family in which gene redundancy has been observed is 14-3-3. In Arabidopsis, single mutant phenotypes have been found for flowering time and root length. In this Chapter we validate T-DNA insertion lines for six non-epsilon family members corresponding to three closely related gene pairs; kappa, lambda, nu, upsilon, phi and chi. No full length transcripts were found for all six lines. However, in frame truncated versions of kappa, lambda and phi were found before the T-DNA insertion. Yeast-two-hybrid assays have shown that these truncated versions do not bind known 14-3-3 targets, and therefore all lines are considered loss-of-function mutants. Moreover, higher order double, triple and quadruple 14-3-3 mutants were generated and verified. In addition, we show that the expression of KAPPA and LAMBDA within the quadruple mutant upsilon/nu/phi/chi, of NU and UPSILON in kappa/lambda/phi/chi, and of PHI and CHI in kappa/lambda/upsilon/nu, is at wild-type level which indicates that 14-3-3’s do not affect transcription of related 14-3-3 genes.
Introduction
While the “classical” forward genetics approach starts with a specific mutant phenotype and searches for the genomic mutation causing it, the reverse genetic approach initiates from a known mutation and tries to assign the respective phenotypic aberrations. Essential to reverse genetics is the occurrence of an observable phenotype, whereat the phenotype can be linked to the disruption of a single gene function either caused by incomplete transcription or by mutation of essential amino acids within the gene product.

In organisms such as *Escherichia coli*, yeast and mice, homologous recombination with null gene constructs is used to replace functional alleles. However, this method does not work efficiently in flowering plants (Puchta 2002), which restricts plant geneticists to transcript alteration methods like RNAi, insertion mutants and inducing agents like ethyl methane sulfonate. RNAi is an example of directional gene alteration. This method uses the introduction of a sequence complementary to the gene of interest into the plant genome in order to produce short double-stranded RNA (ds-RNA) transcripts. These RNA fragments stimulate the RNA-induced silencing complex, inducing faster RNA turnover and subsequently a reduced expression (Alonso and Ecker 2006; Zhang et al. 2013). Drawbacks of RNAi are the unpredictability of whether constructs work and to what degree the expression is altered. Moreover, multiple ds-RNA may be generated from one construct and thus off-target effects can occur (Alonso and Ecker 2006).

Random mutagenesis methods can be divided by the nature of the inducing agent, e.g. into chemical, physical and biological agents. In *Arabidopsis*, ethyl methane sulfonate (EMS) and nitrosomethylurea (NMU) are used as chemical agents to generate point mutations. Although chemical mutagenesis is an easy method to use, finding the point mutation causing the phenotypes on genome level can be challenging. Besides, multiple point mutations are generated which should be separated to link the phenotype to a single point mutation (Greene et al. 2003; Alonso and Ecker 2006). Radiation is an example of physical mutagenesis which results in large chromosomal rearrangements, insertions and deletions affecting multiple genes, and so the phenotypes observed after radiation do not result from a single gene (Alonso and Ecker 2006).

Transposons and large non-coding transposon fragment (T-DNA) are biological mutagenesis methods that can be used for random insertions where the mutations can easily be detected on genomic scale (Krysan et al. 1999; Alonso and Ecker 2006). The defined sequence of the inserted T-DNA provides a useful marker to identify the exact location of its insertion into the genome by simple PCR. In addition, T-DNAs are stably inserted into the genome over many generations and confer a stable genetic trait, unlike transposons which might change their location in the genome rapidly (Krysan et al 1999; Alonso et al 2006). A drawback of T-DNA insertion mutagenesis is that the exact insertion site within a gene may determine the severity of the resulting transcriptional aberration. Hence, different insertions within one gene may give rise to different phenotypes (Valentine et al. 2012). In addition, multiple T-DNA insertion events may occur in one plant and could potentially give rise to phenotypes that are not related to the gene of interest (O’Malley and Ecker 2010). Taken together, T-DNA insertion mutants may serve as tools to investigate the consequences of the loss of a single gene function. Furthermore, the genetic combination of T-DNA lines for members of
a gene family allows the dissection of redundancy and isoform specificity between
the different family members. For *Arabidopsis* several large collections of sequence-
indexed insertion mutants (e. g. SALK, SAIL, GABI and FLAG collections, (Samson et
al. 2002; Sessions et al. 2002; Alonso et al. 2003; Kleinboelting et al. 2012)) have been
generated by insertional T-DNA mutagenesis and subsequent insertion-site discovery.
These collections have been made available to the research community.

Although single mutant plants showing a phenotype have been published, a large
number of single mutant plants lack a phenotype. In these situations redundancy, where
a family member takes over the role of the mutated gene, could result in the absence of
a phenotype. In these cases higher order mutants must be obtained by combining the
respective single mutants of a gene family. Three types of redundancy can be found
in plants: 1) full redundancy, in which neither parental plant line shows a phenotype
and the homozygous double mutant does, 2) partial redundancy, in which both parental
plants show a phenotype that is enhanced in their double mutant offspring and 3)
unequal redundancy, seen in paralogous genes with a single mutant of the ancestral
gene showing a phenotype while the single mutant of the duplicated gene does not show
a phenotype. However, the offspring of these two mutants shows an enhanced ancestral
phenotype (Briggs et al 2006).

One protein family that shows redundancy is the 14-3-3 protein family (van
Heusden et al. 1995; van Heusden et al. 1996; Jaspert et al. 2011; Paul et al. 2012). In
yeast, two 14-3-3 genes are present and both genes are needed for normal cell function.
Removing one gene shows no phenotype, however, when both genes are mutated the
phenotype becomes lethal (van Heusden et al. 1995). In *Arabidopsis*, 14-3-3 single
mutant phenotypes have been found in both flowering time and root length. Both *mu*
and *upsilon* single mutants show a delay in flowering time under long day conditions
(Mayfield et al. 2007) and *mu* shows a shorter primary root phenotype (Mayfield et
al. 2012). In addition, 14-3-3 LAMBDA and not its closely related gene KAPPA is
necessary for PHOT2 mediated stomatal opening (Tseng et al. 2012). 14-3-3’s Bind
phosphorylated target proteins through three canonical binding motives: internal mode-I
((R/KXXpS/pTXP), internal mode-II (R/KXXXpS/pTXP) and C-terminal mode-III pS/
pTX1,2-COOH). *Arabidopsis* possesses 13 expressed members which, based on their
amino acid sequence, can be grouped into an epsilon and non-epsilon group. The epsilon
group consists of IOTA,OMICRON,MU, EPSILON and PI. The non-epsilon group can
further be divided into psi, omega and kappa group. The psi group contains PSI and the
closely related genes NU and UPSILON, the omega group comprises the closely related
genes PHI, CHI and OMEGA. The kappa group is formed by the closely related genes
KAPPA and LAMBDA.

The work in this thesis focuses on 14-3-3 genes of the non-epsilon family.
The epsilon group is found in all eukaryotic organisms and is most likely necessary
for basal eukaryotic cell functions. The non-epsilon group members potentially have
organism specific functions (Jaspert et al. 2011). In this chapter, we show the analysis
of the SALK-lines for kappa, lambda, nu, upsilon, phi and chi. Loss-of-function T-DNA
insertion mutant lines were only found for these six non-epsilon group members
(personal communication Prof. Dr. Claudia Oecking). Although truncated transcripts of
KAPPA, LAMBDA and PHI are found within the SALK-lines, a yeast-two-hybrid
protein interaction assay shows that the respective truncated proteins do not bind 14-3-3 targets as homo-dimers. In addition to the single mutant lines, higher order mutants were generated viz. double, triple and quadruple mutants. Moreover, we show that the expression level of PHI and CHI within the quadruple mutant kappa/lambda/upsilon/nu (klun), of UPSILON and NU in kappa/lambda/phi/chi (klpc) and of KAPPA and LAMBDA in upsilon/nu/phi/chi (unpc) remain at the wild-type level, which indicates that these 14-3-3’s do not affect their own transcription.

Results
Characterization of 14-3-3 T-DNA insertion lines
14-3-3 Proteins have been shown to be key regulators in diverse signalling pathways ranging from metabolic processes to stomatal opening (Toroser et al. 1998; Cotelle et al. 2000; Tseng et al. 2012). Even though isoform specificity has been found for MU and UPSILON, no phenotypes were found in other 14-3-3 insertion mutants like the double mutant kappa/lambda (kl) (Deng et al. 2007; Jaspert et al. 2011). This raises the question whether there is isoform specificity and/or redundancy among Arabidopsis 14-3-3 family members. In a multiple gene family genetic redundancy is more likely to occur between two highly related gene pairs when compared to more distant genes. To answer this question, a reverse genetic approach was used, obtaining homozygous single mutant T-DNA insertion lines for the closely related gene pairs KAPPA-LAMBDA, NU-UPSILON and PHI-CHI (Fig. 1A). The presence and location of the T-DNA within the 14-3-3 genes were confirmed by PCR and sequencing (Fig. 1B). The T-DNA insertion for NU, UPSILON, PHI and CHI lie within the 5'UTR or exons of the respective 14-3-3 gene. For KAPPA and LAMBDA T-DNA insertion resides within introns. The presence of a T-DNA insertion within a gene does not guarantee that these lines are loss-of-function mutants. By means of semi-quantitative reverse transcriptase PCR (RT-PCR) we confirmed that full length KAPPA, LAMBDA, NU, UPSILON, PHI and CHI transcripts are not present in the respective T-DNA insertion lines (Fig. 1C).

Characterization of truncated transcripts for KAPPA, LAMBDA and PHI
Although kappa, lambda and phi do not express full length transcripts, in frame transcripts were found covering the open reading frame before the T-DNA insertion (Figs. 1C and D). These truncated transcripts could be translated into truncated but partially functional proteins. For example, C-terminal truncation mutants of Arabidopsis 14-3-3 OMEGA have shown an increased affinity for target proteins (Shen et al. 2003). To study the binding properties of these truncated 14-3-3’s, a yeast-two-hybrid assay (Y2H) with known interactors was performed. The interactors selected were HvH'-ATPase and AtTPK1 since both interactors have different 14-3-3 binding motifs. TPK1 has an internal binding motif while Hv-ATPase has a C-terminal binding motif. For KAPPA and LAMBDA, the N-terminus of the vacuolar ion channel TPK1 was used which has been shown to interact with 14-3-3 LAMBDA (Latz et al. 2007) and KAPPA (Chapter 5, this thesis). Although PHI does interact with TPK1 (Chapter 5, this thesis), we have chosen to use the barley H'-ATPase as PHI target protein. Figure 1E shows that the possible truncated versions of KAPPA, LAMBDA and PHI do not bind 14-3-3 target proteins in a Y2H assay. This result indicates that the truncated KAPPA,
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LAMBDA and PHI do not bind with target proteins. Accordingly, the mutants kappa, lambda, nu, upsilon, chi and phi are considered loss-of-function mutants.

**Higher order 14-3-3 mutants and WT 14-3-3 transcripts in quadruple mutants**

One way to investigate possible isoform specificity and/or redundancy, under different growth conditions when single mutants do not show a phenotype, is to obtain higher order mutants through genetic crossing. The highly related gene pairs were crossed and selfed to obtain double mutant lines. These double mutants were used to generated 12 triple and three quadruple mutant lines. Figure 1F shows the generated combinations of 14-3-3 mutants. The triple mutants kappa/phi/chi (kpc), lambda/phi/chi (lpc), nu/phi/chi (npc) and upsilon/phi/chi (upc) were generated by crossing the double mutants phi/chi with the respective single mutants (Fig. S1A). Plants of the segregating F2 were genotyped by PCR and selected accordingly. The mutants containing lambda and upsilon (lambda/upsilon/nu (lun), kappa/lambda/upsilon (kun) and kappa/lambda/upsilon/nu (klun)) were generated by crossing the double mutants kappa/lambda (kl) and upsilon/nu (un) (Fig. S1B). The remaining triple and quadruple mutants were generated by crossing the two double mutants and back-crossing with one of the two double mutants (Fig. S1C). T-DNA insertions were monitored using PCR and the final mutants were scored on full length 14-3-3 transcripts (Fig. S2).

The loss of a particular gene function might induce transcriptional up-regulation of redundant genes (Barratt et al. 2009; Geiger et al. 2010; Franz et al. 2011). To check whether the expression levels of potentially redundant non-epsilon 14-3-3’s are enhanced in response to the loss of four 14-3-3’s, we analysed transcription levels of two non-epsilon group members in the respective quadruple mutants. Therefore KAPPA and LAMBDA transcription levels were analysed in upsilon/nu/phi/chi (unpc), NU and UPSILON transcription levels were tested in kappa/lambda/phi/chi (klpc) and PHI and CHI transcription levels were analysed in klun. In none of the quadruple mutants promoter activity of the two non-epsilon 14-3-3’s checked was altered, in response to the loss of the other four genes (Fig. 1G). This indicates, that the loss of function of any of these six 14-3-3’s is not buffered by transcriptional up-regulation of the others. However, we cannot exclude the possibility that the transcription of the seven remaining 14-3-3’s may show differences.

--- Figure 1. 14-3-3 T-DNA insertion mutants.
A) Phylogenetic tree of *Arabidopsis* 14-3-3’s with boxed the three pairs of highly related 14-3-3 proteins of which T-DNA insertion lines were used in this study. B) Position of T-DNA insertion within the six *Arabidopsis* 14-3-3 genes used in this study. Grey boxes represent UTRs, black boxes exons, lines represent introns and inverted triangles indicated T-DNA insertion. C) Expression of 14-3-3 in insertion mutants. Total RNA was isolated from 14 day after stratification (DAS) plants and RT-PCR to amplify 14-3-3 transcripts. No full length 14-3-3 transcripts are found in the single mutants. D) In frame truncated transcripts have been found in the single mutants kappa, lambda and phi. E) Yeast-two-hybrid (Y2H) assay using wild-type 14-3-3’s and truncated 14-3-3’s (14-3-3 T) showing that the truncated 14-3-3’s do not bind to 14-3-3 target proteins while wild-type 14-3-3 does. F) Overview of the mutants used and generated during this study. The inner triangle depicts the single mutants in grey and at the corners in orange are the double mutants. The outer triangle shows at the sides in blue 12 triple mutants and at the corners in red the quadruple mutants. G) The quadruple mutants do not show differences in expression of two wild-type 14-3-3’s tested.
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A

B

C

D

E

F

G

14-3-3 transcripts

ACTIN

WT

mutant

empty pGAD7

AitPK1 M1-N73

empty pGAD7

HvH+ ATPase S584-V626

14-3-3 expression/actin

WT

quadraple
Discussion

14-3-3 Isoform specificity has been observed in only two 14-3-3 single mutants. The single mutant mu, but not upsilon, shows a reduction in root growth under continuous light (Mayfield et al. 2007; Mayfield et al. 2012). Another example of 14-3-3 isoform specificity was found between the closely related gene products KAPPA and LAMBDA. Only LAMBDA is necessary for PHOT2 mediated stomatal opening while KAPPA did not show an effect (Tseng et al. 2012). Although single mutant phenotypes have been recorded, so has the absence of phenotypes under normal growth conditions for single mutants kappa, lambda and double mutant kappa/lambda (kl) (Deng et al. 2007; Jaspert et al. 2011). The lack of phenotype under normal growth conditions could have two causes. 1) There is gene redundancy whereby the function of one or more 14-3-3 gene products is taken over by the remaining members of the 14-3-3 protein family, 2) the growth conditions used for these studies did not trigger a phenotype. It could be that only under certain growth conditions phenotypes in single mutants will be observed.

To investigate 14-3-3 function further we obtained single, double, triple and quadruple mutants of six non-epsilon group members. The non-epsilon group was chosen for its potential species specific functionality. In addition, phenotypes have been observed for mutants with members of this group as described above. Possibly truncated transcripts occur in T-DNA lines that give rise to a phenotype while a loss-of-function mutant does not show such phenotype (O’Malley and Ecker 2010). The T-DNA insertion lines used in this thesis for KAPPA, LAMBDA and PHI have shown detectable transcripts of in-frame ORFs located before the T-DNA insertion (Fig. 1D). Research has been conducted on Arabidopsis 14-3-3 OMEGA in relation to C-terminal truncations. Removing the C-terminal tail from OMEGA enhances the binding capacity of 14-3-3 to their targets (Shen et al. 2003). However, removing helix 9 and 8 changed the structure of the 14-3-3 protein in such a way that 14-3-3’s were unable to bind target proteins and do not form dimers (Athwal and Huber 2002). The T-DNA insertion causing a truncation for phi lies within the 9th helix, for lambda it resides within helix 8 and for kappa in helix 7 (Fig. S3). Our Y2H assay, where no binding occurs between truncated forms of 14-3-3 and its target, is consistent with the publication of Athwal and Huber 2002.

We can only speculate whether the truncated versions form stable dimers with WT 14-3-3’s and what the outcome of such a binding will be. So far, only a truncation between helix 8 and 9 of human 14-3-3 SIGMA was shown to form homo-dimers and to reduce the binding capacity of WT SIGMA (Li et al. 2005; Xin et al. 2010). However, 14-3-3 SIGMA is only found in epithelial cells and forms preferentially homo-dimers (Fu et al. 2000; Wilker et al. 2005). As a result, the outcome of a dimerization between a truncated plant 14-3-3 form and another WT 14-3-3 member is unknown. In the Y2H we show that truncated versions do not bind targets, but whether a dominant negative effect occurs within the SALK-line mutant of kappa, lambda and phi is unknown.

Our results show that the 14-3-3 mutants generated for this study are loss-of-function mutants. The truncated transcripts of KAPPA, LAMBDA and PHI do not bind to interactors in the Y2H, although the outcome at plant level is unknown since in mammalian cells a truncated version of 14-3-3 SIGMA has a dominant negative effect on WT 14-3-3 SIGMA. Moreover, the transcript of two WT non-epsilon 14-3-3’s remains unchanged in the quadruple mutants. This indicates that no cross-regulation
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occurs between these six non-epsilon 14-3-3’s since no difference is seen in WT 14-3-3 transcript in the quadruple mutants. We cannot exclude the possibility of differences in expression of the remaining seven 14-3-3’s.

**Methods & Materials**

**Plant growth and material**

Seeds (*Arabidopsis thaliana* Columbia ecotype (Col-0)) were surface sterilized by rinsing the seeds in 70% ethanol (10 min), followed by 10 min 25% bleach - 0.1% Tween20. Thereafter, the seeds were washed 3 times with sterilized MQ and resuspended in 0.1% sterile agarose. Seeds were plated on 0.5 x MS medium (pH 5.8) and stratified for 3 days at 4°C. Plates were placed vertically in a growth chamber with 14 hours light (22°C) / 10 hours dark (18°C), 170 µmol.m⁻².sec⁻¹.

All single and double mutant T-DNA insertion lines were provided by Prof. Claudia Oecking (Univ. Tübingen). The SALK numbers can be found in Table S1. T-DNA lines were genotyped using primers mentioned in Table S2. For each T-DNA insertion a WT and mutant (MT) PCR was performed. For *kappa*, *lambda*, *nu* and *phi* the annealing temperature was 58°C, extension 1:30 min. For *upsilon* the annealing temperature was 55°C, extension 2:30 min. For *chi* the annealing temperature was 45°C, extension 2:00 in the presence of 5% DMSO. All PCR products were cloned into pGEMT-easy (Promega) and sequenced. The primers used for RT-PCR can be found in Table S3. Higher order mutants were created by crossing the double mutants (Fig. S1). Young flower buds were opened and emasculated before dehiscence of the anthers. The following day the remaining gynoecia were manually pollinated. Seeds were harvested when the silique almost burst open.

**RT-PCR**

Plants were harvested from plate 14 days after stratification (DAS) and snap frozen in liquid nitrogen. Total RNA isolation from plant (NucleoSpin® RNA plant) according to manufactures manual. 1 µg of RNA, an oligo(dT) primer and SuperScript™-II Reverse Transcriptase (Invitrogen) was used to convert RNA into first strand cDNA. Primers used for RT-PCR can be found in Table S3. The annealing temperature used was 58°C, extension 2:00 min, 30x. All PCR products were cloned into pGEMT-easy (Promega) and sequenced.

**Yeast-two-hybrid**

WT 14-3-3’s in pGBK7T vectors were provided by Prof. Dr. C. Oecking. Truncated 14-3-3 transcripts were cloned into pGBK7T vectors and the targets AtTPK1 and barley HvH⁺-ATPase into the pGADT7 vectors (both vectors Clontech). In short, PJ694A yeast strain was selectively grown overnight in Dropout supplement-Lys (MP Biomedicals) at 30°C, 220 rpm, after which a 1:500 dilution was used to grow yeast in YAPD medium o/n at 30°C, 220 rpm. An 0.5 ml aliquot per transformation was transferred into an eppendorf and centrifuged, supernatant was removed and 2.5 µl of pGADT7, 2.5 µl of pGADT7 and 5 µl of boiled and sonicated salmon sperm (Stratagene) were added and resuspended. Next, 100 µl 40% PEG-4000/0.2M LiAc was added and vortexed. Thereafter, samples were placed at 30°C, 220 rpm for 30 min followed by 15 min incubation at 42°C. Samples were plated on selective SD-DDO (-LW) medium and placed in a 30°C incubator for 3-5 days. Four colonies were resuspended in 50 µl MQ of which 5 µl was spotted on SD-DDO, SD-TDO (-LWH medium supplemented with 5 mM 3-Amino-1,2,4-triazole (3-AT)) and SD-QDO (-LHWA). Plates were incubated for 1-3 days after which viability was checked. At least three independent colonies had to show viability to consider interaction.

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References


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Xin, Y., Q. Lu and Q. Li (2010). 14-3-3sigma controls corneal epithelial cell proliferation and differentiation through the Notch signaling pathway. Biochem Biophys Res Commun 392: 593-598.

### Supplementary materiaal Chapter 2.

#### Table S1. T-DNA insertion lines

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#### Table S2. Genotyping primers for T-DNA insertion lines

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<td>lambda RP</td>
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<td>phi RP</td>
<td>GAAAGGCAAAACATTAGATCTC</td>
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#### Table S3. 14-3-3 transcript primers

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<tr>
<td>chi LP</td>
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Figure S1. Crossing schemes used to obtain the higher order 14-3-3 mutants.

Two capital letters denote two WT alleles, one capital letter and one lower case denotes heterozygous alleles, two lower case denote two mutant alleles. A) Crossing scheme for generating kappa/phi/chi, lambda/phi/chi, nu/phi/chi and upsilon/phi/chi where X or x denotes KAPPA, LAMBDA, NU or UPSILON. P or p stands for PHI, and C or c stands for CHI. B) Crossing scheme used to generate kappa/lambda/upsilon, lambda/upsilon/nu and kappa/lambda/upsilon/nu. K or k is KAPPA, L or l is LAMBDA, N or n is NU, U or u is UPSILON C) Example crossing scheme used to obtain the mutants phi/kappa/lambda, chi/kappa/lambda, kappa/lambda/phi/chi, nu/kappa/lambda, kappa/upsilon/nu, phi/upsilon/nu and chi/upsilon/nu.
Figure S2. Transcript PCRs of the higher order 14-3-3 mutants.
Total RNA was isolated and transcripts were detected using RT-PCR. The mutated 14-3-3 genes show no full length transcripts and are therefore considered being loss-of-function mutants.
Figure S3. T-DNA insertion on protein level for KAPPA, LAMBDA and PHI.
The position of the T-DNA insertion is depicted by the arrow. Black boxes represent the nine 14-3-3 α-helixes.