The Arabidopsis 14-3-3 family -target protein specificity and expression of isoforms

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Abstract
14-3-3 proteins comprise a family of highly conserved proteins. 14-3-3 proteins have been found in all organisms examined except for members of the prokaryotic kingdom. 14-3-3s are involved in numerous processes in the cell and they typically bind to phosphorylated motifs in other proteins and regulate their activities.

In plants, 14-3-3 proteins are recognized as key regulators of primary metabolism and membrane transport. In Arabidopsis, there are 15 genes coding for 14-3-3s and hence several 14-3-3 isoforms may be present simultaneously in the plant. The aim of my work has been to understand why there are so many 14-3-3 isoforms.

To investigate if there is specificity in 14-3-3/target protein interaction, the H^+-ATPase/14-3-3 interaction was used as a model system. The study indicated some specificity but also a wide redundancy. To further analyse the question of specificity at different levels promoter:GUS fusions were utilized. The results clearly indicate a developmental, cell-, tissue- and organ-specific expression for all of the 14-3-3 isoforms in Arabidopsis. There is not a single case where the promoter of one isoform shows an expression that is identical to the expression of another isoform.

Key words
14-3-3, H^+-ATPase, Arabidopsis, GUS, protein-protein interaction, expression

Classification system and/or index terms (if any)

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Mum and dad for always believing in me

My sons Jonatan and Alexander, you make life worth living

My husband Niklas, for everything. I love you

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THIS THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

Paper I

Alsterfjord M, Sehnke P C, Arkell A, Larsson H, Svennelid F, Rosenquist M, Ferl R J, Sommarin M and Larsson C (2004) Plasma membrane H^+-ATPase and 14-3-3 isoforms of Arabidopsis leaves: Evidence for isoform specificity in the 14-3-3/H^+-ATPase interaction. Plant Cell Physiol 45: 1202-1210

Paper II

Alsterfjord M, Arkell A, Larsson C and Sommarin M (2010) Expression patterns of Arabidopsis 14-3-3 isoforms. Manuscript in preparation.

1

INTRODUCTION

The 14-3-3 proteins constitute a family of highly conserved proteins with a molecular mass of about 30 kDa. The first 14-3-3 proteins were discovered in 1967 as part of an examination of brain tissue proteins (Moore and Perez 1967). The 14-3-3s were given their name based on their fraction number on DEAE-cellulose chromatography and migration position on starch gel electrophoresis. For a long time 14-3-3s were thought to be brain-specific proteins but in 1992 14-3-3s were found in several plants (Brandt *et al* 1992, Hirsch *et al* 1992, Lu *et al* 1992) as well as in the yeast *Saccharomyces cerevisiae* (van Heusden *et al* 1992). 14-3-3s have now been found in all organisms examined except for members of the prokaryotic kingdom. In unicellular organisms as yeast there are only few 14-3-3 isoforms whereas in multicellular organisms, such as Arabidopsis, there may be as many as 15 isoforms (van Heusden *et al* 1995, Rosenquist *et al* 2000, Rosenquist *et al* 2001).

The first functional properties of 14-3-3s described were their ability to bind to and activate tyrosine and tryptophane hydroxylase in bovine brain in the presence of Ca^{2+}/calmodulin-dependent protein kinase type II (Ichimura *et al* 1988). Over the following years more functions were discovered, such as inhibition of protein kinase C in sheep brain, activation of Raf in *Xenopus* ooccytes and binding to cruciform DNA in humans (Toker *et al* 1990, Aitken *at al* 1995, Todd *et al* 1998). The first 14-3-3 protein identified in plants was found to be involved in gene regulation (Lu *et al* 1992). Now, 14-3-3 proteins are

recognized as key regulators of primary metabolism and membrane transport in plants (Bachmann *et al* 1996, Moorhead *et al* 1996, Toroser *et al* 1998, Huber *et al* 2002). For eukaryotes in general, 14-3-3s have been found to be involved in numerous processes in the cell (Finnie *et al* 1999, Ferl *et al* 2002, Roberts 2003) and over 700 binding partners have been identified and the total number of potential plant 14-3-3 targets is more than 300 (MacKintosh 2004, Oecking *et al* 2009).

The aim of my work has been to understand why multicellular organisms need so many 14-3-3 isoforms. One possibility is that there is specificity in the 14-3-3/target protein interaction, which we have investigated in the model plant *Arabidopsis thaliana* using the plasma membrane H^+-ATPase/14-3-3 interaction as a model system. Another possibility is that there is a specific expression of isoforms (Daugherty *et al* 1996), which we also have investigated in Arabidopsis. This was done using promoter:GUS fusions, which has yielded information on the cell-, tissue- and organ-specific distribution of most of the fifteen 14-3-3 isoforms, as well as on developmental regulation of 14-3-3 expression.

2

STRUCTURE AND FUNCTION

The 14-3-3 proteins are small acidic proteins, which are highly conserved even between kingdoms (Paul *et al* 2009) (Figure 1).

The 14-3-3 proteins assemble as stable homo- or heterodimers (Roberts 2000, Jones *et al* 1995) and, for example, 14-3-3 omega is shown to dimerize with at least 10 of the other 14-3-3 isoforms in Arabidopsis (Chang *et al* 2009). All 14-3-3 proteins appear to share a similar tertiary structure, first defined for the human isoforms tau (Xiao *et al* 1995) and zeta (Liu *et al* 1995) (Figure 2A). Each polypeptide is organized into nine anti-parallel α-helices, each separated by a short loop. The four N-terminal helices lie in a planar array and create an extensive dimerization surface. Parts of helices 1 and 2 from one monomer and parts of helices 3 and 4 from the other monomer form the dimerization domain (Liu *et al* 1995, Xiao *et al* 1995, Aitken 2002). The amino acids in the dimerization domains are not completely conserved which might indicate differences in dimer formation between isoforms.

The crystal structure has been solved for several mammalian and plant 14-3-3s (Liu *et al* 1995, Xiao *et al* 1995, Würtele *et al 2003*) and the extreme conservation of the central core region of the 14-3-3s make it very likely that this structure is a common feature of all 14-3-3s in all eukaryotes. However, all of the known crystal structures fail to resolve the N and C termini, which (along with several small regions within the molecule) are highly divergent among isoforms. Thus it is possible to consider the model as generally applicable to all plant 14-3-3s while recognizing that divergent areas might well contribute to specific structures and regulatory functions (Paul *et al* 2008).

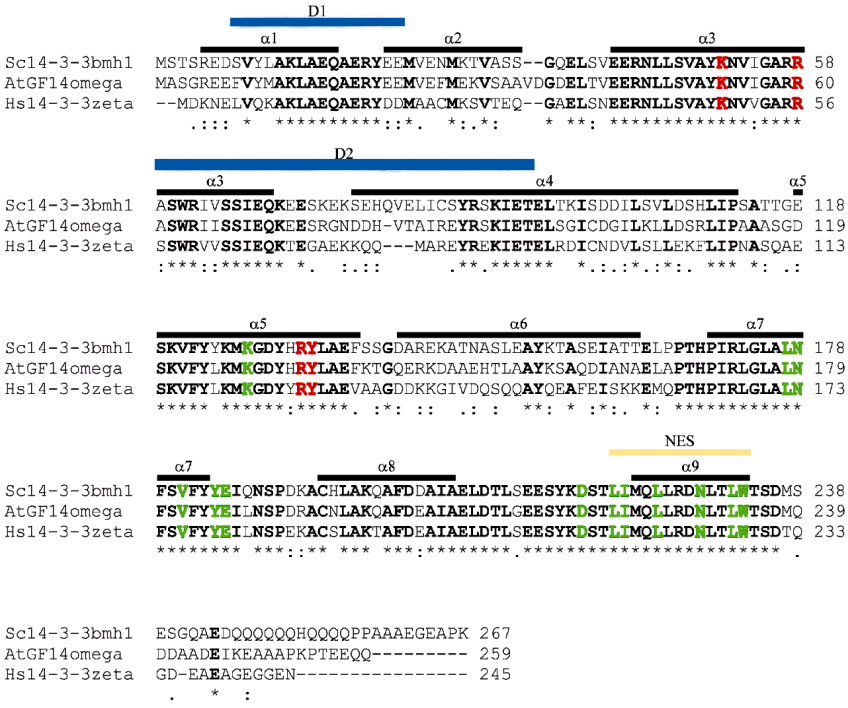

Figure 1 *Amino acid sequence alignment (Clustal W 1.83) of Saccharomyces cerevisiae 14-3-3 bmh1, Arabidopsis thaliana GF14 omega and human 14-3-3 zeta. Black bars indicates α-helices, blue bars indicate dimerization domains, yellow bar indicates the nuclear export signal (NES), amino acids in red are directly interacting with the phosphogroup (pS/pT) of the target protein and amino acids in green are also involved in interactions with the target protein (compare Figure 2). Completely conserved residues are indicated with * and bold letters, substitutions with similar chemistry are indicated with : and less conserved residues are indicated with* **.** *(modified fram Alsterfjord 2006).*

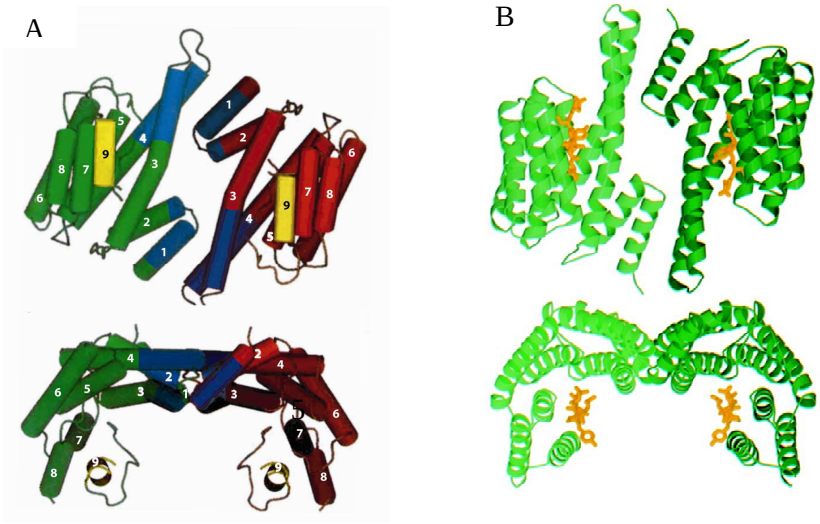

Figure 2 A *Structure of the human 14-3-3 zeta dimer. The numbers indicate helix numbers. Blue and yellow parts on helices indicate dimerization domains and the nuclear export signal (NES), respectively (compare Figure 1) (modified from Liu et al 1995).* ***B*** *A dimeric 14-3-3 protein binding two peptides (in yellow), mimicking the C-terminal end of the plasma membrane H^+-ATPase from tobacco (Würtele et al 2003)* **.**

14-3-3s typically bind to phosphorylated motifs in their target proteins. Thus, the α-helices forms the walls of an amphipathic groove, large enough for a phosphopeptide chain of a target protein to fit in (Figure 2B). The amino acid residues exposed in the binding groove are highly conserved and each subunit of the dimer is able to bind one target protein independently of the target in the other subunit (Liu *et al* 1995, Obsil *et al* 2001, Rittinger *et al* 1999). The C-terminal region of a 14-3-3 was shown to change in conformation when phosphorylated (Obsilova *et al* 2004). This relatively unconserved region of the 14-3-3 has been hypothesized to play an autoinhibitory role in ligand binding (Obsilova *et al* 2004, Truong *et al* 2002) through its high content of negatively charged amino acid residues, possibly in an isoform-specific manner (Figures 1 and 3).

The C-terminal end is the part of the 14-3-3s that differs most, both in sequence and length, and the length of Arabidopsis C termini vary from 8 to 31 amino acids, counting from the last conserved residue (Figure 3). Also, the amino acids facing the outside of the 14-3-3 molecule are relatively less conserved (Liu *et al* 1995, Xiao *et al* 1995).

Figure 3 *The C-terminal amino acid sequences from the last conserved residue of all 14-3-3s in Arabidopsis except the putative products of grf14 (xi) and grf 15. grf is short for G-box regulating factor (see section 3). grf14 is truncated N-terminally of this domain. Acidic amino acid residues are indicated in bold (modified from Alsterfjord 2006*).

The amino acid sequence motifs that 14-3-3s bind to are usually phosphorylated but also non-phosphorylated motifs have been identified. These non-phosphorylated motifs contain negatively charged amino acids which replace and mimic the negatively charged phosphogroup. The phosphorylated motifs have been divided into three modes (Ganguly *et al* 2005, Coblitz *et al* 2006), as shown in Figure 4.

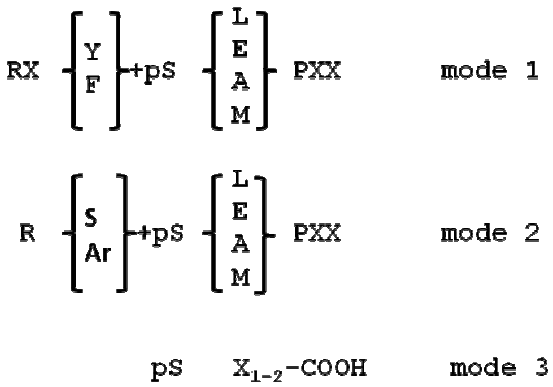

Figure 4 *The phosphorylated motifs that 14-3-3 bind to have been divided into three modes (mode 1, 2 and 3). pS may be replaced by pT.*

The proline in mode 1 and 2 is needed to bend the peptide to exit the binding groove (Rittinger *et al* 1999) (Figure 5). The mode 3 motifs are also called C-terminal binding motifs, since they constitute the C termini of the target proteins (Ganguly *et al* 2005). The motifs in mode 3 do not need a proline to bend the peptide since the target protein ends in the binding groove (Figure 5).

The C-terminal motif was first found in the plasma membrane proton (H^+)-ATPase of plants, Y/H-pT-V/L/I-COOH, which constitutes the last three amino acids in Arabidopsis plant plasma membrane H^+-ATPase isoforms 1 to 11 (Olsson *et al* 1998, Fuglsang *et al* 1999, Svennelid *et al* 1999). In 2003, a crystallized structure of 14-3-3 from tobacco, together with the fungal toxin fusicoccin and a phospopeptide mimicking the mode 3 binding motif of a tobacco H^+-ATPase, showed that the same residues in the binding groove of the 14-3-3 protein are involved in binding the phosphothreonine in mode 3 as in mode 1 and 2 binding to phosphoserine (Würtele *et al* 2003, Ganguly *et al* 2005, Coblitz *et al* 2006). Fusicoccin occupies the hydrophobic end of the binding groove, which is not occupied in mode 3 (Figure 5) and enhances binding strength 100-fold (Würtele *et al* 2003).

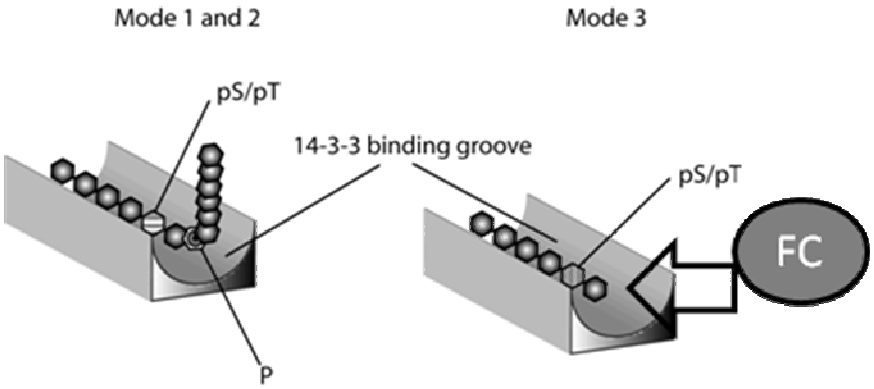

Figure 5 *The different modes of 14-3-3 binding (Modified from Coblitz et al 2006 and Alsterfjord 2006). FC=Fusicoccin.*

All 14-3-3 proteins contain a nuclear export signal (NES) (Figure 2). This domain can bind to the chromosome maintenance region 1 (Crm1), which interacts with the nuclear pore and in this way the NES-containing proteins will be exported from the nucleus (Fornerod *et al* 1997, Fukuda *et al* 1997, Ossareh-Nazari *et al* 1997, Stade *et al* 1997). The NES domain in 14-3-3 proteins includes amino acids that are also involved in target binding and thereby the target will compete with the Crm1 (Rittinger et al 1999). Even if one NES domain is occupied by a target protein, the 14-3-3 proteins assemble as dimers and there will thus be one free NES domain. 14-3-3s can be found in the nucleus and this indicates that the NES signal can be hidden (Cutler *et al* 2000, van Zeijl *et al* 2000). When the NES signal is exposed, the 14-3-3 protein will be exported from the nucleus together with its target. This is a way of removing proteins, such as transcription factors, from the nucleus, as a response to other regulatory mechanisms (Fornerod *et al* 1997, Fukuda *et al* 1997, Ossareh-Nazari *et al* 1997, Stade *et al* 1997).

The number of identified physiological functions involving 14-3-3 proteins has increased rapidly since the discovery of 14-3-3s in 1967 (Moore and Perez 1967). Some of the physiological functions involving 14-3-3 proteins are regulation of signaling pathways, apoptosis, cell cycle entry, intracellular trafficking and metabolism (Darling *et al* 2005) (Figure 6).

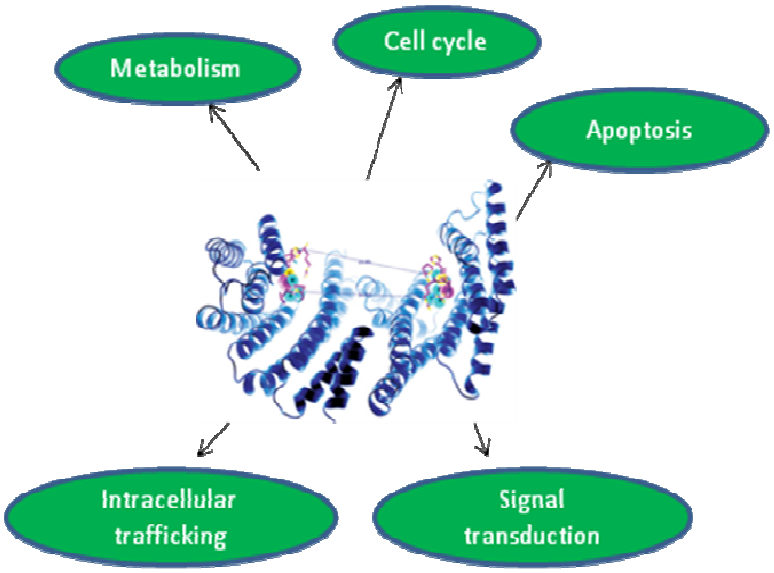

Figure 6 *Some of the numerous physiological functions that involves 14-3-3 proteins (modified from Darling et al 2005 and Johnson et al 2010*)**.**

Figure 7 shows the various proposed mechanisms of action for 14-3-3 proteins (Darling *et al* 2005). **(I)** 14-3-3 proteins have a rigid structure which leads to deformation of the target protein with little or no change in the structure of the 14-3-3 dimer (Yaffe 2002, Obsil *et al* 2001). The deformation of the target protein will lead to a change in activity, an increase or a decrease. Plant ATP synthases in chloroplasts and mitochondria are examples of proteins that are negatively regulated by this mechanism (Bunney *et al* 2001, Moorhead *et al* 1999) whereas the plant plasma membrane H^+-ATPase is upregulated upon 14-3-3 binding (Jahn *et al* 1997, Oecking *et al* 1997, Olsson *et al* 1998, Fuglsang *et al* 1999, Svennelid *et al* 1999). **(II)** 14-3-3s can mask a region of a protein and in that way e.g. hinder protein/protein interaction. **(III)** A 14-3-3 can hold two phosphoproteins close together, stabilizing their interaction. **(IV)** 14-3-3s can bind to phosphorylated targets and prevent either dephosphorylation or proteolysis. One example of prevention of proteolysis is 14-3-3 binding to phosphorylated plant nitrate reductase (Weiner and Kaiser 1999, Cotelle *et al* 2000). **(V)** Binding to 14-3-3 can increase the nuclear export or decrease nuclear import for the target protein (Muslin and Xing 2000).

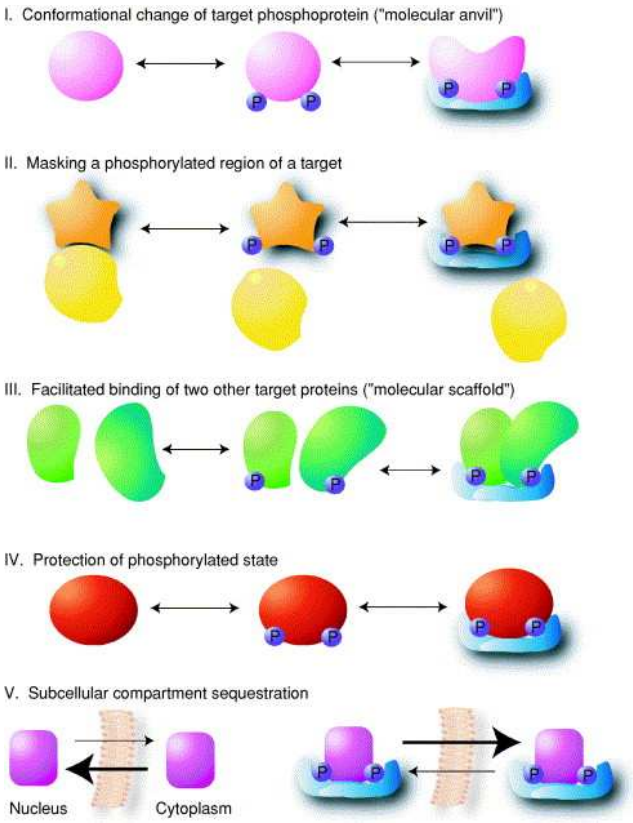

Figure 7 *The various proposed mechanisms of action that 14-3-3 can take on its targets (Darling et al 2005*).

Thus, the functional diversity of 14-3-3 proteins is very high, and may even have facilitated evolutionary changes in target protein families (Johnson *et al* 2010).

3

14-3-3 IN PLANTS

14-3-3s were first discovered in plants (spinach, pea, barley and Arabidopsis) in 1992 (Brandt *et al* 1992, de Vetten *et al* 1992, Hirsch *et al* 1992, Lu *et al* 1992).

Numerous data for 14-3-3 functions and 14-3-3 interactants in plants have come from the study of *Arabidopsis thaliana*, a well-characterized model organism first described by Laibach in 1943. Arabidopsis has five chromosomes and approximately 26,000 genes and was fully sequenced in 2000 (Arabidopsis Genome Initiative). The life cycle of *Arabidopsis* from germination to mature seed is approximately 6 weeks, facilitating its study. It is also susceptible to genetic manipulation with mutagens or *Agrobacterium tumefaciens* infection to create transgenic Arabidopsis plants. Arabidopsis has 15 genes for 14-3-3s (Rosenquist *et al* 2001) which may be divided into two groups dependent on their exon patterns, the non-epsilon group and the epsilon group (Figure 8).

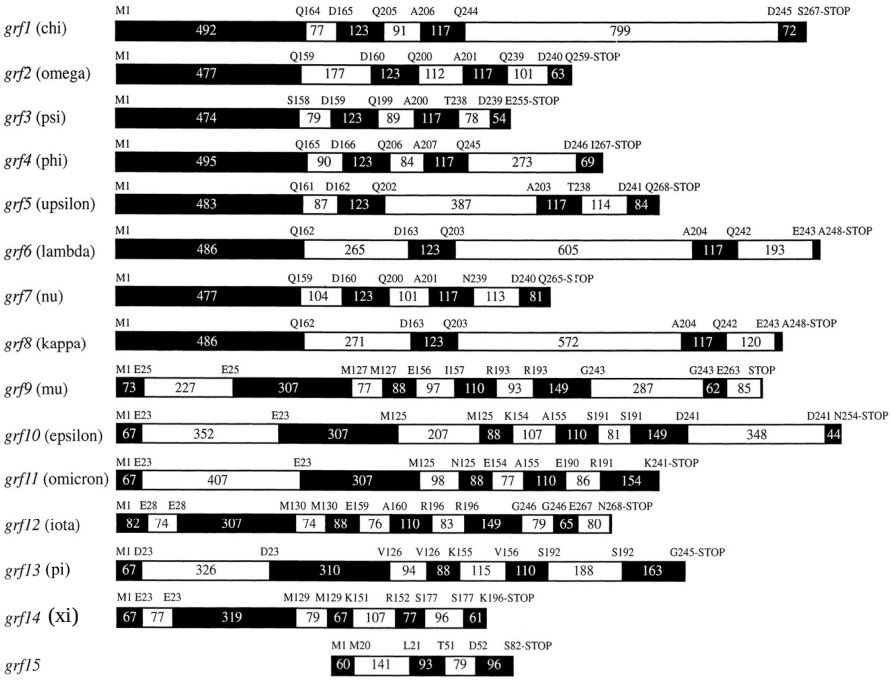

Figure 8 *Gene maps of all Arabidopsis 14-3-3 genes. Exons are indicated as black boxes and introns as white boxes. Exon and intron sizes are indicated with the number of bases within each box. The genes can be divided into two groups based on the exon patterns: grfs 1 to 8 (the non-epsilon group) and grfs 9 to 14 (the epsilon group). grf 15 is aligned N-terminally with its closest neighbors, grfs 3 and 5 (modified from Rosenquist et al 2001). Grfs stands for G-box regulating factors.*

The G-box is a common regulatory element found in many plant gene promoters, and historically 14-3-3s have been named GF14 proteins in Arabidopsis, because the protein was first identified to be a “G-box factor 14-3-3 homologue” (de Vetten *et al* 1992). The 14-3-3 genes in Arabidopsis are named *grfs* which stands for G-box regulating factors. Experiments showed that a plant 14-3-3 was able to function as a mammalian 14-3-3 (Lu *et al* 1994) providing evidence of the conserved nature of 14-3-3s. In 1994, 14-3-3 was identified as the binding protein for the fungal toxin fusicoccin (Korthout and de Boer 1994, Marra *et al* 1994, Oecking *et al* 1994). The binding protein was later shown to be a complex of the plasma membrane H^+-ATPase and 14-3-3 (Jahn *et al* 1997, Oecking *et al* 1997), and binding of 14-3-3 was shown to activate the plasma membrane H^+-ATPase.

Since 1992, 14-3-3s have been discovered to be involved in numerous processes in plants, such as metabolism, the cell cycle, apoptosis, signal transduction and intracellular trafficking.

Thus, 14-3-3 proteins were found to interact with the mitochondrial and chloroplastic ATP synthase, negatively regulating the ATP synthesis in both organelles (Bunney *et al* 2001).

In anti-sense experiments, down-regulation of specific 14-3-3 isoforms resulted in increase in leaf starch accumulation. The starch synthase III family was identified as a possible 14-3-3 target as all members of the family contain a 14-3-3 binding motif. The interaction between the starch synthase III member, DU1 and 14-3-3 was demonstrated, confirming a role for 14-3-3 proteins in regulation of starch synthesis (Sehnke *et al* 2001).

Regulation of key enzymes such as sucrose phosphate synthase, starch synthase (Toroser *et al* 1998), nitrate reductase (Bachmann *et al* 1996, Moorhead *et al* 1996), the plasma membrane H^+-ATPase as well as the mitochondrial and chloroplast ATP synthases implies that 14-3-3 is essential for regulating carbon and nitrogen metabolism in plants (Kulma *et al* 2004, Harthill *et al* 2006, Huber *et al* 2002).

14-3-3s also play diverse roles during seed germination. Two major hormones, abscisic acid (ABA) and gibberellins have opposite functions during germination. Gibberellins generally promote germination, whereas ABA inhibits germination and has a role in inducing and maintaining dormancy. ABA seems to mediate its effect by promotion of the *Em* (embryo) gene, and in the absence of *Em* transcripts, maize produces embryos that germinate while still attached to the parent plant. By use of a yeast two-hybrid system, 14-3-3 dimers were demonstrated to provide a structural link between elements of the transcriptional protein complex and the *Em* promoter to inhibit germination (Schultz *et al* 1998). RSG is a tobacco plant bZIP transcription factor that regulates shoot growth by altering transcription of genes required for gibberellin synthesis. A yeast two-hybrid screen demonstrated that RSG interacts with several isoforms of tobacco 14-3-3 in a phosphorylation-dependent manner (Igarashi *et al* 2001, Ishida *et al* 2004). If a point mutation is done in the phosphorylated motif, the transcriptional activity of RSG is increased due to transcription factor accumulation within the nucleus. In wild-type cells, RSG is distributed throughout the cell. 14-3-3 therefore negatively regulates gibberellin signaling by localizing RSG outside the nucleus.

A recent studie has demonstrated 14-3-3s to be an essential part of brassinosteroid (BR) signaling (Gampala *et al* 2007, Ryu *et al* 2007*)*. Many components in this signaling pathway are already well known in Arabidopsis, including the cell surface receptor kinase BRASSINOSTEROID-INSENSITIVE 1 (BRI1) and its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) as well as the transcription factor BRASSINAZOLE-RESISTANT 1 (BZR1), which directly regulates BR responsive gene expression (Gendron *et al* 2007). The recent studies showed 14-3-3 to bind the BZR1 transcription factor upon phosphorylation by an intracellular kinase which is active in the absence of BR (Gampala *et al* 2007, Ryu *et al* 2007*)*. Comparable to RSG, 14-3-3 association mediates the cytoplasmic retention and/or nuclear export of BZR1, thus efficiently inhibiting its function.

4

ISOFORM SPECIFICITY IN TARGET PROTEIN INTERACTIONS

In multicellular organisms there is a relatively large number of 14-3-3 isoforms and the question arises as to the reason for this. One possibility is that there is specificity in the 14-3-3/target protein interaction, which we have investigated in the model plant *Arabidopsis thaliana* using the plasma membrane H^+-ATPase/14-3-3 interaction as a model system. Another possibility is that there is a developmental, cell-, tissue- or organ-specific expression of isoforms (Daugherty *et al* 1996), which we also have investigated in Arabidopsis (see section 5).

14-3-3 isoform specificity has been shown for some targets. Thus, both plasma membrane H^+-ATPase (see section 4.1, Rosenquist *et al* 2000, Emi *et al* 2001) and nitrate reductase (Bachmann *et al* 1996) show binding specificity to14-3-3 isoforms in Arabidopsis. In barley, 14-3-3B and C are efficient inhibitors of nitrate reductase whereas 14-3-3A is not (Sinnige *et al* 2005). Also the phototropin receptor kinase 1 (phot1) shows specificity in Arabidopsis. 14-3-3 binding to phot1 is limited to non-epsilon 14-3-3 isoforms (Sullivan *et al* 2009).

The C terminus of 14-3-3s may act as an autoinhibitory domain that interferes with ligand binding (Truong *et al* 2002, Shen *et al* 2003, Kubala *et al* 2004, Silhan *et al* 2004). All 14-3-3 C termini contain acidic amino acids (Figure 3) which may mimic phosphorylated target motifs and thus compete with proper target binding. The parts of 14-3-3 that bind to a target protein are very conserved but the C terminus is the part of the 14-3-3 that differs most. This feature may be one factor leading to isoform specificity.

Posttranslational modification (proteolytic cleavage or phosphorylation) could be another way to increase specificity of 14-3-3s (Fuller *et al* 2006). Proteolytic cleavage was shown in barley (van Zeijl *et al* 2000) and also the 43 kD band in Figure 10 is probably a proteolytic cleavage product of a 14-3-3 dimer (Bernfur and Alsterfjord, personal comunication). 14-3-3s may also be posttranslationally modified by phosphorylation. Phosphorylation sites in the dimerization domain of human 14-3-3s have been identified (Aitken 2002).

4.1 The H^+-ATPase/14-3-3-interaction

The plasma membrane H^+-ATPase couples ATP hydrolysis to proton transport. This creates the pH and potential difference across the plasma membrane required by secondary transporters whose activity is directly dependent upon the proton motive force. In plants, the plasma membrane H^+-ATPase also participates in other functions essential for normal plant growth such as salt tolerance, intracellular pH regulation and cellular expansion (Morsomme and Boutry 2000, Palmgren 2001). Given these multiple physiological roles and the high ATP consumption, the H^+-ATPase has to be tightly regulated. The C terminus of the H^+-ATPase acts as an autoinhibitory domain (Palmgren *et al* 1991) and when it is phosphorylated 14-3-3 can bind, the autoinhibitory domain is displaced and the activity of the H^+ pump is increased (Jahn *et al* 1997, Oecking *et al* 1997, Olsson *et al* 1998, Fuglsang *et al* 1999, Svennelid *et al* 1999).

Fusicoccin is a wilt-inducing toxin produced by the fungus *Fusicoccum amygdale.* The natural hosts of *F. amygdale* are almond and peach trees. The toxin is commonly used in plasma membrane H^+-ATPase experiments as it causes an “irreversible” 14-3-3 binding and thus gives a stable H^+-ATPase/14-3-3 complex (Jahn *et al* 1997, Oecking *et al* 1997, Fullone *et al* 1998, Würtele *et al* 2003).

Similar to the Arabidopsis 14-3-3 proteins the Arabidopsis plasma membrane H^+-ATPase belongs to a large gene family, with 12 predicted isoforms of which 11 are expressed (Arango *et al* 2003). The plasma membrane H^+-ATPase is the main 14-3-3 target in the plasma membrane and during conditions requiring full activation of H^+ pumping several percent of total cellular 14-3-3 may be involved in activation of the H^+-ATPase (Paper I). Considering the large number of 14-3-3 and H^+-ATPase isoforms in Arabidopsis, specificity in binding may exist between isoforms. This assumption is supported by the large differences in

amino acids in the C-terminal binding motif of the H^+-ATPase isoforms, which may affect binding (Figure 9). There is also another motif in the C terminus of the H^+-ATPase (DIE/DID/DLE)) that has been suggested as a nonphosphorylated binding motif for 14-3-3 (Visconti *et al* 2003) and this motif is not found in H^+-ATPase isoform 10 (AHA10) which may indicate a difference in binding properties (Figure 9).

Figure 9 *Amino acid sequences of the C termini of the 11 expressed Arabidopsis H^+-ATPase (AHA) isoforms harbouring the 14-3-3 binding motifs. The most conserved part of the mode 3 motif is indicated in red, the more variable beginning of the motif is in green and a proposed nonphosphorylated binding motif (Visconti et al 2003) is in yellow (modified from Alsterfjord 2006*).

Using 12 of the Arabidopsis 14-3-3 isoforms, we could show that they all bind to the H^+-ATPase present in isolated Arabidopsis plasma membranes (Paper I). This was however not the case *in vivo*. Using 14-3-3 isoform-specific antibodies, all isoforms tested (all but omicron, iota, psi and pi for which we do not have access to isoform-specific antibodies) except kappa and phi were identified in the supernatant (Figure 10).

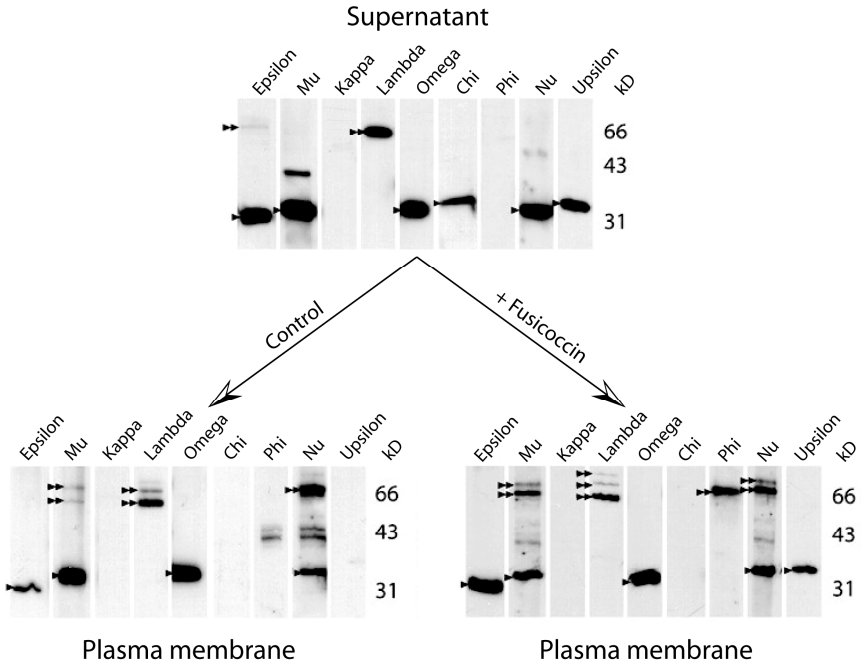

Figure 10 *14-3-3 isoforms in plasma membrane and supernatant fractions visualized by isoform-specific antibodies. Single arrows indicate the position of 14-3-3 monomers and double arrows the position of dimers (modified from Paper I and Alsterfjord 2006). The 43 kD band is probably a proteolytic cleavage product of the 14-3-3 dimer (Bernfur and Alsterfjord, personal communication*).

However, the plasma membrane fraction lacked not only kappa and phi but also chi and upsilon in the absence of fusicoccin (Figure 10, control). Thus, we could detect differences in distribution of the 14-3-3s between isolated plasma membranes from Arabidopsis leaves and a supernatant fraction, representing all soluble proteins. In the presence of fusicoccin which increases the H^+-ATPase/14-3-3 interaction 100-fold (Würtele *et al* 2004) also phi and upsilon were attached to the plasma membrane, but not chi. Thus, phi and upsilon are accessible to the plasma membrane but have either a low affinity for the plasma membrane H^+-ATPase isoforms present or are, in the absence of fusicoccin, occupied by other targets for which they have higher affinity. Chi may be localized in another compartment than the cytosol and may therefore not be available to the plasma membrane. Altogether this suggests that there is some isoform specificity in the 14-3-3/H^+-ATPase interaction in vivo (Paper I).

5

SPECIFICITY IN EXPRESSION

A developmental, cell-, tissue- or organ-specific expression is suggested by the large number of 14-3-3 isoforms in multicellular organisms compared to the very few in unicellular organisms (Rosenquist *et al* 2000, Alsterfjord *et al* 2004). An organ-specific expression was shown for Arabidopsis 14-3-3 iota, which was only expressed in the flower whereas Arabidopsis omicron was expressed in leaf, root and flower (Rosenquist *et al* 2001). Arabidopsis 14-3-3 mu was shown to be expressed in all tissues and developmental stages (Kuromori and Yamamoto 2000) and transcripts for all of the Arabidopsis 14-3-3 isoforms except pi, iota and psi are present in leaves (Paper I). Also in mammalians some 14-3-3 isoforms are widely expressed whereas others show a more specific expression. Human 14-3-3 zeta is present in high levels in the brain grey matter, 14-3-3 gamma is specific for the central nervous system, 14-3-3 epsilon is found in the pineal gland and the retina, and 14-3-3 tau is only present in glial cells (Watanabe *et al* 1993, Takahashi 2003).

There are also differences in subcellular localization between isoforms. Arabidopsis 14-3-3 epsilon, mu, nu and upsilon are present in both the chloroplast and cytoplasm (Sehnke *et al* 2000) and three isoforms in barley were detected in mitochondria (Bunney *et al* 2001), and it has been suggested that some 14-3-3 subcellular localization is driven by both isoform specificity and target interactions (Paul *et al* 2005).

5.1 Promoter analyses of 14-3-3 isoforms

Agrobacterium tumefaciens infection was used to create transgenic Arabidopsis plants to see if there is specificity in developmental expression and localization of the different 14-3-3 isoforms. *A. tumefaciens* has the ability to insert its natural so called T-DNA into the nuclear genome of Arabidopsis. The specific T-DNA is chosen by two short sequences, left border and right border. The DNA fragment that is to be inserted into the genome of the plant is cloned between the left and the right border together with a marker and thus an artificial T-DNA is created and the *A. tumefaciens* can introduce it into the genome of the plant.

The methods to get *A. tumefaciens* to insert the T-DNA into the plant have rapidly changed and today it is relatively simple to get transgenic plants. The Arabidopsis flower is simply dipped into a solution of *A. tumefaciens* approximately five days before the flowers open, the so called floral-dip method (Desfeux *et al* 2000).

The *Escherichia coli uidA* gene encoding β-glucuronidase (GUS) is one of the most effective reporter gene systems used for evaluating transient and stable transformation in plants. Since its description by Jefferson *et al* (1987), the GUS gene fusion system has found extensive application in plant gene expression studies because of the enzyme stability and high sensitivity and suitability of the assay to detection by fluorometric, spectrophotometric or histochemical techniques. The GUS protein is a 68kD homo-tetramer that catalyzes the hydrolysis of β-glucuronides. In most eukaryotic organisms, these are formed to detoxify and excrete xenobiotic and endogenous waste products (Fior *et al* 2009).

To see if there is specificity in expression and localization 1,5kb upstream of the start codon of thirteen of the fifteen 14-3-3 isoforms have been fused to reporter genes coding for GUS and enhanced green fluorescent protein (EGFP) (the EGFP was not used in this work) (Figure 11). This 1,5 kb region represents the 14-3-3 promoter. This construct is transformed into Arabidopsis with *A. tumefaciens*. When the transgenic plants are incubated with the substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt (X-gluc) a blue precipitation is created where the 14-3-3 isoform normally is expressed. This precipitation can easily be seen by the eye or in a microscope. To see if there is any difference in expression of the promoter when the plant matures, samples were taken at four different developmental stages, seedlings (one week old), adult leaves (three weeks old), flowers (four to five weeks old) and siliques (six weeks old).

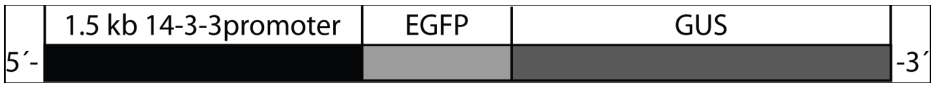

Figure 11 *Schematic view of the promoter-reporter genes fusion used in expression analyses of Arabidopsis 14-3-3 promoters (Alsterfjord 2006*).

5.2 Results from the promoter:GUS analysis

The data presented below demonstrate a specific developmental cell-, tissue and organ distribution of the Arabidopsis 14-3-3 isoforms. Arabidopsis transformed with the 14-3-3 promoter-EGFP-GUS construct makes the study of both tissue distribution and developmental regulation possible. The results support the idea that the various 14-3-3 isoforms have separate and distinctive tissue distribution, suggesting that plant 14-3-3 isoforms may indeed have specific roles within individual tissues (Paper II).

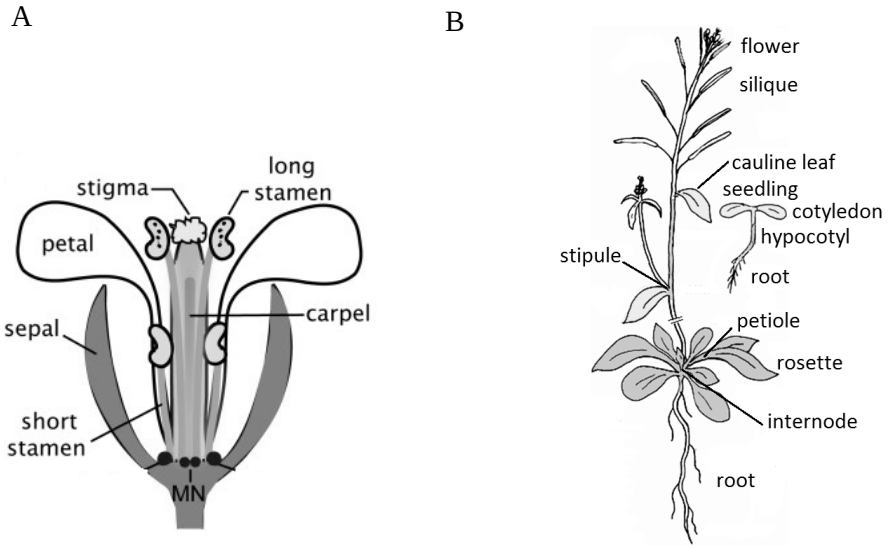

Figure 12 *A schematic Arabidopsis thaliana flower***(A)** *(modified from geochembio.com) and plant* **(B)** *(modified from Winter et al 2007) with different tissues indicated.*

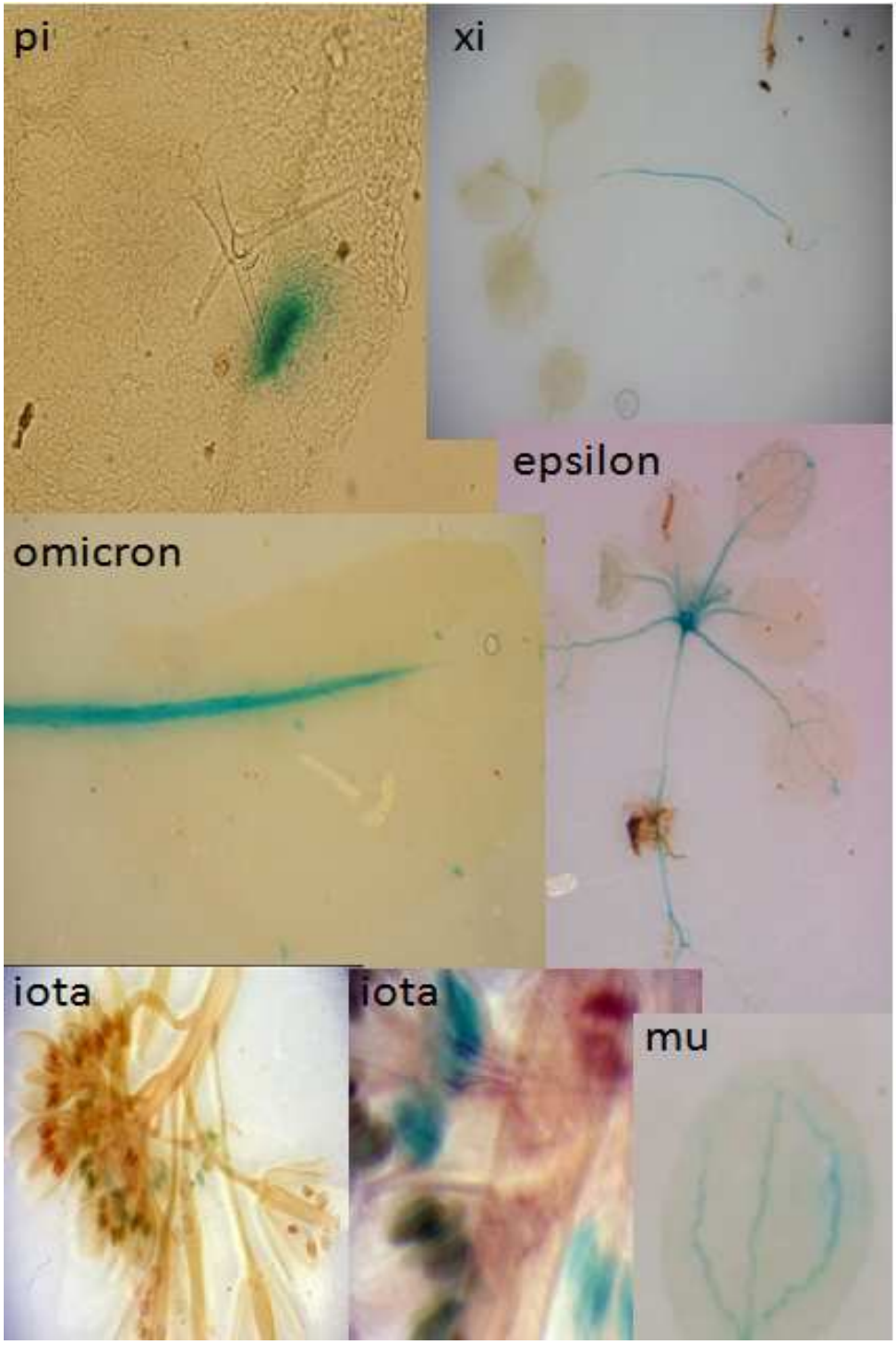
pi
xi
epsilon
omicron
iota
iota
mu

Figure 13 *GUS-staining detecting promoter activity of the Arabidopsis 14-3-3 isoforms in the epsilon group.The promoter of pi shows activity in hydathodes, xi shows promoter activity in the hypocotyls of seedlings, the omicron promoter is active in the petioles of leaves and the promoter of epsilon shows expression especially in vascular tissue. The promoter of iota is expressed in early stamens exclusively and the mu promoter shows expression in green tissue, especially in vascular tissue. Compare figure 12.*

5.2.1 The epsilon group

The promoters of the epsilon group show a more specific expression than the promoters of the non-epsilon group (Figures 13 and 15).

The Arabidopsis 14-3-3 **pi** shows promoter activity in hydathodes, pollen and seeds only.

The isoform **xi** has never been shown to be expressed, however the promoter shows activity in the hypocotyls of seedlings, in roots, in pollen and in seeds.

The **omicron** promoter shows an expression pattern which is different from all the other Arabidopsis 14-3-3 promoters. Activity is found in internodes of vascular tissue, in leaves and in the petioles of leaves. The promoter of omicron also showes expression in pollen and seeds.

The promoter of **epsilon** shows activity in young tissue, especially in vascular tissue, in roots (it is the only isoform that shows expression in the root buds) and in stipules.

The promoter of **iota** is exclusively active in early stamens but not anywhere else in the plant.

The promoter of **mu** shows expression in the green tissues of the plant, especially in the vascular tissues, but also in roots and sepals.

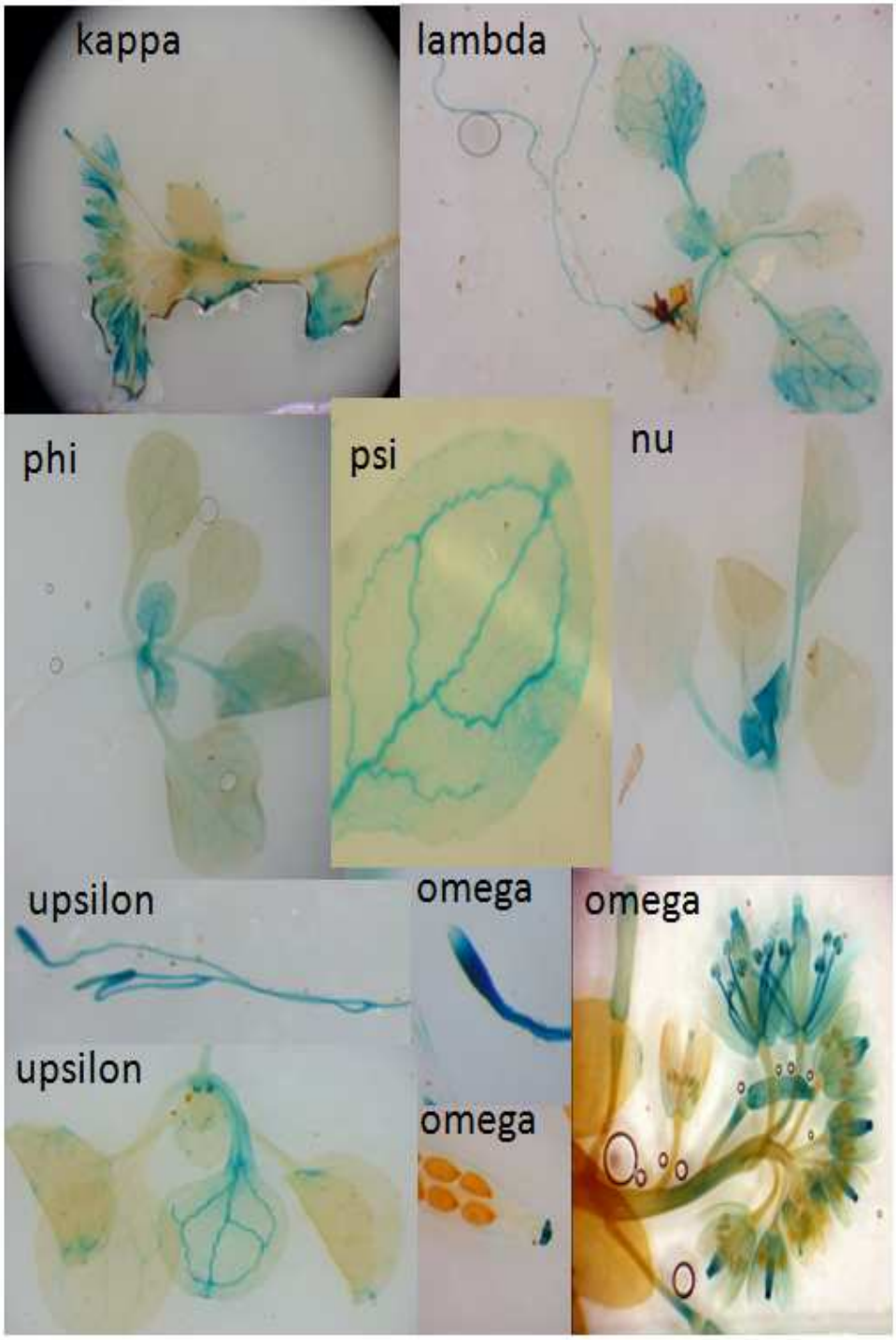
kappa
lambda
phi
psi
nu
upsilon
omega
omega
upsilon
omega

Figure 14 *GUS-staining detecting promoter activity of the Arabidopsis 14-3-3 isoforms in the non-epsilon group. The kappa promoter shows much activity in the flower and in cauline leaf hydathodes, the lamda promoter is active primarily in vascular tissue. The phi promoter shows activity in young tissues, the psi promoter is mainly active in the vascular tissue and the nu promoter shows activity in young tissues and decreases with age. The promoter of upsilon is active throughout the whole plant especially in the vascular tissue and the promoter of omega is active in the flower and the activity in carpels is decreasing but is still present in the siliques. The promoter of omega is also active in the roots with the exception of the root tip. Compare figure 12.*

5.2.2 The non-epsilon group

The promoters of the non-epsilon group are widely active and shows more random expression in leaves, roots and flowers than the promoters of the epsilon group (Figures 14 and 15).

The **kappa** promoter is mainly active in young tissues and in meristematic tissues of seedlings. The roots also show activity, especially the ends of the roots. Kappa is also the only isoform whose promoter shows activity in the cauline leaf hydathodes and it also shows much activity in the flower.

The promoter of **lambda** shows activity primarily in vascular tissue, both in green tissue and in roots. Although the amino acid sequences of kappa and lambda are very much alike they do not show the same expression pattern.

The **phi** promoter is active in the young tissue of seedlings and decreases with age, the activity remains in the vascular tissue whereas activities in other parts disappear in older tissue. The roots as well as the flower show some phi promoter activity.

The promoter of **psi** is mainly active in vascular tissues and in roots.

The **nu** promoter shows activity in young tissue which decreases with ageing, and then appears again in pollen and seeds.

The promoter of **upsilon** is active throughout the whole plant especially in the vascular tissue.

In seedlings, the promoter of **omega** is active in young tissues and especially in meristem and vascular tissue. There is also high activity in the roots, with the exception of the root tip. The omega promoter is also highly active in flowers. In early flowers the activity is limited to parts of the carpels. As the flower matures the activity is changed to include the stamens, especially pollen. The promoter activity in carpels is decreasing but is still present in siliques.

5.2.3 Conclusions regarding specificity of expression

Studied at this level there is clearly cell-, tissue- and organ-specific expression for all of the Arabidopsis 14-3-3 isoforms as well as specific developmental expression. There is not a single case where the promoter of one isoform shows an expression that is identical to the expression of another isoform (figure 12). The overall finding is that the promoters of the non-epsilon group show more random expression in leaves, roots and flowers and the promoters of the epsilon group show more specific expression. For example the promoter of iota shows expression only in the early stamens and the promoter of pi shows expression in hydathodes, pollen and seeds only.

The promoter of Arabidopsis 14-3-3 chi was earlier shown to be active in several tissues of the plant detected by a promoter:GUS fusion (Daugherty et al 1996). Promoter activity was shown in roots of seedlings and mature plants, in root hairs, in the whole bud of immature flowers, in the anthers, stigma and pollen of more mature flowers, in differential style and abscission zone of immature siliques and in mature siliques throughout the tissue. Promoter activity could also be detected in imbibed seeds.

We used the promoter:GUS fusion to see where in Arabidopsis the different 14-3-3 isoforms are expressed. It should be noted that a quantitative measure of the expression was not obtained due to the fact that inhibitors of GUS activity are ubiquitous in organ tissues of Arabidopsis, tobacco and rice (Fior *et al* 2009). In order to achieve reliable quantitative results, inhibitor activity should be routinley tested during quantitative GUS assays which was not done here.

	Epsilon group						Non-epsilon group							
Isoform / Tissue	Pi	Xi	Epsilon	Omicron	Iota	Mu	Kappa	Lambda	Omega	Chi*	Phi	Psi	Nu	Upsilon
Primordial		+	+				+	+	+		+	+	+	+
Hypocotyls		+		+			+		+		+			+
Vascular hypocotyls		+	+			+		+	+		+	+		+
Petioles				+		+	+		+		+	+	+	+
Vascular petioles						+	+	+	+		+	+	+	+
Cotyledons				+			+				+			+
Vascular cotyledons			+	+		+	+	+			+	+		+
Leaves						+	+	+	+		+	+	+	+
Emerging leaves						+	+	+	+		+	+	+	+
Vascular leaves			+	+		+	+	+	+		+	+	+	+
Trichomes							+		+		+	+	+	+
Hydathodes	+		+	+		+	+	+			+			+
Roots		+		+		+		+	+	+	+	+		+
Vascular roots		+	+				+	+						
Lateral roots		+					+		+			+	+	+
Root branches		+	+											
Root tips		+	+									+	+	
Root buds			+											
Cauline leaf							+							
Stem							+				+		+	+
Stipules			+						+					
Sepals						+	+	+	+		+			
Stamen							+	+	+		+			+
Pollen	+	+		+	+		+		+	+	+		+	+
Carpels							+		+		+		+	+
Pedicels									+		+			+
Pedicel tips							+				+			+
Siliques							+		+	+	+			+
Seeds	+	+		+			+		+				+	+

Figure 14 *Promoter expression of Arabidopsis 14-3-3s in plant organs at different developmental stages (Paper II). Compare Figure 12 .*Data from Daugherty et al 1996.*

Our results are largely supported by the microarray data reflecting mRNA abundance, available at Genevestigator. Notably, these microarray data give a resolution at organ level and developmental stages, where as GUS staining gives a resolution at tissue and sometimes even cellular level.

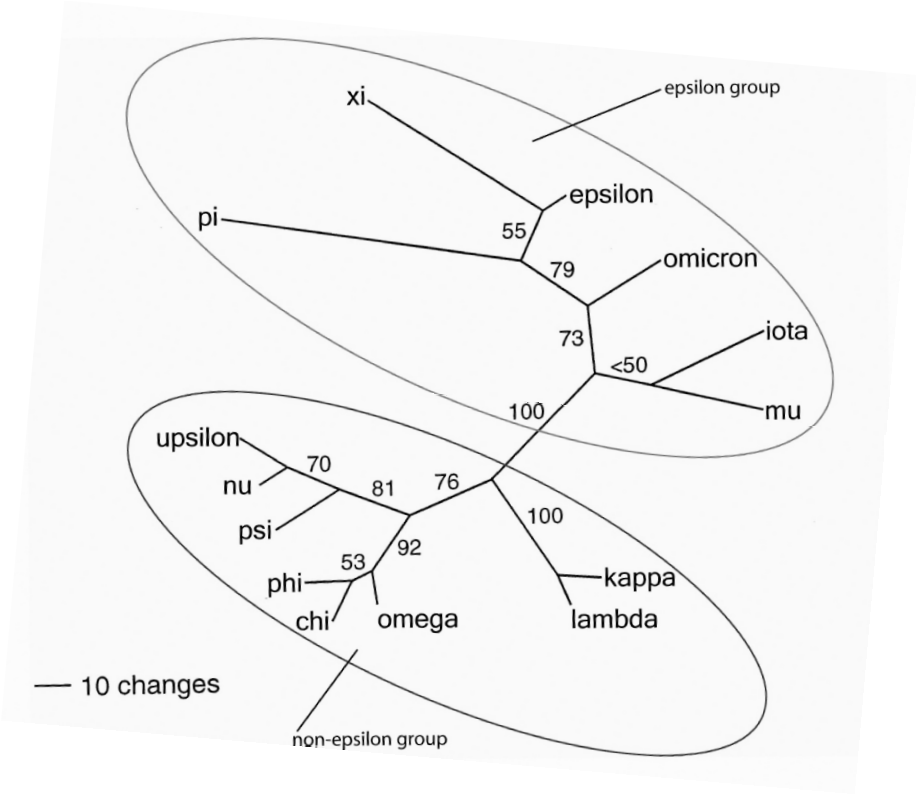

Figure 16 *A phylogenetic tree with topology representative for the Arabidopsis 14-3-3 protein family (modified from Rosenquist et al 2001 and Alsterfjord 2006*).

As seen from Rosenquist *et al* (2001) some of the 14-3-3 isoforms are more closely related than others. For example, lambda and kappa are situated alone on one branch in the phylogenetic tree and also phi, chi and omega are situated on a common branch of the tree (Figure 16). The promoters of phi, chi and omega show similar expression, except that the promoter of chi does not show any expression in green tissues (Daugherty *et al* 1996). Also upsilon, psi and nu are close and they also show similar expression except that the promoter of psi

is not at all expressed in the reproductive tissues. However, the situation is different for kappa and lambda. Although these isoforms are situated close to each other in the phylogenetic tree their promoters do not always show a similar expression.

From the present work it is clear that a phylogenetic tree does not always reveal similarities or differences in where different isoforms are expressed, and this is because a phylogenetic tree is based on the amino acid sequences of the isoforms and not on promoter resemblence.

We have tried to compare the promoter regions but it has not been successful, since it is not easy to determine which bases in the promoter region that are important. A typical promoter contains a TATA-box and a CAAT-box. The function of a TATA-box is mainly the precise initiation of transcription. The CAAT-box is frequently focused on controlling transcription initiation. A typical promoter also harbours some special DNA sequences; cis-acting elements inhibiting or activating gene transcription by combining with the transcription factor (Gou *et al* 2010). Grondal *et al* (1990) were unable to identify conserved sequence element(s) by direct comparison of the promoter region of RNA polymerase I in *Crithidia fasciculata* and similar promoter regions in other eukaryotes, including the promoter region of the most closely related kinetoplastid species. When deciding what to use as the promoter region, a 1,5kb fragment is often choosen (Engelmann *et al* 2008). The fragment contains all the necessary information for the specific expression and it is not too long to work with. Barrero *et al* (2009) showed that a 850bp fragment upstream of the start codon of ZmMRP-1 is sufficient to direct GUS reporter gene activity in maize, but both Guo *et al* (2010) and Engelmann *et al* (2008) reported that they needed at least 1,5kb to capture the promoter of the calcium sensor gene CBL1 in *Ammopiptanthus mongolicus* and the glycine decarboxylase in *Flaveria trinervia* respectively .

6

SUMMARY AND FUTURE WORK

The 14-3-3 proteins are involved in a large number of processes and over 700 target proteins have been identified. In plants, 14-3-3s are involved in regulation of metabolism, membrane transport, and signal transduction. 14-3-3s have an important role in the regulation of nitrogen and carbon metabolism and in regulating the plasma membrane H^+-ATPase.

To investigate if there is specificity in 14-3-3/target protein interaction the model system H^+-ATPase/14-3-3 was used and it indicated some specificity but also a wide redundancy (Paper I). To further analyse the question of specificity the promoter:GUS fusion was utilized. The results clearly indicated developmental, cell-, tissue- and organ-specific expression for all of the Arabidopsis 14-3-3 isoforms. There is not a single case where the promoter of one isoform shows an expression that is identical to the expression of another isoform (Paper II). The results suport the idea that 14-3-3s may indeed have specific roles within individual tissues.

It would be of interest to continue a sequence analysis of the different promoters to see if there are any similarities in the DNA sequence correlating to the similarities in expression between the different 14-3-3 isoforms.

Since promoter:GUS fusions of all isoforms of Arabidopsis H^+-ATPase are also available, it would be of interest to compare the expression of these to the expression of the 14-3-3s and see if there is any correlation between expression of 14-3-3s and expression of one of the 14-3-3 proteins major targets, the H^+-ATPase . For example, promoter activity of the Arabidopsis H^+-ATPase 3 (AHA3) has been shown in vascular tissues similar to many 14-3-3s (DeWitt and Sussman, 1995) and the promoter of Arabidopsis H^+-ATPase 10 has shown activity in developing seeds (Harper *et al* 1994).

7

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Allt levande är uppbyggt av celler, från encelliga organismer som t ex jäst till flercelliga organismer som t ex djur och växter. Alla celler regleras noggrant så att den inre miljön är konstant oavsett vad som händer i den yttre miljön. Cellerna har sensorer så att de alltid vet vad som händer runt dem och kan svara på detta. Dessa reglerande system består till stor del av stora molekyler som kallas proteiner. En sådan grupp av reglerande proteiner kallas 14-3-3.

14-3-3 proteiner utgör en familj av proteiner som är mycket lika varandra. De upptäcktes redan 1967 i hjärna från ko. Det konstiga namnet har 14-3-3 proteinerna fått från sättet de renades fram på. Från början trodde man att dessa proteiner bara fanns i hjärna men sedan hittades de också i andra vävnader hos djur. 1992 hittades 14-3-3 även i växter och i jäst. I encelliga organismer som tex jäst finns få varianter (isoformer) av 14-3-3 medan i djur och växter kan det finnas många. T ex så finns det 15 stycken i modellväxten Arabidopsis. 14-3-3 är med stor sannolikhet det protein som är involverat i flest processer i cellen och har visat sig interagera med mer än 700 andra sorters proteiner.

Växten Arabidopsis heter backtrav på svenska och används mycket inom växtforskningen. I växten har 14-3-3 visat sig vara involverade i många viktiga processer. Tidigare trodde man att de många olika varianterna av 14-3-3 hade samma uppgifter i cellen men nya data visar att de faktiskt kan ha specifika uppgifter trots att de är så lika varandra. Därför är det av intresse att t ex kartlägga var i växten som de olika varianterna finns.

Med hjälp av ett modellsystem där 14-3-3 reglerar ett annat protein som sitter i cellmembranet och pumpar protoner från insidan av cellen till utsidan har vi kunnat visa att det finns skillnader i hur de olika 14-3-3 varianterna binder till protonpumpen.

Det har också visat sig att olika isoformer av ett protein inte behöver finnas (uttryckas) i alla celler i en hel organism och i de fall där det finns många isoformer så är det av intresse att se om man kan hitta var de olika isoformerna uttrycks. Vi har med hjälp av genmodifierade Arabidopsis lyckats ta reda på var 13 av de 15 isoformerna av 14-3-3 uttrycks och visat att de finns på olika ställen i växten och att de då har olika uppgifter.

8

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