PHOSPHORYLATION MEDIATED REGULATION OF 14-3-3 PROTEIN DIMERIZATION IN Arabidopsis thaliana AND THE EFFECT OF DIMERIZATION IN 14-3-3/TARGET INTERACTIONS

By

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To Mustafa Kemal Ataturk, the founder of the Modern Turkish Republic
“I am not leaving a spiritual legacy of dogmas, unchangeable petrified directives.
My spiritual legacy is science and reason.” M. Kemal Ataturk
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LIST OF ABBREVIATIONS

PKA Protein Kinase A
pThr phosphothreonine
pSer phosphoserine
GA Gibberellic Acid
NR Nitrate Reductase
GRF General Regulatory Factor
GFP Green Florescent Protein
ABA Abscisic Acid
BR Brassinosteroids
RSG REPRESSION OF SHOOT GROWTH
BRI1 BRASSINOSTEROID-INSENSITIVE 1
BZR1 BRASSINAZOLE-RESISTANT 1
CO CONSTANS
ROS Reactive Oxygen Species
PCD Programmed Cell Death
HDAC Histone Deacetylases
PKC Protein Kinase C
Difopein Dimeric Fourteen-three-three Peptide Inhibitor
GBF3 G-box Binding Factor 3
14-3-3s are a family of regulatory proteins that are uniquely eukaryotic, evolutionarily conserved across all eukaryotes, and deeply involved in protein-protein interactions that mediate diverse biological processes. The 14-3-3 proteins commonly bind to target proteins containing well defined phosphothreonine (pThr) or phosphoserine (pSer) motifs. Though evolutionarily conserved, most eukaryotes have a range of 14-3-3 genes and proteins that provide functional divergence to the family. The families of 14-3-3 proteins are present in all cell types as homo- and hetero-dimers.

The 14-3-3/target interactions can be regulated at two different levels: First, the phosphorylation status of the target and second, the phosphorylation status of 14-3-3 itself, which has been shown to regulate the dimer/monomer status of the 14-3-3s. The present study demonstrated that in Arabidopsis there is phosphorylation dependent regulation of 14-3-3 dimerization that drastically affects the range of dimerization among 14-3-3s and severely affects target protein interactions.

This phosphorylation dependent regulation of 14-3-3 dimerization has been conserved throughout the other eukaryotic organism. The data in this dissertation
demonstrated that phosphorylation of 14-3-3ω at Ser-62 has the potential regulatory
role in both dimerization and target interactions. In addition, phosphorylation at this
conserved serine residue has the capacity to influence homo- and heterodimerization of
14-3-3 proteins.
CHAPTER 1
THE 14-3-3 PROTEINS

Introduction

14-3-3s are a family of regulatory proteins that are uniquely eukaryotic and evolutionarily conserved across all eukaryotes. Despite this conservation, most eukaryotes have a range of 14-3-3 genes and proteins that provides functional divergence to the family. The 14-3-3 proteins are deeply involved in protein-protein interactions that mediate important biological process such as regulation of metabolic enzymes, signal transduction pathways, cell cycle regulation, cell differentiation and proliferation by interacting directly with numerous target proteins. Some of these targets include histones, kinases, transcription factors and chromatin remodeling enzymes such as histone deaceylases, and naked DNA.

The 14-3-3s were first discovered in mammalian brain tissue (Moore and Perez, 1968). The name “14-3-3” comes from the same study by Moore and Perez, in which they purified brain proteins with unknown function and gave each a numerical designation. The numbers corresponded to their ion exchange chromatography elution profiles and starch gel electrophoresis mobility patterns. The first reported function of 14-3-3s was the activation of two enzymes, tyrosine hyroxylase and tryptophan hydroxylase, involved in the neurotransmitter pathway in the presence of calcium and calmodulin-dependent kinase II or cAMP-dependent kinase (Ichimura et al., 1987). Later, searches for regulatory proteins in diverse cellular processes such as cell cycle regulation, signaling, and metabolic pathways led to the re-discovery of 14-3-3s as regulatory proteins. Currently, it is well known that 14-3-3s play regulatory roles in
various biological processes by direct interaction with diverse target proteins both in animals and plants (Fu et al., 2000).

14-3-3s are characterized as acidic proteins with a pI in the range of 4 to 5.5. The 14-3-3 proteins are present in all cell types as homo- and hetero-dimers with a monomeric mass of 25-32 kDa. However, the dimer/monomer status of 14-3-3 proteins can be regulated by phosphorylation at the dimerization interface. *In vitro* and *in vivo* studies showed that several protein kinases phosphorylate human 14-3-3ζ at Ser-58 located in helix H3 at the dimer interface leading to monomerization (Hamaguchi et al., 2003; Megidish et al., 1998; Powell et al., 2003; Gu et al., 2006; Porter et al., 2006). Powell et al. (2003) and Gu et al. (2006) showed that the mutation at Ser-58 of 14-3-3ζ to mimic phosphorylation prevents homodimer formation. In addition, Woodcock et al., (2003) examined the effect of sphingosine-dependent kinase, (SDK1) mediated phosphorylation and Gu et al. (2006) characterized protein kinase A (PKA) mediated phosphorylation at Ser-58 and showed that phosphorylated Ser-58 also inhibits dimerization.

The 14-3-3 proteins generally bind to target proteins containing well defined phosphothreonine (pThr) or phosphoserine (pSer) motifs (Ferl, 1996). Extensive study of the binding site for mammalian 14-3-3 proteins on serine/threonine kinase Raf-1 revealed that they recognize the consensus sequences Arg(Ser/Ar)X(pSer/pThr)XPro (mode-1: RSXpSXP) and ArgX(Ar/Ser)X(pSer/pThr)XPro (mode-2: RXF/YXpSXP), in which Ar indicates an aromatic residue and X indicates any residue (Table 1-1). This interaction occurs within the conserved core section of the 14-3-3 proteins, which encodes an amphipathic groove in each monomer (Muslin et al., 1996; Yaffe et al.,
Mutational and co-crystallization studies with the mammalian 14-3-3ζ isoform and phosphorylated targets such as a phospho-peptide from Raf-1 kinase and a phospho-peptide representing the 14-3-3 binding epitope of polyoma virus middle T antigen showed that the amino acids Lys49, Arg56, and Arg127 interact with the phosphorylated amino acids of the target proteins (Petosa et al., 1998; Yaffe et al., 1997). Another binding motif, “SWpTX” (motif-3) was characterized by a genetic screen against the C-terminus of the Kir2.1 potassium channel (Coblitz et al., 2005). However, 14-3-3s were also reported to bind to sequences that diverge from those so-called 14-3-3 binding modes. Non-phosphorylated target protein sequences have been identified such as Gly-His-Ser-Leu (GHSL) of the glycoprotein Ib-IX-V complex protein (Andrews et al., 1998) and Trp-Leu-Asp-Leu-Glu (WLDLE) of the R18 peptide, a synthetic 14-3-3 antagonist isolated by phage display assay against human 14-3-3τ (Petosa et al., 1998).

Co-crystallization studies of 14-3-3ζ with R18 revealed that the core region of R18 peptide, WLDLE, is located in the region of the amphipathic ligand binding groove of 14-3-3, where the phospho-serine is located. In addition, hydrophobic residues of R18 core sequence align with hydrophobic residues in the amphipathic ligand binding groove of 14-3-3. The structural features of the 14-3-3/R18 interaction suggest that R18 interacts with 14-3-3 in a very similar manner to natural phosphorylated targets (Masters and Fu, 2001). Table 1-1 summarizes the major 14-3-3 binding motifs characterized in divergent organisms.

For 14-3-3 target proteins that undergo phosphorylation, the interaction between the 14-3-3 and the target protein appears to be regulated by the phosphorylation status of the target protein. Binding of 14-3-3 to its target can have several mechanistic
consequences (Figure 1-1) (Tzivion et al., 2002; Roberts, 2003): (1) The binding may induce conformational changes on the target: Crystal structure of 14-3-3ζ with serotonin N-acetyltransferase (AANAT) revealed that 14-3-3 binding to AANAT regulates the substrate affinity of AANAT by stabilizing the region that is involved in substrate binding (Obsil et al., 2001) (Figure 1-1A). (2) 14-3-3 binding change the nuclear vs. cytoplasmic location of the target protein: Nuclear vs. cytoplasmic localization of REPRESSION OF SHOOT GROWTH (RSG), a plant bZIP transcription factor involved in gibberellic acid (GA) hormone signaling, is regulated by 14-3-3 interaction. Phosphorylation mediated binding of 14-3-3 sequesters RSG in the cytoplasm, which in turn lowers the GA level in the cell (Ishida et al., 2008) (Figure 1-1B). (3) 14-3-3s regulate the intrinsic catalytic activity of the partner protein: The plant plasma membrane H⁺-ATPase enzyme activity is regulated by 14-3-3 binding. Phosphorylation mediated binding of 14-3-3s to the plasma membrane H⁺-ATPase leads to the displacement of the conserved C-terminal autoinhibitory domain which in turn increases the activity of the plasma membrane H⁺-ATPase (Olsson et al., 1998; Fuglsang et al., 1999; Svennelid et al., 1999) (Figure 1-1C). (4) 14-3-3s control turnover of the target protein: Nitrate reductase (NR) is another plant enzyme regulated by 14-3-3 proteins in response to environmental and internal signals. Regulation of NR by phosphorylation 14-3-3s occurs at two levels: First, phosphorylation mediated binding of 14-3-3s at the hinge 1 region of nitrate reductase lowers the intrinsic enzymatic activity of the NR (Kanamaru et al., 1999). Second, 14-3-3 binding regulates the proteolysis of nitrate reductase. Removal of 14-3-3s from plant extracts decreases the NR turnover. This suggests that 14-3-3 binding negatively regulates NR stability (Weiner and Kaiser, 1999) (Figure 1-1D). (5) 14-3-3 binding alters
the affinity of the target to other proteins: Insulin receptor substrate 1 (IRS-1) is a key signaling protein that transmits insulin signaling through activating several signaling pathways such as PI-3 kinase and MAP kinase pathways (Kosaki et al., 1998). 14-3-3 binding to IRS-1 decreases the affinity of IRS-1 to PI-3 kinase, which in turn attenuates its ability to activate PI-3 kinase (Tzivion et al., 2001) (Figure 1-1E).

**Gene Organization and Evolutionary History**

The members of the 14-3-3 protein family from the bovine brain were designated by Greek letters according to their order of elution during reversed phase chromatography. The Arabidopsis 14-3-3 protein family members were also assigned Greek letter designation, but the designation was based on the gene sequence similarity and the name designation starts from the end of the Greek alphabet (Ferl, 1996). The genes encoding the 14-3-3 proteins in Arabidopsis were given a three letter designation GRF (General Regulatory Factor) followed by a number. Table 1-2 summarizes the nomenclature used in Arabidopsis 14-3-3 protein gene family. Protein alignments of 14-3-3s from different species and isoforms from the same species showed that the middle core sections of the 14-3-3 proteins, which consist of the helices involved in dimerization and phosphorylated target binding are highly conserved (Figure 1-2). This high level of conservation makes 14-3-3 from divergent species fit into similar functional and structural models. On the other hand, the amino terminus and the carboxyl terminus are highly divergent and this might contribute to isoform specificity of 14-3-3s (Sehnke et al., 2002). The plant 14-3-3 proteins cluster into two groups when analyzed phylogenetically: These are an epsilon (ε) group and a non-epsilon group (Figure 1-3). The isoforms in the plant non-epsilon group appear to be plant specific and are significantly different from the plant and animal epsilon groups (Ferl et al., 2002). In
Arabidopsis the epsilon group has five members, \( \mu, \varepsilon, \pi, \iota, \) and \( \omicron, \) and the non-epsilon group has eight members, \( \kappa, \lambda, \psi, \upsilon, \omega, \varphi, \) and \( \chi. \) Arabidopsis 14-3-3 proteins are located on all five chromosomes with at least one isoform on each (Figure 1-4). A similar chromosomal distribution was observed for other species that have more than one 14-3-3 isoform (Ferl et al., 2002).

Early three dimensional structure models for 14-3-3 proteins were developed by X-ray diffraction crystallography using mammalian \( \zeta \) and \( \tau \) isoforms (Liu et al., 1995; Xiao et al., 1995). The more highly conserved core region of 14-3-3s makes these structural models valid for other 14-3-3s of diverse species (Ferl et al., 2002). Later, several X-ray crystallography models for plant 14-3-3s were developed (Wurtele et al., 2003; Ottmann et al., 2009). The 14-3-3 monomer consists of nine antiparallel helices that form an L-shaped structure (Figure 1-5). A concave amphipathic groove lies in the interior of the L-shape structure, where the target protein interaction occurs. This highly conserved section of the 14-3-3 proteins (~70% conserved) is made up of four helices: Two of these are the H3 and H5 helices, which are primarily composed of charged and polar amino acids. The two others, H7 and H9, contain hydrophobic amino acids.

The 14-3-3s can form both homodimers and heterodimers. The contact between the monomers occurs at the amino-terminal of helix H1 of one monomer and helices H3 and H4 of the other monomer (Figure 1-5). The high amino sequence conservation of H1 and H3 also allows 14-3-3s to form heterodimers (Jones et al., 1995; Rittinger et al., 1999). The 14-3-3 dimers can bind two or more target proteins simultaneously (Braselmann et al., 1995; Vincenz and Dixit, 1996). This capacity of 14-3-3s suggests that they may be able to act as adaptor proteins linking two or more different target
proteins to one another. If so, they can influence the formation of protein complexes or change the structural conformation of the target proteins by binding to two different regions of the same protein. One example is the role identified for the 14-3-3 that functions as an adaptor protein in the interaction between the plant plasma-membrane proton ATPase and the plant toxin fusicoccin in the presence of magnesium. It was shown that a binding site for the fusicoccin was created when the 14-3-3 protein binds to the carboxy-terminal autoinhibitory (C-TA) domain of the ATPase (Jahn et al. 1997; Würtele et al. 2003). Fusicoccin binding stabilized the complex of 14-3-3 and the ATPase, as well as displacing the C-TA domain, thus allowing the ATPase to become fully active (Chung et al., 1999).

**Localization of 14-3-3 Proteins**

The 14-3-3 proteins are localized throughout the entire cell, suggesting that they can be involved in diverse protein-protein interactions. Subcellular localization of 14-3-3s can provide important clues to possible roles of 14-3-3s in eukaryotic organisms. The first reported mammalian 14-3-3s isolated from brain tissue were cytosolic (Moore and Perez, 1968). Later, 14-3-3s were found in the nucleus, mitochondria, plasma membrane and chloroplast (Bihn et al., 1997; Bunney et al., 2001; Baunsgaard et al., 1998; Sehnke et al., 2000). Although 14-3-3s do not have nuclear targeting sequences, Bihn et al (1997) showed that 14-3-3s are present in both Arabidopsis and maize nuclei. A localization study using four evolutionarily diverse Arabidopsis 14-3-3 isoforms; κ, λ, ω, and φ, fused to green florescent protein (GFP) revealed that 14-3-3s have distinct and differential subcellular localization (Paul et al., 2005). Considering the lack of subcellular localization signals on 14-3-3s, differential sublocalization of the individual
14-3-3 isoforms may be driven by target interactions rather than the intrinsic properties of 14-3-3s. Use of AICAR, a 5’-AMP analog, and R18 peptide, a high-affinity 14-3-3 target, showed that in the absence of the 14-3-3/target protein interactions, 14-3-3s were found to localize throughout the cell without any clear subcellularization (Paul et al., 2005). Milton et al. (2006) reported that the mammalian 14-3-3ε is distributed throughout the cell. The interaction between 14-3-3ε and DP-3δ, a subunit of the E2F family of transcription factors, localizes 14-3-3ε into the nucleus. These results suggest that many 14-3-3 isoforms may have specific targets and distinct regulatory functions. Furthermore, as mentioned earlier, 14-3-3 can also form heterodimers, with a capacity to mediate concurrent interactions between two or more target proteins (Paul et al., 2005).

In Arabidopsis, a study with isoform specific antibodies showed that two 14-3-3s from the epsilon group (µ and ε) and two 14-3-3 isoforms from the non-epsilon group (υ and ν) were the only 14-3-3s prominently located in the chloroplast (Sehnke et al., 2000). This finding suggests that phylogenetically different isoforms can share similar subcellular locations and functions. The expression profiles of 14-3-3 isoforms vary from tissue to tissue and organ to organ. For example, in Arabidopsis, ψ and λ isoforms are expressed in the leaves, stems and flowers, µ is found in the leaves (Chung et al., 1999), and χ is found in pollen grains and stigma papillar cells (Daugherty et al., 1996). Therefore, the tissue specific expression of 14-3-3 isoforms increases the complexity of the 14-3-3 mediated regulation of the target proteins. Earlier GFP fusion studies in Arabidopsis also confirmed the isoform-specific subcellular localization of 14-3-3 proteins. The 14-3-3 κ-GFP fusion protein localizes to the plasma membrane, the 14-3-3
3 υ-GFP fusion tends to be in the cytosol, and the 14-3-3 ε-GFP localizes at nuclear envelopes (Cutler et al., 2000; Sehnke et al., 2002). Localization of the 14-3-3 ω-GFP fusion protein between cytoplasm and nucleus was found to change during the course of the cell cycle. These proteins generally localized out of the nucleus, then entered the nucleus following nuclear division, and finally moved back out of the nucleus just before completion of cytokinesis (Cutler et al., 2000).

The 14-3-3s are also implicated in regulation of subcellular localization for many mammalian target proteins. For example, 14-3-3 proteins regulate the activity of a type II tumor suppressor ING1 by targeting it to cytoplasm. The ING1 affects cell growth regulation, stress signaling, DNA repair and apoptosis by altering chromatin structure and transcriptional regulation. The interaction between 14-3-3 and ING1 is regulated by the phosphorylation status of ING1. Decreased expression and mislocalization of ING1 cause several different human cancers (Gong et al., 2006). The 14-3-3 proteins can also directly regulate subcellular localization of transcription factors such as FoxO forkhead type, which are involved in regulation of numerous genes that control cell proliferation and apoptosis. Interaction with 14-3-3 proteins affects the binding affinity of these transcription factors to their target DNAs (Obsilova et al., 2005).

**Functions of 14-3-3 Proteins in Plants**

In the early plant literature, 14-3-3s were recruited more heavily to central metabolic pathways, hormone signaling, carbohydrate metabolism and stress metabolism (reviewed in Ferl, 1996; Huber et al., 2002), while animal 14-3-3 interactions were more dedicated to signal transduction cascade players such as protein kinases, phosphatases and transcription factors (reviewed in (Mackintosh, 2004)). These include Raf (Freed et al., 1994; Irie et al., 1994; Li et al., 1995), Ras
(Gelperin et al., 1995; Rommel et al., 1996), protein kinase C (Toker et al., 1992; Dellambra et al., 1995), and Bcr (Reuther et al., 1994; Braselmann and McCormick, 1995). In contrast to the early plant 14-3-3 literature where 14-3-3s were characterized as regulators of metabolic enzymes such as nitrate reductase (Bachmann et al., 1996), sucrose phosphate synthase (Moorhead et al., 1999), and starch synthase (Sehnke et al., 2001), recent in vitro and in vivo proteomics, genetics and physiology studies have identified 14-3-3 proteins as essential players in plant signaling pathways. It is becoming increasingly apparent that 14-3-3s are involved in the signal transduction associated with hormones, light responses, stress signaling as well as basic metabolism. Proteome and interactome studies indicate that 14-3-3s interact with a diversity of signaling proteins and metabolic processes. Table 1-2 summarizes the recent studies of plant 14-3-3-binding proteins as signaling mediators.

**Role of 14-3-3 Proteins in Plant Hormone Signaling**

The active roles of 14-3-3s in plant hormone signaling have been described for gibberellic acid (GA), abscisic acid (ABA) and brassinosteroids (BR) (Ishida et al., 2008; Schoonheim et al., 2007; Gendron and Wang, 2007). In gibberellic acid mediated pathways, 14-3-3s are involved in negative-feedback regulation of GA homeostasis by altering the cytoplasmic/nuclear partitioning of a tobacco bZIP transcription factor, REPRESSION OF SHOOT GROWTH (RSG) (Ishida et al., 2004). RSG controls the GA level in cells through transcriptional regulation of genes encoding GA biosynthesis. An increased level of GA causes the phosphorylation of RSG by a Ca$^{+2}$ dependent kinase CDPK1. Upon phosphorylation, 14-3-3 binds to RSG and sequesters it in the cytoplasm, which in turn lowers the GA level in the cell (Ishida et al., 2008) by affecting
transcription. The removal of the 14-3-3 binding site leads to an accumulation of RSG protein in the nucleus, thereby increasing the transcriptional activity of RSG in vivo.

Participation of the 14-3-3s in ABA signaling is also mediated through transcriptional regulation. 14-3-3s are shown to be present physically at the promoters of two Arabidopsis late embryogenesis genes, AtEm1 and AtEm6, which are induced by ABI3, an ABA regulated transcription factor (del Viso et al., 2007). 14-3-3s may act as an adaptor protein in the transcription complex found in Em gene promoters (del Viso et al., 2007). Another example of 14-3-3 being part of transcription complex is described in barley seeds where the ABI5 family of ABA regulated transcription factors in barley interacts with 14-3-3s in yeast two-hybrid assays. In the same study, it was shown that ABA induces the expression 14-3-3s in embryonic barley roots. Furthermore, RNAi mediated silencing of several 14-3-3 members in barley reduces the ABI5 mediated induction of ABA-inducible reporter constructs (Schonnheim et al., 2007).

Brassinosteroid (BR) hormone signaling is another well described signaling pathway in Arabidopsis that plays a crucial role in plant growth and development. The ligand, receptors, protein kinases and transcription factors of the BR signaling pathway have been characterized by several research groups (e.g. Kim and Wang, 2010; Belkhadir et al., 2006). Recent reports showed that 14-3-3s play important regulatory function in BR signaling at several different levels. Two 14-3-3 isoforms are present in a complex with membrane bound BR receptor kinase BRASSINOSTEROID-INSENSITIVE 1 (BRI1). The significance of this interaction is yet to be elucidated (Karlova et al., 2006). BR induced transcription factor BRASSINAZOLE-RESISTANT 1 (BZR1) is also a target for 14-3-3 proteins in Arabidopsis and rice (Ryu et al., 2007; Bai
et al., 2007). BZR1 is a highly phosphorylated transcription repressor whose phosphorylation status determines its nuclear/cytoplasmic localization (Ryu et al., 2007; He et al., 2005). BR induced dephosphorylation and nuclear accumulation of BZR1 causes brassinosteroid-induced growth and feedback regulation of brassinosteroid biosynthesis (Wang et al., 2002). Phosphorylation mediated BZR1/14-3-3 interaction causes cytoplasmic retention or nuclear export of the BZR1. Mutations on putative 14-3-3 interaction binding sites on BZR1s abolish the interactions and increase the nuclear localization of BZR1s (Gampala et al., 2007).

**Role of 14-3-3 Proteins in Light Signaling**

Compared to hormone signaling, involvement of 14-3-3s in light signaling is less well understood. Phototropins are blue light specific plant protein kinase receptors that are autophosphorylated by blue light. They are involved in several different blue light responses such as phototropism, hypocotyl growth inhibition, stomatal opening and chloroplast movement (Christie, 2007). Autophosphorylation of a serine residue in the Hinge1 region of PHOT1 in response to blue light creates a 14-3-3 binding site (Inoue, et al., 2008). However, mutant phot1 plants without the 14-3-3 binding site did not show any phenotypic difference from wild-type plants. A recent study also revealed that 14-3-3s interact with PHOT1 in an isoform specific manner (Sullivan et al., 2009). Only non-epsilon isoforms were shown to interact with PHOT1. The same study also showed that 14-3-3s do not interact with PHOT2 due to the less conservation of the phospho-motifs in the Hinge 1 region of PHOT2 (Sullivan et al., 2008).

A mechanistic link between 14-3-3s and red-light signaling was discovered using a reverse genetic approach. Arabidopsis T-DNA insertion lines for 14-3-3 u and µ isoforms exhibited a delayed flowering time phenotype on long days. Plants also
showed hyposensitive hypocotyl growth inhibition under red light but no difference under blue and far-red light (Mayfield et al., 2007). In the same paper, a direct interaction between these two 14-3-3 isoforms and photoperiodic regulatory protein CONSTANS (CO) was demonstrated using yeast two-hybrid and co-immunoprecipitation assays. Although, 14-3-3s do not appear to interact with the photosensor phytochrome B (phyB) directly, the flowering delay phenotype and direct interaction with CO suggest that 14-3-3s are part of the red light signaling pathway (Folta et al., 2008).

**Role of 14-3-3 Proteins in Biotic and Abiotic Stress Response**

There have been a number of studies suggesting that 14-3-3s play a role in plant stress responses (reviewed in Chevalier et al., 2009). Transcriptome analyses of plant biotic and abiotic treatments reveal that many 14-3-3 isoforms are consistently differentially expressed in response to a variety of stressors (e.g. Chen et al., 2006; Lancien et al., 2006; Xu et al., 2006). Proteomic analyses also revealed an abundance of 14-3-3 partners that are associated with biotic and abiotic stress responses (Paul et al., 2009; Chang et al., 2009; Alexander and Morris, 2006). In addition to this indirect evidence, several groups reported direct involvement of 14-3-3s in stress regulation. Reactive oxygen species (ROS) production by plasma membrane bound NADPH oxidases is an important event in response to both biotic and abiotic stress stimuli (Torres and Dangl, 2005; Elmayan and Simon-Plas, 2007). A yeast two-hybrid screen against the C-terminal section of a plasma membrane NADPH oxidase in tobacco showed that NtrbohD interacts with Nt14-3-3h/omega1 isoform. In addition, stimulation of tobacco leaves with the fungal elicitor cryptogein caused the accumulation of this 14-3-3 transcript. The significance of the 14-3-3/NtrbohD interaction was demonstrated by
transforming the tobacco cells with antisense constructs for this Nt14-3-3h/omega1 isoform. Cells transformed with this antisense construct were not able to produce ROS in response to cryptogein elicitor (Elmayan et al., 2007). So, these results suggest that 14-3-3s may play a pivotal role in ROS mediated programmed cell death in plant defense signaling named hypersensitive response (HR) in an isoform specific manner.

Plants usually trigger localized programmed cell death (PCD) as a defense response against pathogen attack (van Doorn and Woltering, 2005). *Pseudomonas syringae pv tomato*-induced PCD in tomato and tobacco is positively regulated by mitogen-activated protein kinase kinase kinase (MAPKKKα) (del Pozo et al., 2004). A tomato 14-3-3, TFT7, was identified as a MAPKKα-interacting protein in a yeast two-hybrid screen using MAPKKKα as bait (Oh et al., 2010). Virus mediated silencing of the TFT7 gene in tobacco hinders MAPKKKα mediated PCD. On the other hand, co-expression of TFT7 and MAPKKKα causes enhancement in PCD response in tobacco. Phosphorylation dependent direct interaction between TFT7 and MAPKKKα was shown by a site-specific mutation on MAPKKKα. This mutation decreased the PCD elicitation ability and stability of MAPKKKα.

Physiological evidence for 14-3-3 mediated abiotic stress response was shown by over expression of Arabidopsis 14-3-3λ in cotton plants. Compared to wild-type cotton plants, these transgenic cotton plants had a slow wilting phenotype and higher photosynthesis rate under water-stress conditions. Furthermore, under normal growth conditions, these transgenic cotton plants displayed a late leaf senescence phenotype (Yan et al., 2004).
Role of 14-3-3 Proteins in Chromatin-mediated Gene Regulation

In addition, there is evidence to indicate that 14-3-3 proteins are involved in chromatin-mediated gene regulation through interaction with histones (Chen and Wagner, 1994) and histone deacetylases (HDACs) (Grozinger and Schreiber, 2000). Cross-linking experiments and affinity chromatography revealed that 14-3-3 proteins bind to histones in rat cell line PC12. The 14-3-3 proteins enhance the histone phosphorylation mediated by protein kinase C (PKC), which leads to increasing secretion of catecholamine in chromaffin cells. The 14-3-3 proteins were also found to inhibit the rate of histone dephosphorylation (Chen and Wagner, 1994). Transcription is partly controlled by the acetylation status of the histones. Histone deacetylation is mediated by histone deacetylases (HDACs). The interaction between 14-3-3 proteins and HDACs was demonstrated by Grozinger and Schreiber (2000). This interaction triggers nuclear export of HDACs, which permits gene expression (Chang et al., 2005).

Conclusions

In contrast to the early plant 14-3-3 literature where 14-3-3s were characterized as metabolic regulators, recent in vitro and in vivo proteomics, genetics and physiology studies have placed 14-3-3 proteins as essential components of plant signaling pathways. It is becoming increasingly apparent that 14-3-3s are involved in signal transduction associated with hormones, light responses, stress signaling as well as basic metabolism. Proteome and interactome studies indicate that 14-3-3s interact with a diversity of over hundred proteins involved in signaling and metabolic processess. Considering that 14-3-3s are extensively involved in protein-protein interaction, understanding the regulation of 14-3-3/target interactions is essential both at cellular and organismal levels.
14-3-3/target interactions can be regulated at two different levels: first, the level of phosphorylation on the 14-3-3 recognition motif on the target protein; second, the phosphorylation status of 14-3-3 itself. The regulation of 14-3-3/target interactions by phosphorylation of 14-3-3s has been reported previously. Phosphorylation of human 14-3-3ζ at Ser-58 by PKA and B/Akt kinases has been shown to affect target interactions by regulation of the dimer status of the 14-3-3ζ (Gu et al., 2006, Powell et al., 2002). Two other residues of 14-3-3ζ, Ser-184 and Thr-232, have been also demonstrated to be phosphorylated and involved in 14-3-3/target interactions (Aitken et al., 1995; Dubois et al., 1997). The chapters of this dissertation mainly focus on the regulation of 14-3-3 dimerization and the effect of dimerization in Arabidopsis 14-3-3/target interactions. Phosphorylation of a conserved serine residue, Ser-62, in the dimerization interface of 14-3-3ω interferes with the dimerization of the 14-3-3 protein. In vitro and in vivo studies showed that Ser-58 of human 14-3-3ζ, the corresponding serine residue to Ser-62 in Arabidopsis 14-3-3ω, located in helix H3 at the dimer interface, can be phosphorylated by several different kinases (Hamaguchi et al., 2003; Megidish et al., 1998; Powell et al., 2003; Gu et al., 2006; Porter et al., 2006). These studies showed that the phosphorylation of Ser-58 inhibits dimerization of 14-3-3ζ. In addition, phosphorylation mimic mutations, Glu-58 and Asp-58, in the dimerization interface of 14-3-3ζ were shown to prevent homodimer formation (Powell et al., 2003; Sluchanko et al., 2008). The present study showed that a similar mechanism also exists in plant 14-3-3s, specifically in the model plant organism Arabidopsis 14-3-3s. In addition, this study demonstrated that phosphorylation of 14-3-3 in the dimerization interface not only disrupts the dimerization, but it can also change the affinity of a specific isoform to
another 14-3-3 isoform as a dimeric partner. Arabidopsis has 13 different 14-3-3 isoforms, and they can form 91 potential 14-3-3 dimers (13 homodimers, 78 heterodimers). Considering the differential affinities of 14-3-3 isoforms for the 14-3-3 targets, this large number of 14-3-3 dimer combinations may explain the functional divergence of the 14-3-3 protein families. Therefore, an understanding of the regulation of hetero- or homo-dimer formation is crucial for describing how 14-3-3s are involved in dynamic protein-protein interactions with different target affinities.
<table>
<thead>
<tr>
<th>14-3-3 binding motifs</th>
<th>Protein Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode-1</td>
<td>RSxPYSXP</td>
<td>Yaffe et al., 1997</td>
</tr>
<tr>
<td>Mode-2</td>
<td>RXF/YxPYSXP</td>
<td>Yaffe et al., 1997</td>
</tr>
<tr>
<td>Mode-3</td>
<td>SWpTX</td>
<td>Coblitz et al., 2005</td>
</tr>
<tr>
<td>H(+) - ATPase</td>
<td>YpTV</td>
<td>Fuglsang et al., 1999</td>
</tr>
<tr>
<td>Exoenzyme S</td>
<td>DALDL</td>
<td>Henrikkson et al., 2002</td>
</tr>
<tr>
<td>Glycoprotein Ib-IX-V</td>
<td>GSHL</td>
<td>Andrews et al., 1998</td>
</tr>
<tr>
<td>complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R18</td>
<td>WLDLE</td>
<td>Petosa et al., 1998</td>
</tr>
</tbody>
</table>
Figure 1-1. The mode of 14-3-3/target interactions. Binding of 14-3-3 to its target may A) induce conformational changes, B) alter the nuclear vs. cytoplasmic distribution, C) regulate the intrinsic catalytic activity, D) control turnover of the target protein, or E) associate multiple targets together. (Figure adapted from Gökirmak et al., 2010).
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Protein Name</th>
<th>Genomic Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRF1</td>
<td>Chi (χ)</td>
<td>At4g09000</td>
</tr>
<tr>
<td>GRF2</td>
<td>Omega (ω)</td>
<td>At1g78300</td>
</tr>
<tr>
<td>GRF3</td>
<td>Psi (ψ)</td>
<td>At5g38480</td>
</tr>
<tr>
<td>GRF4</td>
<td>Phi (φ)</td>
<td>At1g35160</td>
</tr>
<tr>
<td>GRF5</td>
<td>Upsilon (υ)</td>
<td>At5g16050</td>
</tr>
<tr>
<td>GRF6</td>
<td>Lambda (λ)</td>
<td>At5g10450</td>
</tr>
<tr>
<td>GRF7</td>
<td>Nu (ν)</td>
<td>At3g02520</td>
</tr>
<tr>
<td>GRF8</td>
<td>Kappa (κ)</td>
<td>At5g65430</td>
</tr>
<tr>
<td>GRF9</td>
<td>Mu (μ)</td>
<td>At2g42690</td>
</tr>
<tr>
<td>GRF10</td>
<td>Epsilon (ε)</td>
<td>At1g22300</td>
</tr>
<tr>
<td>GRF11</td>
<td>Omicron (ο)</td>
<td>At1g34760</td>
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<tr>
<td>GRF12</td>
<td>Iota (ι)</td>
<td>At1g26480</td>
</tr>
<tr>
<td>GRF13</td>
<td>Pi (π)</td>
<td>At1g78220</td>
</tr>
</tbody>
</table>
Figure 1-2. Whole protein sequence alignment of human (Hs), Arabidopsis (At), yeast (Sc), Drosophila (Dm) and C.elegans (Ce) 14-3-3s. Protein sequences were aligned using online MultAlin software (http://multalin.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988). High consensus (= 90 %) residues are in "red", low consensus (= 50 %) residues are in "blue" and non-conserved residues are in "black". Consensus symbols: ! is either I or V; $ is either L or M; % is either F or Y; # is either N, D, Q, E, B or Z.
Figure 1-3. Unrooted phylogenetic tree of Arabidopsis 14-3-3 proteins. Whole protein sequences of 13 Arabidopsis 14-3-3s were downloaded from TAIR website (http://www.arabidopsis.org/) by their genomic locus numbers (Table 1-2). Protein sequences were aligned by ClustalW (www.ebi.ac.uk/clustalw) and the phylogenetic tree was drawn with TreeView (Page, 1996) using the UPGMA method.
Figure 1-4. Chromosomal distribution of Arabidopsis 14-3-3 protein genes. Arabidopsis 14-3-3 genes encoding 14-3-3 proteins were placed on Arabidopsis chromosomes by plugging in Arabidopsis 14-3-3 genomic locus numbers (Table 1-2) into the Arabidopsis Information Resource (TAIR) Chromosome Map Tool (http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). The tool allows the display of alternative names by entering the alternative name after the genomic locus name (i.e., entering: “At5g65430 Kappa” will display only “Kappa” on the chromosome).
Figure 1-5. 3D structure model of 14-3-3 proteins. A) Side view of the 14-3-3 dimer. B) Top view of 14-3-3 dimer. The model is derived from _Nicotiana tabacum_ 14-3-3 in complex with the differentiation-inducing fungal agent Cotylenin A (PDB3E6Y) (Figure adapted from Ottmann et al., 2009) using PDB SimpleViewer 3.8 software. Figures were captured by screen shots. Each helix is labeled with a different color. Helix H1 represents the first helix starting from amino terminus. Helix H9 is the last helix near to the carboxy terminus.
**Table 1-3. Summary of recent studies investigating the involvement of plant 14-3-3 proteins in plant protein signaling pathways**

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Target</th>
<th>Mode of Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gibberellic acid (GA)</td>
<td>RSG</td>
<td>Localization</td>
<td>Ishida et al., 2008; Ishida et al., 2004</td>
</tr>
<tr>
<td>Abscisic acid (ABA)</td>
<td>ABI3, ABI5</td>
<td>Association</td>
<td>Schoonheim et al., 2007; del Viso et al., 2007</td>
</tr>
<tr>
<td>Brassinosteroids (BR)</td>
<td>BRI1, BZR1</td>
<td>Localization</td>
<td>Karlova et al., 2006; Ryu et al., 2007; Bai et al., 2007</td>
</tr>
<tr>
<td>Blue light signaling</td>
<td>PHOT1</td>
<td>Unknown</td>
<td>Inoue et al., 2008; Sullivan et al., 2009</td>
</tr>
<tr>
<td>Red light signaling</td>
<td>CO</td>
<td>Unknown</td>
<td>Mayfield et al., 2007</td>
</tr>
<tr>
<td>Hypersensitive response (HR)</td>
<td>NtrbohD</td>
<td>Activity</td>
<td>Elmayan et al., 2007</td>
</tr>
<tr>
<td>Hypersensitive response (HR)</td>
<td>MAPKKKα</td>
<td>Stability</td>
<td>Oh et al., 2010</td>
</tr>
</tbody>
</table>
CHAPTER 2
SER-62 OF ARABIDOPSIS 14-3-3Ω REGULATES DIMERIZATION AND TARGET INTERACTIONS

Introduction

The 14-3-3 proteins are phosphopeptide-binding regulatory proteins that constitute conserved and ubiquitous eukaryotic protein families. 14-3-3 proteins were first discovered in mammalian brain tissue in the late 1960s. Later, 14-3-3 proteins were shown to be involved in important biological processes such as signal transduction, cell cycle regulation, cell differentiation and proliferation and regulation of metabolic enzymes through interacting with target proteins containing well defined phosphothreonine or phosphoserine motifs (Ferl et al., 1996). Binding of 14-3-3s can affect their targets in a variety of different ways: 14-3-3 binding may increase or decrease the enzymatic activity of the target, keep the target in a specific subcellular location, act as an adapter protein by binding to two targets at same time or regulate the stability of the target protein (reviewed in Gökirmak et al., 2010; Tzivion and Avruch, 2002).

Phylogenetic analysis of 14-3-3 proteins from diverse organisms showed that plant 14-3-3 proteins cluster into two major groups: an epsilon (ε) group and a non-epsilon group. Non-epsilon 14-3-3 isoforms from different plant species form a separate distinct phylogenetic cluster, which is very different from animal 14-3-3 isoforms and plant epsilon isoforms (Ferl et al., 2002). In Arabidopsis, the epsilon group has five members, μ, ε, π, ι, and ο and the non-epsilon group has eight members, κ, λ, ψ, ν, υ, ω, φ, and χ (Ferl et al., 2002).

The 14-3-3 proteins have a monomeric mass of 25-32 kDa and they can form homo- and hetero-dimers. Each 14-3-3 monomer consists of nine antiparallel helices
that form an L-shaped structure (Figure 2-1A). 14-3-3 dimerization occurs between
the amino-terminal of the H1 helix of one monomer and the H3 and H4 helices of the other
monomer (Figure 2-1). The sequence conservation of H1 and H3 helices between
isoforms allows 14-3-3s to form hetero-dimers as well as homo-dimers (Jones et al.,
1995; Rittinger et al., 1999). However, there is still substantial sequence diversity in
Helix 1 and Helix 3, which might be responsible for the differential affinity of one 14-3-3
isoform to another as a dimeric partner (Figure 2-1B). For instance, with 13 different 14-
3-3 isoforms, Arabidopsis can form 91 potential 14-3-3 dimers (13 homodimers, 78
heterodimers). This large number of 14-3-3 dimer combinations may explain the
functional divergence of the 14-3-3 protein families. Considering the differential affinities
of 14-3-3 isoform to the 14-3-3 targets, each 14-3-3 dimer combination may exhibit
distinct affinity to target proteins. Therefore, understanding the regulation of hetero- or
homo-dimer formation is crucial for describing how 14-3-3s are involved in dynamic
protein-protein interactions in response to specific time and input.

The 14-3-3/target protein interactions occur in a concave amphipathic groove that
lies in the interior of the L-shape structure in each 14-3-3 monomer (Muslin et al., 1996;
Yaffe et al., 1997) (Figure 2-1A). Mutational and co-crystallization studies with the
mammalian 14-3-3ζ isoform and phosphorylated targets such as a phospho-peptide
from Raf-1 kinase and a phospho-peptide representing the 14-3-3 binding epitope of
polyoma virus middle T antigen showed that the amino acids Lys-49, Arg-56, and Arg-
127 of 14-3-3ζ interact with the phosphorylated amino acids of the target proteins
(Petosa et al., 1998; Yaffe et al, 1997). However, 14-3-3s were also reported to bind to
non-phosphorylated target protein sequences such as Gly-His-Ser-Leu (GHSL) of the
glycoprotein Ib-IX-V complex protein (Andrews et al., 1998) and Trp-Leu-Asp-Leu-Glu (WLDLE) of the R18 peptide, a synthetic 14-3-3 antagonist isolated by a phage display assay against human 14-3-3τ (Petosa et al., 1998). Co-crystallization studies of 14-3-3ζ with R18 revealed that the core region of the R18 peptide, WLDLE, is located in the amphipathic ligand binding groove of 14-3-3, where the phospho-serine of a phosphorylated target is normally located. Furthermore, hydrophobic residues of the R18, the core sequence aligns with hydrophobic residues in the amphipathic ligand binding groove of 14-3-3. The structural features of the 14-3-3/R18 interaction suggest that the R18 peptide interacts with 14-3-3 in a manner similar to natural phosphorylated targets (Wang et al., 1999; Masters and Fu, 2001). A synthetic higher affinity 14-3-3 antagonist, difopein (dimeric fourteen-three-three peptide inhibitor), was designed based on the dimeric R18 peptide (Masters and Fu, 2001). Expression of difopein in human cell lines led to apoptosis, indicating that 14-3-3ζs are involved in the regulation of anti-apoptotic pathways.

14-3-3/target interactions can be regulated by phosphorylation at two fundamentally different levels: first, level of phosphorylation at the 14-3-3 binding motif of the target protein (Figure 2-2A); second, level of the phosphorylation status of 14-3-3 itself, which can determine the dimeric status of the 14-3-3 protein (Figure 2-2B). In vitro and in vivo studies showed that human 14-3-3ζ Ser-58 located in helix H3 at the dimer interface can be phosphorylated by several different kinases (Hamaguchi et al., 2003; Megidish et al., 1998; Powell et al., 2003; Gu et al., 2006; Porter et al., 2006). Woodcock et al. (2003) and Gu et al. (2006) examined the effect of sphingosine-dependent kinase (SDK1) mediated phosphorylation and protein kinase A (PKA)
mediated phosphorylation at Ser-58, respectively. They showed that phosphorylation of Ser-58 inhibits dimerization of 14-3-3ζ. In addition, phosphorylation-mimic mutations, Glu-58 and Asp-58, in the dimer interface of 14-3-3ζ were shown to prevent homodimer formation (Powell et al., 2003; Sluchanko et al., 2008). Alignment of human 14-3-3ζ with Arabidopsis 14-3-3s showed that the Ser-58 residue is conserved in all Arabidopsis isoforms except 14-3-3κ and 14-3-3λ (Figure 2-4A).

In this study the effect of phosphorylation of Ser-62 in Arabidopsis 14-3-3ω dimerization was investigated. This residue corresponds to the Ser-58 of human 14-3-3ζ. The present study showed that the 14-3-3ω Asp-62 (ωS62D) phospho-mimic mutation disrupts 14-3-3ω homodimerization, which in turn inhibits the 14-3-3ω/target interactions. The data also showed that mutant ωS62D can form heterodimers with 14-3-3ε, 14-3-3ο, 14-3-3χ, 14-3-3ω, 14-3-3ν, 14-3-3ψ and 14-3-3u, but not with 14-3-3ι, 14-3-3μ, 14-3-3π, 14-3-3φ, 14-3-3κ and 14-3-3λ. It was also demonstrated that when ωSer-62 is phosphorylated, the affinity of 14-3-3ω hetero-dimerization to certain 14-3-3 isoforms is significantly increased. The present study characterized a phosphorylation-mediated de novo regulation of 14-3-3 hetero-dimerization that clearly affects target interactions in plants.

**Materials and Methods**

**3D Model and Protein Alignment**

The 14-3-3 dimer model was derived from *Nicotiana tabacum* (PDB3E6Y) (Ottmann et al., 2009) using PDB SimpleViewer 3.8 software. Whole protein sequences of all 13 Arabidopsis 14-3-3 proteins were aligned using online MultAlin software ([http://multalin.toulouse.inra.fr/multalin/multalin.html](http://multalin.toulouse.inra.fr/multalin/multalin.html)) (Corpet, 1988).
Plasmids

The full open reading frames (ORF) of all thirteen Arabidopsis 14-3-3 isoforms and the N-terminal half of nitrate reductase (NR1-562aa) (residues 1-562) were PCR amplified with Gateway tagged primers from an Arabidopsis leaf and flower cDNA library, a gift from Dr. Kevin Folta (University of Florida). EYFP-difopein and EYFP-R18 (Lys) in pEYFP were kindly provided by Dr. Haian Fu (Emory University). EYFP-Difopein and EYFP-R18 (Lys) were PCR amplified from these vectors with Gateway tagged primers. PCR fragments were subcloned into pDONR221 vector using Gateway BP Clonase II (Invitrogen, CA). The phosphorylation-mimic mutation ωS62D and non-phosphorylatable ωS62A in 14-3-3ω, and S534L mutation that disrupts 14-3-3 binding to the nitrate reductase NR1-562aa (residues 1-562) (NR1-562aaS534L) (Kanamura et al., 1999), were created by site-directed mutagenesis and overlapping PCR (Ho et al., 1989). Mutant PCR fragments were subcloned into the pDONR221 vector as described above. All thirteen wild-type Arabidopsis 14-3-3s and the point mutation (ωS62D and ωS62A) cDNAs in pDONR221 were cloned into the pDEST22 yeast two-hybrid prey vector via Gateway LR Clonase II (Invitrogen, CA) reactions. The 14-3-3ω, 14-3-3ωS62D, 14-3-3ωS62A and NR1-562 and NR1-562S534L cDNAs in pDONR221 were cloned into the pDEST32 yeast two-hybrid bait vector via Gateway LR Clonase II reactions. EYFP-Difopein and EYFP-R18(Lys) fragments in pDONR221 were transferred into E.coli expression vector pDEST15 by LR Clonase II recombination reaction.

The 14-3-3ω in pET15b vector for expressing his-tagged recombinant protein in E.coli was described previously (Lu et al., 1992; Wu et al., 1997). 14-3-3ωS62D and 14-3-3ωS62A coding sequences were PCR amplified from pDONR221-14-3-3ωS62D and
pDONR221-14-3-3ωS62A vectors with primers containing Ndel-BamHI restriction sites and cloned into the pET15b E.coli expression vector.

The constitutive yeast expression vector p415GPD (Amp^r LEU2 ARS/CEN) (Mumberg et al., 1995) was a gift from Dr. Paul van Heusden (Leiden University). Arabidopsis 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A coding sequences were cloned into BamHI and HindIII restriction sites of p415GPD using primers with BamHI and HindIII restriction sites.

Split YFP vectors, pHNYFP (YFP_N: N-terminal fragment) and pFCYFP (YFP_C: C-terminal YFP fragment), for bimolecular fluorescence complementation (BiFC) assays were developed by Akhtar et al. (2008). 14-3-3ω and 14-3-3ωS62D fragments were PCR amplified with primers that introduce Ndel/Ncol restriction sites. Digested PCR fragments were ligated into Ndel/Ncol sites of pHNYFP and pFCYFP plasmids to create C-terminal 14-3-3/split-YFP peptides.

**Yeast Two-hybrid System**

*S. cerevisiae* strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_UAS-GAL1_TATA-HIS3, GAL2_UAS-GAL2_TATA ADE2,URA3::MEL1_UAS-MEL1_TATA-lacZ, MEL1) was co-transformed with bait (pDEST22) and prey (pDEST32) vectors by using the standard lithium acetate/polyethylene glycol method (Gietz and Woods, 2006). Co-transformed yeast cells were selected on synthetic complete dropout, SC-Leu-Trp-, plates. The spot assays were performed using yeast cells cultured overnight in SC-Leu-Trp- medium at 30°C. Concentrations were adjusted to A600 of 0.2, and 10μl of the culture and its ten-fold serial dilutions were spotted on SC-Leu-Trp- and SC-Leu-Trp-His- plates. Due to the self activation of 14-3-
3ω and 14-3-3ωS62A proteins fused to the GAL4 DNA binding domain, background yeast growth on SC-Leu-Trp-His- plates was inhibited by inclusion of 0.5 mM 3AT in the media. SC-Leu-Trp- plates were incubated at 30°C for 36 hr, and SC-Leu-Trp-His- plates were incubated at 30°C for 48 hr.

**Expression and Purification of Recombinant Proteins in Bacteria**

_E. coli_ strain BL21-AI (Invitrogen) was transformed with pET15b-14-3-3ω, pET15b-14-3-3ωS62D, pET15b-14-3-3ωS62A, pDEST15-EYFP-difopein and pDEST15-EYFP-R18 (Lys) plasmids. Transformed cells were cultured in Luria-Bertani (LB) medium containing 50μg/ml of carbeniciline until $A_{600}$ between 0.5 and 1 was reached. Recombinant protein expression was induced with 0.2% arabinose (final concentration), and induced cultures were incubated for 3 h at 37°C and harvested by centrifugation at 5,000 x g for 30 minutes. Pellets of cells expressing GST-tagged EYFP-difopein and EYFP-R18 (Lys) were resuspended in GST binding buffer (25mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and protease-inhibitor mixture (Calbiochem)). Pellets of cells expressing His-tagged 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A were re-suspended in equilibration buffer (50 mM sodium phosphate, pH 8.0, with 0.3 M sodium chloride and 10 mM imidazole and protease-inhibitor mixture (Calbiochem)). Cells were lysed by French press at 1260 psi. Cellular debris was removed by centrifugation at 12,000 X g for 30 min at 4 °C. The recombinant GST fusion proteins were affinity purified using a column loaded with glutathione-Sepharose resin (Calbiochem) and eluted from the beads by adding the elution buffer (100mM Tris-HCl, pH8.0, containing 20mM glutathione). The His-tagged recombinant proteins were affinity purified using column loaded with HIS-Select® HF Nickel Affinity Gel resin
(Sigma) and eluted from the column by adding the elution buffer (50 mM sodium phosphate, pH 8.0, with 0.3 M sodium chloride and 250 mM imidazole). Samples were dialyzed overnight at 4°C in phosphate buffered saline (PBS).

**Determining the Affinity and the Binding Kinetics of 14-3-3ω and 14-3-3ωS62D for Targets**

The interaction kinetics of 14-3-3ω and 14-3-3ωS62D with two analytes; EYFP-difopein and EYFP-R18(Lys) were investigated using the Octet QK platform with amine reactive biosensors (Fortebio). Recombinant 14-3-3ω and 14-3-3ωS62D proteins (35 µg/ml) in MES buffer (pH 6) were immobilized onto the amine reactive biosensors surface as ligands. The kinetics parameters of 14-3-3ω and 14-3-3ωS62D with GST-tagged analytes, EYFP-Difopein and EYFP-R18(Lys), were measured against four two-fold dilutions of 2 µM of analytes using the Octet QK platform at 30°C.

**Subcellular Fractionation and Localization 14-3-3ω**

Arabidopsis suspension cells were harvested on day 3 by filtration through MiraCloth (Calbiochem). Suspension cells (10g) were treated with gently overnight (12-14 h) with 50 ml of protoplasting solution (0.1% celluysin, 0.1% macerase and 0.1% pectolyase in 10% mannitol, 0.5X MS salts, pH 5.7) on a horizontally rotating platform (Belly Dancer). Protoplasts were filtered through the Miracloth and pelleted by centrifugation at 900 rpm for 5 min in a swinging bucket centrifuge. Pelleted cells were washed and resuspended gently in 10 ml MMS (10% mannitol with 0.5 X MS salts, ph 5.7) in the presence of phosphatase and protease inhibitors (Calbiochem). Protoplasts were repelleted and resuspended in 5 ml of 1X nuclear isolation buffer (NIB) (Sigma) in the presence of 25 mM NaF, phosphatase and protease inhibitors. An aliquot of protoplasts, representing the whole cell fraction, was removed. The cell membrane of
the remaining protoplats was disrupted with 0.15% Triton X-100 on ice for 5 min and the nuclei pelleted at 2000xg, (4°C) for 10 min. The supernatant, representing the cytoplasmic fraction, was removed and kept on ice. The nuclear pellet was resuspended in 5 ml of NIB buffer and an aliquot representing the whole nuclear fraction was removed. The nuclei were re-pelleted and resuspended in 2 ml of NIB. A portion (500 μl) of nuclear fraction was pelleted at 2000xg, (4°C) for 5 min and resuspended in 450 μl of NHB (5 mM Hepes, pH7.4, 2 mM EDTA, 2 mM KCl, 1 μM DTT and 0.1% digitonin) with phosphatase and protease inhibitors. 50 μl of 50 mM lithium 3,5-diiodosalicylate (LIS) was added to the nuclear fraction to a concentration of 5 mM to extract the soluable proteins and gently rocked on ice for 15 min. The insoluble nuclear fraction was pelleted at 12,000xg, (4°C) for 5 min, and the supernatant was kept as the soluble nuclear fraction. The pellet was washed and resuspended in 500 μl of NHB and kept as the insoluble fraction.

**Fluorescent Spectroscopy Analyses**

The effect of the phosphorylation-mimic mutation, S62D, on the structure of 14-3-3ω was studied by measuring the changes in the intrinsic tryptophan fluorescence of 14-3-3ω using a fluorometer (Jobin Yvon HORIBA FluoroMax-3). *E.coli* expressed 14-3-3ω and its mutants (ωS62D and ωS62A) (25 μg/ml) (pH 7.5) were excited at 295 nm (slit width of 5 nm) in PBS buffer and the intrinsic tryptophan fluorescence was recorded in the range of 300–400 nm (slit width of 2.5 nm).

Hydrophobicity of wild type 14-3-3ω and mutant 14-3-3ωS62D and 14-3-3ωS62A was measured with bis-ANS (Invitrogen), a fluorescent hydrophobic probe that binds to hydrophobic residues in proteins (Takashi et al., 1977). Recombinant proteins (25
μg/ml) in PBS buffer (pH 7.5) were incubated with indicated concentrations of bis-ANS (Figure 2-5) and were excited at 295 nm (slit width of 5 nm). Fluorescence values were recorded in the range of 305-575 nm (slit width of 5 nm).

**Cross-linking Studies**

*S. cerevisiae* strain INVSc1 (*MATa his3D1 leu2 trp1-289 ura3-52 MATAlpha his3D1 leu2 trp1-289 ura3-52*) was transformed with p415GPD-14-3-3ω, p415GPD-14-3-3ωS62D and p415GPD-14-3-3ωS62A plasmids by using the standard lithium acetate/polyethylene glycol method (Gietz and Woods, 2006). Transformed cells were selected on SC-Leu- plates. A single colony from each transformation was picked and cultured overnight in SC-Leu- media to express recombinant 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A proteins. Overnight cultures were spun down and re-suspended in yeast breaking buffer (50 mM sodium phosphate, pH 7.4, 5% glycerol, 1 mM PMSF 1mM DTT, phosphatase-inhibitor mixture and protease-inhibitor mixture (Calbiochem)) to OD600 of 75. Cell-free yeast lysates were prepared by homogenization with acid-washed glass beads and subsequently centrifuged at maximum speed for 10 minutes. Recombinant 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A in cell-free yeast lysates and endogenous Arabidopsis 14-3-3ω localized in the cytoplasmic fraction of protoplasts were cross-linked with varying concentrations of bis[sulfo succinimidyl] suberate (BS3) (Pierce) at room temperature for 30 minutes. The Cross-linking reaction was quenched by adding Tris-HCl, pH 7.5 to a final concentration of 20-50 mM.

**SDS-PAGE and Western Blot Analysis**

Recombinant proteins, and plant and yeast lysates, were resolved using a discontinuous SDS-PAGE gel (4% stacking gel, 12% resolving gel). Gel resolved proteins were transferred to nitrocellulose membranes for western blot analysis using a
Mini-Trans Blot cell (BioRad). Membranes were blocked overnight with PBS containing 0.05% (v/v) Tween 20 and 5% (w/v) non-fat milk powder. Blocked membranes were washed with PBS containing 0.05% Tween 20 and incubated for 1 h with polyclonal anti-14-3-3\(\omega\) antibody, anti-ADH antibody and anti-Histone H3 antibody (1:2000) in PBS containing 0.05% Tween 20. Membranes were washed with PBS containing 0.05% Tween 20 and incubated for 45 min with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:4000) in PBS containing 0.05% Tween 20. Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo-Pierce).

**Bimolecular Fluorescence Complementation (BIFC)**

Protoplasts isolated from Arabidopsis suspension cells were transformed with bimolecular fluorescence complementation (BIFC) vectors as described in Akhtar et al. (2008). Briefly, protoplasts were prepared as described in the subcellular fractionation method. Protoplasts were incubated in W5 solution (154 mM NaCl, 5 mM KCl, 125 mM CaCl\(_2\), 5 mM Glc, 2 mM MES-KOH, pH 5.7) for 30 min on ice before the transformation according to the protocol described in Yoo et al., (2007).

**Results**

**14-3-3 Dimerization Interface Shows Sequence Diversity**

The 3D structure models developed for both animal and plant 14-3-3s (Liu et al., 1995; Xiao et al., 1995; Wurtele et al., 2003; Ottmann et al., 2009) indicate that the dimerization of 14-3-3s occurs between helix H1 of one monomer and helices H3 and H4 of the other monomer (Figure 2-1A). Protein sequences around the dimerization interface in Helix H1, Helix H3 and Helix H4 of Arabidopsis 14-3-3s were aligned with online MultAlin software (http://multalin.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988). The alignment showed generally high conservation around the dimerization interface.
domains, but a substantial level of amino acid sequence diversity is also present, which might be responsible for differential affinity in heterodimer formation (Figure 2-1B).

**Arabidopsis 14-3-3ω Exists as Dimers and Monomers in the Cytoplasm of Arabidopsis Tissue Culture Cells**

The dimeric status of 14-3-3ω in protoplasts isolated from Arabidopsis tissue culture cells was investigated by cross-linking with BS3. Western blot analysis with subcellular fractionations of Arabidopsis protoplasts showed that 14-3-3ω is predominantly cytoplasmic. However, the evolutionarily distinct 14-3-3ι isoform was localized in the non-soluble nuclear fraction of Arabidopsis protoplasts (Figure 2-3A). These data suggest that heterodimerization of some 14-3-3 isoform combinations may not occur in vivo due to the different subcellular compartmentalization. To study the dimer/monomer status of the 14-3-3ω, the cytoplasmic fraction was cross-linked with indicated concentrations of bis(sulfosuccinimidyl) suberate (BS3), and western blotting was performed with polyclonal isoform-specific 14-3-3ω antibody (Figure 2-3B). This experiment showed that 14-3-3ω was present in both monomeric and dimeric conformations. Considering that previously the native forms of 14-3-3 proteins were reported to be homo- and hetero-dimers (Ferl. 1996), this result suggests that there might be a cellular mechanism that regulates the monomer/dimer status of 14-3-3s in Arabidopsis.

**Effects of ωS62D Phosphorylation-mimicking Mutation on Arabidopsis 14-3-3ω Structure**

Several groups showed that the phosphorylation of Ser-58 in Helix 3 inhibits dimerization of human 14-3-3ζ (Powell et al., 2003; Woodcock et al., 2003; Gu et al., 2006). 14-3-3 protein sequence alignment of human 14-3-3ζ (zeta) with Arabidopsis 14-3-3s showed that this serine residue is conserved in eleven Arabidopsis 14-3-3 isoforms
(Figure 2-4A). Here, the structural effects of phosphorylation-mimic mutation ωS62D in Arabidopsis 14-3-3ω were examined. The ωS62D residue corresponds to Ser-58 in human 14-3-3ζ. Sluchanko et al. (2008) studied the effect of several phosphorylation-mimic mutations, including Ser-58 to Glu-58 (S58E), on the structure of human 14-3-3ζ. They showed that S58E increased the monomeric status and the susceptibility of 14-3-3ζ to proteolysis. Arabidopsis 14-3-3ω has a total of two trytophans (W-63 and W-234), one of which, W-63, is in the dimerization interface. The intrinsic tryptophan fluorescence properties of 14-3-3ω and 14-3-3ωS62D were compared by exciting at 295 nm. The florescent emission was recorded in the range of 300-400 nm. Both proteins showed the same emission maxima at 341 nm, but 14-3-3ωS62D had almost 2-fold the relative fluorescence intensity of wild type 14-3-3ω. On the other hand, the intrinsic tryptophan fluorescence of wild-type 14-3-3ω and 14-3-3ωS62A were essentially the same (Figure 2-4B). The higher intrinsic fluorescence of the ωS62D mutation suggests that tryptophans in 14-3-3ωS62D are more accessible to the solvent than those in wild-type 14-3-3ω.

If the ωS62D point mutation causes structural changes in 14-3-3ω, it may affect the hydrophobic nature of the protein as well. The effect of ωS62D point mutation on the hydrophobicity of 14-3-3ω was investigated by bis-ANS fluorescence. Bis-ANS is a molecule that binds to hydrophobic residues on the surfaces of proteins and becomes fluorescent (Andley et al., 2008). The mutant 14-3-3ωS62D protein exhibited higher bis-ANS fluorescence compared to wild-type 14-3-3ω (Figure 2-5A and 2-5B), and a significant blue shift of the emission maximum from 520 nm to 480 nm (Figure 2-5B). These data suggest that the ωS62D phosphorylation-mimic mutation results in higher
exposure of hydrophobic residues to bis-ANS binding (Figure 2-5B). The intensity of the fluorescence and the emission maxima of the nonphosphorylation-mimic control mutant 14-3-3ωS62A protein were not significantly different than wild type 14-3-3ω (Figure 2-5C).

**14-3-3ωS62D Cannot Form Homo-dimers in Yeast**

The impact of the ωS62D mutation in dimerization of 14-3-3ω in yeast was investigated by two different approaches. First, we employed the yeast two-hybrid assay by cloning 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A each into bait (pDEST32) and prey (pDEST22) vectors. Yeast two-hybrid assays for homodimerization of 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A were performed by co-transforming *S. cerevisiae* AH109 yeast cells with bait and prey vectors. A single colony from each co-transformation was picked and cultured overnight in liquid SC-Leu-Trp- media. Homodimerization was indicated by yeast growth on SC-Leu-Trp-His- dropout media. This assay showed that 14-3-3ω and 14-3-3ωS62A can each form a homodimer, whereas 14-3-3ωS62D cannot (Figure 2-6A).

Second, the effect of the ωS62D mutation on the ability of 14-3-3ω to homodimerize was studied by cross-linking. The 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A coding sequences were cloned into p415GPD constitutive yeast expression vectors and transferred into the yeast strain INVSc1, a fast-growing diploid strain used for protein expression. Transformed cells were selected on Sc-Leu- plates. A single colony from each transformation was picked and cultured overnight in SC-Leu- liquid media. Cell lysates were cross-linked with indicated concentrations of BS³ (Figure 2-6B). This cross-linking study showed that the ωS62D mutation interfered with dimer formation dramatically compared to levels exhibited by wild-type 14-3-3ω and 14-3-
3ωS62A as noted by the reduced band at 29 kDa (Figure 2-6B). These results show that 14-3-3 dimerization has the potential for regulation in vivo through a mechanism involving phosphorylation at the conserved Ser-62 of 14-3-3ω.

**Heterodimerization of Arabidopsis 14-3-3s Can Be Regulated by Ser-62 Phosphorylation**

With thirteen 14-3-3 isoforms, Arabidopsis can have potentially 91 different (13 homo- and 78 hetero-) 14-3-3 dimer combinations. However, the regulation of how isoforms chose their dimer partners is still a mystery in 14-3-3 biology. Here we discovered the potential for phosphorylation-mediated regulation of 14-3-3 dimer partner selection. The coding sequences of 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A were cloned into the DNA binding domain fusion (bait) vector, pDEST32, and all thirteen Arabidopsis 14-3-3 isoforms were cloned in to the activation domain fusion (prey) vector pDEST22. Yeast two-hybrid assays were performed to characterize the dimerization profiles of 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A. Colony growth on triple dropout plates (Sc-Leu-Trp-His-) demonstrated that the wild-type 14-3-3ω can dimerize with all thirteen isoforms with various interaction affinities (Figure 2-7A). However, phosphorylation-mimic mutation ωS62D was not able to form dimers with 14-3-3ι, 14-3-3μ, 14-3-3π, 14-3-3φ, 14-3-3κ and 14-3-3λ. In addition, 14-3-3ωS62D monomer has an increased dimerization affinity for 14-3-3ω, 14-3-3χ, 14-3-3ω, 14-3-3υ, 14-3-3ψ and 14-3-3υ (Figure 2-7B). These results showed that phosphorylation of Ser-62 may influence both the selection of a dimerization partner and dimer affinity. On the other hand, the dimerization profile of 14-3-3ωS62A was not significantly different than wild-type 14-3-3ω (Figure 2-7C).
14-3-3ωS62D Cannot Form Homodimers in Arabidopsis Protoplasts

The interference of the ωS62D mutation with 14-3-3ω homodimerization was further investigated by a biomolecular fluorescence complementation (BiFC) assay in Arabidopsis protoplasts using a transient expression system (Akhtar et al., 2008). The principle of BiFC assay is summarized in Figure 2-8A. 14-3-3ω and 14-3-3ωS62D coding sequences fused to N-terminal (YFP<sub>N</sub>) and C-terminal (YFP<sub>C</sub>) fragments of the yellow fluorescent protein (YFP) were co-transformed into Arabidopsis protoplasts (Figure 2-8B). YFP signal was detected when 14-3-3ω-YFP<sub>N</sub> and 14-3-3ω-YFP<sub>C</sub> were co-transferred into protoplasts indicating 14-3-3ω homodimerization in Arabidopsis protoplasts. In contrast, protoplasts co-transferred with 14-3-3ωS62D-YFP<sub>N</sub> and 14-3-3ωS62D-YFP<sub>C</sub> did not have strong YFP fluorescence, indicating that the ωS62D mutation interferes with 14-3-3ω homodimerization in protoplasts (Figure 2-8B). The dimerization of 14-3-3ωS62D and 14-3-3ω observed in the yeast two-hybrid assay was also tested (Figure 2-7B) with the BiFC assay in protoplasts. We detected a very strong YFP fluorescence when Arabidopsis protoplasts were co-transferred with 14-3-3ωS62D-YFP<sub>N</sub> and 14-3-3ωS62D-YFP<sub>C</sub> (Figure 2-8B). This result suggested that 14-3-3ωS62D can still interact with non-phosphorylated 14-3-3ω with higher affinity than 14-3-3ω.

Phosphorylation of Ser-62 Regulates Target Interactions

Dimerization is essential for most 14-3-3 interactions. There are several reports showing that 14-3-3/target interactions are regulated by the dimer status of the 14-3-3s (Tzivion et al., 1998; Shen et al., 2003). Recently, Zhou et al. (2009) showed that phosphorylation of mammalian 14-3-3ζ at Ser-58 interferes with the 14-3-3/ASK1 interaction, which leads to ASK1-mediated oxidant stress-induced cell death. In this part
of the study, the effect of the \( \omega S62D \) mutation on 14-3-3/\( \omega \)/target interactions was investigated using two known 14-3-3 targets, difopein (dimeric fourteen-three-three peptide inhibitor) and nitrate reductase, representing the non-phosphorylated and phosphorylated targets respectively. A non-phosphorylated synthetic 14-3-3 target, R18 peptide, was isolated from a phage display screen against mammalian 14-3-3\( \tau \) isoform (Wang et al., 1999). Later, another high affinity 14-3-3 target, difopein, was created by Masters and Fu (2001) by combining two R18 peptides separated by a short linker sequence. In the same study, they also developed a negative control, R18 (Lys), by changing two acidic residues (D12 and E14) to lysine, which caused a disruption of 14-3-3 binding. We received EYFP fusions of difopein and R18(Lys) in the pEYFP vector as gifts from Dr. Haian Fu (Emory University) and inserted the coding sequences into the yeast two-hybrid bait vector, pDEST32 and GST-tagged pDEST15 using Gateway cloning. First, the impact of the \( \omega S62D \) mutation on the 14-3-3\( \omega \)/difopein interaction was studied using a yeast two-hybrid assay. The yeast two-hybrid assay indicated that wild-type 14-3-3\( \omega \) and 14-3-3\( \omega S62A \) mutant proteins did interact with EYFP-difopein in yeast. However, the \( \omega S62D \) mutation disrupted the 14-3-3/difopein interaction (Figure 2-9A). In contrast, 14-3-3\( \omega \), 14-3-3\( \omega S62A \) and 14-3-3\( \omega S62D \) did not interact with EYFP-R18(Lys), the negative control (Figure 2-9B).

Second, the effect of \( \omega S62D \) mutation on the kinetics of 14-3-3\( \omega \)/EYFP-difopein and 14-3-3\( \omega \)/EYFP-R18(Lys) (the negative control) interactions were characterized. Wild-type 14-3-3\( \omega \) and 14-3-3\( \omega S62D \) proteins were covalently immobilized onto Amine Reactive Biosensors and the real time interaction with EYFP-difopein and EYFP-R18 (Lys) were recorded using the Octet QK platform (ForteBio) (Figure 2-10). The kinetic
values were calculated using Octet software (Table 2-1). Both 14-3-3ω and 14-3-3ωS62D proteins showed no significant interaction with the negative control peptide EYFP-R18(Lys), indicating that interaction between 14-3-3ω and EYFP-difopein was specific (Figure 2-10C and 10D, Table 2-1).

The sensogram obtained with wild-type 14-3-3ω and EYFP-difopein (0.25μM-2μM) interaction demonstrated a strong binding affinity (Figure 2-10A) with an association rate ($k_{on}$) of $1.07 \pm 0.01 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and a dissociation rate ($k_{off}$) of $5.96 \pm 0.05 \times 10^{-5} \text{s}^{-1}$. The equilibrium dissociation constant ($K_D = k_{off}/k_{on}$) for the 14-3-3ω/EYFP-difopein interaction was 55.9 nM (Table 2-1). On the other hand, monomeric 14-3-3ωS62D showed a lower affinity to EYFP-difopein (Figure 2-10B). Although the association rate of 14-3-3ωS62D with EYFP-difopein, $1.47 \pm 0.05 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, was very similar to wild-type 14-3-3ω, the 14-3-3ωS62D dissociated from EYFP-difopein 12-fold faster than 14-3-3ω with a dissociation rate of $5.05 \pm 0.06 \times 10^{-6} \text{s}^{-1}$, resulting in an equilibrium dissociation constant $K_D$ of 345 nM (Table 2-1). In summary, the kinetics data showed that the monomeric 14-3-3ωS62D has 6 times lower affinity than wild-type 14-3-3ω to EYFP-difopein.

Finally, the effect of ωS62D mutation on binding of 14-3-3ω to the N-terminal half of nitrate reductase (NR1-562aa) was characterized. Nitrate reductase is a well studied phosphorylated 14-3-3 target (Kanamaru et al., 1999) by yeast two-hybrid assays. Nitrate reductase is phosphorylated at Ser-534, and this phosphorylation is required for 14-3-3 interaction (Kanamaru et al., 1999). The point mutation S534L on NR1-562aa was created to use as a negative control for the yeast two-hybrid interaction assay. The greatly reduced growth of colonies in the yeast two-hybrid assay showed that the
ωS62D mutation significantly decreased the affinity of 14-3-3ω for NR1-562aa (Figure 2-11A). As expected, the negative control, the S534L mutation of NR1-562aa did not interact with 14-3-3ω or 14-3-3ωS62D (Figure 2-11B).

Discussion

The 14-3-3 proteins are essential regulatory proteins that are involved in multiple essential cellular processes through interacting with over one hundred different phosphorylated target proteins both in plants and animals. Some of the important 14-3-3 target proteins include transcription factors, metabolic enzymes, cell cycle regulators, proteins involved in programmed cell death and signaling proteins (Mackintosh, 2004; Jin et al., 2004; Oecking and Jaspert, 2009; Paul et al., 2009; Chang et al., 2009). Regulation of 14-3-3/target interaction can happen at two different levels: 1) phosphorylation at the level of the target (Figure 2-2A), and 2) phosphorylation at the level of 14-3-3 protein itself (Figure 2-2B). The regulation of 14-3-3/target interactions by phosphorylation of 14-3-3s has been reported previously. Phosphorylation of human 14-3-3ζ at Ser-58 by PKA and B/Akt kinases has been shown to affect target interactions by influencing the capacity of 14-3-3ζ to dimerize (Gu et al., 2006, Powell et al., 2002). Two other residues of 14-3-3ζ, Ser-184 and Thr232, have been shown to be phosphorylated and to affect 14-3-3/target interactions (Aitken et al., 1995; Dubois et al., 1997).

This study demonstrated that Arabidopsis 14-3-3ω is mainly localized in cytoplasm and is present both in dimer and monomer conformations (Figure 2-3B). Possible role of Ser-62 phosphorylation in the dimerization interface of Arabidopsis 14-3-3ω in the regulation of dimerization and target interactions was investigated. A site directed mutagenesis approach was used to imitate the phosphorylation at Ser-62 by replacing
this residue with Asp. The phosphorylation-mimic mutant ωS62D caused significant biophysical changes to the structure of 14-3-3ω as evidenced by the higher intrinsic tryptophan fluorescence, increased hydrophobicity, of 14-3-3ωS64D, might be due to the destabilization of the dimer conformation. This hypothesis was confirmed by several different in vivo studies such as yeast two-hybrid, cross-linking and BiFC in Arabidopsis protoplasts.

Although the S62D point mutation abolished the homo-dimerization ability of 14-3-3ω, the yeast-two hybrid assay showed that 14-3-3ωS62D can still form hetero-dimers with seven Arabidopsis 14-3-3 isoforms (Figure 2-7B). In addition, the dimerization affinity of the mutant 14-3-3ωS62D for some isoforms was higher than wild-type 14-3-3ω. The capacity of Ser-62 phosphorylation to influence dimerization may explain how 14-3-3s choose their dimer partner in response to certain signals and in different cellular locations.

Finally, inhibition of dimerization of 14-3-3s by phosphorylation significantly reduced the interaction of 14-3-3ω with phosphorylated and non-phosphorylated targets such as nitrate reductase and EYFP-difopein respectively. In summary, phosphorylation of Ser-62 has a potential regulatory role in both dimerization and target interactions.
Figure 2-1. 14-3-3 proteins consist of 9 helices. A) Top view of 14-3-3 dimer model derived from *Nicotiana tabacum* 14-3-3 in complex with the differentiation-inducing fungal agent Cotylenin A (PDB3E6Y) (Figure adapted from Ottmann et al., 2009) using PDB SimpleViewer 3.8 software. B) Whole protein sequence alignment of Arabidopsis 14-3-3 protein family. Protein sequences were aligned using online MultAlin software (http://multalin.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988). The helices in dimerization interface were indicated with boxes. High consensus (= 90 %) residues are in “red”, low consensus (= 50 %) residues are in “blue” and non-conserved residues are in “black”. Consensus symbols: ! is anyone of I and V; $ is anyone of L and M; % is anyone of F and Y; # is anyone of N, D, Q, E, B and Z.
Figure 2-2. Regulation of 14-3-3/target interactions by phosphorylation. A) The phosphorylation state of the target protein carrying the 14-3-3 binding motif regulates the 14-3-3 interactions. B) Phosphorylation of 14-3-3 at the dimerization interface disrupts dimer structure, which also inhibits target interaction.
Figure 2-3. Dimer/monomer status of 14-3-3ω in the cytoplasmic fraction of Arabidopsis protoplasts. A) Subcellular fractionations of Arabidopsis protoplasts and localization of 14-3-3ω and 14-3-3ι. 1. Total protoplast, 2. Cytoplasmic fraction, 3. Total nuclear fraction, 4. Soluble nuclear fraction, 5. Insoluble nuclear fraction. Arabidopsis alcohol dehydrogenase (AtADH) and histone H3 antibodies are used as cytoplasmic and nuclear controls, respectively. B) The cytoplasmic fraction of Arabidopsis protoplasts was cross-linked with indicated concentrations of BS³. 14-3-3ω was detected with western blotting using 14-3-3ω-specific antibody.
Figure 2-4. The effects of ωS62D mutation on the intrinsic fluorescence of 14-3-3ω. A) Protein sequence alignment of Helix H3 of human 14-3-3ζ and Arabidopsis 14-3-3 protein family (At14-3-3). The phosphorylatable Ser-58 that is conserved in human 14-3-3ζ and 11 Arabidopsis 14-3-3 isoforms is indicated. B) Intrinsic tryptophan fluorescence of At14-3-3ω (blue), 14-3-3ω S62D (red) and 14-3-3ω S62A (green). Recombinant proteins (25 μg/ml) were excited at 295 nm and recorded from 300 nm to 400 nm.
Figure 2-5. Phosphorylation-mimic mutation ωS62D increases the hydrophobicity of 14-3-3ω. Interaction of 14-3-3ω and its mutations with the hydrophobic probe bis-ANS. The Y-axis represents fluorescence resonance energy transfer (FRET) from excited Trp residues at 295 nm to bis-ANS interacting with the hydrophobic surfaces of A) 14-3-3ω, B) 14-3-3ωS62D and C) 14-3-3ω S62A. All recombinant proteins were at 25 μg/ml, and bis-ANS concentrations were indicated.
Figure 2-6. Dimer status of 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A expressed in yeast cells. A) Yeast two-hybrid assays showed that 14-3-3ω and 14-3-3ωS62A monomers interact in yeast (top and bottom panels respectively). However, 14-3-3ω S62D monomers did not interact (middle panel). B) 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A over-expressed in INVSc1 yeast strain were cross-linked with indicated concentrations of BS³. The Dimer status of the proteins was detected by western blot using 14-3-3ω specific antibody.
Figure 2-7. The effects of dimerization interface mutations of Ser-62 to Asp-62 (ωS62D) and to Ala-62 (ωS62A) of Arabidopsis 14-3-3ω with thirteen Arabidopsis 14-3-3 isoforms in yeast two-hybrid assay. A) Dimerization profile of wild-type 14-3-3ω. B) Dimerization profile of 14-3-3ωS62D. C) Dimerization profile of 14-3-3ωS62A.
Figure 2-8. ωS62D phospho-mimic mutation interferes with 14-3-3ω homodimerization in Arabidopsis protoplasts. A) Schematic representation of the of BIFC assay for 14-3-3 dimerization. N-terminal YFP fragment (YFPN) and C-terminal YFP fragment (YFPC) in blue and orange, respectively, were fused to the C-terminal ends of 14-3-3ω and 14-3-3ωS62D proteins. B) BIFC fluorescence and bright-field photos of Arabidopsis protoplast transiently expressing 14-3-3ω-YFPN/14-3-3ω-YFPC, 14-3-3ωS62D-YFPN/14-3-3ωS62D-YFPC and 14-3-3ωS62D-YFPN/14-3-3ω-YFPC fusion proteins.
Figure 2-9. Phosphorylation mimic mutation of Ser-62 to Asp-62 of 14-3-3ω inhibits 14-3-3/target interaction. A) Protein-protein interaction using yeast two-hybrid assay with 14-3-3ω, 14-3-3ωS62D and 14-3-3ωS62A in activation domain fusion vector and EYFP-difopein in DNA binding domain fusion vector interaction and B) EYFP-R18(Lys). pDEST22 represents the empty control vector.
Figure 2-10. Kinetic analyses of effect of Ser-62 to Asp (ωS62D) phosphorylation-mimic mutation in 14-3-3ω on binding to EYFP-Difopein (2000-250 nM) and EYFP-R18 (Lys) (2000-250 nM). A) 14-3-3ω/EYFP-Difopein binding kinetics, B) 14-3-3ωS62D/EYFP-Difopein binding kinetics, D) 14-3-3ω/EYFP-R18(Lys) binding kinetics, C) 14-3-3ωS62D/EYFP-R18(Lys) binding kinetics.
Figure 2-11. Phosphorylation mimic mutation S62D in the dimerization interface of 14-3-3ω inhibits 14-3-3ω/phosphorylated target interaction. A) Yeast two-hybrid assay to show protein-protein interactions between 14-3-3ω (in the activation domain fusion vector) and A) NR1-562aa (in the DNA binding domain fusion vector) or B) NR1-562aaS534L (in the DNA binding domain fusion vector). pDEST22 represents empty control vector.
Table 2-1. Kinetic analysis of 14-3-3ω and 14-3-3ωS62D against non-phosphorylated targets

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Analyte</th>
<th>$K_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_{off}$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3ω</td>
<td>EYFP-Difopein</td>
<td>$(1.07 \pm 0.01) \times 10^3$</td>
<td>$(5.96 \pm 0.05) \times 10^{-5}$</td>
<td>55.9</td>
</tr>
<tr>
<td>14-3-3ωS62D$^b$</td>
<td>EYFP-Difopein</td>
<td>$(1.47 \pm 0.05) \times 10^3$</td>
<td>$(5.05 \pm 0.06) \times 10^{-6}$</td>
<td>345</td>
</tr>
<tr>
<td>14-3-3ω$^c$</td>
<td>EYFP-R18(Lys)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>14-3-3ωS62D$^d$</td>
<td>EYFP-R18(Lys)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$K$_D$ values of each interaction are calculated as $k_{off}/k_{on}$. $^b$Kinetic parameters of this interactions are calculated with 2000nm and 1000nm concentration of analytes. Lower concentrations (500nm and 250nm) of anaylte did not give any significant kinetics parameters. $^c$ and $^d$ no significant interaction determined.
CHAPTER 3
DIRECT INTERACTION BETWEEN ARABIDOPSIS 14-3-3Ω AND G-BOX BINDING FACTOR 3 (GBF3)

Introduction

Gene regulation is a complex multistep cellular task that involves integration of several cellular processes such as signal transduction pathways, protein-protein interactions, subcellular compartmentalization of proteins, chromatin structure remodeling, RNA synthesis and RNA processing. Transcription factors have fundamental roles in gene regulation, which controls all major events of the organisms such as cell cycle, development, metabolism and environmental responses. The availability of the full sequence for the Arabidopsis genome has led to the realization that there are more than 1500 transcription factors, which represents approximately 5% of the Arabidopsis genome. These have been classified into several different families, one of which is the basic leucine zipper (bZIP) family, which is the eighth largest in Arabidopsis (Riechmann et al., 2000). With more than eighty members, bZIPS participate in the regulation of diverse biological processes such as light and stress signalling, pathogen response, hormone response, seed maturation and flower development (Marc Jakoby et al. 2002).

G-box binding factor 3 (GBF3) is a member of the bZIP transcription factor family that binds to a cis acting element called G-box (5'-CCACGTGG-3'). The G-box is present in many plant gene promoters (Yamaguchi-Shinozaki and Shinozaki, 2006; Lu et al., 1996) and is involved in the regulation of the response to diverse environmental stimuli, such as cold, salt, light, dehydration, hypoxia and abscisic acid (ABA) (Mallappa et al., 2008; Menkens and Cashmore, 1999,
Shinozaki et al., 1997). In addition to GBF3, the G-box sequence is recognized by a variety of other bZIP and also basic helix-loop-helix (bHLH) transcription factors (de Pater et al., 1997; Martinez-Garcia et al., 2000; Malaba et al., 2006; Kim et al., 2007, Alanso et al., 2009). The binding affinity of bHLH transcription factors is not exclusive to the whole G-box, but rather to the central CANNTG sequence (Siberil et al., 2001). The flanking sequences of the G-box have a significant role in determining the specificity and affinity for the range of the GBF interactions with diverse to the entire G-box sequences (Williams, et al., 1992; Schindler et al., 1992a; Izawa et al., 1993). The term GBF is, therefore, closely linked to these bZIP factors that bind to the G-box.

GBF proteins have three major domains: The N-terminal proline-rich domain, the central bZIP domain and an undefined C-terminal domain (Figure 3-1). The N-terminal proline-rich domain is involved in either in transactivation, as in GBF1 (Schindler et al., 1992b), or repression as with soybean SGBF-2 (Liu et al., 1997). The central bZIP domain consists of a basic region for DNA binding and a nuclear localization signal (Hurst, 1996; Terzaghi et al., 1997) together with a leucine zipper domain for dimerization (Figure 3-1). The C-terminal region does not, as yet, have an assigned function.

Transcripts encoding Arabidopsis GBF3 and several G-box binding bZIPs, from other plant species such as bean ROM1 and ROM2, rice OsBZ8, and maize ZmBZ-1 and EmBP-2 have been shown to be up-regulated in response to ABA treatment (Lu et al., 1996; Chern et al., 1996a; Chern et al., 1996b; Nakagawa et al., 1996; Nieva et al., 2005; Suzuki et al., 2003). Another study also showed that
GBF3 transcript is up-regulated in the dark (Schindler et al., 1992c). Arabidopsis GBF3 transcript profile using the Genevestigator Response Viewer (http://www.genevestigator.com) Arabidopsis microarray database showed that GBF3 transcription is highly induced by osmotic stress, drought, ABA, salt stress, cold and light (Figure 3-2). These data suggest that, as a transcription factor, GBF3 may play a very important role in regulation of expression of genes involved in environmental response.

The G-box of alcohol dehydrogenase (Adh) is among the well studied cis elements in plants and is known to be occupied by a multiprotein complex that includes 14-3-3 proteins (Ferl and Laughner, 1989; DeLisle and Ferl, 1990; McKendree et al., 1990; Lu et al., 1992; Lu et al., 1994). In addition, GBF3 can interact in vitro with the G-box in the Adh promoter specifically. The in vivo footprint profile of the Adh G-box and in vitro footprint profile of the GBF3/G-box were essentially the same (Lu et al., 1996). However, the 14-3-3 proteins do not directly interact with the G-box element (Lu et al., 1992). It was proposed that 14-3-3s can be part of the G-box protein complex through interaction with GBF3 or another protein (Lu et al., 1996; Paul and Ferl, 1997). The abscisic acid-VIVIPAROUS1 (VP1) response complex that interacts with a G-box in the rice Em promoter is another example where 14-3-3 proteins are part of the G-box binding protein complex (Schultz et al., 1998). In addition, an in vitro cross-linking study showed that 14-3-3 proteins directly interact with EmBP1, a GBF-like bZIP transcription factor, and VP1, a tissue specific regulatory factor. It has been
proposed that 14-3-3 protein has a scaffolding type of function in the assembly of transcription complexes involving VP1 and EmBP1 (Schultz et al., 1998).

The 14-3-3 proteins are phosphopeptide-binding proteins that have been associated with many important biological processes in eukaryotes such as signal transduction, cell cycle regulation, apoptosis, metabolic pathways and cell differentiation (Fu et al., 2000; Ferl et al., 2002). 14-3-3s exist in multiple isoforms and can form homo- and hetero-dimers. 14-3-3s were first discovered in plants to be part of G-box binding complexes and thought to regulate the Adh promoter (Lu et al., 1992). Later they were shown to be involved in regulation of important plant metabolic enzymes such as nitrate reductase, ATP synthase, sucrose phosphate synthase, glutamate synthase starch synthase and ascorbate peroxides (Ferl, 1996). Although much of the 14-3-3 related plant research is focused on the role of 14-3-3s in the regulation of metabolic enzymes, several studies have revealed a role of 14-3-3s in plant signaling and gene regulation (reviewed in Oecking and Jaspert, 2009; Gokirmak et al., 2010).

Several studies showed that 14-3-3 proteins may be actively involved in stress management in plants. Four out of eight rice 14-3-3 genes, GF14b, GF14c, GF14e and Gf14f, are differentially regulated by abiotic stresses such as salinity, drought, wounding and abscisic acid (Chen et al., 2006). In tobacco, T14-3-3 is induced by salt stress (Chen et al., 1994). The 14-3-3 proteins function as regulators of target proteins that are involved in several abiotic stress responses. In Arabidopsis the activity of mitochondrial and chloroplast ATP synthases are regulated by 14-3-3 proteins during the light/dark transition.
(Bunney et al., 2001). A transgenic study with cotton showed that constitutive expression of a cotton 14-3-3 protein, GF14λ, conferred drought tolerance, probably through controlling the stomatal opening by H+-ATPase regulation (Yan et al., 2004). The 14-3-3 proteins also play an essential role in ABA signaling pathway. Schoonheim et al. (2007) showed that 14-3-3s are intermediate players in ABA signaling during barley seed germination. The interaction between 14-3-3 proteins and ABI5 family members increases the trans-activation capacity of ABI5.

Considering the well established role of both GBF3 and 14-3-3s in stress regulation in plants, the present study was designed to demonstrate a genetic and direct interaction between 14-3-3ω and GBF3 using a heterologous model system S. cerevisiae. Expression of GBF3 in Saccharomyces cerevisiae causes cellular toxicity. Serial N-terminal and C-terminal deletions showed that this toxicity can be released by deletion of an N-terminal proline rich domain or the C-terminal undescribed domain. This suggests these two domains are essential for GBF3 function. Co-expression of 14-3-3s in yeast rescue GBF3 mediated cellular toxicity through direct 14-3-3/GBF3 interaction. This suggests that 14-3-3 proteins have an antagonistic role in GBF3 mediated regulation of transcription in plants.

Materials and Methods

Yeast Strains and Media.

*S. cerevisiae* strain INVSc1 *(MATa his3D1 leu2 trp1-289 ura3-52)*

*MATAlpha his3D1 leu2 trp1-289 ura3-52* was used for galactose-inducible protein expression. *S. cerevisiae* strain AH109 *(MATa, trp1-901, leu2-3, 112, 112)*
ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3,GAL2UAS-GAL2TATA ADE2,URA3::MEL1UAS-MEL1TATA-lacZ, MEL1) was used for yeast two-hybrid assays. Yeast cells cultured in rich medium (YPD: 1% yeast extract, 2% bactopeptone, and 2% glucose) were transformed by the standard lithium acetate/polyethylene glycol method (Gietz and Woods, 2006). Transformed cells were selected on synthetic dropout media (SD) (Clontech, CA) plates deficient in specific amino acids for plasmid selection. Spot assays, as a measure of growth, were performed with yeast cells cultured overnight in appropriate SD-glucose medium. Cells were pelleted and washed in sterile water. Concentrations of overnight cultures were adjusted to OD600 of 0.2. Ten-fold serial dilutions of each normalized culture were prepared. Aliquots of 10 µl from each culture and dilutions were spotted on SD-glucose or SD-galactose plates and incubated at 30°C for the indicated times.

**Plasmids**

The full length ORF of Arabidopsis GBF3 (At2g46270) and 14-3-3ω (At1g78300) ORFs were PCR amplified with Gateway tagged primers (supplemented table 1) from a flower and leaf cDNA library (a gift from Dr. Kevin Folta). The point mutation 14-3-3 ωS62D mimicking phosphorylation of 14-3-3ω was generated by site-directed mutagenesis and overlapping PCR (Ho et al., 1989). PCR fragments were first cloned into the pDONR221 vector to create entry vectors using Gateway BP Clonase II (Invitrogen, CA). N-terminal and C-terminal deletions of GBF3 were PCR amplified with Gateway tagged primers using GBF3 in pDONR221 as template and subcloned into pDONR221 as
described earlier. The 14-3-3s, full length GBF3 cDNA and its N-terminal deletions were cloned into pDEST22 (GAL4 AD), pDEST32 (GAL4 DBD) and Gal inducible pYES-DEST52 vectors from pDONR221 clones via Gateway LR Clonase II (Invitrogen, CA) reactions. The GBF3-GFP C-terminal fusion construct was created by sequential cloning. GFP was PCR amplified with a forward primer containing a multi-cloning tag and GFP\_attB2 primer. The multi-cloning tagged primer had the following sequence and the restriction sites: 5’-AAGCTT GAGCTC GAATTC ATGGTGAGCAAGGGCGAG-3’ (HindIII site is underlined, SacI site is bold and EcoRI site is italic). The second round of PCR was performed with a forward primer (mcsGFP\_attB1, a primer specific to the multi-cloning site and the first codon of GFP), and the GFP\_attB2 primer. The PCR product was gel purified and cloned into the pDONR221 vector using Gateway BP Clonase II. GFP with the multi-cloning site was cloned into the pYES-DEST52 vector via the Gateway LR Clonase Reaction II. GBF3 was cloned into HindIII and EcoRI sites upstream of the GFP in pYES-DEST52.

**Monitoring Growth Curves and Cell Viability Assay**

Three colonies from yeast cells transformed with pYES-DEST52 empty vector and GBF3 in pYES-DEST52 (pYES-GBF3) were picked and cultured overnight in SD-Ura liquid medium containing 2% glucose at 30°C. Concentrations were normalized for OD\_600 = 0.2 in SD-Ura containing 2% galactose and growth at 30°C was monitored at every 12 hr. Cell viability was measured every 24 hr after induction of GBF3 by galactose. The same numbers of cells transformed with pYES-GBF3 and pYES-DEST52 (vector control) were
plated on three SD-Ura containing 2% glucose plates to repress GBF3 expression. After three days of incubation at 30ºC, the colonies were counted and the percent ratios determined.

**Yeast His-tag Pull-down**

The his-tag pull-down protocol for GBF3 and 14-3-3 interaction was modified from Gong et al., 2006. Briefly, INVSc1 cells co-transferred with pYES-GBF3/p415GPD-14-3-3ω or pYES-GBF3/p415GPD-14-3-3ω S62D were grown in SC-Leu-Ura with 2% glucose overnight at 30ºC. Cells were washed in sterile water and resuspended in 30 ml of SC-Leu-Ura with 2% galactose to induce protein expression from the GAL1 promoters. After 48 hr of culturing at 30ºC with shaking, cells were cross-linked with 1% formaldehyde (final concentration) at room temperature for 20 mins. Cross-linking was quenched with 0.5 M glycine (final concentration) at room temperature for 10 mins. After cross-linking, the cells were washed first in cold Tris-buffered saline (TBS) and then in cold lysis buffer (50 mM HEPES-KOH (pH 7.5), 200 mM KCl, 10 mM MgCl2, 0.1 mM EDTA, 10% (v/v) glycerol and 1% (v/v) Triton X-100). Finally, cells were resuspended in 1 ml of cold lysis buffer containing 1mM PMSF, 1mM DTT, phosphatase-inhibitor mixture and protease-inhibitor mixture (Calbiochem). The cells were broken open by vortexing with an equal volume of acid-washed glass beads. Each 300μl cell-free extract was mixed with 0.1 ml Ni-NTA agarose beads (Sigma) and incubated overnight at 4ºC with agitation. The Ni-NTA agarose beads were gently washed three times with lysis buffer and three times with lysis buffer with 20 mM imidazol and twice with 1 X PBS buffer. Beads were re-suspended in 1X SDS-PAGE buffer, and the samples were boiled for 10 min.
Western blots were performed using a 14-3-3ω isoform-specific polyclonal antibodies and the protein bands visualized by ECL chemiluminescence substrate (Thermo Scientific).

**Fluorescence Microscopy**

Yeast cells co-transferred with pYES-GFP and pYES-GBF3-GFP were cultured overnight in SC-Leu-Ura with 2% glucose at 30°C. Cells were washed in sterile water and protein expression was induced in equal volume of SC-Leu-Ura with 2% galactose for 24 hours. For fluorescence microscopy, cells were fixed in 70% ethanol for 1 hour at room temperature. Nuclei were stained with 1 μg/ml-1 DAPI (Sigma). GFP and DAPI localizations were examined with Olympus BX51 fluorescent microscope coupled to an Evolution MP cooled charge-coupled device camera with Q-capture 2.60 software (Quantitative Imaging, Burnaby, British Columbia, Canada).

**Results**

**GBF3 Has A Toxic Phenotype in Yeast That Can Be Suppressed by 14-3-3s**

Expression of GBF3 severely inhibits yeast growth. Colonies of yeast cells transformed with GAL4 AD-GBF3 were significantly smaller than colonies of cells transformed with vector controls (Figure 3-3A). However, co-expression of certain Arabidopsis 14-3-3s suppressed GBF3 toxicity. Yeast cells co-transformed with AD-GBF3 and Arabidopsis 14-3-3ω, 14-3-3μ or 14-3-3υ fused to the GAL4 DNA binding domain in the pDEST32 vector (DBD-Omega, DBD-Mu and DBD-Upsilon) had normal sized colonies compared to cells co-transformed with empty pDEST22 and pDEST32 vectors (Figure 3-3A). On the other hand, yeast cells co-transformed with AD-GBF3 and DBD-14-3-3π had intermediate
sized colonies (Figure 3-3A). These results suggested that suppression of GBF3 mediated toxicity in yeast by 14-3-3s is isoform specific. When the cells were co-transformed with AD-GBF3 and DBD-14-3-3ωS62D (DBD-ωS62D), the colonies were only slightly larger than the cells expressing only AD-GBF3 (Figure 3-3B), suggesting that, the dimeric status of the 14-3-3 is essential for suppression of the GBF3 toxicity phenotype.

**GBF3 Directly Interacts with 14-3-3ω in Yeast**

The direct interaction of 14-3-3ω with GBF3 was demonstrated with his-tag pull-down assay in yeast. Yeast cells co-expressing GBF3 or GBF3 C-terminal his-tag (GBF3-his tag) and 14-3-3ω or 14-3-3ωS62D were cross-linked with formaldehyde and lysed. The cell lysates were incubated with nickel-his resin overnight at 4°C. The nickel-his resin was then excessively washed and the proteins were eluted. Crosslinks were reversed with boiling in SDS sample buffer. 14-3-3ω and 14-3-3ωS62D bound to GBF3 were analyzed with western blotting (Figure 3-4). As a negative control, GBF3 with no his-tag was used to show that the 14-3-3 pulled from the resin was attached to GBF3 and not due to non-specific binding to the nickel-his resin (Figure 3-4). These data showed that 14-3-3ω and 14-3-3ωS62D directly interact with GBF3.

**GBF3 Toxicity Is Not Related to Yeast Two-hybrid Constructs and Is Likely Due to The Nuclear Function**

To confirm that GBF3 is toxic in yeast cells without the GAL4 AD, full length GBF3 and GBF3-GFP were cloned into pYES-DEST52, a galactose inducible high copy yeast expression vector. INVSc1 cells were transformed with pYES-DEST52, pYES-GBF3 and pYES-GBF3-GFP vectors and plated on Sc-Ura
plates containing 2% glucose to select the transformed cells. A single colony for each transformation was picked and cultured overnight in glucose-containing SD-Ura medium. Cells were spotted on SD-Ura containing glucose and galactose plates. Spot assays on galactose-containing agar plates showed that cell growth was severely inhibited upon galactose induction compared with cells transformed with an empty plasmid and a plasmid expressing GFP alone (Figure 3-5A). Fluorescence microscopy showed that GBF3 was localized into nucleus (Figure 3-5B) suggesting that the toxic phenotype of GBF3 may be linked to its nuclear functions.

We further analyzed GBF3-mediated toxicity by comparing cell growth and cell viability of cells expressing GBF3 versus cells transformed with the empty pYES-DEST52 vector in galactose-containing liquid media culture. The presence of GBF3 not only severely inhibited cell growth but affected cell survival as well (Figure 3-6A). After 48 hr, 56 %, and after 72 hr only 15 % of the cells expressing GBF3 could form colonies on glucose-containing agar plates (Figure 3-6B).

**N-terminal Proline-rich Domain and C-terminal Domain Are Required for GBF3 Toxicity**

GBF3 consists of several domains: 1) an N-terminal proline-rich domain, which is thought to be involved either in transactivation or repression; 2) a central bZIP domain consisting of a basic region for DNA binding, a nuclear localization signal (Hurst, 1996; Terzaghi et al., 1997), and a leucine zipper domain for dimerization; and 3) a C-terminal region that does not have an assigned function (Figure 3-7A). To determine which regions are necessary for GBF3 toxicity a
A panel of N-terminal and C-terminal GBF3 deletions were generated and these deletion series were expressed in yeast cells using the galactose-inducible pYES-DEST52 vector (Figure 3-7A). Spot assay were performed to determine which domains are required for the GBF3 mediated toxicity (Figure 3-7B).

Deletion of the first 70 aa from the N-terminal end, which partially removes the proline-rich domain, caused a dramatic drop in cellular toxicity compared to the full-length GBF3 (Figure 3-7B, construct II). However, deletion of the whole proline-rich domain completely (Δ1-108 aa) completely removed the GBF3 toxicity in yeast cells (Figure 3-7B, construct III). This result suggests that the N-terminal proline rich domain is essential for GBF3-mediated cellular toxicity. This observation was further confirmed with several other N-terminal deletions, which removed additional residues up to the bZIP domain. These deletions also showed no toxicity (Figure 3-7B, constructs IV and V).

To identify other domains that may be critical for GBF3 toxicity, several C-terminal deletions were performed. Removal of last 27 residues (Δ355-382 aa) had no effect on GBF3 toxicity. However, further removal of the C-terminal region up to the bZIP domain (Δ329-382 aa) completely abolished (Figure 3-7B, constructs VII) demonstrating that this region (aa 329-355) is important for GBF3 toxicity. Finally, the role of the basic region localized in the bZIP domain of GBF3 in cellular toxicity was studied with an internal deletion construct (Figure 3-7B, construct IX). Removal of the DNA binding region (basic region, Δ261-280 aa) only slightly repressed GBF3 toxicity (Figure 3-7B, constructs IX). This result
suggests that GBF3 toxicity is not mediated by specific interactions with yeast DNA, but through an unknown mechanism.

**Discussion**

The 14-3-3 proteins have been shown to be involved in cell cycle progression, cell proliferation and differentiation by directly interacting with histones, chromatin modifying enzymes, kinases and cruciform DNA (Chen and Wagner, 1994; Grozinger and Schreiber, 2000). In addition, they are also found to be part of transcriptional G-box DNA binding complexes in several plant species, including Arabidopsis, rice and maize (Lu et al., 1992). Our current knowledge of 14-3-3 proteins suggests that they play a central role in eukaryotic gene regulation. The presence of 14-3-3s in transcription protein complexes has been reported previously. 14-3-3 proteins interact with several groups of plant transcription factors such as the PHDF-HD family in Arabidopsis, maize, and parsley (Halbach et al., 2000), and bZIP transcription factors such as RSG in tobacco, which controls shoot development by regulating the genes involved in gibberellin biosynthesis (Igarashi et al., 2001). In addition, 14-3-3s bind to general transcription factors including human and Arabidopsis TATA box binding protein (TBP), transcription factor IIIB (TFIIB) and human TBP-associated factor hTAFII32 (Pan et al., 1999). There is also evidence indicated that 14-3-3 proteins are involved in chromatin-mediated gene regulation through interaction with histones (Chen and Wagner, 1994) and histone deacetylases (HDACs) (Grozinger and Schreiber, 2000; Chang et al., 2005).

GBF3 transcription has been shown to be up-regulated by several environmental stimuli and stress responses such as osmotic stress, drought,
ABA, salt stress, cold and light (Figure 3-2). In agreement with the previous literature, discussing the role of 14-3-3 proteins and GBF3 in gene regulation and in stress management, the present study showed that there is a direct interaction between 14-3-3 proteins and GBF3. Heterologous expression of GBF3 in yeast caused severe cellular toxicity that was partially suppressed by co-expression of 14-3-3s (Figure 3-2). In addition, the his-tag pull down assay showed that 14-3-3_ω and 14-3-3_ωS62D interact directly with GBF3 (Figure 3-4). Although 14-3-3_ωS62D was pulled down with GBF3, the level of repression by 14-3-3_ωS62D was not significant (Figure 3-3B). These data suggest that the dimeric status of 14-3-3s is required for the antagonistic role of 14-3-3s in GBF3 toxicity. These findings confirm the previous model that 14-3-3s may indirectly interact with the G-box through binding to GBF3. GBF3 has a potential 14-3-3 binding motif (265-RKQS-268) localized in the DNA binding domain (Aitken, 1996). Although not directly confirmed, the binding of 14-3-3 proteins to this site has the potential to alter GBF3 function by keeping GBF3 out of the nucleus, decreasing the half life, or changing the activity of GBF3. Future work needs to focus on the mechanism of the rescue of GBF3 toxicity by 14-3-3s. A model describing how direct interaction between 14-3-3s and GBF3 may antagonistically affect function is given in Figure 3-8. We also utilized this toxic phenotype in yeast to characterize the domains of GBF3 protein. Deletions of N-terminal proline-rich and C-terminal uncharacterized domains release the toxic phenotype, suggesting that these domains are essential for GBF3 function.
Figure 3-1. Three-dimensional structure model of the GBF3 bZIP transcription factor. The model is derived from *Schizosaccharomyces pombe* bZIP transcription factor PAP1. PAP1 is in complex with the DNA oligomer 5'-TTACGTAA-3' (PDB1GD2) (Figure adapted from Fujii et al., 2000) using RCSB - Protein Workshop Viewer for PDB software. Figure was captured by screen shot. Pro-rich domain is located in the amino-terminus. Leucine zipper is involved in dimerization and basic region binds to the target DNA sequence.
Figure 3-2. Arabidopsis GBF3 (At2g46270) transcript profile in response to different treatments. Genevestigator Response Viewer (http://www.genevestigator.com) Arabidopsis microarray database showed that GBF3 transcription is significantly up-regulated by environmental stimuli such as osmotic stress, drought, ABA, salt stress, cold and light. Screen shot shows only the treatments that show two fold changes. Fold changes on the figure is in log scale.
Figure 3-3. Small colony formation in yeast transformed with GBF3 fused to GAL4 transcriptional activation domain (AD-GBF3). A) AD-GBF3 mediated toxicity is suppressed by co-transformation with Arabidopsis 14-3-3s fused to GAL4 DNA binding domain (DBD) in pDEST32 vector. B) Monomeric 14-3-3ωS62D does not rescue AD-GBF3-mediated toxicity as effective as 14-3-3ω. AD/DBD: empty pDEST22 and pDEST32 vectors respectively. DBD-Lambda: 14-3-3λ, DBD-Mu: 14-3-3μ, DBD-Pi: 14-3-3π and DBD-Upsilon: 14-3-3υ.
Figure 3-4. 14-3-3ω and 14-3-3ωS62D interacts with GBF3 in yeast. His-tag pull down assay was performed in yeast cells co-transformed with pYES-GBF3-his tag/empty p415GPD vector, pYES-GBF3-his tag/14-3-3ω and pYES-GBF3-his-tag/14-3-3ωS62D. In the control experiment, GBF3 had a stop codon, which prevents the fusion the C-terminal 6x His-tag fusion. Pull-down experiment was analyzed with Western blotting using 14-3-3ω specific antibody (α-14-3-3ω).
Figure 3-5. Arabidopsis GBF3 and GBF3-GFP fusion proteins cause growth inhibition in yeast likely through nuclear function. A) Spot assays of yeast expressing GBF3. Yeast cells were transferred with galactose-inducible empty pYES-DEST52, pYES-DEST52/GBF3 (pYES-GBF3), pYES-DEST52/GFP (pYES-GFP) or pYES-DEST52/GBF3-GFP (pYES-GBF3-GFP) vectors. Equal volume of ten-fold serial dilutions of transformed cells were spotted on glucose or galactose containing SC-Ura plates and were incubated at 30°C for 2 days or 3 days respectively. B) GBF3 is localized to the nucleus. Yeast strain INVSc1 expressing GFP or GBF3-GFP from galactose inducible GAL1 promoter was grown in synthetic complete media without uracil, stained with DAPI and examined by a fluorescent microscopy. BF, bright field.
Figure 3-6. Over-expressing GBF3 is toxic in yeast cells. A) Yeast growth curves in SD-Ura liquid media containing 2% galactose. Cells were cultured overnight in SD-Ura containing 2% glucose, washed and resuspended in SC-Ura containing 2% galactose to an initial OD<sub>600</sub> of 0.1. OD<sub>600</sub> was monitored every 12 hours. Each point represents the mean OD<sub>600</sub> of three different cultures ± SE. B) Cell viability assay. After galactose induction, every 24 hours OD<sub>600</sub> of 1/30,000 cells per ml were plated on SC-Ura containing 2% glucose to determine whether GBF3 slows down the cell growth or it affects the cell viability. The percent survival refers the ratio of number of colonies harboring pYES-GBF3 relative to those harboring empty pYES-DEST52.
Figure 3-7. Dissection of the GBF3 domains causing growth inhibition in yeast. A) Schematic representation of the domain structures in full length GBF3 and various deletion constructs expressed. B) Spot assays of yeast expressing GBF3. GBF3 deletion constructs showed that N-terminal proline-rich domain is required for growth inhibition in yeast. Partial deletion of the proline-rich domain (construct II) yields reduced growth inhibition.
Figure 3-8. The model of GBF3 mediated toxicity in yeast. A) Over-expression of GBF3 in yeast causes severe cellular toxicity. The potential 14-3-3 binding site is located in DNA-binding domain (265-RKQS-268). B) Co-expression of 14-3-3s with GBF3 in yeast partially rescues the toxic phenotype through direct interaction. Pro-rich domain (in orange) and an uncharacterized C-terminal domain (in red) are essential for GBF3-mediated toxicity in yeast.
CHAPTER 4
ARABIDOPSIS 14-3-3 EPSILON IS LOCALIZED IN THE PLASMA MEMBRANE
AND SECRETED IN RESPONSE TO FUNGAL ELICITOR

Introduction

The 14-3-3 proteins are highly conserved, ubiquitous eukaryotic proteins. 14-3-3s are characterized as acidic, dimeric proteins. They form homo and heterodimers with a monomeric mass of 25-32 kDa. The 14-3-3 proteins usually bind to target proteins containing well defined phosphothreonine or phosphoserine motifs (Muslin et al., 1996; Rittinger et al., 1999). 14-3-3s are involved in a diversity of protein-protein interactions, which allows them to mediate a range of biological functions. In plants, 14-3-3 proteins have been shown to play important biological roles through regulation of certain key enzymes such as nitrate reductase, sucrose phosphate synthase, starch synthase, glutamate synthase, ATP synthase and ascorbate peroxides. In mammals, 14-3-3s have been shown to be involved in cell cycle progression, cell proliferation and differentiation by directly interacting with histones, chromatin modifying enzymes, kinases and naked DNA (reviewed in Ferl, 1996; Huber et al., 2002, Oecking and Jaspert, 2009; Gokirmak et al., 2010). In addition, they are also part of transcriptional G-box DNA binding complexes in several plant species, including Arabidopsis, rice and maize (Lu et al., 1992)

The plant 14-3-3 proteins cluster into two groups when analyzed phylogenetically: These are an epsilon (ε) group and a non-epsilon group. The isoforms in the plant non-epsilon group appear to be significantly different from the plant and animal epsilon groups (Ferl et al., 2002). In Arabidopsis, the epsilon group has five members, μ, ε, π, ι, and ο and the non-epsilon group has eight
members, κ, λ, ψ, ν, ω, φ, and χ. Subcellular localization of specific 14-3-3s can provide important clues to possible roles of 14-3-3 family members. The first reported mammalian 14-3-3s isolated from brain tissue were cytosolic (Moore and Perez, 1967). Later, 14-3-3s were found in the nucleus, mitochondria, plasma membrane and other organelles. Although 14-3-3s do not have nuclear targeting sequences, Bihn et al. (1997) showed that 14-3-3s are present in both Arabidopsis and maize nuclei. A localization study using four evolutionarily diverse Arabidopsis 14-3-3 isoforms; κ, λ, ω, and φ, fused to green florescent protein (GFP) revealed that 14-3-3s have distinct and differential subcellular localization. Considering that 14-3-3 proteins lack subcellular localization signals, differential sublocalization of the individual 14-3-3 isoforms may be driven by target interactions rather than the intrinsic properties of 14-3-3s. Use of AICAR, a 5’AMP analog, and R18 peptide, a high-affinity 14-3-3 target, showed that in the absence of the 14-3-3/target protein interactions, 14-3-3s were found to localize throughout the cell without any clear subcellularization (Paul et al, 2005). These results suggest that many 14-3-3 isoforms may have a defined subset of targets and distinct functions.

In Arabidopsis, a study with isoform specific antibodies showed that two 14-3-3s from the epsilon group (μ and ε) and two 14-3-3 isoforms from the non-epsilon group (υ and ν) were the only 14-3-3s prominently located in the chloroplast (Sehnke et al., 2000). This finding suggests that phylogenetically different isoforms can share similar subcellular locations and functions. The expression profiles of 14-3-3 isoforms vary from tissue to tissue and organ to
organ (Chung et al., 1999 and Daugherty et al., 1996). Therefore, the tissue specific expression of 14-3-3 isoforms increases the complexity of the 14-3-3 mediated regulation of the target proteins.

Several studies reported the presence of 14-3-3s in the extracellular space. 14-3-3s are shown to be secreted into the extracellular space of Chlamydomonas and involved in cross-linking of hydroxyproline (Hyp)-rich glycoproteins in the Chlamydomonas cell wall (Voigt and Frank, 2003). Another study showed that 14-3-3s are secreted from human fibroplasts (Ghahary et al., 2005). 14-3-3s were also identified in a pea root cap secretome analysis in response to inoculation with a pea pathogen, *Nectria haematococca* (Wen et al., 2007). This study suggested that extracellular 14-3-3s may be involved in plant pathogen response. The present study also showed that a 14-3-3 (epsilon) is secreted from Arabidopsis suspension cells by fungal elicitation, probably from the plasma membrane.

**Materials and Methods**

**Plant Material and Elicitors**

Arabidopsis Col-0 hypocotyl suspension cell cultures were generated and maintained according to Ferl and Laughner, 1989. Cells were transferred to fresh media every three days to ensure that they were in log growth phase. Arabidopsis Col-0 ecotype seeds were surface sterilized with 40% household bleach with 2% Tween 20 and washed five times with sterile water. Sterile seeds were cold-treated at 4°C for 3 days to enhance germination and plated on vertical 0.5X Murashige and Skoog (MS) medium agar plates containing 0.05% MES hydrate, 0.6 % sucrose, Gamborg vitamins (Sigma) and 0.45% phytagel (Sigma).
Roots of 2 weeks old plants were used for fluorescence microscopy. Yeast elicitors were prepared by dissolving 0.5% yeast extract (YE) (Difco) in MMS media (1 X MS salts, 10% mannitol, pH 5.7) and autoclaving.

**Specificity Test for Isoform Specific 14-3-3 Antibodies Using ELISA**

The cDNAs of twelve Arabidopsis isoforms (μ, ε, π, ι, o, κ, λ, ψ, ν, u, ω, φ, and χ) were cloned into the pET15b expression vector and the recombinants his-tagged proteins were expressed in *E.coli* and purified through his-tag/nickel columns (Sigma) as previously described (Wu et al., 1997). The wells of an ELISA plate were coated with 10ng/μl of recombinant 14-3-3 proteins overnight at 4°C. The next day, the plate was washed four times with ELISA wash buffer (1X PBS with 0.02% sodium azide and 0.5% Tween 20) and blocked with ELISA blocking solution (1% milk powder in 1X PBS with 0.02% sodium azide) for 1 hr at room temperature followed by 4 washes ith ELISA wash buffer. Plate was washed with ELISA wash buffer four times. Seven isoform specific polyclonal rabbit anti-At14-3-3 antibodies (14-3-3 μ, ε, ι, ν, u, ω, and χ; diluted 1:2000) were applied for 1 hr followed by 4 washes. The wells were incubated with horseradish peroxidase conjugated secondary antibodies (diluted 1:4000) for 45 min and washed as before. Labeled wells were identified using SuperSignal West Pico Chemiluminescent Substrate according to the supplier’s instructions (Thermo Scientific).

**Cellular Fractionation**

Arabidopsis suspension cells were harvested on day 3 by filtration through MiraCloth (Calbiochem). Suspension cells (10g) were treated gently overnight (12-14 hr) with 40 ml of protoplasting solution (0.1% celluysin, 0.1% macerase
and 0.1% pectolase in 10% mannitol, 0.5X MS salts, pH 5.7) on a rotating platform (Belly Dancer). Protoplasts were filtered through Miracloth and pelleted by centrifugation at 900 rpm (120 x g) for 5 min in a swinging bucket centrifuge. The supernatant was kept as the cell wall fraction. Pelleted cells were washed and resuspended gently in 10 ml MMS (10% mannitol with 0.5 X MS salts, pH 5.7) in the presence of phosphatase and protease inhibitors (Calbiochem, CA). Protoplasts were re-pelleted and re-suspended in 5 ml of NIB buffer (Sigma) in the presence of 25 mM NaF, phosphatase and protease inhibitors. An aliquot representing the whole cell fraction was removed. The cell membrane was disrupted with 0.15% Triton X-100 on ice for 5 min and the nuclei pelleted at 2000 x g, (4°C) for 10 min. The supernatant representing the cytoplasmic fraction was removed and kept on ice. The nuclear pellet was resuspended in 5 ml of NIB buffer and an aliquot representing the whole nuclear fraction was removed. The nuclear fraction was stained with Trypan blue and visualized with a light microscope. The nuclei were repelleted and resuspended in 2 ml of NIB. The nuclear fraction (500 μl) was pelleted at 2000 x g, (4°C) for 5 min and resuspended in 450 μl of NHB (5 mM Hepes, pH7.4, 2 mM EDTA, 2 mM KCl, 1 μM DTT and 0.1% digitonin) with phosphatase and protease inhibitors. 50 μl of 50 mM lithium 3,5-diiodosalicylate (LIS) was added to the nuclear fraction to a concentration of 5 mM and gently rocked on ice for 15 min. The insoluble nuclear fraction was pelleted at 12,000 x g, (4°C) for 5 min and the supernatant kept as the soluble nuclear fraction. The pellet was washed and resuspended in 500 μl of NHB and kept as the insoluble nuclear fraction.
Western Blot Analysis

Loadings of all cellular fraction protein samples, except the digested cell wall fraction (8-fold more dilute) were adjusted against each other according to their starting volume and mixed with 2X sample buffer. The proteins were run in discontinuous SDS-PAGE and transferred to nitrocellulose using a Minifold I Dot-Blot system (Schleicher & Schuell BioScience). 14-3-3-epsilon specific polyclonal antibody was used to detect the localization of each 14-3-3 isoform in cellular extracts, and labeled bands were identified using SuperSignal West Pico Chemiluminescent Substrate according to the supplier’s instructions (Thermo Scientific). AtADH1 and histone H1 antibodies were used as cytoplasmic and nuclear control antibodies, respectively.

Plasmolysis and Detection of 14-3-3 Epsilon-GFP by Fluorescence Microscopy

Transgenic Arabidopsis plants constitutively expressing 14-3-3 epsilon-GFP fusion protein were planted on vertical plates. GFP fluorescence signal in root cells of two week old plants was visualized before and after plasmolysis with an Olympus BX51 fluorescence microscope. Plasmolysis was induced in 0.5 M NaCl solution for 5 min.

Results and Discussion

Polyclonal 14-3-3 Antibodies Recognize 14-3-3 Proteins with High Specificity

Since antibody cross-reactivity can lead to false-interpretation of the western blot and confocal microscopy data, we decided to characterize the specificity of each antibody against recombinant 14-3-3 isoforms using ELISA. The assay showed that isoform specific antibodies, chi, epsilon, iota, omega and
upsilon have very specific affinity for the corresponding recombinant 14-3-3. Nu and mu antibodies bind their corresponding isoform but they also have some level of cross-reactivity to other isoforms. The nu antibody cross-reacts with 14-3-3 kappa isoform. The 14-3-3 mu antibody cross-reacts with 14-3-3 omega and psi isoforms (Figure 4-1). These results were further investigated with western blot analysis and showed that the cross-reactivity was not as prominent as that seen with ELISA (data not shown).

**Cellular Fractionation and Western Blot Analysis**

Intact protoplasts from Arabidopsis hypocotyl tissue culture cells were isolated by protoplasting enzyme solution. Treatment of protoplasts with 0.15% Triton X-100 disrupts the cell membrane, but it keeps the nuclear envelope intact at this concentration. The insoluble nuclear fraction was separated from the soluble fraction using 50 mM of lithium 3,5 diiodosalicylate (LIS), a hypotonic detergent.

The identity of the cellular fractionation was confirmed with SDS-PAGE/western blot assays using histone H3 and alcohol dehydrogenase polyclonal antibodies (Figure 4-2A). These western blots showed that the cellular fractions were separated from each other without any major contamination. Western blot with 14-3-3 epsilon specific antibody showed that this isoform is present in all cellular fractions (Figure 4-2B). Considering the 8-fold more dilute loading of the cell wall fraction, the 14-3-3 epsilon is mainly localized in the digested cell wall fraction (Figure 4-2B, lane1). The large amount of the 14-3-3 epsilon in protoplast is present in the cytoplasmic fraction (Figure 4-2B, lane 2 and 3). A small amount of isoform is also found in the nuclear fractions (Figure 4-
2B, lane 4) and the majority of these proteins are shown to be present in the soluble nuclear fraction (Figure 4-2B, lane 5).

Arabidopsis 14-3-3 epsilon is secreted in response to fungal elicitation

14-3-3 epsilon was detected in the digested cell wall fraction of Arabidopsis suspension cells during the isolation of Arabidopsis protoplasts for subcellular fractionation (Figure 4-2B). Considering the fact that the protoplasting enzymes cellulose, macerase and pectolyase were isolated from fungi and they are not completely pure, we inquired whether the 14-3-3 epsilon is localized in the cell wall or secreted in response to fungal elicitors present in protoplasting enzyme cocktail. We detected the 14-3-3 epsilon secretion after incubation of cells with boiled enzyme cocktail overnight (data not shown). To support the secretion hypothesis, suspension cells were also treated with 0.5% yeast extract (YE) overnight and a substantial amount of 14-3-3 epsilon secretion was detected with Western blotting (Figure 4-3). Finally,

14-3-3 epsilon-GFP Is Localized in the Plasma Membrane

Two week old transgenic Arabidopsis plants expressing 14-3-3 epsilon-GFP fusion protein were used to characterize the plasma membrane and/or cell wall localization of 14-3-3 epsilon-GFP. GFP fluorescence was detected at the plasma membrane/cell wall interface (Figure 4-4A). To determine the localization of 14-3-3 epsilon-GFP in the plasma membrane or cell wall, Arabidopsis roots were plasmolysed with 0.5 M NaCl for 5 minutes. After plasmolysis, GFP fluorescence signal and plasma membrane was pulled away from the cell wall (Figure 4-4C and 4D). This result suggested that 14-3-3 epsilon is localized in plasma membrane but not in cell wall.
Figure 4-1. Specificity test for isoform specific 14-3-3 antibodies using ELISA. Each column was coated with 12 recombinant 14-3-3 proteins and each row was incubated with 7 isoform specific polyclonal antibodies.
Figure 4-2. Localization analysis of 14-3-3 epsilon by western blot. Arabidopsis cell suspensions were used to create sub-cellular and sub-nuclear fractions: lane1 – cell wall, lane2 – whole cell (protoplast), lane3 – cytoplasm, lane 4 – whole nuclei, lane5 – soluble nuclei, lane6 – halos. A) Western blot with control antibodies, alcohol dehydrogenase 1 (AtADH1) and histone H3. B) Western blot with 14-3-3 epsilon specific polyclonal antibody. Loadings of proteins samples in lane 2, 3, 4, 5 and 6 were approximately equal, but the loading of lane 1 was 8-fold more dilute.
Figure 4-3. Elicitor-induced secretion of 14-3-3 epsilon from Arabidopsis tissue culture cells. 8 grams of Arabidopsis tissue culture cells were treated with 40 ml of 0.5% YE in MMS media overnight. Western blot was performed with 14-3-3 epsilon isoform specific antibody. YE, yeast extract (Difco).
Figure 4-4. Localization of 14-3-3 epsilon-GFP fusion protein in Arabidopsis root cells. A) Detection of the localization of 14-3-3 epsilon by GFP fluorescent, B) Bright field image of A), C) Detection of the localization of 14-3-3 epsilon after plasmolysis by GFP fluorescent, D) Bright field image of B). PM stands for plasma membrane. CW stands for cell wall.
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BIOGRAPHICAL SKETCH

Tufan Gokirmak was born in Istanbul, Turkey, in 1979. He received his undergraduate degree from Bogazici University, Department of Molecular Biology and Genetics, in the Summer of 2002. After receiving his Bachelor of Science degree, he moved to Oregon, United States, to pursue a Master of Science degree at Oregon State University (OSU), Department of Horticulture under the supervision of Dr. Shawn Mehlenbacher. After he received his M.S. degree from OSU in June 2005, he moved to Florida to start his Ph.D. at the University of Florida, Plant Molecular and Cellular Biology Program under the supervision of Dr. Robert J. Ferl. During his doctoral studies he investigated the role of phosphorylation-mediated regulation of 14-3-3 protein dimerization in Arabidopsis thaliana and the effect of this regulation in 14-3-3/target interactions.