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The Functional Role of 14-3-3 Proteins in Plant-Stress Interactions

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ABSTRACT

14-3-3 proteins are important components in signal transduction pathways affecting multiple plant functions by mediating protein-protein interactions through post-translational modification via phosphorylation. Homodimers or heterodimers of 14-3-3 proteins bind to other proteins inducing site-specific targeting and alteration of the conformation of client proteins, which is an essential regulatory element for plant function and response. This protein family is involved in a wide range of cellular functions including the regulation of hormonal induction in response to stress stimuli, mediation of symbiotic relationships between the plant host and symbionts, as well as the mediation of functions between cellular components and enzymes. Identification of the function and mechanism of 14-3-3 proteins in plants offers insight into how crops can be made more efficient via the manipulation of these proteins.

INTRODUCTION

14-3-3 proteins are an important component in biological pathways involved in the mitigation of biotic and abiotic stresses. 14-3-3 proteins govern multiple signal allocations among plant biological aspects in response to external incitements and the facilitation of internal chemical systems. 14-3-3 proteins were first isolated in 1967 from mammalian brain tissue (Moore & Perez 1967), yet the name 14-3-3, which is based on the elution (14^{th}) and migration pattern (3, 3) on ion-exchange column and starch gel, has not changed. This protein family is implicated in the regulation of distinctive signal transduction cascades (Visconti et al. 2008; Purwestri et al. 2009) and function as activators, chaperones, binding agents, repressors, and other intermediaries (Berg et al. 2003; Sun et al. 2014). 14-3-3 proteins are conserved among many eukaryotic organisms (Aitken et al. 1992) and interact with other target proteins to trigger the transcriptional regulation of genes that generate specific modifications. These adjustments serve to circumvent damage caused by stress or they help facilitate essential cellular functions. Interactions may influence plant phenotypes such as establishment and development of nodules (Radwan et al. 2012). Additionally, phosphorylation or alteration of the 14-3-3 protein or client proteins can be activated by the stress to induce plant cell death

(PCD) and alter biochemical intermediaries and products (Shin et al. 2007; Oh & Martin 2011). The study of functional roles and gene expression of this gene family is important because many of these 14-3-3 proteins are essential intermediaries in eliciting a functional or physical response. This review summarizes current research involving the role of 14-3-3 proteins and investigates the molecular prospects of 14-3-3 proteins as credible adjuncts for molecular crop improvement. Responses are categorized under abiotic, biotic and symbiotic interactions. Once function is detailed, research can be done to determine how 14-3-3 proteins interact with constituents within a pathway to improve upon the biochemical system with molecular techniques.

FUNCTIONAL MECHANISMS AND PROTEIN STRUCTURE

14-3-3 proteins and proteins with forkhead-associated (FHA) domains specifically recognize phospholipids and bind them to elicit post-translational modifications (Lorenzo-Durán & Robatzek 2015; Gökirmak 2010). 14-3-3 proteins commonly form homo- or heterodimers consisting of antiparallel alpha helical amphipathic groves for target protein binding interaction (Chevalier et al. 2009). A phosphorylated target is recognized by a 14-3-3 protein, which facilitates additional modifications to regulate signal transduction within plant cells. Common phosphorylation target motifs occur on phosphotyrosine (pTyr), phosphothreonine (pThr) or phosphoserine (pSer) residues (Chevalier et al. 2009).

14-3-3 proteins are highly conserved eukaryotic-specific proteins. Animals typically contain less 14-3-3 proteins than plants though this protein family was first discovered in mammalian brain tissue (Moore & Perez 1967). 14-3-3 proteins were first classified as pSer/pThr-binding proteins which recognized R(S/Ar)XpSXP and RX(Ar/S)XpSXP consensus sequences (Yaffe et al. 2001). Possible modes of action 14-3-3 proteins exhibit during an interaction with client proteins (illustrated by figure 1) include control over localization, binding, interaction, catalytic activity, and post-translational modifications of the client protein (Lorenzo-Durán & Robatzek 2015). Consequently, 14-3-3 proteins can also interact symbiotically with pathogen-specific client proteins to alter host transcriptional gene expression or induce defense responses to stress stimuli.

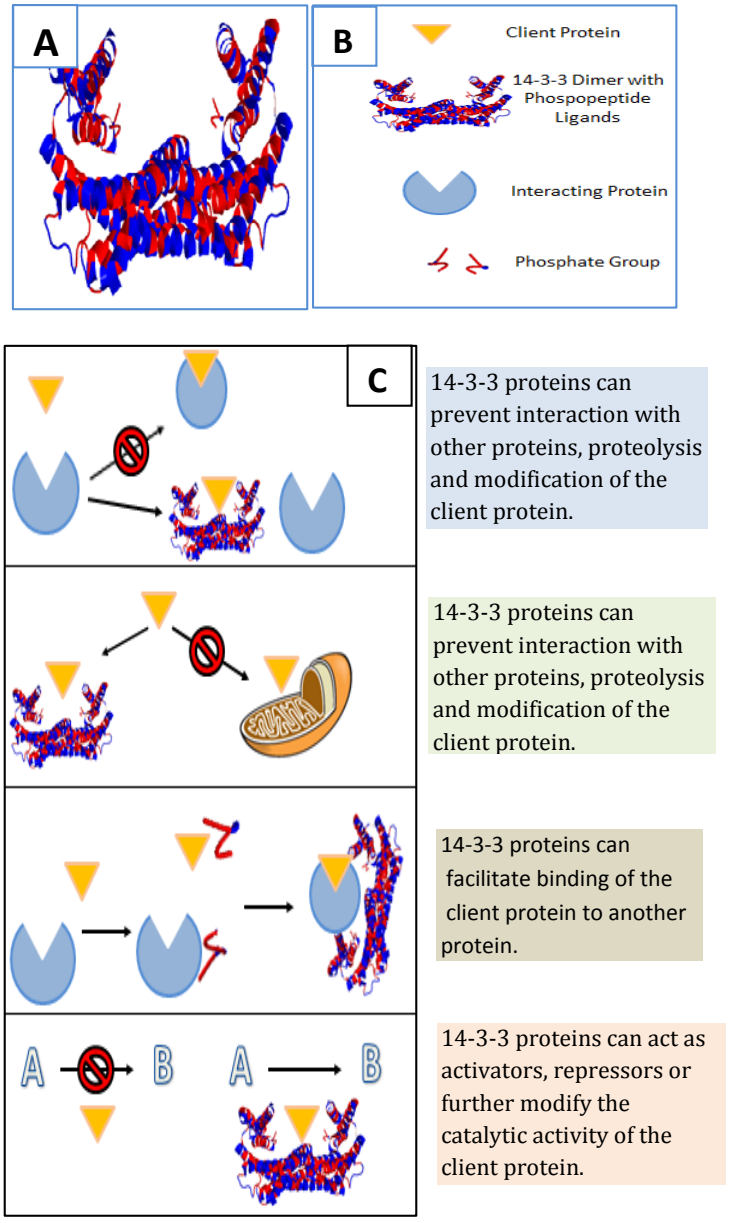

Figure 1. Structure of a 14-3-3c homodimer and modes of action of 14-3-3 proteins. **A.** 14-3-3c homodimer made using a PDB viewer; asterisks indicate phosphopeptide-binding pockets, the monomers are blue and yellow. **B.** Legend of symbols used in C. **C.** General modes of action of 14-3-3 proteins (Lorenzo-Durán & Robatzek 2015; Reinhardt et al. 2013).

Functional Roles of 14-3-3 Proteins in Plant Response to Biotic Stress

The functional roles of 14-3-3 proteins in plant response to biotic stress were reported from many plant pathosystems. Quantitative trait loci (QTL) are sometimes influenced by 14-3-3 proteins that function in accordance with several other genes to defend the plant against a pathogen. It is often difficult to dissect the mechanism by which the 14-3-3 protein operates in QTL traits due to the complexity and magnitude of interactions between genes underlying QTL. For example, five defense-related genes of a race were found to be associated with resistance QTL and diseased leaf area (DLA) variation in response to sheath blight disease (Liu et al. 2004) . These genes encode putative oxalate oxidase, dehydrin, PR-1, chitinase, and a 14-3-3 protein, and each attributed some effect towards DLA variation. Recombinant Inbred Lines (RILs) were mapped to quantify phenotypic effects of these genes. Linkage areas between SHZ-2 alleles near chitianse and 14-3-3 proteins were found to account for 21% out of the 60.3% reduced DLA. RILs containing alleles of SHZ-2 with oxalate oxidase, dehydrin, PR-1, chitinase, and a 14-3-3 protein were the most resistant to sheath blight suggesting a cumulative effect of these genes for plant resistance. This study demonstrated natural variation and contribution of a few genes is substantial for obtaining some level of resistance to an external stimuli. The linkage effect between 14-3-3 and SHZ-2 has yet to be determined as well as how each gene contributes to resistance.

In rice, 14-3-3 proteins also regulate complex defense responses and interact with cellular components to consequently induce defense responses to biotic and abiotic stresses. 14-3-3 proteins GF14b and GF14f interact with the mitogen-activated protein (MAP) kinase BIMK1 to activate systemic resistance against the rice blast fungus (Cooper et al. 2003). GF14b, GF14c and GF14e transcription levels were upregulated in salt and drought stresses

indicating roles during stress response. 14-3-3 genes GF14b, GF14c, GF14e and GF14f were also expressed in response to the inoculation with rice fungal and bacterial pathogens suggesting functions in defense signaling responses (Chen et al. 2006). This study provided potential functional roles of 14-3-3 proteins in response to both biotic and abiotic stresses.

14-3-3 proteins can also be involved in plant responses to damage caused by other organisms. A gene expression analysis of resistant soybean in response to soybean cyst nematode (SCN) syncytium formation and maintenance identified genes involved in conferring resistance to this pathogen. The transcription of key genes involved in the phenylpropanoid and jasmonic were upregulated in response to syncytium formation (Klink et al. 2009). Highly induced genes included S-adenosylmethionine synthetase, dehydration response, and 14-3-3 gene GENERAL REGULATORY FACTOR 2 (GRF 2) (Klink et al. 2009). Defense response study of GRF 2 in tomato showed that it is involved in mechanical damage signaling and is expressed when Avr9 elicits a defense response that activates *Cladosporium fulvum* 9 (Cf-9) (O'Donnell et al. 1998). This illustrates that 14-3-3 genes may not be directly activated by a biotic factor and instead may function as important intermediaries in a signal cascade caused by stress. High levels of GRF 2 protein were detected during syncytium formation suggesting a potential role in morphological modification of the roots as a plant defense response.

When pea (*Pisum sativum*) roots were inoculated with the pathogen *Nectria haematococca*, extracellular proteins in pea root tips and border cells release chemical defense signals and proteins to counteract the infection (Wen et al. 2007). Study of extracellular microbial biofilms revealed that the border cells released 14-3-3 proteins and root cap secretome species following inoculation. Wen et al. (2007) inferred that 14-3-3 protein ability to facilitate signal transduction has implications on the operation of other proteins in extracellular space. Roots treated with R18, an antagonist of 14-3-3 proteins, were more susceptible to the fungal infection. The mechanistic involvement and interaction of 14-3-3 proteins and other cellular components in the secretome is not characterized but they have implications on root tip ability to confront infections.

14-3-3 proteins also can have immunity-based interactions which trigger programmed cell death (PCD). Tomato 14-3-3 protein TFT7 was found to interact with a MAP kinase to regulate PCD triggered by the recognition between pathogenic effector protein and the corresponding plant resistance (R) protein (Oh et al. 2011). Interaction between MAPKKKα and TFT7 positively regulates Pto-mediated PCD in tomato and *Nicotiana benthamiana*. This is caused by multiple interactions between MAPKK and SIMKK2 downstream of SIMAPKKKα required by R proteins for PCD. Silencing TFT7 in *N. benthamiana* revealed that TFT7 recruits client proteins for signal transduction by forming a homodimer when it binds to SIMAPKKKα and SIMKK2, promoting interaction between the two proteins (Oh et al. 2011).

Functional Roles of 14-3-3 Proteins in Plant-Abiotic Stress Interactions

The function of a 14-3-3 protein can be detected by fluctuations in expression levels in response to stress, indicating that these proteins have some implications on plant defense responses. Further studies overexpressing and knocking out these proteins produce visible phenotypes and information as to how particular 14-3-3

proteins function. *Arabidopsis* 14-3-3 protein GF14-λ was overexpressed in cotton, which improved plant tolerance to water stress and produced a "stay-green" phenotype (Yan et al. 2004). Multiple interactions with this protein make it hard to identify the mechanism that confers drought tolerance. It is thought that interaction between transporter H(+)-ATPase and the overexpression of GF14-λ affected stomatal conductance, resulting in higher photosynthesis under drought conditions (Yan et al. 2004). The manipulation of 14-3-3 proteins has major implications for certain crops to improve performance under environmental stress.

In another study, the overexpression of a 14-3-3 protein was utilized to determine its involvement in drought tolerance. The overexpression of *Glycine soja* 14-3-3 protein GsGF14o in *Arabidopsis thaliana* was shown to be involved in plant development and drought response via induced reduction of plant tolerance at seed germination and seedling growth stages (Sun et al. 2014). Decreased stomatal development reduced net photosynthesis and produced a growth penalty to the plant under drought stress although it also reduced water loss and transpiration rate. These results provided evidence that the 14-3-3 gene GsGF14o functions as a negative regulator of drought tolerance. GsGF14o overexpression also affected root hair formation and development reducing the water uptake capacity of the plant. When the homologous 14-3-3 gene of GsGF14o in *Arabidopsis thaliana*, AtGF14u, was silenced, transgenic lines showed increased drought tolerance suggesting a strong correlation between the activity of this 14-3-3 protein and the plant tolerance to drought stress.

The overexpression of GsGF14o down-regulated transcription levels of drought-responsive marker genes, especially KIN1, P5CS and RD29A (Sun et al. 2014). P5CS is involved in proline biosynthesis, which functions in osmoregulation responses to hyperosmotic stresses (Delauney et al. 1993). These marker genes are activated by osmotic stress (Huh et al. 2010; Li et al. 2013; Sun et al. 2014) and are negatively controlled by GsGF14o, which decreases drought tolerance (Sun et al. 2014). Silencing GsGF14o may have a similar outcome as silencing of AtGF14u and reverse effect may be produced by over-expressing these genes to elicit increased drought tolerance in future study.

A common method of analysis and identification of key 14-3-3 proteins is the utilization of gene profiling techniques such as microarrays, RNA-Seq and qRT-PCR. Gene expression of *Ta14R1* and *Ta14R2* using qRT-PCR in wheat tissues revealed that these 14-3-3 mRNAs were up-regulated during seed germination (Meng et al. 2014). When plants were exposed to abscisic acid (ABA), extreme temperatures, and drought, these genes were induced indicating a possible link to ABA mediated stress responses as well as abiotic stress response. *Ta14R1* and *Ta14R2* were identified as 14-3-3 proteins with highly conserved phosphorylation-dependent binding motifs, alfa-spirals, and function domains common among 14-3-3 structural features (Meng et al. 2014). Phylogenetic analysis revealed commonalities to 14-3-3 proteins in *Arabidopsis thaliana* and cereal crops which are classified within the non-epsilon group (Sehnke et al. 2002). Expression profiles of root and leaf stress responses showed that 14-3-3 transcripts were up-regulated though expression profiles differed among each tissue, reflecting multiple biochemical signal transduction pathways. *Ta14R1* and *Ta14R2* were also up-regulated in response to ABA treatment, suggesting the

participation of 14-3-3 protein in hormone signaling. The functions of 14-3-3 target proteins as well as the biochemical network in which these stress responses are facilitated needs to be expanded on to explain how these 14-3-3 proteins function in response to internal and external stimuli.

14-3-3 signal transduction was studied in tomato (*Solanum lycopersicum*) mitigation against salt stress and potassium and iron deficiency (Xu and Shi, 2006). Twelve 14-3-3 genes of tomato (*TFT1-TFT12*) were analyzed using qRT-PCR under high salt, potassium and iron deficiency conditions. Among twelve genes, the mRNA expression level of *TFT7* was highly induced in response to iron deficiency and had moderate response to salt stress and potassium deficiency. *TFT1, TFT4, TFT7* and *TFT10* were upregulated under salt stress conditions in young roots, suggesting involvement of 14-3-3 in salt stress signal facilitation (Xu and Shi, 2006). The other 14-3-3 genes had varying levels of expression in response to the imposed stresses though the function of each was not explained and further research needs to be done on how these proteins interact.

A different study focusing on how 14-3-3 TFT genes are upregulated in alkaline stress response (ASR) revealed that these proteins are implicated in the regulation of the plasma membrane (PM) H^+-ATPase. Expression of *TFT1*, *TFT4*, *TFT6*, and *TFT7* were studied in tomato under ASR and overexpressed in Arabidopsis. PM H^+-ATPase activity was upregulated in root tips of *TFT4* overexpression (*TFT4o*) lines. Only *TFT4o* lines were tolerant to alkaline stress while the other 14-3-3 genes were implicated in other signal transductions with low phosphorous levels. However, *TFT7* displayed some involvement in IAA transport by increasing H^+ efflux. Studies done in tomato found that *TFT7* interacts with a MAP kinase to regulate plant cell death (Oh et al. 2011) and it may also be involved in regulating iron deficiency (Xu & Shi 2006). Under ASR, *TFT4* is involved with H^+ efflux integration, but unlike *TFT7*, it facilitates basipetal auxin (IAA) transport in root tips. *TFT4* interacts with PKS5-J3 (PM-H + -ATPase-mediated H + efflux regulators) facilitating root apex signaling and primary root growth (Xu et al. 2013). Additionally, *TFT4* reduces alkaline stress by regulating H^+ responses though research is still being done on how *TFT4* functions in tomato plants.

The interaction of 14-3-3 protein with a target protein can also be altered by increasing external stimuli to upregulate defense responses within the plant. An example is the mechanism by which magnesium (Mg) works to alleviate aluminum (Al) toxicity in broad bean (*Vicia faba*. L) roots to increase Al-induced citrate exudation. Micromolecular Mg application can enhance Al-induced citrate exudation and increase the activity of the plasma membrane H^+-ATPase by upregulating phosphorylation levels of VHA2 under Al stress (Chen et al. 2015). The 14-3-3 protein vf14-3-3b interacts with the phosphothreonine-containing sequence of the PM H^+-ATPase to control phosphorylation of this protein and regulate its activation (Fuglsang et al. 1999; Svennelid et al. 1999). Immunoprecipitation results suggest that interaction between the MATE-like gene VHA2 and vf14-3-3b is upregulated when the plant is stressed with Al and treated with Mg. This treatment decreased Al rhizotoxicity via Mg induced alteration in the phosphorylation rates of VHA2 when Al induces the expression of MATE-like gene and vha2 (Chen et al. 2015). Mg may also competitively inhibit Al at binding sites resulting in reduced Al toxicity. The upregulated citrate exudation from the roots

chelates the organic acids and Al to form stable complexes in the rhizosphere, thus reducing toxicity.

Gene Expression and Functional Roles of 14-3-3 Proteins in Plant-Symbiont Interactions

SGF14c and its paralog SGF14l belong to the 14-3-3 protein family and facilitate symbiotic interaction between soybeans and bacteria. Elevated mRNA levels of *SGF14c* in soybean were found to be induced by inoculation with *Bradyrhizobium japonicum* (Brechenmacher et al. 2008). In a recent work, Radwan et al. (2012) examined the transcriptomic and proteomic levels of SGF14c in correspondence with inoculation with *B. japonicum*. Additionally, Radwan et al. (2012) studied the phenotypic effect and ultrastructure defects in nodule establishment and development due to silencing 14-3-3 in soybean roots. SGF14c/SGF14l RNAi-silenced roots displayed lowered SGF14c mRNA and protein levels, a lower quantity of standard mature nodules, and an increased number of arrested nodule primordia (Fig. 2).

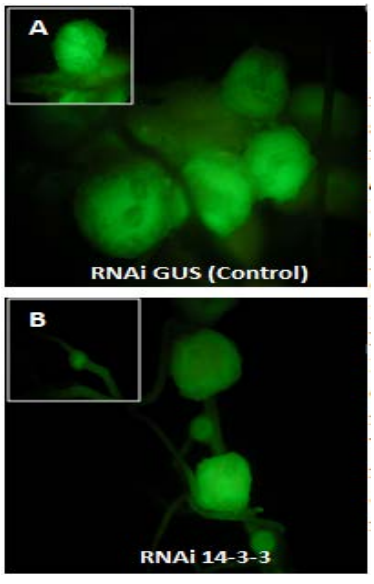

Figure 2. Nodulation response in transgenic soybean roots after inoculation with *B. japonicum*. A and B, Five-week-old GFP-expressing root nodule phenotypes for RNAi GUS (A; control); and RNAi 14-3-3 in soybean hairy roots (B). The RNAi 14-3-3 roots exhibited poor nodulation (as small bumps or empty nodules), whereas the control show many fully matured nodules. (Reproduced from Radwan et al. 2012).

Additionally, transmission electron microscopy was used to demonstrate ultrastructure defects in nodule cells due to the silencing of 14-3-3. These ultrastructure defects include severe degradation of the host cytoplasm and membranes, except the

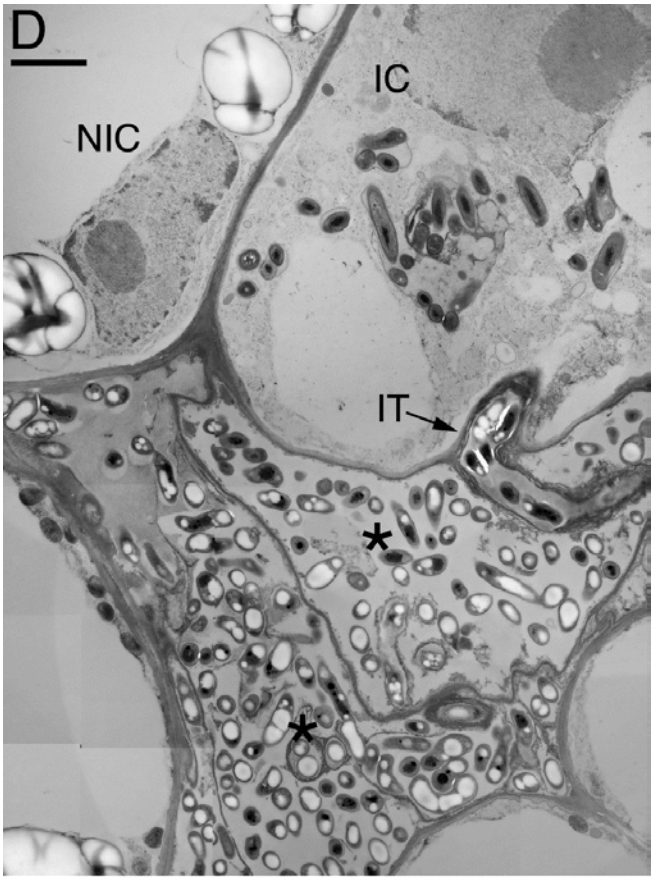

Figure 3. Ultrastructure defects in empty nodule cells due to the silencing of 14-3-3. A recently infected cell (IC) has an infection thread (IT) and bacteroids. The cytoplasm and organelles of this cell, and the adjacent non-infected cell (NIC) have a loss of electron density likely due to early degradation of these components. Two infected cells (asterisks) have completely lost the host cell components and have collapsed, leaving remnant bacteria. Bars= 2µm. (Reproduced from Radwan et al. 2012).

symbiosome membrane. In addition, infected cells have completely lost the host cell components and collapsed (Fig. 3).

These data ascertained a connection between the establishment of mature soybean nodules and SGF14c, while demonstrating a possible symbiotic connection between SGF14c and rhizobia pertaining to nodule development. Thus the presence of SGF14c and its functional activity confer the plant's ability to mitigate infection by *B. japonicum* while eliciting a response that allows inoculation. However, further research needs to be done to decipher the mechanism by which SGF14c interacts with other proteins to moderate nodule formation.

A class of flavonoid phenolic compounds called isoflavonoids can also have implications on stress responses as well as symbiotic relationships with nitrogen fixation in legume species. In soybean (*Glycine max*) the GmMYB176 R1 MYB transcription factor regulates CHS8 gene expression and consequently isoflavonoid biosynthesis when SGF14d and GmMYB176 interact (Li et al. 2012). All 16 of the SGF14 proteins regulate nuclear-cytoplasmic movement of GmMYB176 to mediate isoflavonoid biosynthesis, though each isoform displayed varying expression levels. Interaction between the D2 binding motif on GmMYB176 and 14-3-3 proteins triggered the phosphorylation of the S29 residue. This causes the activation of GmMYB176 and retains it to the cytoplasm where it impacts isoflavonoid biosynthesis.

CONCLUSIONS and Perspectives

Recent genomic profiling and sequencing tools have facilitated the discovery of 14-3-3 proteins as well as their classification among species. However, the functions of 14-3-3 proteins as well as the biochemical network in which they operate needs to be expanded on to explain how they function in response to internal and external stimuli. This can be accomplished with experiments targeting over expression or silencing of the 14-3-3 protein homo- or heterodimers with consequent gene expression data to identify interacting phosphorylation targets. As shown in figure 4, many of the client proteins interacting with 14-3-3 proteins are still unknown requiring more analysis of protein-protein interactions and signal transductions implicating 14-3-3 proteins.

14-3-3	Client Protein	Abiotic Stress	Biotic Stress	symbiosis
GF14b and GF14f	H^+-ATPase	Conformati-onal change of client protein		
Ta14RI, Ta14R2			?	
	MAPKK		?	
TFT1-12	MAPKK Kα		Stabilizes Client protein	
	SHZ-2		Conformat ional change of client protein	
SGF14	D2 Binding motif on GmMY B176		Phosphory lation for gene expression control	Rhizobia
GRF2	?	?	?	
GsGF14o	KINI, P5CS, RD29A		?	

Figure 4: Summary of the 14-3-3 and client proteins discussed in this review implicating a response to a stimuli.

The 14-3-3 protein SGF14c has been shown to moderate nodule formation though the biochemical intermediaries and target proteins are still being identified. Silencing this 14-3-3 protein caused loss of target protein function resulting in impaired nodulation displayed by the phenotype and ultrastructure imaging (Radwan et al. 2012). We are currently overexpressing this gene in soybean via stable transformation to study different mechanisms employed by SG14c during soybean responses to the symbiotic bacteria and important soybean pathogens. Additionally, immunoprecipitation and RNA-Seq are being used to identify interacting proteins with SGF14c. Possible mechanisms of function may include the phosphorylation of target proteins involved in soybean nodule development or a signal transduction between the rhizobia and soybean genes.

The 14-3-3 gene family *TFT1-TFT12* in tomato highlights the complex role of 14-3-3 proteins in biochemical systems. *TFT7* is involved in mediated plant cell death and recruitment of proteins for MAP kinase activity in tomato but it is also upregulated under alkaline stress conditions to mediate H+ efflux and IAA transport when studied in Arabidopsis. Additional proteomic studies need to be done to further define the role and regulation of 14-3-3 proteins in native species. Arabidopsis is commonly used as a model organism but 14-3-3 proteins may display alternative functions if transformed into other species.

The systematic approach to detailing a 14-3-3 protein is to first create a phylogeny tree within the organism of interest. This will highlight similar homologs and identify proteins containing the characteristic phosphorylation target motifs occurring on phosphotyrosine (pTyr), phosphothreonine (pThr) and phosphoserine (pSer) residues. Then gene expression levels can be monitored by targeting the 14-3-3 gene with techniques such as RNAi silencing, stable knockouts, overexpression, and gene expression quantification. These should also produce a cascade response that is phenotypic (irregular cells and organelles or plant structures) or something that is harder to visualize like the binding ability of implicated proteins.

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