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## Molecular Responses to Osmotic Stresses in Soybean

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### 1. Introduction

Soybean is an important economic crop for food and feed worldwide and currently has become an important raw material for biodiesel due to its high protein and oil contents. The global shrinkage of arable lands as a result of human activities and environmental factors has limited the expansion of soybean acreage. Exploring soybean cultivation on marginal lands has caused much attention; and yet the productivity of soybean under adverse environment is significantly limited by osmotic stresses (including salinity, drought, and cold), which impose negative impacts on growth, nitrogen fixation, agronomy traits, seed quality, and yield.

It is therefore important to acquire a better understanding of the molecular responses to osmotic stresses in soybean. In the past decade, related reports in this area have been accumulated rapidly. The recent completion of a reference soybean genome (Schmutz et al., 2010) has provided comprehensive genomic information that will expedite the identification of stress responsive genes and their functions. In view of this recent development, the purpose of this book chapter is to provide a framework for the current understanding of this research area and hence to facilitate future discussion. Here, we summarize and integrate the findings from individual reports and put forth a working model with reference to studies in higher plants. Common and specific components of various types of osmotic stresses and potential tolerant germplasm-specific components are highlighted. We also discuss the current obstacles in this research area and the forward-looking research strategies to tackle these problems.

While the knowledge on soybean is still limited, extensive researches have been carried out to elucidate the mechanisms of osmotic stress signal transduction in higher plants (especially in the model plant *Arabidopsis thaliana*) (for previous reviews, see Zhu, 2001; Mahajan and Tuteja, 2005; Chinnusamy et al., 2006; Phang et al., 2008; Chavez and Gonzalez, 2009; Agarwal and Jha, 2010). The major findings are summarized in Fig. 1. This helps to form a framework for our following discussion on related findings in soybean.

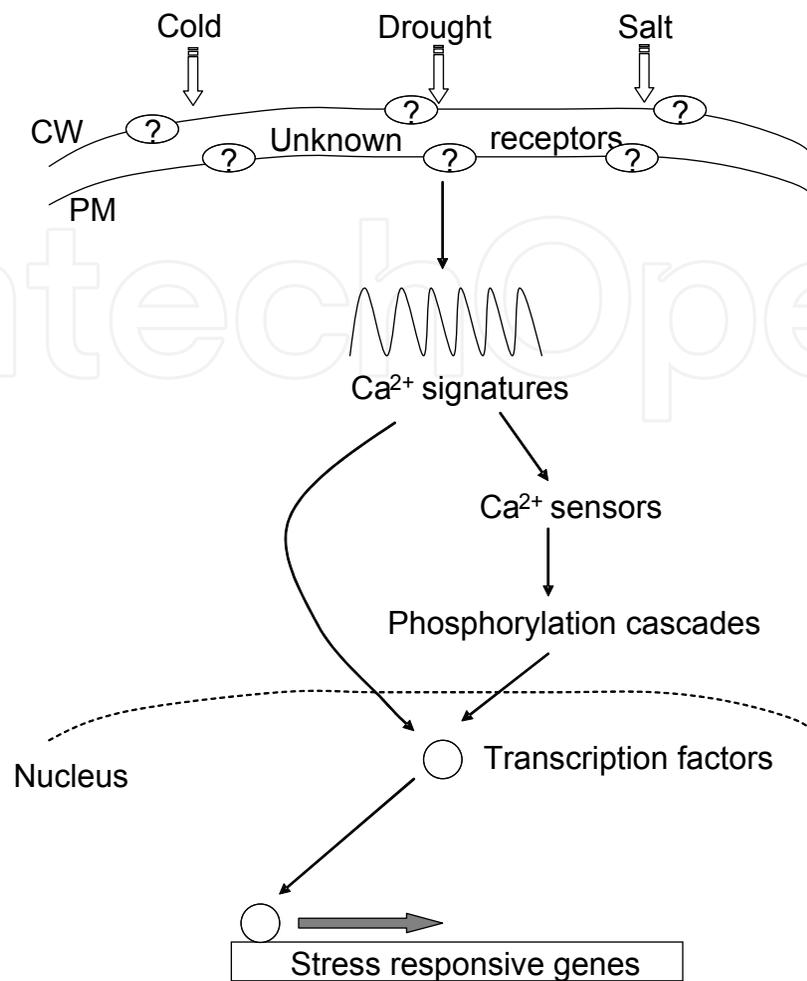


Fig. 1. A schematic model on the current understanding of osmotic stress signal transduction pathway in higher plants. CW: Cell wall; PM: Plasma membrane.

#### Stress sensors

In higher plants, osmotic stresses are presumed to be perceived by unidentified sensors in plasma membrane and/or cell wall. Based on the stress nature and severity, multiple sensors are expected to perceive various types of osmotic stresses (Xiong and Zhu, 2002; Kacperska, 2004). Cold sensors may be involved in the detection of membrane physical state/fluidity, changes in cytoskeleton, and cell wall structure (Heidarvand and Amiri, 2010). Drought sensors may be associated with membrane dehydration (Mahajan and Tuteja, 2005) and turgor pressure (Reiser et al., 2003). Salt signals may be perceived by drought sensors, together with sensors related to ion toxicity and imbalance (Mahajan and Tuteja, 2005). Protein components in cell wall (e.g. receptor kinases) are also potential candidates of osmotic stress sensors (Humphrey et al., 2007).

#### Ca<sup>2+</sup> signatures

Once the stress signals are perceived, consequential spatial and temporal changes in cytosolic Ca<sup>2+</sup> concentration (known as 'Ca<sup>2+</sup> signature') will be triggered (McAinsh and Pittman, 2009). Reactive oxygen species (ROS), inositol-1,4,5-trisphosphate (IP<sub>3</sub>), inositol hexakisphosphate (IP<sub>6</sub>), nicotinic acid adenine dinucleotide phosphate (NAADP),

phosphatidylinositol 3- and 4-phosphate (PI3P and PI4P), cyclic adenosine 5'-diphosphoribose (cADPR), and sphingosine-1-phosphate (S1P) are implicated to control the activities of diverse members of Ca<sup>2+</sup> channels and Ca<sup>2+</sup> transporters which are evolved to regulate the specificity of Ca<sup>2+</sup> signatures (period, frequency, and amplitude) (Ng and McAinsh, 2003; McAinsh and Pittman, 2009). Different strength of environmental stimuli can also generate differential spatial-temporal Ca<sup>2+</sup> waves (Goddard et al., 2000).

#### Ca<sup>2+</sup> sensors

The Ca<sup>2+</sup> signatures are detected and decoded by Ca<sup>2+</sup> sensor proteins (Dodd et al., 2010), which exhibit different Ca<sup>2+</sup>-binding characteristics, subcellular localizations, and downstream signalling interactions. The molecular features enable the sensor proteins to decode and process the information embedded within Ca<sup>2+</sup> signatures into alterations of cell functions (Dodd et al., 2010).

Ca<sup>2+</sup> sensor proteins can be classified into sensor responders and sensor relays (Dodd et al., 2010). Sensor responder proteins combine the sensing function (mediated by Ca<sup>2+</sup>-binding domains) and the response activity (e.g. kinase activity) within a single protein (Dodd et al., 2010). In contrast, sensor relay proteins (e.g. most calmodulins) only possess Ca<sup>2+</sup>-binding domains that can undergo Ca<sup>2+</sup>-induced conformational changes to interact and regulate the activity of target proteins (Dodd et al., 2010).

Calcineurin B-like protein (CBLs) are sensor relay proteins, sensing Ca<sup>2+</sup> by four Ca<sup>2+</sup>-binding EF-hands. They form complex with CBL-interacting protein kinases (CIPKs) in the conserved NAF (Asn-Ala-Phe) domain to release the C-terminal (autoinhibitory) domain from the kinase domain; thereby transforming the CIPKs into their active state. CBLs can form independent complex with CIPKs for transmitting the Ca<sup>2+</sup> signal to activate different subset of stress-responsive genes.

Calcium-dependent protein kinases (CDPKs) are typical sensor responders that are activated after binding of Ca<sup>2+</sup> to the C-terminal EF-hand-containing regulatory domain, causing conformational changes that relieve the active site of the kinase domain from masking by an autoinhibitory domain. They are then fully activated by autophosphorylation. Activated CDPKs will phosphorylate downstream kinase and phosphatase components and transmit the signals via phosphorylation. The roles and regulation of CDPKs in higher plants were reviewed previously (Ludwig et al., 2004).

Calmodulins (CaMs), forming a large protein family in higher plants (McCormack and Braam, 2003), are another group of Ca<sup>2+</sup> signature decoders. In response to osmotic stress, this calcium sensor may transmit the calcium signal by functioning as a transcription factor to regulate gene expression directly (as sensor responders) (Kushwaha et al., 2008), working as sensor relays through the interaction with transcription factors and transcription factor-binding protein, or modulating phosphorylation status of transcription factors (Kim et al., 2009).

#### Phosphorylation cascade

Protein phosphorylation cascade, regulated by kinases and phosphatases, plays central role to link Ca<sup>2+</sup> sensors to cellular responses. The mitogen-activated protein kinase (MAPK) pathway that has received much attention is composed of three kinase modules: MAPK, MAPKK and MAPKKK (Jonak et al., 2002).

MAPKs are serine/threonine kinases that modulate a variety of downstream gene expression and physiological responses (Jonak et al., 2002; Zhu, 2002). The substrates include transcription factors, protein kinases, and cytoskeletal proteins. MAPKKs are dual-specificity kinases which

activate MAPKs by phosphorylation of both tyrosine and threonine residue in the T-X-Y activation motif (Jonak et al., 2002; Zhu, 2002). MAPKKs are serine/threonine kinases that catalyze the phosphorylation of MAPKs through the two serine/threonine residues in a conserved S/T-X3-5-S/T motif (Jonak et al., 2002). Multiple MAPKs, MAPKKs and MAPKKKs can be found in higher plant genomes (Ichimura et al., 2002). These kinases form a phosphorylation cascade in higher plants in response to osmotic stress stimuli (Ichimura et al., 1998; Ichimura et al., 2000; Teige et al., 2004; Nakagami et al., 2005).

In contrast, phosphatases play negative roles in the regulation of osmotic signalling by down-regulating MAPK and abscisic acid (ABA) pathways (Huang et al., 2000; Xiong et al., 2001; Gupta and Luan, 2003; Schweighofer et al., 2007).

#### Transcriptional regulation network

The relationship between osmotic stresses and plant transcription factors has been extensively investigated in model plants (Singh et al., 2002; Mahajan and Tuteja, 2005; Agarwal et al., 2006; Kim et al., 2006; Nakashima and Yamaguchi-Shinozaki, 2006; Tran et al., 2007; Tuteja, 2007; Bhatnagar-Mathur et al., 2008; Saibo et al., 2009; Agarwal and Jha, 2010). The transcriptional machinery in response to osmotic stress is controlled by various transcription factors/regulons, and can be classified into ABA-dependent and ABA-independent pathways. The two pathways are, however, not mutually exclusive. Four major regulons are involved in osmotic stress responses: C-repeat binding factor/dehydration responsive element binding factor (CBF/DREB) regulon (in cold stress responses), ABA-responsive element binding protein and ABA-responsive element binding factor (AREB/ABF) regulon (in ABA, drought, and salinity responses), NAC and zinc finger homeodomain (NAC/ZF-HD) regulon (in ABA-independent, drought and salinity responses), and MYC/MYB regulon (ABA-dependent and responsive to different abiotic stresses).

In the following sections, we will provide a summary of findings in soybean with reference to the knowledge from other higher plants.

## **2. Potential sensors for osmotic stress signals in soybean**

Ca<sup>2+</sup> channels, two-component histidine kinases, receptor-like protein kinases, G-protein coupled receptors have been proposed to work as sensors to initiate signalling cascades in higher plants (Xiong and Zhu, 2002; Kacperska, 2004; Solanke and Sharma, 2008). A direct proof for the presence of osmosensors in soybean is still missing. However, several receptor-like protein kinases that are important candidates of osmosensors have been identified in soybean, including GmCLV1A, GmCLV1B, GmRLK1, GmRLK2, rlpk1, rlpk2 and rlpk3 (Yamamoto et al., 2000; Yamamoto and Knap, 2001; Ma et al., 2006). Except the rlpk3 protein, all of the above proteins belong to the leucine-rich repeat (LRR) protein superfamily that can respond to a wide range of extracellular signals and transduce the signals into intracellular responses. On the other hand, the rlpk3 protein belongs to the RLCK (receptor-like cytoplasmic kinases) family (Ma et al., 2006).

Making use of available sequence resources, which include large EST (expressed sequence tag) databases, full length-cDNA collections, and the recently completed soybean genomic sequence, more potential osmotic sensors can be identified. For example, 605 putative RLK genes in soybean were identified by a large-scale ESTs survey of the database (Liu et al., 2009b). Based on the phylogeny of the kinase domain, these soybean RLKs can be classified

into 58 different small subfamilies and are presumed to perform different functions (Liu et al., 2009b). Further works are needed to determine their exact roles in osmotic stress sensing.

### 3. Intracellular secondary messengers for osmotic stress signalling in soybean

#### Calcium signalling

Different signals may activate different  $\text{Ca}^{2+}$  sensors/receptors that will work independently or co-operatively to trigger the signalling pathways for stress responses (Xiong and Zhu, 2002). In soybean, the application of external  $\text{Ca}^{2+}$  could ameliorate salt-induced inhibitory effects on primary root elongation (An et al., 2004). Calcium oscillation has been observed in soybean, although osmotic stresses specific oscillation has yet to be reported. For example,  $\alpha$ -1,4-linked oligogalacturonides (OGs), a well-known elicitor of defense responses, can trigger rapid and transient changes in the cytosolic  $\text{Ca}^{2+}$  in an aequorin-transformed soybean cell line (Navazio et al., 2002). This implies that soybean can transmit signals by generating  $\text{Ca}^{2+}$  signatures. Moreover, homologues of  $\text{Ca}^{2+}$  sensors and several downstream components have been identified in soybean.

In soybean, SCA1 is a plasma membrane-localized  $\text{Ca}^{2+}$ -ATPase (encoded by *GmSCA1*) that belongs to a novel family of plant type IIB  $\text{Ca}^{2+}$  pump. It is stimulated by CaM (Chung et al., 2000) and can be rapidly and dramatically induced by NaCl stress and fungal elicitor (Chung et al., 2000) although its role in shaping  $\text{Ca}^{2+}$  signatures awaits further studies.

On the other hand, the soybean gene *GmSTL* encodes a calcineurin-like protein (Li et al., 2006) which shares 63.6% protein sequence identity with its homologue *AtSTO* in *A. thaliana*. Expression of *AtSTO* can functionally complement yeast calcineurin deficient mutants (Lippuner et al., 1996) and enhance salt tolerance in transgenic *A. thaliana* (Nagaoka and Takano, 2003). *AtSTO* interacts with the H-protein promoter binding factor (an MYB transcription factor) (Nagaoka and Takano, 2003). Since many MYB transcription factors involve in the osmotic stress signalling pathways in higher plants (Abe et al., 1997), it is suggested that *AtSTO* and *GmSTL* may act as calcium sensors.

Another group of putative  $\text{Ca}^{2+}$  sensors, the CaM proteins are encoded by a large gene family (at least 56 loci in *A. thaliana*; McCormack and Braam, 2003). A large gene family may signify their diverse physiological roles related to  $\text{Ca}^{2+}$  signalling. Five CaM cDNA clones (*GmCaM1* to 5) have been obtained from soybean (Lee et al., 1995). *GmCaM-1* and *GmCaM-3* are CaM isoforms with an identical amino acid sequence to *CAL1*, an alfalfa CaM (Barnett and Long, 1990). *GmCaM-2*, with two amino acid residues different from *GmCaM-1* (Barnett and Long, 1990), was identical to a barley CaM (Ling and Zielinski, 1989). *GmCaM-4* and *GmCaM-5* form a novel group of CaMs that has not yet been identified in other plants or animals (Lee et al., 1995). *GmCaM-1* and *GmCaM-4* activate  $\text{Ca}^{2+}$ /CaM-dependent molecules, including CaM-dependent protein kinase II, calcineurin,  $\text{Ca}^{2+}$ -ATPase, plant NAD kinase, and nitric-oxide synthase (Park et al., 2004). The expression of *GmCaM-4* in soybean can be up-regulated by treatment with  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , glycol chitin, and *Pseudomonas syringae*, but not with  $\text{K}^+$ , mannitol, hydrogen peroxide, salicylic acid, jasmonic acid, or ABA (Park et al., 2004). Its promoter can be induced by pathogen or NaCl (Park et al., 2009). *GmCaM-4* can also activate an R2R3-type MYB transcription factor which is an upstream regulator of a number of salt- and dehydration-responsive genes (Yoo et al., 2005). Ectopic expression of *GmCaM4* in *A. thaliana* induces the expression of marker genes for osmotic stress responses, such as *P5CS1*, *ADH1*, and *RD22*.

Furthermore, the homologue of a member of CaM-binding protein family was identified from a suppression subtractive hybridization (SSH) cDNA library from nodular tissue of soybean under drought stress (Clement et al., 2008). Interestingly, nodules development and maintenance are affected by osmotic stresses via a  $\text{Ca}^{2+}$  mediated pathway (Guenther et al., 2003)

Under salt and drought stresses, a CDPK residing on the soybean symbiosome membrane can phosphorylate nodulin 26, a water channel on the same membrane, to control its water permeability (Guenther et al., 2003). Phosphorylated nodulin 26 increases the water permeability of the membrane, and may be responsible for regulating the accumulation and compartmentalization of compatible solutes.

#### Phospholipid signalling

The osmotic stress-induced phospholipid signalling in higher plants involves the generation of InsP6 and the subsequent release of  $\text{Ca}^{2+}$  from an intracellular store. The molecule phosphatidic acid (PA) functions as the plant lipid secondary messenger (reviewed in (Munnik and Vermeer, 2010)). In soybean, PA is an upstream activator of the wound-induced MAPK pathway (Lee et al., 2001).

Diacylglycerol (DAG) is one of the key members in the phospholipid signalling pathway in response to stress. Phosphatidylinositol transfer proteins (PITPs) transfer phosphatidylinositol (PtdIns) or phosphatidylcholine between membrane bilayers and maintain the integrity of a critical Golgi DAG pool (Wirtz, 1991; Kearns et al., 1997). Functional rescue of PITP-deficient yeast strains by two soybean proteins (Ssh1p and Ssh2p) proved that they are functional homologues of yeast PITPs (Kearns et al., 1998). Osmotic stress (NaCl or sorbitol) can induce the expression of *Ssh1p* and the phosphorylation of Ssh1p proteins (Kearns et al., 1998), suggesting its role in the phospholipids signalling of osmoprotective responses.

Further studies revealed that two soybean serine/threonine protein kinases, SPK1 and SPK2, are responsive to hyperosmotic stress by phosphorylating Ssh1p (Monks et al., 2001). Immunoprecipitation assays demonstrated that the two protein kinases do not belong to MAP kinases, indicating that Ssh1p is not part of the well-known osmotic stress-induced MAPK pathway. On the other hand, Ssh1p may activate PtdIns 3-kinase and PtdIns 4-kinase to regulate the synthesis of PtdIns, which acts as a secondary messenger for the downstream responses to osmotic stresses. A speculative model of such osmosensory signal transduction has been proposed (Monks et al., 2001).

Two other putative components for phospholipids signalling have been identified in soybean. A nodulin gene (*G93*) is down-regulated by drought in nodules. The *G93* protein shares a high homology with the Arabidopsis PARF-1 that binds to phosphatidylinositol 3-phosphate (Clement et al., 2006). On the other hand, salt can induce the expression of a soybean gene encoding an oxysterol-binding protein (OSBP)-homologue (Li et al., 2008). Members of the OSBP family may function as regulators of cellular lipid metabolism, vesicle transport, and signal transduction (Li et al., 2008). Whether *G93* or OSBP from soybean takes part in the phospholipid signalling remains unclear at this point.

#### ROS signalling

Osmotic stress often results in the upset of ion homeostasis, which in turn leads to ROS formation and cellular damage (Moran et al., 1994; Zhu, 2001). The expression of a number of Arabidopsis CaMs, protein kinases, and transcription factors are induced by hydrogen

peroxide (Desikan et al., 2001; Neill et al., 2002). Therefore, the ROS-mediated pathway is potentially connected to  $\text{Ca}^{2+}$ -mediated osmotic stress responses.

In soybean leaves, the expression and the activities of catalase, superoxide dismutase, and haem oxygenase-1 are affected by NaCl treatment (Zilli et al., 2009). In a separate work, it was found that NaCl treatment can enhance the expression of haem oxygenase-1 and thus the production of hydrogen peroxide and superoxide. Such induction will be inhibited by inhibitors of NADPH oxidase, guanylate cyclase, and calcium channel - three important candidates in the oxidative stress responsive pathway (Balestrasse et al., 2008). Therefore, the soybean haem oxygenases may play dual roles in the protection machineries against salt and oxidative stresses.

#### 4. Phosphorylation cascades for osmotic stress signalling in soybean

Several putative protein kinases have been identified in soybean. *GmAAPK*, a gene encoding a putative serine/threonine protein kinase was cloned using a cDNA array (Luo et al., 2006). Its expression is enhanced by  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , polyethylene glycol (PEG), and ABA, suggesting the possible involvement of *GmAAPK* in osmotic stress signalling. Another gene for a putative serine/threonine protein, *GmSTY1*, was identified by screening the cDNA GAL4 activation domain fusion library of soybean (Xu et al., 2006). *GmSTY1* can be induced by salt and drought treatments but not exogenous ABA, indicating that this protein kinase may respond to abiotic stresses in an ABA-independent pathway.

From a soybean SSH library (Clement et al., 2008), a putative Ste-20 related kinase was identified. In yeast, Ste-20 is a kinase essential for osmotic stress signalling via the SHO1 branch of the high osmolarity glycerol (HOG) MAPK pathway, as shown by complementation tests using yeast mutants (Raitt et al., 2000). Ste-20 may be an upstream component that, under osmotic stress, phosphorylates an MAPKKK (Ste-11) and then trigger the subsequent activation of members in the MAPK cascade (Raitt et al., 2000).

On the other hand, the late nodulin G93 (see Part 3) might also take part in a MAPK cascade in soybean nodule during osmotic stress, since the protein sequences also resembles the ZR1 and RCC1 proteins which involve in nuclear trafficking of MAPKs (Clement et al., 2006).

There are also some clues coming from the rich genetic resource of wild soybean (*Glycine soja*). At least 20 kinase ESTs in *G. soja* are up-regulated by salt and/or dehydration stress (Yang et al., 2010). A CaM-binding receptor-like kinase (*GsCBRLK*) was isolated from *G. soja* (Yang et al., 2010). This *GsCBRLK* gene is induced by cold, salt, drought, or ABA stress and the encoded protein is localized on the plasma membrane. *In vitro* and *in vivo* assays suggested that *GsCBRLK* proteins can bind to CaM and exhibit kinase activities in a  $\text{Ca}^{2+}$ -dependent manner. Ectopic expression of *GsCBRLK* in *A. thaliana* can increase the expression of a number of salt stress or ABA-related marker genes (*RD29A*, *RD22*, *KIN1*, *COR15A*, and *NCED3*) and enhance the tolerance toward high salinity (Yang et al., 2010). *GsCBRLK* is therefore a possible molecular link between osmotic stress- and ABA-induced  $\text{Ca}^{2+}$ /CaM signalling pathways (Yang et al., 2010).

Another study identified the *GmGSK* gene that encodes a novel glycogen synthase kinase-3 in soybean (Zhang et al., 2010a). Glycogen synthase kinase-3 is a serine/threonine kinase conserved in plants and animals. *GmGSK* behaves similar to *GsCBRLK*, including the inducibility toward cold, salt, drought, or ABA treatment, the localization on plasma membrane, and the ability to enhance multiple stress tolerance ( $\text{Na}^+$ , sorbitol, and low/high temperature in transformed yeast cells). The results suggested a possible role of *GmGSK* in

osmotic stress signalling in soybean though an extensive functional analysis is yet to be completed.

The WNK (With No Lysine) protein kinases in human belong to a serine/threonine kinase family involved in the signalling pathway regulating epithelial ion transport and cell volume homeostasis in response to osmotic stress (Richardson and Alessi, 2008). In soybean, *GmWNK1* encodes a root-specific WNK protein kinase. Expression studies and bimolecular fluorescence complementation experiments (Wang et al., 2010a) demonstrated that *GmWNK1* can be down-regulated by ABA and osmotic stresses (NaCl, PEG, mannitol and glucose) and interacts with the ABA 8'-hydroxylase (an important enzyme for ABA catabolism) (Wang et al. 2010a). Overexpression of *GmWNK1* in soybean will lead to reduction in total and lateral root length, and increase in endogenous ABA level. This novel protein kinase in soybean may mediate the control of root system architecture by ABA and osmotic signals (Wang et al., 2010a).

## 5. Transcription factors participated in osmotic stress signalling in soybean

Reprogramming of transcriptome is an effective and durable mean to cope with environmental stresses. The release of a reference soybean genome sequence enables a quick survey of transcription factors in soybean (Mochida et al., 2009; Schmutz et al., 2010; Wang et al., 2010b). There are >4000 transcription factor gene loci occupying 6.56% of non-redundant gene loci in the soybean genome, more than a double of that in *A. thaliana* (~1900 out of 27235, ~7%) and rice (~2000 out of 56797, ~3.5%; Mochida et al., 2009). Categorizing the annotated loci according to GO terms suggests that more than 500 transcription factors in soybean would probably respond to osmotic stresses (Mochida et al., 2009). There are rapid accumulations of information on soybean transcription factors and their putative functions related to osmotic stresses (Tian et al., 2004; Mochida et al., 2009; Schmutz et al., 2010; Wang et al., 2010b). A summary of the recent findings is presented below and in Table 1.

### AP2/ERF

AP2/ERF transcription factors can be classified into 5 subfamilies including the AP2 (*APETALA2*), ERF (ethylene-responsive transcription factor), DREB (dehydration-responsive element-binding protein), RAV (related to ABI3/VP1), and Soloist (Sakuma et al., 2002; Zhuang et al., 2009) depending on the number of AP2/ERF domain. More than 380 AP2/ERF genes are present in the soybean genome (Mochida et al., 2009; Wang et al., 2010b) while only a few of them have been characterized (Gao et al., 2005; Li et al., 2005; Chen et al., 2006; Chen et al., 2007; Mazarei et al., 2007; Wang et al., 2008; Zhang et al., 2008b; Chen et al., 2009a; Zhang et al., 2009a; Zhang et al., 2009b; Zhuang et al., 2009; El Ouakfaoui et al., 2010; Jin et al., 2010).

More than 10 members of the gene family encoding DREB transcription factors present in the soybean genome (Phang et al., 2008), and 7 of the *GmDREB* genes are induced by ABA, salt, drought, and/or cold stress (Table 1) (Gao et al., 2005; Li et al., 2005; Chen et al., 2006; Chen et al., 2007; Chen et al., 2009a; Jin et al., 2010). Ectopic expression of *GmDREB* (in wheat) and *GmDREB2* (in *A. thaliana*) can enhance salt and drought tolerance. While *GmDREB3* confers salt, drought, and cold tolerance in transgenic *A. thaliana*, *GmDREB1* can only enhance salt tolerance in alfalfa (Table 1) (Gao et al., 2005; Chen et al., 2007; Chen et al., 2009a; Jin et al., 2010) These transgenic studies suggested that members of *GmDREB* family may have different roles in osmotic stress responses.

Eleven *GmERF* genes encoding ERF transcription factors in soybean exhibit differential expression under salt, drought, cold, and phytohormone treatments (JA, SA, ET and ABA)

(summarized in table 1) (Zhang et al., 2008b; Zhang et al., 2009a; Zhang et al., 2009b). GmERF3 and GmERF4 are localized in nucleus and bind specifically to the GCC box and the DRE/CRT element (Zhang et al., 2009a; Zhang et al., 2009b). Transgenic tobacco ectopically expressing *GmERF057* can increase salt tolerance whereas *GmERF3*, *GmERF4* and *GmERF089* can confer both salt and drought tolerances (Zhang et al., 2008b; Zhang et al., 2009a; Zhang et al., 2009b). Surprisingly, *GmERF3* and *GmERF4* impose opposite effect on the expression of biotic and abiotic stress responsive genes (*PR1*, *PR2*, *PR4*, *osmotin* and *SAR8.2*) in transgenic tobacco, suggesting that they may function in different branches of osmotic stress signalling cascade (Zhang et al., 2009a; Zhang et al., 2009b).

### bZIP

Expression of ABRE/ABF regulon is regulated by basic leucine zipper (bZIP) transcription factors in plants (Liao et al., 2008a; Liao et al., 2008c). Nearly 150 bZIP transcription factors have been annotated from the soybean genome (Mochida et al., 2009), in which nearly one third of them are induced under ABA, salt, drought, and/or cold stresses (Aoki et al., 2005; Liao et al., 2008a; Liao et al., 2008c). *SGBF-1* and *SGBF-2* are two bZIP homologues cloned from soybean (Hong et al., 1995) that are induced by cold and ABA, and *SGBF-1* has been shown to interact with a C<sub>2</sub>H<sub>2</sub>-type zinc finger protein, *SCOF-1* (Kim et al., 2001b); and yet there is no evidence showing the direct involvement of these two proteins in osmotic stress response. Expression of *SCOF-1* is induced by ABA and low temperature but neither by high salinity nor dehydration (Kim et al., 2001b). Ectopic expression of *SCOF-1* in tobacco improves tolerance toward chilling. While *SCOF-1* itself has no DNA binding ability, it interacts with *SGBF-1* and enhances the binding ability of the transcriptional factor *SGBF-1* to the *cis*-element ABRE found in the promoter of some cold responsive genes (Kim et al., 2001a; Kim et al., 2001b).

Other studies were performed to elucidate the functions of five bZIP transcription factors in relation to osmotic responses, including *GmbZIP44*, *GmbZIP46*, *GmbZIP62*, *GmbZIP78* and *GmbZIP132* (Liao et al., 2008a; Liao et al., 2008c). Except *GmbZIP132*, the other four bZIP transcription factors can bind to the GCN4-like motif (GLM: GTGAGTCAT), ABRE (CCACGTGG), and PB-like element (TGAAAA). On the other hand, *GmbZIP132* can only loosely associate with GLM. *GmbZIP46* can form homodimers or heterodimers with *GmbZIP62* or *GmMYB76*. Ectopic expression of *GmbZIP44*, *GmbZIP62*, or *GmbZIP78* in *A. thaliana* will result in reduction of ABA sensitivity and salt/ freezing tolerance, implicating their roles in ABA-dependent, salt and cold stress responses (Liao et al., 2008c). *GmbZIP132* is induced by salt and drought stresses. Transgenic *A. thaliana* expressing *GmbZIP132* becomes less ABA sensitive and more tolerance to salt in germination stage, but not in seedling stage. Abiotic stress-related genes, such as *rd29B*, *DREB2A*, and *P5CS*, are up-regulated in these transgenic *A. thaliana* plants suggesting that *GmbZIP132* probably participated in ABA-dependent, salt and drought stress responses (Liao et al., 2008a).

### NAC

NAC is an acronym referring to petunia no apical meristem (NAM), Arabidopsis ATAF1, ATAF2 and CUC2 (cup-shaped cotyledon) (Aida et al., 1997; Olsen et al., 2005a). It is a group of plant specific transcription factors involved in various biological functions (Olsen et al., 2005b). Overexpression of NACs in *A. thaliana* or rice can confer drought tolerance (Tran et al., 2004; Hu et al., 2006; Gao et al., 2010). More than 100 NAC genes have been found in the soybean genome (Mochida et al., 2009; Pinheiro et al., 2009), in which the expression of 31 NAC genes under drought treatment has been studied (Tran et al., 2009).

Nine of them (*GmNAC002*, *003*, *004*, *010*, *012*, *013*, *015*, *020*, *028*) show inducibility under drought (Tran et al., 2009), but exhibit diverse responses toward salt, cold, and ABA treatments. This indicates their non-redundant roles in osmotic stress responses (Tran et al., 2009). Although several members of *GmNACs* are responsive to osmotic stresses (Pinheiro et al., 2009; Tran et al., 2009), their physiological roles are still largely unknown.

### MYB

About 800 MYB-type transcription factor genes have been predicted in the soybean genome (Wang et al., 2010b). Among 156 soybean genes encoding GmMYBs, 43 of them respond to salt, drought, cold and/or ABA treatments (Liao et al., 2008b). GmMYB76, GmMYB92, and GmMYB177 were studied in more detail (Liao et al., 2008b). Using the yeast system, it has been shown that GmMYB76 and GmMYB92 are capable to form homodimers and exhibit transactivation activity, while GmMYB177 forms heterodimers with GmMYB76 and lacks transactivation activity. GmMYB76, GmMYB92, and GmMYB177 can bind to *cis*-MYB binding sites but with different affinities (Liao et al., 2008b). Ectopic expression of any one of the three genes in *A. thaliana* can enhance growth under salt stress; whereas only GmMYB177 can significantly improve cold stress tolerance in the host plants (Liao et al., 2008b). Some ABA- or abiotic stress-responsive genes are commonly regulated by GmMYB76, GmMYB92, and GmMYB177 while some exhibit differential responses, suggesting that they are involved in common and specific pathways in osmotic stress responses (Liao et al., 2008b).

### GT factors

Trihelix transcription factors, also known as GT factors, are signified with the highly conserved helix-loop-helix-loop-helix (trihelix) protein structure. The soybean genome contains 13 putative members (Tian et al., 2004). GT-2 type factors contain twin trihelix DNA binding domains (Ni et al., 1996), with the N-terminal one preferentially binding to GT3-bx and the C-terminal one to GT2-bx (Kuhn et al., 1993). Two soybean genes (*GmGT-2A* and *GmGT-2B*) encoding the homologues of the GT-2 factor were identified. They are induced under ABA, NaCl, cold, and drought treatments (Xie et al., 2009) (Table 1). GmGT-2A and GmGT-2B proteins are localized in nucleus (Xie et al., 2009). GmGT-2B, but not GmGT-2A exhibits transactivation activities in yeast and Arabidopsis protoplast (Xie et al., 2009). Ectopic expression of *GmGT-2A* and *GmGT-2B* in *A. thaliana* confers tolerance to salt, freezing, and drought stresses (Table 1); and leads to the induction of downstream stress tolerance-related genes such as the MYB genes, *LTP3*, *LTP4*, *PAD3*, *UGT71B6*, *DREB2A*, *STZ*, *AZF1*, and *RHL41/Zat12* (Xie et al., 2009).

### PHD finger containing proteins

PHD finger containing proteins are a group of transcription factors that was first identified in *A. thaliana* (Schindler et al., 1993). Six GmPHD genes (*GmPHD1* to *6*) were found in soybean. They are nuclear proteins and can bind to the *cis*-element 'GTGGAG' via an N-terminal domain. They express differentially under NaCl, drought, ABA, and cold treatments. The expression pattern is also distinct in stress tolerant versus sensitive soybean varieties (Table 1) (Wei et al., 2009). GmPHD1 to 5 are likely transcription repressors, as shown by a study using the *LUC* reporter gene in an Arabidopsis protoplast system (Wei et al., 2009). GmPHD6 can form homodimers or heterodimers with other GmPHDs except GmPHD2.

Ectopic expression of *GmPHD2* in *A. thaliana* confers salt and oxidative stress tolerance. This can also repress the expression of genes encoding negative regulators of stress tolerance (such as *CBF2/DREB1C*, *STRS1*, *STRS2*, and *At1g73660*) and induces the expression of genes

encoding putative ROS scavenging enzymes (Wei et al., 2009). Consensus sequences were identified in the promoter regions of the target genes: 'GTGG(A6/T7/G2/C2)G' for down-regulated genes and 'GTGG(A3/T1/G2/C3)G' or 'G(A1/G1/C4)GGTG' for up-regulated genes (Wei et al., 2009).

GmPHDs (except GmPHD1) respond differently in stress-tolerant germplasm JD23 versus stress-sensitive germplasm HBZ (Table 2; (Wei et al., 2009). Their differential expression suggests that they may be potential modulators determining tolerance in different soybean germplasms. More in-depth studies are required to test this hypothesis.

### WRKY

WRKY transcription factors are signified by the WRKY DNA binding domain (Rushton et al., 2010) which binds to the W box (TTGACC/T) in the promoters of their target genes. WRKY transcription factors participate in various biological functions and have been extensively reviewed (Rushton et al., 2010). Nearly 200 WRKY transcription factors are predicted from the soybean genome (Mochida et al., 2009; Wang et al., 2010b). Differential gene expressions toward salt, drought, or cold are exhibited by 25 (out of 64 tested) soybean WRKYs (Zhou et al., 2008). Among those, seven *GmWRKY* genes are responsive to all three treatments while 14 are responsive to both salt and drought stresses (Zhou et al., 2008). Five (out of 9 tested) GmWRKY proteins show transactivation activities in yeast (Zhou et al., 2008).

Ectopic expression of different *GmWRKY* in *A. thaliana* will lead to different effects: *GmWRKY13* increases sensitivity toward salt and mannitol stresses, upregulates *ABI1* and *ARF6*, and results in an increase of lateral roots; *GmWRKY21* confers cold tolerance; *GmWRKY54* enhances salt and drought tolerance, possibly through the regulation of *DREB2A* and *STZ/Zat10*. These GmWRKYs may therefore play differential roles in osmotic stress tolerance (Zhou et al., 2008).

### General and stress-specific components

Based on the expression studies summarized in Table 1, we noticed that the transcription factors are differentially responsive to ABA, drought, salt, and cold stresses. Those respond to osmotic stress are probably common components involved in cold, drought, and salt stress responses, while those respond differentially may be components specific to a particular stress (Table 2 & Table 3).

## **6. Other modes of gene expression regulations of stress signals in soybean**

### The ubiquitin 26S proteasome pathway

The ubiquitin-dependent protein degradation pathway involves the orderly action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The pathway is related to various physiological processes and responses, ranging from floral development, senescence, pathogen defence, to abiotic stress responses (reviewed in (Zhou et al., 2010). Ubiquitin ligases are likely negative regulators in the osmotic stress response in higher plants, since loss-of-function Arabidopsis mutants are more drought-tolerant while overexpressors are hypersensitive to drought stress (Cho et al., 2008). In another study (Qin et al., 2008), it was found that two RING (Really Interesting New Gene) ubiquitin ligases can cause ubiquitination of a drought stress-related transcription factor DREB2A (Qin et al., 2008).

Transcription Factor	Responsive To <sup>c</sup>				Transactivation (Host) <sup>d</sup>	Transgenic study <sup>e</sup>			
	N	D	C	ABA		Host	N	D	C
<b>AP2/ERF</b>									
GmDREBa	+	+	+	+	Yes (Yeast)				
GmDREBb	+	+	+	nc	Yes (Yeast)				
GmDREBc	+	+	nc	+	No (Yeast)				
GlyDREB1		+							
GmDREB	+	+				Wheat	T		T
GmDREB1						Alfalfa	T		
GmDREB2	+	+	+	+		Arabidopsis	T		T
GmDREB3	nc	nc	+	nc	Yes (Yeast)	Arabidopsis	T		T
GmERF3	+	+	nc	+	Yes (Yeast)	Tobacco	T		T
GmERF4	+	+	+	-		Tobacco	T		T
GmERF039	+	+	nc	+					
GmERF056	+	+	nc	+					
GmERF057	+	+	nc	+		Tobacco	T		
GmERF061	+	+	+	+					
GmERF069	+	+	+	-					
GmERF079	+	+	+	+					
GmERF081	+	nc	+	+					
GmERF089	+	+	nc	+		Tobacco	T		T
GmERF098	+	+	+	+					
<b>bZIP</b>									
SGBF-1			+	+					
GmbZIP44	+	+	nc	+	No (Yeast)	Arabidopsis	T		T
GmbZIP46	-	-	nc	nc	No (Yeast)				
GmbZIP62	+	+	nc	nc	No (Yeast)	Arabidopsis	T		T
GmbZIP78	+	nc	nc	nc	No (Yeast)	Arabidopsis	T		T
GmbZIP132	+	+	+	+		Arabidopsis	T		
<b>NAC</b>									
GmNAC002	+	+	+	nc	Yes (Yeast)				
GmNAC003	+	+	+	+	Yes (Yeast)				
GmNAC004	+	+	+	+	Yes (Yeast)				
GmNAC010	+	+	-	nc	Yes (Yeast)				
GmNAC012	+	+	nc	nc	Yes (Yeast)				
GmNAC013	+	+	+	nc	Yes (Yeast)				
GmNAC015	+	+	+	nc	Yes (Yeast)				
GmNAC020	nc	+	-	nc	Yes (Yeast)				
GmNAC028	+	+	+	nc	No (Yeast)				

Table 1. Characterized osmotic stresses related transcription factors in soybean <sup>a,b</sup>

<b>MYB</b>									
GmMYB76	+	nc	nc	nc	Yes (Yeast)	Arabidopsis	T		
GmMYB92	+	nc	+	nc	Yes (Yeast)	Arabidopsis	T		
GmMYB177	+	+	nc	nc	No (Yeast)	Arabidopsis	T		
<b>Trihelix transcription factors</b>									
GmGT-2A	+	+	+	+	No (Yeast)	Arabidopsis	T	T	
GmGT-2B	+	+	+	+	Yes (Yeast)	Arabidopsis	T	T	
<b>PHD</b>									
GmPHD1	+	+	nc	+	Inhibition (At)				
GmPHD2	+	+/- <sup>f</sup>	-	+/- <sup>f</sup>	Inhibition (At)	Arabidopsis	T		
GmPHD3	-	+	+/- <sup>f</sup>	+	Inhibition (At)				
GmPHD4	+	+	+/- <sup>f</sup>	+/- <sup>f</sup>	Inhibition (At)				
GmPHD5	+	+	+/- <sup>f</sup>	+	Inhibition (At)				
GmPHD6	+	+	+/- <sup>f</sup>	+/- <sup>f</sup>	No (At)				
<b>WRKY</b>									
GmWRKY13	+	+	nc		No (Yeast)	Arabidopsis	S	S	
GmWRKY21	+	+	+		Yes (Yeast)	Arabidopsis	nc	nc	
GmWRKY54	+	+	nc		No (Yeast)	Arabidopsis	T	T	

<sup>a</sup> N: NaCl treatment; D: Dehydration/drought treatment; C: low temperature/freezing treatment

<sup>b</sup> Empty space: not included in the study

<sup>c</sup> +/-: upregulated/downregulated; nc: no change

<sup>d</sup> At: Arabidopsis protoplast

<sup>e</sup> T: Tolerant; nc: no change; LS: Less sensitive

<sup>f</sup> +/-: Differential expression among salt/drought tolerant and sensitive soybean varieties

ABA responsive		
Osmotic stress	Salt & drought	Salt & cold stress
GmDREBa	GmDREBc	GmERF081
GmDREB2	GmERF3	
GmERF061	GmERF039	
GmERF079	GmERF056	
GmERF098	GmERF057	
GmbZIP132	GmERF089	
GmNAC003	GmbZIP44	
GmNAC004	GmPHD1	
GmGT-2A		
GmGT-2B		
GmPHD2		
GmPHD3		
GmPHD4		
GmPHD5		
GmPHD6		

Table 2. Soybean transcription factors that are responsive to ABA.

ABA irresponsive					
Osmotic stress	Salt only	Cold only	Salt & drought	Salt & cold	Drought & cold
GmDREBb	GmbZIP78	GmDREB3	GmbZIP46	GmMYB92	GmNAC020
GmERF4	GmMYB76		GmbZIP62		
GmERF069			GmNAC012		
GmNAC002			GmMYB177		
GmNAC010					
GmNAC013					
GmNAC015					
GmNAC028					

Table 3. Soybean transcription factors that are irresponsive to ABA.

A novel RING ubiquitin ligase gene (*GmRFP1*) was identified from soybean (Du et al., 2009). *GmRFP1* is up-regulated by ABA and salt stress, but down-regulated by drought and cold stress. The role of the ubiquitin-conjugating enzyme E2 (encoded by *GmUBC2* that was identified from a salt-induced cDNA library) in soybean has been studied in more details (Zhou et al., 2010). *GmUBC2* is an ubiquitin-conjugating enzyme homologous to the yeast RAD6. Ectopic expression of *GmUBC2* confers tolerance to salt and drought stresses with the transgenic *Arabidopsis* plants, which exhibit higher superoxide dismutase (SOD) activity as well as proline and Na<sup>+</sup> content, when challenged with NaCl (Zhou et al., 2010)

#### Post-transcriptional regulation by micro RNAs (miRNAs)

miRNAs are short non-coding RNAs (18-24 nt) that inhibit gene expression through promoting mRNAs degradation or interfering translation after perfect or near perfect complementarily binding to their target mRNAs (Zhang et al., 2005). The diversity,

biogenesis, identification, and function of plant miRNAs were summarized previously (Yang et al., 2006; Zhang et al., 2006; Dong et al., 2008). The miRNAs are widespread in plant species. In a large-scale *in silico* survey, 338 potential miRNAs were identified in 60 plant species (Zhang et al., 2005). About one quarter of the EST contigs containing these potential miRNAs are stress-induced. Among the stress-induced EST, 22% are associated with water stress, 6% with cold stress, 3% with salt stress, 3% with oxidative stress, and 5% with hormone treatment (ABA, SA or JA) (Zhang et al., 2005). Such a high portion of osmotic stress-associated miRNAs implies their important roles in osmotic stress responses.

Up-to-date, there is no publication focused on the searching and functional study of soybean miRNAs that tackle osmotic stresses. Nevertheless, soybean miRNAs searches have identified some potential candidates (Zhang et al., 2005; Zhang et al., 2008a; Chen et al., 2009b) (Table 4).

miRNA	Predicted Target	References
gso-miR3	alcohol dehydrogenase-like protein	Chen et al., 2009b
GmMiR319m-o	--	Zhang et al., 2005
miR-159	glutathione S-transferase	Zhang et al., 2008a
miR-398	superoxide dismutase	Zhang et al., 2008a
miR-414	cytochrome C reductase	Zhang et al., 2008a

Table 4. Potential soybean miRNAs that may participate in osmotic stress responses.

## 7. A hypothetical model

To put the available information discussed above in perspective, we constructed a hypothetical model to position known molecular components in osmotic stress responses from soybean (Fig 2).

Cold, drought, and salt are perceived by multiple, unknown sensors that are located in cell wall or on plasma membrane. Upon perception, stress specific  $Ca^{2+}$  signature may be triggered. GmSCA1, a salt responsive plasma membrane-localized  $Ca^{2+}$ -ATPase (Chung et al., 2000), may play roles in shaping the  $Ca^{2+}$  signatures.

SPK1 and/or SPK2 are activated by upstream osmosensors. They will phosphorylate Ssh1p and reduce its binding affinity toward the plasma membrane, potentially redirecting its PtdIns 3-kinase and PtdIns 4-kinase-stimulating activities to a different subcellular location; and subsequently, result in the alternation of phosphoinositide metabolism. Such alternation may affect the stress responses by affecting  $Ca^{2+}$  signature or acting on cellular transcription events.

GmSTL and GmCaMs are candidates of  $Ca^{2+}$  sensors to decode and transmit signals to transcription responses via the phosphorylation cascade or by acting on the transcription factors directly. Several kinases, including GmAAPK, GsCBRLK, GmGSK, GmWINK1, and GmSTY1 are putative components that may participate in the phosphorylation cascade.

Different subsets of stress responsive genes are controlled by various transcription factors, such as GmDREBs, GmERFs, GmbZIPs, GmNACs, GmMYBs, GmGTs, GmPHDs, and GmWRKYs. In response to a particular stress signal, the activated transcription factors will bind to the corresponding *cis* elements on the promoters to turn on/off the target genes and eventually lead to enhanced tolerance.

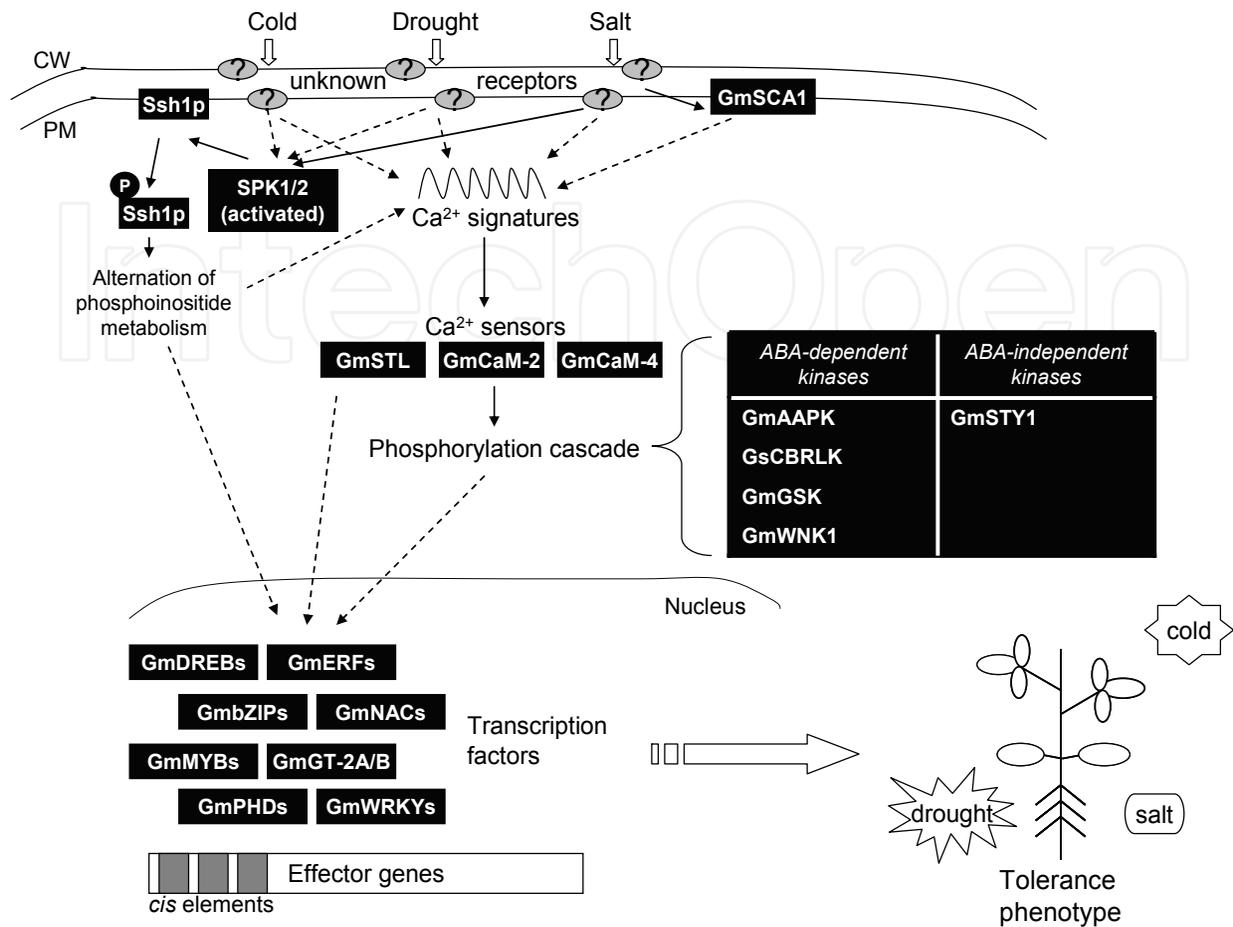


Fig. 2. Hypothetic model to position known molecular components involved in osmotic stress responses in soybean. Dotted line: hypothetical connection between components; Solid line: connection proven by experiments; CW: cell wall; PM: plasma membrane; GmSCA1: a plasma membrane-localized Ca<sup>2+</sup>-ATPase of soybean; SPK1/4: soybean serine/threonine protein kinase 1 and 4; Ssh1p: soybean P1TP1; GmSTL: soybean calcineurin-like protein; GmCaM-2: soybean calmodulin isoform 2; GmCaM-4: soybean calmodulin isoform 4; GmAAPK: a putative serine/threonine protein kinase in soybean; GsCBRLK: soybean calmodulin-binding receptor-like kinase; GmGSK: a novel glycogen synthase kinase-3 in soybean; GmWNK1: soybean WNK (With No Lysine) protein kinase; GmSTY1: a putative serine/threonine protein in soybean; GmDREBs, GmERFs, GmbZIPs, GmNACs, GmMYBs, GmGT-2A/B, GmPHDs, and GmWRKYs: osmotic stress responsive transcription factors in soybean. Details refer to text.

## 8. Future perspectives

Despite the rapid accumulating scientific reports in the past decade, the understanding of osmotic stress responses in soybean is still quite preliminary. Most results are just correlation studies that try to link the expression patterns of candidate genes to their responses toward osmotic stresses. Gain-of-function test in the model plant *A. thaliana* becomes an important tool for function tests. The progress of this research area is hindered by the absence of a systematic mutant collection and the inefficiency of soybean

transformation system. Functional proofs of candidate genes in the native system remain to be a difficult task.

On the other hand, there are a large collection of soybean germplasms which constitutes a natural mutant library. Wild soybean accessions (*Glycine soja*) often carry unique stress tolerance mechanisms that may provide novel ways to elevate tolerance capability. Various genetic populations (by artificial crossing) have been constructed and can be used to map functional genes. In the past, the application of these genetic materials was restricted by the lack of high-density physical markers in the soybean genome. Recently, the chromosome-scale draft sequence of a reference soybean genome has been released (Schmutz et al., 2010). Based on the information on full length cDNAs, ESTs, annotated domains, introns and transposons, 46,430 high-confidence protein-coding loci were identified (Schmutz et al., 2010). In addition, *de novo* sequencing of a wild soybean genome and re-sequencing of soybean germplasms have been finished (Lam et al., 2010 and unpublished data). A combined use of the genomic data, germplasms resources, and unique genetic populations will open up new ways to identify functional genes related to osmotic stress tolerance.

The low efficiency of soybean transformation (by *Agrobacterium*-mediated or biolistics approaches (Olhoft et al., 2007; Rech et al., 2008) remains a major obstacle in functional tests, despite the vast success in the production of transgenic soybean globally. There is a urgent need for the soybean researchers to optimize the transformation system or to explore alternatives, including the use of the soybean hairy root transformation system (Cao et al., 2009), the ovary-drip transformation system (Liu et al., 2009a), and the transformation systems of other legumes such as alfalfa (Zhang et al.) and *Lotus japonicus* (Aoki et al., 2002; Lombari et al., 2003).

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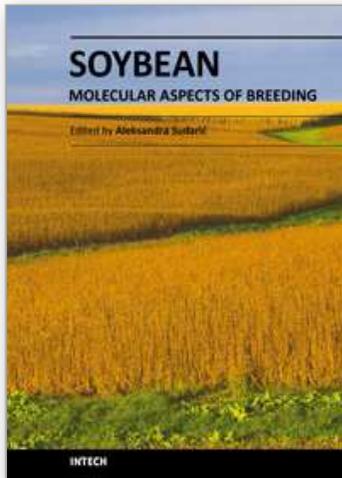
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