Chapter 6
Plant Protein Phosphatase 2C: Critical Negative Regulator of ABA Signaling

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6.1 Introduction

Plants are commonly encountered with various environmental stresses in their life span, and they thrive due to well-developed and efficient sensory system to the constantly changing environment. Environmental stress includes both biotic such as bacteria, fungi, viruses, and insects and abiotic stresses like cold salinity, and drought. Abiotic stress is becoming a major worldwide threat to food security due to climate change, which hampers the growth and development in plants as well as in animals (Huang et al. 2013). Abiotic stress influences the crop productivity and yield by affecting the plant health. Plants have developed complex signaling network to sense environmental cues and respond by changing their physiological and biochemical processes (Droillard et al. 2002; Franz et al. 2011).

Plants synthesize numerous diffusible hormonal signals like abscisic acid, brassinosteroids, and methyl jasmonate which work together to maintain growth, development, and cellular physiology and also to respond against environmental stresses (Lumba et al. 2010). Abscisic acid (ABA) is the carotenoid derivative, which functions as the key abiotic stress signal in plants. ABA is a ubiquitous phytohormone, which regulates growth and development of plants such as maturation of embryo, inhibition of seed germination via maintaining dormancy, and inhibition of post-germination growth and transition from vegetative growth to reproductive growth of plants (Cutler et al. 2010). ABA has a significant role to play in responding to environmental stimuli and in triggering modifications in a number of plant physiological and developmental processes like stomatal development and function, resulting in adaptation to stress circumstances (Melotto et al. 2006). In response to environmental stimuli, ABA biosynthesis takes place in the cell which leads to the activation of the signaling pathway. There are three major components of ABA signaling in

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plants: ABA receptors (PYR/PYL/RCAR), type 2C protein phosphatases (PP2Cs), and SNF1-related protein kinases 2 (SnRK2s). These core components of ABA signaling function in a double negative regulatory mechanism in which ABA receptors inhibit protein phosphatase and protein phosphatases inhibit protein kinases (Ikegami et al. 2009). Here, we will attempt to provide a basic understanding toward the core ABA signaling, mainly focusing onto the role of protein phosphatases in ABA signaling.

6.2 ABA Signaling: Major Stress Signaling Pathway of Plants

Abscisic acid (ABA) is one of the most important plant hormones for the regulation of various elements of plant life. ABA is a significant phytohormone that is essential in plant reaction to abiotic stress variables like cold, drought, salinity, and heat. Abscisic acid was identified to control the water status and acclimatization to a variety of stresses in crop plants (Koornneef et al. 1984). In Arabidopsis, its core signaling pathway is comprised of ABA receptor family, protein phosphatase 2Cs (PP2Cs), and SnRK2 (SnRK2 family members are plant-specific serine/threonine kinases) protein kinases (Fig. 6.1). In normal condition, the physiologically active concentration is in sub-micromolar range, while its working concentration increases to low micromolar range during stress (Ikegami et al. 2009). At the early stage of ABA signaling, ABA is sensed by PYR/PYL/RCAR (Pyrabactin Resistance/Pyrabactin Resistance Like/Regulatory Component of ABA Receptors) receptor family which is comprised of 14 members in Arabidopsis (Table 6.1) (Park et al. 2009; Ma et al. 2009).

Protein phosphatase 2Cs (PP2Cs) are the upstream component of this pathway which negatively regulates ABA signaling via dephosphorylating the downstream component like SnRK2s that hence keep ABA-responsive genes and transcription factors silent in the absence of ABA (Fig. 6.3). The interaction between ABA receptors and PP2Cs contributes to negative feedback regulation of PP2Cs at the moment of ABA perception. In the presence of sufficient concentration of ABA, SnRK2s act as a critical positive regulator in the ABA signaling, generally activated by autophosphorylation mechanism (Fujii and Zhu 2009; Fujita et al. 2009).

ABA signaling in plant cell leads to change in the gene expression of approximately 5–10% genes of genome mainly involved in stress tolerance (Nakashima et al. 2009b). Promoters of these genes possess many *cis*-regulatory elements named as ABA-responsive elements (ABREs). ABRE-binding protein (AREB), also known as ABRE-binding factor (ABF), was identified by using yeast 1-hybrid (Y1H) screening (Uno et al. 2000; Choi et al. 2000). The ABFs belong to group A subfamily of basic leucine zipper (bZIP) domain transcription factors. This family is comprised of nine homologues in Arabidopsis that share conserved C-terminal bZIP domain and N-terminal regions (Jakoby et al. 2002). Several reports suggest that

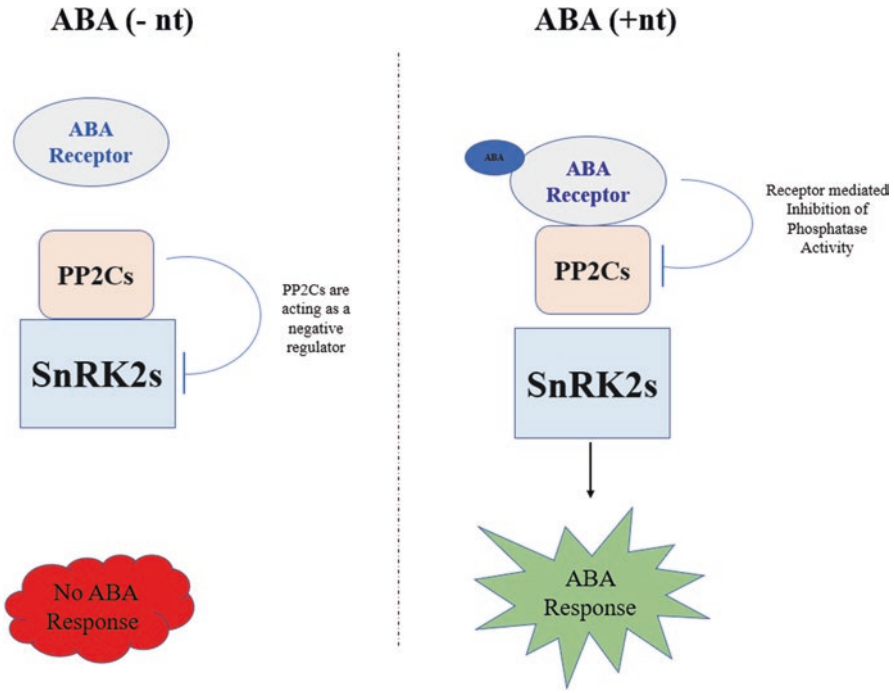

Fig. 6.1 Core component of ABA signaling pathway in plants. In the absence of ABA, PP2Cs prevent the accumulation of active SnRK2s by dephosphorylating them, but in the presence of ABA, receptor-mediated inhibition of PP2Cs results in the activation or accumulation of SnRK2s and ultimately leads to ABA responses

ABFs require ABA-dependent phosphorylation at the conserved domain for their full activation (Furihata et al. 2006; Fujii et al. 2007). So, phosphorylation and activation of ABFs by SnRK2s are essential for the ABA-induced gene expression.

6.2.1 ABA Receptors: Site for ABA Perception

In 1990s, studies on implication of ABA analogs have suggested that ABA-binding proteins are generally present either on plasma membrane or inside the cell (Allan et al. 1994; Schwartz et al. 1994). Isolation of ABA-binding protein in plants was considered to build a key step toward the identification of ABA receptors. The protein having ability to bind with ABA was identified from broad bean (*Vicia faba*) epidermal protein extracts via affinity chromatography (Zhang et al. 2002). In Arabidopsis, it was named as ABAR (ABA receptor) having ABA binding ability. Protein sequence studies revealed that ABAR was a component of Mg-chelatase, which is a multi-subunit complex present on plastid. T-DNA insertion mutants of ABAR were responsive to ABA (Shen et al. 2006).

Table 6.1 Identified interaction between ABA receptors and PP2Cs

ABA receptors	Interactive PP2Cs	References
PYL1/RCAR12	ABI1 and 2, HAB1 and 2, PP2CA	Fujii et al. (2009), Park et al. (2009), Ma et al. (2009), Hao et al. (2011)
PYL2/RCAR14	ABI1, HAB1 and 2, PP2CA	Fujii et al. (2009), Park et al. (2009), Hao et al. (2011)
PYL3/RCAR13	ABI1, HAB1 and 2, PP2CA	Fujii et al. (2009), Park et al. (2009), Hao et al. (2011)
PYL4/RCAR10	ABI1, HAB1 and 2, PP2CA	Fujii et al. (2009), Park et al. (2009), Hao et al. (2011), Pizzio et al. (2013)
PYL5/RCAR8	ABI1 and 2, HAB1 and 2, PP2CA	Fujii et al. (2009), Ma et al. (2009), Hao et al. (2011), Santiago et al. (2009)
PYL6/RCAR9	ABI1, HAB1 and 2, PP2CA	Fujii et al. (2009), Hao et al. (2011), Santiago et al. (2009)
PYL7/RCAR2	ABI1	Fujii et al. (2009)
PYL8/RCAR3	ABI1 and 2, HAB1 and 2, PP2CA	Fujii et al. (2009), Ma et al. (2009), Hao et al. (2011), Santiago et al. (2009)
PYL9/RCAR1	ABI1 and 2, HAB1 and 2, PP2CA	Fujii et al. (2009), Park et al. (2009), Ma et al. (2009), Hao et al. (2011)
PYL10/RCAR4	ABI1, HAB1 and 2, PP2CA	Fujii et al. (2009), Hao et al. (2011)
PYL11/RCAR5	ABI1	Fujii et al. (2009), Hao et al. (2011)
PYL12/RCAR6	PP2CA/AHG3	Fujii et al. (2009), Park et al. (2009)
PYL13/RCAR7	ABI1 and 2, PP2CA/AHG3	Zhao et al. (2013), Fuchs et al. (2014)
PYR1/RCAR11	ABI1 and 2, HAB1 and 2, PP2CA/AHG3	Fujii et al. (2009), Park et al. (2009), Hao et al. (2011)

Many groups have worked to isolate and identify the PYR/PYL/RCAR proteins via different approaches (Ma et al. 2009; Park et al. 2009; Santiago et al. 2009; Nishimura et al. 2010). The characterization of a synthetic ABA agonist named Pyrabactin has made the connection between PYR1 and ABA signaling. *In vitro* studies revealed that both Pyrabactin and ABA help PYR1 to interact and inhibit the clade A protein phosphatase 2C (PP2C) such as *ABA Insensitive 1* (*ABI1*), *ABI2*, and *Hypersensitive to ABA 1* (*HAB1*) (Park et al. 2009). Regulatory component of ABA response 1 (RCAR1), identical to PYL9, was discovered by using Y2H method. This investigation was leading to the identification of remaining 13 members of ABA receptor family (Ma et al. 2009). By using HAB1 as bait in Y2H screening, Rodriguez group identified PYL5, PYL6, and PYL8 (Santiago et al. 2009). In another *in planta* study, nine PYR/PYL/RACR proteins were identified as interactor proteins of ABI1 by using affinity chromatography followed by mass spectrometry (Nishimura et al. 2010).

PYR/PYL/RCAR proteins are total 14 in number which are the members of soluble ligand binding superfamily named as START (steroidogenic acute regulatory protein (StAR)-related lipid transfer)-domain superfamily (Iyer et al. 2001). The crystal structure of PYR/PYL/RCAR ABA receptors has revealed that all PYLs share similar helix grip structure which is composed of a seven-stranded β-sheet

flanked by two α-helices. The characterization of START-domain proteins is based on the presence of "helix grip" which is required for the formation of central hydrophobic ligand-binding pocket (Iyer et al. 2001). Crystal structures of ternary complexes have revealed that six members of the PYR/PYL/RCAR family of proteins (PYR1/RCAR11, PYL1/RCAR12, PYL2/RCAR14, PYL3/RCAR13, PYL8/RCAR3, PYL9/RCAR1) have been shown to bind to protein phosphatase 2C in the presence of ABA (Ma et al. 2009; Melcher et al. 2009; Park et al. 2009; Zhang et al. 2012b; Antoni et al. 2013).

Genetic evidences have confirmed the ABA receptors as a central regulator of ABA signaling. Since *pyr1* mutant did not show sensitivity to ABA, maybe because of functional redundancy of other family members of ABA receptor family. However, the reduced ABA sensitivity was observed in the triple and quadruple mutant of *pyr1pyl1pyl4* and *pyr1pyl1pyl2pyl4*, respectively (Park et al. 2009). Overexpression lines of RCAR1, PYL5, and PYL8 showed enhanced ABA sensitivity and stress tolerance to drought (Ma et al. 2009; Santiago et al. 2009). Thus, ABA receptors can be targeted for the better tolerance for abiotic stresses such as drought in crop plants.

6.2.2 Protein Kinases: Positive Regulator of ABA Signaling

Protein phosphorylation is the major event in almost all signal transduction pathways. Many protein kinases have been isolated and characterized as an important component of ABA signaling pathway (Hirayama and Shinozaki 2007, 2010). Major protein kinases involved in ABA signaling belong to SNF1-related kinase 2 (SnRK2) kinase family. Some other kinase families like SNF1-related kinase 3 (SnRK3; CBL-interacting protein kinases (CIPKs)), calcium-dependent protein kinase (CDPK), and mitogen-activated protein kinase (MAPK) family were also found to be involved in ABA signaling (Colcombet and Hirt 2008; Jammes et al. 2009; Cutler et al. 2010; Kudla et al. 2010). In Arabidopsis *thaliana*, there are ten members in SnRK2 family which are characterized into three subclasses (I, II, and III). SnRK2 family is conserved in all land plants, and their involvement has been shown in ABA signaling in barley, maize, pea, and rice (Li et al. 2000; Shen et al. 2001; Kobayashi et al. 2004; Huai et al. 2008). SnRK2.2/3/6/7/8 were found to be activated by ABA. Interestingly, members of subclass III of SnRK2 family, SnRK2.2/3/6, have been exhibiting strongest activation by ABA. These groups of kinases are activated within 30 mins of ABA treatment which suggests their involvement in early signal transduction for initiation of ABA signaling (Mustilli et al. 2002; Yoshida et al. 2002, 2006a; Boudsocq et al. 2004). Phenotypic analysis of mutants and interaction capability of CIPKs including CIPK1/3/8/14/15/20/23/24 with PP2Cs revealed their involvement in ABA signaling (Ohta et al. 2003; Kudla et al. 2010). Calcium-dependent protein kinases (CDPKs) such as CPK3/4/6/11/32 were also found to be involved in ABA signaling. *CPK4* and *CPK11* have sequence similarity, and both phosphorylate ABF1 and ABF4, which are known as

ABA-responsive transcription factors (Choi et al. 2005; Mori et al. 2006; Zhu et al. 2007).

Several Ca^{2+}-independent (SnRK2s) and Ca^{2+}-dependent kinases (SnRK3s/CIPKs and CDPKs/CPKs) have been characterized and known to regulate ABA signaling. The first identified kinase was an SnRK2 named PKABA1 (Wheat Abscisic Acid-Responsive Protein Kinase), which was originally isolated from wheat and was highly upregulated by ABA (Gomez-Cadenas et al. 1999). PKABA1 phosphorylates TaABF1 (a transcription factor of AREB family which binds on ABRE sequence) and mediates ABA suppression in the gibberellic acid-induced gene expression (Gomez-Cadenas et al. 1999). SnRK2.6/Open Stomata 1 (OST1) was the first functionally characterized kinase in guard cells, which led to closing of stomata in response to ABA (Mustilli et al. 2002).

Accumulation of ABA in plant cells leads to the activation of protein kinases, which regulate the phosphorylation status of downstream component of the ABA signaling. Although the molecular mechanism underlying autoactivation of SnRK2s is not very well known, a very recent report suggests that SnRK2 is phosphorylated at a specific OST1 site by MAPKK kinase (M3Ks) for ABA-induced activation (Takahashi et al. 2020).

Some reports using genetic approach have deciphered the importance of subclass III SnRKs in ABA signaling in Arabidopsis. The triple mutant of SnRK2.2/2.3/2.6 has shown the ABA-insensitive phenotype on higher concentration of ABA in the context of seed germination, seed dormancy, post-germination growth, and stomatal movement. These triple mutants did not show any significant expression of ABA-responsive genes (Fujii and Zhu 2009; Fujita et al. 2009; Nakashima et al. 2009a; Umezawa et al. 2009). Based on above studies, it can be concluded that subclass III of SnRK2 kinases functions as a central hub in ABA signaling.

6.2.3 Protein Phosphatase 2C: Negative Regulator of ABA Signaling

Reversible phosphorylation process is a well-understood event which is comprised of protein phosphorylation and dephosphorylation catalyzed by the protein kinases and protein phosphatases, respectively. Previous research was more focused on protein kinases, but nowadays, extensive research is ongoing on protein phosphatases to decipher the mechanism of reversible phosphorylation. Reversible phosphorylation regulates many biological processes in eukaryotes by modulating stability, activity, conformation, and localization of the target substrate in growth, development, and signal transduction pathways (Hunter 1995). Protein kinases and phosphatases are the key players of cellular phosphorylation processes in almost all the organisms under normal and stressed conditions. Protein kinases have been thoroughly studied for their structure and evolution in many organisms (Manning et al. 2002; Kerk et al. 2008).

6.2.4 *Classification and Evolution of Protein Phosphatases in Plants*

Protein phosphatases are evolutionary conserved from bacteria to complex organism. They have been classified into different subclasses on the basis of structural similarity (Cohen 1989). In higher plants like Arabidopsis and rice, there are 76 and 90 members of protein phosphatases 2C, respectively, which are further subdivided into ten or more subgroups (Xue et al. 2008; Singh et al. 2010). Protein phosphatases are basically divided into two major categories on the basis of their substrate specificity: serine/threonine (Ser/Thr) phosphatases and tyrosine (Tyr)-specific phosphatases. Ser/Thr phosphatases dephosphorylate the phosphoserine/phosphothreonine residue, whereas Tyr-specific phosphatases dephosphorylate the phosphotyrosine residue of substrate protein. Ser/Thr phosphatases are further classified into two families on the basis of molecular, biochemical and genomic analysis: (1) phosphoprotein phosphatases (PPs) consisting of PP1, PP2A, PP2B, and other distantly related phosphatases like PP4, PP5, PP6, and PP7 and (2) metallo-dependent phosphatase (PPM) family. PPM family requires metal ion for their activation or catalysis process, comprising PP2C and other Mg^{2+}-dependent phosphatases (Singh et al. 2010). However, a Ca^{2+}-dependent phosphatase, PP2B, is found in animals and also known as calcineurin A (CNA) but could not be found in plants so far (Uhrig et al. 2013). On the other hand, plant protein Tyr phosphatases (PTPs) are also classified into Tyr-specific phosphatases (PTPs) and dual-specificity phosphatases (DSPs). DSPs have ability to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine (de la Fuente van Bentem and Hirt 2009; Shankar et al. 2015) (Fig. 6.2).

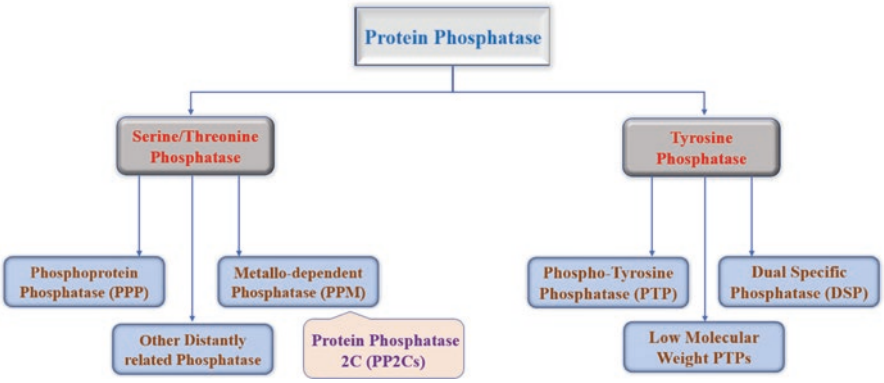

Fig. 6.2 Classification of plant protein phosphatase based on its substrate specificity. Protein phosphatases are categorized into Ser/Thr and Tyr phosphatase. Ser/Thr phosphatases are further subcategorized into three families: PPP, PPM, and other distantly related phosphatases. PPM family is also referred to as PP2Cs. Tyr-specific phosphatases are also subcategorized into PTP, DSP, and LMWPs

Plant genomes have a large number of protein phosphatase-encoding genes. Arabidopsis genome encodes 126 protein phosphatases (Kerk et al. 2008), while other plant species like rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), and hot pepper (*Capsicum annuum*) have 132, 113, and 102 protein phosphatases, respectively (Singh et al. 2010; Kim et al. 2014). The major group of protein phosphatase is PP2Cs having 80, 88, 90, and 91 genes in the Arabidopsis, hot pepper, rice, and tomato genome, respectively (Kim et al. 2014; Singh et al. 2010). In Arabidopsis, PP2Cs are further subdivided into 11 sub-clades, clade A to clade K (Singh et al. 2016). The presence of clade A PP2Cs in unicellular algae (e.g., *Chlamydomonas reinhardtii*) and clade B PP2Cs in *Selaginella moellendorffii* suggests that these PP2Cs are conserved throughout the plant kingdom (Fuchs et al. 2013). Interestingly, six out of nine clade A PP2Cs have been found to be involved in ABA signaling and have been characterized as negative regulators of ABA signaling.

6.2.5 PP2Cs as a Fine Modulator of ABA Signaling

Protein phosphatases are the largest efficient group of proteins in all eukaryotes suggesting their involvement in almost all of the signaling processes in eukaryotic organism. Through genetic studies, several Arabidopsis mutants have been identified which showed insensitivity to ABA. These ABA-insensitive (*abi*) mutants have mutations in numerous genes, which are essential for ABA signaling. Interestingly, instead of kinases, two protein phosphatases were also found to be a critical component in transmission of ABA signal. These findings illustrated a paradigm that protein phosphatase also plays an important role in regulation of protein kinases by dephosphorylating them or their substrate (Luan 1998). Clade A PP2Cs have been very well documented in ABA signaling. Apart from these PP2Cs, a dual-specificity protein phosphatase (DSP), Propyzamide Hypersensitive 1 (PHS1), has been identified and characterized as a critical regulator of ABA signaling, since the mutation in PHS1 gene resulted in ABA hypersensitivity during seed germination and reduction in stomatal aperture (Quettier et al. 2006).

Earlier, we have discussed that there is receptor-mediated inhibition of PP2Cs that takes place in ABA signaling in the presence of ABA. Now, most of the ABA receptors have shown to interact with PP2Cs. ABA receptors interact and inhibit the phosphatase activity of almost all the PP2Cs. Table 6.1 shows the interactions of ABA receptors with PP2Cs. Some interactions have been well characterized in the context of activity inhibition, whereas others still need detail investigation.

6.2.6 Clade A PP2Cs and ABA Signaling

There are nine PP2C members in clade A; most of them have been characterized as modulators of ABA signaling. The first identified ABA-responsive PP2C was ABA Insensitive 1 (ABI1). In Arabidopsis, ABI1 and its homologue regulate

ABA-dependent responses like transpiration, growth, and seed germination (Leung et al. 1994, 1997; Meyer et al. 1994). ABI1 and ABI2 play an important role during early events in ABA signaling (Koornneef et al. 1984). Consequently, six out of nine members of clade A PP2Cs have been emerged as negative regulators of ABA signaling and regulate physiological responses such as germination, stomatal conductance, and root growth (Merlot et al. 2001; Saez et al. 2004; Umezawa et al. 2009). In addition to ABI1 and ABI2, clade A consists of PP2CA/AHG3 (ABA hypersensitive germination 3) (Yoshida et al. 2006b), HAB1 (Homology to ABI1 1) (Rodriguez et al. 1998b), HAB2 (Homology to ABI1 2) (Umezawa et al. 2009), HAI1 (Highly ABA-induced PP2C 1), HAI2 (Highly ABA-induced PP2C 2), HAI3 (Highly ABA-induced PP2C 3), and AHG1 (ABA hypersensitive germination 1) phosphatases (Nishimura et al. 2007).

Under stressed condition or in response to high ABA, PP2Cs stimulate the ABA biosynthesis in the plant cell (Saez et al. 2006; Rubio et al. 2009). It has been postulated that this upregulation of PP2Cs desensitizes the plant to high level of ABA in a negative feedback loop (Szostkiewicz et al. 2010).

The various PP2C members physically interact with cytosolic or nuclear localized proteins suggesting their multiple role and multiple targets in ABA response (Chérel et al. 2002; Saez et al. 2008; Brandt et al. 2012). Among the interactors of clade A PP2Cs, a group of proteins, which is structurally related to pollen allergen Bet V 1, was identified as soluble ABA-binding proteins and named as ABA co-receptors regulatory component of ABA receptors (RCAR) and ABA receptor proteins named Pyrabactin resistance 1 (PYR1/PYR1-like). RCARs/PYR1-like proteins inhibit the function of protein phosphatase activity in the presence of ABA (Ma et al. 2009; Nishimura et al. 2010). ABA receptors require co-receptors ABI1 or other clade A PP2Cs to sense the sub-micromolar concentration. This trimeric complex formation between ABA ligand and holoenzyme leads to inhibition of PP2C activity in ABA signaling. SnRK2 family (subfamily 2 of SNF1-related kinases) member OST1 (Open Stomata 1) functions as a positive regulator downstream to PP2Cs in ABA signaling (Mustilli et al. 2002; Rubio et al. 2009; Kulik et al. 2011). ABI1 and HAB1 dephosphorylate pSer176 of OST1, which is present in activation loop and necessary for autoactivation of this kinase (Ng et al. 2011; Yunta et al. 2011). Some studies also show that RCARs serve as pseudo-substrate for PP2Cs, which can be locked in the presence of ABA (Klingler et al. 2010). It has been already shown by biochemical and protein interaction analysis that RCAR proteins interact with eight clade A PP2Cs to inhibit the activity of PP2Cs (Nishimura et al. 2010; Antoni et al. 2012; Zhang et al. 2012a).

6.2.7 ABA Insensitive 1 and 2 (ABI1 and ABI2)

The *abi1-1* and *abi2-1* mutants have been isolated from ethyl methane sulfonate-mutagenized Arabidopsis seeds through a genetic screening (Koornneef et al. 1984). These mutations lead to phenotypic alterations such as ABA-tolerant seed germination and seedling growth, abnormality in stomatal regulation, and altered drought

stress responses (Koornneef et al. 1984; Finkelstein and Somerville 1990). Both of these loci encode homologous proteins and are transcriptionally induced by ABA. Substitution from Gly to Asp in the catalytic domain of ABI1 and ABI2 results in significant reduction in phosphatase activity and Mg^{2+} binding affinity in both. Due to this missense mutation, Gly is changed to Asp in both *abi1-1* (G180D) and *abi2-1* (G168D), which leads to a dominant insensitive phenotype (Leube et al. 1998; Rodriguez et al. 1998a). Further studies have revealed that this mutated protein ABI1-1 is unable to bind with ABA receptors (PYL) in the presence of ABA (Ma et al. 2009; Park et al. 2009) but can still bind to SnRK2s (Umezawa et al. 2009; Vlad et al. 2009). Crystal structure of ternary complex (PYLs-ABA-PP2Cs) also showed that the conserved Gly in active site and conserved Ser in the "gate" loop of PP2Cs form a hydrogen bond, but in mutated protein ABI1-1, hydrogen bond is disrupted due to G to D substitution (Yin et al. 2009). So, it can be concluded that receptor-mediated inhibition of PP2Cs is not happening in the *abi1-1* and *abi2-1* mutants; hence, these mutants show ABA insensitivity.

ABI1-1 was found to inhibit ABA signal transduction in isolated protoplasts using ABA-responsive promoters HAV1-GUS and RBCS-GUS (Sheen 1998). The similar type mutation in the alfalfa MP2C results in diminished phosphatase activity of recombinant protein *in vitro* but could not affect its dephosphorylation activity for MAPK substrate *in vivo* (Meskiene et al. 2003). Isolation and analysis of mutants of *abi1-1* and *abi2-1* provided evidence that these both PP2Cs negatively regulate the ABA signaling in plants. Mutations in the catalytic domain of the ABI1 or ABI2 cause loss of function with very low phosphatase activity and also make plants supersensitive to ABA. Near 50% of the ABA-induced PP2C activity is contributed by ABI1 and ABI2 that indicates that other PP2Cs may also involve in ABA signaling (Merlot et al. 2001). Overexpression and microinjection approach has shown that mutant protein abi1-1 but not wild type ABI1 can block ABA, cyclic ADP-ribose, and Ca^{2+}-induced activation of promoters KIN2-GUS and RD29A-GUS (Wu et al. 2003). As per the model suggested by authors, both PP2Cs are functioning at different levels; *abi1-1* acts upstream, whereas *abi2-1* acts downstream of ROS production induced by ABA in guard cells (Murata et al. 2001). Interaction of ABI1 with ABA-inducible transcription factor ATHB6 and promoter reporter expression of ATHB6 was inhibited in *abi1-1* mutant plants, suggesting that ABI1 acts upstream of ATHB6 (Himmelbach et al. 2002).

6.2.8 ABA Hypersensitive Germination 3 (AHG3/AtPP2CA)

Another clade A type PP2C named *PP2CA/AHG3* was found to block ABA signaling when it is expressed transiently in protoplasts (Sheen 1998). Transcriptional upregulation of *PP2CA* is induced in cold, drought, salt, and ABA. Reduction in mRNA level of *PP2CA* in cold and drought in the *aba1-1* mutant suggested that expression of *PP2CA* is ABA dependent. Using antisense approach, downregulation of *AtPP2CA* resulted in better plant development and led to freezing tolerance,

suggesting its negative role in ABA responses during cold (Tähtiharju and Palva 2001). Interestingly, interaction between AtPP2C5 and AKT2/AKT3 in yeast was specific for catalytic domain of phosphatase compared with other potassium (K^+) shaker channels. This interaction enables *AtPP2C5* to regulate inward rectifying K^+ channel AKT2 in *Xenopus* oocytes. The *AtPP2CA* and *AKT2* genes are regulated by ABA and show similar tissue-specific expression with highest expression level in phloem vasculature. This study suggests the regulation of AKT2 channel by AtPP2CA enables K^+ uptake and also maintains membrane potential during stress condition (Vranová et al. 2001; Chérel et al. 2002).

6.2.9 Hypersensitive to ABA (HAB1)

One of the very close relatives of *ABI1* and *ABI2* is *HAB1* (previously known as AtPP2CA), upregulated by ABA and expressed in almost all part of Arabidopsis plant including root, stem, leaf, flower, and silique (Rodriguez 1998; Rodriguez et al. 1998b). ABA insensitivity in seeds and vegetative tissues was seen in constitutive overexpression lines of this gene, which suggests that *HAB1* is also a negative regulator of ABA signaling. Overexpression of *HAB1* results in impaired stomatal activity, ABA insensitive root growth, and reduced ABA-inducible gene expression (Saez et al. 2004).

Previous data suggests that clade A PP2Cs function as co-receptors of ABA, and functional analysis of other PP2Cs revealed their involvement in ABA-dependent stress responses (Jia et al. 2009). Moreover, clade A protein phosphatase (PP2C) is the negative regulator of ABA signaling, but a few reports also suggest that some PP2Cs can also regulate positively to ABA signaling. Splice variant HAB1.2 positively regulates ABA signaling by interacting with OST1 but not inhibiting the kinase activity (Wang et al. 2015). In another study, overexpression of beech (*Fagus sylvatica*) PP2C 2 (FsPP2C2) in Arabidopsis showed the enhanced expression of ABA-responsive genes, suggesting its role as a positive regulator of ABA signaling (Reyes et al. 2006).

6.3 Role of PP2Cs in Various Signaling Pathways in Plants

6.3.1 PP2Cs in Developmental Signaling

Integration of different signaling pathway is required for proper growth and development of plants. Now, it has been established that protein kinases and protein phosphatases play a vital role in plant developmental pathways. MAPK (mitogen-activated protein kinase) signaling pathway is a major developmental pathway, which is also activated in biotic and abiotic stress condition. Clade B protein phosphatase 2C, named AP2C3/PP2C5, interacts with MPK3, MPK4, and MPK6 and

dephosphorylates MPK6. These data suggest that AP2C3 inhibits the MAPK signaling pathway to maintain the balance between stomatal differentiation and pavement cell differentiation (Umbrasaite et al. 2010).

Protein phosphatases are found to play an important role in developmental signaling during different stages of plant development (Singh and Pandey 2012). Most significant PP2Cs include *KAPP* (kinase-associated protein phosphatase) and *POL* (POLTERGEIST); both of these are involved in *CLAVATA 1* (CLV1, a receptor-like kinase) signaling, regulating the Arabidopsis flower development (Luan 2003). KAPP interacts and dephosphorylates the CLV1 *in vitro*, and POL modulates the activity of a transcription factor WUSCHEL (WUS) (Yu et al. 2003). However, genetic analysis of double mutant of *clv/wus* and triple mutant of *pol/clv/wus* showed that POL functions in WUS-dependent and independent pathway (Yu et al. 2003). Besides, double mutant of *POL* and *POL-Like* (PLL) has shown seedling lethality suggesting their implication in shoot and root meristem as well as embryo development (Song and Clark 2005; Wang et al. 2007). Later on, it was also found that POL and PLL1 regulate the stem cell fate by affecting the WUS expression

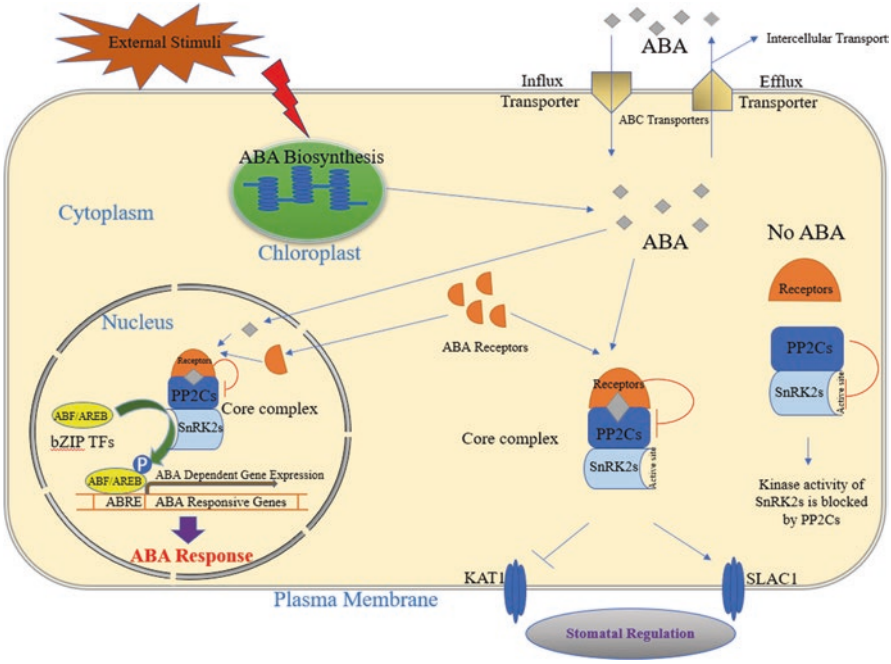

Fig. 6.3 Model of the core ABA signaling pathway in plants. Under normal conditions, PP2Cs remain bound to SnRK2s and inhibit their kinase activity and hence negatively regulate ABA-mediated responses. When ABA is perceived by PYR/PYL/RCAR ABA receptor family, these receptors bind with PP2Cs and block the phosphatase activity. This receptor-mediated inhibition of PP2Cs leads to activation of SnRK2s. These SnRK2s further phosphorylate the downstream components of the signaling pathway to activate or deactivate them like KAT1 and SLAC1 for stomatal regulation

downstream to CLV1. POL and PLL1 modulate the development of early embryo and root meristem by mediating the CLE40 (CLV-related 40)/WOX5 (WUSCHEL-related homeobox-5) pathway (Gagne and Clark 2010; Song et al. 2008). Therefore, POL and PLL1 involved in asymmetric stem cell division and in maintenance of stem cell polarity by controlling the early embryo and root meristem development (Fuchs et al. 2013). Moreover, genetic analysis of PLL4 and PLL5 has suggested their role in leaf development (Song and Clark 2005). On the basis of above data, it can be concluded that PP2Cs regulate the plant growth and development via mediating the receptor kinase signaling pathway.

6.3.2 PP2Cs in Abiotic Stress Signaling

Activation of Ca^{2+} signaling network, which basically consists of calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs), leads to triggering an adaptive mechanism to maintain K^+ homeostasis during K^+ deficiency. Clade A PP2C AIP1 (AKT1-interacting PP2C 1)/HAI2 was found to interact with CIPK23 and also AKT1 (Arabidopsis K^+ transporter 1). CIPK23 promotes the activity of AKT1, and AIP1 downregulates the activity of AKT1, suggesting that *AIP1* is a negative regulator of this pathway and negatively regulating the K^+ uptake during K^+ deficiency condition (Lee et al. 2007). Another clade B PP2C named AP2C1 was also found to interact and dephosphorylate the CIPK9 under K^+ deficiency. *CIPK9* positively regulates whereas *AP2C1* negatively regulates Arabidopsis root growth and seedling development under K^+ deficiency and hence acts as phosphorylation-dephosphorylation-based switch under low K^+ condition (Singh et al. 2018).

6.3.3 PP2Cs in Biotic Stress Signaling

AP2C1, clade B PP2C, is also known to regulate wound and biotic stress response by dephosphorylating/inactivating MAPK4 and MAPK6, which are known as positive regulators of wound and pathogen-triggered signaling (Schweighofer et al. 2007). *AP2C1* expression is upregulated in response to tissue injury and fungal pathogen *Botrytis cinerea*. Overexpression of AP2C1 phosphatase has been shown to reduce the kinase activity of MAPK in response to wounding. Overexpression lines of AP2C1 have shown low level of ethylene and hampered innate immunity against *B. cinerea.* Moreover, *ap2c1* mutants exhibited higher accumulation of jasmonic acid induced by wounding and also resistance to phytophagous herbivore (*Tetranychus urticae*) (Galletti et al. 2011; Fuchs et al. 2013). These findings suggest that *AP2C1* negatively regulates MAPK signaling. Thus, AP2C1 regulates plants innate immunity during pathogen attack via controlling the defense hormone level (Schweighofer et al. 2007; Galletti et al. 2011; Fuchs et al. 2013).

6.4 Conclusions and Future Perspectives

Plants encode a larger number of PP2Cs than yeast, mouse, and human, suggesting PP2Cs as a major group of protein phosphatase, regulating diverse physiological processes in plants. The phytohormone ABA activates a complex signaling that regulates numerous cellular and physiological processes in plants. Recent in-depth studies on ABA receptor uncovered the novel functions of PP2Cs as hormone co-receptors in plants. Now, it has been understood that PP2Cs negatively regulate the kinase function in most of the signaling pathways. But some reports also suggest that PP2Cs can regulate positively to ABA signaling for better adaptive responses against stresses. According to latest paradigm, ABA receptor, PP2Cs, and SnRK2s come together in different combination and interact via specific domain. This coordination regulates the ABA signaling in response to stress and developmental stimuli and enables plants to acclimatize in stress conditions.

Based on functional genomic approaches, novel uncharacterized PP2C candidates can be identified followed by their characterization by various genetic, cellular, and molecular approaches. As PP2Cs are involved in many signal transduction pathways, they can serve as connecting link between different pathways. Also, these can be targeted as potential candidate to decipher the crosstalk between different stress and hormone signaling pathways. Another important aspect for plant biologist is to decipher the role of PP2Cs in regulation of downstream targets of ABA signaling as well as in stress and developmental signaling pathways. Information collected from model plants like Arabidopsis could also be used for generating tools to improve the crop productivity under stress conditions. Functional characterization of PP2Cs should also be explored in other crop plants such as cereal and non-cereal crops to overcome the worldwide food security for constantly growing population.

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