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Cre-lox Univector acceptor vectors for functional screening in protoplasts: analysis of Arabidopsis donor cDNAs encoding ABSCISIC ACID INSENSITIVE1-Like protein phosphatases

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Abstract

The 14,200 available full length *Arabidopsis thaliana* cDNAs in the Universal Plasmid System (UPS) donor vector pUNI51 should be applied broadly and efficiently to leverage a “functional map-space” of homologous plant genes. We have engineered Cre-lox UPS host acceptor vectors (pCR701- 705) with N-terminal epitope tags in frame with the loxH site and downstream from the maize *Ubiquitin* promoter for use in transient protoplast expression assays and particle bombardment transformation of monocots. As an example of the utility of these vectors, we recombined them with several Arabidopsis cDNAs encoding Ser/Thr protein phosphatase type 2C (PP2Cs) known from genetic studies or predicted by hierarchical clustering meta-analysis to be involved in ABA and stress responses. Our functional results in *Zea mays* mesophyll protoplasts on ABA-inducible expression effects on the Late Embryogenesis Abundant promoter Pro*Em*:GUS reporter were consistent with predictions and resulted in identification of novel activities of some PP2Cs. Deployment of these vectors can facilitate functional genomics and proteomics and identification of novel gene activities.

Keywords

functional genomics; pUNI plasmid; systems biology; transient transformation; bioinformatics; PP2C

Introduction

With the completion of the Arabidopsis, rice and poplar genomes, focus has shifted from the acquisition of primary sequence information towards understanding the biological role of genomic sequence (Borevitz and Ecker 2004; Hilson 2006). This is particularly important across species boundaries, where genes of similar sequence may serve unique or different functions and functions specific to plants. Over 8000 loci of the annotated 27,235 Arabidopsis

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Note added in proof

Two reports have appeared of a novel family of ABA receptors that physically interact with some PP2Cs described herein:

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science Express. doi:10.1126/science. 1172408

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, Cutler SR (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science Express. doi:10.1126/science. 1173041

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genes (TAIR8) have similarity to uncharacterized proteins (i.e. hypothetical, predicted, unknown, etc.); another 4000 remain unannotated, and 758 loci have no significant protein similarity to proteins in GenBank based on the three broad ontology categories of molecular function, biological process, and cellular component (Garcia-Hernandez et al. 2002). Superimposed onto knowledge of individual gene functions is the need to understand the regulatory networks comprising genes, RNA, proteins and dynamical control rules among them, comprising the emerging field of contemporary systems biology (Fraser and Marcotte 2004; Kauffman 2004).

One of the challenges in the post-genomics era is to understand and discriminate the regulation and function of closely-related genes that control growth and development Additional tools such as high-throughput vector systems are needed. Multiple alignments of protein sequences is an important bioinformatics method to identify conserved sequence regions that may inform functional experiments, to predict the structures of uncharacterized proteins, and allow evolutionary studies on paralogous genes. Molecular networks represent the backbone of molecular activity within the cell and recent studies have taken a comparative approach toward interpreting these networks (Sharan and Ideker 2006). Integrating computational and functional studies can forge a broader view of complex phenomena such as the mechanisms of hormone "sensitivity" and cross-talk, whereby plants use common responses to acclimate to a range of different stresses after exposure to one stress.

Abscisic acid (ABA) plays a significant role in a variety of physiological processes during plant growth and development and mediates plant responses to environmental stresses. ABA signaling overlaps with other pathways such as hormone-, developmental-, sugar-, and stress-response pathways, suggesting a complex network of interactions that includes targeted proteolysis of effectors (reviewed in Christmann et al. 2006; Finkelstein et al. 2002 and 2008; Israelsson et al. 2006; S. Li et al. 2006; Rolland et al. 2006; Yamaguchi-Shinozaki and Shinozaki 2006). Genetic analysis of germination processes in Arabidopsis have resulted in map-based cloning of *ABA-INSENSITIVE (ABI)* and *ABA HYPERSENITIVE TO GERMINATION (AHG)* genes which encode transcription factors and several PP2Cs among others (Leung et al. 1994, 1997; Meyer et al. 1994; Nishimura et al. 2007; Rodriguez et al. 1998a; T. Yoshida et al. 2006). The *abi1-1* and *abi2-1* alleles (Koornneef et al. 1984) are the most pleiotropic in terms of physiological and tissue-specific ABA processes. *ABI1, ABI2, HOMOLOGY TO ABI-1 (HAB1), HAB2, AHG1/PP2CA* and *AHG3* all encode PP2Cs with partially redundant but distinct tissue-specific functions in negative regulation of ABA-inducible genes and ion channels (Kuhn et al. 2006; Leung et al. 1997; Merlot et al. 2001; Nishimura et al. 2007; Robert et al. 2006; Rodriguez et al. 1998b; Saez et al., 2006; Saez et al. 2008; T. Yoshida et al. 2006). Remarkably, the semi-dominant *abi1-1* and *abi2-1* alleles are both missense mutations of a conserved Gly to Asp (G180D in abi1-1) that result in a dominant-negative phenotype *in vivo* and reduced phosphatase activities *in vitro* (Leung et al., 1997; Rodriguez et al. 1998a; Sheen, 1998). Nuclear localization is an essential aspect of ABI1 and HAB1 function; disruption of the nuclear localization signal in abi1-1 mutant protein rescues ABA-controlled gene transcription in transiently transformed protoplasts and attenuates the semi-dominant abi1-1 insensitivity phenotypes for seed germination, root growth and stomatal movement (Moes et al. 2008). Recent genetic, biochemical, and cell biological evidence demonstrates that nuclear localization of HAB1, ABI1, ABI2 and AtPP2CA and their ABA-dependent interaction with SWI/SNF2 chromatin remodeling complexes is important for ABA regulation of gene expression and seed germination (Saez et al. 2008).

Analysis of the Arabidopsis genome shows that the PP2C family contains 76 members (Kerk et al. 2002; Schweighofer et al. 2004; Xue et al. 2008) which is about an order of magnitude more than in metazoans and yeast, suggesting they play diverse roles in plant signaling networks. In contrast, the moss Physcomitrella has only two ABI1-like PP2Cs (Komatsu et al.

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2009). PP2Cs are monomeric enzymes, a property which lends itself to functional characterization studies. Many Arabidopsis PP2Cs contain the conserved Gly residues (aa174 and aa180 of ABI1 protein) that are required for phosphatase and ABA signaling activities (Leung et al. 1997; Robert et al. 2006; Sheen, 1998), suggesting that they also may function in ABA and/or stress responses.

Here we apply Bayesian statistical approaches using amino acid homologies and expression profiling datasets to identify about 15 uncharacterized ABI1-Like PP2C genes as candidates for functional studies. In the process we were limited by conventional cloning methods in the numbers of genes that could be functionally tested. The Arabidopsis 2010 Initiative (Ausubel 2002) adopted as part of its infrastructure the Univector Plasmid System (UPS) of Elledge and colleagues (Liu et al. 1998) to facilitate functional genomics research. The UPS system is based on the Cre site-specific recombinase of bacteriophage P1 that catalyzes recombination between two 34 bp *loxP* or *loxH* sites (Abremski et al. 1983) and eliminates the need for restriction enzymes, DNA ligases and many *in vitro* manipulations required for subcloning DNA fragments. We engineered Cre-lox UPS host acceptor vectors (pCR701- 705) with N-terminal epitope tags in frame with the *loxH* site and downstream from the maize *Ubiquitin* promoter for use in transient protoplast expression assays and particle bombardment transformation of monocots. We show here that UPS full-length Arabidopsis donor cDNAs encoding PP2Cs can be efficiently recombined and expressed in maize protoplasts, a facile system for functional genomics of signaling (Sheen, 2001) to identify novel ABA effectors. These UPS recombination-based transient gene expression acceptor vectors for plants are a useful tool for parallel analysis of gene sets, a feature that can facilitate functional genomics.

Materials and Methods

Bioinformatics of PP2Cs

The 40 most closely-related paralogues of full length ABI1 from TAIR Release 7 (Garcia-Hernandez et al. 2002) were obtained by BLASTP (Altschul et al. 1997). Multiple sequence alignment was generated with the Pileup program of GCG (Devereux et al. 1984), and a phylogenetic tree was constructed using the GrowTree program of GCG. Global clustering of gene expression patterns was with Genevestigator v.3 (Zimmerman et al. 2005). Secondary structure analysis was with Mfold 3.2 (Zuker 2003).

Plant Materials

Maize seeds FR37cms X FR49 or B73 X MO17 (a gift from Dwight Warfel, Illinois Foundation Seed; http://www.seedgenetics.com) were imbibed in water overnight and sown at a density of 50 seeds per 4 inch square pot containing a 1:1 mix of vermiculite: peat moss and germinated in incandescent light (~100 μE m^{-2} s^{-1}) for 4 days at 23°C and moved to the dark when coleoptiles emerged. Protoplast isolation from etiolated leaves and the transient assay was performed according to Sheen (1998, 2001) with modifications. Details are described in Supplemental Materials and Methods.

Plasmid Constructs

pBM207 contains the Early Methionine-labeled *Em* promoter of wheat driving the expression of GUS (*uidA*) and was the gift of William Marcotte, Clemson University. Plasmid pAHC18 (Christensen and Quail 1996) contains the maize *Ubiquitin* (*Ubi*) promoter driving firefly (*Photinus pyralis*) luciferase (LUC) cDNA and was included in transformations as an internal reference. pDH349 contains VIVIPAROUS1 (VP1)::cMYC cDNA (engineered from a clone from Don McCarty, University of Florida) driven by the *Ubi* promoter with a 35S transcription termination signal from pDH51 (Gampala et al. 2002). Plasmids pG1 and pG2 encode the *Ppdk-35S* promoter chimera driving the Arabidopsis *ABI1*^{null} (G174D) or *abi1-1* dominant-

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negative G180D alleles, respectively, and were the gift of Jen Sheen, Massachusetts General Hospital. Plasmid pG1 is identical to pG2 except that it is wild type at amino acid 180 (Gly) and the phosphatase active site has been mutated (G174D) to express a null mutant (Sheen, 1998). Plasmid pDirect2.6 contains the *Ubi* promoter alone and was used as a control construct to balance the total input DNA. Plasmids were propagated in *E. coli* DH5α, TOP-10 or GC10 (Invitrogen, Carlsbad, CA) and prepared by CsCl density gradient ultracentrifugation (Ausubel et al. 1995).

Parallel Cloning of SSP Arabidopsis Full Length cDNAs using Cre-lox pUNI Recombination System

The recombination-based UPS (Liu et al. 1998) was adopted by the Arabidopsis 2010 Initiative (Ausubel, 2002). The system utilizes the CRE-lox site-specific recombination system of bacteriophage P1 (Abremski et al. 1983). Full length Arabidopsis pUNI cDNA donor clones (Yamada et al. 2003; Table 1) were from the ABRC Stock Center (www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm).

Derivatives of pDH349 were engineered as *loxH*-containing UPS acceptor constructs and were designated pCR701 through pCR705 (Genbank accession numbers FJ750577-FJ750581. The details are described in Supplemental Materials and Methods). Clones of pCR701- 705 (CSXXXXX- CSYYYYY [to be added upon acceptance]) are available from the ABRC at Ohio State University and can be ordered through The Arabidopsis Information Service (TAIR; http://www.arabidopsis.org). These acceptor vectors efficiently recombine *in vitro* with donor pUNI51 vectors to create plasmid dimers, thereby placing the cDNA under control of the *Ubi* promoter (Fig. 4). The GST-Cre fusion protein (ABRC stock #CD3-627) was purified from *E. coli* strain BL21 in mg quantities with glutathione-Sepharose beads (Amershan Biosciences. www.gehealthcare.com) according to the protocol provided by Steve Elledge (Harvard University; selledge@genetics.med.harvard.edu). For recombination reactions, in a 20 μL reaction volume 500 ng each of acceptor and donor vector DNAs were mixed with 2 μL 10x recombination buffer (New England Biolabs. www.neb.com), 2 μL GST::CRE recombinase and incubated at 37° C for 20 min. The DNAs were precipitated with EtOH, the pellet dissolved in 10 μL water and the DNA measured by a Nanodrop spectrophotometer (Wilmington, DE. www.nanodrop.com). 200ng of DNA products were electroporated into electrocompetent *pir*$^{-}$ *E. coli* GC10 cells and the recombinant fusion plasmid was selected on kanamycin-containing LB plates.

Immunoblotting of Transiently Expressed Effector Proteins

Because transient expression of constructs can vary widely due to differences in experimental conditions that affect transformation efficiencies, there is a need to demonstrate that candidate effector proteins are expressed to similar levels between samples. Protein quantification was determined with Coomassie Protein Assay (Pierce. www.piercenet.com). Immunoblotting of SDS-PAGE was according to Ausubel et al. (1995) using a mini vertical gel system (Thermo Fisher Scientific. www.thermofisher.com). Equal amounts (5 μg for LUC immunoblots, 1 μg for ABI1-1) of protein extracts were electroblotted to Immobilon-P PVDF transfer membrane (Millipore, www.millipore.com). Detection was with ECL Advance (Amersham Biosciences, www.gehealthcare.com). Mouse monoclonal antibodies against HA (1:3000 dilution), LUC (1:10,000 dilution), FLAG (1:10,000 dilution), and cMYC (1:5000 dilution) were from Sigma (www.sigmaaldrich.com) and anti-mouse conjugated horseradish peroxidase secondary antibody (1:15000 dilution) was from Amersham.

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Results

Bioinformatic Analysis of ABI1-Like PP2Cs

Basic Local Alignment Search Tool (BLAST) analysis of the *Arabidopsis thaliana* genome shows that the PP2C family contains 76 members (Kerk et al. 2002; Schweighofer et al. 2004; Xue et al. 2008), many of which contain conserved residues shown to be required for ABA signaling (Sheen, 1998; Rock, 2000). We chose to focus our functional genomics of ABA signaling on those ~40 PP2C paralogues most homologous to ABI1 by organizing them into clades using a nearest neighbor joining method with GCG Pileup software (Devereux et al. 1984). Thirty of these paralogues went into further investigation (Table 1) based on an BLASTP E-value cutoff of 1x 10^{-21} similarity of ABI1 to AP2C18, which has previously been shown to be associated with ABA responses (Suzuki et al. 2003). In addition to the 9 member ABI1-Like clade (Finkelstein and Rock 2002; Schweighofer et al. 2004; Xue et al. 2008), we identified two other 5-member (Fig. 1, AP2C11- AP2C15, lower subclade to clade F of Schweighofer et al. 2004) and 4-member clades (Fig. 1, clade B) with strong overall homology ($E < 1e^{-27}$) to ABI1 full length protein sequence. In keeping with the nomenclature for novel PP2Cs in Arabidopsis (Schweighofer et al., 2004), we designate the ABI1 clade genes At1g07430, At2g29380, At5g59220, At5g51760 that are most homologous to PP2CA/ABA HYPERSENSITIVE3 (T. Yoshida et al. 2006) as AP2C7, AP2C8, AP2C9 and AHG1 (erstwhile AP2C10)(Nishimura et al. 2007), respectively, and the next nearest clade members (At5g24940, At5g53140, At4g31750/WIN2, At1g43900, At5g10740) as AP2C11 through AP2C15, respectively (Fig. 1 lower subclade F; Table 1)(Lee et al. 2008). The next-further-distant ABI1-Like clade members (At2g3002, At1g07160, At2g40180, At1g67820) have already been named AP2C1, AP2C2, AthPP2C5, and AP2C4, respectively based on homologies to MP2C, an alfalfa PP2C involved in mitogen-activated kinase and wound signaling pathways (Meskiene et al., 2003; Schweighofer et al., 2004). Although *AP2C1* has been shown to be wound- and nitrate-inducible (Cheong et al. 2002; Gutiérrez et al. 2002; Malek et al. 2000; Navarro et al. 2004; Wang et al. 2003) and to target MPK4 and MPK6 (Schweighofer et al. 2007), no physiological roles for the *AP2C2*- *AP2C5* genes in Arabidopsis have been established. Based on next-nearest homology to ABI1, At2g25620 and At3g17250 were named *AP2C16* and *AP2C17*, respectively. At4g31860 was previously shown to be up-regulated by ABA and by ectopic expression of maize VIVIPAROUS1, the orthologue of the B3-domain transcription factor ABI3, suggesting it may be part of an ABA signaling regulon (Suzuki et al. 2003). Therefore it was given the name *AP2C18*, and its close homologue the name *AP2C19*. Those remaining PP2C genes with greater homology to *ABI1* than *AP2C19* were given the names *AP2C20* through *AP2C27*. These genes variously fall into the *AP2C16* and *AP2C11* clades (Fig. 1, G clade of Schweighofer et al. 2004) whereas *AP2C25* is a relative outlier (Fig. 1; Table 1). Because the cladogram constructed on ABI1-Like amino acid homologies fitted well with known functions or associations of many of these genes with ABA signaling, including relatively distantly homologous PP2Cs like *AP2C18*, we focused on 30 PP2C genes (Table 1) in subsequent analyses.

Recent availability of tools for analysis of comprehensive microarray transcriptome studies in Arabidopsis (Manfield et al. 2006; Menges et al. 2008; Schmid et al., 2005; Zimmermann et al. 2005) provide a means to place genes in a physiological, developmental, and genetic context. Nearest neighbor meta-analysis of gene expression profile datasets for the 28 PP2C paralogues most closely related to ABI1 was performed independently for tissue specificity, developmental stage, stress response, and mutant profiles in order to compare the structure/function relationships of paralogues. The dataset comprised ~200 different experiments and > 3000 Affymetrix® 22k ATH1 gene chips (Schmid et al. 2005; Zimmermann et al. 2005). The results are shown in Fig. 2 and Supplemental Fig. S1. Each gene was expressed above the statistical significance cutoff, however for several genes in each clade (*AP2C8* and *AHG1* in

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the ABI1- A clade; *AP2C1, AP2C2* and *AthPP2C5* in the B clade of Fig. 1; and *AP2C11* in the F clade) the expression levels were low which might have resulted in exaggerated clustering. Nonetheless the hierarchical cluster results based on absolute expression levels showed the *ABI1* gene clustered nearer to previously described functionally-redundant genes *HAB1, HAB2*, and *PP2CA/AHG3* (Fig. 2A and B). The *AP2C11- AP2C15* clade and *AP2C1-*Like clade (Schweighofer et al. 2004, subclade F and B, respectively, Fig. 1) also showed some clustering in smaller blocks. The relative ratio method of clustering gene expression patterns in response to exogenous stress treatments and mutant genotypes resulted in the association of known ABA effectors and showed clustering of all four of the ABI1-Like phylogenetic clades (Supplemental Fig. S1), consistent with previous results suggesting homeostatic feedback mechanisms for ABA sensitivity (Leung et al. 1997; Rodriguez et al. 1998a). Similar computational results have been reported for ABI1-Like homologues from rice (Xue et al. 2008). These results suggest that meta-analysis is a useful method for hypothesis building in functional genomics of complex gene families such as the PP2C genes of plants.

Because more distantly-related PP2C paralogues also showed similar expression profiles to the *ABI1* clade genes, e.g. in senescent leaves (Fig. 2B), these results are consistent with the hypothesis that these paralogues may have redundant and/or overlapping functions in stress responses. Published data in support of this hypothesis is that the evolutionarily-distant *AP2C18* is up-regulated by ABA (Fig. S1) and in VP1-overexpressing transgenic Arabidopsis, along with *ABI1, ABI2, HAB1*, and *PP2CA/AHG3* (Suzuki et al. 2003). Similarly, salt-inducible expression *of ABI1, ABI2, HAB1, HAB2, PP2CA/AHG3, AP2C9*, and *AP2C7* is suppressed in AtMYB44-overexpressing, stress-tolerant transgenic Arabidopsis (Jung et al. 2008). Furthermore, *ABI2, HAB1, HAB2, PP2CA/AHG3, AP2C1*, and *AP2C7,-8* and *-9* are up-regulated by ABA (Fig. S1) and/or in the *abi1-1* mutant (Hoth et al. 2002;Xin et al. 2005); *ABI1, ABI2, AP2C1, AP2C7* and *AP2C9* are up-regulated in response to wounding or pathogen elicitors (Cheong et al. 2002;de Torres-Zabala et al. 2007); *ABI1, ABI2, HAB1, PP2CA/AHG3, AP2C7*, and *AP2C9* are up-regulated in the *esk1* cold stress-resistant mutant (Xin et al. 2007); *AHG3, AP2C1* and *AP2C16* are induced significantly ($P < 0.002$) in roots and shoots by 250 μM nitrate treatment (Wang et al. 2003); and the *SUPERSENSITIVE TO ABA AND DROUGHT* mutant *sad1* (Xiong et al. 2001) suppresses accumulation of *AP2C17* and *ABI1* transcripts. Nitrate has been implicated as a precursor to the secondary messenger nitrous oxide in stomata (Desikan et al. 2002; Garcia-Mata et al. 2003) and in ABA regulation of lateral root development (De Smet et al. 2003). Consistent with the working model, many of the paralogues were induced by ABA, stress-promoting chemicals, high light intensity, and biotic and abiotic stresses (Fig. S1). The protein synthesis inhibitor cycloheximide had strong inductive or suppressive effects on *AP2C1/ -2/AthPP2C5* and *AP2C12* respectively, suggesting these genes may be under regulatory control of short-lived proteins that are post-translationally modified or subject to rapid turnover, e.g. by targeted proteolysis. Some PP2Cs in vertebrates are modified by ubiquitin-related proteins and associated with ubiquitin conjugases (Kashiwaba et al. 2003;Takeuchi et al. 2006) and a Drosophila PP2C is a negative regulator of stress responses (Baril et al. 2009), raising the possibility that mechanisms in animals may also be conserved in plants. Moes et al. (2008) recently demonstrated that application of a proteosomal inhibitor led to both a preferential nuclear accumulation of ABI1 and an enhancement of PP2C-dependent inhibitory action on the ABA response, consistent with the cycloheximide experimental results. Analysis of expression profiles in various mutant backgrounds identified the bacterial and fungal elicitors flagellin and chitin, respectively, to be key triggers for *AP2C1, -2, AthPP2C5* and *AP2C22* expression (Schmid et al. 2005;Zimmermann et al. 2005; data not shown). Taken together these data support the hypothesis that ABI1-Like PP2Cs are likely involved in stress responses, possibly by overlapping and/or redundant functions.

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Functional Analyses of ABI1-Like PP2Cs in Transiently Transformed Maize Protoplasts

Hierarchical clustering by sequence homology or on the basis of organ-, response- or growth-stage-specific expression facilitates the study of gene families. Our convergent computational results supported the hypothesis that more widely divergent PP2Cs may also function in ABA signaling. However, these predictions must be tested directly. We previously demonstrated the conserved nature of ABA signaling effectors among species in transient gene expression assays (Gampala et al. 2001, 2002) and the efficacy of maize mesophyll protoplasts for functional genomics of ABI5-Like basic leucine zipper transcription factor family members from Arabidopsis (Finkelstein et al. 2002; 2005). The maize protoplast transient gene expression assay was therefore employed to screen ABI1-Like PP2Cs for effects on ABA-inducible Pro*Em*:GUS (β-glucuronidase *uidA*) reporter gene expression, focusing on those genes that showed computational clustering (Table 1, Figures 1, 2, S1). Initially, ABI1-like family members amplified from an Arabidopsis cDNA library (Minet et al. 1992)(Supplemental Table S1) were cloned into plasmids driving cDNA expression under the control of strong *Ubiquitin* (*Ubi*)(Christensen and Quail 1996) or *Ppdk-35S* chimaeric promoters (Sheen 1998). Subsequently we developed a Cre-lox recombination method (see below) to produce constructs, and functional data from the Cre-lox system is presented along with conventional construct results in Fig. 3. Consistent with predictions, Pro*35S-Ppdk*:abi1-1, Pro*Ubi*:ABI2, Pro*Ubi*:AP2C7, Pro*Ubi*:AP2C9, and Pro*Ubi*:AP2C18 significantly antagonized the ABA-inducible Pro*Em*:GUS reporter expression more than 50% ($P < 0.012$), while the effect of Pro*Ubi*:HAB2, and Pro*Ubi*:AP2C15 on Pro*Em*:GUS expression were negative but not statistically significant (Fig. 3A and B). The slight antagonism by HAB2 of ABA-inducible Pro*Em*:GUS gene expression was consistent with the results of Yoshida et al. (2006) who showed genetically that HAB2 is a weak negative regulator of ABA-induced chlorophyll loss in seedlings. Interestingly, transient expression of Pro*Ubi*:AP2C1 transactivated Pro*Em*:GUS expression instead of antagonizing it (Fig. 3C). The conserved Gly residue at amino acid position 180 of ABI1 is critical for ABA signaling and the G180→D mutation results in dominant-negative alleles for ABI1 and ABI2 (G168D)(Sheen, 1998). A site-directed mutant derivative of AP2C1 (Pro*Ubi*:AP2C1m) containing four amino acid residue changes, in particular G178E which is analogous to the dominant-negative allele G180D in abi1-1 (Supplemental Fig. S2) did not transactivate Pro*Em*:GUS expression, suggesting that the transactivation effect of AP2C1 was specific (Fig. 3C). Supplemental Figure S3 documents the reproducibility of several PP2C effects obtained with Cre-lox recombination-generated constructs (see below). We extended the work to include functional assays for AthPP2C5 and AP2C13/WIN2 effectors, however these genes did not have an appreciable effect on ABA-inducible gene expression (Supplemental Fig. S3). Taken together, the functional data suggest that genes highly homologous to ABI1 are functionally conserved for ABA-inducible gene expression as previously shown genetically, but genes outside the ABI1-Like clade (e.g. AP2C1, AP2C18) can also affect ABA signaling when overexpressed. We speculate that crosstalk mechanisms between stress pathways may be mediated by PP2Cs either directly by sharing of some targets, or indirectly by altering the homeostatic balances of phosphorylated signaling effectors in regulatory cascades.

Development of Cre-lox Universal Plasmid Acceptor Vectors for Transient Protoplast Expression of Arabidopsis Full Length cDNAs

Because of the facile nature of transient gene expression studies in protoplasts (Sheen, 2001) and the availability of over 14,000 full-length Arabidopsis donor cDNAs in the UPS vector pUNI51 (Yamada et al. 2003), we constructed a series of UPS acceptor plasmids (pCR701-pCR705)(Supplemental Table S2) containing in-frame N-terminal epitope tags recognized by commercially available antibodies (6xHis, 2xHA, 2xFLAG, 2xcMyc) and driven by the maize *Ubi* promoter for expression of tagged cDNA effectors (Fig. 4). Choice of the *Ubi* promoter (Christensen and Quail 1996) was based on its widespread use in transgenic studies and our

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detailed analyses of relative *Ubi, Actin, 35S*, and *Em* promoter activities in transiently transformed rice protoplasts (Hagenbeek et al. 2000; Hagenbeek and Rock 2001; Gampala et al. 2001) that show the promoter strengths of *Ubi, 35S*, and *Em* are similar.

The pioneering work by Sheen (1998, 2001) with maize mesophyll protoplasts was performed with radioactive ^{35}S-Met to assess protein synthesis. In order to test the utility and sensitivity of our expression vectors for protein work, we measured the activities of LUC and abi1-1 proteins in crude extracts of transiently transformed protoplasts and then quantified proteins in those same samples by chemiluminscent immunoblots to establish the limits of detection of our transformed genes. Fig. 5 shows the results from two parallel experiments done with Pro*Ubi*:LUC (Christensen and Quail 1996) and Pro*35S-Ppdk*:HA∷abi1-1 (Sheen 1998). Pro*Ubi*:LUC input DNA concentrations from 0 to 20 μg resulted in exponential increases in LUC activities, with 10 μg of input DNA giving activities which approached a plateau when quantified per unit DNA (Fig. 5A; data not shown). Increases of Pro*Ubi*:LUC input DNA were found to be directly proportional to the amount of LUC protein (60 kD) detected on immunoblots (Fig. 5A). Similarly, the dominant-negative activity on ABA-inducible Pro*Em*:GUS expression by HA∷abi1-1 (43 kD) was proportional to input effector DNA and could be correlated with a single band of the predicted size on immunoblots (Fig. 5B), with a limit of detection below 1 μg of input DNA per μg crude protein extract. Increasing concentrations of input DNA for HA∷abi1-1 to 80 μg could antagonize the ABA-inducible *ProEm*:GUS reporter expression up to 90% (Fig. 5B). These results demonstrate that detection of epitope-tagged effector proteins in a non-radioactive system is practical, which is an important technical consideration because effectors with questionable activities preclude interpretation unless negative functional results can be confirmed by protein quantitation. The presence of the epitope tags can facilitate cell biological assays of gene function, e.g. ABA-mediated nuclear relocalization (Moes et al. 2008) or biochemical analysis of post-translational modifications.

As an example of the utility of the pCR70x series to quickly screen for putative ABA signaling effectors, we recombined with pCR701, pCR703 (HA tag), pCR704 (FLAG tag) and pCR705 (cMYC tag) the UPS donor cDNA clones (u80334 u13940, and c102899) for ABI1 homologues AP2C7, AP2C8, and AP2C2 (Table 1) and performed functional assays for ABA-inducible gene expression in protoplasts. Because numerous ABI1-Like PP2Cs are up-regulated by ectopic expression of VP1 (Suzuki et al. 2003), we included VP1 as a positive control for ABA-inducible gene expression in our transient assays with a view to performing interaction studies in the future. The results shown in Fig. 6A are on a logarithmic scale because the synergies observed between co-transformed VP1 and basic leucine zipper transcription factor ABF3, or between VP1 and ABA (Finkelstein et al. 2002;2005), are a hundred-fold above the modest (seven-fold) transactivation by ABA alone or by AP2C2 (Fig. 6A, compare "control" with "VP1" versus AP2C2). The antagonistic effect of overexpressed AP2C7 using a conventional effector construct (Fig. 3A) was reproduced with the pCR703 HA-tagged construct (Fig. 6A). In addition a FLAG-tagged AP2C2 cDNA had a significant agonist (positive) effect ($P < 0.0005$), similar to that observed with its most closely-related homolog AP2C1 (Fig. 3C). Immunoblots of protein extracts from these and additional functional assays are shown in Fig. 6B-D. AP2C2 was detected as an abundant yet diffuse pair of bands at the predicted size of ~47 kD when tagged by HA (pCR703, Fig. 6C; left panel), FLAG (pCR704, Fig. 6B, E; left panels), and cMYC (pCR705, Fig. 6C; right panel). Other tagged cDNA recombinants were also detected on immunoblots (e.g. FLAG∷AP2C8, Fig. 6D left panel; HA∷PP2CA, 6D right panel) and showed predictable biological activities in antagonizing ABA-inducible gene expression (e.g. HA∷PP2CA, data not shown; HA∷AP2C7 in Fig. 3A). Stripping and reprobing the blots for the co-transformed Pro*Ubi*:LUC reporter protein luciferase was effective (Fig. 6B, right panel). These results demonstrate the utility and potential of our constructs for parallel

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functional assays and quantification of transgenic proteins by immunoblots, which can facilitate biochemical and quantitative analyses e.g. for structure/function studies.

Discussion

Our results showing antagonist and agonist effects of Arabidopsis ABI1-Like PP2Cs in maize protoplasts provide a starting point for detailed and focused molecular, cellular and physiological studies on ABA signaling PP2C effectors (Moes et al. 2008; Saez et al. 2008; Xue et al. 2008). We have demonstrated the pCR701- pCR705 UPS plant expression acceptor vectors for transient protoplast transformation described here can serve to facilitate functional genomics of plant signaling networks. These types of comparative functional studies in turn can lead to insights into molecular evolution. There are longstanding problems associated with overexpression experiments *in planta* such as homologous transgene-mediated silencing, and interpretation of pleiotropic phenotypes due to position effects. Heterologous (interspecies) gene studies, such as testing Arabidopsis genes in maize protoplasts, can circumvent this potential problem, especially when the assay is for gain-of-function effects. In addition, efficient stable transformation of maize by particle bombardment (Shou et al. 2004) of transient vectors like the ones described here is practical, creating scope for broader applications of these acceptor vectors in cereals.

Our results with protoplasts showing PP2C effects are consistent with *in planta* experimental and genetic results to date, as we previously showed for the ABI5-Like clade of basic leucine zipper transcription factors (Finkelstein et al. 2002; 2005). Recapitulation in protoplasts of gene interactions established by conventional molecular genetics studies strengthen our claim that such a systems approach is valid for screening biologically relevant interactions. The novel effects observed could also lead to biotechnological applications in crops, notwithstanding the need to understand molecular mechanisms and the subcellular, spatial, and temporal contexts of gene interactions. We are currently testing the feasibility of transcriptome profiling of transiently transformed maize protoplasts by fluorescence-activated cell sorting (Birnbaum et al. 2006; Hagenbeek and Rock 2001), a method which could advance systems approaches to translational genomics by generating “functional map space” of signaling effectors.

Regarding the biological significance of our finding that AP2C1 and AP2C2 can function in protoplasts as ABA agonists, there is one other report of a PP2C positive effector of ABA responses (Reyes et al. 2006). Those authors concluded that the effect on ABA of beech *FsPP2C2* was indirect because GA application could rescue a dwarf phenotype and GAs were decreased by overexpression of FsPP2C2 in Arabidopsis. In the cases of AP2C1 and AP2C2, both these genes are up-regulated by bacterial pathogens, hydrogen peroxide, and elicitors such as bacterial EF-Tu, chitin, and flagellin22 (de Torres-Zabala et al. 2007; Schweighofer et al. 2007) (Supplemental Fig. S1; data not shown). Recent findings have linked biotic stress responses with ABA signaling (Adie et al. 2007; Asselbergh et al. 2008; Chini et al. 2004; Flors et al. 2008; Fujita et al. 2006; Goel et al. 2008; Hernandez-Blanco et al. 2007; Kaliff et al. 2007; Mohr and Cahill 2003; Thaler and Bostock 2004), consistent with our bioinformatic and functional results showing AP2C1 and AP2C2 may be positive effectors in overlapping ABA and biotic stress signaling pathways. Remarkably, de Torres-Zabala et al. (2007) demonstrated that virulence of the plant pathogen *Pseudomonas syringae* is dependent on modulating host ABA- and stress response pathways through ABI1-Like PP2C expression and increased ABA biosynthesis, but their results raise questions about how negative ABA regulators in the ABI1 clade can work. Schweighofer et al. (2007) showed that AP2C1 overexpression increased susceptibility of transgenic Arabidopsis to infection by *Botrytis cinerea*. A rice PP2C has recently been shown to interact functionally with the XA21 receptor kinase in the innate immune response (Park et al. 2008). Our functional results showing AP2C1 and AP2C2 (Figs. 3C and 6) are positive effectors of ABA sensitivity and which are up-

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regulated by bacterial elicitors and stresses (Supplementary Fig. S1; de Torres-Zabala et al. 2007; data not shown) provide a compelling molecular mechanism (induced positive effectors of ABA) for increased ABA sensitivities required for pathogen infection.

Novel plant proteins are likely phosphorylated and may be preferentially involved in plant-specific processes such as secondary metabolism, responses to hormones and environmental cues, and the identity of specific cell types (DeLong, 2006; El-Khatib et al. 2007). Several proteins have been shown to physically and genetically interact with ABI1, ABI2, HAB1, and PP2CA (Guo et al. 2002; Leung et al. 2006; Miao et al. 2006; Ohta et al. 2003; Saez et al. 2008; Yang et al. 2006; R. Yoshida et al. 2006), consistent with the notion that PP2Cs function as nodes in regulatory networks. Recent results linking plasma membrane-association of G-proteins (Pandey et al. 2009; Zeng et al. 2007) and phospholipase D with ABI1 and GPA1 modulation (Mishra et al. 2006) suggest that gaps in knowledge of ABA signal transduction networks are closing and emerging models of molecular mechanisms can be directly tested. Protoplast systems (including primitive land plants like Physcomitrella)(Komatsu et al. 2009) in conjunction with the transient vectors described here lend themselves to rapid probing of mechanisms by chemical genomics and pharmacological methods, multiparameter-correlated analysis by flow cytometry, cell biology of protein:protein interactions, and subcellular localization (Gampala et al. 2001; Hagenbeek et al. 2000; Hagenbeek and Rock 2001; Huang et al. 2007; Rogers et al. 2006). Application of the systems approach to other response pathways or gene families such as protein kinases and G-protein-coupled transmembrane receptors, which have a complexity comparable to the PP2Cs, are associated with the plasma membrane, and also affect ABA and innate immunity responses (Belin et al. 2006; D'Angelo et al. 2006; Fuglsang et al. 2007; Gudesblat et al. 2007; Hrabak et al. 2003; L. Li et al. 2006; Moriyama et al. 2006; Ortiz-Masia et al. 2007; Pandey et al. 2009; Quan et al. 2007; Trusov et al. 2007; Xu et al. 2006; R. Yoshida et al. 2006) portends advances in functional genomics of plant signaling.

To date only one UPS acceptor vector that exploits Cre-lox fusion events for genetic selection of recombinant clones and expression in plants has been described (pKYLX-myc9-loxP; Guo and Ecker 2003), and only two published studies have resulted, based on citations of the input source papers (Olmedo et al. 2006; Wang et al. 2006; Yamada et al. 2003). This situation suggests a greater need for deployment of tools and associated functional assays with rapid turnaround times to help drive transformational research. Over 10,000 Arabidopsis cDNA/ORF clones from the SSP project originally provided in pUNI51 are now subcloned in the Gateway™ entry vector pENTR223 (TAIR, www.arabidopsis.org), but this system depends on use of proprietary blends of integrase, integration host factor and excisionase enzymes whereas the UPS system is freely available. The UPS allows for the rapid creation of constructs for expression in multiple organisms. It can be used to transfer whole libraries into new vectors (e.g. autofluorescent protein fusions, yeast two-hybrid prey); additional uses include directional PCR cloning and versatile options for N-terminal and C-terminal epitope tags. The UPS system can facilitate protein-protein interaction studies, proteomics, cell biology, protein purification, and structure/function assays. Comparative genomics, especially when combined with functional studies such as demonstrated here for Arabidopsis PP2C genes, can shed light on plant evolution, diversification, and the genetic potential for biotechnological applications for increasing food and fiber through improved stress tolerances (Clark et al. 2007; Fernie et al. 2006).

Supplementary Material

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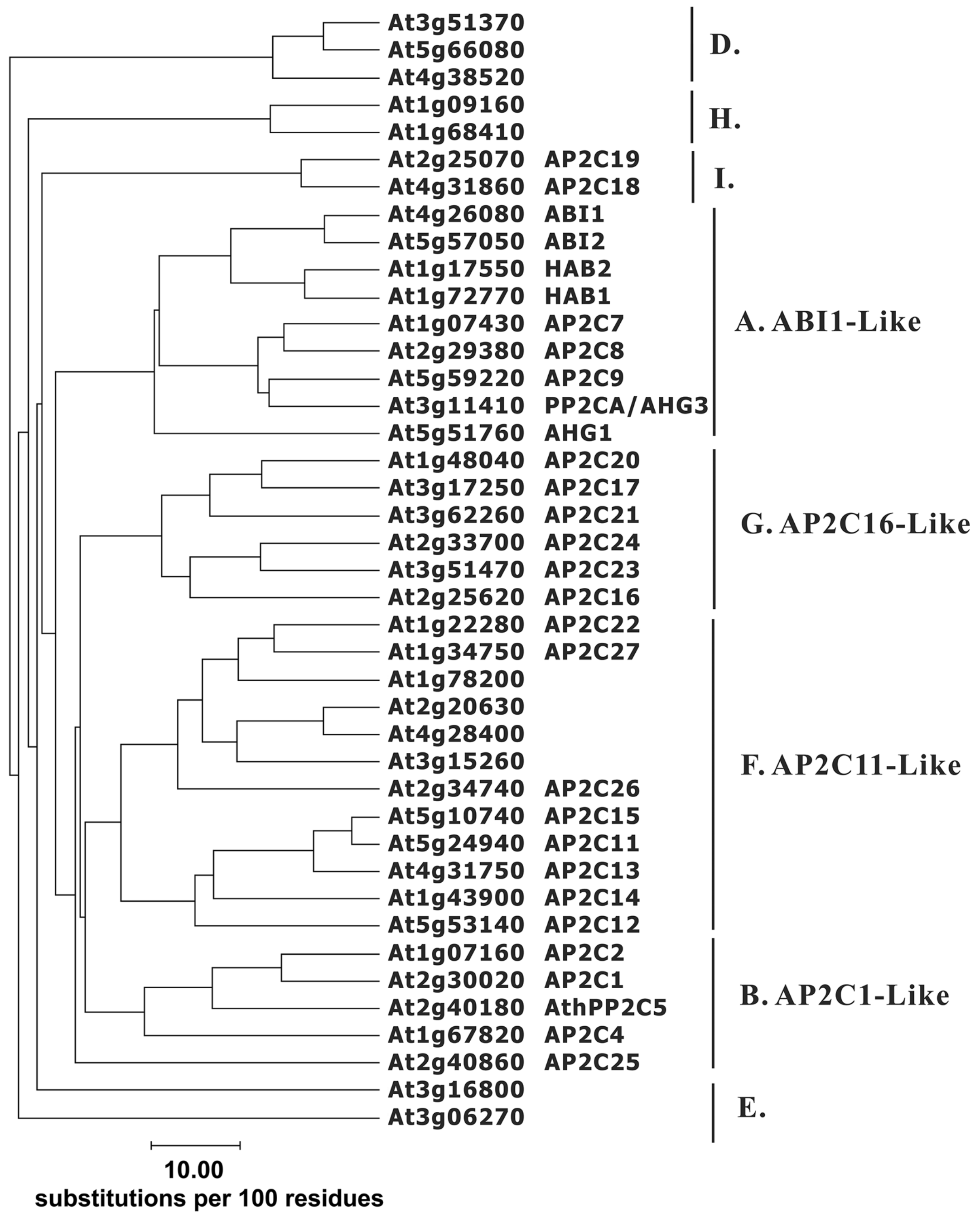

Figure 1.

Homology to ABI1 among Arabidopsis PP2Cs. The 40 most closely-related proteins to ABI1 (BLASTP) were aligned by the Pileup program of GCG using progressive pairwise alignments and the phylogenetic tree was constructed by the GCG GrowTree program. Capital letters on right correspond to clades classified by Schweighofer et al. (2004).

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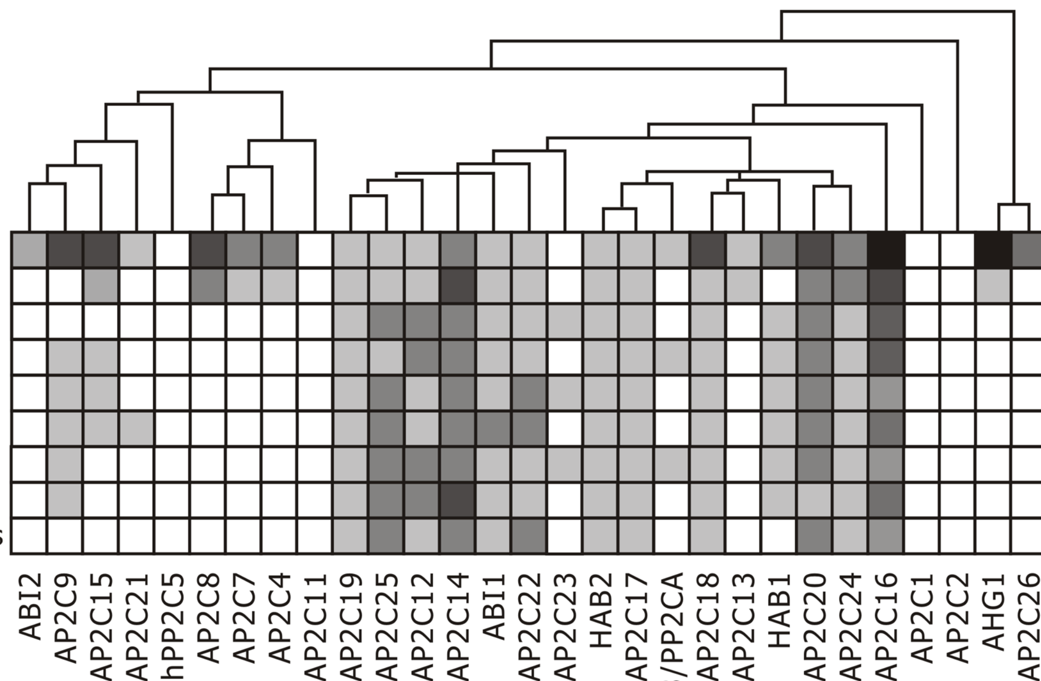

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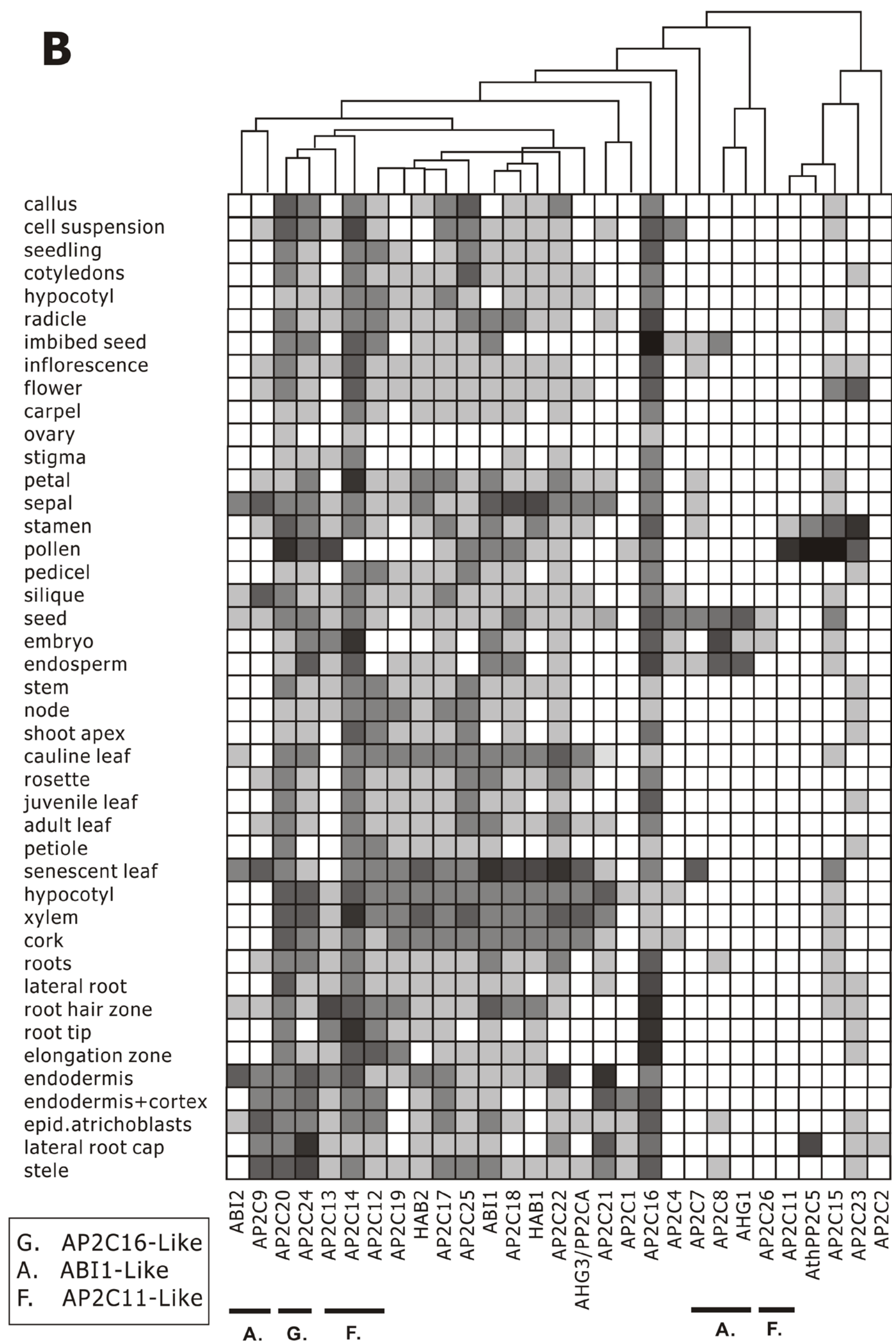

Figure 2.

Hierarchical clustering meta-analysis (Zimmerman et al. 2005) of Affymetrix 22k ATH1 gene expression profiles (Schmid et al. 2005) of the 28 Arabidopsis PP2C gene family members homologous to ABI1. Capital letters spanning genes in the cladogram correspond to clades classified by Schweighofer et al. (2004) shown in Fig. 1. Results are given as heat maps in gray scale coding (absolute signal values, darker = higher expression). (**A**) Growth stages of the life cycle, starting with mature siliques (seeds). (**B**) Plant tissue- and organ-specific expression. Growth stage annotations are based on the Boyes key (2001). The choice of organ terminology is based on vocabulary defined by the Plant Ontology Consortium (http://www.plantontology.org).

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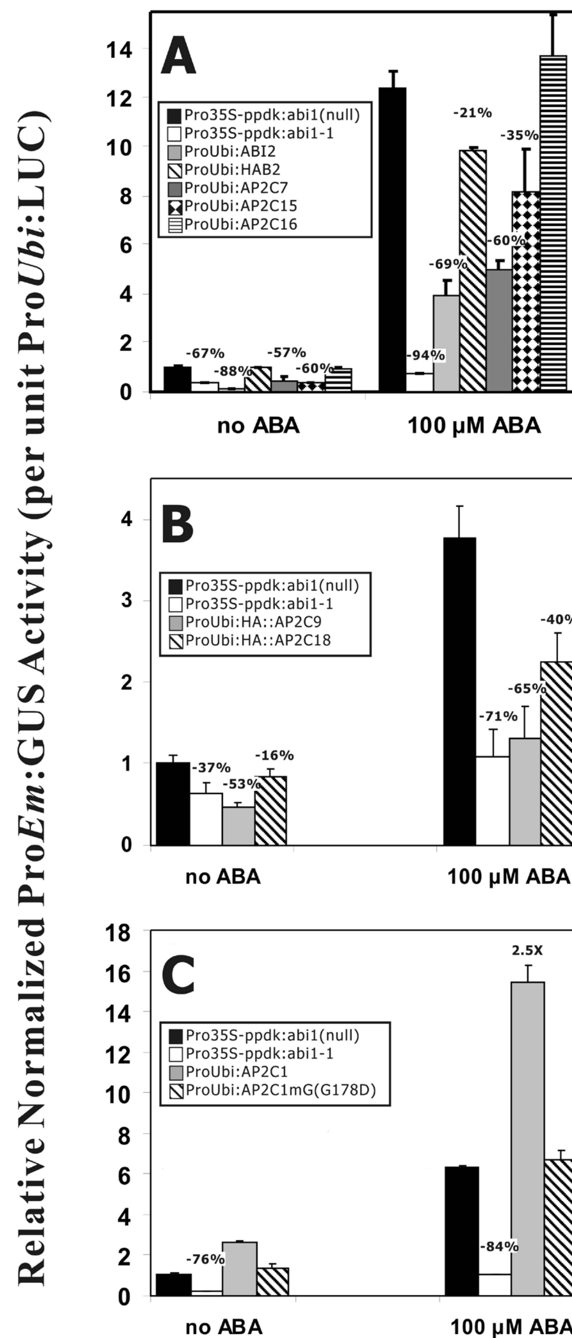

Figure 3.

Functional analysis of Arabidopsis ABI1-Like PP2Cs in transient gene expression assays. The y-axis has been normalized by a scalar applied to all data to reflect fold-induction relative to control (no ABA = 1.0) so that different experimental datasets (**A- C**) are more easily compared. Overexpression of ABI2, HAB2, AP2C7, AP2C15, (**A**), or AP2C9 and AP2C18 (**B**) cDNAs have antagonist effects, whereas overexpressed AP2C1 has agonist (inductive) effects (**C**) on ABA-inducible Pro*Em*:GUS expression in separate maize mesophyll protoplast experiments. Number with symbol “x” represents fold transactivation by AP2C1 relative to ABA-treated control. Negative numbers next to bars indicate the percent inhibition of Pro*Em*:GUS expression relative to controls. The abi1^{null} and abi1-1 effector constructs were included as

negative and positive controls, respectively. Control samples were co-transformed with a Pro*35S-ppdk*:ABI1^{null} expression construct encoding the phosphatase inactive G174D mutant (Sheen, 1998). The AP2C1m construct contains the analogous (G→ D178) point mutation as abi1-1 dominant negative (G→ D180) allele. Data are the average of at least two replicates, ± SE.

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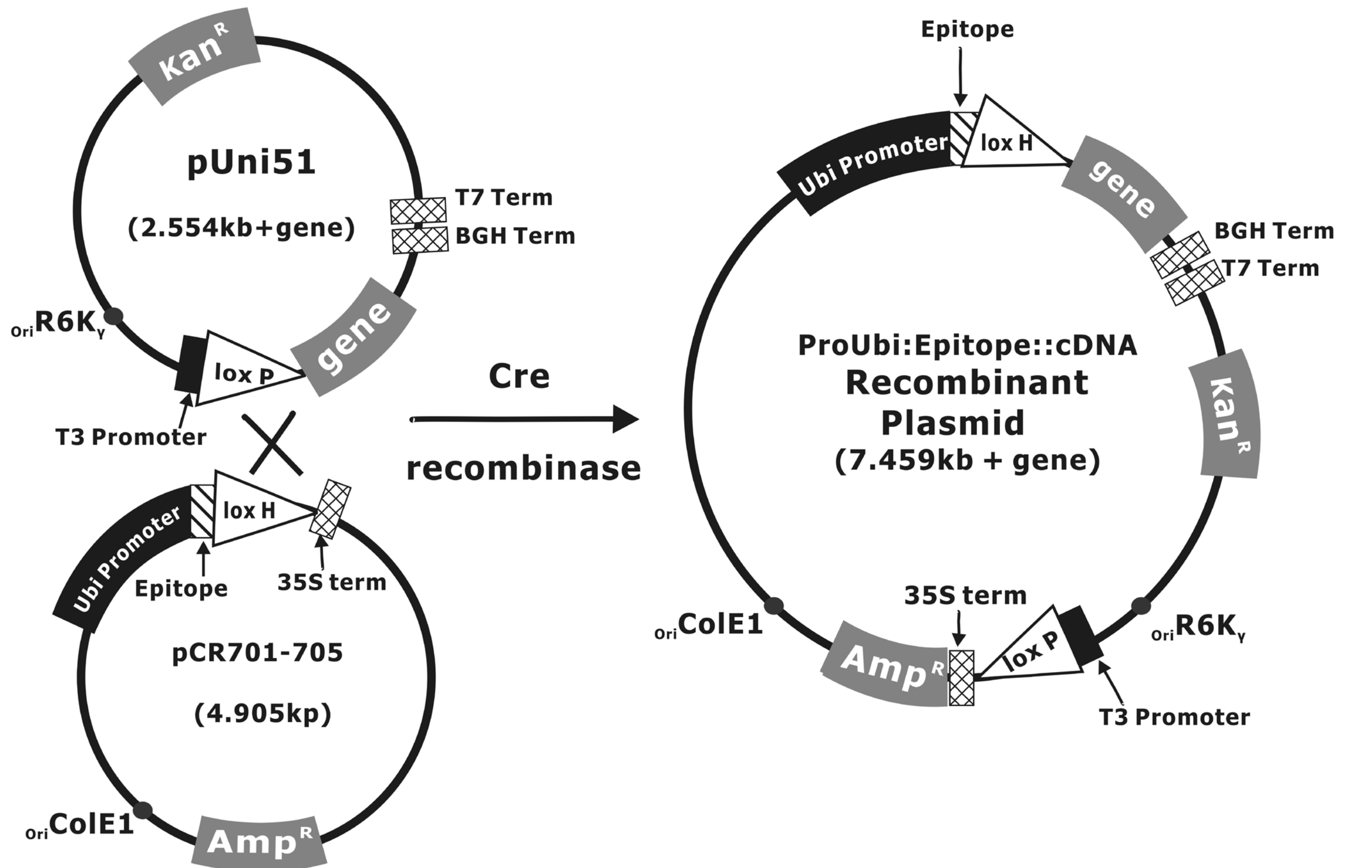

Figure 4.

Cre-lox recombination-ready acceptor vector for transient plant expression assays. pCR701: loxH alone. pCR702- 705 plasmids contain 2xHIS6, 2x-HA, 2x-FLAG, and 2x-cMYC epitope tags, respectively (see Table S2), upstream (5') and in frame with loxH recombination site to give N-terminal fusion peptides with acceptor pUNI cDNA plasmids. *Cis*-acting element symbols are not shown to scale.

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Figure 5.

Immunoblots of protein extracts from maize protoplasts transformed with standard constructs (Pro*Ubi*:HA∷abi1-1, Pro*Ubi*:LUC). (**A**) Increasing amounts of transformed DNA for ProUbi:LUC reporter or (**B**) Pro*Ubi*:HA∷abi1-1 effector show that transgene activities are correlated with protein expression. The amounts of electroporated DNAs and their relative protein activities in the same samples are shown under the lanes of the immunoblots. The relative inhibition of ABA-inducible gene expression by abi1-1 protein (45 kD) was measured by Pro*Em*:GUS/Pro*Ubi*:LUC enzyme assays as shown in Fig. 3.

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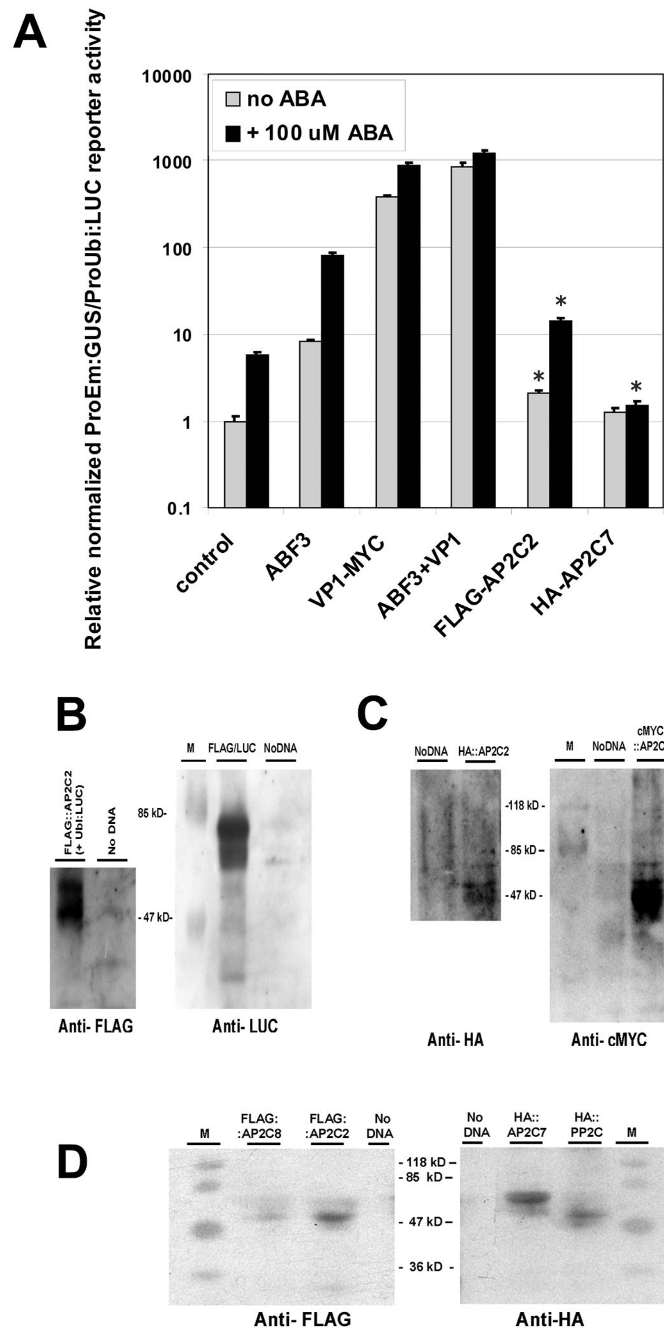

Figure 6.

Functional and expression analyses of select ABI1-Like PP2Cs using the Cre-lox acceptor vectors pCR701, pCR703 and pCR704. (**A**) Transient expression of Pro*Ubi*:FLAG∷AP2C2 and Pro*Ubi*:HA∷AP2C7 effects on ABA-inducible Pro*Em*:GUS reporter gene expression. Asterisk (*) indicates significantly different than control ($P < 0.0008$, two sided Student's t-test, equal variance assumed. The positive control VP1 and ABF3 statistical treatments are not shown to emphasize the test). The y-axis has been normalized by a scalar applied to all data to reflect fold-induction relative to control (no ABA = 1.0). Data are means of four replicates, ± SE. (**B- D**) Immunoblots of protein extracts from samples for panel A. Five μg Pro*Ubi*:LUC and 20 μg Pro*Ubi*:FLAG∷AP2C2 or mock control (NoDNA) were co-electroporated into

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protoplasts and cultured overnight. Fifteen μg protein extract was run on SDS-PAGE, transferred to PVDF membrane, and probed with anti-FLAG antibody (**B**, left side), then stripped and re-probed with anti-LUC antibody (**B**, right side). M= protein standard marker. (**C**) Immunoblots probed for Pro*Ubi*:HA∷AP2C2 (left) and Pro*Ubi*:cMYC∷AP2C2 (right). The predicted recombinant AP2C2 protein is 46 kD. (**D**) Immunoblots probed for Pro*Ubi*:FLAG∷AP2C8 and FLAG∷AP2C2 (left; proteins are predicted 46 kD) and Pro*Ubi*:HA∷AP2C7 and HA∷PP2CA (right; predicted proteins are 55 and 49 kD, respectively).

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Table 1

Genes studied in this work. **Highlighted** genes are reported herein for their effects on ABA-inducible *ProEm*:GUS reporter gene expression.

List of AGI genes under study	Common name; PP2C nomenclature based on Schweighofer et al. (2004) or genetic studies.	Full length cDNA clones available from ABRC[a] or RIKEN[b] (pdx clones)	E value
At4g26080	**ABI1** (Leung et al. 1994)	c104649	0
At5g57050	**ABI2** (Leung et al. 1997; Rodriguez et al. 1998a)	u24491	e-155
At1g17550	**HAB2** (Yoshida et al. 2006)	pda11685	2e-93
At1g72770.1	HAB1 (Rodriguez et al. 1998b; Robert et al. 2006)	u67779	1e-91
At3g11410	**PP2CA/AHG3** (Kuhn et al. 2006; Yoshida et al. 2006)	u10629	3e-67
At1g07430	**AP2C7**	u19116	5e-64
At2g29380	**AP2C8**	u80334	2e-62
At5g59220	**AP2C9**	u13940	3e-58
At5g51760	AHG1 (Nishimura et al. 2007)	pdy22231; pdx59946; pdx60667	8e-56
At5g24940	AP2C11	pda11076; DQ056688	1e-32
At5g53140	AP2C12	u10437; u21197	8e-32
At4g31750	**AP2C13/WIN2** (Lee et al. 2008)	u18734	1e-31
At1g43900	AP2C14	u11600	1e-31
At5g10740	**AP2C15**	u50442	7e-31
At1g67820	AP2C4	pdz88907	7e-31
At2g40180	**Athpp2c5**	u20838[c]	2e-28
At1g07160	**AP2C2**	c102899	3e-28
At2g30020	**AP2C1** (Schweighofer et al. 2007)	pdz34415	1e-27
At4g31860	**AP2C18**	u14631	2e-21
At2g25070	AP2C19	u10848	7e-21
At3g17250	AP2C17	pda19470; pda16083	9e-26
At2g25620	**AP2C16**	u09071; u12815	2e-25
At1g48040	AP2C20	U66309	6e-25
At3g62260	AP2C21	u87348	2e-24
At1g22280	AP2C22/PAPP2C (Phee et al. 2008)	u19121	3e-24
At3g51470	AP2C23	u68291	8e-24
At2g33700	AP2C24	u18901	1e-23
At2g40860	AP2C25	u11316	7e-23
At2g34740	AP2C26	pda13976	1e-21
At1g34750	AP2C27	pda18907	7e-23

[a] http://www.arabidopsis.org

[b] http://www.brc.riken.jp/lab/epd/catalog/cdnaclone.html

[c] not full length according to TAIR8.0; clone missing N-terminal ~230 AAs