

## ZmSnRK2.8 responds to ABA through the SnRK2-PP2C complex

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### Abstract

Understanding the responses of maize to abscisic acid (ABA) dependent drought tolerance is an important topic for the biotechnological application of functional mechanisms of stress adaptation. Key components that control and modulate stress adaptive pathways include SnRK2 (sucrose non-fermenting 1-related protein kinases 2) proteins. Recent studies indicate that SnRK2 are plant specific kinases that together with ABA ligand PYR/PYL/RCAR proteins and type 2C group A protein phosphatases constitute the central core of abscisic acid perception and signal transduction. Here, we study drought responses in maize by analyzing the mechanism of ZmSnRK2.8 in ABA signaling to establish relevant analogies with other plant species. ZmSnRK2.8 is a very closely related protein to *Arabidopsis OST1 (open stomata 1)* kinase with nuclear and cytosolic subcellular localization able to auto-phosphorylate S182 or T183 amino acids on its activation loop suggesting that phosphorylation at these sites may be a general mechanism for SnRK2 activation. In addition, ZmSnRK2.8 is activated by ABA and interacts with PP2C phosphatases in a constitutive, ABA independent manner. Together, our data suggest a conserved mechanism of plant responses to ABA and drought stress in maize and point to the potential use of this kinase in improving programs of drought tolerance in crops.

**Keywords:** ABA signaling, ABA perception, SnRK2 activity, stress response, phosphorylation

### Introduction

An important challenge for current agricultural biotechnology is to cover the increasing demand in crop production. This growing demand is associated with dramatic losses of arable lands due to increasing severity of abiotic environmental conditions. In particular, drought is one of the major determining factors that adversely affect plant growth and development and has a crucial impact in agriculture productivity with major environmental, economic and social consequences. The present climate changes are aggravating these effects and the understanding of plant-water relationship is becoming crucial to better plan for drought, reducing crop vulnerability and the detrimental resulting effects.

The main hormone involved in the responses to osmotic stress is the phytohormone abscisic acid (ABA) with a major role in the regulation of several developmental and physiological processes, such as seed maturation, germination or transpiration. Remarkably, ABA levels increase in late embryo development shortly before the onset of desiccation and in vegetative tissues under water-deficit stress conditions triggering stomata closure and water-deficit tolerance responses (Hirayama and Shinozaki, 2007; Cutler et al, 2010).

Protein kinases appear to play key roles in many signaling cascades and many have already been linked to ABA and/or osmotic stress signaling, either

affecting stomata function and/or gene expression (Sirichandra et al, 2009; Hubbard et al, 2010). Recently, a negative regulatory pathway that controls ABA signaling by inhibiting type 2C protein phosphatases (PP2C) through the direct interaction with a newly described type of ABA receptor (Ma et al, 2009; Park et al, 2009; Melcher et al, 2010; Umezawa et al, 2010; Weiner et al, 2010) have placed sucrose non-fermenting 1-related protein kinases (SnRK2) at the top of the positive response to this plant hormone. In fact, it has long been established that SnRK2 and PP2C proteins have antagonistic effects (Yoshida et al, 2006) but only now do we fully understand the physiological relevance of this fact (Fujii et al, 2009; Umezawa et al, 2009). The current model of ABA signaling includes three core components, the PYR/PYL/RCAR proteins, the type 2C protein phosphatases, and the ABA-activated SnRK2 kinases (Ma et al, 2009; Park et al, 2009). SnRK2 constitutively interact with PP2C phosphatases that dephosphorylate the activation loop of the SnRK2 catalytic domain. When ABA levels increase, for instant during water stress, PYR/PYL/RCAR and PP2C bind through a high affinity to ABA, leaving the activation loop of SnRK2 available for auto-phosphorylation or phosphorylation by unknown kinases (Weiner et al, 2010).

The SnRK2 subfamily has been identified and characterized in various plants. The first SnRK2 gene, PKABA, was isolated from wheat and is up-regulated

by drought and ABA in both seeds and vegetative tissues (Anderberg and Walker-Simmons, 1992). Next, a gene from faba bean, AAPK, was identified to play a key role in regulating ABA-dependent stomata closure in guard cells (Li et al, 2000). Ten SnRK2 genes were isolated from Arabidopsis of which five members are activated by ABA and all members, except SnRK2.9, can be activated by hyperosmotic and salinity stress (Boudsocq et al, 2004). Similarly, ten SnRK2s designated SAPK1-10, were identified in rice of which all are activated by hyperosmotic stress, and SAPK8-10 are also activated by ABA (Kobayashi et al, 2004). Recently, two wheat SnRK2 gene members were characterized, TaSnRK2.4 and TaSnRK2.8, involved in development and tolerance to abiotic stresses (Mao et al, 2010; Zhang et al, 2010). In maize, eleven SnRK2 members were cloned, and most ZmSnRK2 are induced by one or more abiotic stresses with only functional data for ZmSnRK2.8, a protein highly homologous to OST1, involved in diverse stress signal transduction pathways, in particular salt tolerance (Huai et al, 2008; Ying et al, 2011). Although those studies show that SnRK2s play crucial roles in maize abiotic stress responses, knowledge of specific functions of ZmSnRK2s are still in an initial step. Therefore, understanding the molecular basis of ZmSnRK2 function is necessary for the development of genetic improvement of stress tolerance in maize.

With this possibility in mind we have isolated a maize SnRK2 gene, ZmSnRK2.8, and characterized this kinase focusing on its ABA regulation. Here we describe our work determining that ZmSnRK2.8 localizes in the nucleus and cytosol of plant cells and is activated by ABA. In addition, we show that ZmSnRK2.8 is capable of auto-phosphorylating its activation loop and that it directly interacts with ZmPP2C through the regulatory kinase domain. Our results, in conjunction with several other reports (Ying et al, 2011; Hubbard et al, 2010; Hauser et al, 2011), suggest that there is a conserved mechanism of ABA sensing and signal transduction between maize and other plants species with respect to the SnRK2 action.

## Materials and Methods

### Plant material, growth conditions and treatments

The Zea mays W64A line was used for these experiments. For the treatment experiments, plants were germinated and grown in liquid 0.5 x MS medium (Murashige and Skoog, 1962) for one week prior to 100  $\mu$ M ABA application in the medium. Material was deep frozen immediately after treatment and stored at -80°C.

### Phylogenetic alignment of SnRK2 kinases

SnRK2 sequences from maize (Huai et al, 2008), rice (Kobayashi et al, 2004), and Arabidopsis (Boudsocq et al, 2004) were used, together with sequences from other plant species collected from public databases, to perform a phylogenetic alignment. This

alignment was performed with the web-based tool Phylogeny ([www.phylogeny.fr](http://www.phylogeny.fr)) described by Dereeper et al. (2008) based on protein sequence alignment.

### GFP localization and BiFC by confocal microscopy

For Agrobacterium tumefaciens-mediated transient expression in *Nicotiana benthamiana* the full-length ZmSnRK2.8 cDNA sequence (EU676040) was cloned in the PC1302 vector (Clontech) to produce 35S::ZmSnRK2.8-GFP. The regulatory domain of ZmSnRK2.8 (286-366) and the full-length ZmPP2C cDNA sequences (NM\_001154386) were cloned in the GATEWAY-compatible vector pENTRY3C (Invitrogen). The two pENTRY3C plasmids were transferred to a BiFC GATEWAY-modified vector developed by A. Ferrando (<http://www.ibmcp.upv.es/FerrandoLabVectors.php>; López-Paz et al, 2009) to produce 35S::YN-ZmSnRK2.8[286-366]; 35S::YN-ZmPP2C; 35S::YC-ZmSnRK2.8[286-366] and 35S::YC-ZmPP2C. *N. benthamiana* plants were transiently transfected for GFP or YFP detection on a Leica TCS SP confocal laser-scanning microscope. For the co-infiltration, equal volumes of the Agrobacterium cultures were mixed (Dunoyer et al, 2004). Observations were performed 3 days after infiltration.

### Protein purification and in vitro phosphorylation

A cDNA fragment encoding for ZmSnRK2.8 was cloned into the pET28a expression vector (Promega), expressed in *Escherichia coli* BL21 cells and purified as His-tag fusion proteins according to the manufacturers' instructions.

*In vitro* phosphorylation was performed as described by Riera et al (2004) using 100 ng Histone and 100 ng myelin basic proteins as substrates. After incubation for 45 min at 30°C with [ $\gamma$ -<sup>33</sup>P]ATP (3000 Ci mmol<sup>-1</sup>), proteins were separated by SDS-PAGE on a 12.5% acrylamide gel. Radioactivity on dried gels was detected using a Storm 820 imager (GE Healthcare). Relative [ $\gamma$ -<sup>33</sup>P] incorporation was analyzed using the public domain image analysis software ImageJ (<http://rsbweb.nih.gov/ij>).

### Protein immunoprecipitation and in gel kinase assay

Protein immunoprecipitation and in gel kinase assay was performed as described by Lumbroblas et al (2010). Proteins were extracted in 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM  $\beta$ -glycerophosphate, 20 % Glycerol, 1 mM PMSF, 10  $\mu$ M Leupeptin, 1  $\mu$ g/ml Aprotinin and 10  $\mu$ g/ml Pepstatin and 50 mM HEPES-KOH, pH 7.5.

Immunoprecipitation of ZmSnRK2.8 was done with Anti-ZmSnRK2.8 antibody that we produced. Proteins (1.5 mg) were incubated at 4°C overnight with the antibody, loaded on a Protein-A Sepharose CL-4B resin (GE Healthcare) and incubated for 3 h with IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1 mM PMSF, 10  $\mu$ M Leupeptin, 1  $\mu$ g/ml Aprotinin and 10  $\mu$ g/ml Pepstatin). The slurry was washed 3 x 15 min with IP

buffer and the supernatant was removed prior to the in gel kinase assay.

In gel kinase assays were performed according to Fujii et al (2007). Proteins were separated on a 12.5% SDS-PAGE gel containing 0.25 mg/ml of myelin basic protein (Sigma). The gels were washed 3 x 30 min with 0.5 mM DTT, 5 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mg/ml BSA, 0.1% Triton X-100, and 25 mM Tris-HCl, pH 7.5 and proteins were renatured with 1 mM DTT, 5 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 25 mM Tris-HCl, pH 7.5, for 2 x 30 min and 16 h at 4°C prior to the reaction. The gel was incubated for 90 min at room temperature with 2 mM EGTA, 12 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 25 mM Tris-HCl, pH 7.5, supplemented with 50 mCi of [ $\gamma$ -<sup>33</sup>P]ATP and 250 nM cold ATP. Finally, the gel was washed with 5% TCA and 1% sodium pyrophosphate at least five times for 30 min and dried. Radioactivity was quantified using a Storm 820 imager (GE Healthcare).

#### Western blot

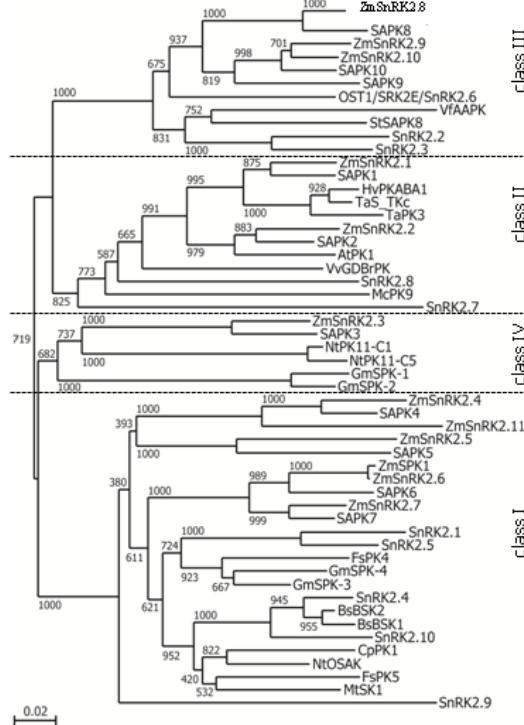
Protein levels of ZmSnRK2.8 and Rab17 were detected by western blot analysis of maize tissue. Approximately 40  $\mu$ g of total protein from several independent maize plants were loaded per lane and transferred to a nitrocellulose membrane. Homogenous protein transfer was confirmed by Ponceau red staining. Anti-ZmSnRK2.8 antibody was used to detect ZmSnRK2.8 and Anti-Rab17 was used to detect Rab17. Rab17 was used as a control for the ABA treatment.

#### Mass spectrometry analyses

Recombinant ZmSnRK2.8 was produced and subjected to in vitro auto-phosphorylation as described previously (see Protein purification and in vitro phosphorylation) without the addition of radioactive [ $\gamma$ -<sup>33</sup>P]ATP. In vitro auto-phosphorylated ZmSnRK2.8 was run on SDS-PAGE gels with 12.5% acrylamide and was subjected to trypsin digestion after separation. Subsequent MALDI-TOF analysis was used to detect phosphorylated peptides on the ZmSnRK2.8 protein sequence at the Proteomics Service of the Centro Nacional de Biotecnología (Madrid, Spain).

## Results and Discussion

Abscisic acid (ABA) is a plant hormone with important functions in numerous physiological processes and particularly in response to abiotic stress situations. The mechanism of ABA action is based on a core of only three different families of proteins: the PYR/PYL/RCAR ABA ligands, the type 2C protein phosphatases (PP2C), and the sucrose non-fermenting 1-related protein kinases (SnRK2) (Fujii et al, 2009; Ma et al, 2009; Park et al, 2009; Melcher et al, 2010; Umezawa et al, 2010; Weiner et al, 2010). It has also been suggested that this PYR/PYL/RCAR-PP2C-SnRK2 complex recently described in *Arabidopsis* may represent a conserved mechanism of ABA and drought response acquired by plants in their

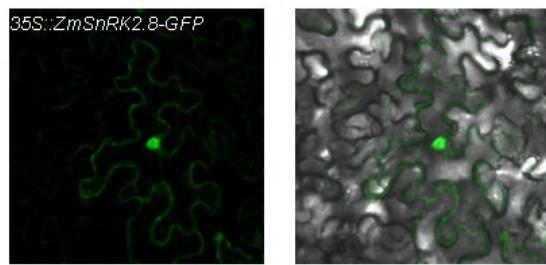


**Figure 1** - Phylogenetic neighborhood analysis of known SnRK2 proteins from different plants. ZmSnRK2.8 aligns in the ABA responsive SnRK2 subgroup next to SnRK2.6 from *Arabidopsis* and SAPK8 from rice.

adaptation to land life to adjust to the limitations in water supply in terrestrial areas (Hauser et al, 2011). Here, we characterize a maize SnRK2 cDNA that corresponds to an ABA responsive ZmSnRK2 kinase.

#### ZmSnRK2.8 is an ABA responsive SnRK2

To explore the phylogenetic relationships of ZmSnRK2.8 with other SnRK2 proteins, we built an unrooted phylogram based on the comparisons of complete amino acid sequences of different subfamily SnRK2 members from several monocots and dicots. Forty-two sequences were recovered from the NCBI database (<http://www.ncbi.nlm.nih.gov/>) by an exhaustive BLASTP search using ZmSnRK2.8 sequence as query. Eleven SnRK2 genes in the maize genome had been identified previously by Huai et al (2008) and were included in these comparisons. The resulting topography suggests that the SnRK2 kinases fall into four groups (Figure 1). Subclasses I, II and III are identical to those according to Kobayashi et al (2004). ZmSnRK2.8 kinase belongs to class III, together with the relatively well-characterized *Arabidopsis* OST1/SRK2E/SnRK2.6 (Mustilli et al, 2002; Yoshida et al, 2002), SnRK2.2 and SnRK2.3 (Fujii et al, 2007), the rice SAPK8, SAPK9 and SAPK10 (Kobayashi et al, 2004), and the *Vicia faba* AAPK (Li et al, 2000). In fact, ZmSnRK2.8 shares an identity of 82% with OST1 and 95% with SAPK8. Both genes have



**Figure 2** - ZmSnRK2.8 localization. ZmSnRK2.8 was fused in phase with GFP under a constitutive 35S promoter and transient transformation of *Nicotiana benthamiana* leaves and was performed. 35S::ZmSnRK2.8-GFP protein localizes in the nucleus and cytosol of plant cells. This localization remains unchanged under different osmotic stress treatments and also ABA application.

been implicated in the response to drought stress, in particular at the level of stomata control (Mustilli et al 2002; Kobayashi et al, 2004) and ABA signaling (Fujii et al, 2009).

The alignment also revealed a new SnRK2 subclass (Class IV). The members of this subclass include maize ZmSnRK2.3 and rice SAPK3. Unlike members in classes I, II, and III, these proteins are characterized by an atypical C-terminal acidic patch in which no Asp or Glu dominates. Furthermore, analysis by MEME revealed that ZmSnRK2.3 and SAPK3 lack motif 3, a sequence conserved in the C-terminal domains of the other SnRK2 proteins (Huai et al, 2008).

**ZmSnRK2.8 localizes in the nucleus and the cytosol**

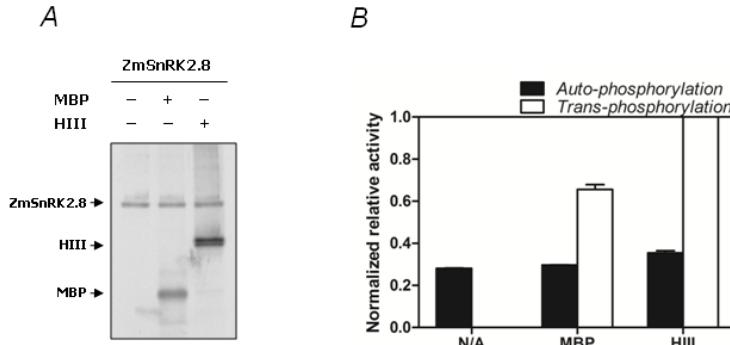
ZmSnRK2.8 shows a typical SnRK2 dual domain structure, characterized by an N-terminal catalytic domain similar to the SNF1/AMPK kinase region and a regulatory C-terminus region with a role in the kinase activation (Hardie et al, 1998) and involved in protein-protein interactions mainly in osmotic responses such as ABA responsiveness and ABA sig-

nal transduction (Kobayashi et al, 2004). The catalytic domain is highly conserved containing the ATP binding site, the protein kinase activating signature and a potential N-myristylation site previously described in wheat (Zhang et al, 2010) suggesting that this protein could be interacting with cell membranes. To determine ZmSnRK2.8 in vivo localization we performed agroinfiltration experiments in *Nicotiana benthamiana* leaves (Figure 2) using a constitutive expression construct fused in frame to GFP (35S::ZmSnRK2.8-GFP) and detected fluorescence through confocal microscopy. As shown, ZmSnRK2.8-GFP was found to localize in the nucleus and the cytosol, however, in our experiments the association of the protein with the cell-membrane system was difficult to determine. We were also able to establish that this localization is unchanged in the presence of ABA, in accordance with the findings of Ying et al (2011) and Zhang et al (2010) on onion epidermal cells and with the localization of SnRK2.6 in *Arabidopsis* (Umezawa et al, 2009). The presence of ZmSnRK2.8 in the cytoplasm and the nucleus of tobacco cells suggests that it may have similar functions in maize.

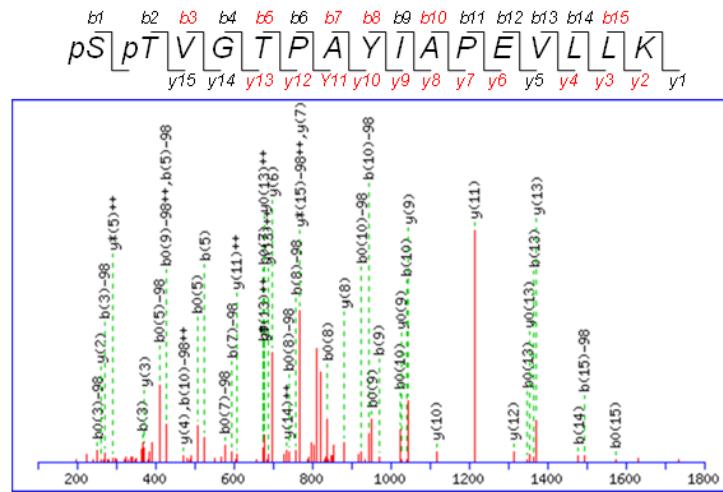
**ZmSnRK2.8 is an active kinase that auto-phosphorylates and responds to ABA**

To study the kinase activity of ZmSnRK2.8 we cloned it on a pET28a vector to allow expression in *E. coli* of a His-tagged N-terminal protein. After recombinant protein production and purification, we performed kinase assays on generic phosphorylation substrates such as myelin basic protein (MBP) and histone-III (HIII). The appearance of two bands (Figure 3) reveals that ZmSnRK2.8 has a kinase activity that is able to trans-phosphorylate both substrates and also to auto-phosphorylate. This auto-phosphorylation is confirmed by the kinase assays performed in the absence of any substrate (Lane 1). These results are in accordance with the findings of Belin et al (2006) for AtSnRK2.6/OST1.

The analysis of the in vitro phosphorylated Zm-



**Figure 3** - Recombinant His-tagged ZmSnRK2.8 protein is an active kinase with auto-phosphorylation and trans-phosphorylation activity on different generic substrates. (A) In vitro phosphorylation of different substrates with His-tagged ZmSnRK2.8 (ND) Lane 1 corresponds to kinase auto-phosphorylation and lanes 2 (MBP) and 3 (HIII) to the trans-phosphorylation activity on myelin basic protein and histone-III respectively. (B) Relative quantification of band intensity for auto-phosphorylation of ZmSnRK2.8 and trans-phosphorylation of myelin basic protein and histone-III (n=3).



**Figure 4** - Mass spectrometry (MALDI-TOF) of ZmSnRK2.8 in vitro auto-phosphorylation. ZmSnRK2.8 auto-phosphorylates in vitro at the level of the activation loop on either the S182 or T183.

SnRK2.8 protein by mass spectrometry showed auto-phosphorylation to either S182 or T183 amino acids (Figure 4). These two residues are part of the conserved catalytic domain of SnRK2 kinases and are located at the activation loop. Other authors have also described that OST1 auto-phosphorylates at this position (Belin et al, 2006; Umezawa et al, 2009). Although it has not yet been proved that SnRK2 auto-phosphorylation is an in vivo relevant phenomenon, the phosphorylation of these amino acids at the activation loop is an essential process for SnRK2 action since directed mutagenesis in those amino acids abolishes its kinase activity (Belin et al, 2006; Boudsocq et al, 2007; Ying et al, 2011).

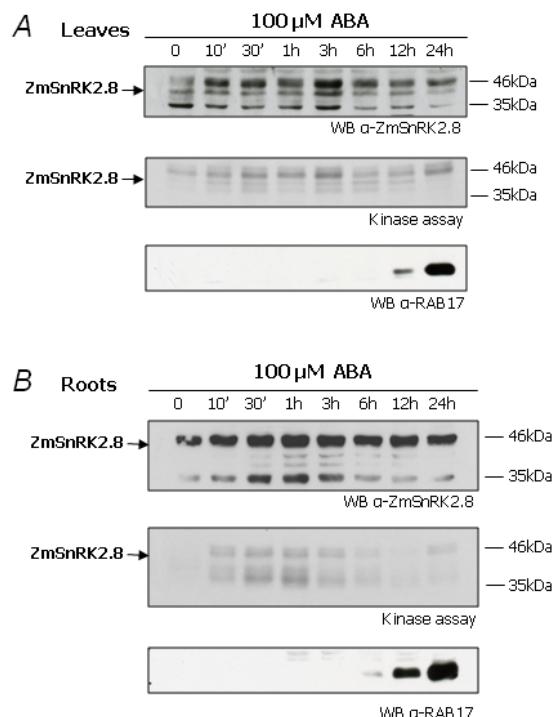
Using in gel kinase assays of immunoprecipitated ZmSnRK2s with an anti-ZmSnRK2.8 antibody that recognizes all the SnRK2 maize family we were able to detect ZmSnRK2 activity in leaves (Figure 5A) and in roots (Figure 5B) of maize seedlings under ABA treatment. In those experiments we also determined that ZmSnRK2 is constitutively present in the plant and that its in vivo activity is regulated by ABA. These results are also consistent with the AtSnRK2 response described by Boudsocq et al (2007) indicating a functional conservation of SnRK2 activity between plant species. As discussed before ZmSnRK2.8 forms part of the SnRK2 subclass III is activated by ABA and also by other osmotic stresses, suggesting that this kinase could be involved in the signal transduction machinery responding to different abiotic stresses. In this way, Ying et al (2011) reported that overexpression of ZmSnRK2.8 in Arabidopsis significantly improved the growth and development at the post-germination stage under salt-treated conditions, suggesting that other type of osmotic stresses such salinity could also be an important regulator of ZmSnRK2.8 activity.

#### **ZmSnRK2.8 interacts in vivo with ZmPP2C through the regulatory domain**

Several studies in Arabidopsis demonstrate functional and physical interactions between PP2C A-type phosphatases and SnRK2-type kinases. The first indication of such interaction came from Yoshida et al (2006) who identified a physical interaction between the ABI1 and ABI2 proteins and OST1 and more recently different groups working in arabidopsis have demonstrated that SnRK2 interactions play a key role in ABA perception and signal transduction (Yoshida et al, 2006; Fujii et al, 2009; Lee et al, 2009). However, there are no data about the conservation of this interaction in other plant species, including crops. As a first step to analyze the conservation of the ABA de-repression mechanism in maize we were interested in determining whether maize SnRK2 kinase regulatory domain interacted in vivo with PP2C phosphatases. We cloned the regulatory domain of ZmSnRK2.8 and the full sequence of a group A ZmPP2C (NM\_001154386) on BiFC vectors to determine if they could interact in planta. After agroinfiltration of both constructs on *N. benthamiana* leaves we were able to determine that these two proteins interact and that this interaction is constitutive and ABA independent (Figure 6), as has been previously reported for the arabidopsis system (Umezawa et al, 2009). This interaction of ZmPP2C with the regulatory domain of ZmSnRK2.8 confirms the conservation in maize of this important mechanism of plant response in adaptation to land habitats as was suggested by Hauser et al. (2011). In this context, the ZmPP2C physical interaction may inactivate SnRK2.8 in maize supporting the plant conservation of the ABA signaling pathway.

#### **Conclusion**

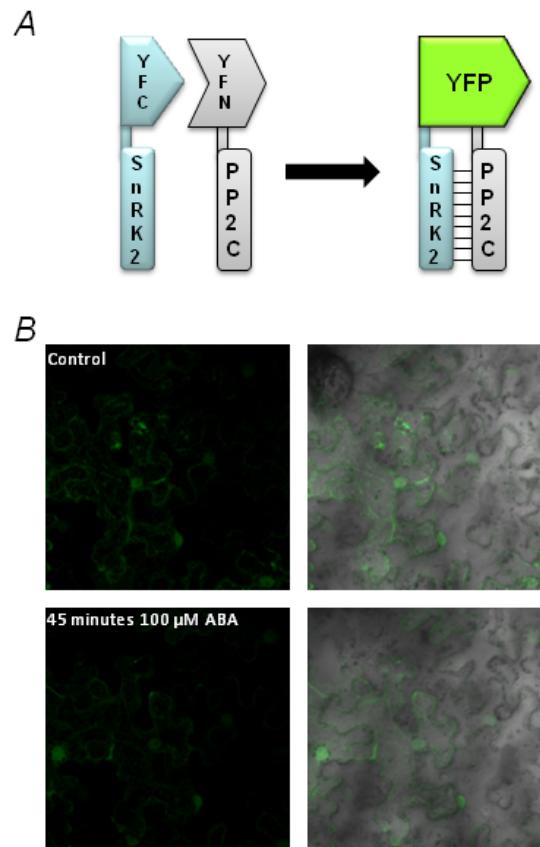
The lack of water is a threat to the production of food for millions of people. Maize is particularly susceptible to drought stress and high yielding pro-



**Figure 5** - ZmSnRK2.8 is activated by ABA at the plant level. ZmSnRK2.8 present in (A) leaves or (B) roots submitted to ABA treatment was immunoprecipitated (IP) using a polyclonal antibody. Western blots and in gel kinase assays of IP ZmSnRK2 revealed that this protein is constitutively present in maize leaves and roots but that its catalytic activity is induced by ABA. Rab17 was used as a control for the ABA treatment.

duction can only be achieved when water supply is adequate. In the near future it is expected that crop varieties continue to provide high yielding rates with reduced irrigation due to the increasing vulnerability of the water resources. In this sense, the identification of the drought-tolerance mechanism and an understanding of the way that crops in general, and maize in particular cope with water scarcity is crucial for food production. Given the relevance of ABA in regulating key processes of plant development from seed germination to stress responses, understanding the molecular components of ABA signaling including the PYR/PYL/RCAR, PP2Cs, SnRKs and its substrates remains an important challenge. In maize, apart from the cloning and characterization of the SnRK2 gene family and the functional characterization of ZmSnRK2.8 using a heterologous arabidopsis system, little is known on this important family of proteins.

The results presented here point to a conserved mechanism of plant responses to ABA and drought stress that seems to be consistent with the adaptation of plants to land habitats. We are confident that unraveling the positive mechanisms of SnRK2 activation by ABA, together with the discovery of new potential



**Figure 6** - ZmSnRK2.8 and ZmPP2C interact *in vivo* through the kinase regulatory domain. (A) Interaction between the regulatory domain of ZmSnRK2.8 and ZmPP2C was tested. YFP fluorescence can only be detected when the two transformed proteins are able to interact, thus reconstituting the YFP protein. (B) 35S::YFC-ZmSnRK2.8 [286-366] and 35S::YFN-ZmPP2C constitutively interact at the nucleus and cytosol of *Nicotiana benthamiana* infiltrated leaves. This interaction is independent of ABA and is unaffected by any osmotic stress treatment.

SnRK2 targets of agronomic interest in different plant species should provide us with the necessary tools to face the future dangers of climate change. SnRK2 engineering research will teach us not only how to engineer a biochemical or metabolic change but also will elucidate much about the drought tolerance pathways themselves.

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