Functional and molecular characterization of maize open stomata 1 protein kinase

Belmiro Vilela

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FACULTAD DE FARMACIA

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PROGRAMA DOCTORAL DE BIOTECNOLOGIA

FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF MAIZE OPEN STOMATA 1 PROTEIN KINASE

Belmiro Vilela

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UNIVERSITAD DE BARCELONA

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Memoria presentada por Belmiro Vilela para optar al grado de Doctor por la Universidad de Barcelona.

Trabajo realizado en el Departamento de Genética Molecular del Centre de Recerca en Agrigenòmica (CRAG)

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Não sou nada.
Nunca serei nada.
Não posso querer ser nada.
À parte isso, tenho em mim todos os sonhos do mundo.

- Fernando Pessoa

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Index

Abbreviations

1D PAGE:	One-dimensional gel electrophoresis
2D PAGE:	Two-dimensional gel electrophoresis
aa:	Amino acid
ABA:	Abscisic Acid
ABI:	ABA insensitive
ATP:	Adenosine triphosphate
BiFC:	Bimolecular fluorescence complementation
bp:	Base pairs
cDNA:	Complementary DNA
CK2:	Casein kinase II
E.coli:	*Escherichia coli*
GFP:	Green Fluorescent Protein
GTP:	Guanosine triphosphate
HA:	human influenza hemagglutinin
HIII:	Histone III
IP:	Immunoprecipitation
IPTG:	Isopropyl-beta-thio galactopyranoside
kDA:	Kilodalton
MALDI-TOF:	Matrix-assisted laser desorption/ionization-Time of flight
MBP:	Myelin basic protein
MS medium:	Murashige and Skoog medium

N. benthamiana:	*Nicotiana benthamiana*
OST1:	Open stomata 1
PCR:	Polymerase Chain Reaction
PEG:	Polyethylene glycol
PP2C:	Protein phosphatase 2C
PYL:	Pyrabactin like
PYR:	Pyrabactin resistance
RCAR:	Regulatory component of ABA receptor
RT-PCR:	Reverse transcription polymerase chain reaction
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SNAC1:	Stress responsive NAC 1
SnRK2:	Sucrose non-fermenting related protein kinase 2
START:	Steroid acute regulatory-related lipid transfer
TFs:	Transcription factors
WT:	Wild-type
YFP:	Yellow fluorescence protein

Figures and Tables

1 - Introduction

1 - Introduction

Plants live in a dynamic setup and are under constantly changing environmental conditions that often impose constraints to their growth, development and yield (Figure 1). Environmental stress factors such as drought (continuous water deficit), high salinity, extreme temperatures, and exposure to pathogens all directly reduce plant survival and agricultural productivity.

Drought stress alone can account for a significant percentage of average yield losses that are reported each year. This is not surprising since over 50% of the Earth's surface area, including the vast majority of agricultural lands, is vulnerable to drought (Hubbard *et al.* 2010). In general, the primary response of plants to water deficit is the inhibition of shoot growth, allowing cellular essential solutes to be diverted from growth requirements to stress-related functions, decreasing plant size and ultimately limiting yield potential. If we consider the global climate changes that are drastically reducing the water availability, exploiting the yield potential and maintaining yield stability of crops in water-limited environments are urgent tasks that must be undertaken in order to guarantee food supply for the increasing world population. To undertake this task, we require a better understanding of the efficiency with which plants draw water from the surrounding soil, the water-retaining capacity within plant tissues or cells, the control of water loss from transpiration, and the developmental adaptations necessary to avoid seasonal water shortage, especially during flowering (Bruce *et al.* 2002).

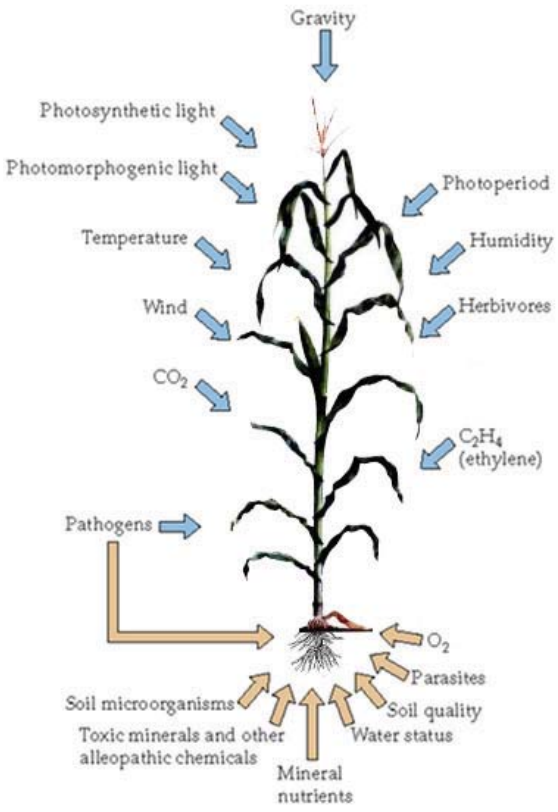

Figure 1 - Environmental challenges. Plants live in a dynamic environment with heterogeneous field conditions and must continuously cope with multiple abiotic and biotic constraints during their life cycle in order to optimize growth. Adapted from Buchanan *et al.* (2005)

Plant adaptive strategies to stress are coordinated and fine-tuned by adjusting growth, development and cellular and molecular activities. Thus, the physiological responses to drought include stomatal closure, decreased photosynthetic activity, altered cell wall elasticity and even the generation of toxic metabolites causing plant death (Ahuja *et al.* 2010). At the molecular level, plants utilize multiple chains of signalling molecules, namely abscisic acid (ABA), which regulate different sets of stress-responsive genes to initiate the synthesis of various classes of proteins, including transcription factors (TFs), enzymes, and molecular chaperones (Valliyodan and Nguyen 2006).

Tremendous efforts have been devoted to identifying key regulators in plant drought response through genetic, molecular, and biochemical studies using, in most

cases, the model species *Arabidopsis thaliana* (Yang *et al.* 2010). However, applicable knowledge relevant to crop cultivation remains scarce.

Maize has a shallow root system which makes it very demanding on irrigation. For this crop, the period during pollination and early grain filling is the most sensitive to water stress as compared with preflowering and late grain-filling growth stages. Since it is an out-crossing species, pollen must move from the anthers at the top of the plant to the exposed silks. In a drought environment pollen can arrive after it has desiccated, when silks have withered or senesced or after ovaries have exhausted their starch reserves (Bruce *et al.* 2002) which drastically reduces corn yield.

1.1 - Abscisic Acid (ABA)

The phytohormone abscisic acid (ABA) plays a central role in plant development. ABA inhibits growth and development under unfavourable environmental conditions, protecting plants against stresses such as drought, salinity, cold, and pathogen exposure (Figure 2).

ABA regulates important aspects of plant growth and development such as embryo and seed development, promotion of seed desiccation tolerance and dormancy, germination, seedling establishment, vegetative development including heterophylly as well as cell growth and reproduction (Cutler *et al.* 2010)

In vegetative tissues, a limiting water supply leads to an immediate hydraulic signal in plants that triggers ABA biosynthesis, enzymatic release from inactive glucose esters, and local increases in transportation (Raghavendra *et al.* 2010). Elevated ABA levels in turn reprogram plants to survive adverse conditions. ABA protective measures

include closure of stomata, synthesis of osmoprotective substances and the transcriptional regulation of several different genes.

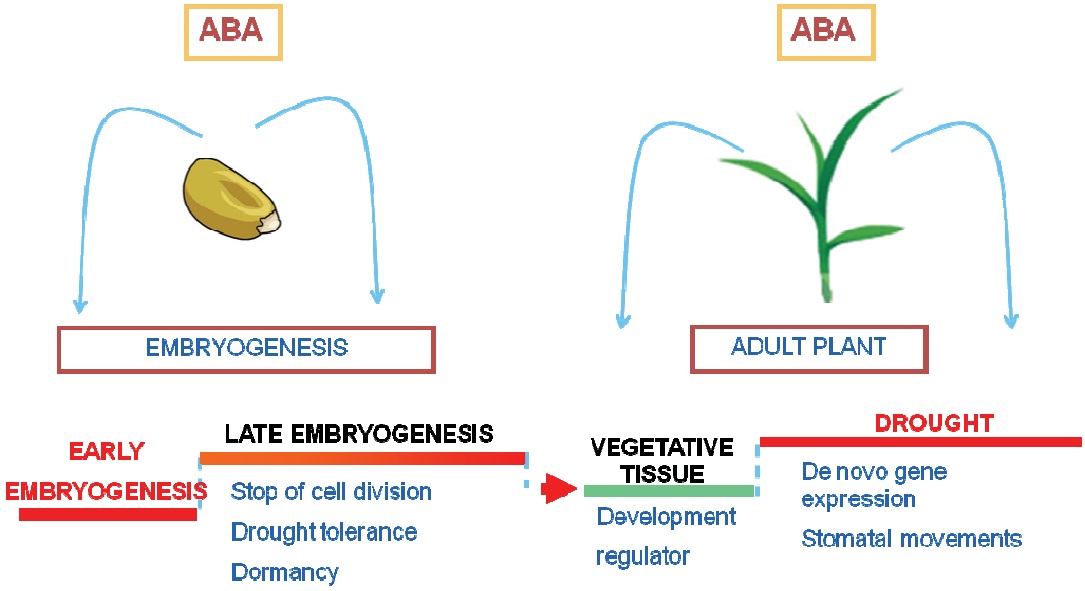

Figure 2 - ABA responses. ABA is a plant hormone that has been known for a long time at the seed level in processes of seed maturation, stop of cell division, acquisition of drought tolerance and seed dormancy. It is also very important in the adult plant as a development regulator and most importantly in stress situations that limit vegetative growth in processes such as stomatal closure and differential gene expression.

In *Arabidopsis* plants, ABA regulates nearly 10% of the protein coding genes, a much higher percentage than other plant hormones. Among other, ABA induces genes that encode proteins associated with stress response and tolerance, including late embryogenesis abundant proteins, TFs, protein kinases and phosphatases, transporters, enzymes involved in osmoprotectant synthesis, phospholipid signalling, fatty acid metabolism, cellular metabolism, carbohydrate metabolism, and secondary metabolism. Conversely ABA down-regulates genes encoding for protein involved in growth and development (Fujita *et al.* 2011).

Even though there is an ample comprehension of the effects of ABA in plant responses to stress or as a developmental factor and multiple proteins were implicated in its signal transduction pathways (namely kinases and phosphatases), the knowledge of ABA perception remained elusive for several decades.

1.2 - ABA perception

In recent years, several proteins have been described that could function as membrane or intracellular ABA receptors (Guo *et al.* 2011). The first ABA binding protein was isolated in barley and its *Arabidopsis* homolog, Flowering time Control protein A (FCA) was proposed to be a ABA receptor (Razem *et al.* 2006). These findings were later retracted by the same authors. The second proposed ABA receptor was the Chloroplastic Magnesium Protoporphyrin-IX Chelatase H subunit (ChlH)/ Abscisic Acid Receptor (ABAR) (Shen *et al.* 2006). Mutations on this gene induce phenotypes consistent with ABAR playing a role in ABA responses but the molecular explanation for these findings is still missing. The third proposed ABA receptor isolated was a G-protein coupled receptor (GCR2), (Liu *et al.* 2007) but there is still some dispute to whether it is involved in ABA signalling.

After all these false starts that brought more questions than answers to ABA perception, two seminal works on a soluble protein that is able to bind ABA (Ma *et al.* 2009, Park *et al.* 2009) made possible the construction of a functional model for ABA signal transduction.

The current working model of ABA signalling includes three core components, the pyrabactin resistance/pyrabactin-like/regulatory component of ABA receptor (PYR/PYL/RCAR), the clade A type 2C protein phosphatases (PP2C), and the ABA-

activated Sucrose non-fermenting-1 related protein kinases 2 (SnRK2) (Ma *et al.* 2009, Park *et al.* 2009). These three protein types are necessary and sufficient to mediate an ABA triggered model signalling cascade *in vitro* (Fujii *et al.* 2009).

The PYR/PYL/RCAR receptor family consists of 14 proteins in *Arabidopsis*, with at least 13 members capable of participating in binding to ABA (Klingler *et al.* 2010). They belong to the steroidogenic acute regulatory-related lipid transfer (START) domain superfamily of soluble ligand-binding proteins (Iyer *et al.* 2001). These proteins are characterized by a structural scaffold that can accommodate a large spectrum of hydrophobic ligands, such as lipids, antibiotics and hormones (Radauer *et al.* 2008).

In the absence of ABA, the PYR/PYL/RCAR receptor exists as an asymmetric dimer which forms a large pocket that in the absence of ABA remains open to the solvent (Klingler *et al.* 2010, Joshi-Saha *et al.* 2011).

The binding of ABA leads to conformation changes that allow the dimer to assume a perfect symmetry, resulting in a more compact structure with a biconcave disc shape that covers the hormone from the solvent. ABA binding to both protomers causes the homodimer to dissociate into monomers and each monomer then binds a PP2C protein, forming a hormone-heterodimer complex. In this complex, the hormone is buried completely by PYR/PYL/RCAR residues and interacts only indirectly with PP2C (Melcher *et al.* 2009, Miyazono *et al.* 2009, Nishimura *et al.* 2009, Santiago *et al.* 2009, Yin *et al.* 2009, Dupeux *et al.* 2011)

The PP2C family consists of 76 *Arabidopsis* genes. Among them, group A contains most of the PP2Cs that are associated with ABA signalling (Schweighofer *et al.* 2004). In fact, it has long been established that clade A PP2C phosphatases are key negative regulators of ABA. Currently, genetic evidence indicates that at least six

Arabidopsis PP2Cs, namely, ABI1, ABI2, PP2CA/AHG3, AHG1, HAB1, and HAB2, act as negative regulators of ABA signalling (Leung *et al.* 1994, Meyer *et al.* 1994, Rodriguez *et al.* 1998, Merlot *et al.* 2001, Leonhardt *et al.* 2004). Specifically, HAB1, ABI1, ABI2, and PP2CA have been shown to affect both seed and vegetative responses to ABA while the others appear to play an essential role in seed development, germination and post-germination growth (Rubio *et al.* 2009). Negative regulatory roles for PP2C in ABA signalling have been demonstrated in some other plant species, suggesting that PP2C functions are well conserved (González-García *et al.* 2003, Komatsu *et al.* 2009, Tougane *et al.* 2010). Group A PP2Cs are functionally redundant at the molecular level, but they have distinctive roles in different tissues and organs, as indicated by tissue-specific expression patterns (Umezawa *et al.* 2010).

Among other *in vivo* targets such as SnRK3 kinases (Ohta *et al.* 2003), potassium transporters (Chérel *et al.* 2002), and chromatin remodelling complexes (Saez *et al.* 2008), clade A PP2C regulate ABA signalling mostly through its binding with SnRK2 kinases (Belin *et al.* 2006, Yoshida *et al.* 2006, Vlad *et al.* 2009, Soon *et al.* 2012), the third component of the ABA signalling model.

Clade A PP2C phosphatases constitutively bind to SnRK2 kinases at the C-terminal region and transiently to the kinase domain (Soon *et al.* 2012). In the absence of ABA, PP2C inactivate SnRK2 both by this physical interaction and the dephosphorylation of the kinase's activation loop at S171 and S175 (Belin *et al.* 2006, Yoshida *et al.* 2006, Vlad *et al.* 2009, Vlad *et al.* 2010, Soon *et al.* 2012). In the presence of ABA-bound PYL/PYR/RCAR, PP2C activity is inhibited and there is a release of the SnRK2 activation loop for autophosphorylation or phosphorylation by other kinases

without a total dissociation from the phosphatase. SnRK2 then becomes active and may transduce the ABA signal through this double repression system (Figure 3).

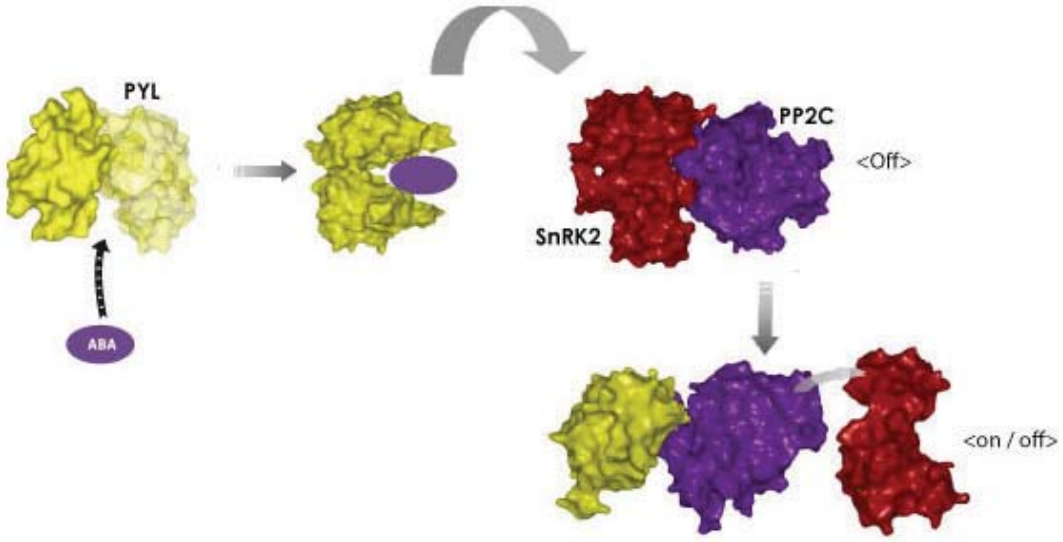

Figure 3 - Early mechanisms of ABA signalling. The binding of ABA to PYR/PYL/RCAR triggers the pathway by inducing structural changes in the receptors that allows them to sequester members of the clade A negative regulating protein phosphatase 2Cs (PP2Cs). This in turn liberates the class III ABA-activated Snf1-related kinases (SnRK2s) to phosphorylate various targets. Adapted from Leung (2012).

1.3 - Sucrose non-fermenting-1 related protein kinases 2 (SnRK2)

The SnRK family is characterized by a catalytic domain that is highly homolog to yeast sucrose non-fermenting1 (SNF1) kinase and AMP-activated protein kinase (AMPK) in mammals (Halford and Hardie 1998). Members of this family are divided into three subfamilies named SnRK1, SnRK2 and SnRK3. SnRK2 and SnRK3 are plant specific serine/threonine kinases.

SnRK2s have been classed into three groups; group 1 comprises kinases not activated by ABA, group 2 comprises kinases not activated or activated very weakly by ABA, and group 3 comprises kinases strongly activated by ABA (Figure 4).

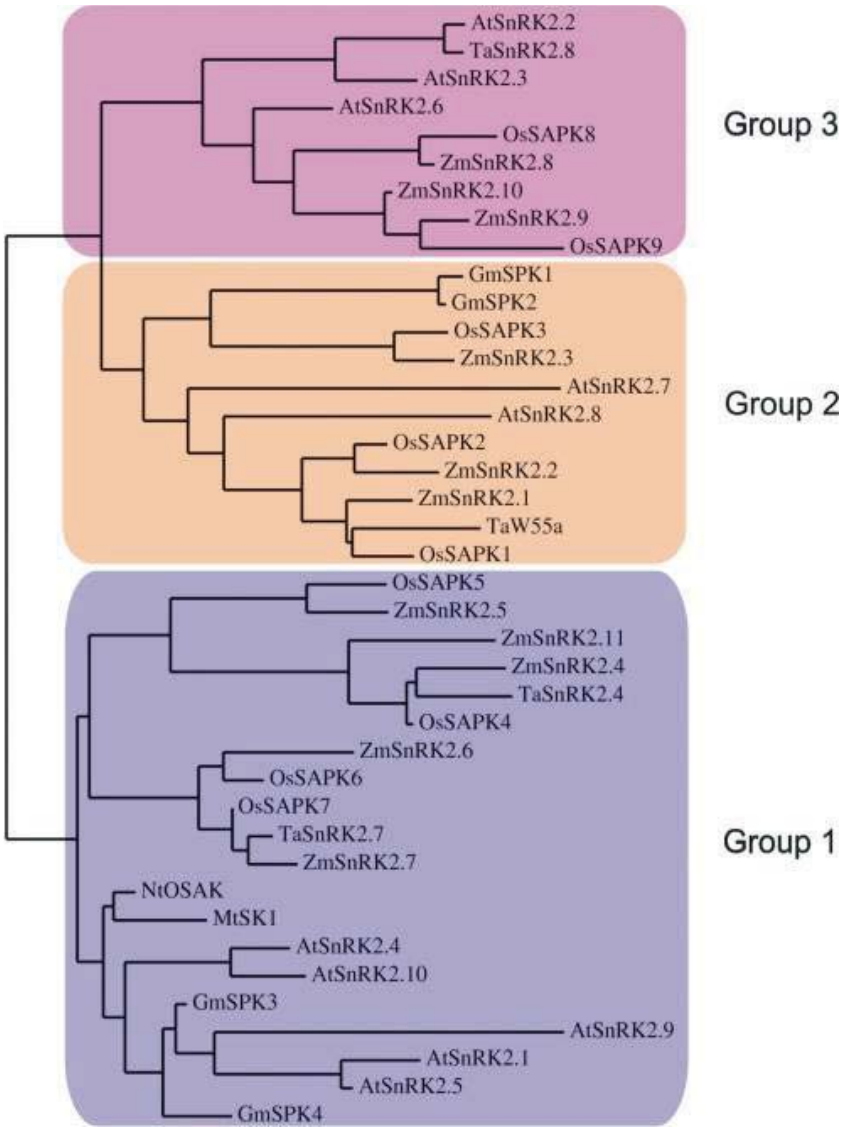

Figure 4 - Phylogenetic tree of higher plant SnRK2s. Group 1— ABA-independent kinases, group 2— kinases not dependent or weakly dependent on ABA, group 3—ABA-dependent kinases. Extracted from Kulik *et al.* (2011)

SnRK2 proteins are formed of a N-terminal catalytic domain highly homolog to SNF1 and AMPK in which the ATP binding domain and the activation loop is present, and a regulatory C-terminal domain containing stretches of acidic amino acids, either Glu (group 1) or Asp (groups 2 and 3) (Kulik *et al.* 2011). The regulatory domain is further subdivided in two subdomains: the SnRK2 domain, the characteristic feature of the SnRK2 family and that is needed for activation by osmotic stress and a ABA box

(Figure 5) that is specific to the ABA-dependent SnRK2 activation (Kobayashi *et al.* 2004, Belin *et al.* 2006, Yoshida *et al.* 2006).

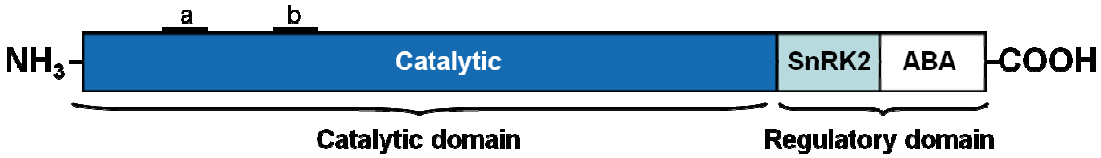

Figure 5 - Schematic representation of SnRK2. SnRK2 proteins are characterized by a N-terminal catalytic domain containing the **(a)** ATP binding domain and the **(b)** activation loop, and a regulatory domain that is divided into the SnRK2 and the ABA subdomains.

Members of the SnRK2 subfamily have been characterized in different plant species (Li *et al.* 2000, Mustilli *et al.* 2002, Yoshida *et al.* 2002). The first SnRK2 gene, *PKABA* from wheat, is up-regulated by drought and ABA in seeds and vegetative tissues (Anderberg and Walker-Simmons 1992). The homolog AAPK from faba bean is essential in relaying the ABA signal in stomatal closure (Li *et al.* 2000). Ten SnRK2 genes exist in the *Arabidopsis* and rice genomes. In *Arabidopsis*, except SnRK2.9, the kinase activity of each member of the family is activated by hyperosmotic stress (Boudsocq *et al.* 2004) with SnRK2.2, SnRK2.3 and SnRK2.6/OST1/SRK2E, also strongly activated by ABA (Boudsocq *et al.* 2004). A similar situation occurs in rice, in that the activities of three of the ten SnRK2 homologs (called SAPKs) are also stimulated by ABA (Kobayashi *et al.* 2004). Studies of mutants deficient in SnRK2.2 and SnRK2.3 activities showed that these kinases are required for ABA-mediated seed germination, dormancy and seedling growth but have minor roles in stomatal control (Fujii *et al.* 2007). Conversely, SnRK2.6/OST1, the ortholog of AAPK, is mainly involved in ABA-mediated stomatal closure in response to humidity decrease but a minor role during seed germination (Mustilli *et al.* 2002, Yoshida *et al.* 2006, Fujii and Zhu 2009, Zheng *et al.* 2010). In

maize, eleven *SnRK2* genes have been reported (Huai *et al.* 2008), although only functional data are available for one, ZmSAPK8/ZmOST1. This kinase, with a primary sequence highly homologous to that of the *Arabidopsis* OST1, is required in diverse stress signal transduction pathways, principally in drought and salt tolerance responses (Huai *et al.* 2008, Ying *et al.* 2011).

SnRK2s are primarily regulated at the posttranslational level, by phosphorylation. However, expression of genes encoding these kinases is also regulated in response to changes of the plant environment. The current model of ABA signalling reconstructed *in vitro* suggests that, in the absence of ABA, the clade A PP2Cs inhibit SnRK2 activity by binding to its kinase catalytic site as well as to the ABA box (Fujii *et al.* 2009, Soon *et al.* 2012). As explained above, ABA triggers the pathway by binding to the PYL/PYR/RCAR receptor, and the changes in its conformation cause the insertion of its "proline gate" domain into the catalytic site of the PP2C phosphatase. This frees the catalytic domain of SnRK2 without dissociating the two proteins completely, as the kinase remains tethered by its ABA box to the phosphatase (Soon *et al.* 2012). The attached protein kinase–phosphatase pair is thought to provide a rapidly reversible phospho-relay in regulating the on/off state of the ABA signalling pathway (Joshi-Saha *et al.* 2011). *In vivo*, however, the association of the PP2C and SnRK2 (particularly OST1) can also be labile, because the complex can only be recovered after treatment of the total soluble protein extract from *Arabidopsis* by chemical cross-linkers (Vlad *et al.* 2009) or transient over-expression of the two proteins in tobacco (Nishimura *et al.* 2009, Hubbard *et al.* 2010).

1.4 - SnRK2 downstream targets

Over the years, multiple groups have tried to identify the proteins that act as targets and are regulated by SnRK2 in response to ABA. Being direct substrates of ABA response, these proteins could be of tremendous biotechnological application for better crop yield with reduced water use.

So far most of the downstream targets for SnRK2 have been described for *Arabidopsis thaliana*. In particular for OST1, which is implicated in stomata control, multiple substrates have been described.

OST1 activates the slow anion channel SLAC1 to trigger plasma membrane depolarization necessary to initiate stomatal closing (Geiger *et al.* 2009, Lee *et al.* 2009, Vahisalu *et al.* 2010); it possibly inhibits the inward-rectifying K+ channel KAT1 (Sato *et al.* 2009); it promotes the generation reactive oxygen species (ROS) that leads to Ca^{2+} spikes via the respiratory burst oxidase homolog F (RbohF) (Sirichandra *et al.* 2009) and it activates the bZIP-class of transcription factors that include ABI5 (Furihata *et al.* 2006, Nakashima *et al.* 2009) and the ABA-responsive element binding factors, ABF2 and ABF3 (Fujii and Zhu 2009, Sirichandra *et al.* 2010). ABFs 2, 3 and 4 together are thought to regulate about 80% of the global ABA-transcriptome (Johnson *et al.* 2002, Furihata *et al.* 2006, Sirichandra *et al.* 2010, Yoshida *et al.* 2010).

In comparison to ion transport across membranes (Blatt 2000, Roelfsema and Hedrich 2005, Joshi-Saha *et al.* 2011), much less is known about the mechanisms of transcriptional control by ABA in guard cell signalling (Leonhardt *et al.* 2004, Wang *et al.* 2011).

Despite the apparent importance of the three ABFs as global regulators of the ABA transcriptome, the *Arabidopsis abf2/abf3/abf4* triple mutant is normal in

transpiration (Yoshida *et al.* 2010). This suggests that the ABF class of transcription factors is not critical for stomatal functions and that OST1 may have additional transcriptional targets (Shinozaki and Yamaguchi-Shinozaki 2007, Fujita *et al.* 2011, Golldack *et al.* 2011, Xue *et al.* 2011b). Various other TFs belonging to several classes including AP2/ERF, MYB, NAC, and HD-ZF, have been reported to engage in ABA-mediated gene expression and should be checked for regulation by phosphorylation in general and SnRK2 activity in particular.

Sirichandra *et al.* (2010) defined the phosphorylation site preferences for OST1 using a combinatorial peptide array to predict targets at the genome scale in *Arabidopsis* guard cells. These authors confirmed that OST1 preferentially phosphorylates the LXRXX(S/T) motif and searched the *Arabidopsis* databases for potential substrates (Table 1). Although several of the previously described targets appear on this list, other are conspicuously absent, indicating that OST1 can phosphorylate proteins both *in vitro* and *in vivo* at sub-optimal sites.

Another study using this peptide array strategy reported that CDPK, SnRK1, SnRK2 and SnRK3 kinases may have related phosphorylation preferences, suggesting that they may share common substrates (Vlad *et al.* 2008). In fact, CDPKs have been implicated alongside OST1 in the regulation of the abovementioned SLAC1 (Geiger *et al.* 2010). In addition, these two kinase types are both known to be downstream targets of PP2C phosphatases and OST1, through the activation of the AtrbohF NADPH oxidase starts the calcium dependent ABA induced signal transduction mechanisms in guard cells which have repercussions at the level of CDPK regulation (Kim *et al.* 2010).

Rank	E-value[a]	AGI	Description	Peptide	p-value[b]
ABA-responsive element binding factors and transcription factors					
18	11	At3g19290	bZIP ABRE Binding Factor ABF4 (AREB2)	LQRQGSLTLP	8,10E-07
	-			LRRTLTGPW*	3,10E-05
	-			LARQSSVYSL	4,40E-05
18	11	At1g45249	bZIP ABRE Binding Factor ABF2 (AREB1)	LQRQGSLTLP	8,70E-07
	-			LTRQGSIYSL	5,60E-06
21	12	At4g34000	bZIP ABRE Binding Factor ABF3 (DPBF5)	LQRQGSLTLP	8,80E-07
	-			LRRTLTGPW*	3,10E-05
	-			LTRQNSVFSL	3,10E-05
29	17	At3g56850	bZIP ABRE Binding Factor AREB3 (DPBF3)	LSRQGSLTLP	1,80E-06
	-			LNRQSSLYSL	4,10E-05
	-			LRRTSSAPF*	6,00E-05
29	17	At2g41070	bZIP Enhanced EM Level (DPBF4)	LVRQGSLTLP	2,10E-06
	-			LTRQNSLYSL	6,70E-06
	-			LRRTNSASL*	4,30E-05
33	18	At1g49720	bZIP ABRE Binding Factor ABF1	LQRQGSLTLP	1,50E-06
	-			LARQSSLYSL	8,80E-06
	-			LRRTLTGPW*	4,90E-05
	-			LERQQTLGEM	9,60E-05
41	23	At3g54320	Transcription factor WRINKLE 1	LRRQSSGFSR	2,10E-06
Dehydrins					
3	1,9	At1g20440	Dehydrin COR47	LHRSNSSSSS	2,40E-07
5	2,4	At1g20450	Dehydrin ERD10/LTI45	LHRSNSSSSS	3,00E-07
9	4,5	At5g66400	Dehydrin RAB18	LHRSGSGSSS	7,80E-07
16	8,9	At2g21490	Dehydrin protein	LRRSGSSSSS	1,60E-06
28	15	At3g50980	Dehydrin protein	LHRSGSSSSS	3,90E-06
Glycine-rich proteins with unknown functions					
1	1,6	At3g09070	Glycine-rich protein	LRRTKSFSAS	7,60E-08
5	2,4	At2g38070	Glycine-rich protein	LRRTKSFSAS	1,20E-07
8	4,3	At5g01170	Glycine-rich protein	LRRTKSFSAK	2,40E-07
Metal binding proteins					
4	2,3	At4g27590	Copper-binding protein	LIRTSSFTWK	4,80E-07
12	6	At2g02835	Zinc ion binding protein	LWRANTISIV	9,90E-07
15	8,8	At1g55915	Zinc ion binding protein	LSRQPSLSFL	6,90E-07
Oligosaccharide metabolism					
10	4,9	At4g19760	Glycosyl hydrolase family 18 protein	LSRAGSFSF*	4,30E-07
36	20	At4g19750	Glycosyl hydrolase protein	LSRAGSFSLT	1,80E-06
41	23	At3g22250	UDP-glucosyl transferase family protein	LERTKSLRWI	1,60E-06
Translational control					
24	13	At4g23620	50S ribosomal protein	LRRAKTLPKT	1,60E-06
41	23	At5g20290	40S ribosomal protein S8 (RPS8A)	LVRTKTLVKS	3,40E-06
41	23	At1g66890	Similar to 50S ribosomal protein-related	LRRRKTLRLL	4,90E-06
Reversible protein phosphorylation					
29	17	At4g08480	MAPKKK9	LLRQGSFGSV	7,10E-07
41	23	At5g66080	PP2C protein	LSRASSLKTP	1,90E-06
Miscellaneous					
7	4,1	At5g24880	Calmodulin-binding protein-related	LSRTKSLGRK	3,00E-07
	-			LLRRRSFDRP	1,10E-05
11	5,3	At1g76690	12-oxophytodienoate reductase OPR2	LTRQKSYGSV	4,50E-07
17	9,2	At5g59200	Pentatricopeptide repeat-containing protein	LSRRKTLISV	5,00E-07
18	11	At3g49150	F-box family protein	LRRTLSLRSL	5,80E-07
	-			LKRSLSSKTL	7,10E-05
21	12	At3g45243	ECA1 gametogenesis protein	LARAPSLTLA	3,50E-06
24	13	At5g10300	Hydrolase, alpha/beta fold protein	LHRQGSFFTE	1,60E-06
33	18	At5g19520	Ion channel domain-containing protein	LVRRKSLSRS	7,90E-07
39	21	At1g10070	Amino acid transaminase, ATBCAT-2	LSRAKSRGFS	1,90E-06
41	23	At1g04540	C2 domain-containing protein	LRRTKSDTSS	1,20E-06
Unknown functions					
2	1,8	At4g40020	Unknown protein	LVRRKSLSFS	9,20E-08
13	6,4	At2g31560	Unknown protein	LTRAKSLTDD	1,00E-06
14	8,1	At5g61710	Unknown protein	LRRRRTSNTR	1,70E-06
21	12	At4g26950	Unknown protein	LRRSRSSSSS	2,70E-06
24	13	At3g12870	Unknown protein	LRRAKSLRVE	2,00E-06
24	13	At2g43340	Unknown protein	LKRTKSLTDD	2,20E-06
29	17	At4g19970	Unknown protein	LTRSKSISFR	7,60E-07
33	18	At5g20900	Unknown protein	LNRAPSFSST	3,10E-06
36	20	At5g06280	Unknown protein	LRRTKSISNM	4,20E-06
36	20	At5g06280	Unknown protein	LRRTKSISNM	4,20E-06
39	21	At1g22110	Unknown protein	LSRTSSSSSS	2,30E-06
41	23	At3g27320	Unknown protein	LSRRNSLGSS	1,60E-06
48	24	At1g68330	Unknown protein	LRRSSSLSSS	2,90E-06

[a]The E-value of a sequence in a database is the expected number of sequences in a random database of the same size that would match the motif as well as the sequence does. Results are displayed for E-value ≤25.
[b]The position p-value is the probability of a single random subsequence of the length of the motif scoring at least as well as the observed match. Only peptides with position p-value ≤0.0001 are displayed.
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Table 1 - Putative OST1 substrates identified in *Arabidopsis*. Extracted from (Sirichandra *et al.* 2010)

Since nine clade A PP2Cs and 14 PYL/PYR/RCAR proteins are encoded by the *Arabidopsis* genome, 126 PYR/PYL/RCAR-PP2C heterodimers could potentially form. In addition, these heterodimers can interact with three ABA-responsive SnRK2s, totalling 378 receptor-kinase combinations. If we also take into consideration the multiple substrates for ABA-activated SnRK2s, the image of a highly sophisticated network of ABA control appears (Figure 6).

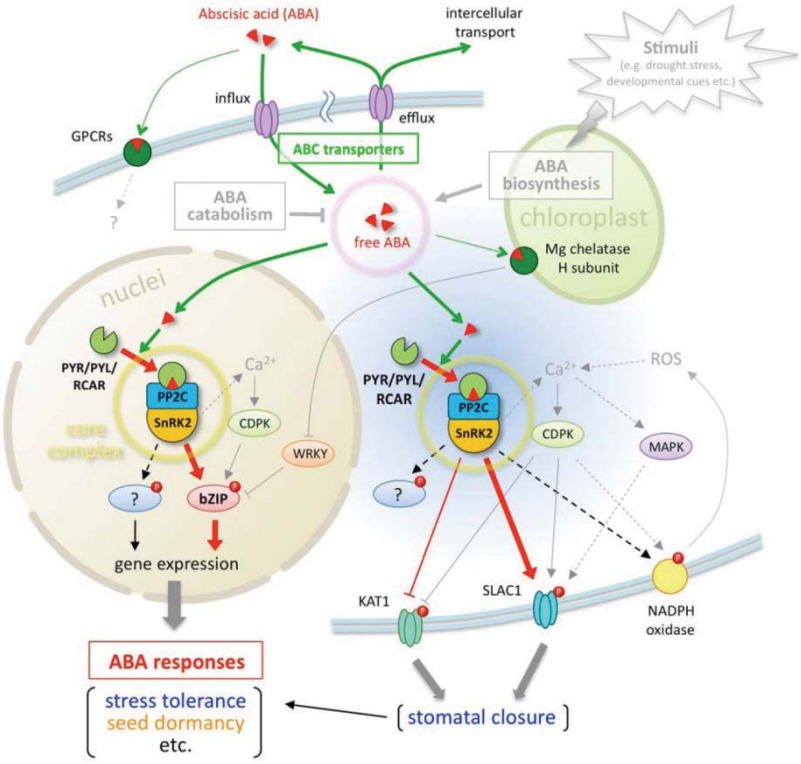

Figure 6 - ABA signalling network. PYR/PYL/RCAR, PP2C and SnRK2 form a core signalling complex (yellow circle). In the nucleus, the core complex directly regulates ABA-responsive gene expression by phosphorylation of AREB/ABF-type transcription factors. In the cytoplasm, the core complex can access the plasma membrane and phosphorylate anion channels (SLAC1) or potassium channels (KAT1) to induce stomatal closure in response to ABA. In contrast, the endogenous ABA level is a major determinant of ABA sensing that is maintained by ABA biosynthesis, catabolism or transport. ABA movements are indicated by green lines and arrows, and major signalling pathways are indicated by red lines and arrows. Dotted lines indicate indirect or unconfirmed connections. Extracted from Umezawa *et al.* (2010)

Objectives

2 - Objectives

Maize is widely cultivated throughout the world, and a greater weight of maize is produced each year than any other grain. Since its root system is generally shallow, maize productivity is very dependent on rain or irrigation especially at the time of silk emergence, when the flowers are ready for pollination. Its sensibility to drought makes maize a crop extremely suitable for improvement in productivity and quality through a better adaptation to environmental conditions, including water use efficiency.

The main purpose of this PhD project was to dissect the molecular mechanisms of drought and ABA signalling focusing on the role of protein phosphorylation. Our primary objective was to functionally characterize maize open stomata 1 (ZmOST1/ZmSnRK2.8) protein and its substrates which have been previously shown to be implicated in signalling events related to osmotic-stress responses in maize.

We subdivided the work in three chapters with specific objectives.

Chapter 1 - ZmSnRK2.8 responds to ABA through the SnRK2-PP2C complex.

The main objective of Chapter 1 was to perform a biochemical analysis of maize OST1 trying to make, whenever possible, a parallel with what is known for the *Arabidopsis* system.

To this purpose we cloned a maize cDNA with high homology to OST1, we determined its subcellular localization in tobacco leaves fused to GFP, we evaluated its catalytic ability to auto and trans-phosphorylate different substrates by both *in vitro* and in gel phosphorylation assays. By MALDI-TOF analysis we determined the residues

on the ZmOST1 kinase that are targets for autophosphorylation and finally we determined the capacity of ZmOST1 to interact with a member of the PP2C phosphatase family by bimolecular fluorescence complementation.

Chapter 2 - The maize OST1 kinase homolog regulates the phosphorylation status of the maize SNAC1-type transcription factor in abiotic stress response.

The main objective of Chapter 2 was to functionally characterize ZmOST1 in an heterologous *Arabidopsis* system, and to describe a novel transcription factor that is a substrate of ZmOST1 (ZmSNAC1).

With this objective in mind we first followed up on the biochemical characterization of ZmOST1 presented in Chapter 1 by profiling its activity by in gel kinase assays during embryogenesis and in vegetative tissues after different treatments. We then used complementation lines overexpressing ZmOST1 under a constitutive 35S promoter in the *Arabidopsis ost1* mutant background to determine whether the maize kinase is a functional homolog to the *Arabidopsis* gene. We finally present results by yeast two-hybrid, *in vitro* pull down assay and bimolecular fluorescence complementation that ZmOST1 co-localizes and interacts *in vivo* with a SNAC transcription factor. We also mapped this interaction to different domains in the kinase and determined the ability of ZmOST1 to phosphorylate the ZmSNAC1 transcription factor.

Chapter 3 - Phosphorylation by CK2 regulates the activity and enhances the proteasome degradation of ZmOST1 kinase during ABA response.

The main objective of Chapter 3 was to characterize ZmOST1 regulation. We describe the first kinase that acts upstream of ZmOST1 and explore the ways in which this phosphorylation regulates ZmOST1 activity and protein stability.

To achieve this objective we started by profiling ZmOST1 phosphorylation in maize and also in the *Arabidopsis* heterolog system described in Chapter 2. By different approaches, namely the use of *Arabidopsis* mutants, we were able to determine one kinase that constitutively phosphorylates ZmOST1. We characterized and mutagenized the target residues for this phosphorylation and tried to determine its biochemical and biological significance by 2D-PAGE and co-localization experiments. We finish this chapter characterizing transgenic lines overexpressing the wild type ZmOST1 and a mutagenized version for stomatal closure under ABA treatment.

3 - Results

3 - Results

3.1 - Chapter 1 - ZmSnRK2.8 responds to ABA through the SnRK2-PP2C complex

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Summary

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.

In this Chapter 1, we study drought responses in maize by analyzing the mechanism of ZmSnRK2.8/ZmOST1 in ABA signalling, trying to establish relevant analogies with other plant species. We cloned a maize cDNA with high homology to OST1, we determined its subcellular localization in tobacco leaves fused to GFP, we

evaluated its catalytic ability to auto and trans-phosphorylate different generic substrates by both *in vitro* and in gel phosphorylation assays. By MALDI-TOF analysis we determined the residues on the ZmOST1 kinase that are targets for autophosphorylation and finally we determined the capacity of ZmOST1 to interact with a member of the PP2C phosphatase family by bimolecular fluorescence complementation (BiFC).

We found that ZmSnRK2.8 is a very closely related protein to *Arabidopsis* OST1 (open stomata 1) kinase. It has a nuclear and cytosolic subcellular localization and a marked catalytic activity on generic substrates. ZmSnRK2.8 is also 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 *in vivo* and interacts with PP2C phosphatases in a constitutive, ABA independent manner.

Together, the data presented in Chapter 1 suggest a conserved mechanism of plant responses to ABA and drought stress in maize and point to the potential use of this kinase in improvement programs of drought tolerance in crops.

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 diverse signalling cascades and many have already been linked to ABA and/or osmotic stress signalling, either affecting stomata function and/or gene expression (Sirichandra *et al.* 2009, Hubbard *et al.* 2010). Recently, a negative regulatory pathway that controls ABA signalling by inhibiting type 2C protein phosphatases (PP2C) through the direct interaction with a newly described type of ABA receptor (Ma *et al.* 2009, Melcher *et al.* 2009, Park *et al.* 2009, 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 signalling 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, 10 *SnRK2s* designated *SAPK1-10*, were identified in rice of which all are activated by hyperosmotic stress, and *SAPK8-10* is 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 these 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 (Hubbard *et al.* 2010, Hauser *et al.* 2011, Ying *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 SnRK2 action.

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, Melcher *et al.* 2009, Park *et al.* 2009, 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 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 7). 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 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 signalling (Fujii *et al.* 2009).

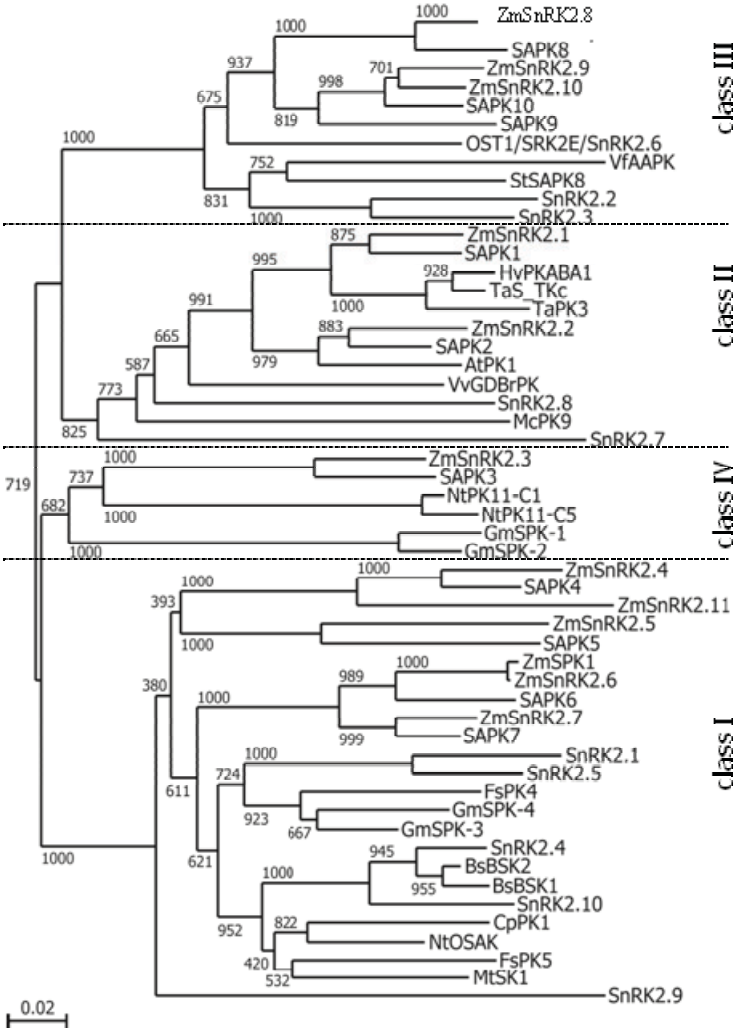

Figure 7 - Phylogenetic neighborhood analysis of known SnRK2 proteins from different plants. ZmSnRK2.8 aligns in the ABA responsive SnRK2 subgroup next to SnRK2.6 from *Arabidopsis* and SAPK8 from rice.

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 kinase activation (Hardie *et al.* 1998) and involved in protein-protein interactions mainly in osmotic responses such as ABA responsiveness and ABA signal 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-myristoylation 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 8) 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.

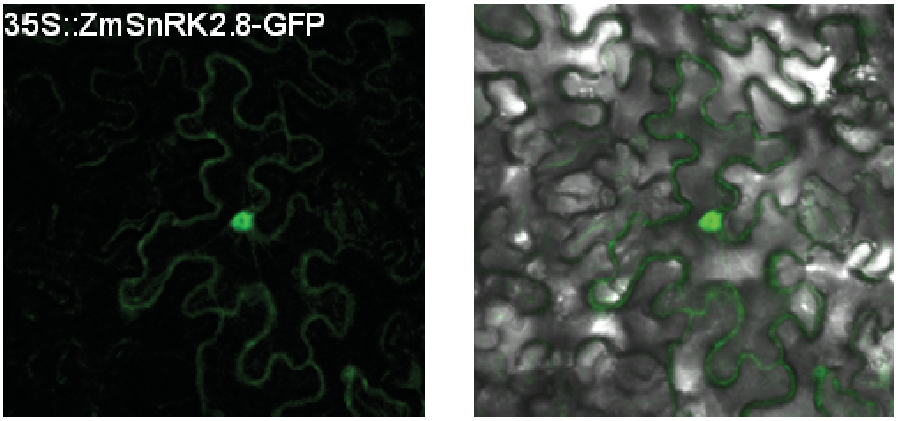

Figure 8 - ZmSnRK2.8 localization. ZmSnRK2.8 was fused in phase with GFP under a constitutive 35S promoter and transient transformation of *Nicotiana benthamiana* leaves was performed. 35S::ZmSnRK2.8-GFP protein localizes in the nucleus and cytosol of plant cells. This localization remains unchanged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

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 subtracts such as myelin basic protein (MBP) and histone-III (HIII). The appearance of two bands (Figure 9) 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.

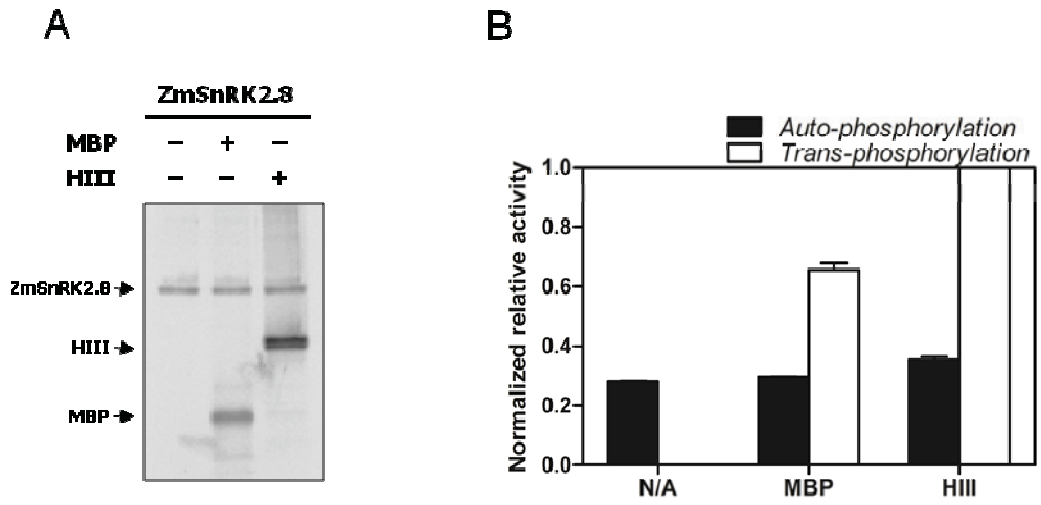

Figure 9 - Recombinant His-tagged ZmSnRK2.8 protein is an active kinase with auto-phosphorylation and trans-phosphorylation activity on different generic substrates. **(A)** *In vitro* phosphorylation of different substrates with His-tagged ZmSnRK2.8. Lane 1 corresponds to kinase auto-phosphorylation 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).

The analysis of the *in vitro* phosphorylated ZmSnRK2.8 protein by mass spectrometry showed auto-phosphorylation to either S182 or T183 amino acids (Figure 10). 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).

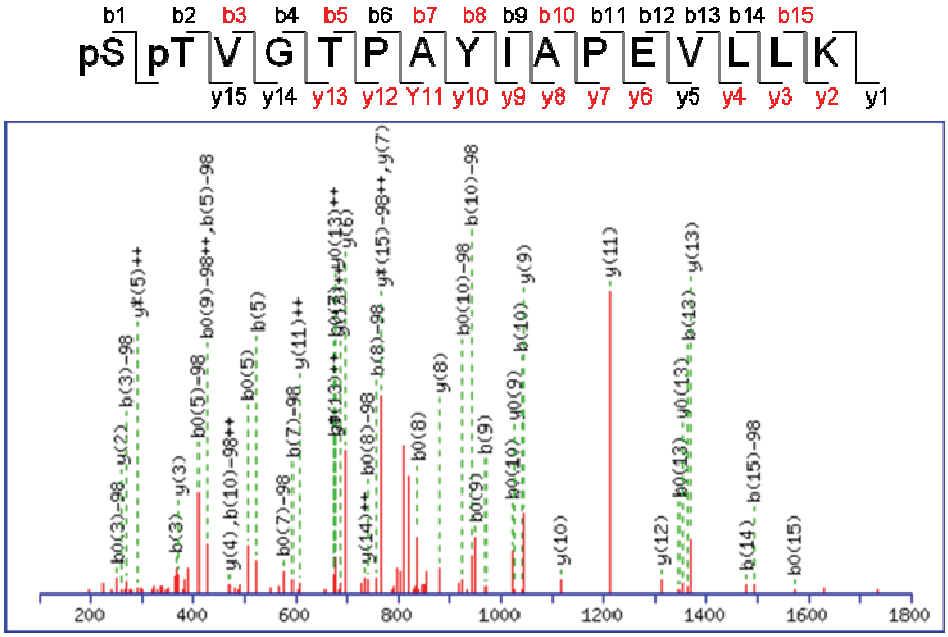

Figure 10 - Mass spectrometry (MALDI-TOF) of ZmSnRK2.8 *in vitro* auto-phosphorylation. ZmSnRK2.8 auto-phosphorylates *in vitro* at the level of the activation loop on either the S182 or T183.

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 11A) and in roots (Figure 11B) 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 which 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 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.

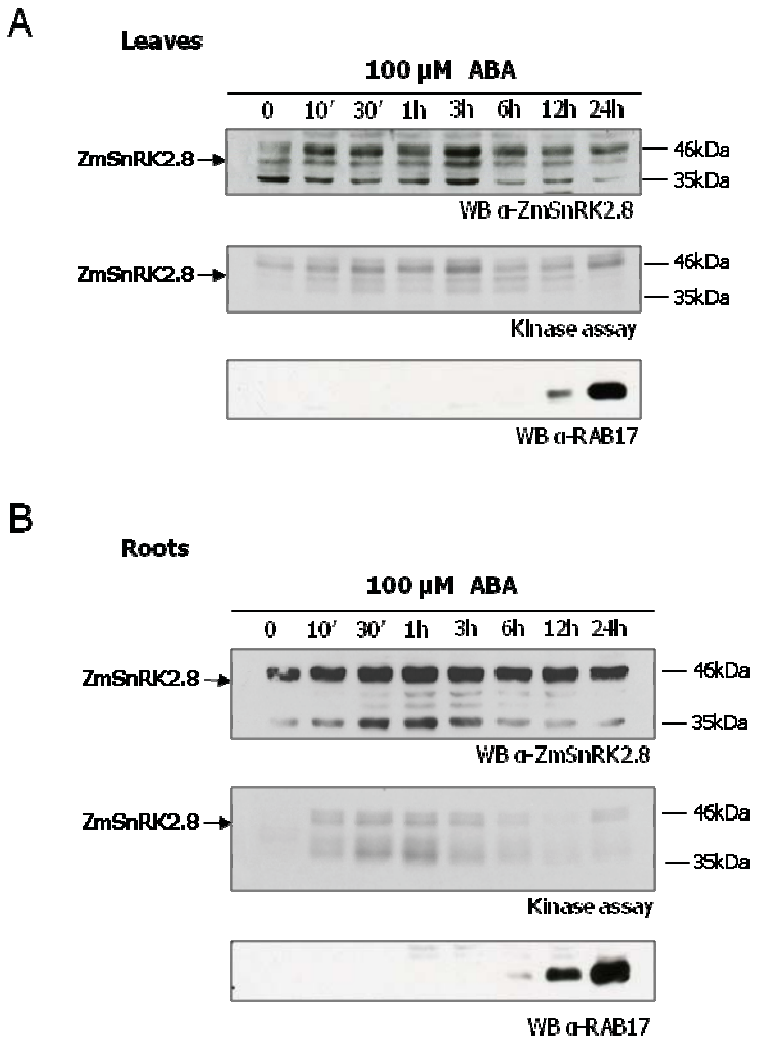

Figure 11 - ZmSnRK2.8 is activated by ABA at the plant level. ZmSnRK2.8 present in **(A)** leaves or **(B)** roots submitted to ABA treatment was immunoprecipitated (IP) using a polyclonal antibody. Western blots and in gel kinase assays of IP ZmSnRK2 revealed that this protein is constitutively present in maize leaves and roots but that its catalytic activity is induced by ABA. Rab17 was used as a control for the ABA treatment.

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 with 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 12), as has been previously reported for the *Arabidopsis* system (Umezawa *et al.* 2009).

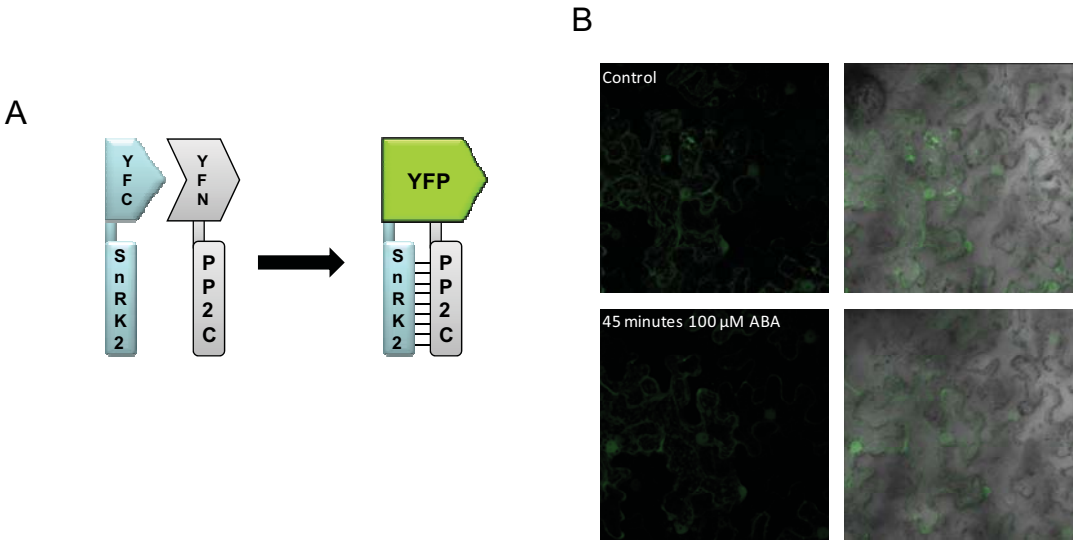

Figure 12 - ZmSnRK2.8 and ZmPP2C interact *in vivo* through the kinase regulatory domain. **(A)** Interaction between the regulatory domain of ZmSnRK2.8 and ZmPP2C was tested. YFP fluorescence can only be detected when the two transformed proteins are able to interact, thus reconstituting the YFP protein. **(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.

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 signalling pathway.

Conclusions

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 production 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 signalling 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 unravelling the positive mechanisms of SnRK2

activation by ABA, together with the discovery of new potential 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.

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 0.5X MS medium for one week prior to 100 µM ABA application in the medium. Material was deep frozen immediately after treatment and stored at -80ºC.

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) 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 30ºC with [γ-^{33}P]ATP (3000 Ci mmol^{-1}), 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 [γ-^{33}P] incorporation was analyzed using the public domain image analysis software ImageJ (http://rsbweb.nih.gov/ij).

Protein immunoprecipitation and in gel kinase assays

Protein immunoprecipitation and in gel kinase assay was performed as described by Lumbreras *et al.* (2010). Proteins were extracted in 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 20 %

Glycerol, 1 mM PMSF, 10 µM Leupeptin, 1µg/ml Aprotinin and 10 µg/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 4ºC 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_3VO_4, 1 mM NaF, 1 mM PMSF, 10 µM Leupeptin, 1µg/ml Aprotinin and 10 µg/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_3VO_4, 0.5 mg/ml BSA, 0.1% Triton X-100, and 25mM Tris-HCl, pH 7.5 and proteins were renatured with 1 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4, and 25 mM Tris-HCl, pH 7.5, for 2 x 30 min and 16 h at 4ºC prior to the reaction. The gel was incubated for 90 min at room temperature with 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, and 25 mM Tris-HCl, pH 7.5, supplemented with 50 mCi of [γ-^{33}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 µg of total protein from several independent maize

plants were loaded per lane and transferred to a nitrocellulose membrane. Homogenous protein transfer was confirmed by Pounceau 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 analyzes

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 [γ-^{33}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).

3.2 - Chapter 2 - The maize OST1 kinase homolog regulates the phosphorylation status of the maize SNAC1-type transcription factor in abiotic stress response.

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* These authors contributed equally for this work

This Chapter in under revision in PLoS One

Summary

The *Arabidopsis* kinase OPEN STOMATA 1 (OST1) plays a key role in regulating drought stress signalling, particularly stomatal closure. In Chapter 1 we cloned a maize protein that is homolog to OST1 and characterized it from the biochemical point of view.

After establishing that ZmSnRK8/ZmOST1 is a functional kinase which localizes to the nucleus and cytosol of plant cells and that it has a closely related regulation to the *Arabidopsis* OST1, we wanted to further characterize this kinase from the functional point of view. In this Chapter 2 we characterize ZmOST1 in an heterologous *Arabidopsis* system, and we describe a novel transcription factor that is a substrate of ZmOST1 (ZmSNAC1).

We first profiled ZmOST1 activity by in gel kinase assays during embryogenesis and in vegetative tissues after different treatments. We then used complementation lines overexpressing ZmOST1 under a constitutive *35S* promoter in the *Arabidopsis ost1* mutant background to determine whether the maize kinase is a functional homolog to the *Arabidopsis* gene. We finally present results on a stress NAC transcription factor (ZmSNAC1) and its relationship with ZmOST1.

Ectopic expression of *ZmOST1* in the *Arabidopsis ost1* mutant restores the stomatal closure phenotype in response to drought. While the endogenous *ZmOST1* mRNA is constitutively expressed at all developmental stages analyzed, its corresponding kinase activity is strongly up-regulated by ABA and osmotic stress treatments.

Furthermore, we identified the transcription factor, ZmSNAC1, which is directly phosphorylated by ZmOST1 under stress conditions. Interestingly, ZmSNAC1 binds to the ABA-box of ZmOST1, which is part of the contact site for the negative-regulating clade A PP2C phosphatases. Competitive binding between ZnSNAC1 and PP2Cs to the ABA-box could provide a mechanisms of sustained ABA signalling required for long-term transcriptional control.

Taken together, our results indicate that ZmOST1 is a functional ortholog of the *Arabidopsis* OST1 and that ZmSNAC1 is a substrate of ZmOST1. These findings delineate a novel osmotic stress transcriptional pathway in maize.

Introduction

Plant growth and crop productivity are compromised by environmental stresses such as extreme temperatures, drought and high salinity. To cope with these adverse situations, plants have developed complex signalling networks to regulate physiological and biochemical acclimation. Reversible protein phosphorylation is one of the major mechanisms for modulating intracellular adaptations, in particular, those involved in ABA and stress signalling (Droillard *et al.* 2002, Chinnusamy *et al.* 2004, Franz *et al.* 2011). The ABA signal can stimulate – within minutes – regulators such as Sucrose non-fermenting related kinase (SnRK)-2 subfamily (Yoshida *et al.* 2002) that are central to diverse physiological responses.

Members of the SnRK2 subfamily have been characterized in different plant species (Li *et al.* 2000, Mustilli *et al.* 2002, Yoshida *et al.* 2002). The first SnRK2 gene, *PKABA* from wheat, is up-regulated by drought and ABA in seeds and vegetative tissues (Anderberg and Walker-Simmons 1992). The homolog AAPK from faba bean is essential in relaying the ABA signal in stomatal closure (Li *et al.* 2000). Ten SnRK2 genes exist in the *Arabidopsis* and rice genomes. In *Arabidopsis*, except SnRK2.9, the kinase activity of each member of the family is activated by hyperosmotic stress (Boudsocq *et al.* 2004) with five, SnRK2.2, SnRK2.3 and SnRK2.6/OST1/SRK2E, SnRK2.7 and SnRK2.8 also activated by ABA (Boudsocq *et al.* 2004). A similar situation occurs in rice, in that the activities of three of the ten SnRK2 homologs (called SAPKs) are also stimulated by ABA (Kobayashi *et al.* 2004). Studies of mutants deficient in SnRK2.2 and SnRK2.3 activities showed that these kinases are required for ABA-mediated seed germination, dormancy and seedling growth but have minor roles in stomatal control (Fujii *et al.*

2007). Conversely, SnRK2.6/OST1, the ortholog of AAPK, is mainly involved in ABA-mediated stomatal closure in response to humidity decrease but only has a minor role during seed germination (Mustilli *et al.* 2002, Yoshida *et al.* 2002, Fujii and Zhu 2009, Zheng *et al.* 2010). In maize, eleven *SnRK2* genes have been reported (Huai *et al.* 2008), although only functional data are available for one, the corresponding protein ZmSAPK8/ZmOST1. This kinase, with a primary sequence highly homologous to that of the *Arabidopsis* OST1, is required in diverse stress signal transduction pathways, mainly in drought and salt tolerance responses (Huai *et al.* 2008, Ying *et al.* 2011).

The molecular mechanisms of OST1 in orchestrating both the "fast" (ion transport across membrane) and "slow" (gene expression) ABA responses are increasingly being understood, which has been particularly aided by the identification of direct targets. OST1 (i) activates the slow anion channel SLAC1 to trigger plasma membrane depolarization necessary to initiate stomatal closing (Geiger *et al.* 2009, Lee *et al.* 2009, Vahisalu *et al.* 2010), (ii) possibly inhibits the inward-rectifying K^+ channel KAT1 (Sato *et al.* 2009), (iii) induces the generation of reactive oxygen species (ROS) that leads to Ca^{2+} spikes *via* the respiratory burst oxidase homolog F (RbohF) (Sirichandra *et al.* 2009) and (iv) activates the bZIP-class of transcription factors that include ABI5 (Furihata *et al.* 2006, Nakashima *et al.* 2009) and the ABA-responsive element binding factors, ABF2 and ABF3 (Fujii and Zhu 2009, Sirichandra *et al.* 2010). ABFs 2, 3 and 4 together are thought to regulate about 80% of the global ABA-dependent transcriptome (Johnson *et al.* 2002, Furihata *et al.* 2006, Shinozaki and Yamaguchi-Shinozaki 2007, Sirichandra *et al.* 2010, Yoshida *et al.* 2010).

In comparison to ion transport across membranes (Blatt 2000, Roelfsema and Hedrich 2005, Joshi-Saha *et al.* 2011), much less is known about the mechanisms of transcriptional control by ABA in guard cell signalling (Leonhardt *et al.* 2004, Wang *et al.* 2011). Despite the apparent importance of the three ABFs as global regulators of the ABA transcriptome, the *Arabidopsis abf2/abf3/abf4* triple mutant is normal in transpiration (Yoshida *et al.* 2010). This suggests that the ABF class of transcription factors are not critical for stomatal functions and that OST1 may have additional transcriptional targets (Xue and Loveridge 2004, Shinozaki and Yamaguchi-Shinozaki 2007, Fujita *et al.* 2011, Golldack *et al.* 2011).

In searching for cognate targets of the maize OST1 ortholog, we focused on the ABA/drought-inducible members of the NAC domain-containing transcription factors (SNAC proteins). Note that SNACs seem to have significant importance in stomatal adaptive regulation and also implications for improving plant stress tolerance (Golldack *et al.* 2011, Nakashima *et al.* 2012). Overexpression of *SNAC* genes in different plant species ranging from *Arabidopsis* to rice leads to enhanced drought and salt tolerance (Zheng *et al.* 2009, Jeong *et al.* 2010, Takasaki *et al.* 2010, Xue *et al.* 2011a). Of particular interest is the rice SNAC1, which is highly induced in guard cells by drought (Hu *et al.* 2006). Similar overexpression of this protein in rice enhances the plant's drought tolerance under field conditions at the reproductive stage. It also improves both drought and salt tolerance at the vegetative stage, and more importantly, without yield penalty (Hu *et al.* 2006) and ZmSNAC1 has been described as a stress-responsive factor acting in positive modulation of abiotic stress tolerance (Lu *et al.* 2012). Thus, SNAC factors are emerging as important nodes in osmotic stress signalling

and as promising tools to engineer enhanced tolerance responses in plants with little compromise in biomass yield.

Maize (*Zea mays*) is an important food and feed crop worldwide, with more than 800 million tons cultivated annually, and about 130 million tons in the USA also being diverted for energy. However, maize requires high water input. For this reason, characterization of osmotic stress signalling pathways and proteins involved in maize water homeostasis are of huge economic importance as bouts of water shortage are becoming more frequent. In the present work, we have characterized the maize ortholog of the *Arabidopsis* OST1. We have also identified its cognate substrate, a SNAC-type transcription factor, ZmSNAC1. ZmSNAC1 represents a founding member of a new class of target that opens the possibility to better understand how ABA mediates transcriptional control of stomatal closure.

Results

The maize OST1 homolog is activated by hyperosmotic stress

OST1 from *Arabidopsis* is one of the three key components in the core signalling complex that includes the ABA receptor and a negative regulating clade A PP2C (Ma *et al.* 2009, Park *et al.* 2009, Cutler *et al.* 2010). Lack of OST1 does not affect seed dormancy nor germination but rather impairs drought and ABA-induced guard cell closure (Mustilli *et al.* 2002, Yoshida *et al.* 2002). To characterize this related signalling pathway in a monocot, we investigated the maize ZmOST1 function by characterizing its profile of expression and kinase activity. *ZmOST1* expression is constitutive during all stages of embryogenesis tested (Figure 13A, upper panel).

However, unlike the transcript, stage-related sets of kinase activities ranging from 37- to 45-kDa were detected by immunoprecipitation using polyclonal antibodies raised against ZmOST1 followed by in-gel kinase assays (Figure 13A, lower panel) (Boudsocq *et al.* 2004). The 45- and 43-kDa activities were first detected 20 days after pollination (dap). Both reached a maximum at 40 dap, concomitant with the appearance of a new 37-kDa activity band. Later, when embryos completed the maturation phase (60 dap), all SnRK2 activities diminished dramatically. Reactivation of these kinases, although weak, was detected in germinating embryos 1 to 2 days after imbibition. This kinase activation pattern during seed development indicates that ZmOST1-related kinases are under differential regulation during embryogenesis and germination, including ZmOST1 with an expected size about 42-43 kDa. It should be noted that the anti-ZmOST1 antibody used, although specific to the two ZmOST1 isoforms present in the maize cultivar used in these experiments, recovers additional activity bands when used in immunoprecipitation experiments.

The *ZmOST1* transcript is constitutively expressed in seedlings regardless of stress treatments (ABA, drought, salt, and mannitol) (Figure 13B). The anti-ZmOST1 antibody also immunoprecipitated at least four major bands as revealed by in-gel kinase assays using MBP as the substrate (Figure 13B) (Huai *et al.* 2008). These ZmOST1-related kinases are strongly activated by ABA and several other osmotic treatments. In shoots, these kinases range from 41- to 45-kDa, whereas in roots they are smaller (37- or 38-kDa) (Figure 13B). Interestingly, we also found SnRK2 activation by glucose in shoots and roots (Figure 14) supporting the previously described putative role of OST1 and other SnRK2 kinase members in carbohydrate regulation (Zheng *et al.* 2010, Zhang *et al.* 2011a).

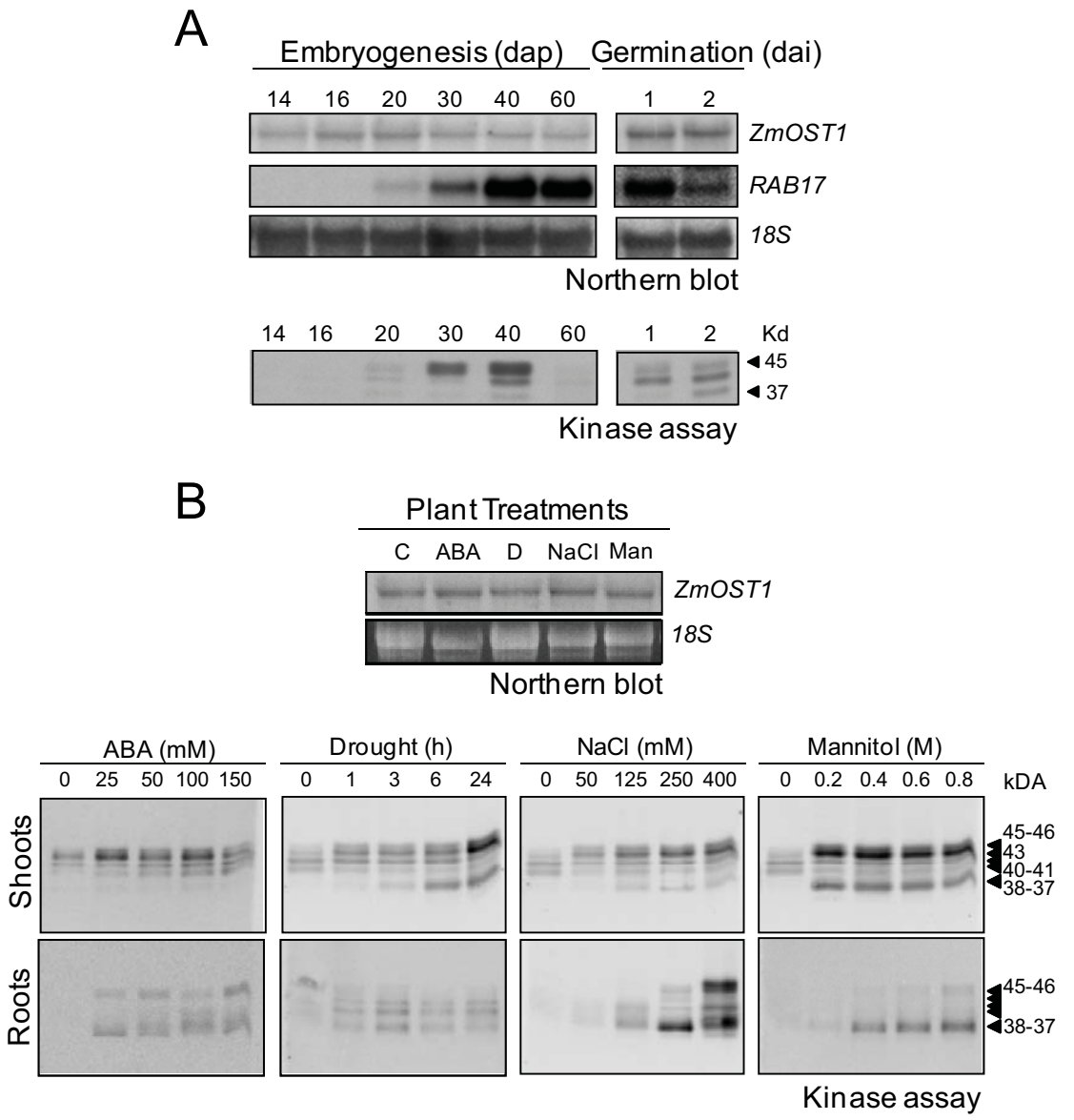

Figure 13 - ZmOST1 activation during seed development and hyperosmotic stress. **(A)** *ZmOST1* expression of maize seeds collected at 14, 16, 20, 30, 40 and 60 days after pollination (dap) and seedlings at 1, 2 days after imbibition (dai). *ZmOST1* expression is analyzed by Northern-blot experiments using *RAB17* and and ethidium bromide-stained ribosomal (*18S*) genes as controls for ABA level and loading, respectively (top panel). Activation of ZmOST1/SnRK2 kinases by in-gel kinase assays after endogenous ZmOST1 immunoprecipitation (bottom panel). Size of the activity bands obtained is indicated on the right. **(B)** *ZmOST1* expression of maize seedlings in response to ABA, drought, NaCl and mannitol treatments (top panel). ZmOST1/SnRK2 kinase activation pattern in shoots and roots of seedlings treated with ABA, drought, salt and mannitol at the indicated concentrations (bottom panel).

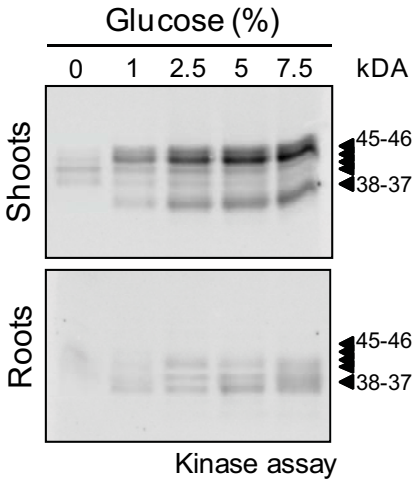

Figure 14 ZmOST1 activation by glucose treatment. Roots and shoots of 5 days-old maize seedlings were treated with glucose at the indicated concentrations. Proteins were immunoprecipitated with anti-ZmOST1 antibody and analyzed by in gel kinase assay using MBP as a substrate. Molecular masses of the obtained bands are indicated on the right.

ZmOST1 protein can substitute the Arabidopsis OST1 in guard cell drought stress signalling

OST1 in *Arabidopsis* limits water loss in leaves through the regulation of stomatal closure (Mustilli *et al.* 2002, Yoshida *et al.* 2002). To test whether ZmOST1 is a functional OST1 ortholog we examined its ability to complement the severe *ost1-2* allele carrying the point mutation (G33R) in the ATP-binding loop domain (Mustilli *et al.* 2002). The expression levels of the *ZmOST1* transgene and protein in *ost1-2* transgenic plants were quantified by RT-PCR and western-blot analyses, respectively (Figure 15A). The ZmOST1 activity in the complemented *Arabidopsis* plants was determined by MBP in-gel kinase assays (Figure 15B). It has been reported that ABA activates OST1 but that this kinase activity is missing in the *ost1-2* allele (Mustilli *et al.* 2002). Comparing MBP phosphorylation from wild-type, *ost1-2* and *ZmOST1*/*ost1-2* seedlings we detected in the *ZmOST1*/*ost1-2* extracts a new specific ABA-dependent kinase activity that is absent in the mutant (Mustilli *et al.* 2002). The 40-kDa activity is

coincidental to the expected size of ZmOST1, suggesting that the maize kinase is active in *Arabidopsis* (Figure 15B).

The ability of ZmOST1 to functionally substitute the *ost1-2* mutation was assessed by comparing the temperature of detached leaves from *ost1-2* and *ZmOST1*/*ost1-2* plants by infrared thermography (Figure 15C and 15D). The *ost1-2* mutant transpires excessively due to its inability to close stomates triggered by drought, which leads to cooling of the leaf temperature (Mustilli *et al.* 2002). We detected a temperature of 22.6 ºC in detached leaves from the mutant. By contrast, leaf temperatures of *35S::ZmOST1/ost1-2* plants were ~0.5 to 1.0 ºC warmer (23.6 °C), indicating the ability of *ZmOST1* to partially limit transpiration in response to drought. Surprisingly, the *ost1-2* mutant expressing the wild-type *Arabidopsis OST1* transgene under the control of its own promoter (*pOST1::At4g33950*) also only rescued partially the leaf temperature phenotype (Mustilli *et al.* 2002).

To confirm these results, water loss kinetics were performed on detached rosettes. Indeed, as compared to *ost1*-2, the water loss susceptibility in *35S::ZmOST1/ost1-2* rosettes was reduced again to 47-53% confirming the rescue of the *ost1-2* phenotype (Figure 15E). The partial complementation obtained in both experiments suggests that the endogenous mutated OST1-2 protein interferes with OST1 and ZmOST1 in the rescue of the ABA signalling pathway. An analogous phenomenon was observed with the inactive AAPK-kinase expressed in wild-type *Vicia faba* guard cells, which interfered with ABA responsiveness (Li *et al.* 2000, Yoshida *et al.* 2002, Fujii and Zhu 2009). Taken together these results confirm that ZmOST1 is

functionally conserved across monocots and dicots and support the hypothesis that ZmOST1 is a positive regulator of water deficit signalling in guard cells.

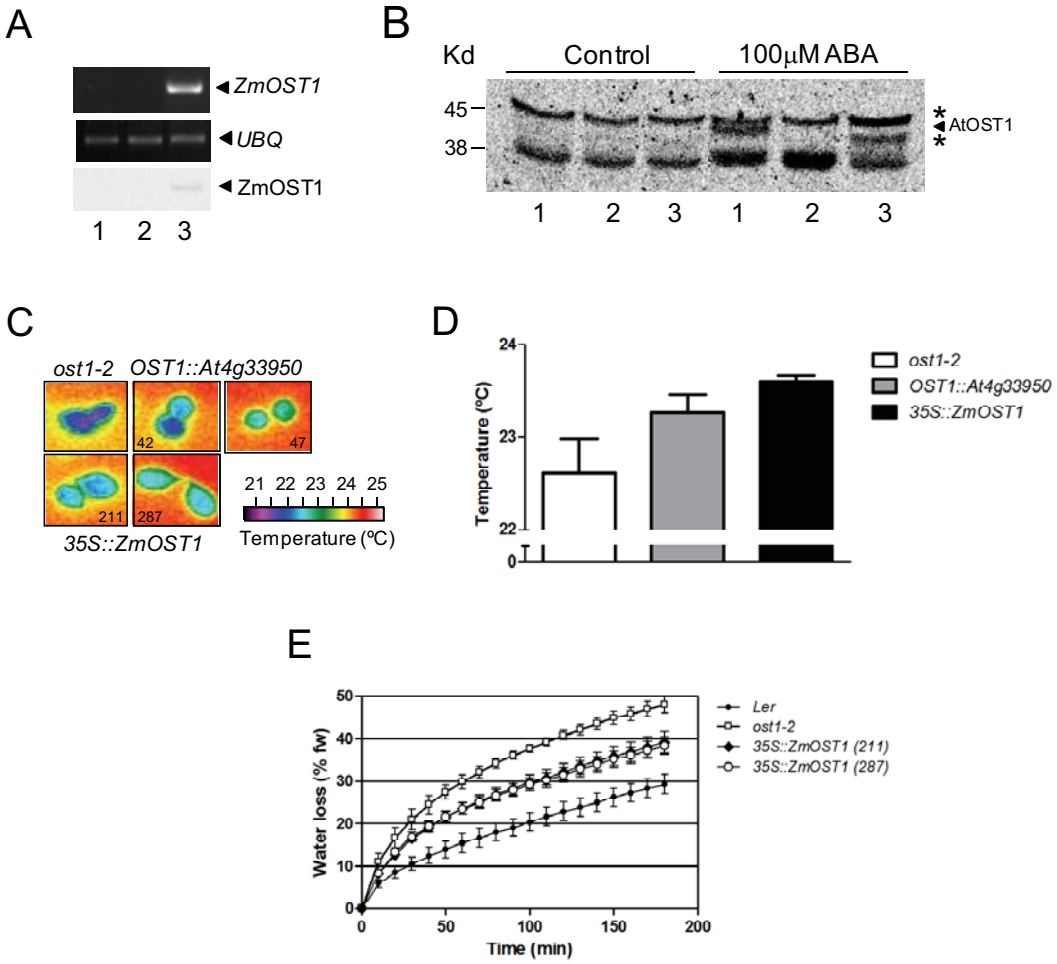

Figure 15 - ZmOST1 complements the lack of OST1 function in drought stress signalling. **(A)** *ZmOST1* expression in different *Arabidopsis* lines analyzed by RT-PCR using *Ubiquitin* expression as control and by western-blot using an anti-ZmOST1 antibody. Lane 1, Ler wild-type seedlings; lane 2, *ost1-2* mutant; lane 3, *35S::ZmOST1/ost1-2* transgenic line (211). **(B)** OST1 activation by ABA in *Arabidopsis* protein extracts analyzed by MBP in-gel kinase assay. Lane 1, Ler wild-type seedlings; lane 2, *ost1-2* mutant; lane 3, *35S::ZmOST1/ost1-2* transgenic line (211). Sizes of molecular markers are shown on the left. Black arrow marks AtOST1 activity. Asterisks mark two new bands of ABA-induced kinase activities resulting from *ZmOST1* transgene expression. **(C)** Phenotypic *ost1-2* complementation by ZmOST1 in drought signalling. Detached leaves from *ost1-2* allele, *OST1::At4g33950/ost1-2* and *35S::ZmOST1/ost1-2* transgenic lines were monitored for foliar temperature by false-colour infrared image subjected to mild drought treatment (Mustilli *et al.* 2002). **(D)** Quantification of infrared images. The average leaf temperatures were 22.61 ± 0.37 for the *ost1-2* allele; 23.27 ± 0.19 for *OST1::At4g33950/ost1-2* and 23.60 ± 0.07 for *35S::ZmOST1/ost1-2*. **(E)** Water loss kinetics using detached leaves of wild-type (closed circles), *ost1-2* mutant (open squares) and two independent *35S::ZmOST1/ost1-2* transgenic lines (line 211, closed diamonds and line 287, open circles). Water loss is expressed as the percentage of initial fresh weight. Values are means ± SD of three independent experiments.

ZmOST1 interacts with a homolog of the rice transcription factor SNAC1 in vitro

To identify unknown ZmOST1 targets, we performed a yeast two-hybrid screen using ZmOST1 as the bait, and as prey, a cDNA library from maize leaves dehydrated for 3 hours. Among several positives clones, we focused on a SNAC1-related transcription factor for further characterization. The *ZmSNAC1* clone encodes a protein of 312 amino acids highly homologous to rice SNAC1 (Figure 16A) which functions mainly in stomatal regulation (Hu *et al.* 2006). Both proteins are almost identical in their DNA-binding NAC domains suggesting that they are functionally conserved.

We then validated the interaction in yeast and found that co-expression of ZmOST1 and ZmSNAC1 proteins permitted yeast growth on selective medium and specific activation of the LacZ reporter system (Figure 16B). In addition, Figure 16C shows that the bacterially-produced GST-SNAC1 fusion protein interacts with ZmOST1 *in vitro* but not with GST alone indicating that ZmSNAC1 is a direct target of ZmOST1.

ZmOST1 interacts with ZmSNAC1 in vivo

We monitored the subcellular localization of ZmOST1-GFP and ZmSNAC1-GFP constructs in *Nicotiana benthamiana* and found that both proteins are localized mainly in the nucleus but also weakly in the cytoplasm of tobacco epidermal cells (Figure 17).

As mentioned above, the upstream negative regulators of OST1 are the clade A PP2Cs. In *Arabidopsis*, the mutated PP2C, *abi1-1*, has been shown to require nuclear localization to block stomatal closure triggered by ABA (Moes *et al.* 2008).

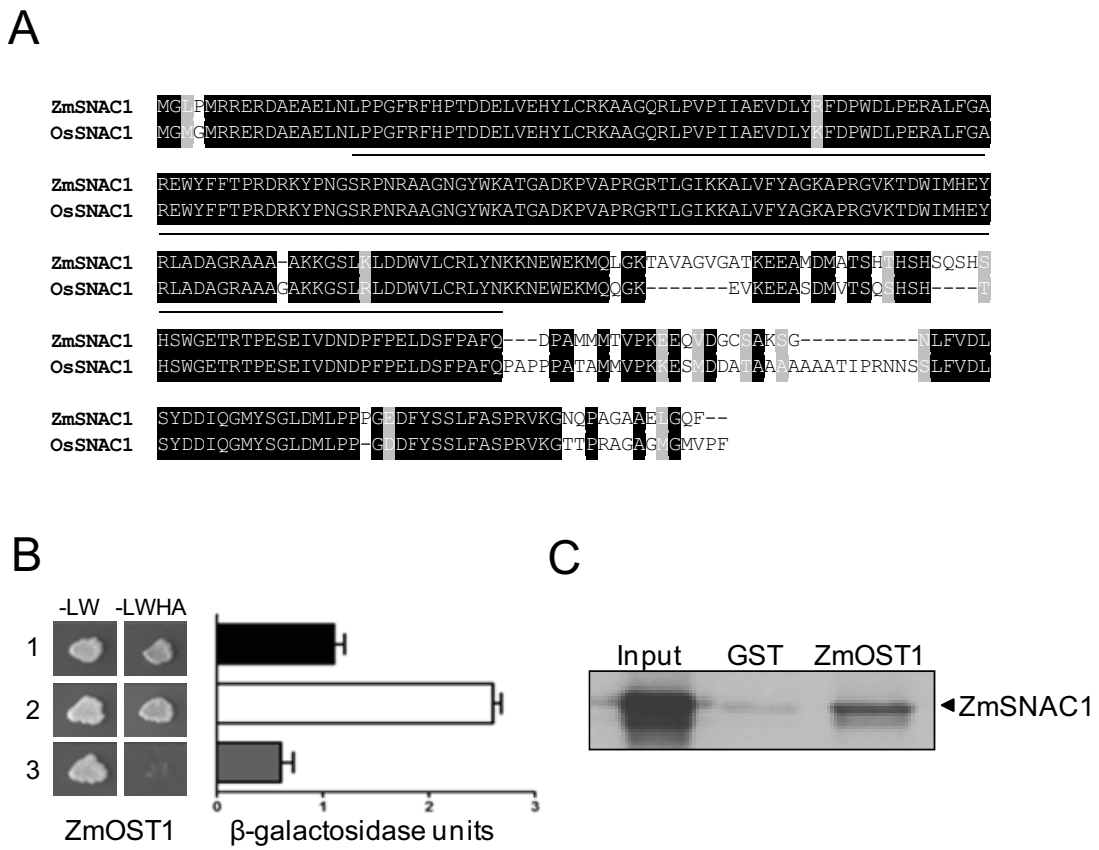

Figure 16 - ZmOST1 interacts with ZmSNAC1. **(A)** Sequence alignment of maize and rice ZmSNAC1 proteins. The NAC-binding DNA domain is underlined below the alignment. **(B)** ZmOST1/ZmSNAC1 yeast two-hybrid interaction by growth in selective medium (left). β-galactosidase activity quantification of the co-transformed yeasts (right). Values are means ± SD of three independent experiments. 1, ZmOST1/ZmSNAC1 interaction; 2, α and β CK2 subunits interaction used as positive control; 3, ZmOST1/pGAD424 interaction as a negative control. **(C)** ZmOST1/ZmSNAC1 interaction is confirmed by *in vitro* pull-down assay. Equal amounts of labelled ZmOST1 were incubated with GST and GST-ZmSNAC1 proteins coupled to gluthathione-sepharose resin obtaining ZmOST1 specific *in vitro* retention.

We thus tested whether the subcellular localization of an inactive ZmOST1 kinase with the point mutation G40R might be altered. This mutated form, however, maintains the same subcellular localization of the wild-type kinase. Unexpectedly, while the level of the wild-type ZmOST1-GFP is low, this mutated form accumulates to higher levels in transformed tobacco cells. Since there is no noticeable altered subcellular localization between the wild-type and the mutant ZmOST1, which is stable, this could explain why the kinases OST1-2 and AAPK similarly mutated in the P-

loop could prevent full phenotypic complementation by their respective wild-type counter parts.

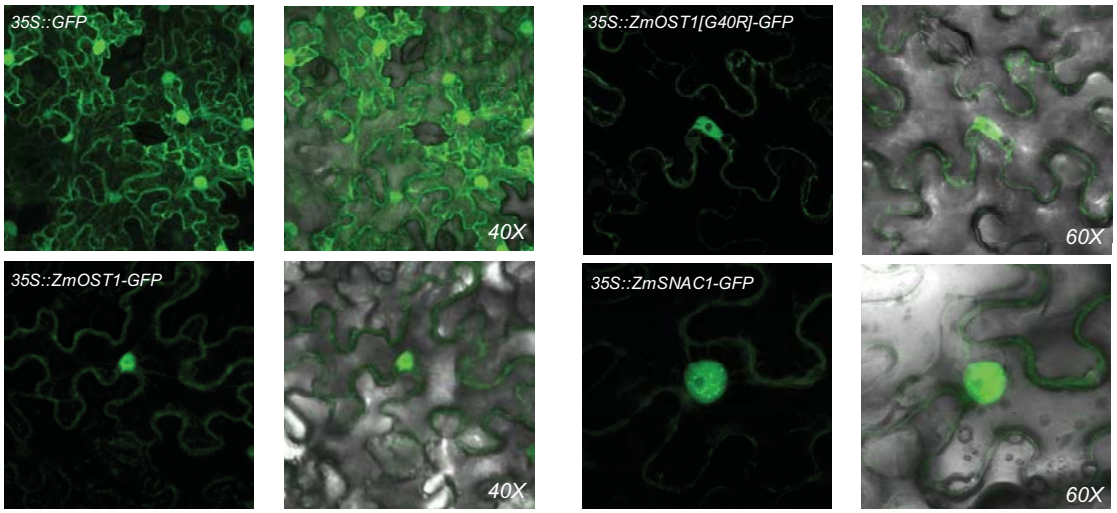

Figure 17 - ZmOST1 and ZmSNAC1 co-localize in the nucleus. ZmOST1-GFP and ZmSNAC1-GFP fusion proteins were localized by transient expression in epidermal tobacco leaves. Left, GFP signals; right light microscope images merged with fluorescence.

Next, we used bimolecular fluorescence complementation (BiFC) (Weinthal and Tzfira 2009) to determine whether and where ZmSNAC1 interacts with ZmOST1 *in planta*, and if so, to characterize the specific ZmOST1 domains involved in this interaction. Full-length ZmOST1[G40R] and different deleted derivatives were fused to the C-terminal half of the YFP while the ZmSNAC1 factor was fused to the N-terminal half (Figure 18A). The results showed that ZmOST1 interaction with ZmSNAC1 is independent of the kinase activity and it is mediated by a site in the C-terminal regulatory domain. This domain is present in ABA-dependent SnRK2 kinases and is important for the negative regulation by the clade A PP2C phosphatases (Belin *et al.* 2006, Yoshida *et al.* 2006, Vlad *et al.* 2008).

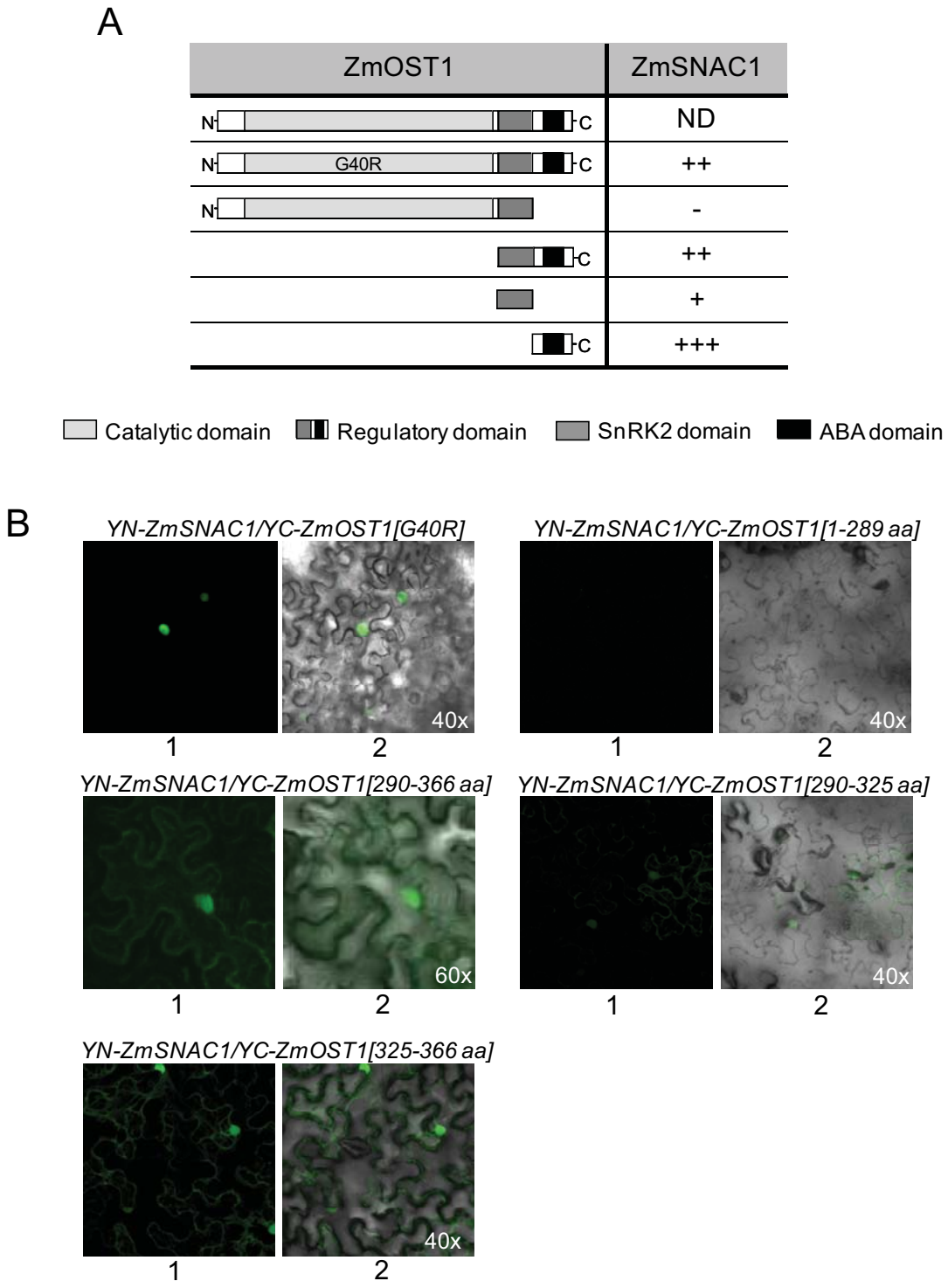

Figure 18 - The interaction of ZmOST1 with SNAC1 depends on the ZmOST1 ABA-box. BiFC analysis of the interaction between ZmSNAC1 and different ZmOST1, ZmOST1 G40R mutant, and deletion forms as depicted on the left. Relative quantification of the BiFC interaction is shown on the right. BiFC fluorescence images analyzed by confocal microscopy are presented on the bottom. 1, YFP signals; 2, light microscope images. Numbers indicate ZmOST1 amino acid regions included in each construct.

As shown in Figure 18B, co-expression of only the regulatory domain or the ABA-box of ZmOST1 alone with ZmSNAC1 reconstituted the YFP signal. No interaction was detected between *YN-ZmSNAC1* and *YC-ZmOST1[1-289]* constructs. Thus, the ABA box is necessary and sufficient for this interaction. While the ABA-box has been shown to form part of the contact site for the negative regulating PP2Cs (Yoshida *et al.* 2006, Soon *et al.* 2012), our results reveal that it is also important for substrate-binding. This raises the possibility that ZmSNAC1 may compete with the clade A PP2C phosphatases sharing the same docking region, highlighting the interesting perspective of substrate occupancy as a mechanism to sustain ABA signalling (Vlad *et al.* 2008).

ZmSNAC1 is phosphorylated by ZmOST1

If ZmOST1 is activated by ABA and hyperosmotic stress, we reasoned that these treatments may lead to ZmSNAC1 phosphorylation. In fact, even though the optimal motif for OST1 phosphorylation, LXRXX(S/T) (Vlad *et al.* 2008), is absent in the ZmSNAC1 primary sequence, we were able to predict potential phosphorylation sites using a web-based bioinformatics tool (Table 2) (Ellis and Kobe 2011). To test ZmSNAC1 phosphorylation we used maize extracts pre-treated or not with either ABA, mannitol or salt to detected kinase activities toward recombinant ZmSNAC1 protein. Using in-gel kinase assays our results revealed a 44-kDa kinase that was rapidly and strongly activated in maize seedlings by mannitol and salt. However, 30 min after ABA stimulation, this activity became barely detectable (Figure 19A) suggesting that ZmSNAC1 is phosphorylated by kinases transiently activated by hyperosmotic stress signals.

Kinase	Substrate	Position	Peptide	Method	Score
ZmOST1	ZmSNAC1	77	YFFTPRD	KSD	68
ZmOST1	ZmSNAC1	87	PNGSRPN	KSD	67
ZmOST1	ZmSNAC1	113	RGRTLGI	KSD	65
ZmOST1	ZmSNAC1	198	DMATSHT	SDR	65
ZmOST1	ZmSNAC1	199	MATSHTH	KSD	67
ZmOST1	ZmSNAC1	201	TSHTHSH	KSD	68
ZmOST1	ZmSNAC1	203	HTHSHSQ	KSD	67
ZmOST1	ZmSNAC1	205	HSHSQSH	KSD	66
ZmOST1	ZmSNAC1	207	HSQSHSH	KSD	69
ZmOST1	ZmSNAC1	209	QSHSHSW	KSD	65
ZmOST1	ZmSNAC1	211	HSHSWGE	SDR	65
ZmOST1	ZmSNAC1	215	WGETRTP	SDR	67
ZmOST1	ZmSNAC1	217	ETRTPES	KSD	83
ZmOST1	ZmSNAC1	233	ELDSFPA	KSD	67
ZmOST1	ZmSNAC1	295	LFASPRV	KSD	72

Table 2 – Predicted ZmOST1 phosphorylation loci on the ZmSNAC1 sequence using the web based bioinformatics tool Predikin (Ellis and Kobe 2011). Two putative peptides are found on SNAC1-A, one on SNAC1-B and 12 on SNAC1-C

We then demonstrated that ZmSNAC1 can be efficiently and directly phosphorylated by ZmOST1 *in vitro* (Figure 19B). To identify the ZmSNAC1 region required for ZmOST1 phosphorylation, we made three deletion constructs: two dividing the NAC-binding DNA domain that lacked the C-terminal region (constructs A and B) and one lacking the N-terminal NAC-binding DNA domain (construct C). As shown in Figure 19B, only domain C was highly phosphorylated by ZmOST1 *in vitro* suggesting that is the regulatory region which is phosphorylated by ZmOST1. These *in vitro* results are in accordance with our *in silico* prediction in which the domain C of ZmSNAC1 contains 12 potential phosphorylated peptides while ZmSNAC1-A and ZmSNAC1-B have two and one peptides, respectively (Table 2).

.

To ascertain whether this ZmSNAC1 phosphorylation by ZmOST1 occurs and if other kinases could also phosphorylate this transcription factor, we assessed ZmSNAC1 phosphorylation by in-gel kinase assays with protein extracts from wild-type *Arabidopsis*, *ost1-2* and *ZmOST1*/*ost1-2* seedlings treated or not with ABA. The *Arabidopsis* OST1 from ABA-treated wild-type plant extracts phosphorylated ZmSNAC1. Note that the same phosphorylation activity was clearly missing in extracts from the *ost1-2* allele and it was recovered in extracts of *ZmOST1/ost1-2* complemented line (Figure 19C).

To validate these results we immunoprecipitated the kinases from the same extracts using an antibody directed against the ZmSnRK2 ABA-box. This indeed confirms that ZmSnRK2 ABA box-containing kinases phosphorylate ZmSNAC1 by an ABA-dependent mechanism, possibly by enhancing the transcriptional activity or stability of ZmSNAC1 in response to ABA as has been shown for some of the ABF factors.

To validate these results we immunoprecipitated the kinases from the same extracts using an antibody directed against the ZmOST1 ABA-box which recognizes OST1 and ZmOST1 proteins. As can be seen by the bottom gel, we recover two proteins by immunoprecipitation using this antibody, the most abundant being OST1/ZmOST1. OST1 is recovered in similar amounts on the wild-type and *ost1-2* backgrounds and ZmOST1 in slightly increased quantities in the *ZmOST1/ost1-2* line used as a result of it being overexpressed under a *35S* promoter. Analyzing the in gel kinase assay presented in the middle section we detected a band that is activated by ABA on the wild-type and *ZmOST1/ost1-2* line which is absent in the *ost1-2* mutant.

This activity can only be identified as OST1/ZmOST1 (Mustilli *et al.* 2002) and indeed confirms that ZmOST1 phosphorylates ZmSNAC1 by an ABA-dependent mechanism, possibly by enhancing the transcriptional activity or stability of ZmSNAC1 in response to ABA or osmotic stresses.

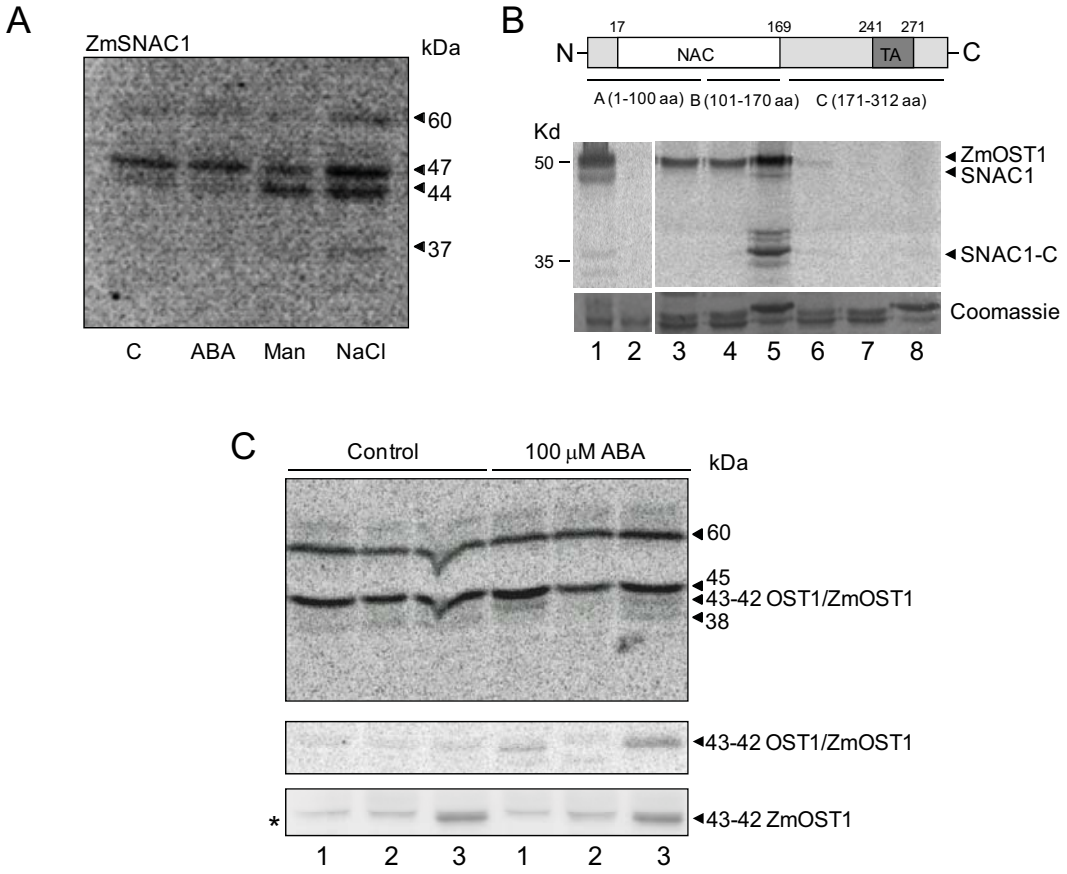

Figure 19 - ZmSNAC1 phosphorylation by osmotic stress is dependent on ZmOST1 activity. **(A)** ZmSNAC1 phosphorylation is analyzed by in gel kinase assay. Protein extracts were prepared from maize young seedlings 30 min after treatments with MS 0.5X medium, 100 µM ABA (ABA), 400 mM mannitol (Man) and 250 mM salt (NaCl). Sizes of the activity kinase bands obtained are shown on the right. **(B)** ZmSNAC1 *in vitro* phosphorylation by ZmOST1. Schematic representation of ZmSNAC1 domains used in the experiment. Numbers indicate ZmSNAC1 amino acid regions included in each construct. Phosphorylation of ZmSNAC1 and deletion forms is tested *in vitro*. 1, ZmSNAC1 phosphorylation by ZmOST1; 2, ZmSNAC1 alone; 3, 4, 5; ZmOST1 phosphorylation of ZmSNAC1 fragments A, B and C, respectively; 6, 7, 8 ZmSNAC1 fragments A, B and C, respectively. The expression of the different constructs is verified by Coomassie-blue protein staining. **(C)** In gel kinase assay with proteins extracted from 7 day-old seedlings treated or not with 100 µM ABA (upper panel). Immunoprecipitation experiment of the same samples with an antibody against the ZmOST1 ABA-domain (medium panel). Western-blot of the immunoprecipitation experiment (lower panel). Lane 1, Ler wild-type seedlings; lane 2, *ost1-2* mutant; lane 3, *35S::ZmOST1/ost1-2* transgenic line. The ZmSNAC1 protein was used as substrate. Sizes of the activity kinase bands obtained are shown on the left.

The above assays not only confirmed the phosphorylation of ZmSNAC1 but also show that several kinases of 60-, 45-kDa can also phosphorylate this transcription factor (Figure 19C). The 60-kDa kinase band probably represents the endogenous AKIN10, another stress-activated kinase of the SnRK1 family (Tsai and Gazzarrini 2012).

We have confirmed this by using ZmSNAC1 as substrate, and performing phosphorylation assays with total protein extracts from wild-type, *35S::AKIN10-HA* and *AKIN10 RNAi* seedlings (Figure 20) (Baena-Gonzalez *et al.* 2007). In contrast to the ABA-dependent phosphorylation of ZmSNAC1 by OST1, AKIN10 phosphorylation occurs in the absence of ABA. Thus, the transcriptional activity or protein stability of ZmSNAC1 might be co-modulated by both ABA-dependent and ABA-independent signalling pathways.

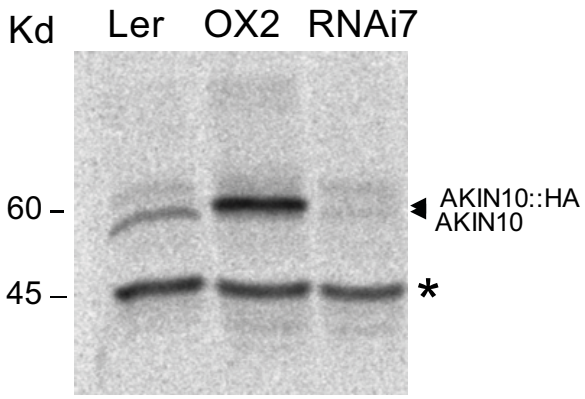

Figure 20 - Phosphorylation of ZmSNAC1 by AKIN10. In gel kinase assay with proteins extracted from seedlings of wild-type (Ler), *35S::AKIN10-HA* (OX2) and AKIN10-RNAi (RNAi7) transgenic lines (Baena-Gonzalez *et al.* 2007) using ZmSNAC1 as substrate. Sizes of activity bands are shown on the left. The 60 Kd band probably represents the kinase activities of the endogenous AKIN10 and the closely related AKIN11, kinases with a similar MW of approximately 60 Kd (Zhang *et al.* 2009). A strong band of activity of about 66 Kd is obtained in extracts from *35S::AKIN10-HA* seedlings suggesting that AKIN10 is likely to phosphorylate ZmSNAC1 protein *in vivo* together with other kinases (45 Kd activity band; asterisk).

ZmOST1 alters the localization and stability of ZmSNAC1 under ABA treatment

Since ZmSNAC1 is phosphorylated by ZmOST1 after being activated by ABA we were interested in determining the effects of this phosphorylation on ZmSNAC1, in particularly during ABA dependent signalling. We transiently co-expressed ZmSNAC1 fused to GFP in maize protoplasts together with ZmOST1 and ZmOST1[G40R] fused to a HA-epitope and checked for fluorescence under a confocal microscope. Using this approach we were able to detect a change of localization of ZmSNAC1-GFP under ABA treatment when co-expressed with ZmOST1-HA to nuclear speckles that is absent when SNAC1-GFP is over-expressed alone or together with the inactive ZmOST1[G40R]-HA (Figure 21). This formation of nuclear speckles is concomitant with a decrease of overall fluorescence that could have implications on protein stability.

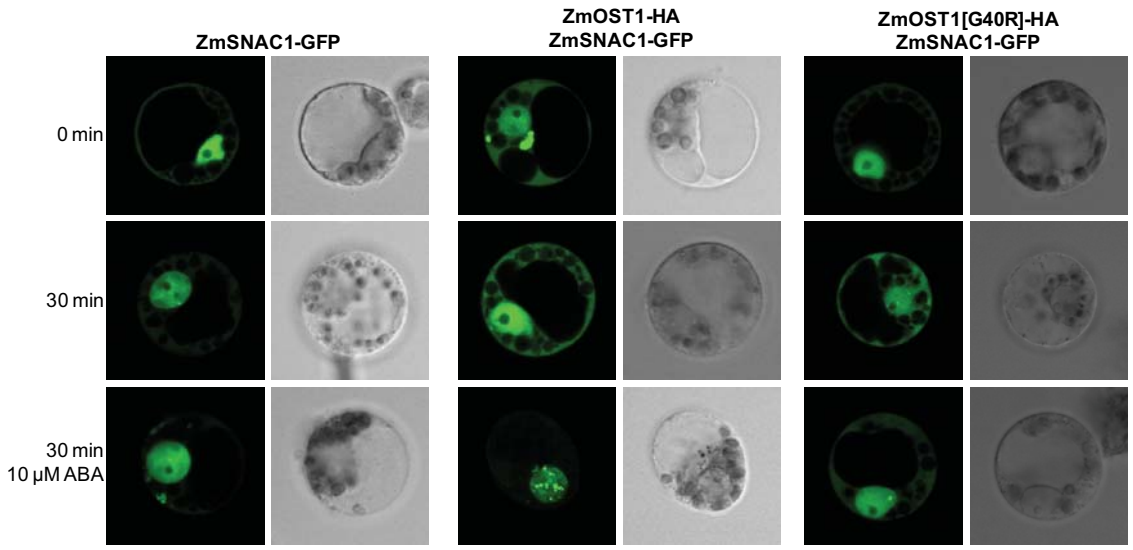

Figure 21 - ZmSNAC1 localizes in nuclear speckles after ABA treatment when co-expressed with ZmOST1. ZmSNAC1-GPF localization alone is presented in the left, co-expressed with ZmOST1-HA in the middle and co-expressed with ZmOST1[G40R]-HA in the right. Upper panel shows the localization of ZmSNAC1-GFP at the beginning of the experiment, centre panel the same localization after 30 minutes and the bottom panel represents ZmSNAC1-GFP localization after 30 minutes ABA treatment (10 µM)

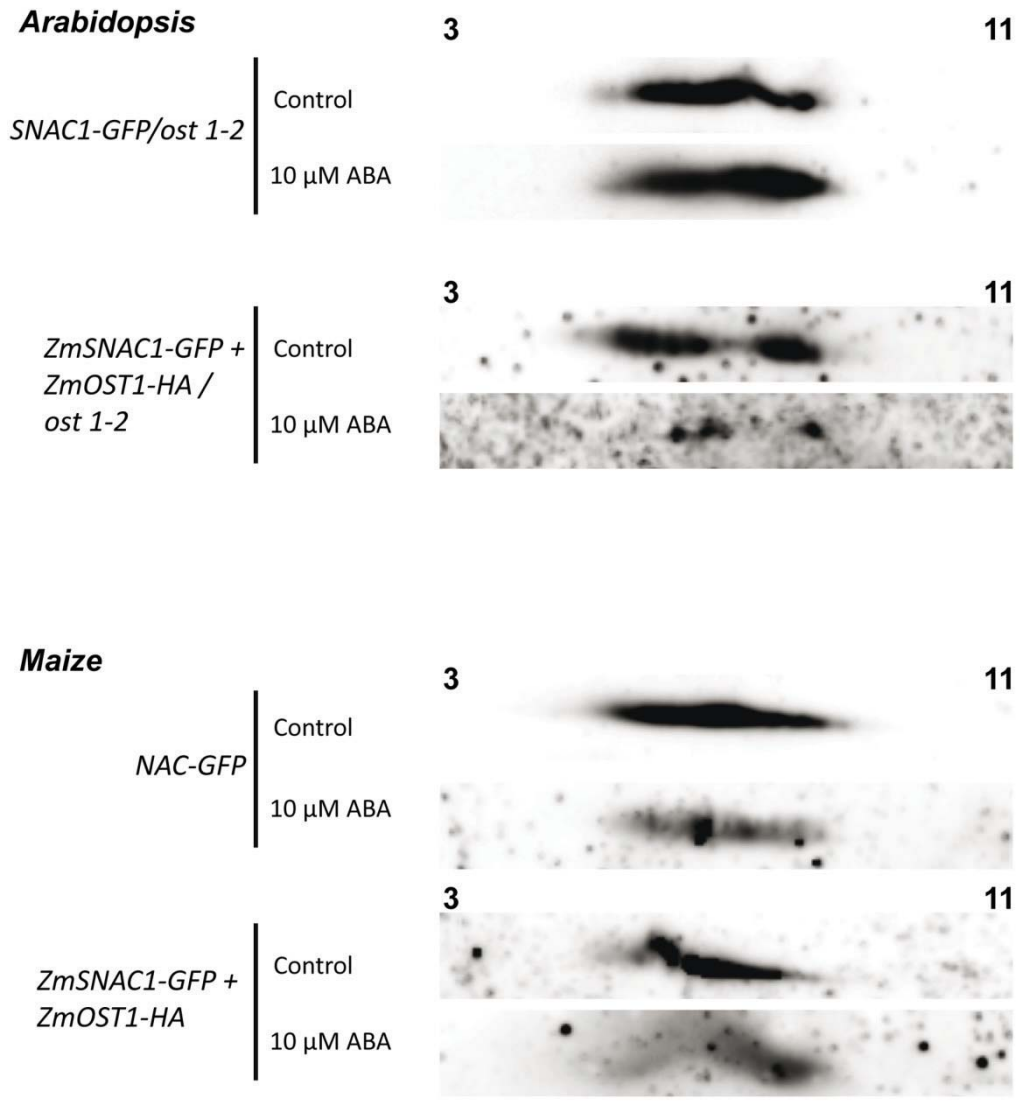

Figure 22 - ZmOST1 alters ZmSNAC1 protein stability under ABA treatment. ZmSNAC1 phosphorylation and protein stability was analyzed by bi-dimensional gel electrophoresis followed by western blot. (A) *Arabidopsis* ost1-2 mutant and (B) Maize B73 protoplasts transfected with ZmSNAC1-GFP alone or with ZmOST1-HA. Upper western blot corresponds to control situations the lower corresponds to 30 min 10µM ABA treatment.

In order to better determine the *in vivo* phosphorylation and protein stability of SNAC1 we performed Bi-dimensional SDS-PAGE experiments comparing ZmSNAC1 protein fused to GFP using the *Arabidopsis* and maize protoplast systems. Even though in this experiment we were not able to clearly detect any protein shift that is consistent with a phosphorylation, the quantity and abundance of ZmSNAC1 spots is

clearly affected by ABA treatment when ZmOST1 is present (Figure 22). While in the *ost1-2* protoplasts ZmSNAC1 quantity is unaffected by ABA, when these protoplasts are co-transformed with ZmOST1, a clear reduction of the most acidic ZmSNAC1 spots is clear (Figure 22A).When repeating the experiment in maize protoplasts we were able to observe the same degradation of ZmSNAC1 under ABA treatment (Figure 22B). These results seem to indicate that ZmOST1 activity has an effect on ZmSNAC1 stability.

Discussion

In recent years, significant progress has been made toward understanding the molecular basis for ABA signal transduction in *Arabidopsis* (Joshi-Saha *et al.* 2011). This has confirmed the SnRK2-related kinases, with OST1 being the founding member in *Arabidopsis*, as key elements in these responses (Raghavendra *et al.* 2010, Umezawa *et al.* 2010, Kulik *et al.* 2011)

The expression of *ZmOST1* can be spatial and temporally regulated, reflecting tissue-specific functions in response to different abiotic stimuli (Huai *et al.* 2008, Ying *et al.* 2011). Up-regulation of this gene by salt, PEG and dehydration treatments has been previously reported (Huai *et al.* 2008, Ying *et al.* 2011). However, similar to OST1, but different from PKABA (Anderberg and Walker-Simmons 1992, Mustilli *et al.* 2002), the *ZmOST1* transcript is constitutively expressed during embryo development and vegetative tissues irrespective of ABA and other stress treatments. We found that this maize kinase is regulated mainly post-translationally at the level of its activity. Thus, during embryogenesis, SnRK2s are activated at around 20 dap and maintain their

activity up to 40 dap, which suggests that these kinases respond to ABA in the transition phase between the morphogenetic and the maturation stage. However, in the final maturation step (>60 dap) the decline in both, ABA endogenous level and sensitivity, coupled to developmental signals could be suppressing SnRK2 activity (Finkelstein *et al.* 1985). Likewise, during germination, SnRK2 activities do not mirror the endogenous ABA levels. Although ABA levels remain high during the first days of germination, SnRK2 induction is markedly low, suggesting that at this stage other hormonal/developmental signals intervene to modulate the activity of these kinases. In seedlings, our results show that ABA and other osmotic stress treatments such as salt, mannitol, drought and glucose activate SnRK2. Salt activation has been previously reported for all rice SnRK2 members and activation by mannitol and low humidity for the *Arabidopsis* kinases confirming that SnRK2s are not exclusively activated by ABA (Kobayashi *et al.* 2004, Yoshida *et al.* 2006, Hauser *et al.* 2011). Interestingly, we also found SnRK2 induction by low concentrations of glucose. It is expected that high levels of exogenous glucose can cause an osmotic stress correlated with ABA increase, which could induce SnRK2 kinases. However, ABA endogenous levels were unchanged in seedlings when treated with low levels of sugars (Garciarrubio *et al.* 1997, Price *et al.* 2003) suggesting that low glucose itself could be creating specific metabolic signals different than those generated through the classical ABA–PYL–PP2C pathway. Therefore, SnRK2 kinases are also involved in carbohydrate homeostasis regulation and energy supply by a mechanism still unknown (Zheng *et al.* 2010, Zhang *et al.* 2011a).

ABA and hyperosmotic stress activation of maize SnRK2 kinases brought us to analyze the cellular function of ZmOST1 in stomatal closure. ZmOST1 complements the

Arabidopsis ost1-2 mutant. This allele carries a missense G33R mutation that renders the kinase catalytically dead, which translates into a strong phenotype of stomatal deregulation in response to both ABA and water stress signals (Mustilli *et al.* 2002). Phenotypic complementation of this allele revealed that ZmOST1 promotes stomatal closure in response to water stress working as a positive regulator in the drought signalling cascade while having no detectable impact on germination. This stomatal closure response might be mediated by direct phosphorylation of ion transporters. However, ABA also elicits a pronounced change in gene expression patterns in guard cell protoplasts (Leonhardt *et al.* 2004, Yang *et al.* 2008, Wang *et al.* 2011). Note that although its predicted major targets, ABF (Sirichandra *et al.* 2010), are also expressed in the guard cell and that together they regulate an estimated 80% of the global ABA transcriptome, the *abf2 abf3 abf4* triple mutant is normal in transpiration (Leonhardt *et al.* 2004, Yang *et al.* 2008, Sirichandra *et al.* 2010, Yoshida *et al.* 2010, Antoni *et al.* 2011, Joshi-Saha *et al.* 2011) suggesting that other intermediates in the OST1-mediated transcriptional cascades in guard cell are still unknown. Among them, we identified ZmSNAC1. Unlike ABF which seem more involved in germination and seedling growth, SNAC proteins have a more prominent role in stomatal regulation. Our results reveal that ZmSNAC1 becomes phosphorylated in response to drought and salt treatments in maize, suggesting that this posttranslational modification is needed to regulate its activation under stress (Takasaki *et al.* 2010). One upstream kinase could well be ZmOST1. ZmOST1/ZmSNAC1 interact in the nucleus (Figure 18), similar to that described for OST1/ABF in *Arabidopsis* (Sirichandra *et al.* 2010). It is possible that ZmOST1 phosphorylates SNAC1 to enhance its transcriptional activity through the regulation of protein stability, in a mechanism that could be similar to what is

described for the ABFs (Fujii *et al.* 2007, Sirichandra *et al.* 2010). Moreover, the results showed that in addition to ZmOST1, an SnRK1-like activity could constitutively phosphorylate ZmSNAC1. SnRK1 protein kinases have also been shown to phosphorylate ABFs and both SnRK1 and SnRK2 kinases share targets of phosphorylation (Kobayashi *et al.* 2005, Furihata *et al.* 2006, Zhang *et al.* 2008). For these reasons, we cannot rule out that SnRK1 may also regulate SNAC1 activity together or alternately with ZmOST1.

The current model of ABA signalling mechanism reconstructed *in vitro* suggests that, in the absence of the hormone, the clade A protein phosphatases 2C inhibit OST1 activity by binding to its kinase catalytic site as well as to the ABA box, an acidic motif of ~25 amino acids at the C-terminus of the kinase. ABA triggers the pathway by binding to the PYL receptor, and the changes in the PYL conformation then allows it to insert its "proline gate" domain into the catalytic site of the phosphatase. This dislodges the kinase but without dissociating the two proteins completely, as the kinase remains tethered by its ABA box to the phosphatase (Soon *et al.* 2012). The attached protein kinase – phosphatase pair is thought to provide a rapidly reversible phospho-relay in regulating the on/off state of the ABA signalling pathway. *In vivo*, however, the association of the PP2C and SnRK2 (particularly OST1) can also be labile, because the complex can only be recovered after treatment of the total soluble protein extract from *Arabidopsis* by chemical cross-linkers (Vlad *et al.* 2009) or transient over-expression of the two proteins in tobacco (Hubbard *et al.* 2010, Nishimura *et al.* 2010). Our current results could explain this labile interaction between the PP2C and OST1 during signalling. If the ABA box is also the contact site for kinase substrates, complete dissociation of PP2C-SnRK would be possible from SNAC1

competition. This may be one mechanism by which the ABA signalling cascade can be sustained required for long-term transcriptional reprogramming in guard cells, as opposed to rapid responses from ion transport.

The rice homologs, *OsNAC5* and *OsNAC6*, highly induced by abiotic stresses are important for stress tolerance acquisition (Ooka *et al.* 2003, Nakashima *et al.* 2007, Takasaki *et al.* 2010, Song *et al.* 2011). Furthermore, over-expression of another homolog, *OsSNAC1*, enhances drought and salt tolerance in transgenic rice. Importantly, over-expressing of SNAC1 did not engender phenotypic changes or yield penalty (Hu *et al.* 2006), which are important considerations for agronomic applications. The identity between rice and maize SNAC1 proteins suggests an evolutionarily conserved functional role in the transcriptional control of stomatal response although further experiments are necessary to demonstrate whether enhanced expression of *ZmSNAC1* would also augment drought tolerance in maize without undesirable phenotypic alterations.

Materials and methods

Plant material

Maize (*Zea mays*) plants of pure inbred line W64A were grown routinely in a greenhouse on soil to collect seeds at the indicated stages of development: 14, 16, 20, 30, 40 and 60 days after pollination (dap). To obtain germinating embryos, mature seeds were sterilized and sown on distilled water-saturated vermiculite layer covered with filter paper for 24 and 48 h (1 and 2 days after imbibition; dai). Daily growing

conditions were the following: 16 h light at 28ºC and 8 h dark at 26ºC. Developing and germinating embryos were extracted from seeds and were immediately frozen in liquid nitrogen. For ABA, salt and mannitol treatments, 5 day-old seedlings were transferred for 3 h to solutions containing 0.5X Murashige and Skoog (MS including vitamins, Duchefa) supplemented with 100 µM ABA, 250 mM NaCl and 400 mM mannitol, respectively. For the drought treatment, the seedlings were taken from the pots and placed on the 3MM paper (Whatman) to dry slowly during 24 h. For activity assays, the leaves and roots from ABA, drought, salt, mannitol and glucose treated seedlings were harvested separately at the selected concentrations. Protein samples were frozen in liquid nitrogen immediately after extraction and stored at -80ºC.

Arabidopsis plants were maintained in controlled growth chambers (24±°C, 16 h:8 h light:dark photoperiod). Seeds were germinated in medium containing 1X MS basal salt mixture supplemented with 0.05% MES and 1% sucrose. Seeds were incubated at 4°C for 3 days to break dormancy prior to germination. ABA treatment was carried out at 100 µM. The *akin10 Arabidopsis* overexpression and RNAi lines were kindly provided by Dr. Elena Baena (Baena-Gonzalez *et al.* 2007).

Full-length ZmOST1 cDNA isolation

A phagemid cDNA library constructed from poly(A)$^+$ RNA maize water-stressed leaves was screened by filter hybridization under high-stringency conditions, using a [α-^{32}P]-labelled partial probe corresponding to the *ZmOST1* gene (ACG36261).

RNA isolation and gel blot analysis

Total RNA was extracted using the method previously described by Logemann *et al.* (1987). RNAs (12 μg for embryos and 20 μg for seedlings) were fractionated by electrophoresis on a formaldehyde-containing agarose gel and blotted to a nylon membrane by standard methods. *ZmOST1* and *RAB17* cDNAs were used as probes in the hybridization.

ZmOST1 polyclonal antiserum production

The recombinant ZmOST1 and ABA-box (325-366 aa) proteins fused to a six histidine tag (6×His) in their amino terminal region were expressed and purified according to the pET manual (Novagen). Purified ZmOST1 fusion protein was used to immunize two rabbits with a primary injection (150 μg) followed by two boosts (150 μg each). Polyclonal antisera were obtained and their titters for detection of ZmOST1 fused protein were estimated with a dot blot analysis on nitrocellulose membranes.

Immunoprecipitation experiments

Maize and *Arabidopsis* proteins were extracted in 50 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM PMSF, 10 μM Leupeptin, 2 μg/ml Aprotinin and 10 μg/ml Pepstatin and cleared twice by centrifugation at 13000 rpm at 4ºC for 15 min. Maize (300 μg) and *Arabidopsis* (500 μg) extracts were incubated with a 1:100 dilution of ZmOST1 or the ABA-box (325-366 aa) antiserum in 300 μl of IP buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 2 mM orthovanadate, 2 mM NaF, 10 mM β-glycerolphosphate, 150 mM NaCl, 0.5% [v/v] Triton X-100, 0.5% [v/v] Nonidet NP40, 1 mM PMSF, 10 μM Leupeptin, 2 μg/ml Aprotinin and 10 μg/ml Pepstatin). After 3 h in

a rotary shaker, 40 µl of protein A-Sepharose CL-4B 50% slurry (GE Healthcare) was added and incubated for another hour. 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 assay

Protein samples were separated on 10% SDS-PAGE gels embedded with 0.25 mg/ml MBP as substrate. In gel kinase assay was performed according to Fujii *et al.* (2007). Gels were washed with 25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg/ml BSA and 0.1% Triton X-100 for 3 × 30 min at room temperature, and proteins were renatured with 25 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF for 2×30 min and overnight at 4ºC. Kinase activity was assayed in 25 mM Tris-HCl pH 7.5, 1 mM DTT, 2 mM EGTA, 0.1 mM Na_3VO_4, 12 mM $MgCl_2$, 250 nM cold ATP and 100 µCi [γ-^{33}P]-ATP at room temperature for 1 h. Finally, gels were washed extensively with 5% [w/v] TCA, 1% Na_2PPi solution at least five times and dried. Radioactivity was detected using a Storm 820 imager (GE Healthcare, http://www.gehealthcare.com). For ZmSNAC1 kinase assay, 0.5 mg/ml of HIS-tagged protein were embedded in the SDS-PAGE gels and 40 µg of maize and *Arabidopsis* extracts were loaded for each lane.

Transgenic Arabidopsis plants generation and analyses

Full-length *ZmOST1* cDNA were amplified by PCR using oligonucleotides 5'-CCGAATTCATGGCAGGGCCGGCGCCG-3' and 5'-GGCTCGAGTCACATTGCGTATACAATCTCAC-3'. The PCR product was cloned into the pGEM-T Easy vector (Promega), digested BamHI /XhoI and subcloned into the pBinAr vector under the CaMV 35S promoter. This construct was used to transform by floral

dip infiltration using *Agrobacterium tumefaciens* ($C_{58}C_1$) wild type (Ler) and *Arabidopsis ost1-2* mutant plants (Mustilli *et al.* 2002). Transgenic seedlings selection was performed in 0.5X MS solid medium supplemented with 1% sucrose, 0.5 g/l MES and 50 mg/l kanamycin at 21ºC under a long-day photoperiod. Five *35S::ZmOST1/ost1-2* homozygous transgenic lines per construct and genetic background were analysed for transgene expression by reverse transcription PCR (RT-PCR) with similar *ZmOST1* expression results opting for lines 211 and 287 for further analyses. The inserted *ZmOST1* transgene was amplified with oligonucleotides 5'-GTAAGAACGTGCGATTCAGTG-3' and 5'-TATCATGCGATCATAGGCGTC-3'. Water-loss experiments were only performed with *ZmOST1* overexpression in the *ost1-2* allele due to the lack of expression obtained in transgenic lines overexpressing *ZmOST1* in the wild-type background.

Water-loss measurements were performed with 2 weeks-old *Arabidopsis* plants grown routinely on soil. For each genotype, three rosettes were detached and weighted during 3 h in intervals of 10 min. Water loss was calculated as percentage of weight at the indicated times in relation to the initial fresh weight. Thermal images of *Arabidopsis* leaves were taken using a Thermacam PM250 infrared camera (Inframetrics) 5 min after they were detached from 1 week old plantlets grown in a phytotron at 21ºC under a long-day photoperiod and 70% relative humidity (Merlot *et al.* 2002).

Yeast two-hybrid screening and in vitro pull-down experiments

Full-length *ZmOST1* cDNA was amplified by PCR and subcloned into the vector pGBT7 (Clontech). A maize cDNA library from 5-day-old leaves water-stressed for 3 h

was constructed using the activation domain expression vector pAD-GAL4 (Kizis and Pages 2002) (Stratagene). *pGBT7-ZmOST1* construct was transformed directly into *Saccharomyces cerevisiae* AH109 strain. Yeast expressing ZmOST1 protein was retransformed with the pADGAL4-cDNA maize library, as previously described (Lumbreras *et al.* 2001, Kizis and Pages 2002). β-galactosidase liquid assays were performed as described by Bhalerao *et al.* (1999). For pull-down experiments, *ZmOST1* and *ZmSNAC1* cDNAs were cloned into pGEX4T (GE Healthcare) and pET28a (Promega) expression vectors, respectively. Expression of ZmOST1-GST fusion protein and binding assays were performed as described by Jimenez *et al.* (1997).

GFP localization and BiFC by confocal microscopy

Full-length *ZmOST1* and *ZmSNAC1* cDNAs were cloned in the PC1302 vector (Clontech) and in the GATEWAY-compatible vector pENTRY3C (Invitrogen). A point mutated G40R ZmOST1 protein and different ZmOST1 protein domains corresponding to the catalytic-osmotic region (1-289 aa), the regulatory domain (290-366 aa), the osmotic SnRK2 box (290-325 aa) and the ABA box (325-366 aa) were also cloned in pENTRY3C vector. The six pENTRY3C plasmids were transferred to pYFN43 and pYFC43 BiFC GATEWAY-modified vectors described in http://www.ibmcp.upv.es/FerrandoLabVectors.php to produce 35S::YC-ZmOST1; 35S::YC-ZmOST1[G40R]; 35S::YC-ZmOST1[1-289]; 35S::YC-ZmOST1[290-366]; 35S::YC-ZmOST1[290-325]; 35S::YC-ZmOST1[325-366] and 35S::YN-ZmSNAC1. *Nicotiana benthamiana* plants were transiently transfected with these constructs. For the co-infiltration, equal volumes of the three *Agrobacterium* cultures (the two truncated YFP

constructs; and the strain expressing the HcPro protein) were mixed (Dunoyer *et al.* 2004). Confocal observations were performed 3 days after infiltration.

In vitro phosphorylation

Purified *E. coli*-expressed ZmOST1-HIS fused protein (500 ng) were incubated at 30ºC for 30 min with either 500 ng of purified ZmSNAC1 and three derivatives corresponding to A, B and C domains (1-100; 101-170; 171-312 aa, respectively) in a final 15 µl volume of 1X kinase buffer (20 mM HEPES pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 5 mM NaF, 125 mM β-glycerolphosphate), 25 µM cold ATP and 5 µCi [γ-^{33}P]-ATP). Relative [α-^{33}P] incorporation was analyzed using the image analysis program ImageJ (http://rsbweb.nih.gov/ij).

Transient Expression Assays Using Maize and Arabidopsis Leaf Protoplasts

Transient expression of maize protoplasts was performed according to Morohashi *et al.* (2012) with some modifications. Protoplasts from 11 to 13 days old etiolated maize seedlings were obtained from kernels of B73 plants. After chopping second or third leaves into small pieces, leaf stripes were digested in 3% cellulase onozuka R10, 0.6% macerozyme R10 (Yakult Honsha Co.), 0.6 M mannitol, 10 mM MES, pH 5.7, 5 mM CaCl2, and 0.1% (w/v) BSA for 15 min under vacuum followed by 2:30 h gentle shaking at 50 rpm in the dark at 28°C. After releasing the protoplasts at 90 rpm for 30 min, the protoplasts were filtered through a 35-mm nylon mesh and collected by centrifugation. The protoplasts were washed twice in 0.6 M mannitol, 5 mM MES, pH 5.7, and 10 mM KCl and counted with a hemocytometer. Electroporation was performed on 1-2x10^5 protoplasts per transformation with 20 µg of plasmid DNA (100

V/cm, 200 µF) with a Biorad Gene Pulser II, high capacitance. After electroporation, protoplasts were incubated for 24 h in the dark at room temperature prior to analysis.

Transient gene expression on *Arabidopsis* mesophyll protoplast was performed acoording to the Sheen lab protocol (Yoo *et al.* 2007) on well-expanded leaves from 3-week-old *Arabidopsis* plants grown on short day conditions (8 h light:16 h dark).

Treatment of maize and *Arabidopsis* protoplast was performed for 30 minutes by adding 10 µM ABA.

Bi-dimensional gel electrophoresis

For two dimensional gel electrophoresis, transfected protoplasts were solubilized in 7 M urea, 2 M thiourea, 4% CHAPS, 4% Triton X-100, 18 mM Tris-HCl pH 8 in the presence of 53 u/ml DNase I, 4.9 u/ml RNaseA, 1 mM PMSF, 50 µM leupeptin, 1 µM pepstatin, 10 µM E-64 and 10 µg/ml aprotinin and cleared by centrifugation at 13000 rpm at 4ºC for 5 min.

Total protein (60µg) was diluted in rehydration solution (8 M Urea, 18 mM Tris-HCl, pH 8.0, 4% w/v CHAPS, 0.5% v/v IPG buffer (pH 3–11), 1.6% v/v DeStreak Reagent (GE Healthcare) and 0.002% w/v Bromophenol Blue) and loaded onto 7 cm IPG strips (NL pH 3–11) (GE Healthcare). Strips were rehydrated for 6 h at room temperature, followed by 6.5 h at 30 V. IEF was performed at 500 V (1 h), 1000 V (1 h) and 8000 V (7 h) using the Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare). Prior to second dimension, strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% v/v glycerol, 2% v/v SDS, a trace of Bromophenol Blue and 10 mg/mL DTT (15 min), followed by a second equilibration step (25 mg/mL iodoacetamide, 15 min).

For the second dimension, proteins were separated on 12% SDS-PAGE gels. Western blot was performed as indicated previously using the Living Colors A. v. Monoclonal JL-8 GFP antibody (Clontech).

3.3 - Chapter 3 - Phosphorylation by CK2 regulates the activity and enhances the proteasome degradation of ZmOST1 kinase during ABA response

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This Chapter is a Manuscript under preparation

Summary

The *Arabidopsis* kinase OPEN STOMATA 1 (OST1) plays a key role in regulating drought stress signalling. This kinase, activated by ABA or osmotic stress, phosphorylates stress-related transcription factors and ion channels, which ultimately leads to the protection of plants from dehydration by guard cell regulation. Many of these functions are apparently regulated by phosphorylation of ZmOST1 although the putative kinases involved in its regulation are still unknown.

In the previous chapters we characterized ZmOST1 both at the biochemical and functional levels and we introduced a new player in the ABA signalling pathway, the ZmSNAC1 transcription factor. In this Chapter 3 we focused our research on the characterization of ZmOST1 regulation, describing the first kinase that acts upstream of ZmOST1 and exploring the ways in which this phosphorylation regulates ZmOST1 activity and protein stability.

We start this chapter by profiling ZmOST1 phosphorylation in maize and also in the *Arabidopsis* heterolog system described in Chapter 2. We determined one kinase that constitutively phosphorylates ZmOST1, characterized and mutagenized the target residues for this phosphorylation and tried to determine its biochemical and biological significance. We then characterized transgenic lines overexpressing the wild type ZmOST1 and a mutagenized version for stomatal closure under ABA treatment.

We show that CK2 directly phosphorylates ZmOST1 at multiple sites in the ABA domain. We mapped four plant conserved serine residues scattered throughout the ABA-box of ZmOST1 and found that serine to alanine mutation at all four sites (ZmOST1[AA]) eliminates CK2-mediated phosphorylation of ZmOST1. Localization studies revealed an interaction between ZmOST1 and CK2α catalytic subunit as well as a re-localization of ZmOST1 by CK2β in nuclear speckles, followed by the degradation of ZmOST1. Moreover, the rate of degradation of ZmOST1[AA] was highly reduced compared with wild-type ZmOST1 in *Arabidopsis* transgenic plants indicating that CK2-mediated phosphorylation enhances degradation of ZmOST1 to regulate stomata closure. Mutation at these four serines also drastically reduces (50%) ZmOST1 interaction with ZmABI1 with implication on kinase activity and confers a higher sensitivity to ABA. Thus, in the plants overexpressing ZmOST1[AA], stomata closure was higher than in the wild-type under ABA treatment suggesting that this mutant is accelerating ABA-dependent stomata closure by increasing ZmOST1 stability and activity. Taken together, these data show that CK2 phosphorylates ZmOST1 and that this posttranslational modification is important for ZmOST1 functions in the ABA signalling pathway.

Introduction

Plant growth and crop productivity are compromised by environmental stresses such as extreme temperatures, drought and high salinity where the phytohormone abscisic acid (ABA) has a protective effect. ABA also regulates seed dormancy, seedling development and stomata aperture (Schroeder and Nambara 2006, Finkelstein *et al.* 2008). The molecular mechanism of ABA perception and signal transduction has been intensively studied in *Arabidopsis* (Klingler *et al.* 2010, Umezawa *et al.* 2010) and reversible protein phosphorylation is one of the major mechanisms for modulating its intracellular adaptations (Droillard *et al.* 2002, Chinnusamy *et al.* 2004, Franz *et al.* 2011). In fact, the ABA signal can stimulate – within minutes – regulators such as Sucrose non-fermenting related kinase (SnRK)-2 subfamily (Yoshida *et al.* 2002), central to achieve physiological responses to acquire drought tolerance.

About ten SnRK2 genes exist in the *Arabidopsis*, rice and maize genomes (Li *et al.* 2000, Mustilli *et al.* 2002, Yoshida *et al.* 2002, Kobayashi *et al.* 2004, Huai *et al.* 2008). In *Arabidopsis*, except SnRK2.9, the kinase activity of each member of the family is activated by hyperosmotic stress (Boudsocq *et al.* 2004) with class three formed by SnRK2.2, SnRK2.3 and SnRK2.6/OST1/SRK2E, also activated by ABA (Boudsocq *et al.* 2004). A similar situation occurs in rice, in that the activities of three of the ten SnRK2 homologs (called SAPKs) are also stimulated by ABA (Kobayashi *et al.* 2004). Studies of mutants deficient in SnRK2.2 and SnRK2.3 activities showed that these kinases are required for ABA-mediated seed germination, dormancy and seedling growth but have minor roles in stomatal control (Fujii *et al.* 2007). Conversely, SnRK2.6/OST1 is mainly involved in ABA-mediated stomatal closure in response to humidity decrease with a

minor role during seed germination (Mustilli *et al.* 2002, Yoshida *et al.* 2002, Fujii and Zhu 2009, Zheng *et al.* 2010). The existence of different mechanisms underlying SnRK2 activation by osmotic and ABA treatments is supported by structural analyses. Thus, the C-terminal part of ABA-dependent SnRK2 contains two conserved subdomains (Figure 5), the SnRK2 domain required for activation by both osmotic and ABA treatments whereas the ABA domain specifically involved in ABA-responsiveness at the biochemical (kinase activation) and physiological levels (limitation of water loss in low-humidity conditions). This ABA domain, only present in class three SnRK2, is involved in the interaction with group A of PP2Cs, whose members include ABI1 and HAB1 in *Arabidopsis* (Yoshida *et al.* 2006, Hirayama and Shinozaki 2007). Genetic and biochemical characterizations revealed that this group of phosphatases are physically associated with and desphosphorylate OST1 at the T-loop hence impeding kinase activity (Yoshida *et al.* 2006, Umezawa *et al.* 2009, Soon *et al.* 2012). A similar mechanism is proposed in maize where ZmOST1 kinase is required in diverse stress signal transduction pathways, principally in drought and ABA responses (Huai *et al.* 2008, Ying *et al.* 2011).

Despite the advancement in the understanding of ABA perception and ABA-regulated inhibition of PP2Cs (Yoshida *et al.* 2006, Umezawa *et al.* 2009, Soon *et al.* 2012), the molecular basis of SnRK2 activation remains poorly understood. OST1 is rapidly phosphorylated in response to ABA suggesting that a mechanism of phosphorylation is required in the control of its function. However, the putative kinase(s) and domains involved are still unknown. So far, only the SnRK2 phosphorylation at the T-loop has been characterized (Blatt 2000, Roelfsema and

Hedrich 2005, Joshi-Saha *et al.* 2011). Mutation in the T-loop changing S175 into alanine completely abolishes the *in vitro* activity of recombinant OST1 and its ability to complement the *srk2e* mutant (Belin *et al.* 2006), and S175 and S171 are necessary for the full activation of the kinase by both hyperosmolarity and ABA (Vlad *et al.* 2010). However, Laurière and co-workers already speculated on the involvement of other kinases and in particular protein kinase CK2 in the regulation of OST1 (Boudsocq *et al.* 2007).

The CK2 protein is a serine/threonine kinase known to be ubiquitously expressed and highly conserved in eukaryotes (Meggio and Pinna 2003). The CK2 holoenzyme consists of two catalytic α subunits and two regulatory β subunits. The *Arabidopsis* genome has four α subunit and four β subunit genes, and members of both the α and β subunit families have been shown to be localized in the cytoplasm, nucleus and also in chloroplasts, although its localization is predominantly nuclear (Salinas *et al.* 2006). In maize, three and four genes for each CK2α and CK2β subunits, respectively, have been described to localize in the nucleus (Dobrowolska *et al.* 1992, Riera *et al.* 2001, Riera *et al.* 2011). Localization studies showed that CK2α is present mainly in the nucleolus whereas CK2β is aggregated in nuclear speckles (Riera *et al.* 2011).

The large number of cellular substrates described for CK2 highlight the important role it plays in maintaining cell homeostasis at different levels. To date, CK2 is known to phosphorylate more than 300 cellular proteins (Meggio and Pinna 2003), among them proteins involved in relevant cellular processes such as plant growth, development and abiotic stress responses (Lee *et al.* 1999, Riera *et al.* 2004, Portolés

and Más 2007, Moreno-Romero *et al.* 2008). In the case of abiotic stress responses, it is also known that the CK2 *α1α2α3* triple mutant is hyposensitive to ABA and NaCl at the level of seed germination and cotyledon greening (Mulekar *et al.* 2012).

Mechanistically, CK2 can use ATP or GTP as phosphoryl donors (Niefind *et al.* 1999). Phosphorylation occurs specifically at serine or threonine residues on target proteins, although CK2 shows a higher propensity to phosphorylate serine residues (Meggio and Pinna 2003). The general consensus motif for a CK2 phosphorylation site was found to be S/T-X-X-E/D. Although maize CK2 proteins are well studied at the structural level due to its biochemical characteristics, high stability and high specific activity (Riera *et al.* 2003), little is known about its putative substrates.

Maize (*Zea mays*) is an important food and feed crop worldwide, with more than 800 million tons cultivated annually, and about 130 million tons in the USA also being diverted for energy. However, maize requires high water input. For this reason, characterization of ZmOST1 function and its regulation directly involved in maize water homeostasis is of huge economic importance as bouts of water shortage are becoming more frequent. In the present work, we have characterized the regulation by phosphorylation of the maize ortholog of the *Arabidopsis* OST1 showing that CK2- interacts and mediates phosphorylation of its ABA domain at multiple sites. CK2 phosphorylation negatively regulates OST1 stability and function, promoting a rapid degradation of ZmOST1 to fine-tune ABA-stomata closure. Thus, CK2 represents a new regulatory member of ZmOST1 that opens the possibility to better understand how ZmOST1 mediates stomatal closure.

Results

CK2 phosphorylates ZmOST1 at the ABA domain

SnRK2s are one of the three key components in the ABA core signalling model that includes the soluble ABA receptors PYR/PYL/RCAR and the negative regulating clade A PP2Cs (Ma *et al.* 2009, Park *et al.* 2009, Cutler *et al.* 2010). These three protein types are necessary and sufficient to mediate an ABA triggered model signalling cascade *in vitro* (Fujii *et al.* 2009). This lead researchers to assume that after repression of the PP2C phosphatases by ABA, SnRK2s are activated by autophosphorylation (Belin *et al.* 2006, Ng *et al.* 2011).

However, so far no efforts were conducted to determine whether SnRK2s could be phosphorylated by other upstream kinases. To test this hypothesis we used maize extracts treated or not with ABA to detect kinase activities toward recombinant ZmOST1 protein. Using in gel kinase assays with ATP as a phosphate donor our results revealed three constitutive bands (Figure 23A), two bands around 60-kDa and an intense band of 42-kDa not inducible by the ABA treatment. These results suggest that in maize OST1 is phosphorylated by upstream kinases. We then demonstrated, by repeating the in gel kinase assay using GTP as a phosphate donor, that the 42-kDa band that appears in these gels is CK2 (Niefind *et al.* 1999)

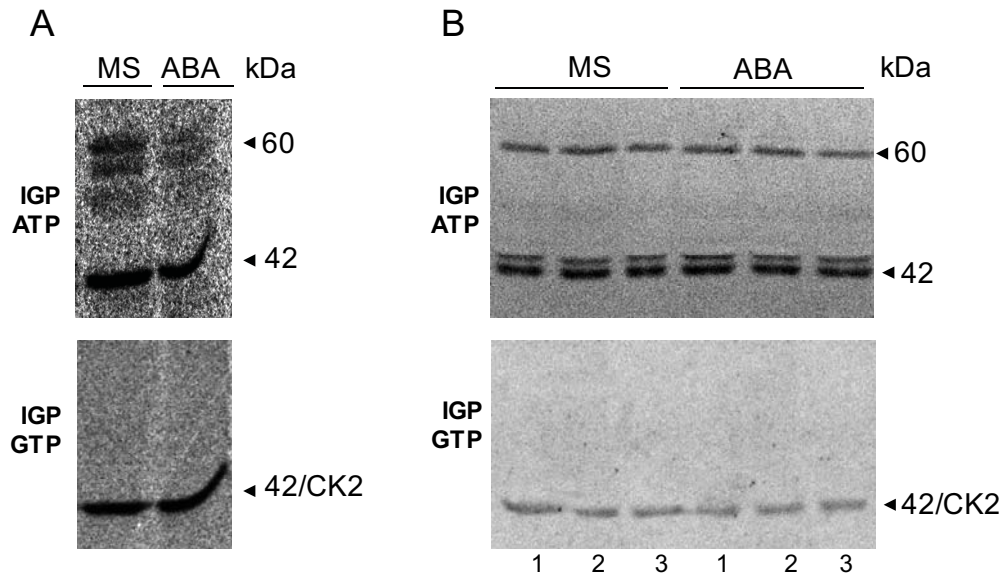

Figure 23 - ZmOST1 is phosphorylated by upstream kinases. Recombinant ZmOST1 was used as a substrate for in gel phosphorylation assays (IGP) with proteins extracted from **(A)** 7 day-old maize or **(B)** *Arabidopsis* seedlings (Lane 1, Ler wild-type seedlings; lane 2, *ost1-2* mutant; lane 3, *35S::ZmOST1/ost1-2* transgenic line) treated or not with 100 µM ABA. Sizes of the activity kinase bands obtained are shown on the right. The lower panel consists of the same extracts run on in gel kinase assays using GTP as a phosphate donor and confirming CK2 as an upstream kinase of ZmOST1 on both systems.

To further validate these results we performed in gel phosphorylation assays using an ortholog *Arabidopsis* system, determining the phosphorylation pattern of recombinant ZmOST1 in Lansberg wild-type, the *ost1-2* mutant (Mustilli *et al.* 2002) and a *35S::ZmOST1/ost1-2* complementation line (Figure 15B).

In these gels, three phosphorylation bands are present, one around 60-kDa and two around 42 kDa (Figure 23B). Again, only the 42-kDa band is maintained when the gel is repeated using GTP as a phosphate donor, confirming that upstream phosphorylation of ZmOST1 by CK2 is conserved between these plant species.

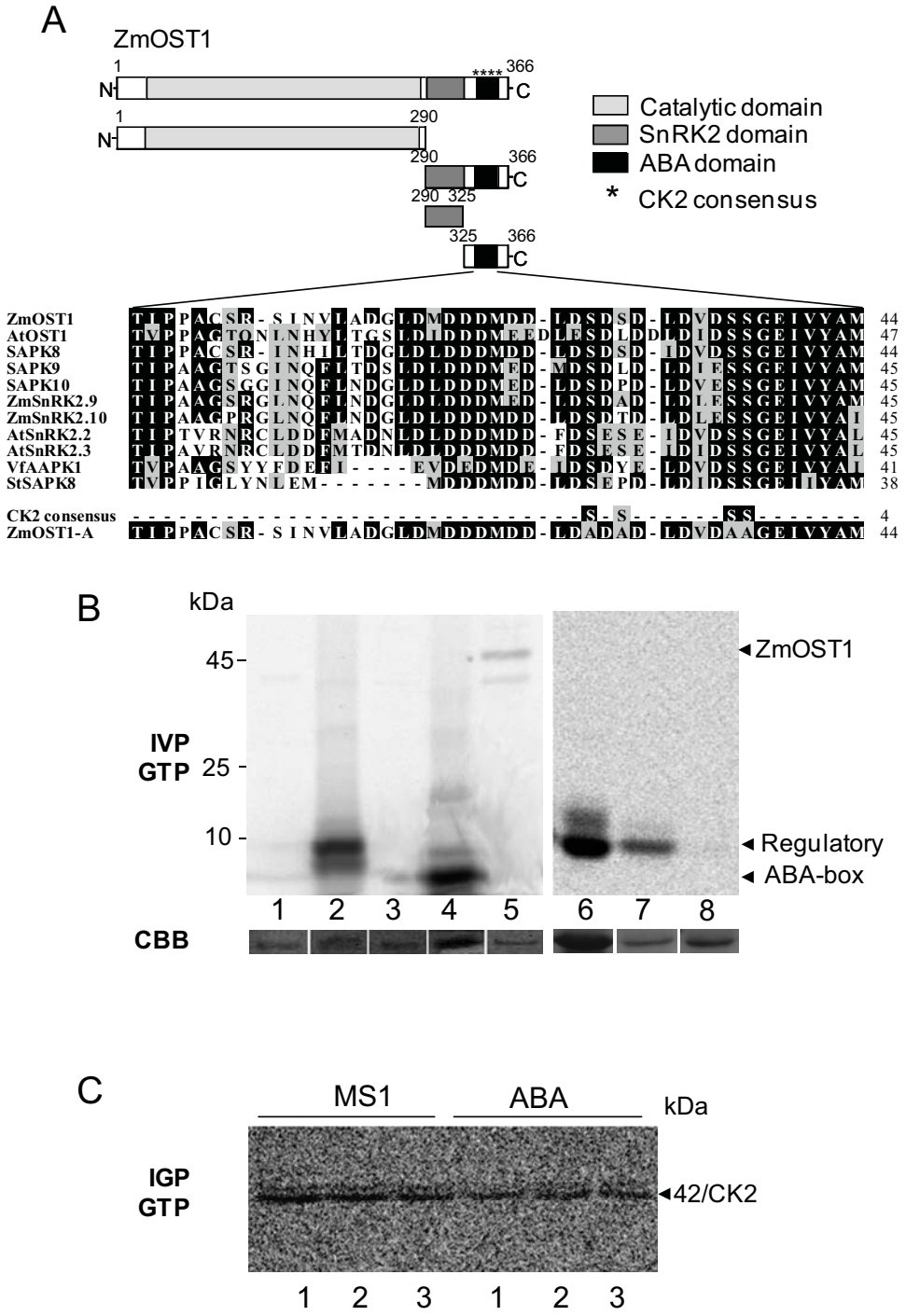

Figure 24 - CK2 phosphorylates ZmOST1 on the ABA regulatory subdomain. **(A)** Schematic representation of ZmOST1 domains used in the experiment and alignment of sequences from different plant species. Asterisks represent consensus sequences for CK2 activity that were mutagenized changing S350A, S352A, S358A and S359A. **(B)** Phosphorylation of ZmOST1 by CK2 and deletion forms is tested *in vitro* (IVP) and corresponding protein Coomasie Brilliant Blue-staining (CBB). 1-8, phosphorylation by CK2 of: 1, ZmOST1[1-290], 2, ZmOST1[290-366]; 3, ZmOST1[290-325]; 4, ZmOST1[325-366]; 5, ZmOST1; 6, AtOST1[316-362]; 8, ZmOST1[290-366]; 9; ZmOST1[290-366AA]. **(C)** In gel phosphorylation assay (IGP) using recombinant ZmOST1[290-325] and GTP as the phosphate donor indicating CK2 maintains its phosphorylation of ZmOST1 at the ABA subdomain. Lane 1, Ler wild-type seedlings; lane 2, *ost1-2* mutant; lane 3, *35S::ZmOST1/ost1-2* transgenic line

We then proceeded to map the phosphorylation domains of ZmOST1 that are targets of CK2. With this purpose we over-expressed in *E. coli* the full length ZmOST1 and the different domains of this kinase (Figure 24A), the catalytic domain (aa 1-290), the regulatory domain (aa 290-366), the SnRK2 subdomain (aa 290-325) and the ABA subdomain (aa 325-366). We also obtained an over-expressed version of the regulatory domain mutagenized in the four putative sites of CK2 activity. After *in vitro* phosphorylation of these different recombinant proteins with ZmCK2α1 using GTP as the phosphate donor, we were able to determine that ZmCK2 activity toward recombinant ZmOST1 occurs mostly at the ABA box (Figure 24B). This phosphorylation is maintained when we repeat the *in vitro* assay using the recombinant AtOST1 regulatory domain (aa 316-362) and is abolished in the mutagenized regulatory domain. Repeating the in gel kinase assay using the ABA subdomain as a substrate we were able to confirm CK2 phosphorylation on this portion of ZmOST1 (Figure 24C).

Casein kinase II is a ubiquitous protein that is involved in multiple plant signalling pathways including ABA and osmotic stress (Riera *et al.* 2004, Mulekar *et al.* 2012). In addition, Boudsocq *et al.* (2007) already speculated on the involvement of CK2 in the regulation of OST1 so we decided to pursue the characterization of ZmOST1 in respect to ZmCK2.

Mutagenesis of ZmOST1 in the ABA domain reduces ZmOST1 phosphorylation under ABA treatment

Since CK2 is capable of phosphorylating ZmOST1 at the ABA-box we were interested in determining whether the post translation modifications of ZmOST1 and of a mutagenized version of the kinase on the potential CK2 phosphorylation loci - ZmOST1[AA] - were different under control situations and ABA treatment.

With this intent, we overexpressed ZmOST1-HA and ZmOST1[AA]-HA in *N. benthamiana* and the degree of phosphorylation attained by these proteins *in vivo* was assessed by western blot analysis of 1-D and 2-D SDS PAGE gels of agroinfiltrated total protein extracts using an anti-HA antibody.

By SDS PAGE followed by western blot we could detect the presence of two bands on the extracts that overexpressed ZmOST1-HA and only one band in the extracts overexpressing ZmOST1[AA]-HA (Figure 25A). This result gives some indication that ZmOST1 undergoes a post-translational modification *in planta* that is highly reduced for the ZmOST1[AA]-HA protein.

To better study this post-translational modification we performed 2-D SDS PAGE analyses of the same extracts and we were able to detect a shift of the ZmOST1 protein in comparison to ZmOST1[AA] (Figure 25B). This shift to the acidic part of the strip indicates a post-translational modification that is consistent with a more phosphorylated wild-type ZmOST1 than the mutagenized kinase, especially under ABA treatment. These results give *in vivo* evidence for ZmOST1 phosphorylation by CK2 and point to an increase of this phosphorylation when ABA is present.

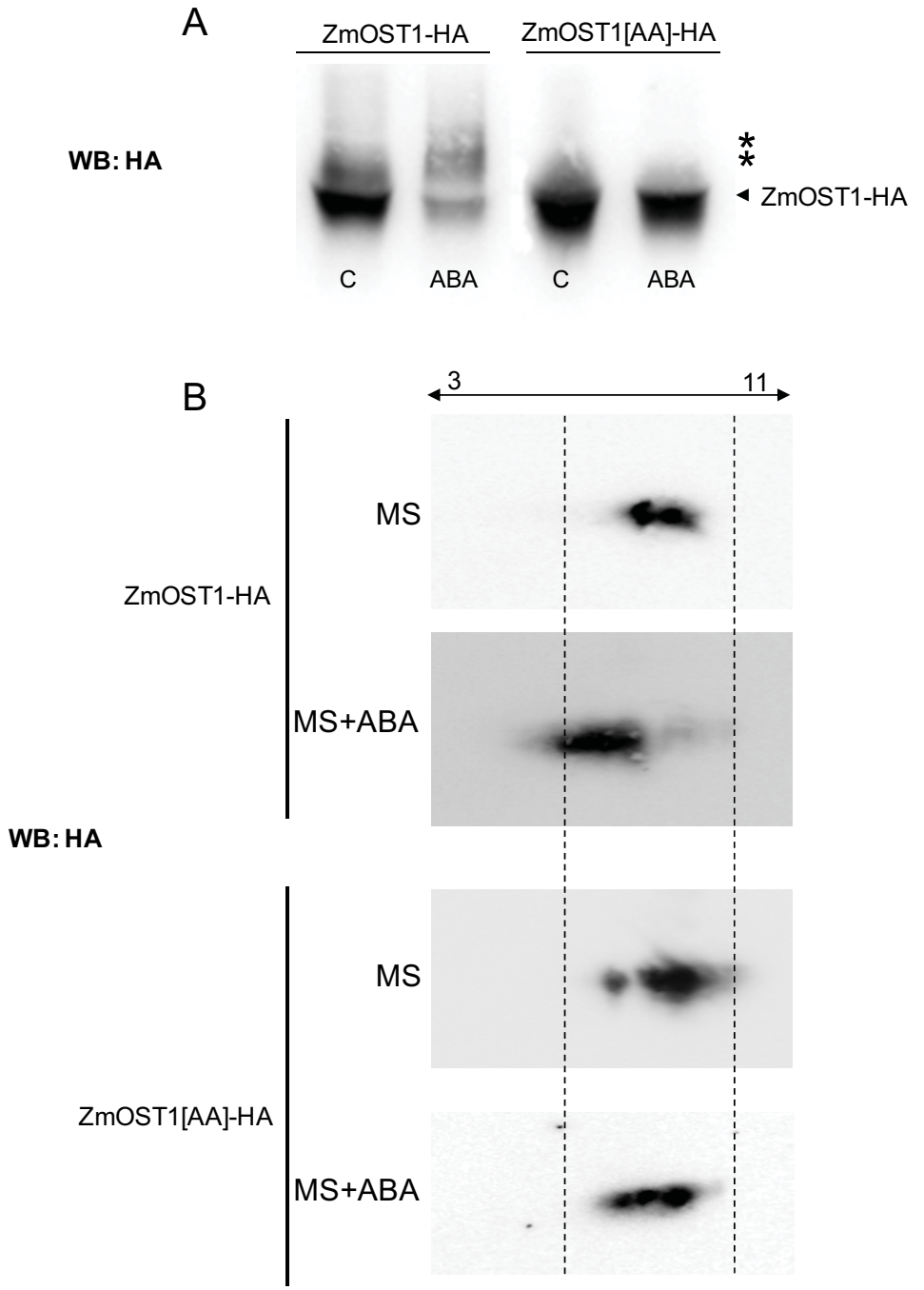

Figure 25 - ZmOST1 is phosphorylated *in vivo* after ABA treatment. **(A)** Western blot (WB) of total protein extracts from tobacco leaves overexpressing either ZmOST1-HA or ZmOST1[AA]-HA. Asterisks represent bands absent on the ZmOST1[AA]-HA construction that are present in the overexpression of the wild-type ZmOST1 kinase indicating its higher phosphorylation status. **(B)** Two-dimensional electrophoresis of the same extracts followed by western blot (WB) analysis. A shift to the acidic part of the strip is clear on the wild-type ZmOST1 kinase that is absence in the mutated ZmOST1[AA], further confirming that the phosphorylation of ZmOST1 after ABA treatment is mostly dependent on these mutagenized residues.

Mutagenesis of ZmOST1 in the ABA domain affects the localization and protein stability when co-expressed with ZmCK2

To determine whether ZmCK2 is involved in ZmOST1 regulation we started by monitoring the changes in the subcellular localization of ZmOST1-GFP and ZmOST1[AA]-GFP. In agroinfiltrated *N. benthamiana* both versions of the OST1 protein localize to the nucleus and cytosol of epidermal cells (Figure 26A, left). It should be noted that the fluorescent levels of the mutagenized ZmOST1[AA]-GFP are higher than the wild-type kinase.

When co-infiltrated with ZmCK2α1-MYC, the appearance of cytosolic aggregates is clear in the case of ZmOST1-GFP but is almost absent in the case of ZmOST1[AA]-GFP (Figure 26A, center). This localization is consistent with the reconstruction of the CK2 holoenzyme that is assembled in the nucleus and exported to the cytosol in insoluble aggregates (Riera *et al.* 2011).

When the two forms of the kinase are co-infiltrated with ZmCK2β1-MYC, ZmOST1-GFP localization is exclusive to the nucleus with both a diffuse pattern and accumulated in small nuclear speckles. ZmOST1[AA]-GFP maintains its cytosolic and nuclear localization even though, in this last organelle, it appears accumulated in large speckles (Figure 26A, right). No nuclear speckles were detected when co-infiltrating ZmCK2β1-MYC with GFP. This change of ZmOST1 localization, together with previous results (Riera *et al.* 2011) that described the same speckled nuclear localization for ZmCK2β2, gives a strong indication that ZmOST1 is indeed an *in vivo* substrate of ZmCK2.

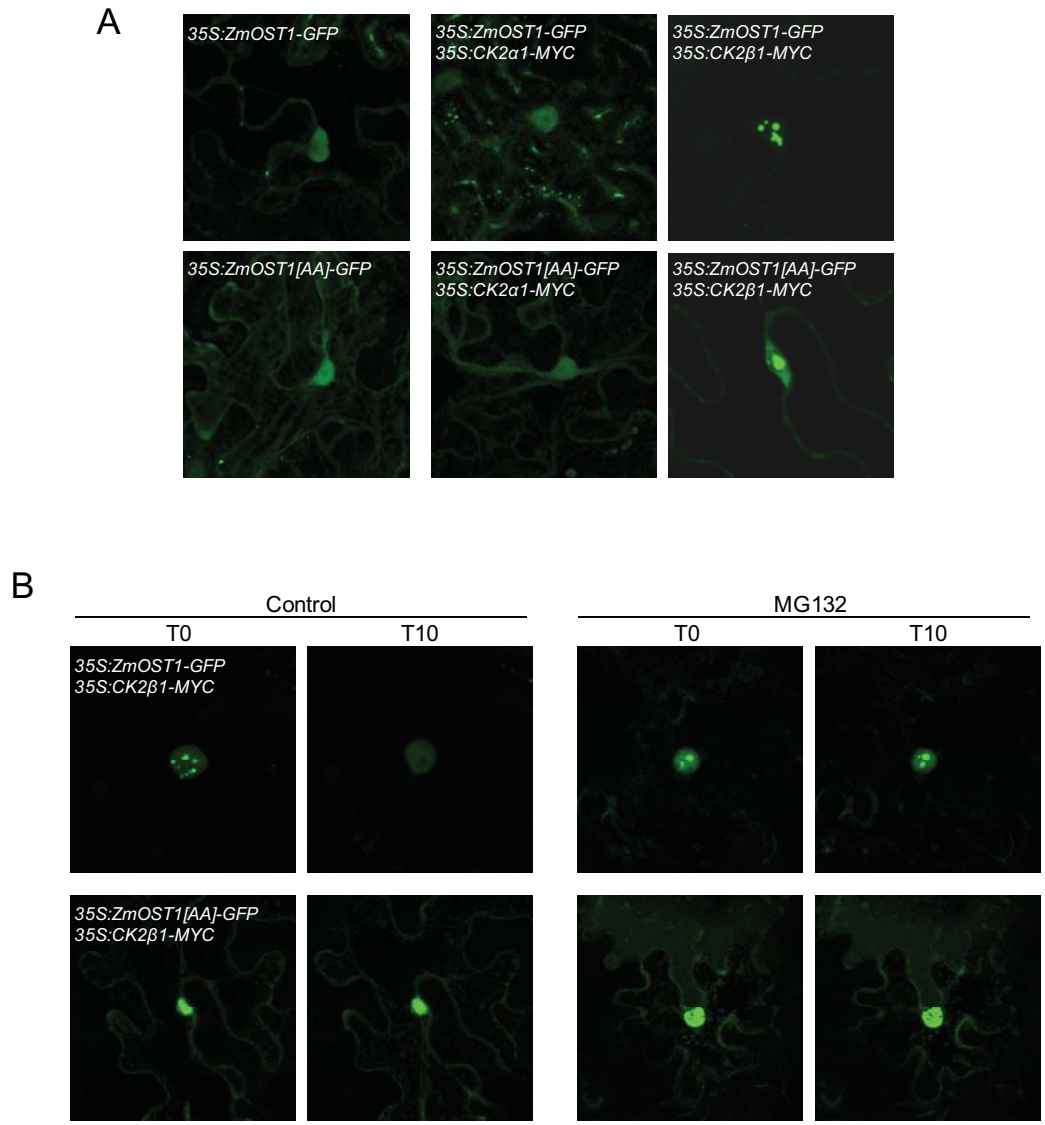

Figure 26 - ZmOST1 localization changes when co-expressed with either ZmCK2α1 or ZmCK2β1. **(A)** Both ZmOST1-GFP and ZmOST1[AA]-GFP have a nuclear and cytosolic localization (left). When co-expressed with ZmCK2α1, ZmOST1-GFP localization remains nuclear and cytosolic but the appearance of cytosolic speckles is clear. This speckled localization is highly reduced in the ZmOST1[AA]-GFP (center). When co-expressed with ZmCK2β1, ZmOST1-GFP localization is exclusively nuclear, accumulating in nuclear speckles. ZmOST1[AA]-GFP maintains its cytosolic and nuclear localization but in this last organelle large speckles of protein accumulation are observable (right). **(B)** Time course of ZmOST1-GFP and ZmOST1[AA]-GFP co-expressed with ZmCK2β1 by confocal microscope imaging (t=10 min). During these 10 min, the nuclear speckled ZmOST1-GFP disappears and fluorescence levels rapidly decrease. No change is observable in ZmOST1[AA]-GFP localization or stability (left). Adding MG132 to the samples during the confocal imaging maintains the speckled nuclear localization of ZmOST1-GFP while unchanging the ZmOST1[AA]-GFP protein. Both proteins seem to have a stronger signal at the end of the observation period (right).

Aside from the change in localization we also detected a different stability of ZmOST1-GFP and ZmOST1[AA]-GFP when co-expressed with ZmCK2β2-MYC. Whereas the speckled localization of ZmOST1-GFP disappears in less than 10 minutes of observation (Figure 26B, upper left) with a marked decrease in total fluorescence, ZmOST1[AA]-GFP fluorescence levels and localization remain constant during the same period (Figure 26B, lower left). This led us to consider that ZmOST1 might be triggered for degradation by CK2 activity on the ABA subdomain residues. We repeated this 10 minute observation of our constructs co-infiltrated with ZmCK2β2-MYC adding the proteasome inhibitor MG132. As expected, protein degradation stops to occur and even an increase in the amount and intensity of the nuclear speckles is visible in this short period for ZmOST1-GFP (Figure 26B, upper left). No visible changes was detected for ZmOST1[AA]-GFP apart from a slight increase of fluorescence (Figure 26B, lower right). These results suggest ZmOST1[AA] is less susceptible to degradation by the proteasome than the wild-type ZmOST1.

Mutagenesis of ZmOST1 in the ABA domain affects the interaction with clade A PP2C phosphatases

ZmOST1, as well as its *Arabidopsis* ortholog, has been known to directly interact with clade A PP2C phosphatases (Belin *et al.* 2006, Yoshida *et al.* 2006, Vlad *et al.* 2009, Soon *et al.* 2012). This interaction is, among other loci (Soon *et al.* 2012), done through the ABA subdomain of the kinase, approximately at the same position of CK2 phosphorylation activity (Yoshida *et al.* 2006, Vlad *et al.* 2009, Vilela *et al.* 2012). We decided to test whether the mutagenesis of ZmOST1 ABA subdomain would alter this

interaction with the PP2C phosphatases by bimolecular fluorescent complementation (BiFC) experiments (Weinthal and Tzfira 2009).

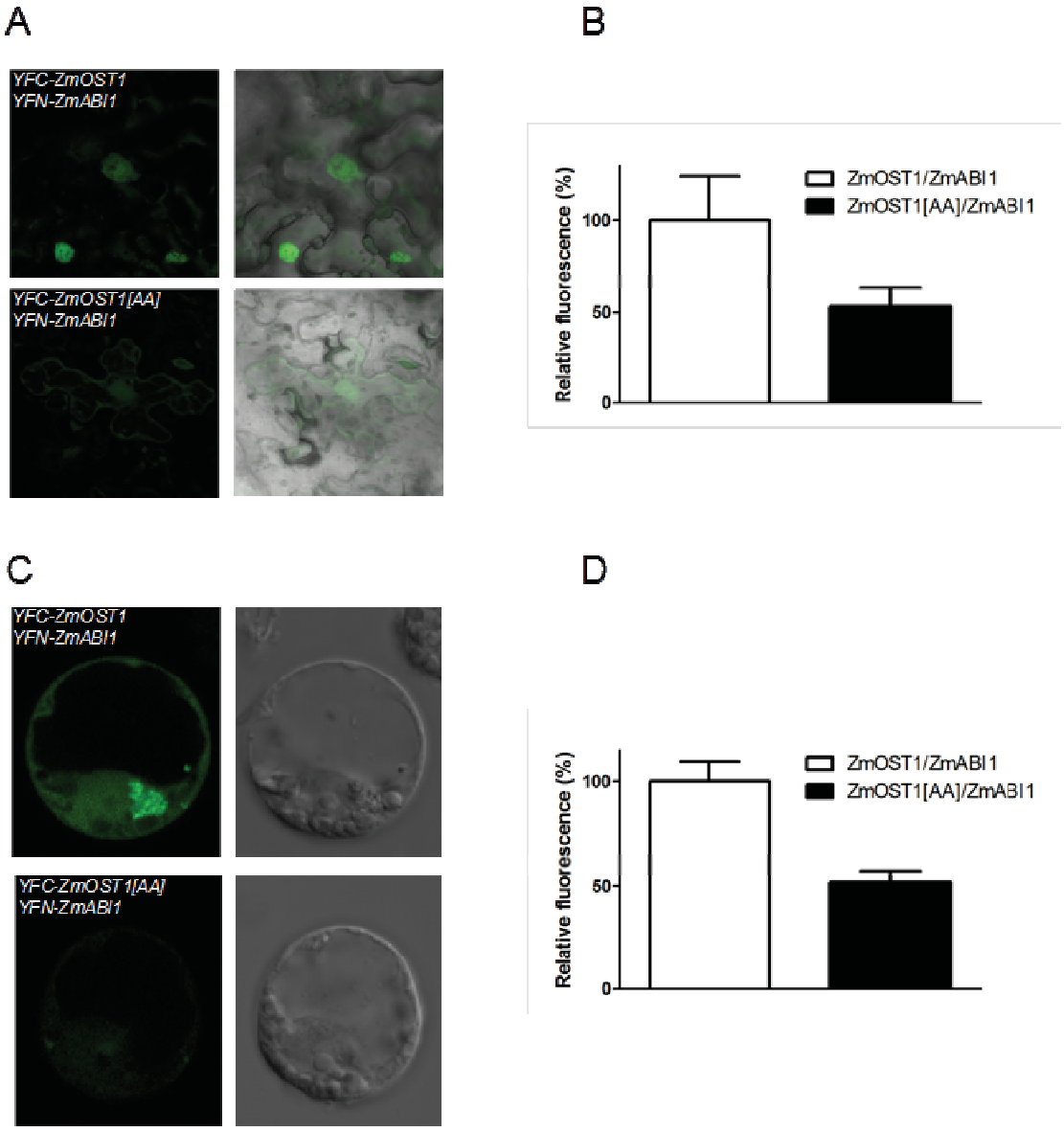

Figure 27 - ZmOST1 interaction with ZmABI1 is reduced by 50% for the mutagenized ZmOST1[AA] kinase. BiFC fluorescence images analysed by confocal microscopy of the interaction between YC-ZmOST1/YC-ZmOST1[AA] with YN-ZmABI1 in agroinfiltrated tobacco **(A)** and transformed maize leaf protoplasts **(C).** Corresponding relative fluorescence quantification **(B, D)** was performed with the ImageJ software on 10 individual microscope fields (n=10).

We co-expressed in *N. benthamiana* (Figure 27A) and maize leaf protoplasts (Figure 27C) ZmOST1 and ZmOST1[AA] fused to the C-terminal half of the YFP and the full length ZmABI1 fused to the N-terminal half and detected the reconstitution of the YFP signal by confocal microscopy. As can be seen in Figure 27A, interaction between ZmOST1 and ZmOST1[AA] with ZmABI1 can be detected at the nucleus and cytosol of agroinfiltrated tobacco epidermal cells. However, the YFP signal is stronger for ZmOST1 than for the mutagenized kinase (Figure 27B), especially in the nucleus. Using tranformed maize leaf protoplasts (Figures 27C and 27D), the same reduction of fluorescence (50%) is detected when comparing ZmOST1 and ZmOST1[AA] interaction with ZmABI1 by BiFC.

These results indicate that ZmOST1 capacity to interact with ZmABI1 is somewhat impaired in the mutagenized kinase and gives additional insight on how CK2 may regulate OST1.

Mutagenesis of ZmOST1 in the ABA domain protects the kinase from degradation and confers hypersensitivity to ABA

To test the physiological relevance of ZmOST1 phosphorylation by CK2 we decided to transform *Arabidopsis* with either a construction overexpressing ZmOST1-HA or ZmOST1[AA]-HA. After checking for the expression of both proteins by western blot we performed a cell free degradation assay to determine whether the tobacco ZmOST1 degradation results were reproducible in the *Arabidopsis* system. Total protein extracts were maintained at room temperature for 0.5, 1, 3, 7 hours with or without MG132. As shown in Figure 28A, ZmOST1-HA starts to get degraded 3h after

protein extraction while the ZmOST1[AA] protein is still present 7 h after the beginning of the experiment. Using MG132, both proteins show the same pattern of degradation. These results further corroborate the evidence that ZmOST1[AA] is more stable and resilient to degradation than the wild-type kinase and that ZmCK2 activity triggers ZmOST1 to degradation.

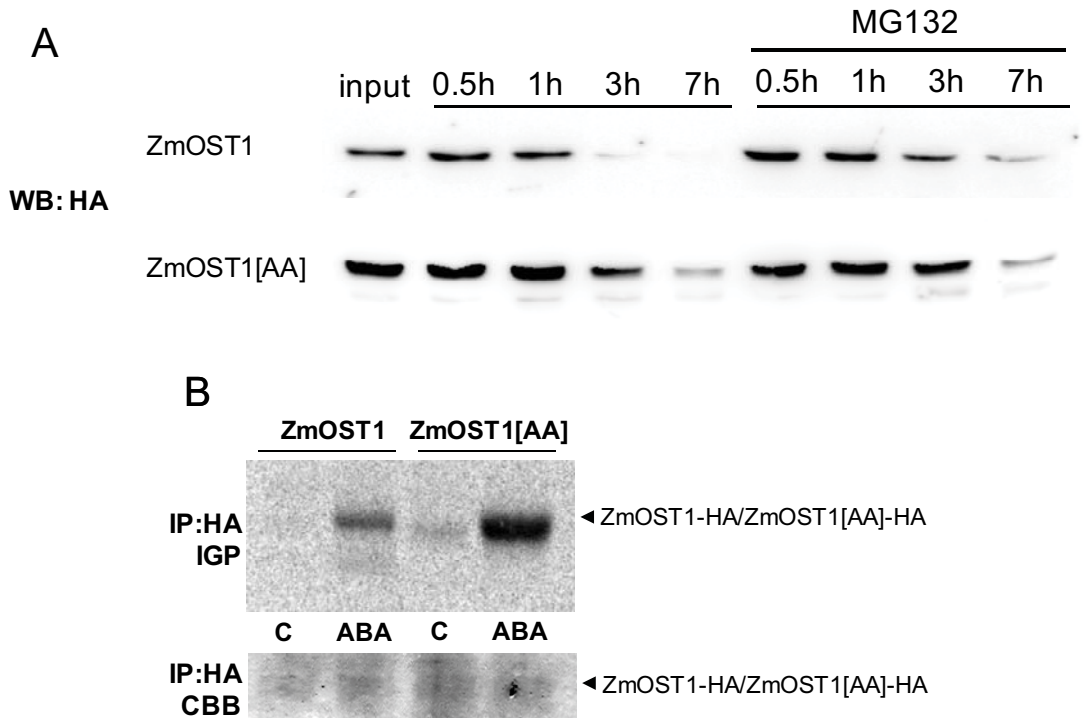

Figure 28 - ZmOST1[AA] is protected from degradation and confers hypersensitivity to ABA when compared with ZmOST1. **(A)** Cell free degradation experiment of transgenic plants overexpressing ZmOST1 or ZmOST1[AA] and analysed by western blot (WB). ZmOST1 degradation after 1h of incubation is dependent on the mutagenized ABA subdomain OST1 residues and ZmOST1[AA] mimics the inhibition of the proteasome by MG132. **(B)** Immunoprecipitation (IP) of ZmOST1-HA and ZmOST1[AA]-HA on transgenic *Arabidopsis* plants overexpressing one of these constructions, followed by in gel phosphorylation assay (IGP) using MBP as the substrate. Coomasie Brilliant Blue staining (CBB) of the IP is used as loading control. ZmOST1[AA]-HA has an increased activity under 100 µM ABA compared to ZmOST1. In control situations, absent to low levels of ABA are sufficient to activate ZmOST1[AA] activity.

To test whether ZmCK2 phosphorylation affects ZmOST1 activity we performed in gel kinase assays using MBP as a substrate. Comparing the activity bands of

immunoprecipitated ZmOST1-HA and ZmOST1[AA] (Figure 28B), an increased phosphorylation band of the ABA activated mutagenized version is clear when compared with the wild-type kinase. Furthermore, this activity band is visible in the control immunoprecipitated ZmOST1[AA], indicating that this form of the kinase is active under absent to low levels of ABA.

Since ZmOST1, as well as its *Arabidopsis* counterpart, has already been implicated in stomatal closure under dehydration conditions (Mustilli *et al.* 2002) we decided to compare the stomata aperture of these transgenic plants overexpressing ZmOST1 or ZmOST1[AA]. The experiments were performed at midday, a time when stomata are more responsive to ABA (Robertson *et al.* 2009). As can be seen in Figure 29A, *Arabidopsis* plants over-expressing ZmOST1[AA]-HA have a higher rosette temperature when compared to plants over-expressing ZmOST1-HA by infrared thermography. Measuring the stomata closure of detached leaves from the same transgenic plants (Figures 29B, 29C and 29D) we were able to detect a hypersensitivity to ABA on the ZmOST1[AA] plants, when compared with the wild-type ZmOST1 kinase. Even in levels as low as 0.5 µM ABA, when no stomata response is visible in the transgenic *Arabidopsis* lines overexpressing ZmOST1, a 20% closure of the stomates is measurable in the transgenic *Arabidopsis* plants overexpressing ZmOST1[AA]-HA.

Taken together these results point to an increased accumulation and activity of ZmOST1[AA] when compared with ZmOST1 probably caused by the higher stability of the mutagenized kinase, with significant results in ABA responsiveness and enhanced stomata response to ABA.

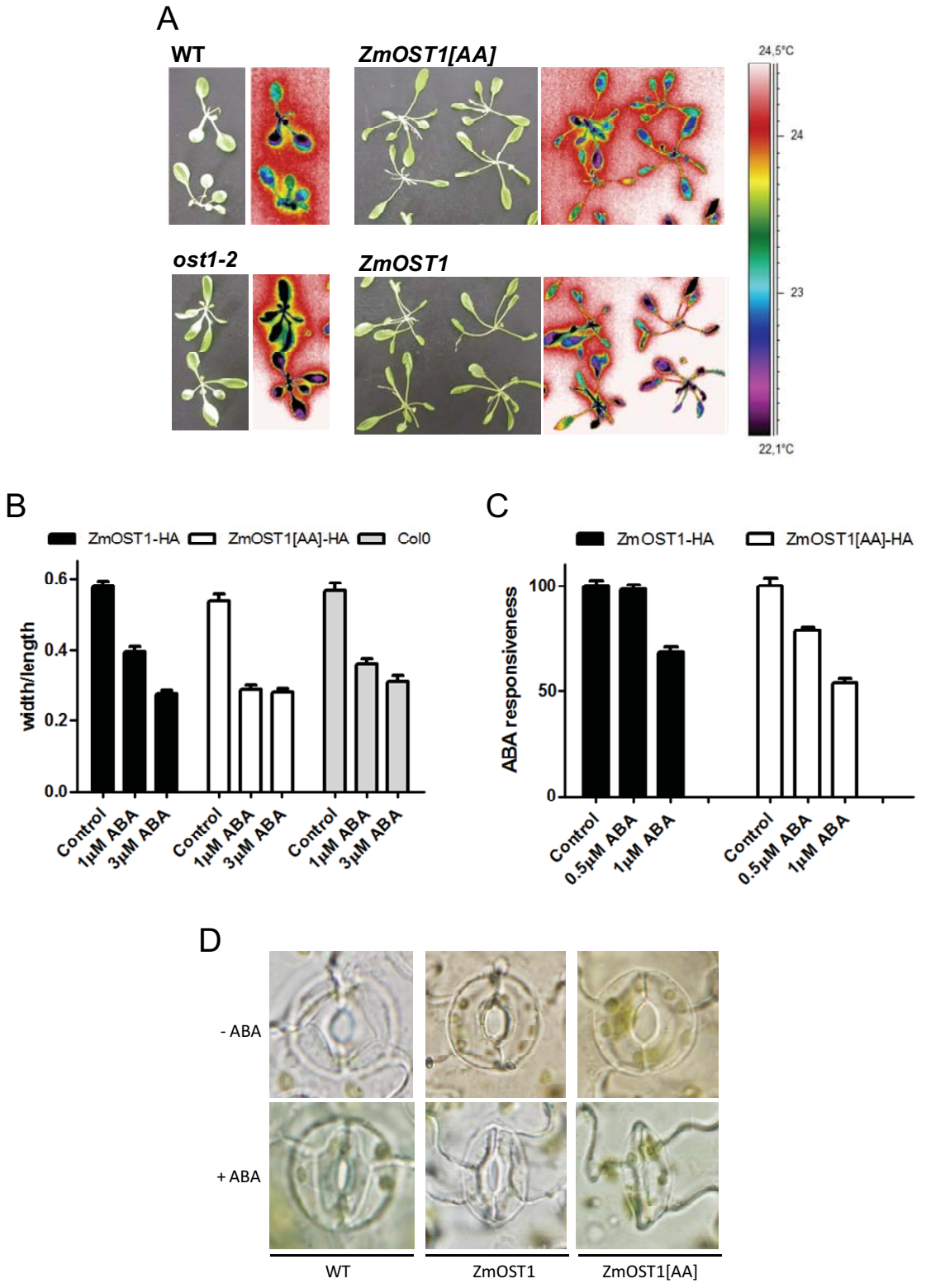

Figure 28 - ZmOST1[AA] confers hypersensitivity to ABA at the level of stomata regulation. **(A)** Infrared thermography comparing rosette temperature of transgenic *Arabidopsis* plants overexpressing ZmOST1 and ZmOST1[AA]. **(B, C, D)** Stomata closure measurement of detached leaves from the same transgenic lines under different ABA treatments.

Discussion

Over the last years, significant progress has been made toward understanding the molecular basis for ABA signal transduction in plants (Joshi-Saha *et al.* 2011). This has confirmed that SnRK2-related kinases, with OST1 being the founding member in *Arabidopsis*, as key elements in these responses (Raghavendra *et al.* 2010, Umezawa *et al.* 2010, Kulik *et al.* 2011). OST1 is rapidly activated by hyperosmotic stress and also by ABA (Boudsocq *et al.* 2004, Kobayashi *et al.* 2004, Boudsocq *et al.* 2007). Here, to study the mechanism by which this kinase is regulated by upstream kinases during ABA responses, we focused on ZmOST1. Between the different kinases that work upstream the identification of CK2 kinase as a ZmOST1 interacting partner raises the possibility that ABA signalling is regulated by phosphorylation of ZmOST1 by CK2. Thus, the results presented in this work support the hypothesis that CK2-mediated phosphorylation contributes to the control of ZmOST1 stability providing a novel route for regulation of ABA responses homeostasis.

Previous studies showed that treatment with alkaline phosphatase completely abolished the activation of OST1 by hyperosmolarity or ABA indicating the essential role of phosphorylation (Boudsocq *et al.* 2007). This result was also supported by an increased phosphorylation level of OST1 after stress activation (Vlad *et al.* 2009). Consistent with this data Umezawa *et al.* (2009) performed immunoprecipitation of OST1-GST overexpressed in *Arabidopsis* mesophyll protoplasts and reported the presence of several phosphorylated sites after ABA treatment. In our study, analysis of endogenous plant kinases allowed the identification of maize CK2 kinase, a ubiquitous

Ser/Thr kinase present in all organisms, upstream of ZmOST1. Phosphorylation by CK2 appears to be necessary for the degradation of ZmOST1 controlled by the proteasome. Several lines of evidence suggest that ZmOST1 is a bona fide substrate for CK2. First, *in silico* studies predicted that ZmOST1 has multiple CK2 phosphorylation sites (Figure 30). Second, in gel kinase assays using ZmOST1 as a substrate and GTP as a phosphor donor indicated CK2 as an upstream kinase of ZmOST1 (Figure 23). Third, *in vitro* kinase assays confirmed that ZmOST1 is strongly phosphorylated by purified CK2α and β subunit holoenzyme combinations (Figure 24). Furthermore, mapping of the phosphorylation sites identified four serines scattered through the C-terminal end of ZmOST1 as a cluster of loci at the ABA domain that is conserved between maize and *Arabidopsis*. Fourth, profiling the changes in post-translational modifications indicated that CK2 activity is important for ZmOST1 phosphorylation under ABA treatment (Figure 25). Fifth, subcellular localization experiments in tobacco co-infiltrated with maize CK2α and β subunits revealed the translocation of ZmOST1 to cytosol aggregates and nuclear speckles, respectively, and subsequent degradation of ZmOST1 protein by a mechanism dependent of the CK2 sites (Figure 26). Sixth, interaction experiments between ZmOST1 and ZmABI1 indicate a potential stabilizing role for CK2 (Figure 27) since the ZmOST1[AA] mutated kinase has a 50% reduced interaction with ZmABI1. Seventh, CK2-mediated phosphorylation promotes ZmOST1 degradation resulting in a reduced protein accumulation when compared with a mutagenized ZmOST1[AA] that lacks CK2 phosphorylation loci, which in turn is reflected in a decreased OST1 activity (Figure 28) and a reduction in ABA–induced stomata closure (Figure 29). Taken together these data provide strong evidence that ZmOST1 is a substrate for maize CK2

and that CK2 phosphorylation plays a role in the ABA-induced stomata closure by regulating ZmOST1 stability.

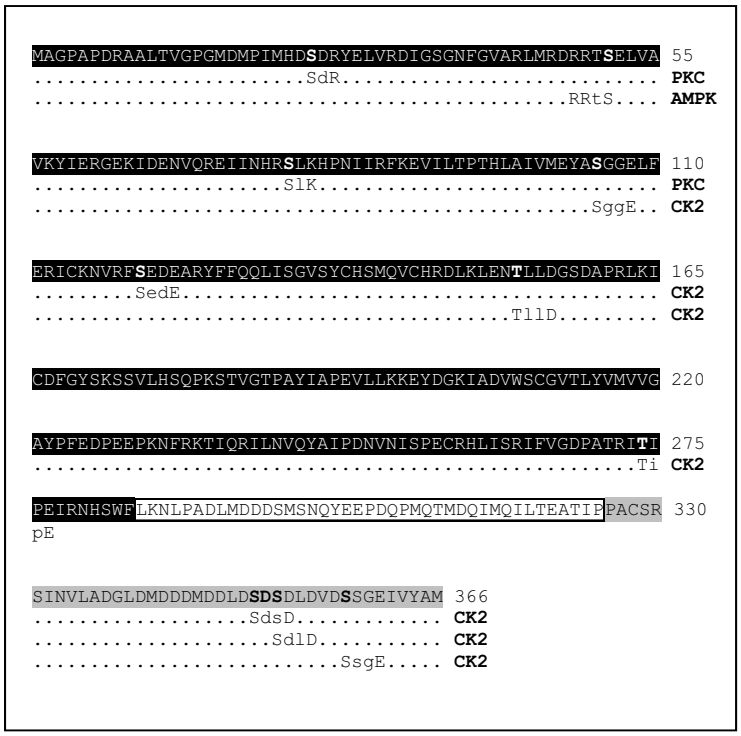

Figure 30 – *In silico* prediction of the putative phosphorylation loci on the ZmOST1 protein sequence done with the web-based software Netphos (Blom *et al.* 1999). CK2 potentially phosphorylates ZmOST1 at multiple sites, especially in the ABA box

Different CK2 substrates have been identified in plants including translation initiation factors (e.g. eIF2α, eIF2β) (Dennis and Browning 2009, Dennis *et al.* 2009), a chromatin remodelling enzyme (histone deacetylase 2B) (Dennis and Browning 2009), circadian clock components (e.g. CCA1 and LHY) (Sugano *et al.* 1999, Daniel *et al.* 2004, Portolés and Más 2007), HMBG proteins from maize and *Arabidopsis* (Stemmer *et al.* 2003), transcription factors involved in light signalling pathway (e.g. HY5, HFR1) (Hardtke *et al.* 2000, Park *et al.* 2008) and the ABA responsive protein Rab17 in maize (Riera *et al.* 2004). In all these cases, phosphorylation by CK2 has been shown to either

stabilize or modulate the activity of these factors (Hardtke *et al.* 2000, Park *et al.* 2008). In contrast, our data show that CK2 promotes the degradation of ZmOST1 through the ubiquitin/26 S proteasome pathway. This is similar to the posttranslational regulation of PIF1 were CK2 promotes the light-induced degradation of this protein also through the proteasome pathways (Bu *et al.* 2011) and to several mammalian examples where CK2-mediated phosphorylation enhanced polyubiquitination and proteasome degradation (Kato *et al.* 2003, Scaglioni *et al.* 2006). Therefore, CK2 mediated stabilization and destabilization of proteins by CK2 might represent an evolutionarily conserved mechanism.

Although our data provide strong evidence that CK2 promotes degradation of ZmOST1 *in vivo*, the ZmOST1 protein is still stable and this is co-related with the increased activity of the mutated ZmOST1[AA] which lacks the majority of the CK2 phosphorylation sites. These data suggest that either CK2 phosphorylates ZmOST1 at other sites than the ones identified and mutated, or a separate kinase is necessary for OST1 activation after its de-repression by the PYL/PYR/RCAR-PP2C complex in the presence of ABA. Our results show that, in addition to CK2, a SnRK1-like activity could constitutively phosphorylate ZmOST1 (Figure 31). It is known that SnRK1 protein kinases share many phosphorylation targets with SnRK2 kinases (Kobayashi *et al.* 2005, Furihata *et al.* 2006, Zhang *et al.* 2008) and that the consensus for SnRK1 and SnRK2 is similar. This could have interesting repercussions in the phosphorylation of the ZmOST1 T-loop and add an additional layer of regulation to this kinase type, asides from autophosphorylation. Further work is still required to completely elucidate this interplay between SnRK1 and SnRK2 kinases.

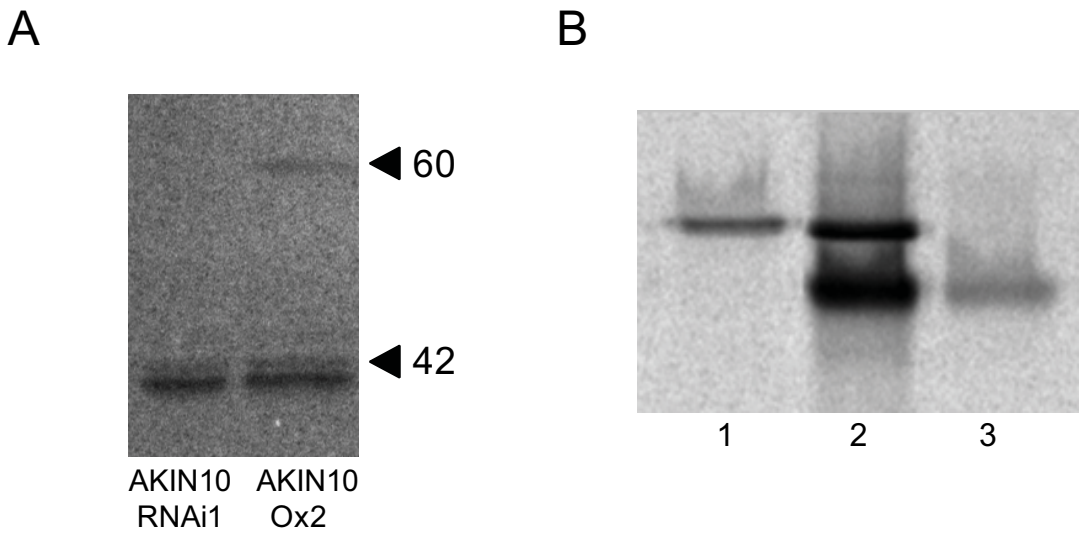

Figure 31 - Phosphorylation of ZmOST1 by SnRK1. **(A)** In gel kinase assay with proteins extracted from seedlings of AKIN10-RNAi (RNAi1) and *35S::AKIN10-HA* (OX2) transgenic lines (Baena-Gonzalez *et al.* 2007) using ZmOST1 as substrate. Sizes of activity bands are shown on the right. The 60 Kd band represents the kinase activity of AKIN10 (Zhang *et al.* 2009), suggesting that it is likely to phosphorylate ZmOST1 protein *in vivo*. **(B)** *In vitro* assay of 1- AMPK authophosphorylation, 2 – AMPK/ZmOST1 transphosphorylation; 3 – ZmOST1 autophosphorylation. The increase in the signal detected in the transphosphorylation experiment indicates that ZmOST1 and AMPK are capable of phosphorylating each other *in vitro*

Because OST1 phosphorylation at the T-loop is necessary for ABA-induced activation (Belin *et al.* 2006, Vlad *et al.* 2009), it is required that OST1 or other upstream kinases directly phosphorylate this site independently of CK2. This contrasts with the results that pointed CK2 as the putative upstream kinase of SnRK2 since its activation by both osmotic and ABA treatments was insensitive to the protein kinase inhibitor staurosporine. However, convincing *in vivo* evidence for the CK2 T-loop phosphorylation is lacking and our data only suggest that CK2 is necessary for induced degradation of ZmOST1 and stabilization of the interaction with PP2C phosphatases. Further work is necessary to identify if CK2 has an additional function in the regulation of ABA induced T-loop phosphorylation.

In addition, the mechanism of CK2-mediated enhanced degradation of ZmOST1 is still unknown. The results show that the CK2 sites at the C-terminal end of ZmOST1 (Ser-350, Ser-352, Ser-358 and Ser-359) play the major role in ZmOST1 degradation. This region corresponds to the ABA-domain where the interaction between SnRK2 and PP2Cs occurs (Yoshida *et al.* 2006, Vlad *et al.* 2009, Soon *et al.* 2012) suggesting that this region could be modulating the interaction with this group of phosphatases. Our results show that ZmOST1 interaction with ZmPP2C is reduced by the mutation of the four serine residues to alanine revealing a potential role of CK2 in modulating OST1 activity. Indeed, this reduced interaction with ZmPP2C of the mutated kinase could also explain its increased activity and hypersensitivity to ABA that we report here. Alternatively, CK2-mediated phosphorylation of ZmOST1 at the ABA-box could enhance the interaction of ZmOST1 with substrate recognition factors responsible for polyubiquitination and subsequent degradation. In fact, enhancement of proteasome degradation of multiple factors by signal-induced phosphorylation has been demonstrated (Karin and Ben-Neriah 2000, Kato *et al.* 2003) and the proteolysis via the *26S* proteasome of critical regulators of other plant hormones was established in the case of gibberellins (Griffiths *et al.* 2006), auxin (Dharmasiri *et al.* 2005), and jasmonate (Chini *et al.* 2007) which made some authors speculate that one or more proteins of the ABA receptor-signal complex and its downstream targets might be regulated by proteolysis (Klingler *et al.* 2010). Identification of factors responsible for recognition and polyubiquitination of ZmOST1 could help to elucidate this point.

Finally, ZmOST1 plant homologues are highly induced by abiotic stresses and important for stress tolerance acquisition in different crop plants (Zhang *et al.* 2010,

Ying *et al.* 2011, Zhang *et al.* 2011b). The identity between the different OST1 genes in the serines responsible for the CK2 activity suggests an evolutionarily conserved role in the regulation of these pathways, in particular the control of stomata response, essential for the adaptation of plants to land environments. Furthermore, the overexpression of ZmOST1 also has implications in carbohydrate homeostasis regulation and energy supply by affecting different sugar pathways increasing oil metabolism, which are important considerations for agronomic applications. The increased accumulation and activity of the CK2 mutated ZmOST1 isoform may have effects not only on the tolerance to drought but also on other biotic and metabolic signalling pathways that are OST1 dependent, vastly enhancing the potential use of this gene in crop improvement programs.

Materials and methods

Plant material and growth conditions

Maize (Zea mays) plants of pure inbred line W64A were germinated in liquid 0.5X MS medium and grown for one week in controlled chambers at 26ºC 16h/8h light/dark photoperiod. Treatment was performed in seven-day-old plants on 0.5X MS medium supplemented with 100 µM ABA.

Arabidopsis plants were maintained in controlled growth chambers (24±°C, 16 h:8 h light:dark photoperiod). Seeds were germinated in medium containing 1X MS basal salt mixture supplemented with 0.05% MES and 1% sucrose. Seeds were

incubated at 4°C for 3 days to break dormancy prior to germination. ABA treatment was carried out at 100 µM on one-week-old plants.

Material was deep frozen immediately after treatment and stored at -80ºC.

Protein extraction and western blot

Maize, tobacco, and *Arabidopsis* proteins were extracted in 50 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 1 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM PMSF, 10 µM Leupeptin, 2 µg/ml Aprotinin and 10 µg/ml Pepstatin and cleared twice by centrifugation at 13000 rpm at 4ºC for 15 min.

For western blot analysis, approximately 40 µg of total protein were loaded per lane and transferred to a nitrocellulose membrane. Homogenous protein transfer was confirmed by Pounceau red staining. Anti-HA antibody (Roche) was used to detect ZmOST1-HA and ZmOST1[AA]-HA. Anti-GFP antibody (Invitrogen) was used to detect ZmOST1-GFP and ZmOST1[AA]-GFP.

In-gel kinase assay

Protein samples were separated on 10% SDS-PAGE gels embedded with 0.25 mg/ml MBP as sustrate. In gel kinase assay was performed according to Fujii *et al.* (2007). Gels were washed with 25 mM Tris-HCl pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg/ml BSA and 0.1% Triton X-100 for 3 × 30 min at room temperature, and proteins were renatured with 25 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF for 2×30 min and overnight at 4ºC. Kinase activity was assayed in 25 mM Tris-HCl pH 7.5, 1 mM DTT, 2 mM EGTA, 0.1 mM Na_3VO_4, 12 mM $MgCl_2$, 250 nM cold ATP and 100 µCi [γ-^{33}P]-ATP at room temperature for 1 h. Finally, gels were

washed extensively with 5% [w/v] TCA, 1% Na_2PPi solution at least five times and dried. Radioactivity was detected using a Storm 820 imager (GE Healthcare, http://www.gehealthcare.com). For ZmOST1 and ZmOST1[290-325] kinase assays, 0.5 mg/ml of HIS-tagged protein were embedded in the SDS-PAGE gels and 40 µg of *Arabidopsis* extracts were loaded for each lane.

In vitro phosphorylation

Purified *E. coli*-expressed ZmOST1-HIS fused protein (500 ng) were incubated at 30ºC for 30 min with 500 ng of CK2-alpha (KinaseDetect) in a final 15 µl volume of 1X kinase buffer (20 mM HEPES pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 5 mM NaF, 125 mM β-glycerolphosphate), 25 µM cold ATP and [γ-^{33}P]ATP (3000 Ci mmol^{-1}). After incubation, 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 [γ-^{33}P] incorporation was analyzed using the public domain image analysis software ImageJ (http://rsbweb.nih.gov/ij).

Transgenic Arabidopsis plants generation and analyses

To determine the effect of CK2 phosphorylation on ZmOST1 we performed multiple-site mutagenesis (primers in Table 3) on the putative loci of CK2 action with the changes S350A, S352A, S358A and S359A, to generate the ZmOST1[AA] sequence.

Full-length *ZmOST1* and *ZmOST1[AA]* cDNAs were cloned in the pMENCHU vector to generate 2x*35S::ZmOST1-HA* and 2x*35S::ZmOST1[AA]-HA* respectively. The pMENCHU cassette containing the desired sequence was subsequently digested with KpnI and cloned into the PC1300 (Clontech) vector for plant transformation. These constructs were used to transform *Arabidopsis* by floral dip infiltration using

Agrobacterium tumefaciens ($C_{58}C_1$). Transgenic seedlings selection was performed in 1X MS solid medium supplemented with 1% sucrose, 0.5 g/l MES and 50 mg/l hygromicine b (Duchefa) at 21ºC under a long-day photoperiod. Two *35S::ZmOST1-HA* and *35S::ZmOST1[AA]-HA* homozygous transgenic lines per construct were selected for further analysis.

Primer	Sequence
ZmOST1 Fw	CCGAATTCATGGCAGGGCCGGCGCCG
ZmOST1 Rev	GGCTCGAGTCACATTGCGTATACAATCTCAC
Mut1 Fwd	GATGTTGACGCCGCCGGTGAGATTGTGTACG
Mut1 Rev	CAATCTCACCGGCGGCGTCAACATCAAGATCTG
Mut2 Fwd	GATCTTGATGCCGACGCAGATCTTGATGTTGAC
Mut2 Rev	CATCAAGATCTGCGTCGGCATCAAGATCATCCATG

Table 3 – List of primers used for mutagenizing the ZmOST1 sequence

Two-dimensional gel electrophoresis

For two dimensional gel electrophoresis, plant tissue was ground in liquid nitrogen and crude protein extracts were solubilized in 7 M urea, 2 M thiourea, 4% CHAPS, 4% Triton X-100, 18 mM Tris-HCl pH 8 in the presence of 53 u/ml DNase I, 4.9 u/ml RNaseA, 1 mM PMSF, 50 µM leupeptin, 1 µM pepstatin, 10 µM E-64 and 10 µg/ml aprotinin and cleared twice by centrifugation at 13000 rpm at 4ºC for 15 min.

Total protein (60µg) was diluted in rehydration solution (8 M Urea, 18 mM Tris-HCl, pH 8.0, 4% w/v CHAPS, 0.5% v/v IPG buffer (pH 4–7), 1.6% v/v DeStreak Reagent (GE Healthcare) and 0.002% w/v Bromophenol Blue) and loaded onto 7 cm IPG strips (NL pH 4–7) (GE Healthcare). Strips were rehydrated for 6 h at room temperature, followed by 6.5 h at 30 V. IEF was performed at 500 V (1 h), 1000 V (1 h) and 8000 V (7 h) using the Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare). Prior to

second dimension, strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% v/v glycerol, 2% v/v SDS, a trace of Bromophenol Blue and 10 mg/mL DTT (15 min), followed by a second equilibration step (25 mg/mL iodoacetamide, 15 min). For the second dimension, proteins were separated on 12% SDS-PAGE gels. Western blot was performed as indicated previously.

GFP localization and BiFC by confocal microscopy

Full-length *ZmOST1* and *ZmOST1[AA]* cDNAs were cloned in the PC1302 vector (Clontech). The CK2α1 and CK2β1 cDNAs were cloned in the pLOLA vector to produce *2x35S::CK2α1-MYC* and *2x35S::CK2β1-MYC*. The pLOLA cassette containing the desired sequence was subsequently digested with KpnI and cloned in the PC1300 (Clontech) vector for plant transformation.

For BiFC experiments, full-length *ZmOST1, ZmOST1[AA]*, and ZmABI1 cDNAs were cloned in the GATEWAY-compatible vector pENTRY3C (Invitrogen). The three pENTRY3C plasmids were transferred to pYFN43 and pYFC43 BiFC GATEWAY-modified vectors described in http://www.ibmcp.upv.es/FerrandoLabVectors.php to produce *35S::YC-ZmOST1*, *35S::YC-ZmOST1[AA]*, and *35S::YN-ZmABI1*.

Nicotiana benthamiana plants were transiently transfected with these constructs and with a *35S::HCPro* construct to inhibit protein silencing in tobacco. For the co-infiltration, equal volumes of the *Agrobacterium* cultures were mixed (Dunoyer *et al.* 2004). Confocal observations were performed 2 days after infiltration using an Olympus FV1000 (Olympus) confocal microscope.

Transient Expression Assays Using Maize Leaf Protoplasts

Transient expression of maize protoplasts was performed according to Morohashi *et al.* (2012) with some modifications. Protoplasts from 11 to 13 days old etiolated maize seedlings were obtained from kernels of B73 plants. After chopping second or third leaves into small pieces, leaf stripes were digested in 3% cellulase onozuka R10, 0.6% macerozyme R10 (Yakult Honsha Co.), 0.6 M mannitol, 10 mM MES, pH 5.7, 5 mM CaCl2, and 0.1% (w/v) BSA for 15 min under vacuum followed by 2:30 h gentle shaking at 50 rpm in the dark at 28°C. After releasing the protoplasts at 90 rpm for 30 min, the protoplasts were filtered through a 35-mm nylon mesh and collected by centrifugation. The protoplasts were washed twice in 0.6 M mannitol, 5 mM MES, pH 5.7, and 10 mM KCl and counted with a hemocytometer. Electroporation was performed on 1-2x10^5 protoplasts per transformation with 20 μg of plasmid DNA (100 V/cm, 200 μF) with a Biorad Gene Pulser II, high capacitance using the above mentioned BiFC constructs (*35S::YC-ZmOST1*; *35S::YC-ZmOST1[AA]* and *35S::YN-ZmABI1*). After electroporation, protoplasts were incubated for 24 h in the dark at room temperature prior to confocal imaging.

Cell free degradation assays

For cell-free degradation assays, seven-day-old seedlings were ground in liquid nitrogen, resuspended in degradation buffer (25 mM Tris–HCl, pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 4 mM PMSF, 10 mM ATP) and cleared twice by centrifugation at 13000 rpm at 4ºC for 15 min. Equal amounts of extracts were transferred to individual tubes and incubated at room temperature with or without 40 μM MG132 (Sigma) for the indicated times. Reactions were stopped by adding protein gel-loading buffer.

Measurements of guard cell dimensions and thermal imaging

Detached rosette leaves from 3-week-old plants grown under short day conditions (8 h light:16 h dark) were incubated for 2 h in a buffer containing 50 mM KCl and 10 mM MES, pH 6.15 with cool white fluorescent light (50 µmol m−2 s−1). ABA was subsequently added in the same buffer solution at different concentrations (0, 0.5, 1 and 3 µM) and leaves were incubated for 2 additional hours with the hormone. Leaf abaxial epidermis were carefully taken with tweezers and mounted with the same solution. Epidermis strips were observed with a Zeiss AxioPhot microscope with a × 63 objective. Approximately 100–200 stomata per sample were photographed using an Olympus DP70 camera attached to the microscope. Stomatal aperture was scored as width:length pore ratio using ImageJ software and normalized to the control situation (100%).

Thermal images of *Arabidopsis* rosettes were taken using a Thermacam PM250 infrared camera (Inframetrics) on 2 week old plants grown in a phytotron at 21ºC under a long-day photoperiod and 70% relative humidity (Merlot *et al.* 2002).

4 - General discussion

4 - General discussion

Plant growth and productivity are compromised by environmental stresses such as pathogens, extreme temperatures, drought and high salinity. Being sessile organisms, plants had to develop different physiologic and biochemical strategies to cope with these potential harmful situations.

Drought in particular is one of the major environmental stresses that plants are forced to endure during their life cycle. The adaptation to water deficit is controlled by a cascade of molecular networks that start with the perception of water shortage which leads to increases in the ABA levels.

The current ABA signalling model described for *Arabidopsis* consists of three types of proteins (Fujii *et al.* 2009, Ma *et al.* 2009, Park *et al.* 2009), the PYR/PYL/RCAR ligands, the PP2C phosphatases and the ABA dependent SnRK2 kinases. In the absence of ABA the PP2C are constitutively bound and dephosphorylate the active centre of the SnRK2, repressing its activity. The binding of ABA to PYR/PYL/RCAR triggers the ABA signal transduction pathway through conformational modifications that allow the repression of the PP2C and the de-repression of SnRK2. This constitutes a double repression model in which the phosphatase represses the kinase which in turn is repressed by ABA bound PYR/PYL/RCAR.

The most studied SnRK2 kinase is the *Arabidopsis* OST1. This protein plays a key role in regulating drought stress signalling and is activated by ABA or osmotic stress (Boudsocq *et al.* 2004). OST1 is involved in the ABA dependent stomata closure (Mustilli *et al.* 2002) and is known to activate the slow anion channel SLAC1 which

triggers the plasma membrane depolarization necessary to initiate stomatal closing (Geiger *et al.* 2009, Lee *et al.* 2009, Vahisalu *et al.* 2010). OST1 also inhibits the inward-rectifying K^+ channel KAT1 (Sato *et al.* 2009), induces the generation of reactive oxygen species (ROS) *via* the respiratory burst oxidase homolog F (RbohF) which triggers the calcium dependent stomata closure (Sirichandra *et al.* 2009) and it activates the bZIP-class of transcription factors that include ABI5 (Furihata *et al.* 2006, Nakashima *et al.* 2009) and the ABA-responsive element binding factors, ABF2 and ABF3 (Fujii and Zhu 2009, Sirichandra *et al.* 2010).

Even though this signalling model is well described for *Arabidopsis*, little is known for other plant species. With this thesis we proposed to increase the knowledge of maize response to drought, focusing on a maize kinase of the SnRK2 family - ZmSnRK2.8/ZmOST1 - which is highly homologous to the *Arabidopsis* OST1.

We divided our work on three main objectives, the biochemical characterization of ZmOST1, the functional characterization of ZmOST1 and the study of ZmOST1 regulation.

ZmOST1 biochemical characterization

The maize *SnRK2.8/ZmOST1* gene was isolated from a library of stressed leaves and we started this thesis by characterizing the kinase that is coded by this gene, focusing our research on its regulation by ABA.

An initial phylogenetic approach, comparing the protein sequence of ZmSnRK2.8 with other SnRK2 from different plant species allowed the identification of

ZmSnRK2.8 in group 3 (Figure 7), which corresponds to the ABA dependent kinases. By this approach we could also determine that ZmSnRK2.8 has a strong homology to the *Arabidopsis* OST1 and the rice SAPK8.

By agroinfiltration of ZmSnRK2.8 fused in frame to GFP under a constitutive *35S* promoter in *N. benthamiana* leaves we determined that this kinase has a subcellular localization in the nucleus and cytosol of plant cells (Figure 8), which is in accordance with the localization of the *Arabidopsis* OST1 protein (Umezawa *et al.* 2009).

Through *in vitro* phosphorylation assays using MBP and Histone-III as general kinase substrates we determined that ZmSnRK2.8 has a catalytic activity capable of auto and transphosphorylation (Figure 9). Using a mass spectrometry system (MALDI-TOF) we were able to ascertain that ZmSnRK2.8 autophosphorylates at the S182 or T183 residues (Figure 10), a loci very similar for what is described for the *Arabidopsis* homolog (Belin *et al.* 2006).

We also established that ZmSnRK2.8 is constitutively expressed and that its catalytic activity is induced by ABA (Figure 11). OST1 from *Arabidopsis* is also a constitutive kinase that is induced by ABA (Boudsocq *et al.* 2007).

Using a bimolecular fluorescence complementation system we were able to reproduce the results obtained for the *Arabidopsis* system that OST1 interacts with PP2C phosphatase (Umezawa *et al.* 2009). As can be seen in Figure 12, the ZmOST1 regulatory domain interacts in a ABA independent manner with a maize PP2C phosphatase.

With Chapter 1 we characterized ZmOST1 at the biochemical level, making parallels with the *Arabidopsis* system whenever pertinent. We found a very close biochemical relationship between the maize and *Arabidopsis* kinases that suggests a conserved mechanism of plant responses to ABA and drought stress and point to the potential use of this kinase in improvement programs of drought tolerance in crops.

ZmOST1 functional characterization

After the biochemical approach presented in the first chapter, we decided to functionally characterize ZmOST1.

We started Chapter 2 outlining ZmOST1 activity in maize during embryogenesis and in 7 day old plants under different treatments (Figures 13 and 14). We were able to determine that ZmOST1 is activated by osmotic stresses in addition to ABA. By profiling ZmOST1 activity during embryogenesis we found that it is more active between 30 and 40 days after pollination and in the first two days after germination.

Since we had previously established that ZmOST1 is closely related to the *Arabidopsis* OST1 we decided to determine whether ZmOST1 could complement the *Arabidopsis ost1-2* phenotype that is deficient in stomata closure under water deficit (Mustilli *et al.* 2002). we prepared transgenic lines overexpressing ZmOST1 in the *ost1-2* line and compared these plants with the Landsberg erecta wild-type and *ost1-2* mutant. By in gel kinase assay, thermal imaging and water loss experiments we were able to detect a complementation of the mutant phenotype on the transgenic lines, indicating that ZmOST1 is a functional ortholog of the *Arabidopsis* kinase (Figure 15).

To further our understanding of ZmOST1 function we were also interested in finding new targets of this kinase with potential biotechnological application. Since the mechanisms of transcriptional control of ABA guard cell signalling are still poorly understood (Leonhardt *et al.* 2004, Wang *et al.* 2011), we were particularly interested in the discovery of new transcription factors that are potential substrates of OST1, apart from the ABFs (Shinozaki and Yamaguchi-Shinozaki 2007, Yoshida *et al.* 2010).

We took a yeast two-hybrid approach using ZmOST1 as bait against a library of maize stressed leaves. Among several interesting candidates, we decided to characterize ZmOST1 relationship with a NAC transcription factor, ZmSNAC1. This transcription factor was selected because it is highly homologous to the rice SNAC1 that is known to be implicated in stomata regulation under abiotic stress (Hu *et al.* 2006). By directed yeast two-hybrid, *in vitro* pull down experiments (Figure 16), co localization (Figure 17) and bimolecular fluorescence complementation (Figure 18) we were able to prove that ZmOST1 and ZmSNAC1 interact both *in vitro* and *in vivo* and that this interaction is done through the kinase's ABA-box.

After establishing that ZmOST1 and ZmSNAC1 interact, we were interested in determining whether the transcription factor was indeed a substrate of the kinase. By *in vitro* phosphorylation and in gel kinase assays in both maize and *Arabidopsis* we established that ZmSNAC1 is phosphorylated by osmotic stresses in general and by ZmOST1 in particular (Figure 19). We were also able to pinpoint ZmOST1 loci of phosphorylation to the C-terminal part of the ZmSNAC1 protein.

By transient transformation of maize protoplasts with ZmOST1 fused to HA and ZmSNAC1 fused to GFP we were able to detect a change of localization of ZmSNAC1 in response to ABA into nuclear speckles (Figure 21). This change in localization is

consistent with the ABA-induced ZmOST1 activity and is followed by protein degradation (Figure 22).

With Chapter 2 we described ZmOST1 as a functional kinase that is activated by different osmotic stresses and that is able to complement the *Arabidopsis ost1-2* mutant with effects on stomata closure. We also present ZmSNAC1 as a novel cognate substrate of ZmOST1 with further implications on stomata regulation.

ZmOST1 regulation

In the first two chapters of this thesis we characterized ZmOST1 at the biochemical and functional levels and we introduced a novel transcription factor that is a substrate of ZmOST1 with potential biotechnological implications. In Chapter 3 we focused on our results on ZmOST1 regulation.

It has been established for some time now that the *Arabidopsis* OST1 protein requires being phosphorylated in order to be active and that the abolishment of phosphorylation either by the use of alkaline phosphatase or by direct mutagenesis of S171 or S175 at the activation loop renders OST1 inactive (Boudsocq *et al.* 2007, Vlad *et al.* 2010). There is not much information available on upstream signals apart from the de-repression by PP2C phosphatases in the presence of ABA (Soon *et al.* 2012). Currently, most authors assume OST1 autophosphorylates after its activation loop is free from the phosphatase (Belin *et al.* 2006, Ng *et al.* 2011) but more research is required to have a full picture of OST1 regulation.

We focused our research on ZmOST1 regulation in protein phosphorylation searching for upstream kinases that may use ZmOST1 as a substrate. We used in gel

kinase assays to profile ZmOST1 phosphorylation under different treatments and plant backgrounds and found two kinases that could act upstream of ZmOST1, namely SnRK1 (Figure 31) and CK2 (Figure 23). This phosphorylation is constitutive and does not require any detectable signal to occur, which is consistent with the double repression characteristic of the central model for ABA perception.

In the remainder of Chapter 3 we describe all the work that was done to characterize the effect of CK2 phosphorylation on ZmOST1. We first determine by in silico analysis (Figure 30) and phosphorylation domain mapping (Figure 24) that CK2 acts on four serine residues on the ABA-box of ZmOST1 that are conserved between plant species and we proceed to mutagenize them.

We then used the mutated version of the kinase (ZmOST1[AA]) to compare it with the wild-type ZmOST1.

By western blot and bi-dimensional SDS-PAGE of ZmOST1 and ZmOST1[AA] fused to HA and agroinfiltrated in tobacco leaves we were able to detect that ZmOST1 is somewhat phosphorylated after ABA treatment while ZmOST1[AA] does not evidence any gel shift that is consistent with this post-translational modification (Figure 25). This result pointed CK2 as implicated in ABA response so we put our efforts into trying to better elucidate its role on ZmOST1 regulation.

We checked for ZmOST1 and ZmOST1[AA] localization in tobacco agroinfiltrated leaves when co-expressed with the alpha and beta subunits of maize CK2 to further determine whether ZmOST1 is indeed an *in vivo* substrate of ZmCK2 (Figure 26). When co-infiltrated with ZmCK2α1-MYC we were able to detect the appearance of cytosolic aggregates in the case of ZmOST1-GFP which is almost absent in the case of ZmOST1[AA]-GFP. When co-infiltrated with ZmCK2β1-MYC, ZmOST1-GFP

localizes exclusively to the nucleus with both a diffuse pattern and accumulated in small nuclear speckles while ZmOST1[AA]-GFP maintains its cytosolic and nuclear localization even though, in this last organelle, it appears accumulated in large speckles. These changes of ZmOST1 localization are consistent with previous results from our lab (Riera *et al.* 2011).

Apart from the changes of localization we were also able to detect that ZmOST1 is rapidly degraded after the formation of the nuclear speckles in a process that is dependent of the proteasome since this degradation is halted when MG132 is added to the system.

Since CK2 phosphorylation seems to occur after ABA treatment and since the loci of CK2 activity are located on the ZmOST1 ABA-box, close to the region responsible for the interaction with PP2C phosphatases, we decided to use a BiFC system to compare ZmOST1 and ZmOST1[AA] interaction with ZmABI1. As presented in Figure 27, ZmOST1 capability to interact with ZmABI1 is reduced by 50% in the mutagenized kinase, highlighting the importance of CK2 for this response.

For the concluding part of this thesis we prepared transgenic lines overexpressing ZmOST1 and ZmOST1[AA] fused to HA on *Arabidopsis thaliana* and we proceeded to characterize them in respect to protein degradation, ABA activation and stomata regulation (Figures 28 and 29).

By cell free degradation assays we could detect that ZmOST1 is slowly degraded by the proteasome while the mutagenized kinase maintains high levels of protein. Performing in gel kinase assays of immunoprecipitated ZmOST1 we could detect an enhanced activity of ZmOST1[AA], even in the absence of ABA treatment.

Finally, checking for stomata regulation both by analyzing leaf temperature and measuring pore opening we were able to detect that mutagenizing ZmOST1 at the loci of CK2 action confers hypersensitivity to ABA.

With the results presented in Chapter 3 a larger picture of ABA signalling appears that implicates new partners on ZmOST1 regulation, specifically the CK2 kinase and the proteasome degradation. Ample evidence is shown suggesting CK2 phosphorylation is implicated in ABA signalling by affecting ZmOST1 localization, protein levels, protein degradation and interaction with PP2C phosphatases. At the plant level, overexpressing ZmOST1 mutagenized on the CK2 loci of phosphorylation grants several potential beneficial traits that could prove important for crop biotechnology, such as higher protein levels, better protein stability, enhanced phosphorylation activity and better stomata regulation.

Working model

Taken the results presented in this thesis together, we propose a change in the current ABA signalling model (Figure 32).

First we believe that there is an important role for CK2 in ABA sensing and SnRK2 activation that could affect the binding of the kinase to the PP2C phosphatases and regulate SnRK2 through degradation.

Second, we propose that, apart from the always off and transiently fast on/off modes of SnRK2 activity, there is a third always on mechanism in which the kinase is fully detached from the phosphatase.

Third we present a novel substrate for SnRK2, the SNAC1 transcription factor, involved in salt and drought tolerance at the stomata level.

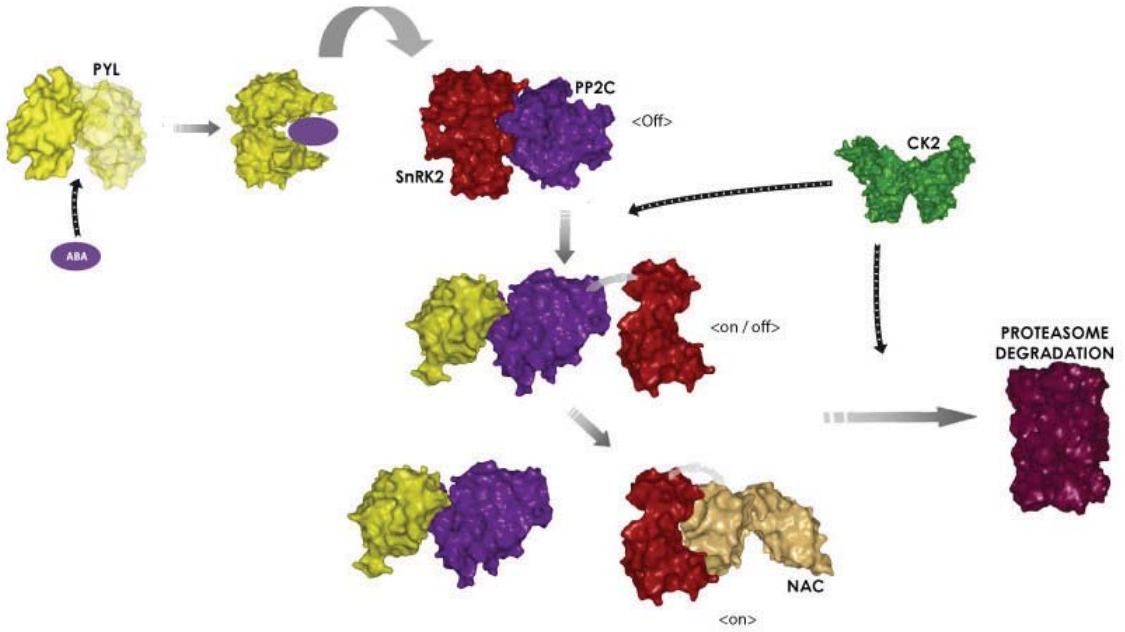

Figure 32 - Proposed changes to the ABA signalling model. CK2 has an important role in ABA sensing and SnRK2 activation that could affect the binding of the kinase to the PP2C phosphatases and regulate SnRK2 through degradation. Apart from the always off and transiently fast on/off modes of SnRK2 activity, there is a third always on mechanism in which the kinase is fully detached from the phosphatase. SNAC1 is a new substrate of SnRK2 that regulates drought and salt responses at the transcriptional level.

5 - Conclusions

5 - Conclusions

With this project we proposed to characterize maize responses to drought, particularly at the level of protein phosphorylation. We focused our work mainly in a protein kinase highly homologous to the *Arabidopsis* OPEN STOMATA 1 (OST1) which was named ZmSnRK2.8/ZmSAPK8/ZmOST1.

We performed both a biochemical and functional characterization of this protein, establishing that it is a ABA activated kinase, capable of auto and trans-phosphorylating different substrates and that it is implicated in stomatal closure. We present a transcription factor (ZmSNAC1) that is a novel cognate substrate of ZmOST1 which has implications in the responses to water deficit, particularly at the stomata level. We implicate the CK2 protein kinase as an upstream regulator of ZmOST1, affecting ZmOST1 protein stability, response to ABA and interaction with ZmPP2C phosphatases.

The following conclusions were made:

Chapter 1

1 - ZmSnRK2.8/ZmOST1 is closely related to *Arabidopsis* OST1 (open stomata 1) kinase with nuclear and cytosolic subcellular localization.

2 - ZmSnRK2.8/ZmOST1 is able to phosphorylate generic kinase substrates *in vitro* and also auto-phosphorylate at S182 or T183 amino acids on its activation loop.

3 - ZmSnRK2.8/ZmOST1 is activated by ABA in planta and interacts with PP2C phosphatases in a constitutive, ABA independent manner.

Chapter 2

1 - Ectopic expression of ZmOST1 in the *Arabidopsis ost1* mutant restores the stomatal closure phenotype in response to drought.

2 - Endogenous ZmOST1 mRNA is constitutively expressed at all developmental stages, however, its corresponding kinase activity is strongly up-regulated by ABA and osmotic stress treatments.

3 - ZmSNAC1 transcription factor interacts *in vivo* with the ABA-box of ZmOST1 and is directly phosphorylated by ZmOST1 under stress conditions.

Chapter 3

1 - CK2 directly phosphorylates ZmOST1 at multiple sites in the ABA domain. Serine to alanine mutation at four plant conserved serine residues scattered throughout the ABA-box of ZmOST1 eliminates CK2-mediated phosphorylation.

2 - ZmOST1 interacts with CK2α and CK2β. There is a change in localization of ZmOST1 to cytosol aggregates and nuclear speckles when co-infiltrated with CK2α and CK2β, respectively.

3 - CK2-mediated phosphorylation promotes the degradation of ZmOST1 to regulate stomata closure and also affects the binding to PP2C phosphatases.

4 - Plants overexpressing a version of ZmOST1 with changes S350A, S352A, S358A and S359A (ZmOST1[AA]) evidence enhanced protein levels, activation of the kinase under absent to low levels of ABA, and hypersensitivity to ABA at the level of stomata.

6 - References

6 - References

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Curriculum Vitae

Personal information

Curriculum Vitae

Name	Belmiro Jorge Peres Bessa Vilela
Address	Centre for Research in Agricultural Genomics. Parc de Recerca UAB. Edifici CRAG, Campus UAB. Bellaterra (Cerdanyola del Vallés). 08193 BARCELONA
Telephone	(+34)665347035
E-mail	belmiro.vilela@cragenomica.es
Nationality	Portuguese
Date of birth	29.09.1978
Gender	Male

Work experience

Dates	***2006-PRESENT DAY***
Position held	*Marie Curie Adonis Early Stage Training Fellowship (2006-2009)* - www.adonis.science.ru.nl/ *Fundação para a Ciência e Tecnologia PhD grant (2009-Present day)*
Project	The functional characterization ZmSnRK2.8 in the response to ABA and abiotic stress - determination of protein and gene targets of interest.
Employer	*Parc de Recerca UAB. Edifici CRAG, Campus UAB. Bellaterra (Cerdanyola del Vallés). 08193 Barcelona, España. http://www.cragenomica.es/*
Dates	***2002-2006***
Position held	*Research Fellowship*
Project	Characterization of the light stress associated with the transfer of *in vitro Vitis vinifera* to ex vitro conditions.
Employer	*Centro de Botânica Aplicada à Agricultura - Instituto Superior de Agronomia. Tapada da Ajuda 1349-017 Lisboa, Portugal.* http://www.isa.utl.pt/cbaa/
Dates	***2001-2002***
Position held	*Undergraduate Student*
Project	Characterization of the effects of cadmium on the *Rhizobium leguminosarum* and *Pisum sativum* symbiosis
Employer	*Departamento de Biologia. Universidade de Aveiro. Campus Universitário de Santiago. 3810-193 Aveiro. Portugal.* http://www.bio.ua.pt/

Education

Dates	***2009***
Qualification	ADONIS training course 5
Principal subjects	*EcoTilling, Genetic crop improvement*
Organisation	*Instituto de Tecnologia Quimica e Biologica, Oeiras, Portugal*

Dates	***2008***
Qualification	ADONIS training course 4
Principal subjects	*Symposium on RNomics; Advanced Microscopy*
Organisation	*Radbound University, Nijmegen, Netherlands*
Dates	***2008***
Qualification	ADONIS training course 3
Principal subjects	*ABA signalling; Stress Signalling in Reporter Plants; Heavy Metal and Xenobiotica Detoxification*
Organisation	*Technical University of Munich, Germany*
Dates	***2007***
Qualification	ADONIS training course 2
Principal subjects	*Mass Spectrometry*
Organisation	*Swiss Federal Institute of Technology, Zurich, Switzerland*
Dates	***2007***
Qualification	ADONIS training course 1
Principal subjects	*Phoshoproteomics;* Arabidopsis *and Barley Transformation; Luciferase Imaging; Confocal Microscopy*
Organisation	*Bonn University and Max Planck Institute for Plant Breeding (Cologne) Germany*
Dates	***2005***
Qualification	Masters Degree
Principal subjects	*Biology of Stress in Plants*
Organisation	*Universidade do Minho – Escola de Ciências (University)*
Classification	18/20
Dates	***2002***
Qualification	Bachelor of Science
Principal subjects	*Biology*
Organisation	*Universidade de Aveiro (University)*
Classification	14/20

Training

Dates	***2009***
Principal subjects	*Getting Funded: Writing an Individual Fellowship Application*
Organisation	*Bioscript – Scientific Communication Consultancy*
Dates	***2008***
Principal subjects	*Scientific PowerPoint Presentation: Presenting with Confidence*
Organisation	*Bioscript – Scientific Communication Consultancy*
Dates	***2007***
Principal subjects	*Advanced Scientific Writing*
Organisation	*Bioscript – Scientific Communication Consultancy*

Dates	***2006***
Principal subjects	*Communicating Science, Technology and Innovation*
Organisation	*Instituto Superior de Engenharia e Gestão (University)*

Dates	***2004***
Principal subjects	*Advanced Light Microscopy in Living Cells*
Organisation	*Instituto Gulbenkian de Ciência (Research Institute)*

Dates	***2003***
Principal subjects	*Teachers Course*
Classification	5/5

Dates	***2002***
Principal subjects	*Short Course on Bioenergetics: the cellular power plants*
Organisation	*Portuguese Society of Biophysics*

Dates	***2001***
Principal subjects	*Course on Epidemiology and statistical analysis of biomedical data*
Organisation	*Faculdade de Medicina da Universidade do Porto (University)*

Dates	***1995***
Qualification	First Certificate in English
Organisation	*Cambridge University*
Classification	B

Personal skills and competences

Mother tongue **Portuguese**

Other language(s)

Self-assessment	**Understanding**				**Speaking**				**Writing**	
European level (*)	Listening		Reading		Spoken interaction		Spoken production			
English	C2	Proficient user	C2	Proficient user	C2	Proficient user	C2	Proficient user	C2	Proficient user
Spanish	C2	Proficient user	C2	Proficient user	C2	Proficient user	C2	Proficient user	C1	Proficient user
French	B2	Independent user	B2	Independent user	B1	Independent user	B1	Independent user	A2	Basic user

(*) Common European Framework of Reference for Languages

Technical skills and competences

- Large experience in manipulating different plant material (*Arabidopsis*, tomato, tobacco, grapevine, pea, maize)
- Good grasp of diverse laboratory techniques, applied in the study of nucleic acids and proteins
- Practical knowledge of state of the art technology such as real-time PCR, microarrays and confocal microscopy

Computer skills and competences	- Good command of Microsoft office / Open office - Appreciable familiarity with the internet and experienced in database queries - Competent working with web based bioinformatics tools (eg. Primer3, Epclust, Genevestigator, Netphos) - Acceptable knowledge of imaging software, namely ImageJ and Adobe Creative Suit - Satisfactory experience with statistical tools (Epi Info, Graphpad Prism, Graphpad InStat) - Basic control over webdesign tools, such as Adobe Dreamweaver - Basic knowledge of programming (Pascal and C++) and networking
Social skills and competences	- Good ability to adapt to distinct environments/situations, through my experiences living, studying and working in different cities - Considerable competence in dealing with others from previous experiences in organising events and being a part of several multidisciplinary and multicultural groups
Organisational skills and competences	- Member of the organising committee of several conferences - Founder and member of the editorial team, webmaster and web designer of the biology department newsletter, Aveiro University (www3.bio.ua.pt/Newsletter/) from 2002 to 2003 - Founder and member of the team responsible for a weekly scientific section in a regional newspaper (Diário de Aveiro) from 2001 to 2003 - PhD student representative at the Molecular Genetics Department of the CRAG from 2006 to 2009
Other skills and competences	Scuba divers licence
Driving licence	I am a holder of a Portuguese drivers licence. Category B

Publications

Thesis

2005

1 - Vilela BJ (2005). *Response of the antioxidative system to photooxidative stress in micropropagated* Vitis vinifera *L., transferred to ex vitro under excess light*. **Masters' Thesis in Biology of Stress in Plants**. Universidade do Minho, Escola de Ciências.

Papers

2012

12 – Vilela B, Moreno A, Capellades M, Pagès M, Lumbreras V (2012). *ZmSnRK2.8 responds to ABA :through the SnRK2-PP2C complex.* **Maydica** 57:11-18

2011

11 – Carvalho L, Vilela BJ, Mullineaux PM, Amâncio S (2011). *Comparative Transcriptomic Profiling of Vitis vinifera Under High Light Using a Custom Made Array and the Affymetrix GeneChip.* **Molecular Plant** 4:1038-1051

2010

10 – Vilela B, Pagès M, Lumbreras V (2010). *Regulation of MAPK signalling and cell death by MAPK phosphatase MKP2.* **Plant Signaling and Behavior** 5:156-159

9 – Lumbreras V, Vilela B, Irar S, Solé M, Capellades M, Valls M, Coca M, Pagès M (2010). *MAPK phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with MPK3 and MPK6*. **The Plant Journal** 63:1017-1030

2009

8 – Lopez C, Vilela B, Riera M, Pagès M, Lumbreras V (2009). *Maize AKINβγ assembles into SnRK1 complexes and dimerizes through the KIS/CBM domain*. **FEBS Letters**. 583:1887-1894

2008

7 - Vilela BJ, Carvalho LC, Amâncio S (2008). *Integration of stress produced reactive oxygen species in the stomatal regulation of micropropagated* Vitis vinifera *L. plantlets impaired in ABA signalling*. **Plant Signaling and Behavior**. 3:558-559.

6 - Carvalho LC, Santos S, Vilela BJ, Amâncio S (2008). Solanum lycopersicon *Mill. and* Nicotiana benthamiana *L. under high light show distinct responses to anti-oxidative stress.* **Journal of Plant Physiology.** 165:1300-1312

5 - Lumbreras V, López C, Vilela B, Moreno A, Pagès M (2008). Respuesta de adaptación al estrés osmótico en plantas: Función de las proteínas quinasas SnRK1/2. **Fitohormonas: Metabolismo y Modo de Acción**. ISBN:978-84-7401-201-9. pp:79-84.

2007

4 - Vilela BJ, Carvalho LC, Ferreira J, Amâncio S (2007). *Gain of function of stomatal movements in rooting* Vitis Vinifera *L plants: regulation by H_2O_2 is independent of ABA before the protruding of roots*. **Plant Cell Reports.** 26:2149–2157

2006

3 - Carvalho LC, Vilela BJ, Vidigal P, Mullineaux P, Amâncio S (2006). *Activation of the ascorbate–glutathione cycle is an early response of micropropagated* Vitis vinifera *L. explants transferred to ex vitro*. **International Journal of Plant Sciences**. 167:759-770

2 - Carvalho LC, Vilela BJ, Mullineaux P, Amâncio S (2006). *Scanning* Vitis vinifera *Transcriptome with an array of heterologous primers for light responsive genes*. **Proceedings International Grape Genomics Symposium**, St. Louis, USA. 13-16

2005

1- Carvalho LC, Vidigal P, Vilela B, Mullineaux P, Amâncio S (2005). *Monitoring the response of grapevine plantlets to excessive light by real-time PCR*. **Acta Horticulturae** (ISHS) 689:177-182

Short communications

2011

12 – Rabissi A, López-Paz C, Vilela B, Pagès M, Lumbreras V (2011). *Characterization of the SnRK1 complex involved in osmotic response.* **XXXIV Congreso de La Sociedad Española de Bioquímica y Biologia Molecular**. Barcelona, Spain

11 – Vilela B, Moreno A, Lumbreras V, Pagès M (2011). *Drought signal transduction in maize: Characterization of SnRK2 kinases.* **XXII Eucarpia Maize and Sorghum Conference**. Opatija, Croatia

2010

10 - Vilela B (2010). *ZmSnRK2.8 is involved in stomatal closure and phosphorylates two maize transcription factors.* **BASF (Plant Sciences) - Centre for Research in Agricultural Genomics Meeting**. Barcelona, Spain

9 – Vilela B, Moreno A, Pagès M, Lumbreras V (2010). *ZmSnRK2.8 is involved in stomatal closure and phosphorylates two maize transcription factors.* **52nd Annual Maize Genetics Conference**. Riva del Garda, Italy

2009

8 - Vilela B (2009). *Functional and Molecular Characterization of Maize SnRK2 Kinases*. **Centre for Research in Agricultural Genomics / Wageningen University PhD students Meeting**. Barcelona, Spain

2008

7 - Vilela B, Moreno A, Pagès M, Lumbreras V (2008). *Caracterización Funcional y molecular de dos quinasas SnRK2 de Maíz y sus interactores*. **VI Jornada de Fisiologia Vegetal**. Barcelona, Spain

2005

6 - Carvalho LC, Vilela BJ, Mullineaux P, Amâncio S (2005). *Analysis of the expression of* Vitis vinifera *light responsive genes.* **COST 858 Viticulture Functional Gene Analysis in Grapevine**. Geilweilerhof, Germany

5 - Carvalho LC, Vilela BJ, Mullineaux P, Amâncio S (2005). *An insight into the response of* Vitis vinifera *to light stress*. **IX Congresso Luso Espanhol de Fisiologia Vegetal**. Évora, Portugal, Abstract pp. 333

4 - Carvalho LC, Vilela BJ, Mullineaux PM, Amâncio S (2005). *Scanning* Vitis vinifera *Transcriptome with an array of heterologous primers for light responsive genes*. **International Grape Genomics Symposium**. St. Louis, USA

3 - Carvalho LC, Vilela BJ, Mullineaux P, Amâncio S (2005). *Transcription of photooxidative stress genes of in vitro grapevine at transfer to ex vitro*. **COST 843 Quality Enhancement of Plant Production through Tissue Culture**. Stará Lesná, Slovakia, Extended abstract pp. 215-217

2004

2 - Carvalho LC, Vidigal P, Vilela BJ, Mullineaux P, Amâncio, S (2004). *Monitoring the response of grapevine plantlets to excess light by real-time PCR.* **Seventh International Symposium on Grapevine Physiology & Biotechnology**, Davis, California, U.S.A., Abstract pp. 76

1 - Carvalho LC, Vilela BJ, Mullineaux P, Amâncio S (2004). *Ex vitro conditions induce photooxidative stress but trigger the expression of genes related to rapid recover*. **COST 843 Quality Enhancement of Plant Production through Tissue Culture**. Saanen, Switzerland, Extended abstract, pp. 35-36

Posters

2011

14 – Vilela B, Nájar E, Moreno M, Pagès M, Lumbreras V (2011). *Understanding drought in maize: characterization of ZmSnRK2.8 and its interactors.* **XXXIV Congreso de La Sociedad Española de Bioquímica y Biologia Molecular**. Barcelona, Spain

13 – Nájar E, Vilela B, Moreno N, Pagès M, Lumbreras V (2011). *SnRK2 family in maize: STOP1 protein regulation.* **XXXIV Congreso de La Sociedad Española de Bioquímica y Biologia Molecular**. Barcelona, Spain

2010

12 - Vilela B, Moreno A, Pagès M, Lumbreras V (2010). *Functional characterization of maize SnRK2.8*. **20th International Conference on Plant Growth Substances.** Tarragona, Spain

2009

11 - Vilela B, Moreno A, Pagès M, Lumbreras V (2009). *Two maize NAC and bHLH transcription factors are substrates of SnRK2*. **2nd EMBO Conference Series: Frontiers of Plant Research**. Cadiz, Spain

2008

10 - Vilela B, Moreno A, Pagès M, Lumbreras V (2008). *Functional and molecular characterization of maize SnRK2 kinases*. **IX Reunión de Biologia Molecular de Plantas**. Santiago de Compostela, Spain

9 - Vilela B, Lumbreras V, Pagès M (2008). *Facing the water problem*. **Marie Curie Conference 2008 – ESOF 2008**. Barcelona, Spain

2006

8 - Vilela BJ, Carvalho LC, Mullineaux P, Amâncio S (2006). *Early peroxidase response in micropropagated grapevine transferred to ex vitro under excess light*. **Peroxidase 2006**. Aveiro, Portugal

2005

7 - Vilela BJ, Carvalho LC, Santos S, Amâncio S (2005). *Stomatal functioning in Vitis vinifera L. transferred from in vitro to ex vitro under light stress conditions*. **IX Congresso Luso Espanhol de Fisiologia Vegetal**. Évora, Portugal

6 - Santos S, Vilela BJ, Carvalho LC, Amâncio S (2005). Establishment of Lycopersicon esculentum L. and Nicotiana benthamiana L. *in vitro* systems and characterization of photooxidative stress symptoms upon transfer to ex vitro. **IX Congresso Luso Espanhol de Fisiologia Vegetal**. Évora, Portugal

2004

5 - Vilela BJ, Carvalho LC, Amâncio S (2004). *Imaging of photooxidative stress in leaves of micropropagated grapevine after transfer to ex vitro.* **XIV Congresso Nacional de Bioquímica. Vilamoura**, Portugal

4 - Carvalho LC, Vilela BJ, Mullineaux P, Amâncio S (2004). *Excess light stress triggers an antioxidative response upon transfer of in vitro grapevine to ex vitro*. **XIV Congresso Nacional de Bioquímica. Vilamoura**, Portugal

2002

3 - Vilela B, Figueira E, Pereira S, Lima A (2002). *Effects of Cadmium on Pisum sativum / Rhizobium leguminosarum Symbiosis*. **XIII Congresso Nacional de Bioquímica**. Lisboa, Portugal

2 - Pereira S, Figueira E, Lima A, Vilela B (2002). *Protein Profile Alterations Induced After Cadmium Exposure in Rhizobium leguminosarum*. **XIII Congresso Nacional de Bioquímica**. Lisboa, Portugal

	1 - Lima A, Figueira E, Pereira S, Vilela B (2002). *Effects of Salinity on Protein Patterns of Rhizobium leguminosarum Strains with Different Salt Tolerances*. **XIII Congresso Nacional de Bioquímica**. Lisboa, Portugal
Present research interests	- The use of molecular techniques to characterize the signalling pathways involved in the response of plants to different stimuli, particularly to stress situations. Other interests: Drought response; Oxidative stress; Proteomics, Transcriptomics, Genomics, Bioinformatics; Senescence, aging and programmed cell death; Stomatal movements
References	- Montserrat Pagès – Centre for Research in Agricultural Genomics – montse.pages@cid.csic.es - Victoria Lumbreras – Centre for Research in Agricultural Genomics – vlrgmm@cid.csic.es - Sara Amâncio – Centro de Botânica Aplicada à Agricultura – samport@isa.utl.pt - Luísa Carvalho – Centro de Botânica Aplicada à Agricultura – lcarvalho@isa.utl.pt