Tesis doctoral:

CHARACTERIZATION OF THE MAIZE PROTEIN ZmSTOP1 AND ITS ROLE IN DROUGHT STRESS RESPONSE

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A mi padre, por
transmitirme la curiosidad por la
ciencia.

A mi madre, por
apoyarme siempre.

A Bel, por acompañarme
durante el camino.

A Sol, que antes de nacer
ya ilumina mi vida

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Por fin llega ese momento. El final de una etapa. Como todas en la vida, uno siente que será eterna, pero siempre se acaba. Para bien o para mal.

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INTRODUCTION

1. Introduction

Humans consume just a small percentage of the eatable plant species available. Nowadays it is believed that human diet is mainly based in 20 crops, especially cereals. In ancient hunter-gatherers societies, the variety of species that human consumed was probably much higher than in farming societies. The plant domestication process coincides with the establishment of sedentary human societies, during the Agricultural Revolution. Interestingly, this period overlaps with the end of the last ice age, which occurred about 12.000 years ago. This period is recognized as the transition from Pleistocene to Holocene, the present era (Vigouroux et al., 2011).

Many studies defend that agriculture originated independently in several regions worldwide, including Mesoamerica, Middle East, Sahelian zone in Africa and China (Vavilov, 1992; Smith, 1998). In each event a different plant was domesticated. Namely, wheat was domesticated in Middle East, rice in China, and maize in Mesoamerica (Lev-Yadun et al., 2000; Matsuoka et al., 2002; Huang et al., 2012).

Maize extended throughout the world after Europeans arrived in the American continent, as they imported some crops back to Europe and spread them worldwide.

Nowadays, Maize (*Zea mays* ssp. *mays*) is one of the most abundantly harvested crop species around the world. It represents an important source of energy either for human and animal consumption, and it is the biggest grain-

producing crop. In 2012, for instance, 872 million tone of maize were collected, approximately a 36% of total production of cereals (www.fao.org).

Modern maize has been continuously selected and modified by humans for thousands of years. The maize domestication event probably happened around 9000 years ago (Piperno and Flannery, 2001; Matsuoka et al., 2002), from the wild plant teosinte (*Zea mays* ssp. *Parviglumis*).

Interestingly, the phenotype of teosinte wild plants varies depending on the climate conditions present at the time of its development (Piperno D. R., 2014). By the time domestication is thought to have taken place, global temperatures were lower by 5 – 7 °C, and annual precipitations were also significantly lower than after the glacial period (20 – 40%). Atmospheric CO_2 levels were also decreased in comparison with current levels (180 ppm at the beginning of the Holocene, 270-280 ppm in pre-industrial Holocene) (Jinho Ahn, 2004). Some experiments carried out with several stocks of teosinte seeds show that plants grown in Late Pleistocene climate controlled conditions present a maize-like phenotype, i.e., short plants (knee high) with apical dominance presenting a tassel (masculine inflorescence) at the tip of the main stem and shorter lateral branches ending with ears (female inflorescences), whereas in control conditions (present climate conditions) teosinte is characterized by multiple lateral branches tipped with tassels and secondary branches bearing female ears with a few small seeds. These plants can also be very tall (2-3 meter high)(Piperno D. R., 2014).

These facts bring to mind several remarkable appreciations:

- First agricultural societies faced a phenotype of the wild teosinte plant more similar to what we understand nowadays as maize than what we usually consider.
- The genotypic-phenotypic plasticity associated to teosinte got lost along maize domestication, giving as a result a modern maize plant less able to adapt to different conditions.

The domestication process along millennia, therefore, shaped a maize plant with traits suitable for human collection and consumption, i.e. short enough plants to easily collect its fruits, with seeds containing a high amount of starch, with kernels all maturing at the same time and with an increasing productivity. The process of crop-species domestication is usually associated to a loss of genetic variability, by both directed selection of specific characteristics and by population bottleneck effect. Bottleneck effects affect the whole genome and completely transform the genetic variation of loci, dramatically diminishing their variability.

To illustrate this idea, it is estimated that fewer than 3500 individuals, or less than 10% of the teosinte population, contributed to the genetic diversity of modern maize inbred lines, resulting in a loss of variability bigger than 40% (Wright et al., 2005).

Genes specifically selected by human intervention present a high decrease in their diversity, and it is considered greater than what should be expected from bottleneck effects. On the contrary, neutral or unselected genes maintain more diversity, whose reduction can be explained by bottleneck effects.

One of the clear examples of selected genes is *Teosinte Branched* (*TB1*), which controls lateral branch morphology. Wang et al. (1999) defined the region selected by domestication, which corresponds to the 5' regulatory region, and not to the protein-coding sequence. This regulatory region showed a drastic decrease in diversity in maize modern crops relative to teosintes.

Although there is no evidence in this respect, genes controlling maize ability to survive drought periods or living in non-well-watered environments were probably indirectly affected by bottleneck effects.

Actually, modern maize is a plant that requires abundant watering, well-drained soils and relatively high temperatures to develop optimally. Despite the fact that maize has proven to be viable in a wide range of latitudes and altitudes, water shortage is highly constricting for the full development of the plant (Araus et al., 2012). In fact, during its development, the water needs of the plant increase almost exponentially, reaching a plateau between flowering and yield formation, in which water requirements are maximum (Fig. 1). In case a water shortage or a drought period occurs during this phase, the yield loss can become really important.
(http://www.fao.org/nr/water/cropinfo_maize.html).

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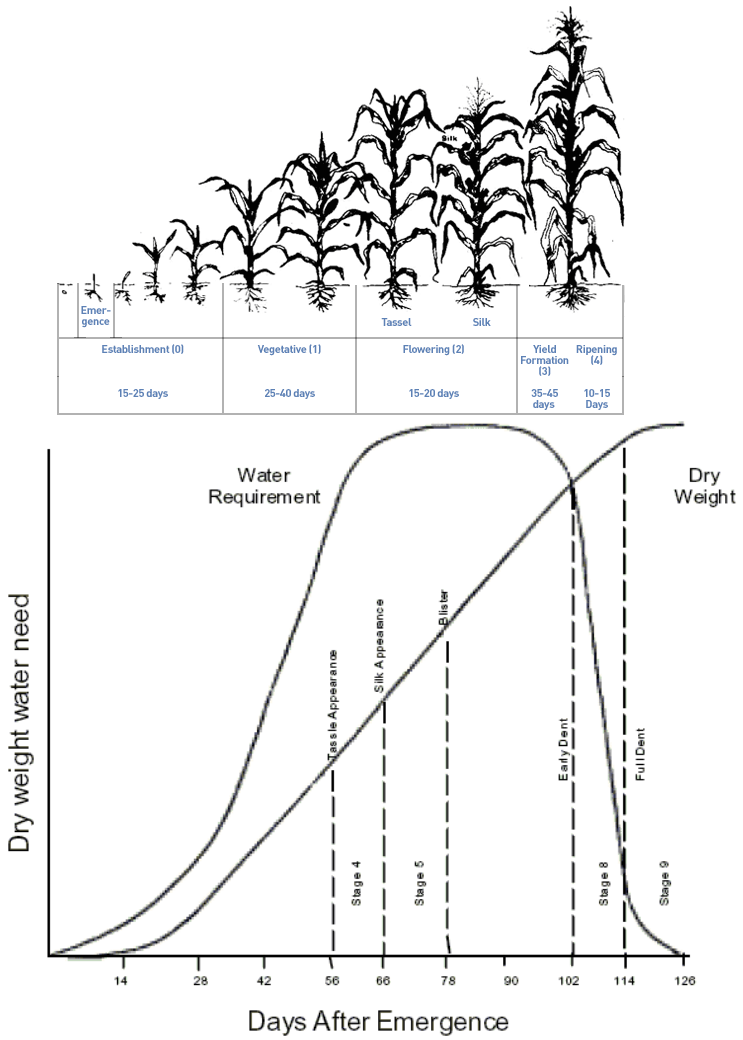


Figure 1. Maize water requirements

Water requirements during maize developmental stages. Higher amount of water is needed from flowering to yield formation. Figure adapted from www.fao.org andwww.ictinternational.com.

Nowadays, it is widely accepted by scientific community that we are facing a substantial change in climate conditions. This alteration implies a variety of local weather modifications in temperature, precipitation frequency and intensity, etc. Thus, the low variability of modern maize represents a handicap

to face the new global situation, threatening food security worldwide (Morari et al., 2015).

A better understanding of molecular mechanisms involved in drought stress and tolerance can provide us a clue to further improve, either by direct genetic modification or by classic breeding, maize capacity to endure water deficiencies derived from climate change.

1.1. Abscisic Acid and Drought Response

Abscisic acid (ABA) is the phytohormone responsible for drought tolerance in plants. It was first isolated in the 1960s from cotton and it was attributed the property of leaf abscission (Ohkuma et al., 1963). Later it was determined that abscission was an indirect consequence of inducing ethylene biosynthesis (Cracker and Abeles, 1969), and ABA has been very well documented since then as a signal molecule in plants (although it is also present in metazoans) (Wasilewska et al., 2008).

ABA acts in and regulates many developmental and physiological processes. ABA accumulates during seed development, inducing cell cycle arrest, which ends in seed dormancy. ABA is also involved in seed longevity, embryo and seedling development, germination, vegetative growth and reproduction (Barrero et al., 2005; Fujii and Zhu, 2009; Nakashima et al., 2009). Many loci associated to ABA response have also been identified as targets of other hormones, signaling pathways, and developmental signals. For example, ABA is able to antagonize gibberellic acid (GA) effects, like promotion of seedling

growth and alpha-amylase synthesis (Thomas et al., 1965). Other studies establish a cross talk with sugar metabolism, with specific transcription factors acting as connection nodes (Teng et al., 2008). ABA has also been found to play an important role in pathogen resistance, interfering with defense responses activated by other signaling pathways. (Finkelstein et al., 2002; Dekkers et al., 2008; Piskurewicz et al., 2009; Ton et al., 2009; Cutler et al., 2010).

Despite the variety of processes in which ABA is involved, stress signaling (in particular drought) is arguably the most documented and important of all (Sirichandra et al., 2009). Salt and specially drought stress result in a strong increase in ABA levels, which leads to the regulation of water relations by stimulating changes in cellular homeostasis and gene expression that will conclude in the physiological readjustment of the plant. Such changes include vegetative growth arrest, root architecture reorganization and particularly, stomatal closure (Deak and Malamy, 2005; Sirichandra et al., 2009).

The adaptation of root growth to water deficit is of dominant importance. In fact, under certain situations, the maintenance of root growth in drought conditions can ensure the finding of new sources of water and, thus, the survival of the plant. Lateral root formation is strongly inhibited in the presence of exogenous ABA (De Smet et al., 2003). Although ABA has a much stronger effect on lateral roots than on primary roots, under water deficit ABA plays a very important role in primary root growth also. At low water potentials seedling growth is arrested, but roots can still grow at a higher rate than shoots. ABA concentrations lower than 1uM are known to promote primary root growth in *Arabidopsis* (*Arabidopsis thaliana)* (Ephritikhine et al., 1999). At higher ABA concentrations, root elongation diminishes at increasing rates (Sharp et al.,

1988; Spollen and Sharp, 1991; van der Weele et al., 2000), preventing the plant to invest too much energy in growth.

The process in which ABA has been most widely studied is stomatal closure (Joshi-Saha et al., 2011). Stomata are very precisely regulated structures fundamental for gas exchange. The essential carbon source in plants, CO_2, enters the organism through stomata, but this process is unavoidably accompanied by water loss, so these structures must be finely tuned to regulate the proper balance between both. The levels of ABA increase under drought conditions provoking a decrease in guard cell turgor pressure (Sirichandra et al., 2009). Thus, ABA triggers stomatal closure by modifying activities of a number of ion channels (Schroeder et al., 2001). Stomatal opening is driven mainly by K^+ uptake in the guard cells. This activity is mediated by voltage-dependent K^+ inward-rectifying channels (like KAT1 and KAT2 among others), which are activated by hyperpolarization of the plasma membrane driven by the proton (H^+) pump ATPase OST2. This increase in intracellular K^+ leads to the increase in turgor by the subsequent entry of H_2O in the cells.On the other hand, stomatal closing is caused by K^+ efflux through outward-rectifying channels (like GORK) activated by membrane depolarization. Blue light stimulates stomatal opening, whereas high concentrations of CO2, darkness and water deficit promote their closure (Sirichandra et al., 2009).

Very low concentrations of ABA can stimulate stomatal closure to prevent water loss through transpiration. The stomatal closure activated by ABA is the result of the depolarization of the plasma membrane, which is mainly driven by the slow anion channel SLAC1 (Vahisalu et al., 2008).

Ca^{2+} is a very important second messenger in stomatal regulation. It has been shown that ABA triggers an increase in cytosolic Ca^{2+} in guard cells prior to stomatal closure, and reactive oxygen species (ROS, in particular H_2O_2, are fundamental for this process (Kwak et al., 2003). Some studies have been published describing the mechanisms through which ABA triggers Ca^{2+} and ROS signaling in plants. ABA induces the activation Ca^{2+} channels in the plasma membrane (Pei et al., 2000), which provokes the release of Ca^{2+} to the cytosol. This Ca^{2+} increase is necessary to activate the NADPH oxidase RBOHD (Ogasawara et al., 2008), and ABA-dependent OST1 kinase activates the NADPH oxidase RBOHF by phosphorylation, through a Ca^{2+} independent pathway (Sirichandra et al., 2009). This activation of NADPH oxidases induces H_2O_2 in the cell (Fig. 2). Ca^{2+} and ROS are able to propagate coordinately (Gilroy et al., 2014) along the plant, generating rapid waves transmitted symplastically and apoplastically (Suzuki et al., 2013; Choi et al., 2014), which will provoke not only stomatal closure but systemic adaptation to drought stress in the whole plant (Mittler and Blumwald, 2015). ROS and Ca^{2+} waves have no stress specificity, but they prime the plant for particular stress signals that will provoke exclusive reactions to certain stresses, like stomatal closure in the case of drought (Suzuki et al., 2013). A newly published research shows how Ca^{2+} and ABA signaling act coordinately to activate SLAC1 channel to achieve stomatal closure through membrane depolarization (Brandt et al., 2015).

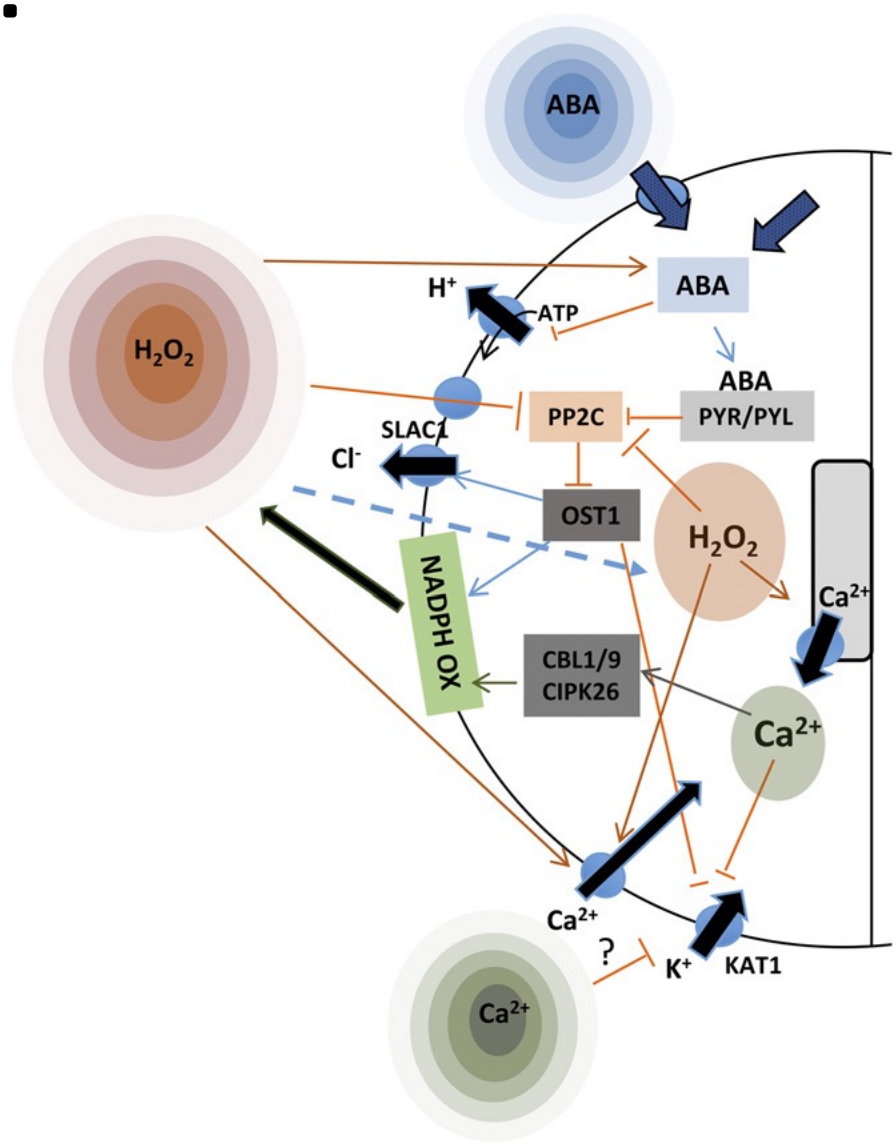


Figure 2. Relationship between ABA, ROS and Ca2+ in guard cells

ABA, synthesized in guard cells or originated in other tissues, can inhibit PP2C phosphatases, resulting in the activation of OST1. This will induce the synthesis of ROS through activation of NADPH oxidases. Apoplastic ROS from ROS wave can also enter the guard cell via aquaporins. ROS can activate Ca^{2+} channels that will lead to an increase in cytosolic Ca^{2+} concentration, promoting more ROS production. All these responses will lead to membrane depolarization and stomatal closure. Extracted from Mittler and Blumwald (2015).

Stomatal movements are driven by changes in ion homeostasis in guard cells, though the uptake and release of water is the final responsible for chang-

es in cell turgor. Recently, PIP2;1 aquaporin has been directly linked to ABA-induced stomatal closure, and its activation is dependent on OST1 phosphorylation (Grondin et al., 2015).

1.2. The Core Signaling Complex of ABA response

The molecular mechanism of ABA action is now well established in *Arabidopsis* (Klingler et al., 2010; Raghavendra et al., 2010; Umezawa et al., 2010; Zhang et al., 2015). ABA triggers downstream responses by binding to the cytosolic receptors pyrabactin resistance/pyrabactin-like/regulatory component of ABA receptor (PYR/PYL/RCAR) (Ma et al., 2009; Park et al., 2009). This interaction provokes conformational changes in the protein, which then sequester the negative regulators clade A type 2C protein phosphatases (PP2C), allowing the activation of Group III Sucrose non-fermenting-1 related protein kinases 2 (SnRK2.2, SnRK2.3 and SnRK2.6/OST1) (Fujii and Zhu, 2009; Umezawa et al., 2009) (Fig. 3). These three protein types are necessary and sufficient to mediate an ABA triggered model-signaling cascade in vitro (Fujii et al., 2009).

Group III SnRK2 kinases are essential for all aspects of ABA signaling, though the results suggest functional segregation between OST1 and SnRK2.2/2.3. Whereas OST1 functions in guard cells (Xue et al., 2011), SnRK2.2 and SnRK2.3 have redundant roles in seed dormancy, seed germination and seedling growth. However, the phenotypes of *ost1* single mutant and *snrk2.2/2.3* double mutant are milder than those of ABA-deficient mutants. This suggests that there is certain redundancy in kinase activity within these mu-

tants. The analysis of the triple *snrk2.2/2.3/2.6* confirms that these three kinases conform the bottleneck for all ABA signaling responses (Yoshida et al., 2002; Fujii et al., 2007; Fujii et al., 2009).

Recent studies have shown that OST1 kinases are able to interact forming homo and heterodimers with other SnRK2s, principally SnRK2.2 and SnRK2.3 (Waadt et al., 2015). Possibly this interaction promotes functional redundancy between the different group III SnRK2s and contributes to their trans-(auto)activation by phosphorylation in their activation loop (Vlad et al., 2010).

SnRK2s phosphorylate many substrates after ABA-dependent activation. For example, OST1 kinase is able to phosphorylate the NADPH oxidase RBOHF, contributing to ROS production and signaling (Sirichandra et al., 2009). Other OST1 substrates are ion channels, which modify guard cell membrane transport upon ABA perception. Some of these transporters are the K^+ uptake channel KAT1, which becomes inhibited, or the K^+ efflux channel GORK and the anion efflux channel SLAC1 (Lee et al., 2013), which become activated, promoting stomatal closure (Hosy et al., 2003; Geiger et al., 2009; Sato et al., 2009).

Recently, SLAC1 activation through Ca^{2+} dependent and independent pathway has been related. ABA- inhibited PP2C protein phosphatases block Ca^{2+} signaling, OST1 and SLAC1 anion channel in non-stress conditions through dephosphorylation. Upon ABA perception, inhibition of PP2C permits SLAC1 phosphorylation by OST1 and Ca^{2+}-dependent CPKs kinases, leading to membrane-depolarization that drives stomatal closure (Brandt et al., 2015).

Some important substrates of SnRK2s are transcription factors (TFs), which alter gene expression to drive the plant to a drought tolerance situation. By an-

alyzing the promoters of ABA-inducible genes, a very well conserved *cis*-element, designated the ABA-responsive element (ABRE; PyACGTGG/TC), was identified (Giraudat et al., 1994; Busk and Pages, 1998). Maize RAB17 ABA-responsive gene was the first promoter region where the ABA responsive element was described (Vilardell et al., 1991). ABRE-BINDING FACTORS (ABFs), a family of three ABA-inducible bZIP transcription factors, have been proposed to mediate drought resistance response after SnRK2 phosphorylation, by binding to the ABRE elements in the promoter regions of ABA-regulated genes. However, the *Arabidopsis abf2*/*abf3*/*abf4* triple mutant presents a normal transpiration (Yoshida et al., 2010), which suggests that other OST1 targets may be involved in ABA transcriptional regulation (Xue and Loveridge, 2004; Shinozaki and Yamaguchi-Shinozaki, 2007; Fujita et al., 2011; Golldack et al., 2011).

Other TFs have been described to act downstream of SnRK2s since the discovery of ABFs. For instance, a maize SNAC-type transcription factor, ZmSNAC1, has been identified as a ZmOST1 substrate, opening the possibility for this TF class to play a role in ABA signal transduction (Vilela et al., 2013). In *Arabidopsis* two basic helix-loop-helix (bHLH) TFs called AKSs, responsible for enhancing stomatal opening by stimulating the transcription of inward K^+ channels, have been shown to be repressed by ABA-dependent OST1 phosphorylation (Takahashi et al., 2013).

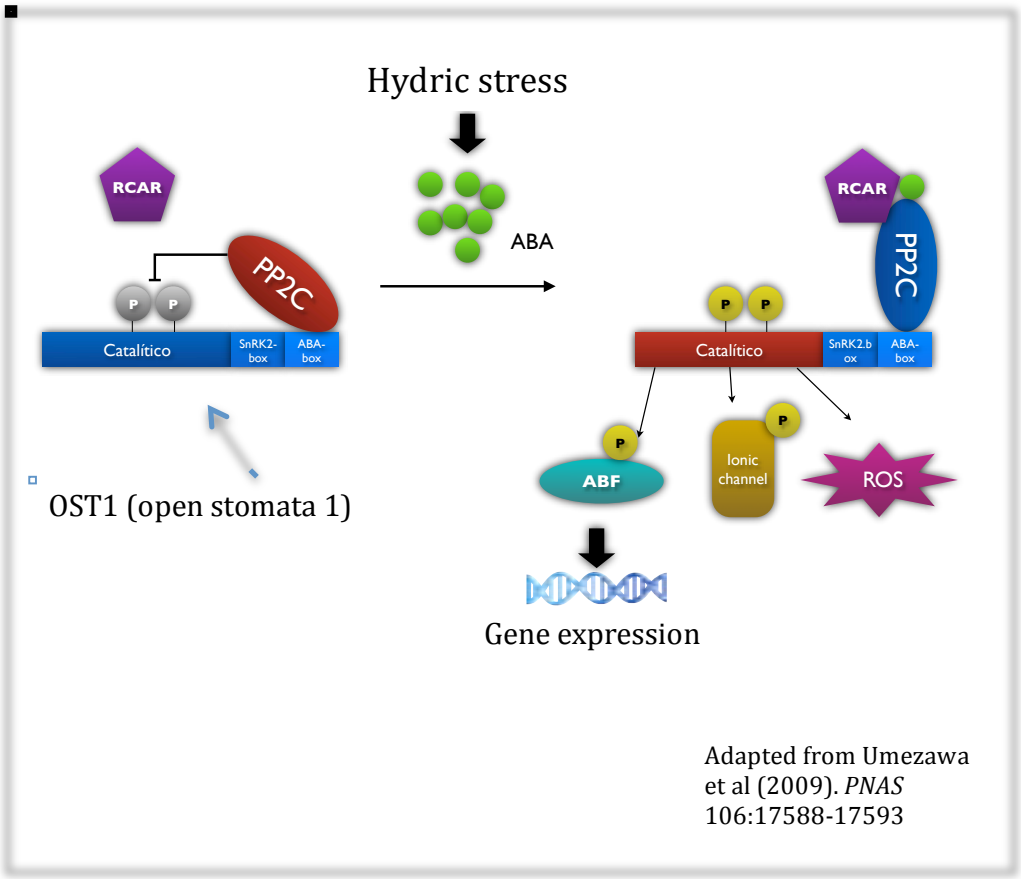


Figure 3. Core complex of ABA signaling.

In non-stress conditions, OST1 is inhibited constitutively by PP2C phosphatase through dephosphorylation. In ABA presence PYL/PYR/RCAR receptors bind ABA and inhibit PP2C phosphatases. This permits OST1 activation through trans-(auto)phosphorylation in its activation loop. Active OST1 phosphorylates its substrates, like transcription factors, ionic channels and NADPH oxidase RBOHF.

Taking into account these latest findings, the study of new OST1 interactors represents an important field of study to better understand drought response in plants.

1.3. STOP1 transcription factor

AtSTOP1 (for sensitive to proton rhizotoxicity1) is a Cys_2-Hys_2 type zinc-finger putative nuclear TF isolated from *Arabidopsis* in 2007. The peptidic sequence is 499 amino acids long, and it contains four potential C_2H_2 zinc-finger domains predicted in silico. Blast search reveals that the protein is present in other plant species, where the four zinc-finger domains are conserved (Iuchi et al., 2007; Iuchi et al., 2008; Sawaki et al., 2009).

AtSTOP1 was first discovered in a root-bending assay designed to detect H^+ hypersensitivity among an ethyl methanesulfonate-mutagenized population. It showed a reduced root length in low pH medium compared to the wild type (Fig. 4). The *stop1* mutation was located in the essential zinc-finger domain 1, where the Hys residue conforming the finger was replaced by Tyr. The phenotype was confirmed in a T-DNA insertion mutant, and it was rescued by overexpressing wild type *STOP1* gene in the mutant background (Iuchi et al., 2007).

Acid soil syndrome is associated with toxicity of other ions. In an attempt to find other rhizotoxicities affecting the *stop1* mutant, ions such as copper, lanthanum, sodium, cadmium, manganese and aluminum (Al) were tested. Al^{3+} was found to induce root growth inhibition in STOP1 defective backgrounds. Complementation assays rescued the wild-type phenotype. The conclusion of these findings is that STOP1 is involved in H^+ and Al^{3+} tolerance (Iuchi et al., 2007).

The genes involved in Al^{3+} hypersensitivity in *stop1* mutant have been identified and described. *AtALMT1*, an Al-activated malate transporter crucial

for Al^{3+} tolerance, was identified as the principal gene regulated by AtSTOP1 to be responsible for Al^{3+} tolerance. Its expression was abolished in *stop1* gene defective backgrounds, and so was the ability of these plants to excrete malate in Al^{3+} medium. Other genes involved in Al^{3+} tolerance were identified as substrates of STOP1, such as *AtMATE*, a multidrug and toxic compound exclusion protein, which is responsible for the exudation of citrate (Liu et al., 2009), or *ALS3*, an Al^{3+} transporter responsible for redistributing Al^{3+} from sensitive tissues (Sawaki et al., 2009).

OsART1, a rice homolog of AtSTOP1, has also been characterized. Analyses have shown how this TF is able to regulate Al tolerance genes in rice, though the mechanism for Al tolerance in rice differs from *Arabidopsis*. In rice, OsART1 regulates *OsSTAR1* and *OsSTAR2* genes, two components of an UDP-glucose transporter complex, which is involved in Al-detoxification through changes in the cell wall (Yamaji et al., 2009). In wheat, a protein with homology to AtSTOP1 has also been described to regulate Al tolerance genes (Houde and Diallo, 2008).

DNA-binding specific sequence has been determined for both OsART1 and AtSTOP1. These studies have used the promoters of Al resistance genes regulated by the TFs to elucidate this target sequence, i.e. OsSTAR1 in the case of OsART1 (Tsutsui et al., 2011), and *AtALMT1* in the case of AtSTOP1 (Tokizawa et al., 2015).

Several studies suggest that Al^{3+} and H^+ tolerance mechanisms seem to be genetically related in some plant species (Rangel et al., 2005; Yang et al., 2005). However neither *ALMT1* nor *ALS3* have an effect in H^+ tolerance in *Ara-*

bidopsis, because the knockout mutants of *AtALMT1* and *AtALS3* do not show H^+ sensitivity (Kobayashi et al., 2007).

Although we have a strong knowledge about toxicity produced by certain ions in acid soils, we lack information about the molecular basis regulating H^+ toxicity. Koyama's laboratory conducted an experiment to identify the genes responsible for low pH hypersensitivity in *stop1* mutant. They performed a microarray on *Arabidopsis* under low pH conditions to detect deregulated genes. The results point to genes involved in GABA shunt pathway such as *GABA-T*, *GAD*, *GDH1* and *GDH2* or genes involved in the biochemical pH stat pathway (Sawaki et al., 2009). K^+ transport and homeostasis has also been identified as a mechanism to regulate pH in some organisms (Zhang and Kone, 2002). *CIPK23*, a kinase regulating one of the major K^+ transporters *AKT1*, was repressed in *stop1* mutant. This kinase could have an effect in pH regulation by STOP1 (Sawaki et al., 2009).

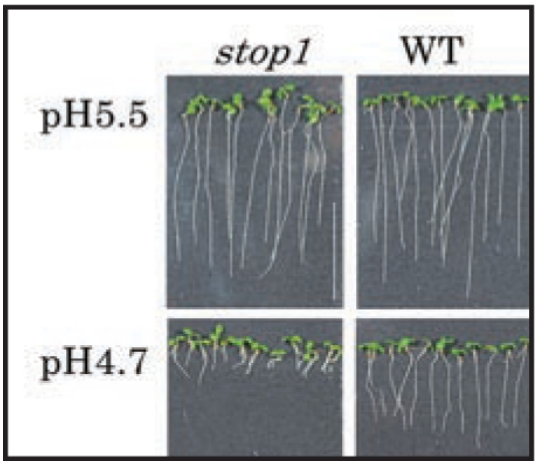


Figure 4. Arabidopsis stop1 mutant phenotype under low pH conditions.

Atstop1 mutant shows a shorter root length than the wild type in low pH conditions. Image extracted from (Iuchi et al., 2008)

Some studies have evaluated the capacity of different STOP1 homologs in several species to complement the *Arabidopsis stop1* mutant's inability to tolerate Al^{3+} and H^+. STOP1 homologs in *Nicotiana tabacum*, *Camelia sinensis*, *Lotus japonicus*, *Populus nigra* and *Physcomitrella patens* were able to recover H^+ tolerance in experiments measuring root growth. However, only some of the homologues were able to activate transcription of Al tolerance genes (Ohyama et al., 2013), meaning that STOP1 pH regulation is well conserved among species.

In fact, a recent work on *Vigna umbellata* VuSTOP1 concludes that although Al tolerance can only be partially restored in complemented *Atstop1* mutants, H^+ tolerance is fully reestablished by VuSTOP1 protein (Fan et al., 2015).

ABA and pH regulation are closely associated. For instance, ABA hyperpolarizes the maize root stellar cells (Roberts and Snowman, 2000). On the contrary, the stomatal closure in response to drought requires the plasma membrane depolarization, provoking the alkalinization of the apoplastic space. This is driven by the inhibition of the plasma membrane H^+-ATPase by dephosphorylation, through a process still unknown, in which the kinase OST1/SnRK2.6 is involved (Merlot et al., 2007; Yin et al., 2013). Some other studies also relate the proton tolerance mechanisms with ABA-induced seedling growth arrest (Planes et al., 2015)

Although pH regulation is impaired in *stop1* mutant, so far no study has implicated STOP1 in drought responses. Here we describe the role of a maize STOP1 homolog in ABA signaling. ZmSTOP1 overexpression has opposite effects depending on the tissue studied. Whereas in plants overexpressing ZmSTOP1

stomata are insensitive to ABA, roots show hypersensitivity to the hormone. We demonstrate that ZmSTOP1 is affecting genes involved in pH regulation, calcium signaling and ion transport. We also show how this TF is phosphorylated by OST1 after ABA treatment. Furthermore, ZmSTOP1 interacts with and is a substrate of ZmOST1, a representative maize SnRK2 from the central ABA signaling complex.

OBJECTIVES

2. Objectives

The long-term goal of our work is to further understand the mechanisms of drought tolerance in maize plants.

Previous work have revealed that ZmOST1 kinase is a central player in drought-tolerance regulation through ABA signaling in maize

Recently ZmOST1 protein-protein interaction experiments showed that this kinase is able to bind a variety of proteins, among them ZmSTOP1.

Here we focused on the role of ZmSTOP1 in drought tolerance in maize. For that purpose, we specified the following objectives:

1. ZmSTOP1 characterization

In this chapter we focused on the molecular characterization of ZmSTOP1. The specific topics of study were:

- Gene characteristics: *In silico* study of inter- and intraspecific conservation of ZmSTOP1 amino acid sequence. Expression pattern of ZmSTOP1 in maize, and its response to stress effectors and hormones.
- Molecular characteristics of ZmSTOP1 protein: cellular localization, dimerization properties, and DNA-binding capacity.

- DNA-binding specificity: determination of in vivo and in vitro DNA targets of ZmSTOP1. Protein-protein interaction study to confer DNA-binding specificity.

2. ZmSTOP1 functional analysis

To address this chapter we used Atstop1 mutant as a tool to understand ZmSTOP1 function. We focused in:

- ZmSTOP1 ability to complement Atstop1 mutant phenotype in low pH conditions (Iuchi et al., 2007).
- ZmSTOP1 overexpression phenotype under drought conditions and after ABA treatment, both in Arabidopsis and maize.
- Study of the genes deregulated by ZmSTOP1 overexpression in the heterologous system Arabidopsis thaliana.

3. ZmSTOP1 regulation by phosphorylation

To study the regulation of ZmSTOP1 we focused on its interaction with ZmOST1. The central subjects were:

- Confirmation of ZmSTOP1 and ZmOST1 interaction both in vitro and in vivo.
- Phosphorylation pattern of ZmSTOP1 by OST1 kinase, paying special attention to ABA signaling.
- Physiological role of OST1 phosphorylation over ZmSTOP1 after ABA treatment and under drought conditions.

RESULTS

3. Results

In our laboratory we focus on drought responses in maize. In previous work we performed a yeast two-hybrid screening using ZmOST1 kinase as a bait against a cDNA library from drought stressed leaves. This experiment yielded several putative ZmOST1 interactors, most of them transcription factors.

In this work we focus on the study of one of these interactors, a maize Zn-finger TF, homolog to the *Arabidopsis* STOP1.

3.1. ZmSTOP1 characterization

ZmSTOP1 is a putative Zn-Finger transcription factor conserved among plant species

In order to study the degree of conservation of STOP1 among different plant species, we performed a phylogenetic tree using the protein sequences of its closest homologs (Fig. 5 A). We detected a high conservation among Poaceae. Brassicaceae and other dicots were grouped in different clades.

To check for homologs in maize, we performed a sequence alignment (Fig. 5 B), with the paralogs obtained using the Blast tool. ZmSTOP1 is located in chromosome five. Four other paralogs were identified in chromosomes one, three, eight and ten. When checking the degree of conservation between

them, we observed four highly conserved Zn-finger domains (Iuchi et al., 2007) with an elevated percentage of identity, highlighted in blue in Fig. 5 B.

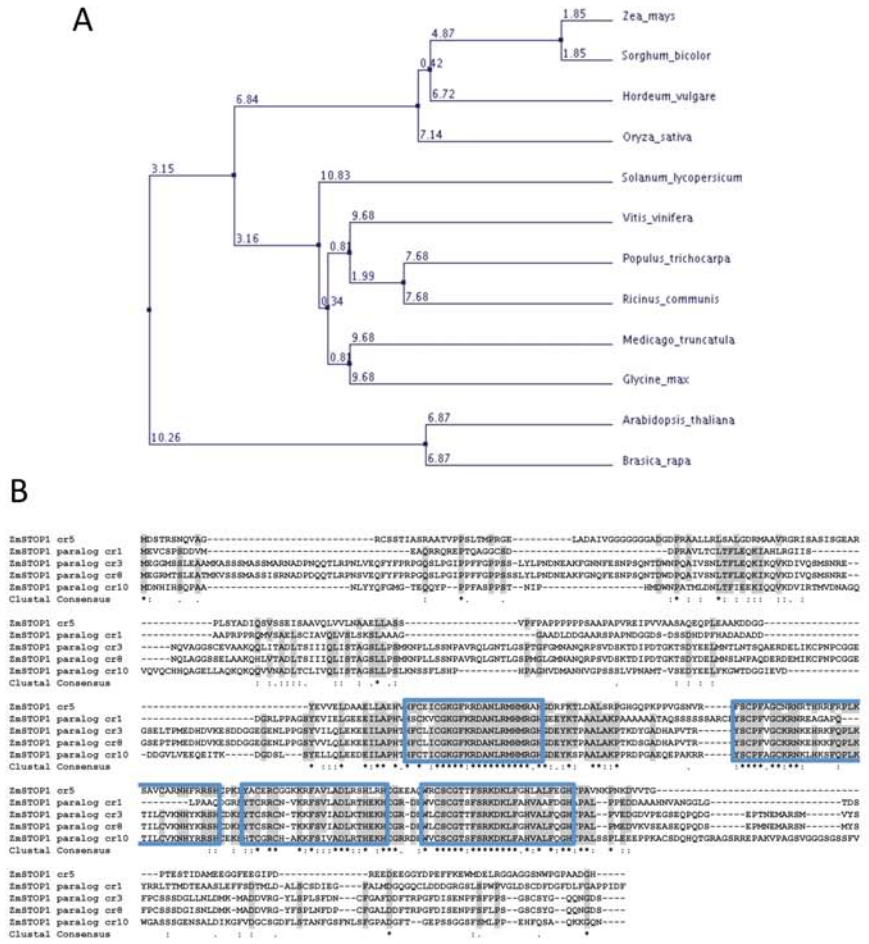


Figure 5. Phylogenetic study of ZmSTOP1

A) Phylogenetic neighbor-joining analysis of various ZmSTOP1 homologues in different species. B) ZmSTOP1 paralogs alignment. ZmSTOP1 aligns with protein sequences from maize and exhibits four highly conserved Zn-finger domains., highlited in blue.

ZmSTOP1 is expressed differentially in shoots and roots, and in response to different treatments

As we identified ZmSTOP1 to be an interactor of ZmOST1, a very important player in stress responses (Vilela, 2012), we decided to continue the characterization of this protein by checking its expression under several stress effectors.

We started by checking ZmSTOP1 expression by qRT-PCR in 10-day-old maize W64A pure inbred seedlings (Fig. 6 A). mRNA copies of this putative transcription factor (TF) could be found both in shoots and roots, showing higher expression in shoots.

Next, we examined its expression after several stress-related treatments, including NaCl, known to induce OST1 activity (Boudsocq et al., 2004; Boudsocq et al., 2007) H_2O_2, an important ROS species whose synthesis is effected by several stresses including drought (Mittler and Blumwald, 2015); methyl jasmonate, a signaling molecule involved in a diversity of defense responses, including wounding (Zhang et al., 2015); and finally ABA, central in stress signaling response (Tuteja, 2007) (Fig 6 B). The measures were taken in a unique experiment after 6 hours, and were expressed relatively to the control conditions. ZmSTOP1 expression was highly enhanced by NaCl and ABA in roots whereas in leaves it only shows a slight increase after methyl jasmonate treatment.

Salinity and drought are both osmotic stresses that provoke the increase of ABA levels in the plant (Finkelstein et al., 2002; Zorb et al., 2013). Thus, is not

surprising that both ABA and NaCl have an effect on ZmSTOP1 expression, as the genes regulated by this putative TF could be decisive under any kind of osmotic stress.

As a control for ABA treatment we checked the expression levels of the *ZmRAB17* ABA-responsive gene (Vilardell et al., 1990) (Fig. 6 C), which was activated in both shoots and roots relatively to ZmRAB17 levels in control treatment.

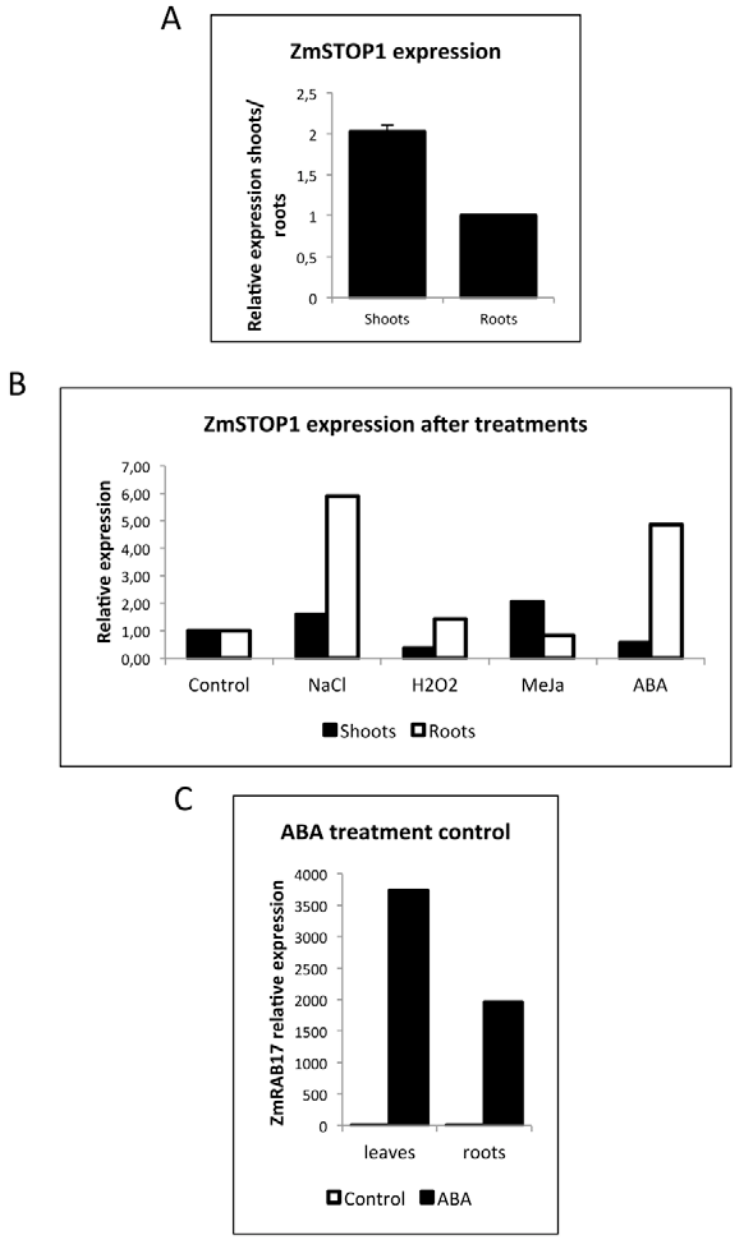


Figure 6. Analysis of ZmSTOP1 expression levels.

Real time qPCR analysis of ZmSTOP1 transcript levels in A) in 9-days-old maize shoot and root, expressed relatively to root expression B) under different treatments (NaCl 250 mM, H_2O_2 10 mM, MeJa 0,01% and ABA 100 mM), expressed relatively to control (0,5x MS buffer) C) *ZmRAB17* expression levels were used as control for the osmotic stress treatments. ZmSTOP1 is expressed in both maize shoots and roots, although at a higher level in shoots. Values expressed relatively to the control treatment (MS1 buffer). ZmSTOP1 expression is increased by NaCl and ABA in roots whereas it is increased by methyl jasmonate in shoots.

ZmSTOP1 localizes in the nucleus and forms dimers

In previous studies it was shown how the *Arabidopsis* homolog AtSTOP1 presents a nuclear localization, indicating that this protein might be involved in transcriptional regulation (Sawaki et al., 2009).

We performed an *in silico* study of the ZmSTOP1 protein sequence in order to identify putative nuclear localization signals (NLSs). The cNLS Mapper software (Kosugi et al., 2009) was used for this purpose. A bipartite NLS was detected in the putative DNA binding domain, corroborating that the nuclear localization could be conserved in maize. (Fig. 7 A).

In order to confirm the subcellular localization of ZmSTOP1 in plants, we agroinfiltrated tobacco leaves with a construct containing ZmSTOP1-GFP fusion under the control of a double CaMV35S promoter. We detected fluorescence in tobacco nuclei, indicating that, like its *Arabidopsis* homolog, ZmSTOP1 is a nuclear protein (Fig. 7 B). Transiently overexpressing the same construct in maize protoplasts also confirms this nuclear localization of ZmSTOP1.

Many Zn-finger transcription factors need to homodimerize to act as transcriptional regulators (Figueiredo et al., 2012). To determine whether ZmSTOP1 is capable of dimerization, we performed a bimolecular fluorescence complementation (BiFC) assay fusing ZmSTOP1 to both halves of the YFP protein, under a constitutive 35S promoter. The presence of fluorescence inside the nucleus when tobacco cells were agroinfiltrated with both constructs indicated that ZmSTOP1 could dimerize (Fig. 7 C).

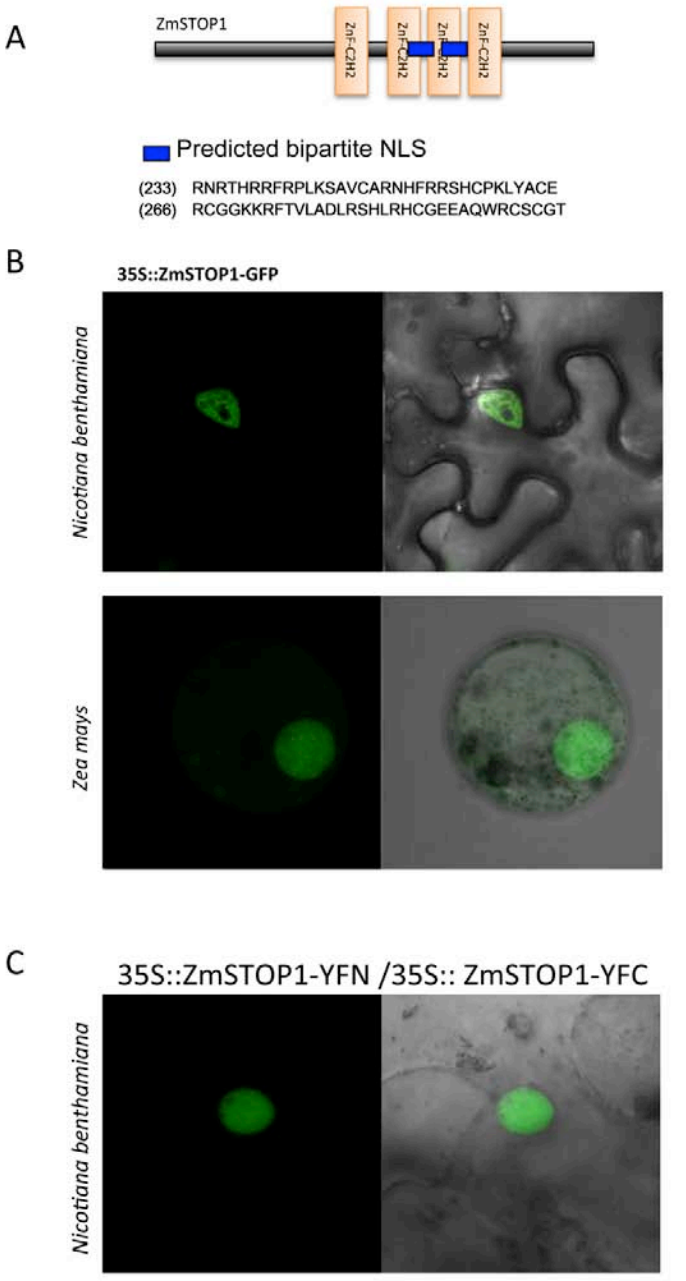


Figure 7. Subcellular localization of ZmSTOP1

A) In silico prediction of nuclear localization signals in the ZmOST1 protein sequence. B) Overexpression of *35S::ZmSTOP1-GFP* in tobacco (upper panel) and maize protoplasts (lower panel) indicates a predominantly nuclear localization for this protein. C) BiFC fluorescence images, analyzed by confocal microscopy, of the interaction between YC-ZmSTOP1 and YN-ZmSTOP1 shows that ZmSTOP1 is capable of dimerization in the nucleus.

ZmSTOP1 binds random DNA

The nuclear localization of ZmSTOP1 and the presence of four zinc-finger domains suggest that this maize protein could be a transcription factor (Sawaki et al., 2009). We decided to perform an electrophoretic mobility shift assay (EMSA) to determine the ability of ZmSTOP1 to bind random DNA (Fig. 8). Fluorescent-labeled random DNA was used as a probe, and purified ZmSTOP1 expressed in *E. coli* (shown in Coommassie Brilliant Blue) was added to the mix. We detected a shift in the DNA band of increasing intensity as the concentration of protein increased, corresponding to the binding of ZmSTOP1 protein to the labeled DNA. After treating the sample with proteinase K the shifted band disappeared, indicating that the shift was due to the presence of the protein. We used LEAFY (LFY) protein extract as a positive control for the experiment.. LFY is a very well documented TF regulating flowering development in angiosperms (Weigel and Nilsson, 1995; Hames et al., 2008),

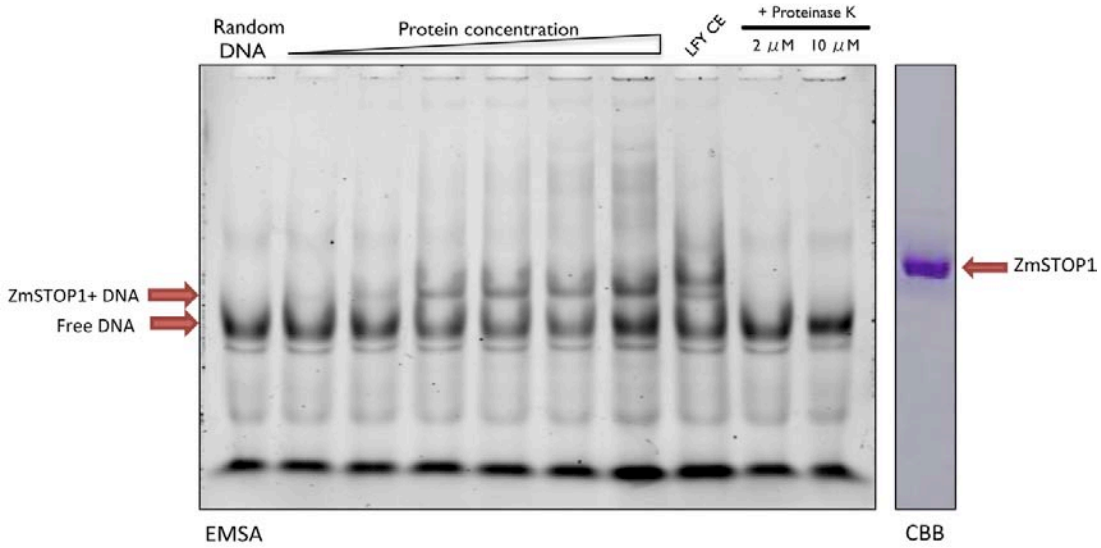


Figure 8. Electrophoretic mobility shift assay

ZmSTOP1 purified protein (CBB) is mixed with random TAMRA-labeled DNA probe to detect a delay in the DNA mobility. Negative control (lane 1) does not contain protein in the mix. Increasing ZmSTOP1 concentration is added to the mix (lane 2 to 7). Crude *E. coli* extract overexpressing LFY TF is used as positive control (lane 8) and proteinase K is added to the mix (lane 8 and 10) as a negative control. Shifts in the DNA band are detected with increasing intensity in lanes 2 to 7, product of DNA-ZmSTOP1 binding. This shift is abolished after proteinase K addition (lanes 9 and 10).

3.2. Searching ZmSTOP1 DNA-binding specificity

The ZmSTOP1 homologues in *Arabidopsis* and rice have been demonstrated to regulate many substrates (Sawaki et al., 2009; Yamaji et al., 2009). Since then both laboratories have been working to determine the DNA binding sequence of STOP1 in the two species. We have also worked in this direction with the maize homolog, trying to identify both the DNA target sequence and the *in vivo* target promoters of ZmSTOP1.

We were unsuccessful in identifying any specific DNA targets for ZmSTOP1 either *in vitro* or *in vivo*, though a very important amount of work was done to achieve this goal. We used different approaches to identify *in vitro* and *in vivo* targets. First we tried *in vitro* methodologies like EMSA with different probes, Selective Evolution of Ligands by Exponential Enrichment (SELEX), and protein array. Later we used *in vivo* techniques, like Chromatin Immunoprecipitation (ChIP), or GUS-reported expression of a promoter region. Finally, we also tried to find protein partners of ZmSTOP1 hoping to identify other TFs that could give specificity to the DNA-binding capacity of ZmSTOP1.

ZmSTOP1 does not bind to specific probes from *Arabidopsis* promoters

We decided to test whether ZmSTOP1 was able to bind a specific sequence present in the promoters of genes regulated by AtSTOP1. For that purpose we collected the promoters from around 60 de-regulated genes in *atstop1* mutant (Sawaki et al., 2009). We looked for overrepresented DNA domains in these promoters using MEME software (Bailey and Elkan, 1994; Bailey et

al., 2009), expecting that these sequences could be the AtSTOP1 targets and also be bound by ZmSTOP1 *in vitro*. We chose several interesting genes for our purpose, and designed specific DNA probes using the overrepresented sequences obtained from the study.

We used the promoters of AtALMT1, a malate transporter responsible for rhizosfere acidification (Hoekenga et al., 2006) described as AtSTOP1 target (Iuchi et al., 2007), *AtUMAMIT14*, a transporter highly deregulated in *Atstop1* mutant, *AtXTH24*, a cell wall enzyme also highly deregulated in *Atstop1* mutant, *AtCIPK23*, a CBL-interacting protein kinase very important for stomatal function (Nieves-Cordones et al., 2012) and *AtSLAH1* a *SLAC1* homologue, which may be important for stomatal regulation (Negi et al., 2008). We labeled the specific probes designed with fluorescent dye and performed an EMSA assay. ZmSTOP1-6xHis purified from *E. coli* (Fig. 9 A and B) was used for the experiment and random DNA was used as a positive control. We did not detect shifts in the DNA-probes designed, indicating that either these were not target sequences of AtSTOP1 or ZmSTOP1 was not able to bind the same target sequences than AtSTOP1.

A

ALMT1 (AT1G08430)	5'GAAGAGTCTCTCTTGGTCTTTATAA 3'CTTCTCAGAGAGAACCAGAAATATT
UMAMIT14 (AT2G39510)	5'GACCCTCCTATAAAACCAACCTCAA 3'CTGGGAGGATATTTTGGTTGGAGTT
XTH24 (AT4G30270)	5'GTAATATCCCTCTAGACCTCTGGTT 3'CATTATAGGGAGATCTGGAGACCAA
CIPK23 (AT1G30270)	5'GCAGAACCTACAGATACAACCTTCC 3'CGTCTTGGATGTCTATGTTGGAAGG
SLAH1 (1) (AT1G62280)	5'GGAGATTCTCTTTTTCTTTCAGCAA 3'CCTCTAAGAGAAAAAGAAAGTCGTT
SLAH1 (2) (AT1G62280)	5'GCAACACTAACTAACTCCCCCTAAA 3'CGTTGTGATTGATTGAGGGGGATTT

B

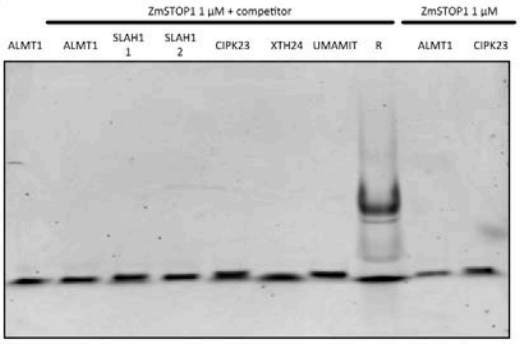


Figure 9. ZmSTOP1 binding to specific promoter sequences.

Cy3-labeled specific DNA probes (A) were designed according to specific promoter sequences present in AtSTOP1-regulated promoters. An EMSA was performed (B) to test the ability of ZmSTOP1 to bind these sequences (lanes 2 to 7). ALMT1 probe was used as a negative control (lane 1) and random DNA as a positive control for the experiment (lane 8). Two different amounts of protein were used (lanes 2-8 and 9-10). No shifts were detected.

ZmSTOP1 does not bind a specific DNA sequence in vitro

In order to define the DNA binding sequence of ZmSTOP1 putative TF we performed a SELEX assay. These experiments were conducted during a short stage at François Parcy's laboratory, as this is a routine technique in this laboratory (Moyroud et al., 2011; Chahtane et al., 2013; Sayou et al., 2014).

The experiment consists of mixing recombinantly-produced ZmSTOP1 protein with a pool of random DNA. Later, ZmSTOP1 is immunoprecipitated carrying random DNA with compatible sequences to ZmSTOP1. This DNA is purified and amplified using specific primers, and the process is repeated at least three times. After each round of enrichment an EMSA is performed to check for stronger shift bands, which correspond to the purified DNA with higher affinity

for ZmSTOP1. When the enrichment is considered strong enough the DNA pool of enriched sequences is sent to sequencing. The resulting sequences are assigned a score depending on the probability to be found in selected DNA (Fig. 10 A).

We performed five rounds of enrichment. After checking the enrichment by EMSA we detected the same intensity of the shift either in random DNA or in five-rounds enriched DNA (fig. 10 B). We concluded that ZmSTOP1 was unable to bind a determined DNA sequence specifically and we stopped the procedure.

We repeated the same experiment with a shorter version of ZmSTOP1, which included the DNA-binding domain [G_{154}-H_{386}]. We obtained similar results with this fragment (data not shown).

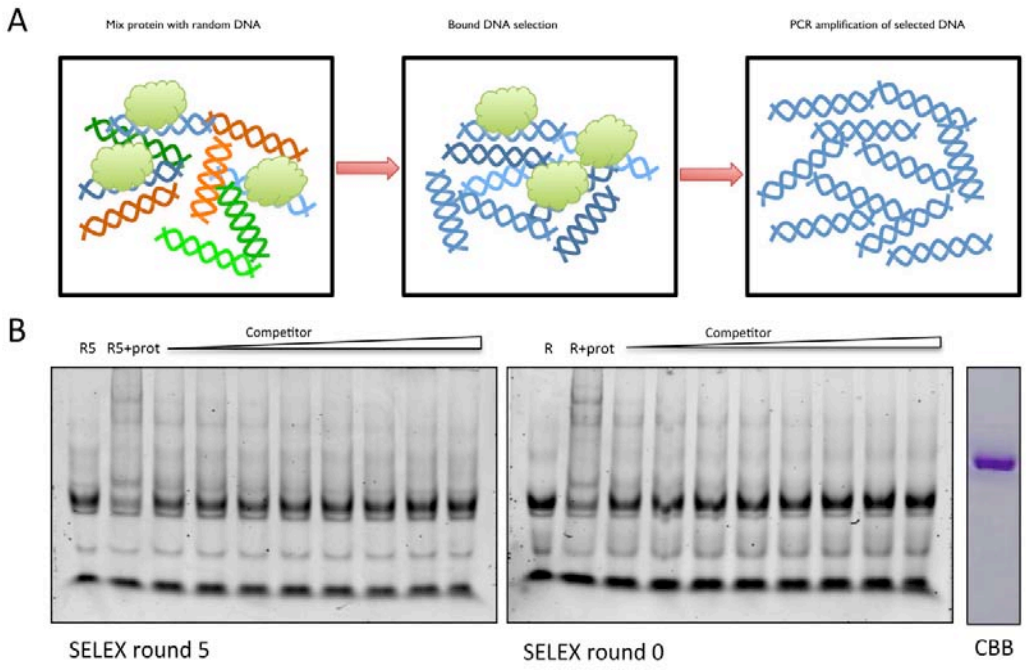


Figure 10. SELEX assay.

A) Representative image of one round of SELEX procedure. Free random DNA is mixed with purified protein (left panel), the protein is purified with the selected sequences of random DNA (middle panel) and selected DNA is amplified (left panel). B) EMSA with purified DNA after 5 rounds of SELEX with ZmSTOP1 (CBB) (left panel) and EMSA with random DNA (right panel). No protein is loaded on lane 1 in both panels. A positive control is loaded on lane 2, with no competitor (fish DNA). Increasing amount of competitor is added to lanes 3 to 10. The intensities of the shifts decrease with the addition of competitor, indicating the lack of specificity of the protein-DNA shifts.

Protein Array with ZmSTOP1

After the lack of success in the SELEX approach, we decided to identify the target DNA sequence for ZmSTOP1 by performing a protein array. This technique incubates recombinantly-produced ZmSTOP1 on a chip with a series of random DNA probes. Depending on the quantity of protein is accumulated the probes are assigned a probability of being ZmSTOP1 target.

Surprisingly this technique yielded no confident results, due to the lack of specificity of ZmSTOP1 for any determined sequence (data not shown).

ZmSTOP1 ChIP-seq

To identify *in vivo* targets of ZmSTOP1 we decided to perform a ChIP followed by sequencing (ChIP-seq) assay. This experiment was conducted during a short stage at Dr. Markus Schmid's laboratory.

We used for this experiment *Arabidopsis* Lansberg Erecta (Ler) 12-day-old plants overexpressing ZmSTOP1-GFP driven by a double CaMV35S promoter for enhanced expression (Fig. 11 A). These plants were obtained by floral dipping transformation technique and driven to homozygosis by self-crossing for three generations. Hygromycin resistant plants at T3 generation were examined for fluorescence in the nuclei using a fluorescence microscope. We treated the plants with and without ABA (100 μM) for 3 hours before the experiment to increase the probability of success, expecting that ZmOST1 could play a role in ZmSTOP1 DNA-binding specificity.

We also transformed maize mesophyll protoplasts obtained from 12-14 day-old seedlings with the same construct, expecting to find the maize targets of ZmSTOP1 using a homolog system (Fig 11 B). The transformation efficiency of the protoplasts obtained ranged between 40 and 50%. The transformed protoplasts were treated with and without 10 μM ABA for 30 minutes. Protoplasts transformed with the empty vector expressing GFP were used as a control.

A qPCR against promoter regions of *AtCIPK23* and *AtROP4* genes (Sawaki et al., 2009) was carried on *Arabidopsis* samples to check for enrich-

ment before continuing with the experiment. SOC1 gene, involved in flowering, was used as a negative control. No enrichment was detected at this point, but we decided to continue with the procedure, as DNA amplification was obtained, and no positive control was available for this experiment.

We constructed libraries with the DNA obtained from immunoprecipitating ZmSTOP1-GFP using an anti-GFP antibody. Before sequencing the libraries we checked the quality of the DNA obtained from all the samples in an automatic electrophoretic assay using Bioanalyzer system (Agilent Technologies, Santa Clara). Though we had obtained previously DNA amplification from ChIP, the results obtained by Bioanalyzer indicated that the quality of the DNA obtained from the libraries was not good enough to continue with the sequencing. There seemed to be too low DNA so the adaptors formed oligomers, which were detected as isolated peaks in the automated electrophoresis plots.

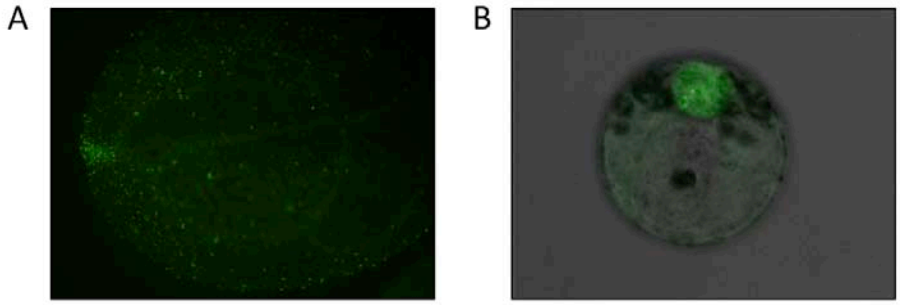


Figure 11. ZmSTOP1 ChIP-seq.

Chromatin immunoprecipitation was carried out on *Arabidopsis* seedlings overexpressing ZmSTOP1-GFP fusion (A) and on maize protoplasts overexpressing the same construct (B).

ZmSTOP1 interactors by Yeast Two-Hybrid

Due to our difficulty in determining *in vivo* targets of ZmSTOP1, we hypothesized the necessity of other proteins to interact with this TF in order to specify a target DNA sequence.

For this purpose we ordered a yeast two-hybrid screening using ZmSTOP1 full-length protein as a bait to Hybrigenics company (Hybrigenics SA, Paris). Unfortunately, the screening yielded no results due to the lack of ZmSTOP1 specificity to bind other proteins.

ZmSTOP1 can activate ScALMT1 promoter

Dr. Juan Carlos Del Pozo kindly provided us with *ScALMT1* promoter (pScSALMT1) inserted into a binary vector and fused to beta-glucuronidase (GUS) reporter gene. He also sent us the cloned ScSTOP1 protein on a binary vector, under the control of CaMV35S promoter. He had detected that ScSTOP1 can activate GUS expression under the control of ScALMT1 promoter in tobacco plants. We conducted an expression experiment to test the possible activation of pScALMT1-GUS by ZmSTOP1.

We co-agroinfiltrated CaMV35S driven ZmSTOP1 and pALMT1-GUS. We used as a positive control the ScSTOP1 construct provided by Dr. Del Pozo. (Fig. 12). We performed a GUS staining after 2h (upper panel) and over-night (lower panel) to detect GUS expression. We could detect GUS expression in both cases when co-infiltrating with ZmSTOP1. The positive control (left panel) showed a

higher intensity of GUS staining, indicating a stronger induction of GUS reporter gene.

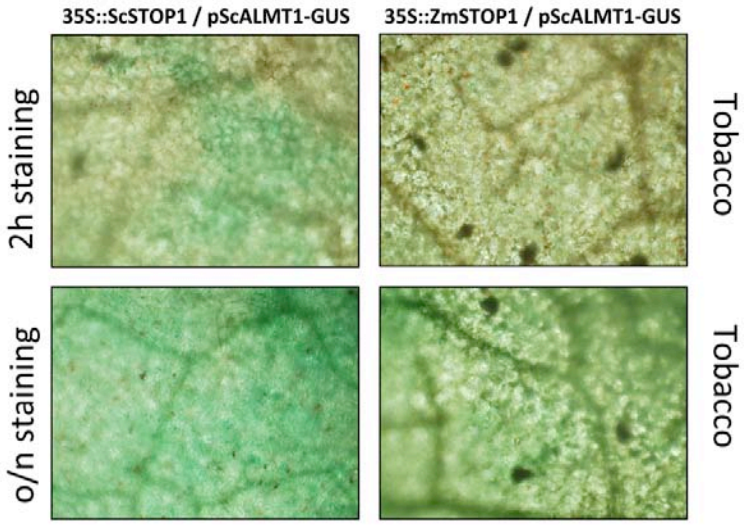


Figure 12. GUS staining of N. benthamiana leaves overexpressing ALMT1 promoter

N. benthamiana was agroinfiltrated with a mixture of ZmSTOP1 and ALMT1 promoter fused to GUS reporter gene (right panel). GUS staining is appreciated after 2 hours of staining (upper panel) and enhanced after over night staining (lower panel). ScSTOP1 was used as a positive control (left panel).

3.3. Functional analysis of ZmSTOP1 in the heterologous system *A. thaliana*

AtSTOP1 has been described to be involved in aluminum (Al) and proton (H^+) tolerance in soils (Iuchi et al., 2007; Iuchi et al., 2008; Sawaki et al., 2009). To check the degree of conservation between the *Arabidopsis* and maize STOP1 proteins, we performed an alignment using Clustal Omega software (Sievers et al., 2011). The most conserved domain is the DNA binding domain, with the four zinc-fingers (mainly domain 1 and 4) showing a higher degree of identity (Fig. 13 A).

To test the implication of ZmSTOP1 in proton tolerance, we decided to use *atstop1* T-DNA insertion mutant as a tool to assess the level of functional redundancy between AtSTOP1 and ZmSTOP1.

Arabidopsis mutant validation

We ordered *Arabidopsis stop1* Salk T-DNA insertion mutant (SALK_100763C) from the NASC seed bank (Scholl et al., 2000).

The T-DNA insertion in *AtSTOP1* gene is located around the nucleotide 238 out of 1500. *Atstop1* possesses one unique exon and no introns (Fig. 13 B).

To confirm that the *Arabidopsisstop1* T-DNA insertion mutant did not express the *STOP1* gene, we performed a quantitative real-time PCR of *Arabidopsis* seedlings. We could see that the expression level in the mutant relative to the

wild type was around 20 times lower (Fig. 13 C). We additionally genotyped the mutant plants. We were unable to amplify the *STOP1* gene in the mutant background whereas we could amplify the T-DNA insertion using specific primers in the same background. The opposite happened in the wild type background (Fig. 13 D).

The *Arabidopsis stop1* mutant is characterized by presenting a shorter root length in low pH than the wild type (Iuchi et al., 2007). We grew 4-day-old *Arabidopsis* seedlings in low pH medium for 7 days and confirmed that the phenotype was conserved, by measuring the root length of the treated plants with low pH relative to the control conditions (Fig. 13 E). We tested the expression of *AtCIPK23,* a gene involved in stomatal regulation (Cheong et al., 2007) and *AtALMT1,* a malate transporter responsible for Al tolerance *(Furuichi et al., 2010)* , both known to be repressed in the A*tstop1* mutant (Iuchi et al., 2007; Sawaki et al., 2009), and established that its expression was also highly diminished in our mutant seedlings relative to the wild type (Fig. 13 F).

Taking into account all this data, we could confirm that the Salk stock was suitable for further experiments.

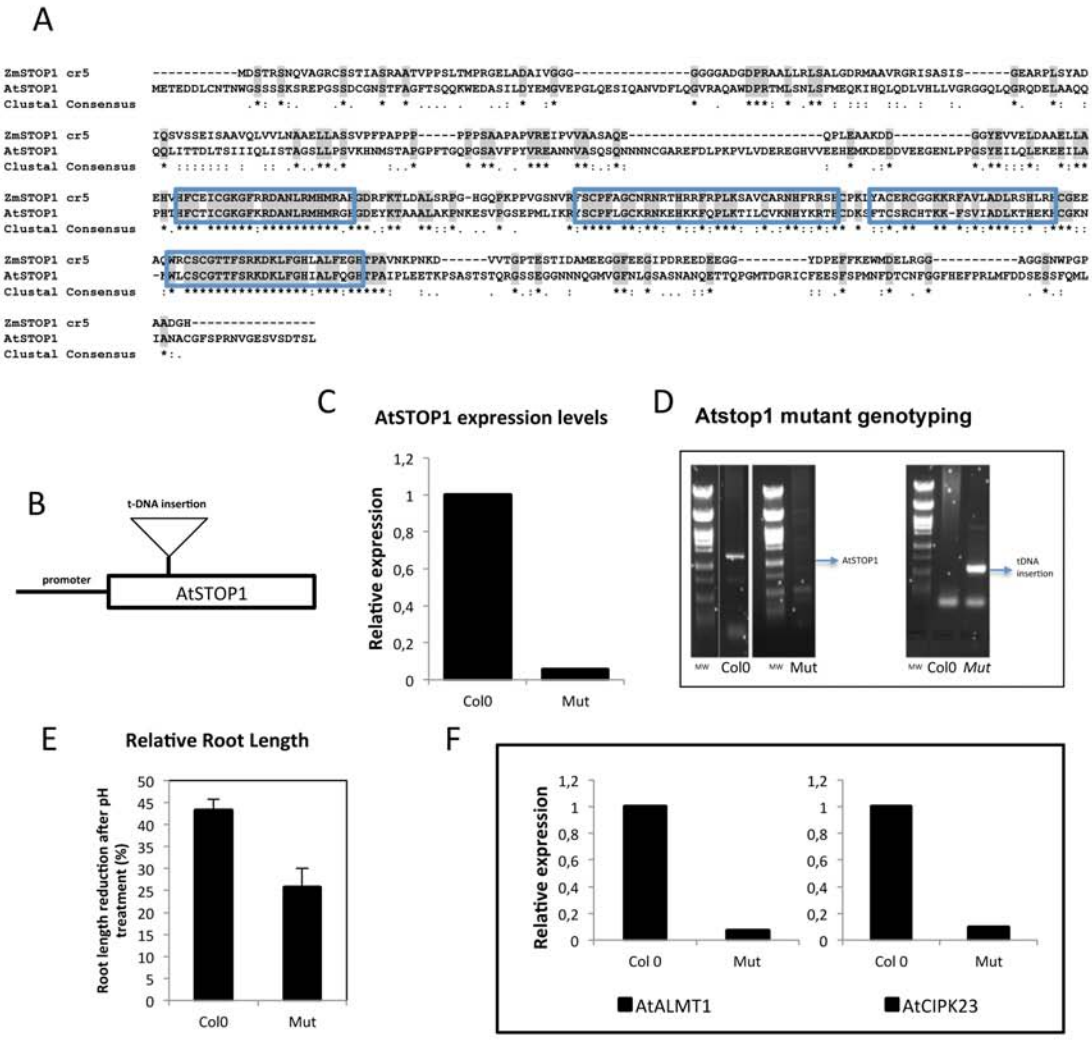


Figure 13. Characterization of the Atstop1 mutant.

A) Alignment of the AtSTOP1 and ZmSTOP1 protein sequences reveals a high identity at the DNA binding site. The SALK_100763C line that has a T-DNA insertion at nucleotide 238 (B) was verified for reduced *AtSTOP1* expression levels (C) and genotyping for the detection of the T-DNA sequence (D). This mutant line was used to verify the results of Iuchi et al. (2007) at the level of root growth under pH 4.7 (E) and relative expression of *AlMT1* and *CIPK23* (F). This mutant line was found suitable for complementation experiments using the *ZmSTOP1* gene.

ZmSTOP1 complements the loss of function mutant in *Arabidopsis*

We performed *in planta* complementation assays, using a double CaMV35S promoter driving a ZmSTOP1-HA fusion construct to transform the *Atstop1* mutant. We also generated overexpression lines, using the same construct, to transform wild type Col0 plants. We first analyzed the protein and expression levels of ZmSTOP1 in these plants by western blot (Fig. 14 A) and quantitative real time PCR (Fig. 14 B), respectively.

We then measured the root length of these plants to assess how ZmSTOP1 overexpression affected to root elongation. We detected that root length was inversely proportional to ZmSTOP1 protein levels, meaning that ZmSTOP1 protein has a detrimental effect on root elongation (Fig. 14 C).

We also tested the behavior of two independent complementation and overexpression lines in low pH medium (Fig 14 D and E). The complementation lines showed a higher root length ratio than the mutant in low pH treatment relative to control conditions, indicating that ZmSTOP1 is able to complement the susceptibility of *Atstop1* mutant to low pH. The two overexpression lines evidenced a higher root length ratio than the wild type, indicating that tolerance to H^+ in the medium depends on the expression levels of ZmSTOP1.

Using the complementation and overexpression lines that had the highest ZmSTOP1 expression, we analyzed the expression levels of different transcripts related to pH homeostasis, such as *GDH1*, *GDH2* and *GAD*, three genes belonging to the GABA-shunt pathway and *CIPK23*, a regulatory kinase of *AKT1*, a very important K^+ transporter, and involved in stomatal regulation (Sawaki et al., 2009). We detected that ZmSTOP1 complemented the reduced expression

of these genes in *atstop1* mutant background (Fig. 14 F). Overexpressing ZmSTOP1 in the wild type background resulted in increased expression of the *GDH2* and *GAD* genes.

Taken together these results indicate that ZmSTOP1 is able to complement the loss of function mutant in *Arabidopsis*, and the overexpression of the maize gene on the wild type background can induce a stronger effect on the phenotype and the expression of certain genes.

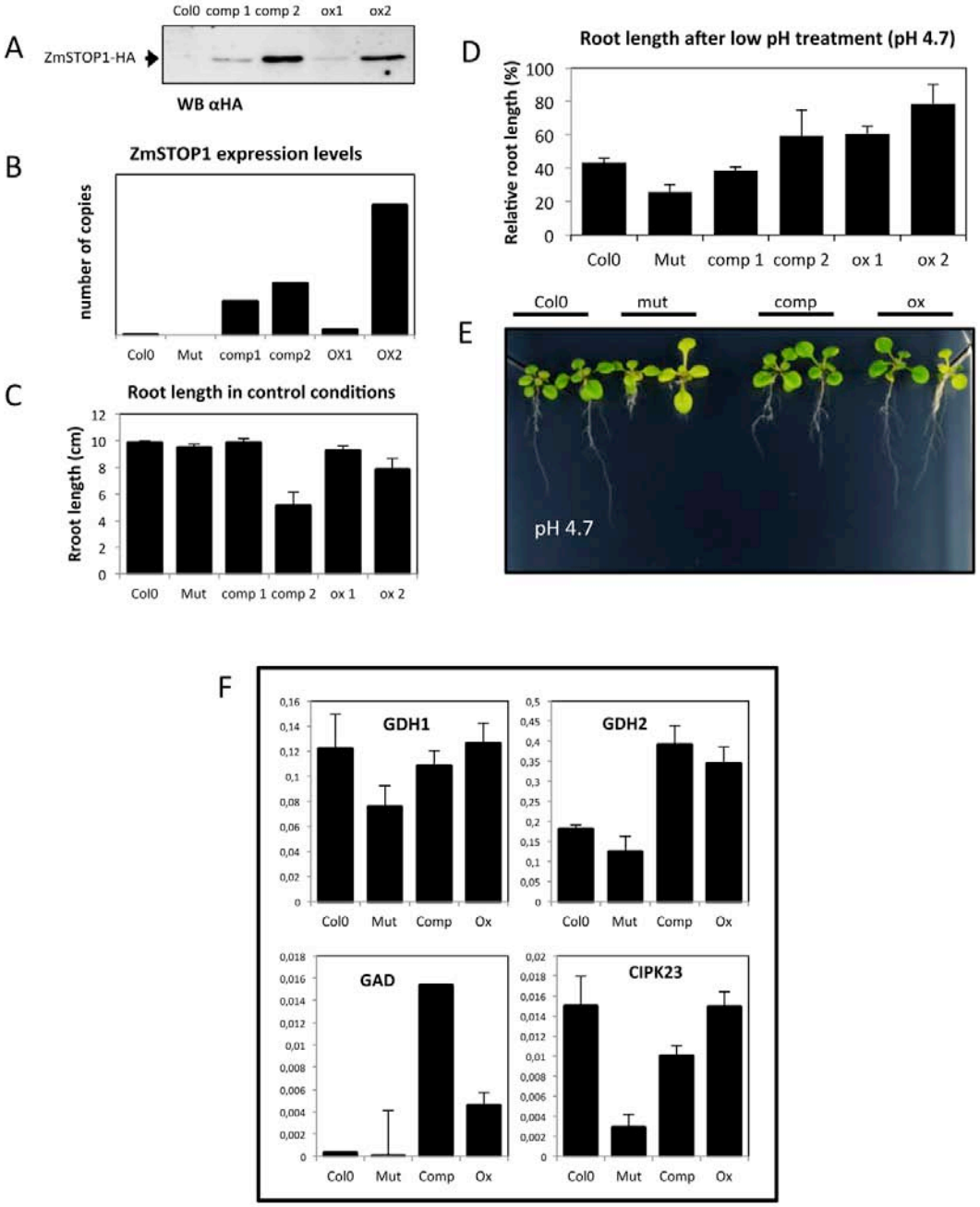


Figure 14. Heterologous expression of ZmSTOP1 in Arabidopsis.

35S::ZmSTOP1-HA was overexpressed in the *Arabidopsis* Col-0 wild-type (ox) and in the stop1 mutant (comp).Two transgenic lines for each background were used for subsequent studies. A) Western blot analysis of ZmSTOP1-HA protein levels.B) Real time qPCR of ZmSTOP1-HA expression levels relative to the wild type. C) Root length measurements of the different lines under control situations reveals that higher levels of ZmOST1 protein correlate with shorter roots. D),Root length measurements of the different *Arabidopsis* linesunder pH 4.7 treatment relative to the control conditions. E) Representative image of the phenotype ob-

served in D. F) Real time qPCR analysis of different genes involved in pH homeostasis that were found to be affected in the *stop1* mutant(Sawaki et al., 2009). The ZmOST1 protein complements the *stop1* mutant phenotype in response to low pH.

ZmSTOP1 confers insensitivity to ABA in stomata and hypersensitivity to ABA in roots

Since we identified ZmSTOP1 through a drought stress screening we decided to study the effect of this TF in drought responses.

To check the phenotypic response of *Arabidopsis* to ABA, we measured root elongation of 12-day-old seedlings in the presence of ABA (Fig. 15 A). The results showed that the *Atstop1* mutant was somewhat insensitive to the hormone, whereas the ZmSTOP1 overexpressing line was more susceptible to the presence of ABA. The overexpression of ZmSTOP1 in *Atstop1* mutant rescued the wild type phenotype.

Stomatal response is finely regulated by ABA (Sirichandra et al., 2009)and we were interested in determining whether ZmSTOP1 was involved in the regulation of stomatal aperture. With this purpose, we directly measured the stomata aperture of our transgenic lines (Fig. 15 B), by measuring stomata in microscope images taken from a nail-polish cast of the leaves. The leaf imprint was performed using dental resin. The *Atstop1* mutant displayed a smaller stomata aperture when compared to Col0, whereas the complementation lines recovered the wild type phenotype. Overexpression of ZmSTOP1 resulted in stomata with a wider pore.

ABA treatment of *Arabidopsis* wild type leaves provokes stomatal closure. The stomatal response varies depending on the ABA concentration used in the treatment (Mustilli et al., 2002). We then checked the changes in stomata aperture of our transgenic lines in response to ABA (Fig. 15 C). The *Arabidopsisatstop1* mutant presented a hypersensitivity to ABA, as stomata closed more dramatically than wild type with the increasing levels of the hormone. ZmSTOP1 overexpressing plants were somewhat insensitive to the application of the hormone. ZmSTOP1 complementation line rescued the wild type phenotype.

Finally, a water loss experiment was conducted to check the role of ZmSTOP1 in drought resistance (Fig. 15 D), by weighting detached rosettes from 3-week-old plants drying on a filter paper during a 100 min period. The ZmSTOP1 overexpression transgenic plants lost more water than the wild-type plants while the *Atstop1* mutant was less susceptible to drought. The phenotype of ZmSTOP1 overexpression plants was probably a consequence of the added effects of reduced root size, insensitivity to ABA in stomata and the wider stomatal aperture.

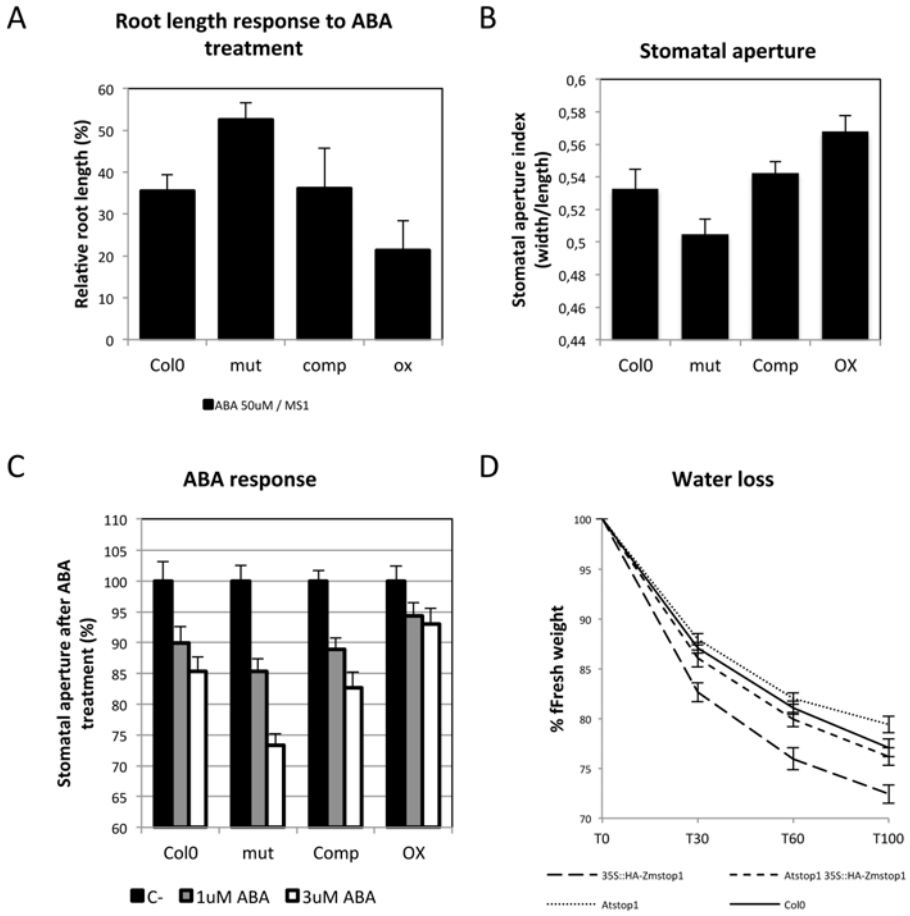


Figure 15. Phenotypical characterization of transgenic Arabidopsis lines overexpressing ZmSTOP1 under ABA and water stress.

Arabidopsis transgenic plants overexpressing ZmSTOP1-HA, together with the Col-0 wild type and Atstop1 mutant, were analyzed in response to ABA. A) Relative root length to wild type of 12-day-old seedlings under 50 µM ABA treatment indicates that the AtSTOP1 is insensitive to ABA while the ZmSTOP1-overexpressing line (ox) is hypersensitive to the phytohormone. B),C) Measurement of stomata aperture shows a hypersensitive response to ABA on the *Atstop1* mutant while the ox line is insensitive to ABA, revealing a differential response from roots. The measurements were done on 3-week-old detached leaves D) Water loss kinetics of 3-weeks-old detached rosettes, expressed as the percentage of initial fresh weight. Values are means ± SE of three independent experiments. The AtSTOP1 mutant loses significantly less water than the ZmSTOP1 overexpressing line.

ZmSTOP1 overexpression effects are conserved in maize transgenic plants

To address the question of whether ZmSTOP1 effects are conserved in maize, we generated maize transformed maize plants by gold-particle bombardment on embryos of the hybrid line Hi-II (A188xB73) (Armstrong et al., 1992). We overexpressed ZmSTOP1 under the control of the maize ubiquitin constitutive promoter. After checking ZmSTOP1 mRNA and protein expression levels of transformed and wild type calli (Fig 16 A), we regenerated transgenic plants from the resulting calli.

With the regenerated transgenic seedlings we performed a root length experiment, growing in parallel control and overexpressing seedlings in 100 µM ABA liquid MS1 medium (Fig. 16 B). ZmSTOP1 overexpressing seedlings showed a shorter root length than control seedlings after two weeks of treatment, suggesting that ABA hypersensitivity of ZmSTOP1 overexpressing plants is conserved between *Arabidopsis* and maize.

Due to the difficulty of directly measuring stomatal opening in maize, we used the indirect measure of stomatal conductance of CO_2 of regenerated wild type and ZmSTOP1 overexpressing plants grown in greenhouse environment, using an Infrared Gas Analyzer (IRGA) (Fig. 16 C). ZmSTOP1 overexpressing plants showed higher stomatal conductance than control plants, indicating that ZmSTOP1 induces stomatal aperture, as observed in *Arabidopsis*.

Finally, to assess the role of ZmSTOP1 in maize resistance to drought, a water loss experiment was conducted in detached leaves from regenerated

seedlings (Fig. 16 D). ZmSTOP1 overexpression plants presented a higher water loss than control plants, maintaining the trend observed in *Arabidopsis*.

Taken together, these results suggest that the phenotype observed in *Arabidopsis* is conserved in maize ZmSTOP1 overexpressing plants.

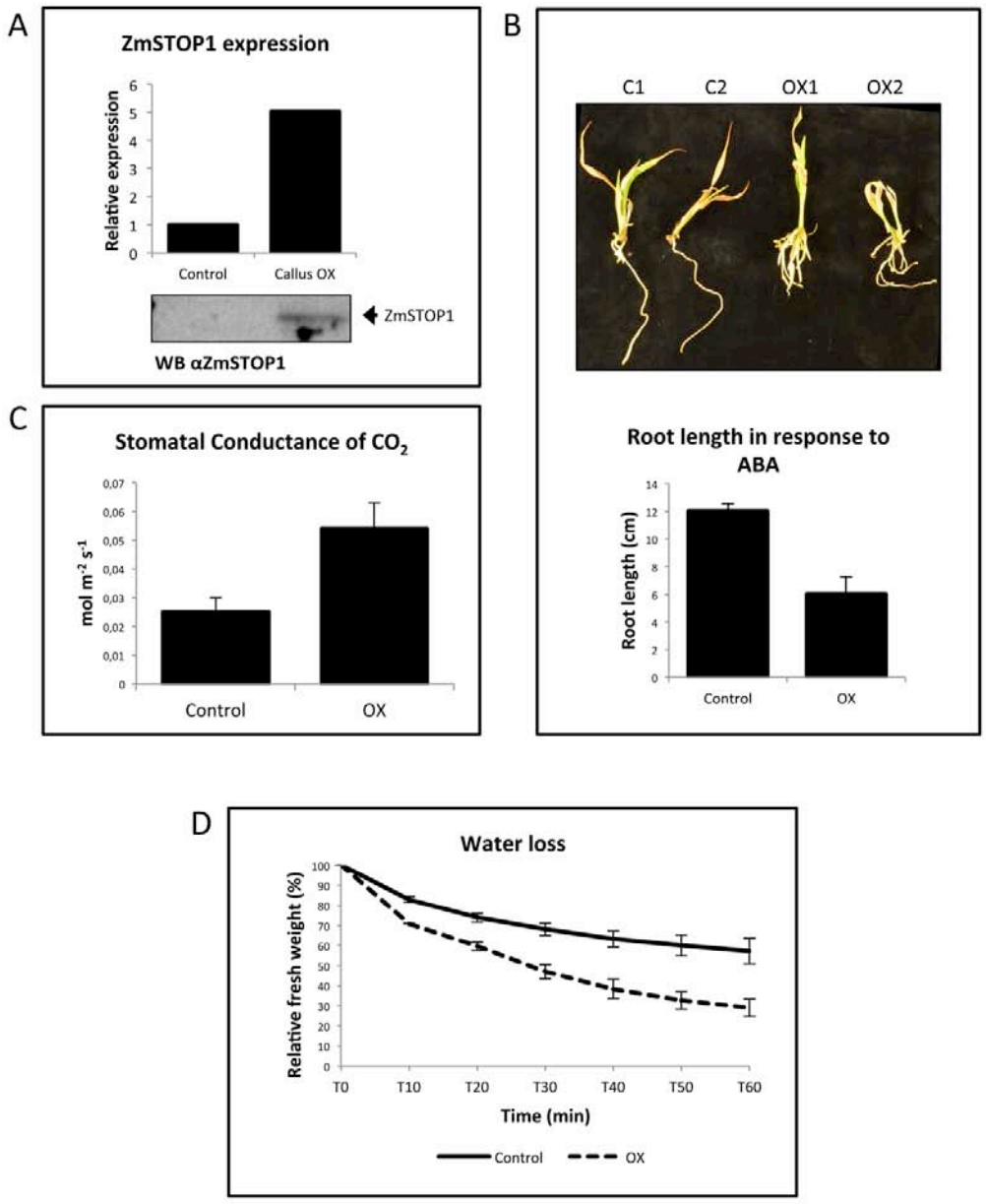


Figure 16. Phenotypical characterization of transgenic maize lines overexpressing ZmSTOP1 under ABA and water stress.

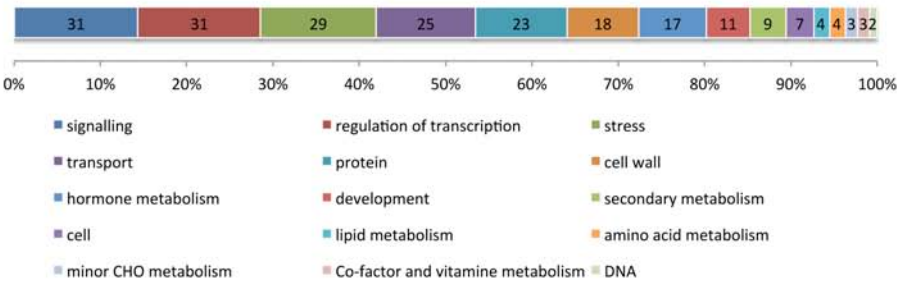
Transgenic maize plants constitutively overexpressing ZmSTOP1 were analyzed in response to ABA. A) Real Time qPCR on ZmSTOP1 overexpressing calli (upper panel) western-blot analysis using anti-ZmSTOP1 antibody of maize control plants and transgenic ZmSTOP1 overexpressing regenerated 9-days seedlings (lower panel). B) Root length measurement under 50 µM ABA, two-weeks treatment indicates that the ZmSTOP1 overexpressing line (ox) is hypersensitive to the phytohormone. C) Stomatal conductance of CO_2 on leaves of maize plantlets treated with 50 µM ABA. D) Water loss kinetics of detached leaves, expressed as the percentage of initial fresh weight. Values are means ± SE of three independent experiments. The ZmSTOP1 overexpressing line loses significantly more water than the maize wild type plants.

ZmSTOP1 regulates genes involved in signaling and stress response

To elucidate the function of the genes affected by ZmSTOP1 we performed a microarray analysis on the overexpression line 2. We clustered the deregulated genes using the Tair gene ontology tool (www.tair.org). The most abundant biological functions of the genes affected by ZmSTOP1 overexpression correspond to signaling, regulation of transcription or stress response, among others. (Fig. 17 A)

We selected some relevant genes from the results obtained in the microarray (Fig 17 B).

A



B

Gene ID	logFC	adj.P.Val	Gene name	Description
AT4G30280	3,39	1,46E-05	XTH18	Xyloglucan endotransglucosylase/hydrolase involved in root growth
AT5G17330	3,14	5,62E-05	GAD	Glutamate decarboxylase member of GABA-shunt pathway
AT3G23250	1,88	4,95E-04	MYB15	Member of the R2R3 factor gene family involved in drought tolerance
AT3G25780	1,72	1,62E-03	AOC3	Allene oxide cyclase, one of the enzymes involved in jasmonic acid biosynthesis
AT1G76650	1,69	1,52E-03	CML38	Calmodulin-like involved in calcium binding
AT3G50770	1,63	2,85E-04	CML41	Calmodulin-like involved in calcium binding
AT5G17860	1,52	1,19E-04	CAX7	Calcium exchanger involved in calcium transport
AT3G50260	1,31	2,24E-04	CEJ1	Member of the DREB subfamily A-5 of ERF/AP2 transcription factor family, involved in stress responses
AT2G46400	1,28	3,04E-02	WRKY46	WRKY transcription factor involved in stomatal movement
AT5G47910	1,20	3,16E-03	RBOHD	Respiratory burst oxidase involved in stomatal movement
AT3G54420	1,13	5,98E-03	EP3	EP3 chitinase involved in stress tolerance
AT2G40330	1,12	1,53E-02	PYL6	Abscisic acid sensor
AT1G79110	1,09	9,59E-04	BRG2	RING E3 ligases that involved in regulation of stress responses
AT2G47000	1,07	6,11E-03	ABCB4	ABC transporter involved in the redirection of auxin from the root tip
AT4G30190	0,86	1,33E-03	HA2	H(+)-ATPase involved in root architecture
AT1G08430	0,73	2,06E-03	ALMT1	Aluminum-activated malate transporter, also induced by ABA
AT5G07440	0,69	4,42E-03	GDH2	Glutamate dehydrogenase member of GABA-shunt pathway

Gene ID	logFC	adj.P.Val	Gene name	Description
AT4G22200	-1,01	6,95E-04	KT2/3	Inward rectifying potassium channel responsible for the Ca2+ sensitivity of the K+ uptake channel in guard cells

Figure 17. Microarray analysis of the transgenic Arabidopsis lines overexpressing ZmSTOP1-HA.

Arabidopsis transgenic plants overexpressing ZmSTOP1-HA were analyzed by microarray, to determine genes that have altered expression levels when compared with the Col-0 wild type. A) The most abundant biological functions of the genes affected by ZmSTOP1 overexpression are mostly involved in signaling processes, regulation of transcription, and stress responses. B) Relevant genes obtained in the microarray analysis.

Several genes are responsible for the phenotype observed in ZmSTOP1 overexpressing plants

In order to identify genes affected by ZmSTOP1 overexpression that could have a role in the phenotype observed in the *Arabidopsis* ortholog under ABA treatment, we selected several genes from the microarray data (Fig. 17 B) and we added *CIPK23* for its particular interest in stomatal regulation (Cheong et al., 2007). These genes were grouped in five different categories: stomatal regulation, root architecture, calcium signaling, pH regulation, and response to stress. We measured their expression by qRT-PCR in 12-days-old seedlings (Fig. 18 to 23, left panel). We also decided to test for differences in their expression levels between shoots and roots (Fig 18 to 23 right panel).

The first gene we checked was *ZmSTOP1*, to verify that both ZmSTOP1 complemented and overexpressing lines were overexpressing the TF (Fig. 18). The *Arabidopsis* endogenous *AtSTOP1* gene was also tested, resulting in down-regulation of its expression both in *Atstop1* mutant and complemented lines (data not shown).

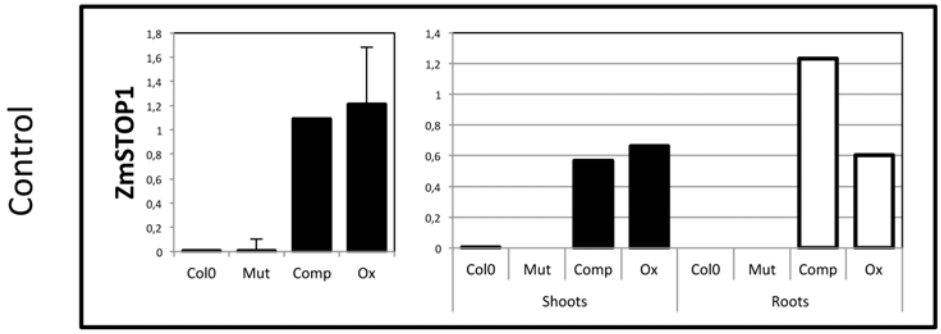


Figure 18. Expression analysis of genes involved in ZmSTOP1 overexpression phenotype

ZmSTOP1 expression is used as a control for the experiment. Whole *Arabidopsis* 12-day-oldseedlings were used to have a global reference of the genes behavior (left panel) in wild type, A*tstop1* mutant, complementation line and overexpression line of ZmSTOP1. The same genetic backgrounds were used to analyze separately shoots and roots (right panel) in 12-day-old *Arabidopsis* seedlings.

Some important genes for stomatal regulation in *Arabidopsis* were significantly deregulated by the expression of ZmSTOP1 (Fig. 19), such as the *CIPK23* kinase that regulates stomatal aperture by phosphorylating *AKT1* K^+ inward channel (Nieves-Cordones et al., 2012). *CIPK23* gene expression is decreased in the *Atstop1* mutant and ZmSTOP1 can partially recover the wild type expression of this gene. *CIPK23* can also be implicated in root processes since we were able to detect its expression in these structures.

KT2/3, a stomatal K^+ inward rectifying channel able to co-assemble with *KAT1* (Baizabal-Aguirre et al., 1999), expression is down-regulated in the presence of ZmSTOP1.

An important TF for stomatal regulation, *WRKY46*, which overexpression results in ABA insensitivity of stomatal closure, and is also involved in stomatal aperture (Ding et al., 2014), is up-regulated by ZmSTOP1 in both roots and

shoots. *WRKY46* TF has also been implicated in *ALMT1* repression (Ding et al., 2013).

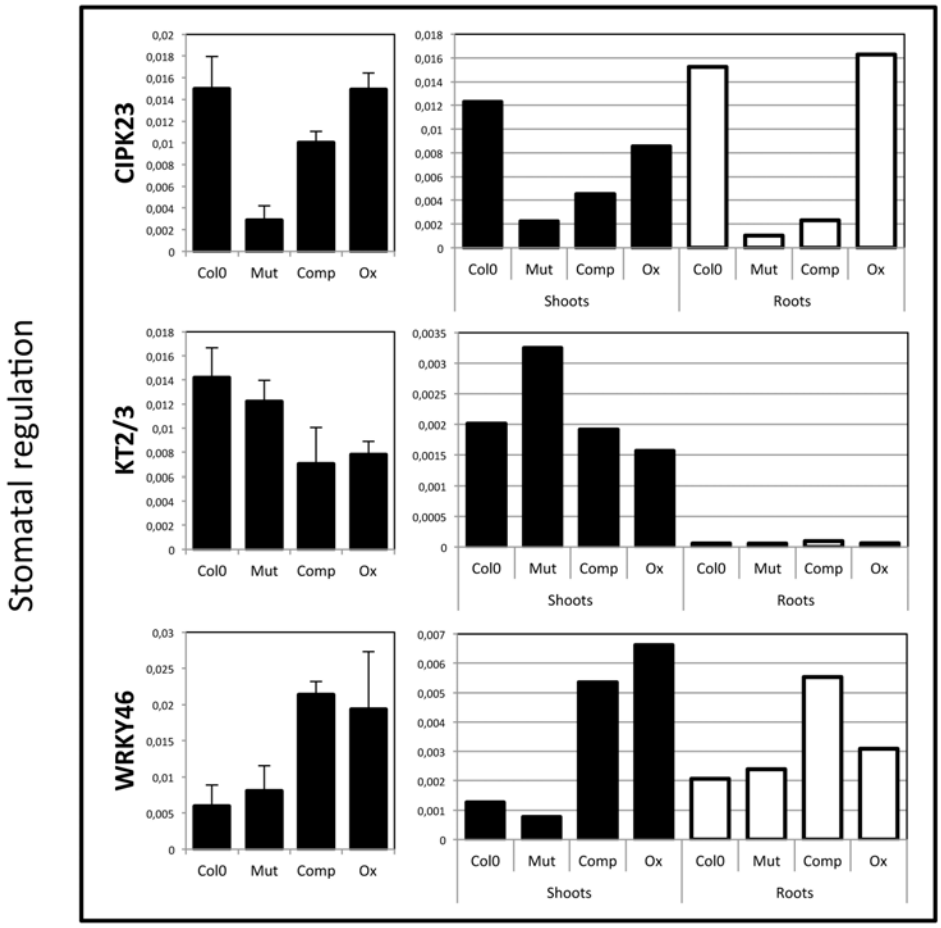


Figure 19. Effect of ZmSTOP1 on genes involved in stomatal regulation

qRT-PCR analysis of expression of genes involved in stomatal regulation. Whole *Arabidopsis* 12-day-oldseedlings were used to have a global reference of the genes behavior (left panel) in wild type, A*tstop1* mutant, complementation line and overexpression line of ZmSTOP1. The same genetic backgrounds were used to analyze separately shoots and roots (right panel) in 12-day-old *Arabidopsis* seedlings.

Genes involved in root architecture were also found to be deeply affected by ZmSTOP1 overexpression (Fig 20).

The H^+ pump ATPase *HA2*, involved in root growth and development (Mlodzinska et al., 2015); *RBOHD*, a NADPH oxidase responsible for producing H_2O_2 (Li et al., 2015) , the root auxin transporter *ABCB4* (Kubes et al., 2012); *AOC3*, an enzyme involved in methyl jasmonate biosynthesis that is also involved in root architecture (Cai et al., 2015); and *XTH18*, an enzyme crucial for primary root elongation (Osato et al., 2006), all have their expression levels enhanced in the transgenic *Arabidopsis* lines overexpressing ZmSTOP1.

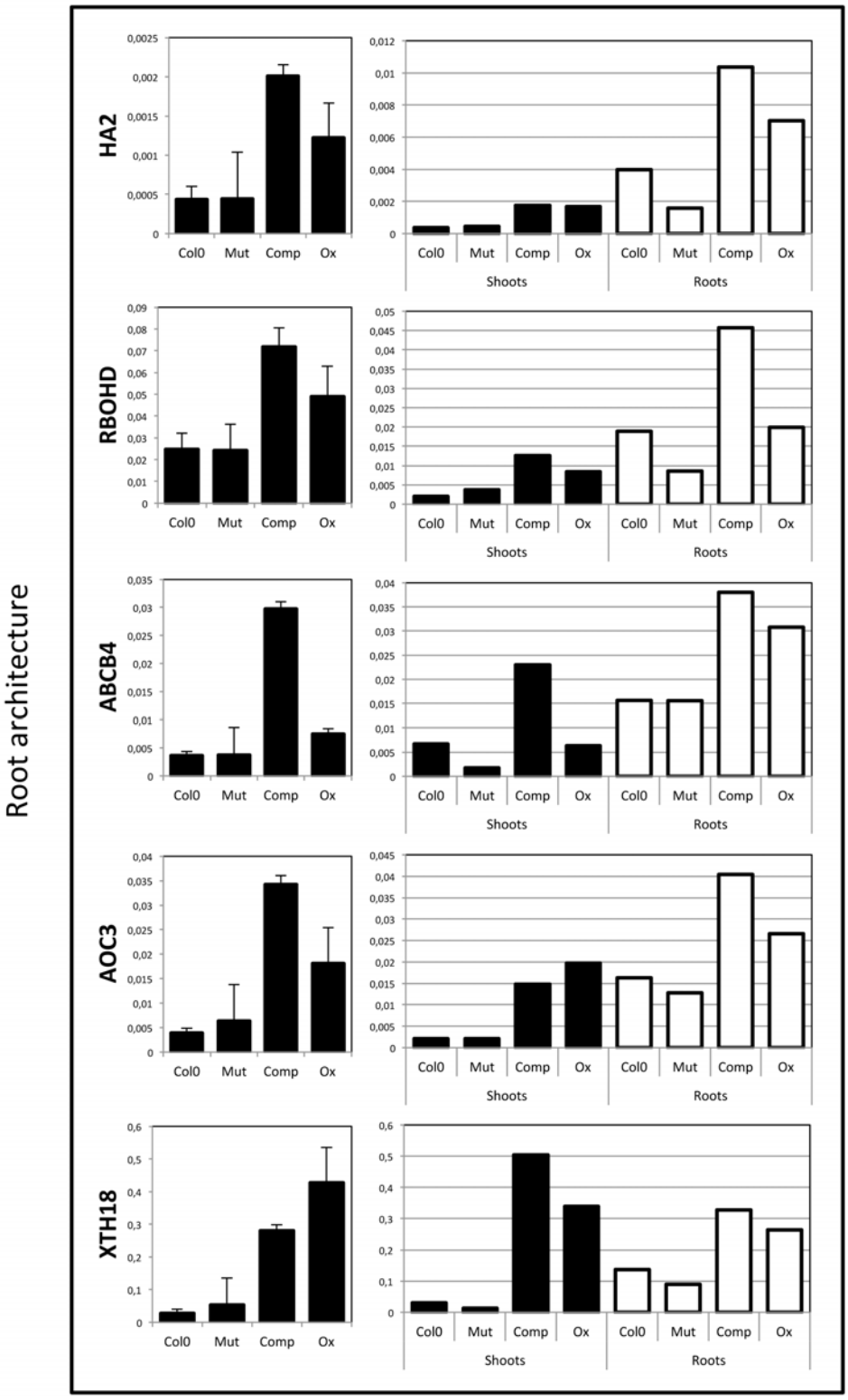


Figure 20. Effect of ZmSTOP1 on the expression of genes involved in root architecture

qRT-PCR analysis of expression of genes involved in root architecture. Whole *Arabidopsis* 12-day-old seedlings were used to have a global reference of the

genes behavior (left panel) in wild type, A*tstop1* mutant, complementation line and overexpression line of ZmSTOP1. The same genetic backgrounds were used to analyze separately shoots and roots (right panel) in 12-day-old *Arabidopsis* seedlings.

We also identified an effect of ZmSTOP1 overexpression on genes involved in Ca^{2+} signaling (Fig 21).

CAX7 is a Ca^{2+} transporter. Its pattern of expression shows that this gene is expressed mainly in shoots. Although little is known about the function of this gene, we observed in our analysis that *CAX7* expression was clearly increased in shoots of overexpressing ZmSTOP1 plants and ZmSTOP1- complemented mutant plants

CML38 and *CML41* are calmodulin-binding proteins, involved in the Ca^{2+} signal transduction. At present there is no functional characterization available for these genes. ZmSTOP1 enhances the expression of both genes in shoots, especially *CML41*. We also observed that ZmSTOP1 enhances *CML38* expression in roots.

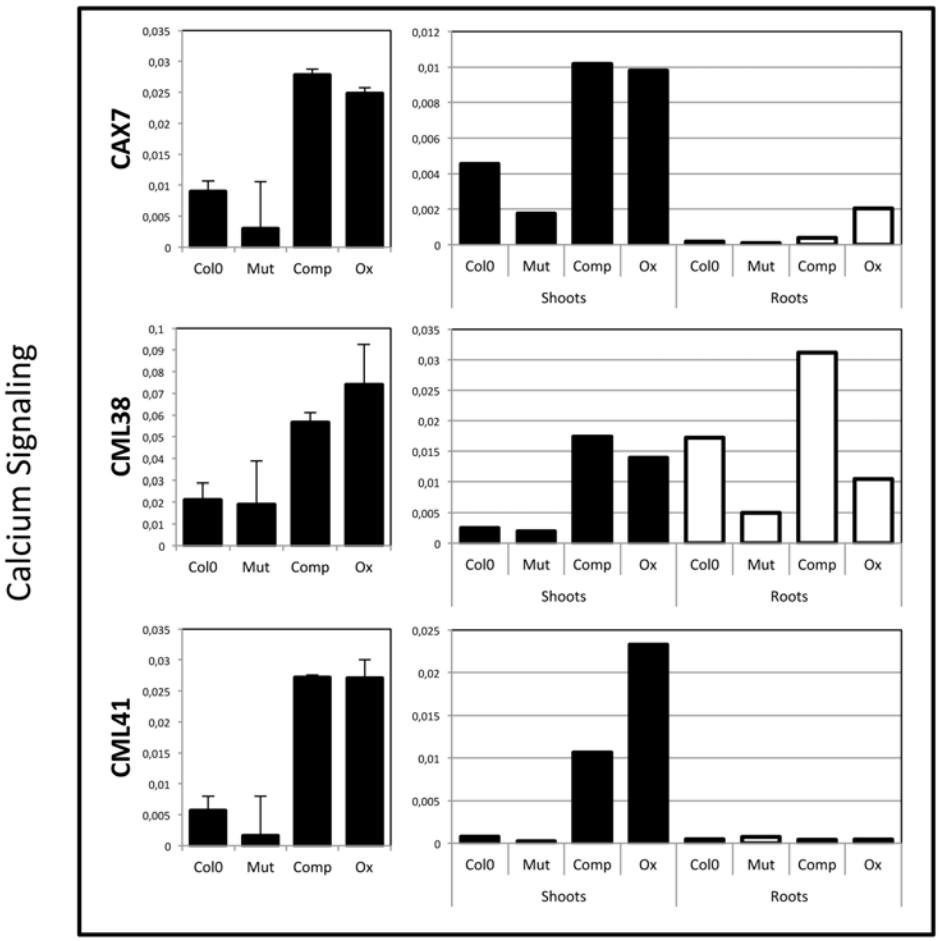


Figure 21. Effect of ZmSTOP1 on the expression of genes involved in Ca^{2+} signaling

Analysis of genes involved in Ca^{2+} signaling. Whole *Arabidopsis* 12-day-old seedlings were used to have a global reference of the genes behavior (left panel) in wild type, A*tstop1* mutant, complementation line and overexpression line of ZmSTOP1. The same genetic backgrounds were used to analyze separately shoots and roots (right panel) in 12-day-old *Arabidopsis* seedlings.

Another category of genes with altered expression in the transgenic *Arabidopsis* lines overexpressing ZmSTOP1 are involved in pH regulation (Fig. 22). As stated above, *GAD* and *GDH2* are genes that are deregulated in the *atstop1*and that are complemented by ZmSTOP1 overexpression.

We focused in two of them in this experiment, namely *GAD* and *GDH2*. *GAD* is a glutamate decarboxylase, and *GDH2* a glutamate dehydrogenase. Both are involved in the GABA shunt pathway, which is also involved in pH regulation (Bouche and Fromm, 2004). *GAD* has been described as root specific (Bouche et al., 2004), and ZmSTOP1 clearly enhances its expression in this tissue. However, a slight induction is also detected in shoots.

GDH2 is present both in shoots and roots, and ZmSTOP1 overexpression enhances its expression in both tissues.

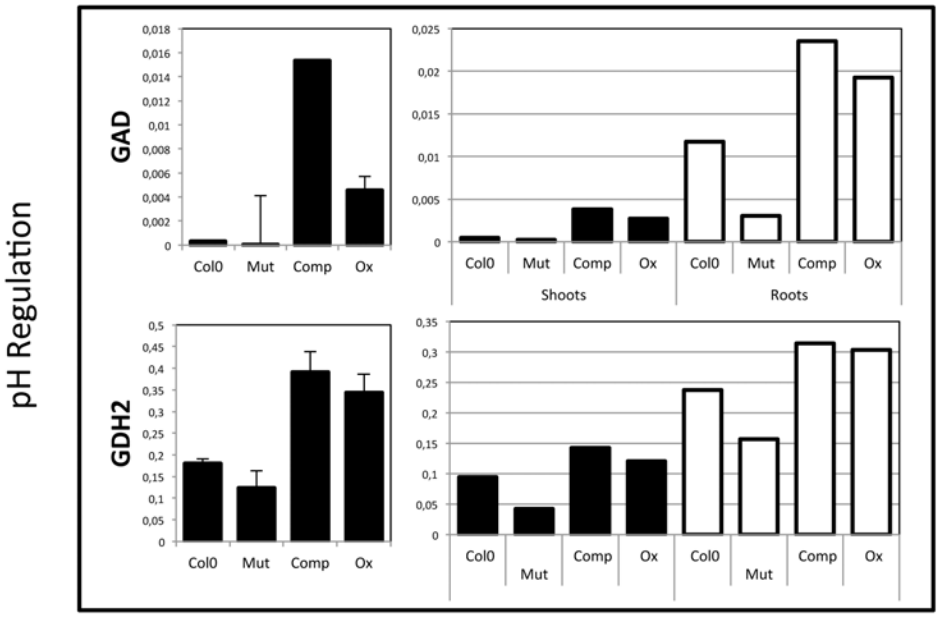


Figure 22. Effect of ZmSTOP1 on the expression of genes involved in pH regulation

Analysis of genes involved pH regulation. Whole *Arabidopsis* 12-day-old seedlings were used to have a global reference of the genes behavior (left panel) in wild type, *Atstop1* mutant, complementation line and overexpression line of ZmSTOP1. The same genetic backgrounds were used to analyze separately shoots and roots (right panel) in 12-day-old *Arabidopsis* seedlings.

Most of the genes mentioned above are stress-related genes and, therefore, they can be clustered by a characteristic function. Some of the genes that we studied did not fit in broader categories, so were grouped under "Response to Stress" function (Fig 23).

In this category we can find genes as *PYL6*. This gene is a component of the 14-member *PYR/PYL/RCAR* family, which have been described as ABA receptors (Park et al., 2009). ZmSTOP1 overexpression seems to enhance its expression in the whole seedling especially in the complementation line. When distinguishing the expression between roots and shoots, we detect an increase in shoots on the complemented line, and surprisingly its expression is down regulated in roots. This gene shows a differential response towards ZmSTOP1 expression in roots and shoots.

CEJ1 gene is a DREB TF involved in defense and freezing stress responses (Tsutsui et al., 2009). Its expression pattern indicates that this gene is mainly expressed in roots . ZmSTOP1 enhances its expression in roots, predominantly in the complementation line. However, its expression in shoots clearly increases with ZmSTOP1 overexpression.

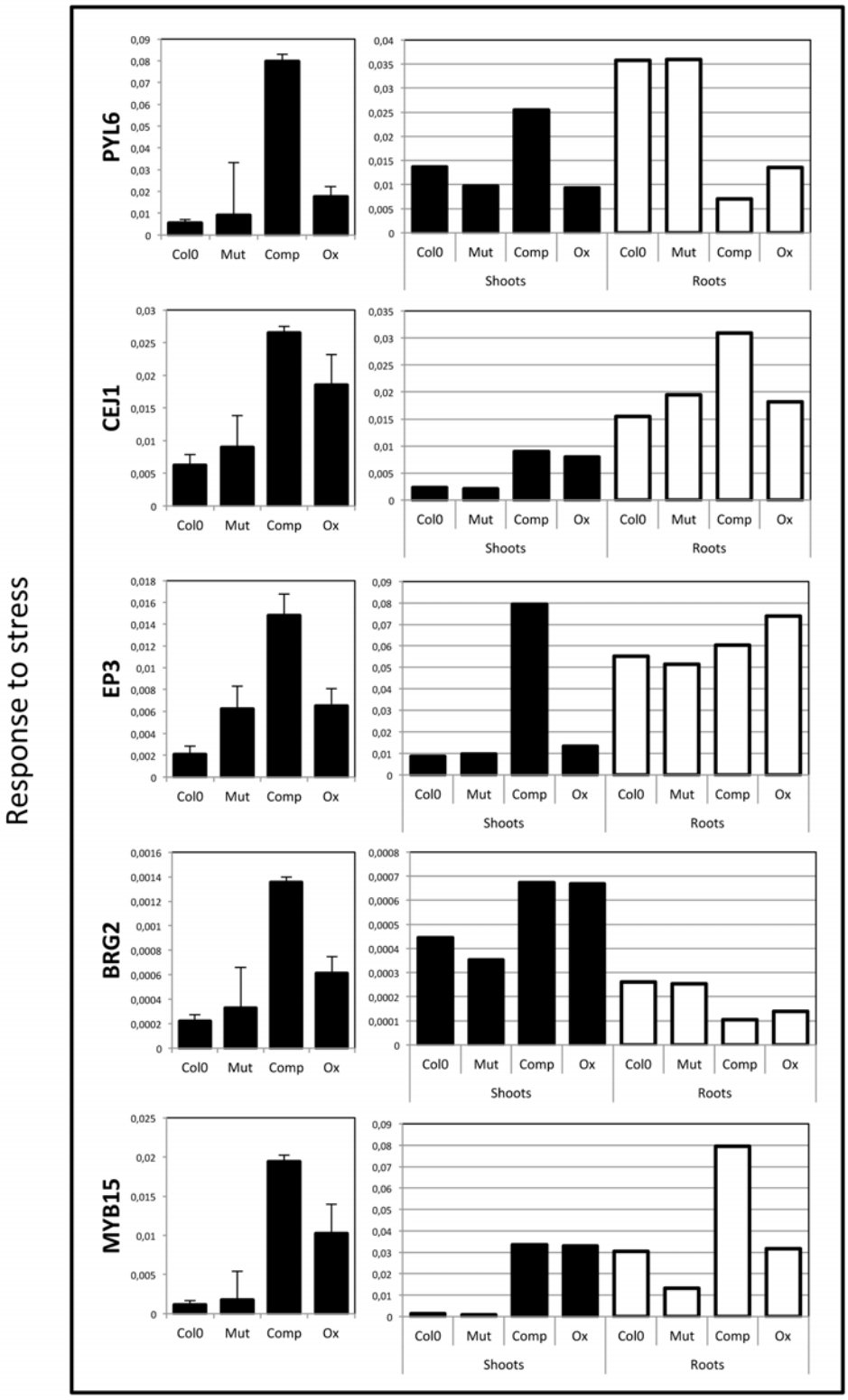
The chitinase *EP3*, which is involved in stress tolerance (Takenaka et al., 2009), shows a clear increased expression in the complementation line exclusively in shoots.

The ubiquitin E3-ligase *BRG2* is involved in biotic and abiotic tolerance (Luo et al., 2010). We observed a differential effect in shoots and roots promoted by ZmSTOP1 presence. It shows enhanced expression in shoots, and diminished expression in roots.

MYB15 is also included in this category. *MYB15*, which encodes a R2R3 MYB TF, plays a relevant role in drought tolerance in response to ABA (Ding et al., 2009). ZmSTOP1 enhances its expression in both shoots and roots.

Finally, *ALMT1*, a malate transporter mentioned repeatedly in this work, has been demonstrated to be essential for Al^{3+} tolerance (Hoekenga et al., 2006). This gene, besides being involved in biotic responses (Kobayashi et al., 2013) has also been reported to increase its expression after several treatments, including ABA (Kobayashi et al., 2007). ZmSTOP1 clearly enhances its expression in roots and shoots, although the expression of this gene seems to be more relevant in the root system.

All the genes analyzed here were also tested after ABA treatment, and no clear response based on ZmSTOP1 presence was observed (data not shown).


Response to stress
PYL6
CEJ1
EP3
BRG2
MYB15
Col0
Mut
Comp
Ox
Shoots
Roots

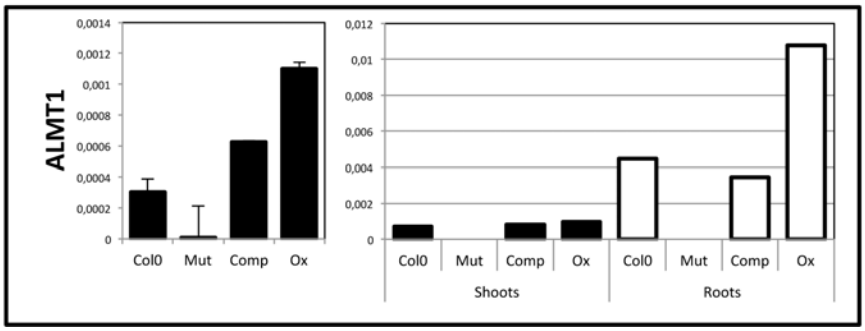


Figure 23. Effect of ZmSTOP1 on the expression of genes involved in response to stress

Analysis of genes involved in response to stress. Whole *Arabidopsis* 12-day-old seedlings were used to have a global reference of the genes behavior (left panel) in wild type, A*tstop1* mutant, complementation line and overexpression line of ZmSTOP1. The same genetic backgrounds were used to analyze separately shoots and roots (right panel) in 12-day-old *Arabidopsis* seedlings.

3.4. Interaction and phosphorylation of ZmSTOP1 by OST1

As mentioned before, we identified ZmSTOP1 in a yeast two-hybrid screening using ZmOST1 as bait. The OST1 kinase is a key component in ABA signaling pathway (Mustilli et al., 2002; Raghavendra et al., 2010) and is known to phosphorylate multiple transcription factors involved in ABA response (Yoshida et al., 2002; Takahashi et al., 2013).

ZmSTOP1 interacts *in vitro* and *in vivo* with OST1

To validate the interaction between ZmSTOP1 and ZmOST1 we performed a direct yeast two-hybrid experiment (Fig. 24 A and B). We found that co-expression of pGBT7-ZmOST1 and pGAD424-ZmSTOP1 proteins permitted yeast growth on selective medium and specific activation of the LacZ reporter system.

To check the in vivo interaction between the TF and the kinase we conducted a bimolecular fluorescent complementation using ZmSTOP1 fused to the C-terminal part of YFP and ZmOST1[G40R] fused to the YFP N-terminus (Fig. 24 C). This inactive construct of ZmOST1, unable to bind ATP, was chosen due to its abundant expression (Vilela et al). We detected fluorescence in the nucleus of co-transformed tobacco epidermal cells, indicating that the interaction takes place in this compartment.

To check whether ABA has any effect on the localization of ZmSTOP1 in the presence of ZmOST1, we transformed a double 35S driven ZmSTOP1-GFP fusion protein in maize mesophyll protoplasts with ZmOST1[G40R]-HA, also under the control of a double 35S promoter (Fig. 24 D). As a control we used the construct with 35S::ZmSTOP1-GFP alone. The presence of ZmOST1 seems to increase the quantity of ZmSTOP1-GFP in the cell, as observed by the increase in fluorescence and by the presence of cytoplasmic aggregates. This could suggest a modification in the stability of the TF provoked by the kinase. The application of ABA (lower panel) does not seem to induce a change in the localization of ZmSTOP1-GFP alone. However, in the presence of ZmOST1-HA, ABA seems to trigger the reorganization of ZmSTOP1-GFP into nuclear speckles, which indicates the possible implication of the kinase OST1 on ZmSTOP1 action after ABA signaling.

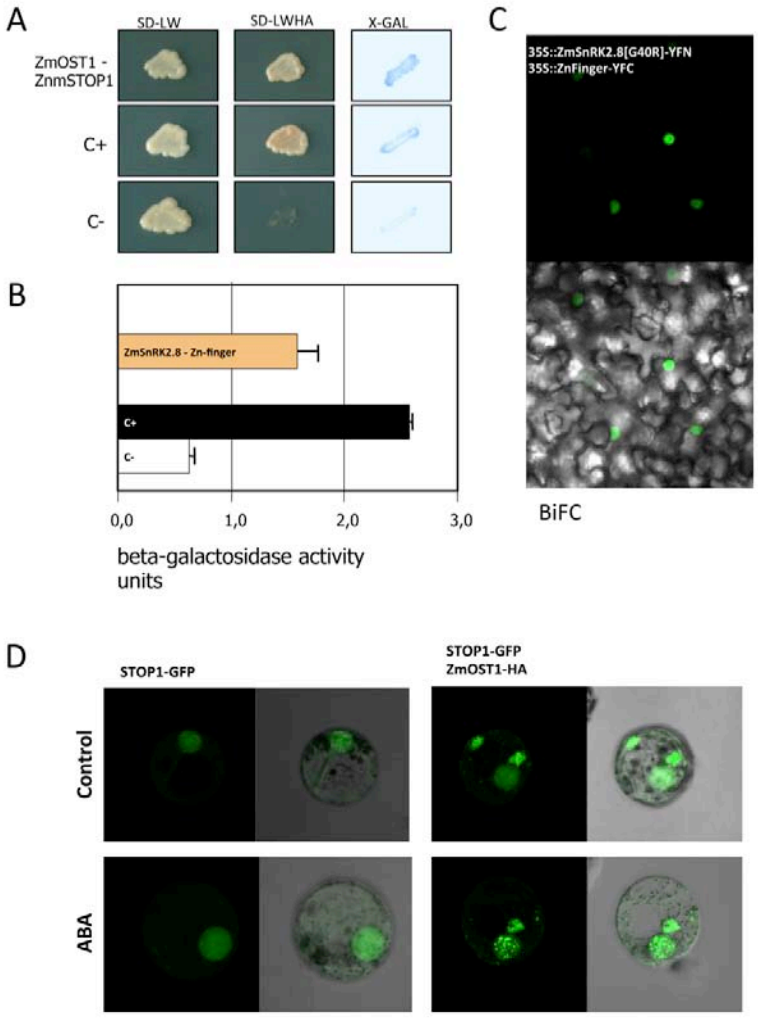


Figure 24. ZmSTOP1 interacts with ZmOST1

(A) ZmSTOP1/ZmOST11 yeast two-hybrid interaction by growth in selective medium (left) and specific activation of the LacZ reporter system (right). B) β-galactosidase activity quantification of the co-transformed yeasts. Values are means ± SD of three independent experiments. C) BiFC analysis of the interaction between YFC-ZmSTOP1 and an inactive form of ZmOST1 (YFN-ZmOST1[G40R]) indicates their ability to interact in the nucleus of agroinfiltrated tobacco leaves. D) ZmSTOP1-GFP overexpressed in maize protoplasts has a nuclear localization (left). When co-transforming ZmSTOP1-GFP with ZmOST1[G40R]-HA, ZmSTOP1 protein is visible in both the nucleus and in cytosolic aggregates (right). After ABA treatment (10 µM, 30 min), ZmSTOP1 is further reorganized into nuclear speckles when overexpressed with ZmOST1.

OST1 phosphorylates ZmSTOP1

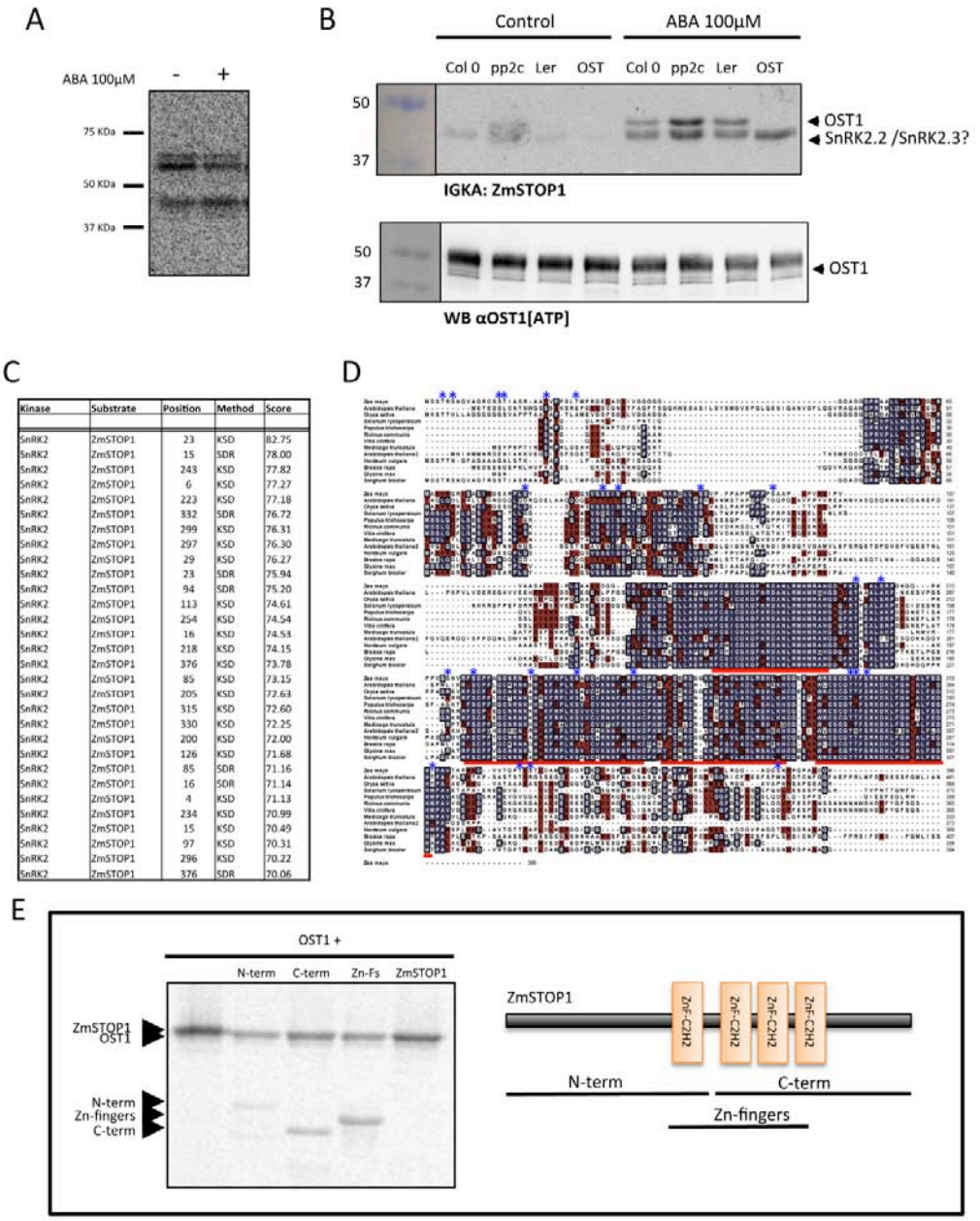
To assess the phosphorylation of ZmSTOP1 by OST1 we performed an in gel kinase assay using recombinant ZmSTOP1 protein as the substrate and crude plant extracts from Ler wild type plants treated with or without ABA (Fig. 25 A). We detected several phosphorylation bands corresponding with several unknown kinases able to phosphorylate ZmSTOP1. We detected a band between 37 and 50 KDa with increased intensity after ABA addition. This band could correspond to a mix of kinases containing OST1.

To verify if OST1 is the kinase detected, we immunoprecipitated OST1 using an antibody against the ABA domain of ZmOST1 (Vilela et al., 2013). We used extracts from *Arabidopsis* Col0 wild type, a PP2C quadruple mutant *Qabi2-2, hypersensitive to ABA* (Antoni et al., 2013), the *Arabidopsisost1*-2 mutant (Mustilli et al., 2002) and its wild type Ler. We detected ZmOST1 phosphorylation band toward ZmSTOP1 in all the plant extracts except for the *Arabidopsis Atost1* mutant (Fig. 25 B). In the quadruple PP2C mutant the intensity of the phosphorylation band is increased, a result that is in accordance with the current model of ABA signaling, in which these phosphatases control OST1activation by ABA. The lower band appearing in all the ABA extracts corresponds to a mix between SnRK2.2 and SnRK2.3 (Vilela et al., 2013; Vilela et al., 2015).

The optimal phosphorylation consensus of the serine/threonine kinase OST1 has been described previously as LXRXX(S/T) (Vlad et al., 2008). However we were not able to find this motif in ZmSTOP1 sequence. In order to identify the aminoacids susceptible to be phosphorylated by OST1, we performed an *in silico* analysis of ZmSTOP1 sequence using the online prediction software Predi-

kin (Ellis and Kobe, 2011) (Fig. 25 C). Multiple phosphorylation sites were detected. The sites obtaining a score over 70 points were plotted in a figure (Fig 25 C and D). Most of the potential sites are conserved among different species, indicating that its regulation by OST1 homologues could be conserved evolutionarily. A high number of the conserved predicted sites are located in the zinc-finger domains, suggesting that this DNA binding domain could be modified and thus regulated by OST1, modulating the possibility of ZmSTOP1 to bind its gene targets.

In order to verify the putative phosphorylation sites in ZmSTOP1 sequence, we performed an *in vitro* kinase assay using purified ZmOST1 and ZmSTOP1 produced in *E. coli*. We used three ZmSTOP1 fragments to better localize the putative phosphorylation sites (Fig. 25 E). We detected phosphorylation in all of the fragments, being the one corresponding to the zinc-finger domains the most intense. This corroborates the hypothesis that the most conserved sites present in the DNA-binding domain are more likely phosphorylated by ZmOST1.



Kinase	Substrate	Position	Method	Score
SnRK2	ZmSTOP1	23	KSD	82.75
SnRK2	ZmSTOP1	15	SDR	78.00
SnRK2	ZmSTOP1	243	KSD	77.82
SnRK2	ZmSTOP1	6	KSD	77.27
SnRK2	ZmSTOP1	223	KSD	77.18
SnRK2	ZmSTOP1	332	SDR	76.72
SnRK2	ZmSTOP1	299	KSD	76.31
SnRK2	ZmSTOP1	297	KSD	76.30
SnRK2	ZmSTOP1	29	KSD	76.27
SnRK2	ZmSTOP1	23	SDR	75.94
SnRK2	ZmSTOP1	94	SDR	75.20
SnRK2	ZmSTOP1	113	KSD	74.61
SnRK2	ZmSTOP1	254	KSD	74.54
SnRK2	ZmSTOP1	16	KSD	74.53
SnRK2	ZmSTOP1	218	KSD	74.15
SnRK2	ZmSTOP1	376	KSD	73.78
SnRK2	ZmSTOP1	85	KSD	73.15
SnRK2	ZmSTOP1	205	KSD	72.63
SnRK2	ZmSTOP1	315	KSD	72.60
SnRK2	ZmSTOP1	330	KSD	72.25
SnRK2	ZmSTOP1	200	KSD	72.00
SnRK2	ZmSTOP1	126	KSD	71.68
SnRK2	ZmSTOP1	85	SDR	71.16
SnRK2	ZmSTOP1	16	SDR	71.14
SnRK2	ZmSTOP1	4	KSD	71.13
SnRK2	ZmSTOP1	234	KSD	70.99
SnRK2	ZmSTOP1	15	KSD	70.49
SnRK2	ZmSTOP1	97	KSD	70.31
SnRK2	ZmSTOP1	296	KSD	70.22
SnRK2	ZmSTOP1	376	SDR	70.06

Figure 25. ZmSTOP1 is a substrate of OST1

A) ZmSTOP1 phosphorylation is analyzed by in gel kinase assay, using ZmSTOP1 as the substrate. Protein extracts were prepared from *Arabidopsis* seedlings treated or not with ABA. Sizes of the activity kinase bands obtained are shown on the left. B) In gel kinase assay using ZmSTOP1 as the substrate after immunoprecipitation with anti-OST1 antibody of Col-0 wild type, pp2c mutant, Ler wild type and ost1-2 mutant with and without exogenous ABA. Western-blot of the immunoprecipitation experiment serves as a loading control. C) In silico prediction of ZmOST1 phosphorylation sites in the ZmSTOP1 protein sequence (C) are plotted in a ZmOST1 alignment between plant species (D). E) ZmSTOP1 in vitro phosphorylation by ZmOST1. Schematic representation of ZmSTOP1 domains used in the experiment are shown on the right. ZmOST1 is capable of phosphorylating all ZmSTOP1 deletion forms.

OST1 phosphorylation of ZmSTOP1 promotes stomatal opening, whereas it has no effect in root elongation.

We decided to check whether the phosphorylation of ZmSTOP1 by OST1 had any effect on the phenotypes we described above for the transgenic *Arabidopsis* plants overexpressing ZmSTOP1. It has been described that AtOST1 has no effect on root length response to ABA (Fujii and Zhu, 2009). We measured the root length of *Atost1* mutant in low pH and ABA medium to confirm that OST1 had no effect on roots (Fig. 26 A). As expected, it did not show any difference, so we decided to focus on aerial parts for further experiments. We transformed *ArabidopsisAtost1-2* mutant with a ZmSTOP1-GFP fusion construct under a 35S promoter.

We measured the stomatal aperture in response to ABA and performed a water loss kinetics experiment (Fig. 26 B and C). We detected that ZmSTOP1 overexpressing plants in *Atost1* background (and thus not phosphorylated by the kinase) lost slightly less water and their stomata responded to ABA more than the *Atost1* mutant, meaning that the phosphorylation of ZmSTOP1 by OST1 modulates drought responses in plants.

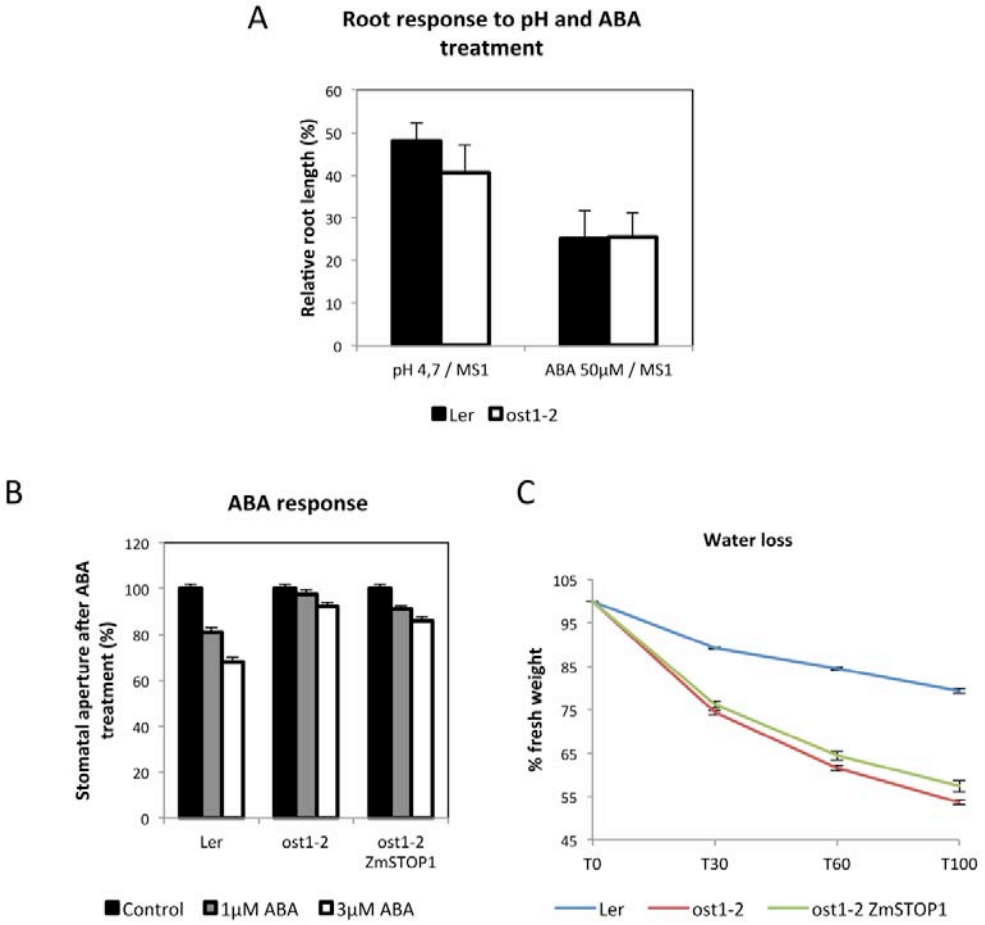


Figure 26. Phenotypic characterization of transgenic lines overexpressing ZmSTOP1 in the ost1-2 mutant background.

A) Relative root length of Ler wild type and ost1-2 mutant under pH 4.7 and 50 µM ABA shows no significant difference. B) Relative stomata aperture after ABA treatment in the Ler Wild-type, the ost1-2 mutant and a transgenic line overexpressing ZmSTOP1 in the *ost1-2* background..C) Water loss experiments of detached leaves from the same *Arabidopsis* lines. Plants overexpressing ZmSTOP1 in the *ost1-2* background show a slightly improved response to ABA at the level of stomata and have an enhanced response to water loss.

DISCUSSION

4. Discussion

The maize homolog of STOP1 was detected in a yeast two-hybrid screening using ZmOST1 as bait (Vilela et al., 2013). ZmOST1 is a key player in drought response, being a fundamental part of the ABA core signaling complex in maize (Vilela, 2012; Vilela et al., 2013). Since these kinases are central in ABA signal transduction, and taking into consideration that ionic and proton transport is crucial for the proper functioning of roots and stomata, we focused our analysis of ZmSTOP1 in determining its role in drought tolerance and ABA signaling.

STOP1-like proteins are present in a high number of plant species. In rice and maize, there are several STOP1-like members composing a family, and usually these proteins group together with other proteins from closely related plant species. For instance, broadleaf trees STOP1-like proteins group together and monocots also do the same. The four zinc-fingers are the most conserved domains in the protein, whereas the N and C terminus differ in a higher degree (Ohyama et al., 2013).

All the STOP1 homologs studied to date have been shown to regulate H^+ and Al toxicity to some extent, being able to complement *Atstop1* mutant up to different levels in both Al and H^+ phenotypes. For instance, tea CsSTOP1 is able to complement H^+ toxicity but not Al toxicity, whereas tobacco NtSTOP1 can complement both toxicities (Ohyama et al., 2013).

Arabidopsis STOP1 has been very well described as an Al tolerance TF (Iuchi et al., 2007). It is known to regulate several genes involved in malate and citrate exudation as *ALMT1* and *AtMATE*, which help sequester Al in the rhizosfere, or genes involved in Al distribution to different tissues as *ALS3*. In rice, ART1 has also been described in Al tolerance (Yamaji et al., 2009), but it has been shown to regulate genes like *STAR1* and *STAR2*, two components of an ABC transporter which transports UDP-glucose, affecting root cell wall and contributing to Al tolerance through a different mechanism than *Arabidopsis* (Huang et al., 2009). These findings are relevant for the management of ion toxicity in acid soil syndrome, which is a very important crop yield constraint. Acidic soils (pH<5.5) represent a big extension of arable lands in the world and thus, affect a great variety of crops. Soil acidity is detrimental for plant growth and productivity, as it provokes nutrient deficiencies. Moreover, it increases the solubility of certain ions like manganese and Al, maximizing their potential toxicity (Kochian et al., 2004). Management of pH homeostasis both intra and extracellularly is one of the mechanisms to cope with soil acidity (Pineros et al., 2005). AtSTOP1 is involved in regulating pH-related genes belonging to the GABA-shunt pathway or the biochemical pH stat pathway, like for example *GAD*, *GDH1* and *GDH2* or *GABA-T* (Sawaki et al., 2009). Some of these genes, and many other resulting from the *Atstop1* mutant microarray are involved in ABA signaling, like *CIPK23* (Cheong et al., 2007), or are affected in their expression by ABA, such as *ALMT1* (Kobayashi et al., 2013), suggesting that AtSTOP1 could have a role in drought stress as well.

ZmSTOP1 is a nuclear regulator of transcription, as it affects the expression of many genes. We have seen that its expression is higher in shoots than in

roots, although it is extressed in both tissues, ABA has an enhancing effect on ZmSTOP1 expression levels in roots (Fig. 6).

The presence of the four inter- and intra-specifically conserved zinc-finger domains, its nuclear localization, its dimerization and its random DNA-binding capacity suggested that this protein could be a DNA-binding transcription factor. In fact, the activation of pScALMT1-GUS by ZmSTOP1 demonstrates that ZmSTOP1 binds DNA. Other homologues like AtSTOP1 or OsART1 are able to bind DNA and their DNA-target sequence has been identified *in vitro (Tsutsui et al., 2011; Tokizawa et al., 2015)*. In our case, no specific DNA-target has been identified. A possibility is that ZmSTOP1 is a pioneer transcription factor, and its DNA-binding specificity is present only in a short period of time after its entrance to the nucleus, the so called "Hit-and-Run" model (Varala et al., 2015). This possibility should be further studied by using an inducible promoter to overexpress ZmSTOP1. Another possibility is that ZmSTOP1 is not working alone, but interacts with other proteins to bind specific sites. As ZmSTOP1 interacting proteins, particular attention should be paid on its five family members. For this purpose, the pattern of expression of all maize paralogues should be thoroughly studied to determine possible co-expressions at specific circumstances and tissues. When performing a blast search against the NCBI database with ZmSTOP1 sequence, a longer isoform of this protein appears. We haven't been able to clone this longer isoform in maize and none other laboratory has. At present, no homolog of this longer isoform has been cloned in any species. Probably this isoform is a hypothetical protein, or an error in the annotation system. Other interpretation could be that this isoform is present under certain developmental or environmental circumstances, justifying the difficulty to clone it. We cannot discard

that ZmSTOP1 and its longer isoform form dimers that can bind specific DNA (Pose et al., 2013).

Putative post-translational changes in the protein, such as phosphorylation, should not be excluded as a possible source of DNA-binding specificity. I*n vivo* approaches performed were expected to incorporate these modifications, as they were performed on wild type background and after ABA treatment, both in maize protoplasts and *Arabidopsis* seedlings (Fig 11).

We have established that OST1 phosphorylates ZmSTOP1 after ABA treatment (Fig. 25 B). However, the effects that this post-translational modification has on ZmSTOP1 at the molecular level are still unknown. The alteration in subcellular localization of ZmSTOP1 on ZmOST1 presence after ABA treatment (Fig. 24 D) suggests that a re-arrangement on ZmSTOP1 protein is taking place. Several options are possible to explain this change in subcellular localization. On one hand, we have seen that the most conserved putative OST1 phosphorylation sites in ZmSTOP1 sequence are located in the zinc-finger domains of the protein (Fig. 25 D). This suggests that addition of negative charges in specific residues, as the introduction of phosphate groups, might substantially change the conformation of this region, promoting DNA affinity modifications and increasing the specificity for its targets (Stemmer et al., 2002; Smykowski et al., 2015). However, the zinc-finger domains are not only able to bind DNA (Wolfe et al., 2000), but also to bind RNA (Lu et al., 2003) and protein-protein interaction (Mackay and Crossley, 1998). Though we have not found protein interactors in a yeast two-hybrid assay. Thus, the phosphorylation in these sites might have broader consequences on ZmSTOP1 action. On the other hand, OST1 phosphorylation might regulate the turnover of ZmSTOP1 by targeting it for deg-

radation as was reported for ZmSNAC1 (Vilela et al., 2013), or by increasing its stability in a similar way to ABF phosphorylation (Sirichandra et al., 2010). An *in silico* study to identify possible PEST sequences in ZmSTOP1 protein should be conducted to assess this possibility. In fact the ZmSTOP1 sequence includes one potential PEST sequence at the C terminus of the aminoacid sequence (between positions 323 and 349), which is putatively phosphorylated by OST1 kinase (Fig. 25 D). This would reinforce the possibility of proteasomal degradation targeting by OST1 phosphorylation.

One of the main objectives of this work is to determine ZmSTOP1 function on drought tolerance. To functionally characterize ZmSTOP1 we transformed both the A*tstop1* mutant and wild type Col-0 with ZmSTOP1 under a constitutive promoter. Using this *Arabidopsis* ortholog system, we have demonstrated that ZmSTOP1 is able to complement the *Atstop1* loss of function mutant phenotype at low pH conditions, restoring wild type levels of root growth arrested under low pH in *Atstop1* mutant (Fig. 14 D and E). Moreover, it also complements the down-regulation in gene expression of genes like *CIPK23* or *GDH1* (Fig. 14 F). ZmSTOP1 overexpression also complements the previously undescribed phenotype of *Atstop1* mutant after ABA treatment in both roots and stomata. Our results indicate that ZmSTOP1 overexpressing plants are hypersensitive to ABA in the roots and insensitive to the hormone in stomatal response (Fig 15). This differential response is remarkable, as ABA is known to promote stomatal closure. ABA triggers membrane depolarization and K^+ efflux through outward-rectifying channels, in order to avoid water evaporation and therfore, enhancing drought tolerance (Sirichandra et al., 2009). On the other hand, ABA inhibits primary root growth at high concentrations, to prevent energy waste in a wa-

ter-shortage situation (van der Weele et al., 2000). In the case of ZmSTOP1, not only its overexpression promotes stomatal aperture in control conditions (Fig. 15 B), but also reduces the stomatal response to ABA as compared to the control (Fig 15 C). This effect contrasts with some results described by other authors (Schroeder et al., 2001). This reverse effect could be considered detrimental for the plant in drought conditions. However, taking into account that the stomatal closure in response to drought conditions varies depending on the stress severity but it never reaches 100% closure, we suggest that ZmSTOP1 could be involved in the fine-tuning of stomatal regulation to ensure the correct balance between water loss avoidance and CO_2 entry into the leave for the plant's correct photosynthetic activity, as evidenced before with the mitochondrial pyruvate carrier NRGA1 (Li et al., 2014). We have shown that the ZmSTOP1 transcript levels after ABA treatment in wild type maize leaves vary in a very insignificant manner (Fig 6 B). This suggests that ABA treatment is not enhancing ZmSTOP1 transcription in leaves, which would not induce ZmSTOP1-dependent stomatal opening.

The root system is very important for the plant to face water deficit stress. A very strict balance between root growth and energy saving is necessary to ensure the discovery of new water sources without investing excessive energy that would compromise the plant survival (Claeys and Inze, 2013). The *Atstop1* mutant shows insensitivity to ABA in roots, and ZmSTOP1 can complement this phenotype (Fig. 15 A). Conversely, ZmSTOP1 overexpression in the wild type background displays hypersensitivity to ABA, with a higher rate of root length reduction after the treatment when compared to the wild type. The expression levels of ZmSTOP1 in wild type maize roots are enhanced substantially after ABA

treatment, indicating that this protein might have an important role in shaping root architecture upon drought stress.

The OST1 kinase phosphorylation of ZmSTOP1 is responsible, at least in part, for the fine stomatal regulation dependent on ZmSTOP1 overexpression, as seen in figure x B and C. The absence of ZmSTOP1 phosphorylation by OST1 in the kinase mutant background provokes a slight but significant stomatal closure when overexpressing ZmSTOP1, relative to the *ost1-2* mutant behavior (Fig. 26 B). The overall performance of the plant before water deficit shows a slight recovery of *ost1-2* mutant overexpressing ZmOST1, compared to the kinase mutant background (Fig. 26 C). Therefore, OST1 can modulate the activity of STOP1 in drought conditions.

As mentioned before, the OST1 kinase does not play an important role in the regulation of root growth in response to drought stress (Fig. 26 A)(Fujii and Zhu, 2009). So the regulation of ZmSTOP1 effect in this tissue must be carried out by other kinases. SnRK2.2 and SnRK2.3 are known to play an important role in root growth (Fujii et al., 2007). ZmSTOP1 is very likely a substrate of both SnRK2.2 and SnRK2.3, in addition to OST1, as these three kinases belong to Group III SnRK2 and often share substrates, apart from having the same phosphorylation consensus sequence. Moreover, we observed that a band containing both kinases appears in all extracts in the kinase assay carried out with the immunoprecipitates using antibody against the ABA domain of OST1 (Fig. 25 B). Some interesting experiments could be carried out in the future by overexpressing ZmSTOP1 on the double mutant *snrk2.2/snrk2.3* background, to test the function of these two kinases on the regulation of ZmSTOP1.

In summary, ZmSTOP1 overexpression provokes different responses to ABA in roots and shoots, and its regulation by phosphorylation after ABA treatment also differs between these two tissues: while OST1 plays a role in stomatal regulation, it does not in root architecture.

We performed some experiments to test whether the ZmSTOP1 function observed in *Arabidopsis* is conserved in maize. The phenotypic results observed in the transgenic maize plants regenerated from calli suggest that ZmSTOP1 effects on stomata and roots are conserved in maize (Fig. 16). The overall performance of these plants tested in the water loss experiment (Fig. 16 D) also shows that ZmSTOP1 overexpressing plants are less resistant to drought than the control.

To look deeper into ZmSTOP1 function, we investigated the effects of its overexpression on the transcriptome. ZmSTOP1 overexpression on the Col-0 wild type background significantly modifies the expression levels of *Arabidopsis* genes involved in signalling. Many of these genes are transcription factors and most of them are involved in stress responses and ion transport (Fig 17 A). After checking the expression of some of these genes by qRT-PCR in whole seedlings and in roots and shoots separately, and also on several genetic backgrounds, we could detect some interesting features (Fig 18 to 23). First, some of the tested genes affected by ZmSTOP1 don't seem to be de-regulated in the *Arabidopsis* mutant background. This suggests that ZmSTOP1 regulation might be slightly different than that of AtSTOP1. Secondly, the deregulation of some genes in *Arabidopsis* mutant background is in most cases higher than that in the wild type background. The levels of expression of ZmSTOP1 in both backgrounds could explain this effect, as in both situations the expression of the pro-

tein driven by a constitutive promoter. ZmSTOP1 effects on expression are possibly higher in the complemented line due to the lack of AtSTOP1 expression, which could provoke a deficiency in the turnover mechanisms of this protein in *Arabidopsis* to stimulate its accumulation, and this way maintaining physiological levels of AtSTOP1 protein. This would result in an over-accumulation of the maize homologue. Finally, we can see that ZmSTOP1 overexpression alters the pattern of expression of some genes in non-specific tissues. As mentioned before, many of these genes deregulated by ZmSTOP1 overexpression are related with ion transport and signaling, and several of them are stress genes (Fig. 19 to 23). This ectopic expression could induce important changes in the homeostasis-maintenance mechanisms of the plant, inducing the phenotypes detected in this work. For instance, overexpression of WRKY46 TF shows a phenotype of stomatal insensitivity to ABA provoked by the reduction in ROS species in stomata (Ding et al., 2014). *KT2/3* is a Ca^{2+} regulated K^+ channel (Held et al., 2011), that is able to modulate K^+ homeostasis by co-assembling with KAT1 (Baizabal-Aguirre et al., 1999), critical for stomatal closure (Sato et al., 2009; Acharya et al., 2013). *HA2*, a plasma membrane H^+-ATPase, also associated with Ca2+ regulation (Fuglsang et al., 2007), has an important role in modulating H^+ homeostasis in stomata, contributing to stomatal opening (Kim et al., 2015), but also affecting root architecture (Mlodzinska et al., 2015). Moreover, this H^+-ATPase is regulated by ABA through dephosphorylaton mediated by the PP2C phosphatase ABI1 (Hayashi et al., 2014).

We tested the expression of the studied genes after ABA treatment, to detect possible influences of ZmSTOP1 overexpression on the hormone signaling. We did not observe significant changes in ABA response on the studied

genes upon overexpression of the protein, meaning that the regulation of ZmSTOP1 on these genes is not related to ABA. However, due to the ZmSTOP1-dependent deregulation of these genes, their ABA response is intrinsically altered, as their expression pattern is distorted in the baseline scenario.

We also have to take into account that all these genes mentioned are at some point related with Ca^{2+} signaling. Ca^{2+} signaling is fundamental both in stomata and roots, as it modulates ionic channel activity, essential for the correct metabolic and physiologic performance of the plant. ZmSTOP1 overexpression contributes to an altered homeostasis of Ca^{2+} (Fig. 21). Several studies have shown that Ca^{2+} signaling is crucial for systemic acclimation to stress responses like drought, and this signaling is mediated by a Ca^{2+} wave that travels all along the plant trough symplastic and apoplastic medium (Choi et al., 2014). ROS production by RBOHD is also essential for systemic acclimation, as it is responsible for producing a ROS wave that travels cell to cell and contributes to propagate Ca^{2+} wave (Suzuki et al., 2013). These two waves respond to ABA, and prime the plant for subsequent signals that will contribute to the plant adaptation to water deficit situation (Mittler and Blumwald, 2015). RBOHD is also deregulated by ZmSTOP1 overexpression (Fig. 20). Taken all together, we can imagine a situation of alteration, or even disruption of signaling pathways including Ca^{2+} and ROS, followed by ionic homeostasis deregulation provoked by ZmSTOP1 overexpression, that cannot respond in a correct manner to ABA signaling. This panorama could explain the phenotypes observed in this work.

Altogether these findings show that ZmSTOP1 plays an important role in drought response under the control of OST1 kinase (Fig 26). As mentioned before, maize is a very important crop for human and animal feed. So the

knowledge of new players in drought response is of major importance for crop improvement, as it broadens the comprehension of water-deficit responses and helps focusing further genetic engineering or breeding.

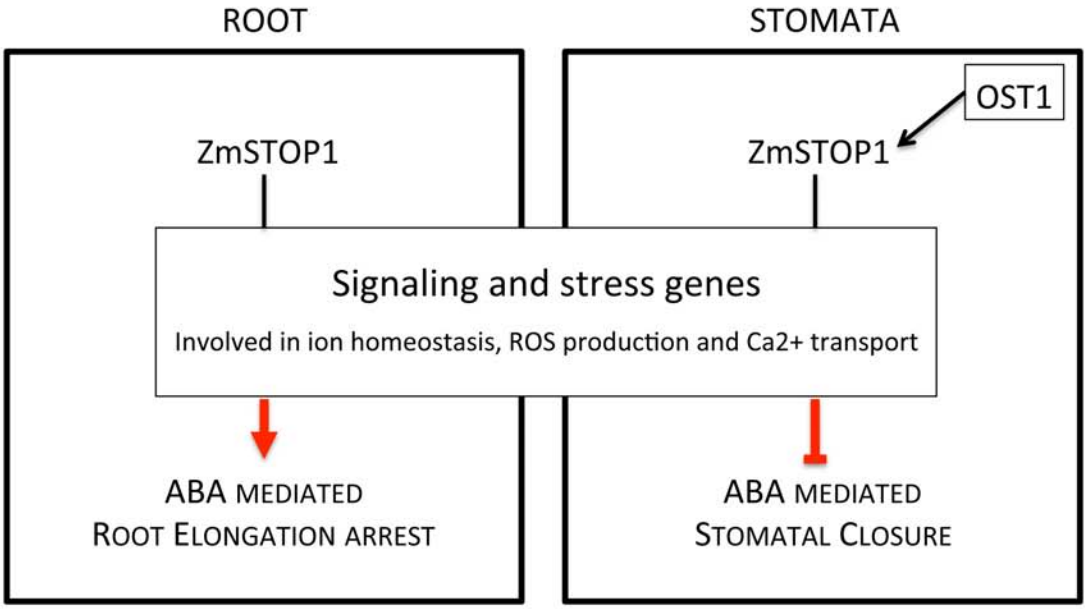


Figure 27. ZmSTOP1 working model

ZmSTOP1 regulates the expression of signaling and stress genes fundamental for ionic homeostasis and ROS and Ca^{2+} dependent signaling, which provoke ABA-mediated root elongation arrest, and inhibit ABA-mediated stomatal closure. ZmSTOP1 activity is modulated by OST1 kinase in stomata.

CONCLUSIONS

5. Conclusions

1. ZmSTOP1 is a nuclear protein belonging to a five-member family in maize, which is very well conserved between plant species and is characterized by possessing four zinc-finger domains. ZmSTOP1 is expressed in maize leaves and roots, where its expression is ABA-inducible.

2. ZmSTOP1 protein dimerizes and possesses DNA-binding capacities, though it does not bind to a specific DNA sequence either in vitro or in vivo, and no protein-protein interaction specificity has been found. It is able to induce pScALMT1-GUS expression in transiently transformed tobacco cells.

3. ZmSTOP1 overexpression complements low pH phenotype of Atstop1 mutant in roots, recovering the standard response in root length to low pH treatment.

4. ZmSTOP1 interacts and is a substrate of ZmOST1 kinase. This phosphorylation is ABA-dependent and PP2C phosphatases are involved in its regulation.

5. Plants overexpressing ZmSTOP1 show a reduction of root length after ABA treatment. This effect is ABA-dependent and independent of ZmOST1 phosphorylation..

6. The overexpression of ZmSTOP1 reduces the capacity of the plants to close stomata upon ABA treatment. OST1 phosphorylation can modulate this ZmSTOP1 activity in stomata.

7. ZmSTOP1 overexpression alters the pattern of expression of stress and signaling related genes, and can increase the expression of these genes in several tissues.

8. The activity of ZmSTOP1 regulated by ABA plays an important role in drought response, modulating stomatal aperture and affecting root architecture.

MATERIALS AND METHODS

6. Materials and Methods

Plant Materials and Culture Conditions

Maize (*Zea mays*) of the pure inbred line W64A was germinated in water and grown at 26ºC with a 16/8 h light/dark photoperiod.

Maize calli were obtained from embryos isolated from the hybrid line Hi-II (A188xB73) (Armstrong et al., 1992). They were transformed by gold-particle bombardment using pANIC5D vector (ABRC, Columbus, OH) overexpressing ZmSTOP1 under ZmUBIQUITIN1 promoter. Maize calli and regeneted pants were handled by CRAG greenhouse facilities.

Arabidopsis seeds were gas sterilized for three hours, sown in solid Murashige and Skoog medium (MS) supplemented with 10% w/v of sucrose (MS1), and vernalized for 48-72 hours at 4ºC before being transferred to a 24ºC chamber, with 16/8 h light/dark photoperiod.

N. benthamiana plants were grown at 28ºC under a long-day photoperiod (16/8 h light/dark).

Generation of transgenic plants

To determine the functionality of ZmSTOP1, full length ZmSTOP1 was cloned in the pMENCHU vector to generate 2x35S::HA-ZmSTOP1 fusion protein, and subsequently cloned into the PC1300 (Clontech) vector for *Agrobacterium*-mediated transformation by floral dip of *Arabidopsis* Col0 and *atstop1* plants. Full length ZmSTOP1 was cloned in pPK100 vector to generate 2x35S::ZmSTOP1-GFP and subsequently cloned into the PC1300 (Clontech) vector for *Agrobacterium*-mediated transformation by floral dip of *Arabidopsis* plants (Clough and Bent, 1998).

Col0 and Atstop1 mutant backgrounds were transformed with 35S::HA-ZmSTOP1 construct, while Ler and ost1-2 mutant backgrounds were transformed with 2x35S::ZmSTOP1-GFP construct. Homozygous T2 plants were selected for further analysis.

Maize Mesophyl Protoplasts isolation and transformation

Maize mesophyll protoplasts were isolated from 12-14 day-old plants grown in darkness (Sheen, 2001). Central section (6-8 cm) of the first real leaf was selected and cut in 0,5 mm-wide slices. They were vacuum- exposed for 30 min with enzymatic solution (0,6 M manitol, 10mM MES pH 5,7, 1,5 % celulase RS (Yakult), 0,3% macerozyme R-10 (Yakult), 1 mM $CaCl_2$, 5mM ß-mercaptoethanol, 0,1 % BSA) and then kept in dark at 37ºC, 40 rpm for 2 h. Resulting protoplasts were filtered using a 35 µm nylon filter and centrifuged for 2

min at 150 g. Pelleted protoplasts were washed with WI buffer (0,5 M manitol, 4 mM MES pH 5,7, 20 mM KCl) twice.

To transform maize protoplasts, 20 µg of DNA (2 µg/µl) were added to 1-2·10^5 cells in 150 µl, counted in a microscope using a Malassez chamber. 160 µl of PEG/Ca solution (4 g PEG4000, 3 ml H_2O, 2,5 ml 0,8 M manitol 1 ml 1M $CaCl_2$) was added to the mix and incubated at room temperature (rt). After 30 min the mix was diluted with 640 µl of WI buffer. Transformed protoplasts were pelleted for 2 min at 1000 rpm, resuspended in 450 µl of WI buffer and kept over night in darkness.

Sequence Analysis

All sequences were retrieved using BLAST in the NCBI database (http://ncbi.nlm.nih.gov/blast).

The phylogenetic tree was completed using Clustal Omega (Sievers et al., 2011) Jailview tool, using neigbour-joining (% ID) algorithm.

The alignments were performed using BioEdit software (Hall, 1999).

The following sequences were selected to be included in the phylogenetic tree and protein alignment: *Zea mays* ZmSTOP1 (NP_001152939), *Oryza sativa* Os02g0572900, *Arabidopsis* AtSTOP1 (NP_174697.1), *Arabidopsis thaliana* AtSTOP2 (NP_197680.2), *Solanum lycopersicum* (NP_001265961.1), *Populus trichocarpa* (XP_002310559.1), *Ricinus communis* (XP_002532839.1), *Vitis vinifera*

(XP_002267529.1), *Brassica rapa* (XP_009124410.1), *Glycine max* (XP_003552051.1), *Sorghum bicolor* (XP_002454046.1).

Expression analysis in maize plants

9-day-old maize seedlings grown in greenhouse facilities under long day conditions (16/8 h light/dark) at 28ºC were transferred to 0,5xMS liquid buffer and treated with NaCl 250 mM, H_2O_2 10 mM, MeJa 0,01% and ABA 100 mM for 6 h.

At least two plants were pooled to obtain one sample. RNA from each sample was extracted using RNeasy Mini Kit (Qiagen) and cDNA was obtained using QuantiTect Reverse Transcription kit (Qiagen), following the manufacturer's instructions. One independent replicate was measured with three technical repetitions.

GFP and BiFC Imaging

Full length ZmSTOP1 was cloned in a pPK100 intermediate vector to produce 2x35S::ZmSTOP1-GFP. The pPK100 cassette was subsequently cloned in the binary vector PC1300 (Clontech) for plant transformation. For BiFC experiments, full-length ZmSTOP1 was cloned in the pYFN43 BiFC and pYFC43 and ZmOST1[G40R] was cloned in the pYFN43 GATEWAY-modified vector (Lumbreras et al., 2010) to produce CaMV35S::ZmSTOP1-YFN, 35S::ZmSTOP1-YFC

and 35S::YFN-ZmOST1[G40R]. *N. benthamiana* plants were transiently transfected with these constructs and with 35S::HCPro to inhibit protein silencing in tobacco. Confocal observations were performed 2 days after infiltration on an Olympus IX81 inverted microscope (Olympus, Center Valley, PA).

Recombinant Protein Purification

Recombinant ZmSTOP1 full sequence was cloned in the pETM11 expression vector (obtained from F. Parcy) (Dummler et al., 2005) containing an N-terminal 6xHis tag, expressed in *E. coli* Rosetta2 cells (Merk KGaA, Darmstadt, Germany). After induction by 0.5 mM IPTG, cells were grown in LB-NaCl medium (triptone 10 g/l, yeast extract 5 g/l, 0,5 M NaCl, pH 7,5) supplemented with $ZnSO_4$ 0,5 mM, overnight at 17°C. For cell lysis, the pellet of 1 l culture was sonicated in 30 ml lysis buffer (20mM Tris pH 8, 1 M NaCl, one protease inhibitor cocktail tablet Complete EDTA-free (Roche, Meylan, France) and centrifuged for 30 min at 13000 rpm. The clear supernatant was run through a column with 1 ml Ni-NTA resin (Qiagen, Courtaboeuf, France) in binding buffer (20mM Tris pH 8, 1 M NaCl). The resin was transferred into a column, washed with 10 column volumes (CVs) of binding buffer, binding buffer + 20 mM imidazole (10 CV) and eluted with binding buffer +300 mM imidazole. The eluted protein was concentrated using a Amicon Ultra-15 3500 MWCO (Merk KGaA, Darmstadt, Germany). Protein concentration was estimated using Nanodrop (Thermo Fisher Scientific, Waltham, MA).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay was performed using 81 basepairs (bp) DNA strand with 38 bp of random DNA. Probes were labeled with TAMRA (Sigma). Binding reactions were performed in 20 μl binding buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1% glycerol, 0.25 mM EDTA, 3 mM TCEP). 10nM random DNA was mixed with 6xHis-ZmSTOP1 protein. Protein concentration ranged from 50nM to 10 mM. 100 μg/mL of proteinase K (Promega) was added to control reactions, and incubated for 1h at 37-56°C. Binding reactions were loaded onto native 6% polyacrylamide gels 0.5 × TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA pH 8) and electrophoresed at 90 V for 80 min at 4°C. Gels were scanned on a Typhoon 9400 scanner (Molecular Dynamics, Sunnyvale, CA).

Specific promoter probes design

Single-stranded oligonucleotides, 5′-labelled with Cy3-dCTP (GE Healthcare) using Klenow polymerase (Promega), were annealed to non-fluorescent complementary oligonucleotides in annealing buffer (10 mM Tris pH 7.5, 150 mM NaCl and 1 mM EDTA).

SELEX assay

In vitro selection of ZmSTOP1 target sequence was performed with fluorescent random DNA and and recombinantly produced 6xHis-ZmSTOP1 folowing the protocol published by Moyroud et al. (2011).

Briefly, ZmSTOP1 was inmunoprecipitated using histidine affinity magnetic beads. Random DNA containing a sequence compatible to ZmSTOP1 remains attached to the TF during the inmunoprecipitation. This DNA is purified and amplified using specific primers for known flanking sequences, of around 20 bp each, while random sequence is around 40 bp long. Each round is validated by EMSA, until the enrichment is considered strong enough. The DNA pool of enriched sequences is cloned in a vector and transformed into E. coli. The vectors are isolated from the resulting colonies and sequenced. The resulting sequences are assigned a score depending on the probability to be found in selected DNA.

ChIP assay

The chromatin inmunoprecipitation assay was performed using 1g of 12-day-old Ler ecotype *Arabidopsis* seedlings expressing 2x35S::ZmSTOP1-GFP. Seedlings were grown in long day conditions (16/8 h light/dark) at 24ºC for 12 days and sprayed with 100 μM ABA, supplemented with 0,01 % Tween. The treated and non-treated plants were left in the same light and temperature

conditions for 3 h before being formaldehyde fixed and frozen in liquid nitrogen to store at -80ºC. Ler wild type plants were used as a control.

Maize mesophyll protoplasts from 12-14 day-old plants were isolated and PEG-transformed with 2x35S::ZmSTOP1-GFP construct. Later they were treated with 10 μM ABA for 30 minutes before being formaldehyde fixed (0,5M Manitol, 1% formaldehyde, 0,25 % BSa 1 mM PMSF) in vacuum, centrifuged 2x 5min at 150g, frozen in liquid nitrogen and stored at -80ºC. Transformation efficiency was calculated by couning transformed (fluorescent) cells using a Malassez counting chamber in a fluorescence microscope. 2x35S::GFP transformed protoplasts were used as a control. ChIP was performed on $3 \cdot 10^6$ total cells (40-50 % transformed).

Ab290 anti-GFP antibody (Abcam) was used to inmunoprecipitate ZmSTOP1-GFP.

The protocol used to perform ChIP experiment was published by Kaufmann et al. (2010).

DNA was fragmented and precipitated using a Covaris sonicator (Covaris, Woburn, MA). The resulting immunoprecipitated DNA was tested for enrichment by qPCR using putative ZmSTOP1 targets such as AtCIPK23 and AtROP4 and a negative control locus from SOC1. Libraries for high throughput sequencing were prepared as previously described (Yant et al., 2010).

GUS expression assay

N. benthamiana plants were agroinfiltrated with pGWB3 vector containing pScALMT1::GUS, pGWB502Ω vector expressing ScSTOP1 and pCambia 1300 vector expressing 2x35S::HA-ZmSTOP1. 35S::HcPro was co-transformed with the constructs to ensure high levels of expression.

Transformed *N. benthamiana* leaves were treated with GUS staining solution (1 mM 5-bromo- 4-chloro-3-indolyl-b-glucuronic acid solution in 100 mM sodium phosphate, pH 7, 0.1 mM EDTA, 0.5 mM ferricyanide, and 0.5 mM ferrocyanide, and 0.1% Triton X-100). After applying vacuum for 5 min, they were incubated at 37°C for 2h and over-night. Chlorophyll was cleared from the plant tissues by immersion in 70% ethanol.

ZmSTOP1 polyclonal antiserum production

6xHIs-ZmSTOP1 was recombinantly produced and purified as described before. Protein was loaded in acrylamide gels and the band corresponding to 6x-His-ZmSTOP1 was isolated. Acrylamide bands were finely sliced and protein was eluted from the gel using elution buffer (0,125 M Tris pH 8, 1 mM EDTA, 0,1 % SDS). The protein was concentrated by precipitation, adding one volume of buffer of pure acetone at -20ºC. With the resulting protein (0,5 mg) one rabbit was inoculated in CID antibody production facilities.

Protein Extraction and Western Blot

Proteins for In Gel Phosphorylation assay were extracted as described by Vilela et al. (Vilela et al., 2015). Briefly, treatments with 100 μM ABA (Sigma, St. Louis, MO) were applied to 12d *Arabidopsis* seedlings after transfer to liquid MS1 medium. Plant material was ground with liquid nitrogen and incubated with kinase extraction buffer (5 mM EDTA, 5mM EGTA, 2 mM DTT, 25 mM NaF, 1 mM Na3VO4, 50 mM ß-glycerophosphate, 20 % glycerol, 1mM PMSF, 50 mM HEPES-KOH pH 7.5) for 30 min on ice. Later, plant extracts were centrifuged at 14000 rpm and supernatant was used for the experiment.

Proteins for HA-ZmSTOP1 detection were extracted in TM 2x buffer (125 mM Tris-HCl pH 6,8, 4% SDS, 20% glycerol, 0,04% bromophenol blue dye and 10% β-mercaptoethanol).

Proteins for ZmSTOP1 detection in maize overexpressing seedlings regenerated from transformed calli were extracted from roots using TM 2x buffer.

For Western blot analysis, 40 μg of total protein were loaded per lane. Anti HA high-affinity antibody (Roche, Mannheim, Germany) was used to detect HA-ZmSTOP1, and anti ZmSTOP1 polyclonal antibody was used to detect ZmSTOP1 overexpression in maize transgenic plants.

Root growth assays

4-day-old seedlings grown in solid MS1 medium were transferred to plates containing solid MS1 medium adjusted to pH 4.7, or to MS1 plates supplemented with 50 μM ABA. After 8 days growing vertically at 24ºC in long day conditions (16/8 h light/dark), roots were photographed and measured using ImageJ software (Schneider et al., 2012).

Relative root length was obtained by calculating the ratio between root length (cm) in treatment conditions and length in control conditions.

Root growth assays were conducted at least three times with similar results.

Stomatal aperture measurements and water loss kinetics

Arabidopsis plants were grown on soil for 3 weeks under short-day conditions (8/16 h light/dark), and stomatal aperture and water loss measurements were performed at least three times.

For stomata aperture measurements, detached *Arabidopsis* leaves were incubated for 2h in 50 mM KCl and 10 mM MES (pH 6.15) with cool white fluorescent light (50 μmol/m^2/s) to induce maximum stomata opening. ABA was added in the same buffer solution at concentrations of 0, 1 and 3 μM, for 2 h. Imprints of treated leaves were performed on Genie Light Body Standard Set dental resin (Sulthan Healthcare, York, PA), a cast of the leaf was obtained us-

ing nail polish, and bright-field microscopic photographs of the cast were taken with a 40x objective lens (Delgado et al., 2012). Stomatal aperture was scored as width/length pore ratio of at least 200 stomata (*n* = 200), using Image J software (Schneider et al., 2012). Student's T-test was used to determine degrees of variation between samples under the same treatment.

Water loss was scored by weight loss over time of at least five fully expanded detached rosettes from transgenic lines overexpressing ZmSTOP1-HA and ZmSTOP1-GFP.

Maize phenotype experiments

Stomatal conductance of CO_2 was performed on regenerated maize plants from transformed calli overexpressing pZmUBI::ZmSTOP1. Plants regenerated from non-transformed calli were used as a control. Plants were acclimated to greenhouse conditions (long day and 28°C) and grown until at least three real leaves were present. An infrared gas analyzer (IRGA) was used to execute the measurements. At least three different plants were measured for each experiment. The experiments were conducted three times with similar results.

Root length was measured directly on regenerated plants grown in MS1 medium supplemented with 100 µM ABA for 14 days. At least two different plants were used for each background.

Water loss experiment was performed for 60 min on detached leaves of maize regenerated seedlings showing at least one real leaf. At least four different plants were used for each background.

Microarray

Microarray was performed on 12-day old *Arabidopsis* seedlings overexpressing ZmSTOP1-HA. Seedlings were grown in long day conditions (16/8 h light/dark) at 24ºC.

RNA was extracted using Maxwell Simply RNA kit in Maxwell-16 automated extractor (Promega). RNA from three independent replicates was transferred to CRAG genomic facilities for quality assessment in a BioAnalyzer system (Agilent Technologies). Labeling, hybridization and scanning was, then performed following the manufacturer recommendations for Affymetrix GeneChip Arabidopsis ATH1 Genome Array.

The data analysis was performed by Sequentia Biotech company (Barcelona).

Quantitative Real time PCRs of stress-related genes

12-day-old Arabidopsis seedlings of Col 0, Atstop1 mutant, Atstop1 2x35S::HA-ZmSTOP1 (complementation) and Col0 2x35S::HA-ZmSTOP1 (overexpression), grown in long day conditions (16/8 h ligh/dark) at 24ºC were used for qRT experiments.

RNA was extracted using Maxwell Simply RNA extraction kit in Maxwell system (Promega) following manufacturers instructions. cDNA was obtained with QuantiTect Reverse Transcription kit (Qiagen). cDNA samples from three independent replicates were analyzed by FluidiGM system (San Francisco, CA) in CRAG genomic facilities.

Mean +/- SE (standard error) from 3 independent replicates of Full seedlings qRT-PCRs was plotted in Fig. 18 to 23.

One representative replicate out of three was plotted in Fig. 18 to 23 in qRT-PCRs from roots and shoots measured separately.

Yeast two-hybrid assay

Yeast two-hybrid experiments were performed by Alicia Moreno and published by Vilela et al. (2013). For yeast interaction experiments ZmSTOP1 was cloned in the vector pGAD424 and ZmOST1 in pGBT7 vector and were transformed directly into *Saccharomyces cerevisiae* AH109 strain. Positive colonies in medium SD-LW were selected in medium SD-LTWA. b-galactosidase liquid assays were performed as described by Bhalerao et al. (1999).

In-Gel phosphorylation assay

Protein samples (40 µg) were separated on 10% SDS-PAGE gels embedded with 0.5 mg/ml recombinant HIS-tagged ZmSTOP1 protein.

OST1 kinase was inmunoprecipitated from protein extracts using anti-OST1 (ATP box) antibody (Vilela et al., 2015). 500 µg of protein were incubated with a 1:150 dilution of the antibody in 300 µl of IP buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 2 mM Na3VO4, 2 mM NaF, 10 mM b-glycerophosphate, 150 mM NaCl, 0.5% [v/v] Triton X-100, 0.5% [v/v] Nonidet NP40, 1 mM PMSF, 10 mM leupeptin, 2 mg/ml aprotinin, and 10 mg/ml pepstatin). After 3 h in a rotary shaker, 40 ml of protein A–Sepharose CL-4B 50% slurry (GE Healthcare, Piscataway, NJ) was added and incubated for another hour. The slurry was washed 3x 15 min with IP buffer and the supernatant was removed prior to the in-gel kinase assay. Proteins were recovered from the beads by adding 20 ml loading buffer and heating at 95ºC for 5 min.

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 x 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 (Perkin Elmer) at room temperature for 1 h. Finally, gels were washed extensively with 5% (w/v) trichloroacetic acid and 1% sodium pyrophosphate solution, at least five times, and dried. Radioactivity was detected using a STORM 820 imager (GMI).

In vitro kinase assay

cDNA fragments encoding for ZmOST1, ZmSTOP1, and three ZmSTOP1 derivatives corresponding to N-terminal, C-terminal and zinc-fingers domains (1–198; 199–386; 108–280 aa, respectively) were 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. Purified E. coli- expressed ZmOST1 (500 ng) were incubated at 30°C for 30 min with either 500 ng of purified ZmSTOP1 or of the truncated N-terminal, C-terminal and zinc-fingers domains in a final 15 ml volume of 1X kinase buffer (20 mM HEPES pH 7.5, 1 mM DTT, 10 mM MgCl2, 5 mM NaF, 125 mM b-glycerolphosphate), 25 mM cold ATP and 5 mCi [c-33P]-ATP).

Primers used for qRT-PCR

The primers used for qRT-PCR during this work have been the following:

Name	Forward sequence	Reverse sequence
ZmSTOP1	AAGGACAAGCTCTTCGGTCA	CCCTTCCTCCATAGCATCAA
ZmCICLO	GTGTGGATCTGTGAACCCCAT	CAGGTGAAACACGAATCAAGCA
ZmRAB17	CGTCAAAGCCGTAATGTTCA	TTCAAGTAAAGCCACTCGCA
AtSTOP1	AGATTCTCGCACCGCATACT	ATGCCCTCTCATATGCATCC
HA2	GCAAACCTCGCATCCATTCC	TCACCACCTTTGCAATGAACA
PYL6	GTCTGGTCGATCCTAAGCCG	TCTGACCTCTCTCACCGACC
WRKY46	CATCACATCCCCGAAGACGA	TTCGGACTTGGTCGGTTTCA
KT2/3	ATCACTGGCACATCCACGTT	AGTCGAAAGCAGCGAATCCA
ABCB4	ATCGGTTAAGCACTGTGCGA	ATAAGCTCCTTCCGGGTCCT
AOC3	CGGCAAGAAACCAACAGAGC	CAGAGACCTGCCGTGATTCC
XTH18	GTTTCCTCGAGGTGTTCCTGT	CACCGCATACATATGAGCAACA
CAX7	CCGCCTGGTTGTTTGTTCTG	TGCCAAGAGAGAGAAGCGTG
CML41	CTTCAGCCATTTCGACAGCG	TTATCGCCTCTTGAGCTGCC
GAD	GTTAGAAGAGGCGGAGACCG	CCTTCAGCTTTGCGCTTGTT
GDH1	GGATACTCGCCTGCAGTTGT	ATGGTCTTTCCGTGCTCGTT
GDH2	AGCTGATCCACGAGAAAGGC	TGAGACTTCCAGTTGCGTCC
CEJ1	GTAGATGCGATAGGGGCGAC	CGAGATTTTCCGGGTCAGGT
EP3	CCTTCAAAACCGCCTTGTGG	CTTGAACGGTGGCTGTGTTG
BRG2	TCGATTCGTCTCCCTTCACTTAT	GCTCTATCGCCTCCATTATCGT
MYB15	AAGCGAATCGGAGCTAGCAG	CCGTCGTGGCTTATGAGTGT
CML38F	TCAGCCGGAGAGATACAACA	AACAGCAGCTACGGCTTCTT
CIPK23	TCCGCACACGAGAGTTGAAT	GGGATTTCGCAACGCTCATC
RBOHD	ACGTGCGTCCAAGAAAAACG	GTCGTCCCTGATGTCTAGCG
ALMT1	CTTGAGAGAGCTGAGTGACCA	GCAAGTCCCACTTTGAAAGCA
UBIQUITIN	GTCTCCGTGGTGGTGCTAAG	ATGACTCGCCATGAAAGTCC

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ANNEXES

8. Annexes

Annex 1 - Table of up-regulated genes in ZmSTOP1 *overexpressing Arabidopsis thaliana* plants.

Gene ID	logFC	adj.P.Val	Gene name
AT1G66270	3,77	2,34E-03	BGLU21
AT4G30280	3,39	1,46E-05	ATXTH18
AT1G21120	3,36	1,74E-04	
AT4G19800	3,28	1,70E-02	
AT3G54150	3,25	1,83E-05	
AT5G17330	3,14	5,62E-05	GAD
AT2G41230	3,07	1,62E-04	
AT4G12545	3,02	1,06E-03	
AT4G31900	2,89	4,32E-04	PKR2
AT1G49860	2,87	7,93E-05	ATGSTF14
AT1G73120	2,87	1,71E-04	
AT4G21680	2,82	2,47E-04	NRT1.8
AT2G37430	2,82	6,20E-04	ZAT11
AT1G65390	2,74	7,73E-04	ATPP2-A5
AT4G30170	2,72	6,49E-03	PER45
AT3G22240	2,65	7,62E-04	
AT5G66640	2,63	2,07E-04	DAR3
AT2G20142	2,50	2,16E-04	
AT5G39580	2,47	2,16E-04	PER62
AT3G01830	2,44	1,18E-02	CML40
AT5G44630	2,42	1,74E-04	BS
AT3G49620	2,42	3,31E-03	DIN11
AT2G30770	2,37	2,46E-02	CYP71A13
AT4G24110	2,37	7,93E-05	
AT4G15975	2,36	3,86E-04	ATL17
AT4G31800	2,34	1,71E-04	WRKY18
AT4G18250	2,33	4,32E-04	
AT4G10380	2,33	1,46E-05	NIP5;1
AT2G26560	2,32	1,56E-04	PLP2
AT4G21390	2,23	2,86E-04	B120
AT1G79680	2,19	6,95E-04	WAKL10
AT1G24140	2,15	3,66E-04	
AT2G18210	2,09	1,08E-04	
AT2G15390	2,06	1,55E-04	FUT4
AT4G22470	2,06	3,41E-03	

AT1G07160	2,06	4,24E-03	
AT2G46750	2,06	4,41E-02	
AT5G37740	2,04	2,23E-05	
AT5G52760	2,01	2,60E-03	
AT4G13420	2,00	8,64E-03	HAK5
AT1G54120	1,99	1,46E-03	
AT1G14880	1,96	7,93E-05	PCR1
AT4G16260	1,95	4,73E-02	
AT4G14365	1,95	6,20E-04	XBAT34
AT5G61160	1,92	2,87E-03	AACT1
AT4G21850	1,91	3,41E-04	ATMSRB9
AT1G02400	1,89	2,83E-03	ATGA2OX4
AT5G64905	1,88	9,99E-03	PROPEP3
AT3G23250	1,88	4,95E-04	MYB15
AT5G07550	1,88	3,61E-02	GRP19
AT5G18470	1,87	3,32E-04	
AT2G01520	1,85	1,86E-02	MLP328
AT5G05300	1,84	4,06E-03	
AT4G33050	1,83	2,81E-05	EDA39
AT2G33710	1,82	3,73E-04	ERF112
AT3G02840	1,81	2,90E-02	
AT2G32030	1,80	2,09E-03	
AT4G25810	1,79	2,59E-03	XTR6
AT3G02550	1,78	7,06E-03	LBD41
AT1G26380	1,78	1,27E-02	
AT5G50760	1,76	3,73E-04	
AT5G65600	1,75	5,53E-04	LECRK92
AT2G35930	1,75	2,60E-03	PUB23
AT4G28085	1,74	2,06E-04	
AT5G57220	1,73	1,74E-04	CYP81F2
AT4G23200	1,73	2,07E-04	CRK12
AT5G52750	1,73	9,12E-04	
AT3G05320	1,73	1,06E-03	
AT5G07560	1,72	3,73E-04	GRP20
AT3G25780	1,72	1,62E-03	AOC3
AT3G23550	1,71	1,03E-03	LAL5
AT1G76650	1,69	1,52E-03	CML38
AT5G01540	1,69	6,63E-04	LECRKA4.1
AT5G15120	1,67	1,11E-02	
AT2G43590	1,67	4,48E-03	
AT4G14130	1,66	4,17E-02	XTR7
AT1G78410	1,65	4,51E-04	
AT1G71400	1,64	3,86E-04	AtRLP12
AT4G27460	1,63	3,42E-03	CBSX5
AT3G50770	1,63	2,85E-04	CML41
AT3G50930	1,63	2,36E-02	BCS1

AT1G01340	1,62	5,62E-05	ATCNGC10
AT5G25250	1,62	1,08E-02	FLOT1
AT3G01290	1,60	2,81E-05	HIR3
AT5G25930	1,59	1,19E-04	
AT2G46440	1,58	4,17E-04	ATCNGC11
AT2G24600	1,57	9,11E-04	
AT3G55840	1,56	1,03E-02	HSPRO1
AT5G28610	1,56	1,10E-02	
AT3G08720	1,56	2,11E-03	ATPK19
AT1G67980	1,56	5,10E-04	CCOAMT
AT3G16530	1,55	2,27E-04	
AT2G16660	1,55	4,29E-02	
AT2G34930	1,53	1,79E-03	
AT5G17860	1,52	1,19E-04	CAX7
AT2G23680	1,52	5,62E-05	
AT3G48520	1,51	1,31E-02	CYP94B3
AT1G18570	1,50	4,70E-04	MYB51
AT1G18390	1,50	1,46E-03	
AT1G26250	1,49	3,25E-02	
AT1G61360	1,49	2,07E-04	
AT3G61190	1,49	2,98E-02	BAP1
AT5G14700	1,49	1,39E-03	
AT1G16130	1,48	8,15E-04	WAKL2
AT3G23230	1,48	7,48E-03	ERF098
AT2G35710	1,48	7,74E-03	
AT2G36690	1,47	7,70E-03	
AT1G21100	1,47	3,79E-03	
AT3G29250	1,47	4,46E-02	SDR4
AT3G22200	1,47	2,23E-05	POP2
AT3G25610	1,47	8,51E-05	ALA10
AT1G32450	1,47	6,03E-03	NRT1.5
AT1G19020	1,46	1,05E-03	
AT3G09020	1,45	9,85E-04	
AT1G29270	1,45	9,34E-03	
AT1G56060	1,45	1,09E-02	
AT1G17250	1,45	2,07E-04	AtRLP3
AT4G32800	1,44	7,62E-04	ERF043
AT1G58420	1,44	9,23E-03	
AT5G41740	1,44	5,39E-03	
AT5G49520	1,43	7,75E-04	WRKY48
AT4G20780	1,43	5,49E-04	CML42
AT5G37540	1,42	4,01E-04	
AT3G26200	1,42	7,59E-04	CYP71B22
AT2G31230	1,42	3,88E-04	ATERF15
AT1G73805	1,41	7,45E-04	
AT1G10070	1,41	4,88E-03	ATBCAT-2

AT4G37290	1,40	1,76E-03	
AT5G58610	1,40	2,83E-03	
AT5G13320	1,40	3,81E-02	PBS3
AT5G64260	1,40	8,26E-05	EXL2
AT5G47960	1,39	6,62E-03	SMG1
AT2G26440	1,38	3,10E-04	PME12
AT2G32140	1,38	6,03E-03	
AT3G12910	1,37	3,71E-02	
AT1G19530	1,36	1,47E-02	
AT4G23700	1,36	3,98E-02	ATCHX17
AT4G26200	1,36	3,07E-02	ACS7
AT1G61340	1,35	3,53E-02	
AT1G51620	1,35	1,67E-03	
AT1G35210	1,35	2,27E-02	
AT5G07580	1,33	1,49E-04	ERF106
AT5G33355	1,33	2,53E-03	
AT5G39670	1,33	1,16E-02	CML45
AT1G07000	1,33	4,11E-03	ATEXO70B2
AT5G26260	1,31	8,61E-03	
AT1G51270	1,31	1,90E-03	PVA14
AT4G30270	1,31	3,05E-04	MERI5B
AT3G50260	1,31	2,24E-04	CEJ1
AT3G11840	1,31	1,21E-02	PUB24
AT1G14370	1,30	3,86E-04	APK2A
AT5G66650	1,30	3,13E-03	
AT1G10990	1,30	7,93E-05	
AT1G08630	1,30	6,71E-03	THA1
AT2G41380	1,29	3,66E-04	
AT3G04210	1,29	9,62E-04	
AT3G28340	1,29	1,40E-03	GATL10
AT1G01560	1,29	5,63E-03	ATMPK11
AT2G17040	1,29	5,61E-03	anac036
AT5G46050	1,28	2,59E-03	ATPTR3
AT4G39675	1,28	7,75E-04	
AT1G74360	1,28	8,08E-03	
AT2G46400	1,28	3,04E-02	WRKY46
AT3G25250	1,28	3,71E-02	AGC2-1
AT1G67810	1,28	2,04E-02	SUFE2
AT3G10930	1,27	2,40E-02	
AT5G62520	1,27	3,75E-02	SRO5
AT4G21410	1,27	2,02E-03	CRK29
AT1G29050	1,27	4,79E-03	TBL38
AT1G30700	1,27	1,72E-02	
AT2G32190	1,27	1,26E-03	
AT3G46110	1,26	3,29E-04	
AT3G02800	1,26	2,00E-02	

AT1G23090	1,26	1,25E-04	AST91
AT1G33030	1,26	2,60E-03	
AT2G18690	1,25	4,24E-03	
AT1G05575	1,25	5,51E-03	
AT5G46295	1,25	1,02E-03	
AT1G66090	1,25	1,90E-02	
AT2G22880	1,24	3,04E-02	
AT1G70740	1,23	6,20E-04	
AT5G65300	1,22	3,53E-02	
AT2G15480	1,22	1,68E-03	UGT73B5
AT4G01750	1,22	2,11E-03	RGXT2
AT5G39020	1,21	1,29E-03	
AT5G22690	1,21	3,57E-04	
AT4G08950	1,21	1,44E-03	EXO
AT5G54720	1,21	3,21E-02	
AT1G22400	1,21	8,38E-03	UGT85A1
AT5G47910	1,20	3,16E-03	RBOHD
AT5G27420	1,20	8,38E-03	CNI1
AT5G28630	1,20	8,95E-03	
AT1G19180	1,20	3,15E-03	TIFY10A
AT4G29780	1,19	3,34E-02	
AT5G26920	1,19	3,79E-03	CBP60G
AT2G27080	1,19	3,98E-03	
AT1G28190	1,19	7,07E-03	
AT5G14180	1,19	2,80E-03	MPL1
AT1G64170	1,18	1,69E-03	ATCHX16
AT1G35350	1,18	3,86E-04	PHO1-H8
AT3G28930	1,18	2,55E-03	AIG2
AT1G72900	1,18	3,86E-03	
AT1G33760	1,18	2,77E-02	ERF022
AT5G26280	1,18	4,23E-03	
AT5G55450	1,18	6,43E-03	
AT2G39330	1,17	1,68E-02	JAL23
AT5G35735	1,16	1,67E-03	
AT3G62150	1,16	1,49E-04	PGP21
AT3G44970	1,16	1,64E-02	
AT3G45970	1,16	2,08E-03	ATEXLA1
AT5G09800	1,15	9,40E-03	PUB28
AT4G15390	1,15	2,26E-03	
AT1G19380	1,15	6,20E-04	
AT4G24120	1,15	3,03E-03	YSL1
AT4G24570	1,15	2,28E-02	DIC2
AT1G69760	1,15	9,19E-04	
AT3G50140	1,14	6,28E-03	
AT1G29195	1,14	3,81E-02	
AT4G11850	1,14	2,88E-04	PLDGAMMA1

AT4G02330	1,14	8,75E-03	ATPMEPCRB
AT1G66180	1,14	5,15E-04	
AT2G19800	1,14	1,24E-02	MIOX2
AT1G53920	1,14	7,75E-04	GLIP5
AT1G02930	1,13	6,45E-03	GSTF6
AT3G54420	1,13	5,98E-03	ATEP3
AT1G76930	1,13	9,68E-03	ATEXT4
AT1G66160	1,13	2,83E-03	ATCMPG1
AT1G77640	1,13	2,75E-02	ERF013
AT4G02200	1,13	9,22E-04	DI19-5
AT4G12720	1,12	1,98E-03	NUDT7
AT4G01010	1,12	1,74E-04	ATCNGC13
AT2G40330	1,12	1,53E-02	PYL6
AT1G09970	1,12	1,19E-04	LRR XI-23
AT1G59960	1,11	1,06E-03	
AT3G20370	1,11	2,39E-03	
AT5G20400	1,11	4,51E-04	
AT5G61560	1,11	5,64E-04	PUB51
AT2G38465	1,11	8,64E-04	
AT4G12280	1,11	8,27E-03	
AT4G23810	1,11	9,23E-03	WRKY53
AT3G18400	1,10	3,32E-02	anac058
AT1G24150	1,10	6,47E-04	ATFH4
AT4G40070	1,10	2,48E-03	ATL32
AT5G04340	1,10	7,92E-03	C2H2
AT2G41640	1,10	2,38E-02	
AT3G26440	1,10	8,65E-04	
AT4G09030	1,10	2,73E-03	AGP10
AT1G80840	1,10	3,93E-02	WRKY40
AT1G13210	1,10	6,95E-04	ACA.l
AT1G27730	1,09	2,92E-02	STZ
AT1G79110	1,09	9,59E-04	
AT4G11000	1,08	3,85E-04	
AT1G05000	1,08	4,19E-03	
AT2G47000	1,07	6,11E-03	MDR4
AT3G46930	1,07	2,39E-03	
AT3G16670	1,07	1,18E-02	
AT5G59820	1,07	2,66E-02	RHL41
AT5G23490	1,07	1,44E-03	
AT4G23190	1,06	2,83E-03	CRK11
AT1G05300	1,06	7,75E-04	ZIP5
AT1G33260	1,06	2,78E-04	
AT5G58660	1,06	1,86E-02	
AT2G22860	1,06	7,67E-04	ATPSK2
AT3G21070	1,06	2,07E-02	NADK1
AT4G39890	1,06	6,31E-03	AtRABH1c

AT5G01040	1,06	4,59E-03	LAC8
AT4G33740	1,06	6,92E-04	
AT2G40140	1,06	1,72E-02	CZF1
AT1G16370	1,05	8,22E-03	ATOCT6
AT1G62300	1,05	5,53E-04	WRKY6
AT3G52400	1,05	6,14E-03	SYP122
AT1G69810	1,05	7,11E-03	WRKY36
AT4G14450	1,05	8,59E-03	
AT5G40540	1,05	1,34E-03	
AT5G11670	1,04	2,07E-04	ATNADP-ME2
AT1G80830	1,04	7,42E-04	NRAMP1
AT3G09830	1,04	9,94E-04	
AT3G44400	1,03	4,82E-04	
AT2G19130	1,03	3,90E-04	
AT4G26260	1,03	3,96E-02	MIOX4
AT4G08770	1,03	2,36E-03	Prx37
AT5G59090	1,03	1,87E-03	ATSBT4.12
AT5G03380	1,02	3,85E-04	
AT5G17350	1,02	4,54E-02	
AT5G24210	1,02	7,59E-04	
AT5G12340	1,02	1,65E-02	
AT5G48540	1,02	9,38E-03	CRRSP55
AT1G35140	1,02	2,83E-02	PHI-1
AT4G21400	1,02	3,57E-04	CRK28
AT4G35480	1,02	2,81E-03	RHA3B
AT4G37370	1,01	2,13E-02	CYP81D8
AT4G17490	1,01	2,53E-02	ATERF6
AT2G43140	1,01	1,93E-02	BHLH129
AT3G16860	1,01	1,31E-02	COBL8
AT3G04720	1,01	2,03E-03	PR4
AT2G22470	1,01	1,26E-02	AGP2
AT4G30210	1,00	1,09E-03	ATR2
AT3G26680	1,00	3,37E-04	SNM1
AT5G04310	1,00	5,70E-03	
AT1G03740	1,00	4,63E-03	
AT4G35180	1,00	2,94E-03	LHT7

Annex 2 - Table of down-regulated genes in ZmSTOP1 *overexpressing* *Arabidopsis thaliana* plants.

Gene ID	logFC	adj.P.Val	Gene name
AT1G18710	-2,01	4,19E-04	AtMYB47
AT5G39860	-1,65	5,86E-04	PRE1
AT1G13650	-1,51	1,79E-02	
AT2G21320	-1,41	3,19E-04	
AT3G22210	-1,38	5,62E-05	
AT5G58770	-1,35	5,65E-03	
AT4G10160	-1,29	4,30E-03	ATL59
AT1G60590	-1,29	2,28E-03	
AT5G18060	-1,28	7,67E-04	
AT2G20750	-1,26	5,51E-03	ATEXPB1
AT2G40610	-1,21	6,30E-03	ATEXPA8
AT1G10640	-1,20	4,77E-03	
AT3G03840	-1,18	3,76E-03	
AT2G01890	-1,14	3,22E-02	PAP8
AT1G06080	-1,14	3,73E-04	ADS1
AT5G01390	-1,12	2,81E-05	
AT4G24700	-1,11	2,06E-03	
AT3G53250	-1,09	5,11E-03	
AT4G25780	-1,08	4,43E-03	
AT5G10930	-1,06	1,39E-03	CIPK5
AT1G10370	-1,05	2,36E-03	GST30
AT2G44230	-1,04	7,93E-05	
AT2G29300	-1,03	1,69E-03	
AT3G54070	-1,03	1,46E-03	
AT5G03130	-1,03	8,15E-04	
AT4G09350	-1,02	6,20E-04	
AT1G65490	-1,01	4,73E-03	
AT4G22200	-1,01	6,95E-04	AKT2/3
AT5G03230	-1,00	7,18E-04	
AT1G18810	-1,00	6,96E-04	PKS3
AT3G02380	-1,00	5,73E-04	COL2

Annex 3 – Publications

Please cite this article in press as: Vilela et al., Casein Kinase 2 Negatively Regulates Abscisic Acid-Activated SnRK2s in the Core Abscisic Acid-Signaling Module, Molecular Plant (2015), http://dx.doi.org/10.1016/j.molp.2014.12.012

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Casein Kinase 2 Negatively Regulates Abscisic Acid-Activated SnRK2s in the Core Abscisic Acid-Signaling Module

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ABSTRACT

SnRK2 kinases, PP2C phosphatases and the PYR/PYL/RCAR receptors constitute the core abscisic acid (ABA) signaling module that is thought to contain all of the intrinsic properties to self-regulate the hormone signal output. Here we identify Casein Kinase (CK)2 as a novel negative regulator of SnRK2. CK2 phosphorylates a cluster of conserved serines at the ABA box of SnRK2, increasing its binding to PP2C and triggering protein degradation. Consequently, CK2 action has implications on SnRK2 protein levels, as well as kinase activity and its response to abiotic stimuli.

Key words: abiotic stress, maize, protein phosphorylation

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INTRODUCTION

The Snf1-Related Kinases 2 (SnRK2s) are largely conserved, with 10 gene members in each of the genomes for *Arabidopsis* and rice, and 11 for maize. In addition to hyperosmotic stress, three of the gene members (group III: SnRK2.2, SnRK2.3, OST1/srk2e/SnRK2.6) are also strongly activated by abscisic acid (ABA) (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002; Boudsocq et al., 2004; Kobayashi et al., 2004; Huai et al., 2008).

Activation of these SnRK2s by osmotic stress and ABA is probably mediated by distinctive mechanisms, as suggested by the C- terminal structural organization of these kinases. That is, in contrast to the kinase catalytic domains, their C-termini are more divergent in sequences. They nonetheless possess two recognizable motifs: the "SnRK2 box" required for the activation by osmotic treatments and/or the "ABA box" specifically involved in ABA responsiveness. This ABA box is also the docking site for the negative regulator PP2Cs (Yoshida et al., 2006; Hirayama and Shinozaki, 2007), which can bind and dephosphorylate group III SnRK2s to impede their kinase activity (Yoshida et al., 2006; Umezawa et al., 2009; Soon et al., 2012).

In *Arabidopsis*, OPEN STOMATA 1 (OST1) is the best genetically and biochemically characterized SnRK2, functioning as a key regulator in the core ABA signaling module (Cutler et al., 2010). Besides responding to low humidity and ABA, the kinase also modulates stomatal response to high atmospheric CO_2 through the slow anion efflux channel SLAC1 (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009; Lee et al., 2009; Xue et al., 2011; Brandt et al., 2012), inhibits blue-light receptor-mediated phosphorylation of downstream H^+-ATPases required for stomatal opening (Hayashi et al., 2011), and activates the ABA gene network through phosphorylation of bZIP transcription factors (Furihata et al., 2006; Sirichandra et al., 2010).

Because OST1 expressed in *Escherichia coli* is catalytically active *in vitro*, it has long been assumed that kinase activation *in planta* is mediated by autophosphorylation (Belin et al., 2006; Vlad et al., 2010; Ng et al., 2011) coupled to the sequestration of the negative regulating PP2Cs by the ABA-receptor complex. Additional OST1 regulators, however, cannot be excluded, as pharmacological studies have predicted the implication of *trans*-activating kinases (Boudsocq et al., 2007). Furthermore, the OST1 protein is detectable in several tissues outside the guard cells from unstressed plants, and changing the level of OST1 expression has an impact on sucrose metabolism and fatty acid accumulation in seeds (Zheng et al., 2010). Thus it is likely that OST1 is subjected to other types of positive or negative controls besides those from the core ABA signal complex.

Here, we identify casein kinase 2 (CK2) as a regulator of OST1 protein stability. CK2 is a multi-subunit serine/threonine kinase

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ubiquitously expressed and highly conserved in eukaryotes (Meggio and Pinna, 2003). The CK2 holoenzyme has two catalytic α subunits and two regulatory β subunits. Both the *Arabidopsis* and maize genomes have four α and four β subunit genes. In maize CK2α are nucleolar proteins except for CK2α4, which is chloroplastic, whereas CK2β are aggregated in nuclear speckles, with CK2β3 also present in the cytosol (Salinas et al., 2006; Riera et al., 2011, 2013). Among kinases, CK2 is distinctive for that it can use either ATP or GTP as phosphoryl donors (Niefind et al., 1999) and efficiently phosphorylate proteins bearing the target motif S/T-X-X-E/D. Although maize CK2 are well characterized both structurally and biochemically (Riera et al., 2003), much remains speculative about their functions in diverse physiological contexts.

In animals, CK2 is purported to phosphorylate more than 300 cellular proteins (Meggio and Pinna, 2003). So far, several targets of CK2, involved in plant growth, development, and abiotic stress responses, have been identified in plants (Lee et al., 1999; Riera et al., 2004; Portoles and Mas, 2007; Moreno-Romero et al., 2008). In the case of abiotic stresses, CK2 has been described as a potential enhancing factor, since *ck2*α mutants are hyposensitive to ABA concerning seed germination, cotyledon greening, and stomatal opening (Mulekar et al., 2012; Wang et al., 2014). These effects were attributed to the down-regulation of ABA-responsive genes, including *OST1* (Wang et al., 2014), but the biochemical relationship between CK2 and OST1 was unclear. By characterizing the regulation of the maize ortholog of *Arabidopsis* OST1, we discovered that CK2 is a direct negative regulator of ZmOST1. Indeed, CK2 can phosphorylate the ABA box of ZmOST1 to accelerate the latter's turnover and promoting binding to PP2Cs. These results therefore uncover a previously unknown function of CK2 in dampening OST1 signal output.

RESULTS

CK2 Phosphorylates ZmOST1 at the ABA Box

Apart from autophosphorylation of the recombinant protein produced in *E. coli*, there has been one pharmacological study showing that the activation of SnRK2 in plants might also be mediated through *trans*-phosphorylation by staurosporine-resistant kinases, such as CK2 (Boudsocq et al., 2007), although direct evidence is still lacking.

We used protein extracts from maize plants treated with or without ABA to detect kinase activities toward recombinant ZmOST1, which is used as substrate. In-gel kinase assays revealed three constitutive bands (Figure 1A), two around 60 kDa, one around 42 kDa showing intense activity, however, no ABA-inducible band was observed. Using GTP as a diagnostic phosphate donor, we were able to identify the 42 kDa band with CK2 activity. These results suggest that maize OST1 might be a target of several upstream kinases, including CK2 (Niefind et al., 1999).

To check whether these upstream kinases also appear in *Arabidopsis*, the phosphorylation patterns of recombinant ZmOST1 were again compared using protein extracts from the Landsberg wild-type and the *ost1-2* mutant, which specifically lacks endogenous OST1 kinase activity (Mustilli et al., 2002). One 60 kDa and two 42 kDa phosphorylation bands were still present in both genotypes, confirming *trans*-phosphorylation of ZmOST1 (Figure 1B). Again, only one of the two 42 kDa bands remained when using GTP as a phosphate donor. These combined results suggest that the upstream kinases of OST1 also exist in *Arabidopsis*, one of which corresponds to a CK2, a kinase known to be well conserved in plants.

We mapped the phosphorylation sites on the functional domains of ZmOST1 as a first step toward understanding the biochemical consequences by this post-translational modification. We used, as *in vitro* substrates, (1) the full-length ZmOST1, (2) the catalytic domain (amino acids [aa] 1–290), (3) the regulatory domain comprising both the SnRK2 and ABA boxes (aa 290–366), (4) the SnRK2 (aa 290–325), and (5) the ABA box (aa 325–366) subdomains (Figure 1C). We found that ZmOST1 was not phosphorylated by ZmCK2 holoenzyme in the catalytic domain, encompassing the T loop required for activation, but exclusively in the ABA box (Figure 1D). We also determined that this phosphorylation pattern is conserved for the *Arabidopsis* ortholog AtOST1 (aa 316–362). We then mutagenized the ZmOST1 sequence to generate a kinase variant, ZmOST1[*ck2A*], with all serines mutated to alanines at potential CK2 phosphorylation sites present in the ABA box (Supplemental Figure 1). ZmCK2 *trans*-phosphorylation was no longer detectable on this ZmOST1 variant (Figure 1D, right).

CK2 Interacts with OST1 and Affects Its Subcellular Localization

After establishing OST1 as an *in vitro* target of CK2, we set out to study their interaction *in planta*. We co-immunoprecipitated (co-IP) the OST1 protein complexes from *Arabidopsis Col-0* wild-type, *ck2* $\alpha 1\alpha 2\alpha 3$ triple mutant that has only residual CK2 activity (Mulekar et al., 2012), and maize protein extracts. We then used both total protein extracts and co-IP complexes for detecting CK2 catalytic (α) subunit activity by in-gel kinase assay, and the regulatory (β) CK2-1 subunit by Western blot. Both the catalytic activity and the regulator protein can be detected in OST1 immunocomplexes (Figure 2A), indicating that they may be involved in the same processes.

To determine how CK2 regulates ZmOST1, we compared the subcellular localization of ZmOST1, ZmOST1[*ck2A*], and another phosphomimetic (serines mutated to glutamates) variant of the kinase ZmOST1[*ck2E*] fused to GFP. Transient expression in tobacco epidermal cells revealed a diffused pattern of distribution in the nucleus and cytosol for all three proteins (Figure 2B, left). We noted, however, that the signal was consistently stronger for the mutagenized ZmOST1[*ck2A*]-GFP than for the wild-type counterpart, and fluorescence was the lowest for ZmOST1[*ck2E*].

We then co-expressed these constructs with non-fluorescent MYC-tagged CK2α1 and CK2β1 to investigate how CK2 affects ZmOST1 subcellular localization. In maize, CK2α is present mainly in the nucleolus, whereas CK2β is aggregated in nuclear speckles (Riera et al., 2011). ZmOST1-GFP formed cytoplasmic aggregates when co-expressed with CK2α1 (Figure 2B, center), and changed from its diffused pattern to nuclear speckles when

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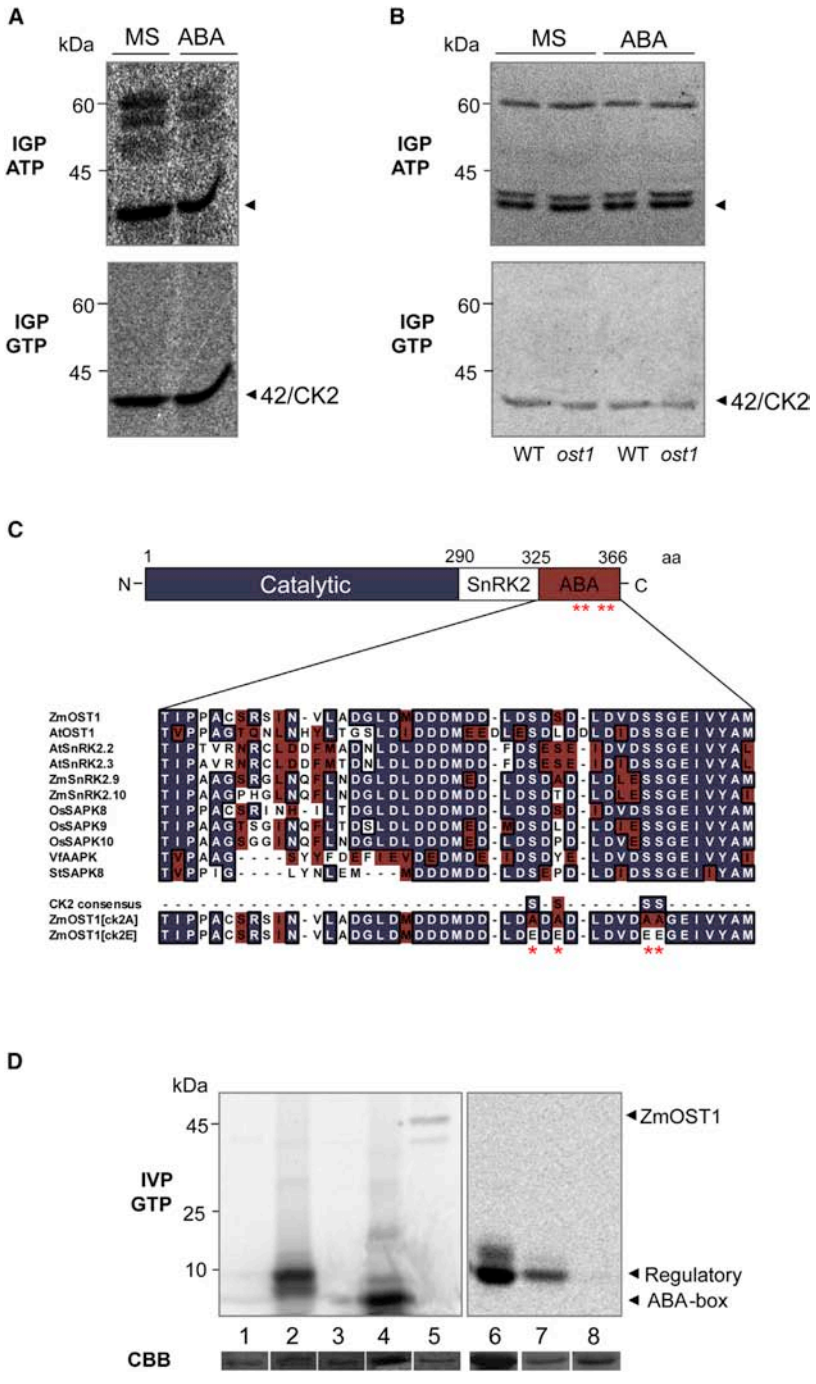

Figure 1. ZmOST1 Is Phosphorylated by CK2 at the ABA Box.

(A and B) Recombinant ZmOST1 was used as a substrate for in-gel phosphorylation assays (IGP) with proteins extracted from 7 day-old **(A)** maize and **(B)** *Arabidopsis* seedlings (Ler wild-type; *ost1-2* mutant) with and without ABA (100 μM) treatment. Sizes of the activity bands obtained are shown on the left. The lower panel shows the same extracts run on in-gel kinase assays using GTP as a phosphate donor and confirms that CK2 is an upstream kinase of ZmOST1 in both systems.

(C) Schematic representation of ZmOST1 domains and alignment of sequences from different plant species. Asterisks indicate consensus sequences for CK2 activity that were mutagenized, changing serines S350, S352, S358, and S359 to alanine to produce ZmOST1[*ck2A*] or glutamate to produce ZmOST1[*ck2E*].

(D) *In vitro* phosphorylation (IVP) of ZmOST1 and deletion forms by CK2, with corresponding protein Coomassie brilliant blue (CBB) staining. 1–8, phosphorylation by CK2 of: 1, ZmOST1[1–289]; 2, ZmOST1[290–366]; 3, ZmOST1[290–325]; 4, ZmOST1[325–366]; 5, ZmOST1; 6, AtOST1[316–362]; 7, ZmOST1[290–366]; 8, ZmOST1[*ck2A*] [290–366]. CK2 phosphorylates ZmOST1 in the ABA box and this activity is abolished in the ZmOST1[*ck2A*] mutagenized protein lacking consensus serines.

co-expressed with CK2β1 (Figure 2B, right). In comparison, the subcellular localization of ZmOST1[*ck2A*] remained mostly unchanged in the presence of the two co-expressed CK2 subunits, with the exception of a clear nuclear accumulation of fluorescence when ZmOST1[*ck2A*] was co-expressed with CK2β1 (Figure 2B, middle). Lastly, ZmOST1[*ck2E*] protein levels are absent in the presence of CK2α1 and have a very mild cytosolic and nuclear speckled signal in the presence of CK2β1 (Figure 2B, bottom). The fluorescence intensities observed are consistent with the results of protein levels analyzed by Western blot (Figure 2C).

The ABA Box Controls ZmOST1 Protein Stability

The stronger fluorescence emanating from ZmOST1[*ck2A*]-GFP than from ZmOST1-GFP, together with the extremely low fluorescence levels of ZmOST1[*ck2E*], suggested that the mutations in the CK2 phosphorylation sites might confer altered protein stability, regardless of whether ZmOST1 was expressed alone or co-expressed with the two CK2 subunits. Furthermore, continuous microscopic observation of ZmOST1-GFP co-expressed with CK2β1-MYC revealed the disappearance of both the diffuse and speckled fluorescence signal in less than 10 min suggesting protein degradation (Figure 3A, upper left, and Supplemental Video 1). In contrast, ZmOST1[*ck2A*]-GFP fluorescence remained constant during the same time span (Figure 3A, lower left).

This led us to consider that ZmOST1 might be tagged for degradation by CK2 phosphorylation at the ABA box. In fact, OST1 was recovered as an ubiquitylation target in an *Arabidopsis* large-scale screening (Kim et al., 2013). These authors also reported that treating plants with the proteasome inhibitor MG132

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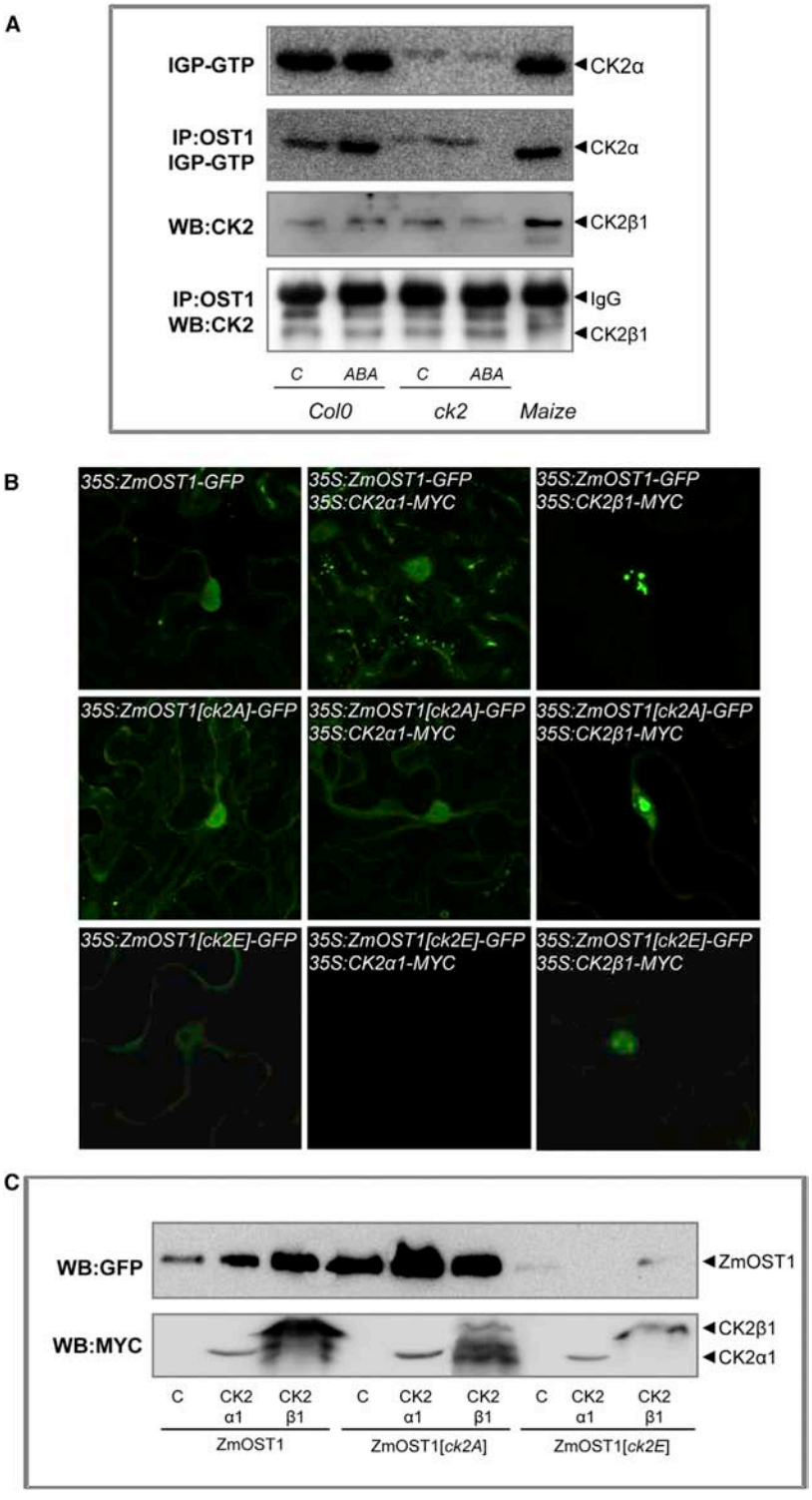

Figure 2. ZmOST1 Interacts with ZmCK2 *In Planta* with Effects on Protein Localization.

(A) Protein extracts from 7-day-old maize and *Arabidopsis* seedlings (*Col-0*; *ck2* $\alpha 1\alpha 2\alpha 3$) treated or not with 100 μM ABA were used for in-gel kinase assays (IGP) using casein as the substrate to detect CK2α activity and Western blot (WB) to detect CK2β protein after co-immunoprecipitation with OST1 (IP:OST1). Both a catalytic CK2α activity and CK2β1 protein can be recovered in OST1 protein complexes, indicating a direct relationship between proteins.

(B) ZmOST1-GFP, ZmOST1[*ck2A*]-GFP, and ZmOST1[*ck2E*]-GFP are accumulated in the nucleus and cytosol of tobacco cells, although at different levels (left). When co-expressed with ZmCK2α1 (center), ZmOST1-GFP localization remains unchanged but the appearance of cytosolic aggregates is clear. This accumulation is not detectable in the ZmOST1[*ck2A*]-GFP while ZmOST1[*ck2E*]-GFP fluorescence disappears completely. When co-expressed with ZmCK2β1 (right), ZmOST1-GFP localization is exclusively nuclear, accumulating in nuclear speckles. ZmOST1[*ck2A*]-GFP maintains its cytosolic and nuclear localization, but in this last organelle large patches of protein accumulation are observable. ZmOST1[*ck2E*]-GFP has a cytosolic and nuclear speckled localization when overexpressed with ZmCK2β1.

(C) Western blot (WB) of proteins extracted from the same plants as for the microscopic observations confirms the presence of ZmOST1 variants at different concentrations, and CK2 α1 and β1 subunits.

resulted in increased OST1 levels, indicating that OST1 could be targeted for proteasome degradation. We repeated the observation of our constructs co-infiltrated with CK2β1-MYC using the proteasome inhibitor MG132 to better assess ZmOST1 degradation. As seen in Figure 3 (upper right), the decrease of ZmOST1-GFP fluorescence stopped while there was a concomitant increase in the amount and intensity of the nuclear speckles. No visible change was detected for ZmOST1[*ck2A*]-GFP apart from a slight increase in fluorescence (Figure 3A, lower right). These results suggest that ZmOST1[*ck2A*] is significantly less susceptible to the degradation by the proteasome than the wild-type ZmOST1, a process that could be regulated by CK2.

To further investigate the stability and the resilience to degradation of these different versions of ZmOST1, we overexpressed ZmOST1-HA, ZmOST1[*ck2A*]-HA, or ZmOST1[*ck2E*]-HA constructs in *Arabidopsis* and performed a cell-free degradation assay. Total protein extracts were maintained at room temperature for the indicated times with or without MG132. As shown in Figure 3B, degradation of ZmOST1-HA began to be noticeable at 1 h after protein extraction while the ZmOST1[*ck2A*]-HA protein was still present after 7 h. ZmOST1[*ck2E*], apart from being expressed at

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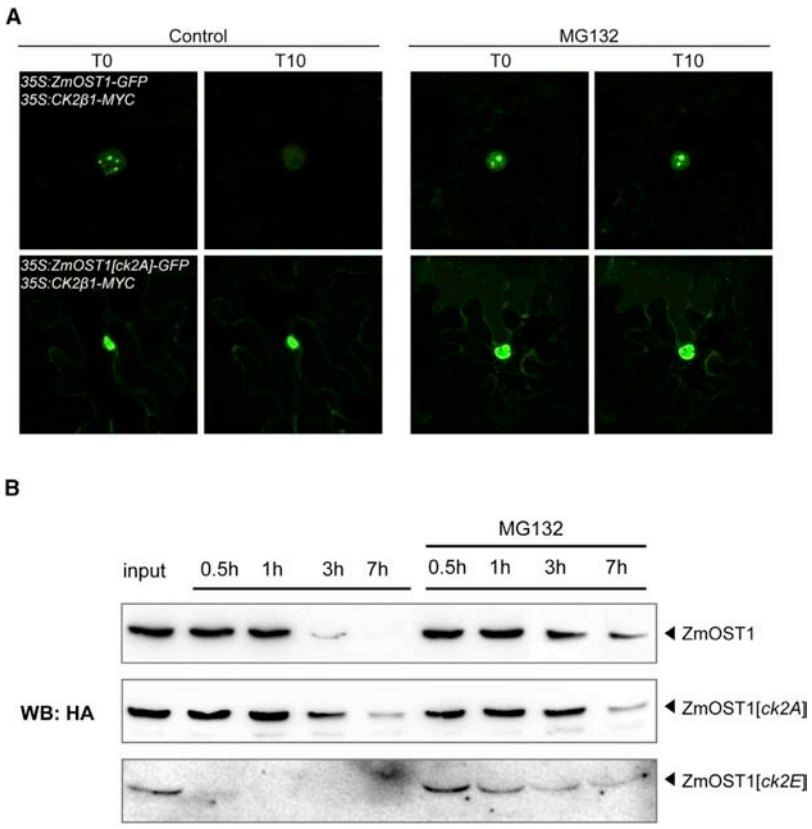

Figure 3. ZmOST1 Protein Stability Is Affected by CK2.

(A) Time course of ZmOST1-GFP and ZmOST1 [*ck2A*]-GFP co-expressed with ZmCK2β1 by confocal microscope imaging (t = 10 min). During the observation period, the speckling in the nucleus of ZmOST1-GFP disappears and fluorescence levels rapidly decrease. No change is observable in ZmOST1[*ck2A*]-GFP localization or stability (left). Adding MG132 to the samples during the confocal imaging maintained the speckled nuclear localization of ZmOST1-GFP while not affecting the ZmOST1[*ck2A*]-GFP protein. Both proteins seem to have a stronger signal at the end of the 10 min period (right).

(B) Cell-free degradation of transgenic *Arabidopsis* plants overexpressing ZmOST1-HA, ZmOST1 [*ck2A*]-HA, or ZmOST1[*ck2E*]-HA, and analysis by Western blot (WB). ZmOST1[*ck2E*]-HA is degraded after 0.5 h of incubation and ZmOST1-HA after 1 h, while ZmOST1[*ck2A*]-HA is still present at the end of the experiment. Adding the proteasome inhibitor MG132, all proteins display the same time course of degradation, indicating that ZmOST1 degradation is dependent on the mutagenized ABA box residues.

low levels, was almost completely degraded after 30 min. However, when MG132 was included, the time course of degradation was the same for all proteins, further establishing that ZmOST1 may be targeted for proteasome degradation by the CK2-mediated phosporylation at the ABA box.

ZmOST1 ABA Box Mutants Have Altered Interaction with Clade A PP2C and Kinase Activity

ZmOST1, and its *Arabidopsis* ortholog, have been shown to directly bind *clade* A PP2C phosphatases (Belin et al., 2006; Yoshida et al., 2006; Vlad et al., 2009; Soon et al., 2012). This interaction is primarily through the ABA box, approximately at the same position of the CK2 phosphorylation sites (Yoshida et al., 2006; Vlad et al., 2009; Vilela et al., 2012). We tested whether the ZmOST1 ABA box mutants would alter their interaction with PP2C phosphatases by bimolecular fluorescent complementation (BiFC) (Weinthal and Tzfira, 2009). We transiently co-expressed, in *Nicotiana benthamiana* epidermal cells, the ZmOST1, ZmOST1[*ck2A*], ZmOST1[*ck2E*], and ZmOST1[1–325] (a version of the kinase lacking the ABA domain) fused to the C-terminal half of YFP, separately, with ZmABI1 fused to the N-terminal half of YFP. As expected, interaction between ZmABI1 and all ZmOST1 variants, apart from the negative control ZmOST1[1–325], was detected in the nucleus and cytosol (Figure 4A). However, the YFP signal was significantly stronger (35%) for the version of the kinase that mimics CK2-mediated phosphorylation (ZmOST1[*ck2E*]) than for the wild-type kinase (Figure 4B). ZmOST1[*ck2A*] interaction with ZmABI1, in contrast, was reduced by 40%, especially in the nucleus. These results indicate that the capacity of ZmOST1 to interact with ZmABI1 is affected by the substitutions at the CK2 phosphorylation loci in the ABA box, and supports that CK2 may phosphorylate OST1 as a priming step to enhance its physical interaction with the PP2Cs. Western blots identifying YFC- and YFN-conjugated proteins reveal that all constructs were transiently overexpressed at similar levels (Figure 4C).

Since the clade A PP2Cs are well-recognized negative regulators of SnRK2 kinases, we were interested in assessing the biochemical relevance of the altered interaction we described. In-gel kinase assays, with maltose binding protein (MBP) as the substrate, were used to compare the relative activity of ZmOST1-HA, ZmOST1[*ck2A*]-HA, and ZmOST1[*ck2E*]-HA, immunoprecipitated from transgenic *Arabidopsis* lines. As shown in Figure 4D, the intensity of the kinase activity from the ABA-activated ZmOST1[*ck2A*] and ZmOST1[*ck2E*] was higher than that from the wild-type kinase. The ZmOST1[*ck2A*] kinase activity was also detectable in the control immunoprecipitation, indicating that this mutant version of the kinase escaped the negative regulation, thereby acquiring constitutive activity or perhaps extreme hypersensitivity to the background ABA.

We tried to verify these results in the *Arabidopsis* ortholog system using the CK2 specific inhibitor 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBB) (Pagano et al., 2008) to mimic ZmOST1[*ck2A*] response. AtOST1 immunoprecipitated from the *Arabidopsis* *Col-0*, *ck2* $\alpha 1\alpha 2\alpha 3$, *Ler*, and *ost1-2* lines treated with or without ABA (to activate SnRK2 activity) and TBB (to inhibit CK2 activity) were subjected to in-gel kinase assays with MBP as the substrate (Figure 4E). Inhibiting CK2 activity with TBB

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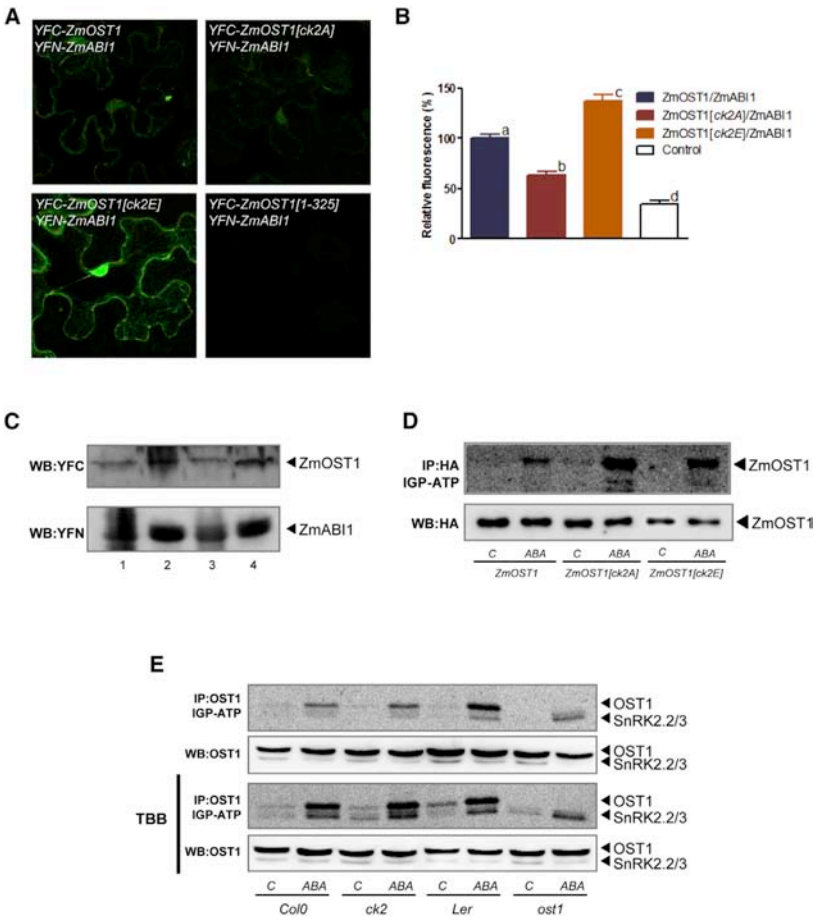

Figure 4. ZmOST1 Interaction with ZmABI1 Is Affected by Substitutions at CK2 Phosphorylation Consensus Serines, with Effects on Protein Activity.

(A) BiFC fluorescence images, analyzed by confocal microscopy, of the interaction between YFC-ZmOST1/YFC-ZmOST1[*ck2A*]/YFC-ZmOST1[*ck2E*] and YFN-ZmABI1 in agro-infiltrated tobacco leaves. YFC-ZmOST1[1–325] was used as a negative control for the interaction.

(B) Corresponding relative fluorescence quantification performed using ImageJ software on 30 individual microscopic fields (n = 30). ZmOST1 interaction with ZmABI1 is reduced by 40% for ZmOST1[*ck2A*] and is increased by 35% for ZmOST1[*ck2E*]. Different letters are used when values differ significantly ($P < 0.05$).

(C) Western blot (WB) indicating the relative quantities of the ZmOST1 variants fused to YFC and ZmABI1 fused to YFN: 1, YFC-ZmOST1/YFN-ZmAbi1; 2, YFC-ZmOST1[*ck2A*]/YFN-ZmAbi1; 3, YFC-ZmOST1[*ck2E*]/YFN-ZmAbi1; 4, YFC-ZmOST1[1–325]/YFN-ZmAbi1.

(D) Immunoprecipitation (IP) of ZmOST1-HA, ZmOST1[*ck2A*]-HA, and ZmOST1[*ck2E*]-HA from transgenic *Arabidopsis* overexpressing lines, followed by in-gel phosphorylation assay (IGP) using MBP as the substrate. ZmOST1[*ck2A*]-HA and ZmOST1[*ck2E*] have increased activity under 100 μM ABA compared with ZmOST1. In control situations, ZmOST1[*ck2A*] activity is initiated without or at low levels of ABA. Western blot (WB) of the same samples was used as a load control.

(E) IGP of immunoprecipitated OST1, and corresponding WB, from different *Arabidopsis* lines (*Col-0*, *ck2* $\alpha 1\alpha 2\alpha 3$, *Ler*, *ost1-2*) with or without 100 μM ABA and 25 μM TBB (CK2 inhibitor). When CK2 activity is inhibited (lower panels), OST1 activity is enhanced, mimicking ZmOST1[*ck2A*] activity, even in the absence of exogenous ABA application.

resulted in an enhanced activity of OST1 in addition to the other ABA-responsive SnRK2s that are immunoprecipitated with the antibody used (Vilela et al., 2013). We also detected that treating *Arabidopsis* with TBB activates these group III SnRK2s in control situations, where only physiological ABA is present, in a manner similar to that of ZmOST1[*ck2A*]. Taken together, these results indicate that CK2 may have an important function in the regulation of group III, ABA-responsive SnRK2s by affecting their interaction with *clade* A PP2C phosphatases and their catalytic activity.

ZmOST1 ABA Box Mutants Have Altered Stomatal Response to ABA

Since OST1 is required for stomatal closure under drought conditions (Mustilli et al., 2002), we decided to compare the water loss kinetics and the stomatal aperture of transgenic *Arabidopsis* lines overexpressing ZmOST1, ZmOST1[*ck2A*], or ZmOST1[*ck2E*] (Figure 5A). The experiments were performed at midday, when stomata are apparently the most responsive to ABA (Robertson et al., 2009). Plants overexpressing ZmOST1 [*ck2A*] are significantly less susceptible to water loss, measured by percentage of fresh weight of detached rosettes, than plants overexpressing ZmOST1 (Figure 5B). In contrast, plants overexpressing ZmOST1[*ck2E*] lose significantly more water over the same period.

By direct measurement of the stomatal closure of detached leaves from these transgenic plants (Figure 5C, D, and E), we detected a hypersensitivity to ABA for ZmOST1[*ck2A*] plants when compared with the wild-type ZmOST1 kinase. Even when ABA was applied at levels as low as 0.5 μM, a concentration that caused little visible stomatal response in the transgenic *Arabidopsis* lines overexpressing ZmOST1, a 25% reduction of the stomatal aperture was clearly detected in the transgenic plants expressing ZmOST1[*ck2A*] (Figure 5C). Plants overexpressing ZmOST1[*ck2E*] had a phenotype of stomata closure similar to wild-type, with no enhanced response to ABA. These results are consistent with the increased accumulation and activity of ZmOST1[*ck2A*] compared with ZmOST1 reported above, resulting in significantly enhanced stomatal response to ABA and resilience to water loss. Furthermore, treating ZmOST1 transgenic *Arabidopsis* plants with TBB, the CK2 inhibitor, enhanced stomatal closure to the

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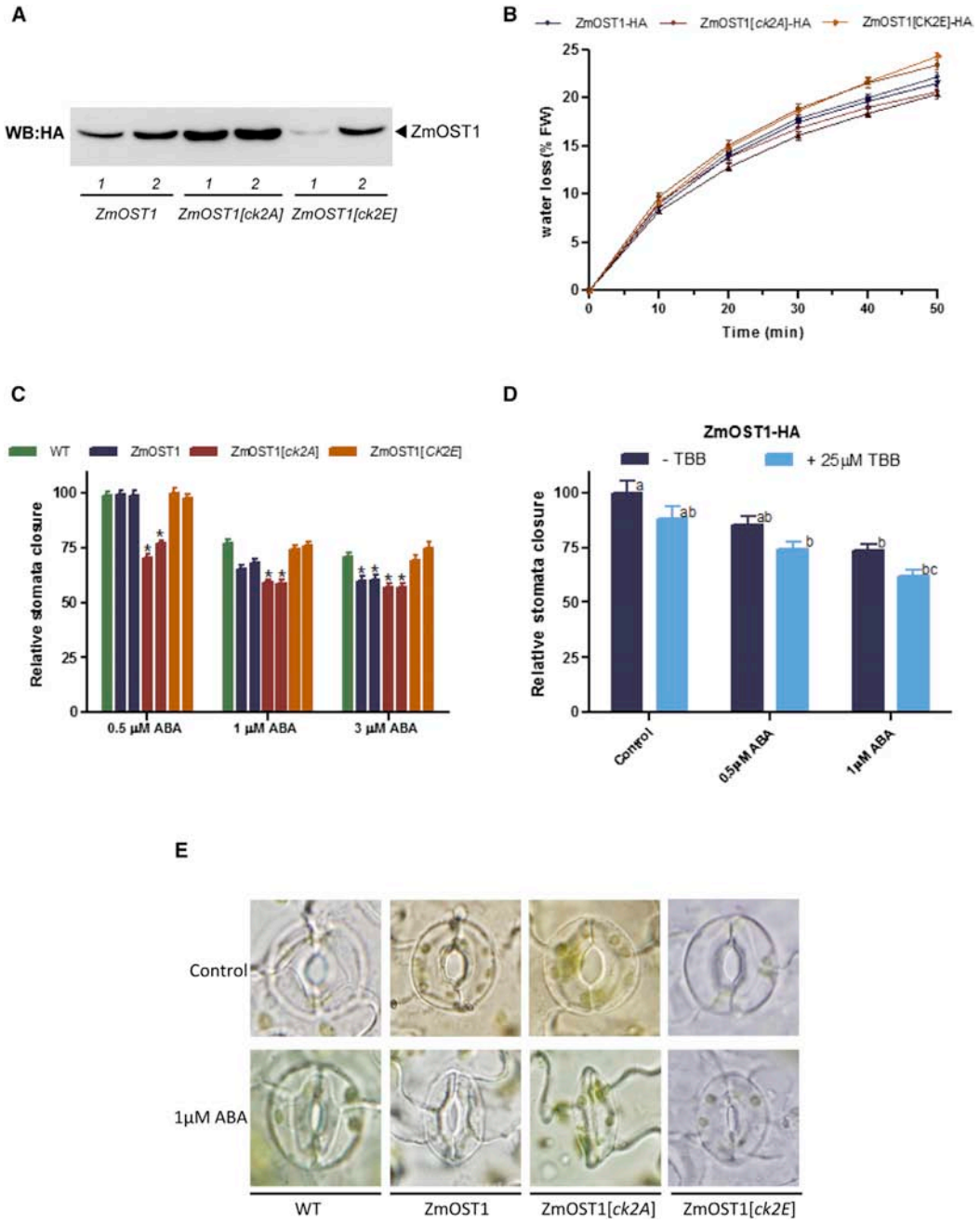

Figure 5. ZmOST1[*ck2A*] Confers Enhanced Drought Resistance and Stomatal Response to ABA.

(A) Western blot (WB) of transgenic *Arabidopsis* lines overexpressing ZmOST1-HA, *ZmOST1[ck2A]-HA*, and *ZmOST1[ck2E]-HA*.

(B) Water loss kinetics using detached leaves of two independent *ZmOST1-HA* wild-type lines (blue), two independent *ZmOST1[ck2A]-HA* lines (red), and two independent *ZmOST1[ck2]-HA* transgenic lines (orange). Water loss is expressed as the percentage of initial fresh weight. Values are means ± SE of three independent experiments (n = 15). The ZmOST1[*ck2A*] transgenic lines lose significantly less water than the lines overexpressing the wild-type kinase, while the lines overexpressing ZmOST1[*ck2E*] lose more water.

(C) Stomata closure measurements of detached leaves from the same transgenic lines under different ABA treatments (n = 200). Mutating ZmOST1 at CK2 phosphorylation sites confers altered sensitivity to ABA at the level of stomata in transgenic *Arabidopsis* lines. The ZmOST1[*ck2A*] mutation confers hypersensitivity to ABA while the ZmOST1[*ck2E*] mutation does not improve the wild-type phenotype. Values are expressed as mean ± SE. Asterisks represent significantly low values of relative stomata closure for each treatment ($P < 0.05$).

(D) Treating ZmOST1 overexpressing plants with the CK2 inhibitor TBB confers the same level of hypersensitivity as in transgenic lines overexpressing the wild-type protein. Different letters are used when averages vary significantly ($P < 0.05$, n = 50).

(E) Representative images by light microscopy of stomata response to 1 µM ABA.

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levels similar to ZmOST1[*ck2A*] at all the ABA concentrations tested (Figure 5D). Treating ZmOST1[*ck2A*] transgenic lines with TBB further enhanced stomatal responsiveness to ABA, an effect likely due to the regulation by the other group III SnRK2s (Supplemental Figure 2).

DISCUSSION

In both animals and plants, CK2 is known to phosphorylate a large number of proteins with a wide spectrum of functions. Despite the broad physiological contexts in which CK2 seems to be required, the *Arabidopsis* mutant lacking all three genes encoding the cytosolic catalytic subunits ($\alpha 1\alpha 2\alpha 3$) is still viable; although morphologically compared with the wild-type, the mutant has smaller cotyledons, shorter roots and hypocotyls (Lu et al., 2011; Mulekar et al., 2012).

There had been preliminary data indicating that CK2 is linked, either directly or indirectly, to ABA signaling. The maize CK2 can phosphorylate RAB17, whose corresponding transcript is up-regulated by ABA (Riera et al., 2004). The phosphodeficient mutant form of RAB17, when overexpressed in transgenic *Arabidopsis*, led to a failure of seed germination arrest in high salt. *Arabidopsis* mutated for either all three CK2 α subunits (as mentioned above) or doubly mutated in all possible combinations ($\alpha 1\alpha 2$, $\alpha 1\alpha 3$, $\alpha 2\alpha 3$) showed hyposensitivity to ABA and high salt when tested by the criteria of seed germination and cotyledon greening (Mulekar et al., 2012).

Here, we have identified OST1 as a direct target of CK2. Moreover, as the kinase activities of SnRK2.2 and SnRK2.3 were actually higher in the plant extracts, in which CK2 had been inhibited, all three ABA-activated SnRK2s are likely *in planta* targets of CK2-mediated phosphorylation. Our results thus indicate a direct link between CK2 and the core ABA signaling complex (Cutler et al., 2010; Nishimura et al., 2010), in which coordinated interactions among OST1 (probably also SnRK2.2 and SnRK2.3), specific PP2Cs (e.g. ABI1, ABI2, HAB1), and the ABA receptors (e.g. PYR, PYL) initiate most of the downstream ABA-dependent responses that include *trans*-membrane ion transport and stress-related gene expression (Cutler et al., 2010; Hubbard et al., 2010). OST1 is activated by the phosphorylation at S175 (Vlad et al., 2010). However, because the OST1 produced in *E. coli* is autophosphorylated and catalytically active, it has been generally assumed that the trio PYR-PP2C-OST1 is necessary and sufficient to coordinate the activity of this complex, depending only on the presence or absence of ABA as the stimulus. Indeed, a functional ABA signaling complex comprising the above three proteins or their homologs has been successfully reconstituted *in vitro* and in *Xenopus* oocytes (Fujii et al., 2009; Geiger et al., 2009; Brandt et al., 2012).

CK2 phosphorylates four specific serines in the ABA box within a negatively charged region of ZmOST1. This region in ZmOST1 has homology to the PEST sequences, whereby it can regulate the intrinsic stability of the protein by its phosphorylation status (Kato et al., 2003). In our case, CK2 phosphorylates ZmOST1 to promote its degradation likely through the ubiquitin/*26S* proteasome pathway, as deduced from the cell-free assays in which the wild-type ZmOST1 was stabilized by the proteasome inhibitor MG132. This mechanism is reminiscent of the post-translational regulation of the plant PIF1 by CK2-mediated phosphorylation promoted by light (Bu et al., 2011). In animals , CK2-mediated phosphorylation at the PEST motifs of IP6K2 and IkBα enhances the ubiquitylation of these targets and subsequent degradation (McElhinny et al., 1996; Chakraborty et al., 2011).

Although the CK2 holoenzyme is a tetramer composed of two α and two β subunits, it has been proposed that each of the two types of subunits could also have independent functions (Filhol et al., 2004). Individual CK2α and CK2β are nuclear localized proteins (with the exception of a chloroplastic CK2α4) (Salinas et al., 2006; Riera et al., 2013). The heterotetrameric holoenzyme forms aggregates in both the nucleus (nuclear speckles) and cytoplasm (Riera et al., 2011). Co-transformation of either CK2α or CK2β with ZmOST1 changed the uniform subcellular localization of ZmOST1 to cytosol aggregates and nuclear speckles, respectively. In animals, CK2 is known to participate in the formation and clearance of aggresomes in response to misfolded protein stress (Watabe and Nakaki, 2011), a process that could also occur in plants. This re-localization of ZmOST1 when co-expressed with CK2 subunits, together with the fact that we could recover CK2 protein and catalytic activity in OST1 protein complexes, strongly suggests that these proteins can form complexes *in vivo*.

The ABA box containing a cluster of CK2-phosphorylatable serines coincides with the docking site for the group A PP2C phosphatases (Yoshida et al., 2006; Xie et al., 2012). Apparently only one-third (aa 320–331) of the ABA box in OST1 was needed for HAB1 occupancy *in vivo* (Vlad et al., 2009). However, increasing evidence obtained *in vitro* and in *Xenopus* oocytes (Geiger et al., 2009; Lee et al., 2009; Soon et al., 2012) support that group III SnRK2 and PP2C can form direct complexes, as a mechanism of negative regulation through the mutual packing of their catalytic sites. Here, we report that altering the phosphorylation state of CK2 consensus serines present in the ZmOST1 ABA box has important consequences for PP2C binding *in vivo*. In fact, a 40% reduction in interaction was observed between the mutated ZmOST1[*ck2A*] and ZmABI1. In contrast, the kinase version that mimics the CK2-mediated phosphoylation increased its binding to a PP2C phosphatase by 35%. Thus, CK2 phosphorylation at the ABA box of SnRK2 contributes to the down-regulation of both the ABA-stimulated and constitutive signaling output of SnRK2, through disrupting SnRK2–PP2C interaction and controlling protein turnover. Presumably these multiple modes of regulation (Figure 6) serve to fine-tune adaptive responses in accordance with the strength and duration of the stress to which the plant is exposed.

ZmOST1 plant homologs have been shown to be important for stress tolerance acquisition in various crop plants (Zhang et al., 2010; Ying et al., 2011; Zhang et al., 2011). ZmOST1[*ck2A*], mutated in the ABA box, results in enhanced ABA-induced stomatal closure, which is essential for plant adaptation to low soil and atmospheric humidity. This PEST-like motif, with the four characteristic serine residues, is also conserved in SnRK2.2 and SnRK2.3 in *Arabidopsis*, suggesting that all members of group III SnRK2 kinases are under control by CK2 phosphorylation. This hypothesis is further supported by our experiments

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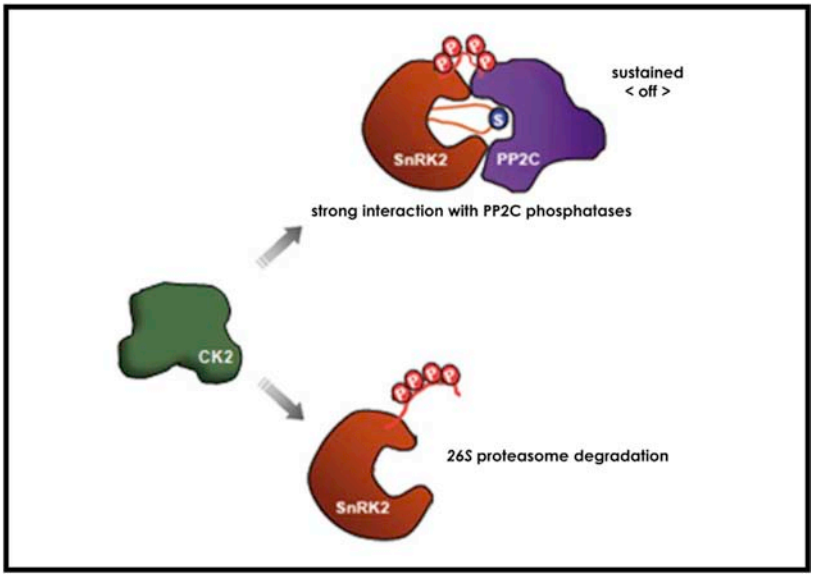

Figure 6. CK2 Negatively Regulates ABA-Activated SnRK2s in the Core ABA Signaling Module.

CK2 regulates SnRK2 kinases through the phosphorylation of several conserved serines present in their ABA box. This negative regulation is twofold: enhancing the interaction with the negative regulator of the core ABA signaling module (PP2C phosphatases), probably causing a sustained off state of kinase activity; and priming SnRK2 protein for controlled degradation through the *26S* proteasome pathway. Consequently, CK2 action has implications on SnRK2 protein levels and on kinase activity and response to abiotic stimuli.

with plants treated with the CK2 inhibitor TBB, in which we detected not only an increase in OST1 activity but also that of the other ABA-responsive SnRK2s recovered by our antibody. The triple mutant, defective in SnRK2.2, SnRK2.3, and OST1, displays severe phenotypes in all elementary ABA signaling pathways, indicating that the three kinases have important contributions to the global regulation of stomatal reactivity to stress, seed germination, root growth, and the ABA transcriptome (Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009). OST1 has other roles outside of ABA signaling, notably carbohydrate homeostasis, with consequences on energy supply by affecting sugar pathways and increasing seed oil metabolism (Zheng et al., 2010). The results here reinforce that the protein stability of OST1, its interaction with PP2Cs and/or catalytic activity will likely be influenced by the broad physiological contexts in which CK2 functions. Thus, the output signals of ABA core complex would also be dependent on the diverse developmental cues linked to CK2. All of these are important considerations for agronomic ameliorations. The controlled expression of ZmOST1[*ck2A*] and other similarly modified SnRK2 homologs may therefore help heighten drought-protection and improve carbohydrate metabolism in crops.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Treatments

Maize (*Zea mays*) of the pure inbred line W64A was germinated in liquid 0.5× Murashige and Skoog (MS) medium and grown for 1 week at 26°C with a 16 h/8 h light/dark photoperiod. *Arabidopsis* plants were maintained at 24°C, with a 16 h/8 h light/dark photoperiod on MS plates. Treatments with 100 μM ABA (Sigma, St. Louis, MO) and 25 μM TBB (Sigma) were carried out on 1-week-old plants after transfer to liquid MS medium. *N. benthamiana* plants were grown at 28°C under a long-day photoperiod (16 h/8 h light/dark).

Sequence Alignment

Group III SnRK2 from different plant species were collected from public databases by protein blast of ZmOST1 sequence. The following sequences were selected to be included in a protein alignment: ZmOST1 (NP_001149657.1), AtOST1 (NP_567945.1), AtSnRK2.2 (NP_190619.1), AtSnRK2.3 (NP_201489.1), ZmSnRK2.9 (ACG32779.1), ZmSnRK2.10 (NP_001130868.1), OsSAPK8 (NP_001051371.1), OsSAPK9 (NP_001067155.1), OsSAPK10 (NP_001050653.1), VfAAPK (AAF27340.1), and StSAPK8 (ABA40436.1).

The regulatory domain of these group III SnRK2s was aligned by ClustalW multiple alignment.

Recombinant Protein Purification and *In Vitro* Phosphorylation

Recombinant ZmOST1 full sequence (EU676040) and deletion forms (for primers see Supplemental Table 1) were cloned in the pET28a expression vector (Promega, Madison, WI), expressed in *E. coli* BL21 cells, and purified as His-tag fusion proteins according to the manufacturer's instructions.

In vitro phosphorylation of *E. coli*-expressed ZmOST1 and deletion forms fused to a HIS tag (500 ng) was done as previously described (Vilela et al., 2013). The different ZmOST1 proteins were incubated with *Z. mays* CK2 α (KinaseDetect, Arslev, Denmark) for 45 min at 30°C with [γ-^{33}P]GTP (PerkinElmer, Waltham, MA). Proteins were separated by SDS–PAGE on 12.5% acrylamide gels. Radioactivity on dried gels was detected using a Storm 820 imager (GMI, Ramsey, MN).

In vitro phosphorylation was performed twice.

Protein Extraction and Western Blot

Proteins were extracted in 50 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 20% glycerol, 1 mM phenylmethanesulfonylfluoride (PMSF), 10 μM leupeptin, 2 μg/ml aprotinin, and 10 μg/ml pepstatin, and cleared twice by centrifugation at 13 000 rpm at 4°C for 15 min.

For Western blot analysis, 40 μg of total protein were loaded per lane. Anti-HA high-affinity antibody (Roche, Mannheim, Germany) was used to detect ZmOST1-HA protein variants; Living Colors JL-8 Monoclonal antibody (Clontech, Mountain View, CA) was used to detect GFP-conjugated proteins; Anti-c-MYC antibody (Santa Cruz Biotechnology, Dallas, TX) was used to detect MYC-conjugated proteins; Anti-GFP N-terminal antibody (Sigma) was used to detect YFN-ZmABI1; anti-GFP ab290 antibody (Abcam, Cambridge, MA) was used to detect YFC-conjugated proteins; anti-ABA box antibody (Vilela et al., 2013) was used to detect SnRK2s; and anti-CK2 antibody was used to detect CK2β1. Both the anti-ABA box and the anti-CK2 antibody were produced as an external service

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(CID-CSIC, Barcelona, Spain) after recombinant expression in *E. coli* and purification of the ZmOST1[325–366] peptide or the full CK2β1 protein fused to a 6× histidine tag in their amino terminal region.

Every Western blot was performed at least twice.

Immunoprecipitation and Co-Immunoprecipitation

Maize pure inbred line W64A and *Arabidopsis Col-0*, *CK2 α1α2α3* triple mutant (Mulekar et al., 2012), *ost1-2* mutant (Mustilli et al., 2002), and the lines overexpressing *ZmOST1-HA*, *ZmOST1[ck2A]-HA*, and *ZmOST1[ck2E]-HA* were used for immunoprecipitation and/or co-immunoprecipitation experiments.

For immunoprecipitation, plant extracts (500 μg) were incubated either with a 1:300 dilution of the HA or a 1:150 dilution of the ABA box antisera in 300 μl of IP buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 2 mM Na_3VO_4, 2 mM NaF, 10 mM β-glycerophosphate, 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, Piscataway, NJ) was added and incubated for another hour. The slurry was washed 3 × 15 min with IP buffer and the supernatant was removed prior to the in-gel kinase assay. Proteins were recovered from the beads by adding 20 μl loading buffer and heating at 95°C for 5 min.

For co-immunoprecipitation, 50 μl of Dynabead Protein A (Life Technologies, Waltham, MA) were equilibrated according to the manufacturer's instructions and incubated overnight with 2 μl of anti-ABA box antibody (Vilela et al., 2013) at 4°C. Proteins extracts, supplemented with 1 mM PMSF, 10 μM leupeptin, 2 μg/ml aprotinin, 10 μg/ml pepstatin, 50 μM MG132, and 2 mM DTT were incubated for 2 h in a rotary shaker. The slurry was washed three times with extraction buffer and protein immunocomplexes were recovered with loading buffer at 95°C for 5 min, prior to Western blot or in-gel kinase assays.

In-Gel Phosphorylation Assay

Protein samples (40 μg) were separated on 10% SDS–PAGE gels embedded with 0.5 mg/ml recombinant HIS-tagged ZmOST1 protein, casein, or myelin basic protein (Sigma). 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. 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 4°C. 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 or GTP, and 100 μCi [γ-^{33}P] ATP or [γ-^{33}P]GTP (PerkinElmer) at room temperature for 1 h. Finally, gels were washed extensively with 5% (w/v) trichloroacetic acid and 1% sodium pyrophosphate solution, at least five times, and dried. Radioactivity was detected using a Storm 820 imager (GMI).

Every In-gel phosphorylation assay was performed at least twice.

Generation of Transgenic *Arabidopsis* Plants

To determine the effect of CK2 phosphorylation on ZmOST1, we performed multiple-site mutagenesis (for primers see Supplemental Table 1) with the changes S350A, S352A, S358A, and S359A to generate ZmOST1[*ck2A*], and S350E, S352E, S358E, and S359E to generate ZmOST1[*ck2E*]. Full-length *ZmOST1*, *ZmOST1[ck2A]*, and *ZmOST1[ck2E]* cDNAs were cloned in the pMENCHU vector to generate *2x35S::ZmOST1-HA*, *2x35S::ZmOST1[ck2A]-HA*, and *2x35S::ZmOST1[ck2E]-HA*, respectively, and subsequently cloned into the PC1300 (Clontech) vector for *Agrobacterium*-mediated transformation by floral dip of *Arabidopsis* (Clough and Bent, 1998). Two homozygous transgenic complementation lines per construct were selected for further analysis.

GFP and BiFC Imaging

Full-length *ZmOST1*, *ZmOST1[ck2A]*, and *ZmOST1[ck2E]* cDNAs were cloned in the PC1302 vector (Clontech). The *CK2α1* (GRMZM2G143602) and *CK2β1* (GRMZM5G857992) cDNAs were cloned in the pLOLA vector to produce *2x35S::CK2α1-MYC* and *2x35S::CK2β1-MYC*. The pLOLA cassette was subsequently cloned in the PC1300 (Clontech) vector for plant transformation. For BiFC experiments, full-length *ZmOST1*, *ZmOST1[ck2A]*, *ZmOST1[ck2E]*, and *ZmABI1* (EU956794) cDNAs were cloned in the pYFN43 and pYFC43 BiFC GATEWAY-modified vectors (Lumbreras et al., 2010) to produce *35S::YFC-ZmOST1*, *35S::YFC-ZmOST1[ck2A]*, *35S::YFC-ZmOST1[ck2E]*, and *35S::YFN-ZmABI1*. *N. benthamiana* plants were transiently transfected with these constructs and *35S::YFC-ZmOST1[1–325]* as a negative control, and with *35S::HCPro* to inhibit protein silencing in tobacco. Confocal observations were performed 2 days after infiltration on an Olympus IX81 inverted microscope (Olympus, Center Valley, PA).

Quantification of fluorescence was performed by scoring the "mean gray value" using ImageJ software (Schneider et al., 2012) on 30 microscopic fields, over three independent experiments (n = 30). Analysis of variance (ANOVA) with Tukey's multiple comparison test was used to determine degrees of variation between samples.

Cell-Free Degradation Assays

Seven-day-old seedlings from the *Arabidopsis* lines overexpressing ZmOST1-HA, ZmOST1[*ck2A*]-HA, and ZmOST1[*ck2E*]-HA were ground in liquid nitrogen and 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). Extracts (40 μg) were incubated at room temperature with or without 40 μM MG132 (Sigma) for the indicated times. Reactions were stopped by adding protein gel-loading buffer. Protein levels over the time course were assessed by Western blot using anti-HA high-affinity antibody (Roche).

Cell-free degradation assays were performed three times.

Water Loss and Measurements of Stomatal Apertures

Arabidopsis plants were grown on soil for 3 weeks under short-day conditions (8 h/16 h light/dark), and water loss and stomata aperture measurements were performed at least three times.

Water loss was scored by weight loss over time of at least five fully expanded detached rosettes from transgenic lines overexpressing ZmOST1, ZmOST1[*ck2A*], and ZmOST1[*ck2E*].

For stomata aperture measurements, detached *Arabidopsis* leaves were incubated for 2 h in 50 mM KCl and 10 mM MES (pH 6.15) with cool white fluorescent light (50 μmol/m^2/s) to induce maximum stomata opening. ABA was added in the same buffer solution at concentrations of 0, 0.5, 1, and 3 μM, for 2 h. TBB treatment (25 μM) was also added at this step. Imprints of treated leaves were performed on Genie Light Body Standard Set dental resin (Sulthan Healthcare, York, PA), a cast of the leaf was obtained using nail polish, and bright-field microscopic photographs of the cast were taken with a 63× objective lens (Delgado et al., 2012). Stomatal aperture was scored as width/length pore ratio of at least 200 stomata (n = 200), using ImageJ software (Schneider et al., 2012). ANOVA with Tukey's multiple comparison test was used to determine degrees of variation between samples under the same treatment.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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