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**Rice SUMO protease *Overly Tolerant to Salt 1* targets the transcription factor, OsbZIP23 to promote drought tolerance in rice**

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**Key words:** SUMO protease, rice, abiotic stress, ABA signalling

## **SUMMARY**

Conjugation of SUMO (Small Ubiquitin-like Modifier) protein to cellular targets is emerging as a very influential protein modification system. Once covalently bound SUMO conjugation can change the stability or functionality of its cognate target proteins. SUMO protease can rapidly reverse SUMO conjugation making this modification system highly dynamic. A major factor in the variation of SUMO-target function is the balance between the conjugated/de-conjugated forms. The mechanistic role of these regulatory SUMO proteases in mediating stress responses has not been defined in any crops. In this study, we reveal the role of the SUMO protease, OsOTS1 in mediating tolerance to drought in rice. *OsOTS1* depleted transgenic plants accumulate more ABA and exhibit more productive agronomic traits during drought whilst *OsOTS1* overexpressing lines are drought sensitive but ABA insensitive. Drought and ABA treatment stimulates the degradation of OsOTS1 protein indicating that SUMO conjugation is an important response to drought stress in rice achieved through down-regulation of OTS1/2 activity. We reveal that OsOTS1 SUMO protease directly targets the ABA and drought responsive transcription factor OsbZIP23 for de-SUMOylation affecting its stability. *OsOTS*-RNAi lines show increased abundance of OsbZIP23 and increased drought responsive gene expression while *OsOTS1* overexpressing lines show reduced levels of OsbZIP23 leading to suppressed drought responsive gene expression. Our data reveals a mechanism where rice plants govern ABA dependant drought responsive gene expression by controlling the stability of OsbZIP23 by SUMO conjugation through manipulating specific SUMO protease levels.

## **INTRODUCTION**

Rice is the staple food source for majority of the world's population. Rice crops lose 75% of their yield potential due to environmental stresses (Araus *et al.*, 2002). Drought can be accompanied by salinity therefore yield losses to salt stress are also increasing globally (Boyer, 1982).

Plant molecular signalling networks that are important in responding to drought stress overlap considerably with those for other abiotic stresses, such as high salinity stress (Barnabas *et al.*, 2008, Ahuja *et al.*, 2010). This indicates that development of drought and salt tolerant crop cultivars is possible and essential for adapting agriculture to climate change. Data from crop models indicate that yield and quality is critically dependent upon the complex perception and signalling mechanisms that generate an integrated reaction to environmental stress (Zhu, 2002, Vinocur and Altman, 2005). Therefore, identifying molecular mechanisms that perform as 'Master' co-ordinators and influence multiple stresses will be vital for increasing crop productivity. Crop improvement programmes that target these 'Master' co-ordinators will have the greatest potential to increase yield under stress.

Post-translational modifications (PTMs) are central actors of responses to stress by either stimulating (positive factors) or disabling (negative factors) stress perception mechanisms and downstream transcription factors that regulate the expression of thousands of genes. Protein phosphorylation and ubiquitination are the most established PTMs involved stress signalling. Many key transcriptional regulators including DREB2, ICE1 (controlling cold, heat, salt and drought stress) and ABI5 (regulator of stress hormone ABA) have been shown to undergo such PTMs in order to be effective in promoting plant stress adaptation (Miura *et al.*, 2005, Agarwal *et al.*, 2006, Dong *et al.*, 2006).

SUMO conjugation (SUMOylation) to target proteins may act as a rapid response mechanism to manipulate substrate behaviour during stress and is beginning to emerge as a critical post-translational apparatus in plants (Gill, 2004, Downes and Vierstra, 2005, Hay, 2005, Vierstra, 2012). As in ubiquitination, protein SUMOylation is determined by the action of three enzymes (E1, E2 and E3). The E1, SUMO-Activating Enzymes AtSAE1 and AtSAE2, operates as a heterodimer, to generate an ATP-dependent thiol-ester bond between SAE2 and SUMO. A transesterification process leads to the transfer of SUMO onto the E2 SUMO-Conjugating Enzyme, AtSCE1. AtSCE1 can generate a SUMO isopeptide bond to target proteins on its own or via the E3 SUMO ligases, HIGH PLOIDY2, AtHPY2 /AtMMS21, or SAP and MIZ1 AtSIZ1 (Kurepa *et al.*, 2003, Miura *et al.*, 2005, Miller *et al.*, 2013).

Abiotic and biotic stresses alter the dynamics of cellular SUMOylation and this can modify target protein stability or interactions thereby affecting protein functionality (Kerscher *et al.*, 2006). SUMO proteases can reverse the SUMOylation process by cleaving SUMO off target proteins, so called DeSUMOylation. This is in contrast to the process of SUMO maturation where in some cases the SUMO proteases cleave a short c-terminal peptide from pro-

SUMO to expose the terminal glycine residue for SUMO conjugation. (Jentsch and Pyrowolakis, 2000, Kerscher *et al.*, 2006, Capili and Lima, 2007). DeSUMOylating proteases cleave the isopeptide bond between the terminal Glycine of SUMO and the Lysine of the conjugated substrate, releasing free SUMO for further cycles of conjugation. These proteases then control the equilibrium in SUMO mediated signalling (Conti *et al.*, 2014). SUMO proteases remain largely understudied especially in crop plants.

Previously we revealed in rice the SUMO protease gene family and showed that *OsOTS1* has a key role in tolerance to high salinity (Srivastava *et al.*, 2016). Here, we report that drought stress and ABA stimulates the degradation of *OsOTS1* protein. *OsOTS1* depleted transgenic rice plants are drought tolerant and exhibit more productive agronomic traits whilst rice transgenics with enhanced levels of *OsOTS1* are drought sensitive. We identify the *OsbZIP23* as a direct SUMO target for *OsOTS1* de-SUMOylation and reveal a mechanism for regulating *OsbZIP23* stability via *OsOTS1* SUMO proteases activity. Our data indicates that manipulating *OsOTS1* levels in transgenic rice allows the modulation of *OsbZIP23* dependant gene expression to confer drought tolerance in rice.

## RESULTS

### ***Rice SUMO protease OTS1 is degraded in response to abiotic stresses***

Previously, we demonstrated an important role for OTS family of SUMO proteases in growth and development of rice seedlings in high salinity (Srivastava *et al.*, 2016). A key finding of this study was that salt stress treatment induced the degradation of *OTS1* protein. We wanted to ascertain if this was also the case during drought stress. Tolerance to desiccation is a key parameter for measuring drought resilience in crops (Ray *et al.*, 2007) therefore we subjected transgenic HA-tagged *OsOTS1* rice lines to desiccation treatment by growing these lines at elevated temperatures (28°C) (Figure 1A and B) . This treatment resulted in a near complete degradation of *OsOTS1* protein within 4 hours indicating that rice plants stimulate the degradation *OsOTS1* proteins as an early response to desiccation. Mannitol is frequently employed as chemical treatment to induce drought responses in plants as they mimic osmotic constraints experienced during periods of drought (Skirycz *et al.*, 2010, Claeys *et al.*, 2014). To expand the osmotic stress analysis, we treated the same transgenic lines with 300mM mannitol and observed a reduction of protein levels but not as severe as with desiccation treatment (Figure 1C).

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Abscisic acid (ABA) signalling plays major roles in the drought stress, and is well established as a major regulator of responses to drought in plants (Zhao *et al.*, 2013) and not surprisingly we observed near complete degradation of OsOTS1 SUMO protease after 4 hours of ABA (100 $\mu$ M) treatment (Figure 1D). Indeed, OTS1 protein levels begin to decrease after 1 hour of ABA treatment (Figure 1D). Our findings reveal a role for ABA in regulating cellular SUMOylation by stimulating the degradation of SUMO proteases.

### ***OsOTS1 negatively regulates ABA signalling***

The rapid degradation of OTS1 protein by ABA prompted us to ascertain the sensitivity of *OsOTS1* overexpression and *OsOTS*-RNAi rice transgenic lines to this phytohormone. ABA treatment of 10-day old transgenic seedlings severely inhibited growth and development of *OsOTS*-RNAi lines but not *OsOTS1-OX* compared to control vector only plants. In particular, shoot length of *OsOTS*-RNAi lines was significantly impeded (31.1%) compared to control, wildtype Nipponbare transformed with empty vector (Figure 2 A-D). This growth inhibition in *OsOTS1*-RNAi lines was also apparent in the roots where significant differences were also detected relative to the wild-type control. (Figure 2E). ABA promotes the induction and maintenance of seed dormancy (Finkelstein *et al.*, 2002). To assess the impact of *OsOTS1* SUMO protease of rice seed germination rates, germination kinetics was also measured in presence of ABA. Our data show that the germination rates of *OsOTS*-RNAi lines are significantly more inhibited compared to control and *OsOTS1-OX* seeds (Supplemental Figure 1). Our data demonstrates that *OSOTS1* is a novel negative regulator of ABA signalling pathways in rice.

### ***Manipulating OsOTS1 affects rice productivity during drought***

Our desiccation assays and ABA treatment indicates that OTS SUMO proteases are likely to have a role in drought responses in rice. Therefore, we subjected *OsOTS1-OX* and *OsOTS*-RNAi transgenic rice lines at flowering stage to drought stress by withdrawing water for 10 days. Compared with the empty vector controls, *OsOTS1* overexpressing lines indicated earlier and more severe wilting symptoms. However, the *OsOTS*-RNAi did not show such severe symptoms (Figure 3A). After re-watering, about 55% of the wild type plants recovered, whereas only 27% of the *OsOTS1-OX* plants recovered (Figure 3B). In contrast, *OsOTS*-RNAi plants were considerably more drought tolerant and 84% recovered after re-

watering. (Figure 3C). These results suggest that rice plants with reduced SUMO protease activity are more tolerant to drought stress.

It is known that plants often adapt to water deficit conditions by manipulating the properties of stomata on the epidermis (Galmes *et al.*, 2007, Xu and Zhou, 2008, Kollist *et al.*, 2014). *AtOTS1 (ulp1d)* mutants in *Arabidopsis* displayed increased stomatal aperture size but the rate of water loss was not significantly different from wildtype *Col-0* (Castro *et al.*, 2016). Since OTS SUMO proteases are highly conserved between rice and *Arabidopsis* (Srivastava *et al.* 2016) we did not focus on stomatal aperture. Therefore, to further explore the mechanism/s behind changes in response to drought in the transgenic lines we assayed stomatal densities from the leaves of the empty vector control, *OsOTS1-OX* and *OsOTS-RNAi* lines (Supplemental Figure 2). Surprisingly, despite obvious differences in drought tolerance, we did not detect any differences in stomatal density between any of the transgenic lines and the empty vector control. An additional screen was next developed using infrared thermal imaging to understand water loss from the different transgenic lines after drought stress as reflected in leaf temperature levels which correlates with the amount of transpired water via stomata (Merlot *et al.*, 2002). It was clear from the screen that *OsOTS-RNAi* lines were actively transpiring more post-drought and so had lower leaf temperatures than either the wild type or *OsOTS1-OX* lines (Supplemental Figure 3). To ascertain whether such responses were linked with water loss during drought we measured water loss as plants were being droughted and found that in tillering plants that *OsOTS1* overexpressing plants always lost more water relative to the wild type control and *OsOTS-RNAi* plants (Figure 3D).

Since drought during anthesis has a critical impact on yield in rice, we next measured the impact of manipulating *OsOTS1* levels on agronomic traits in rice. A number of different agronomic traits were observed at harvest of each line grown under well watered and in drought conditions. Results showed that in non-stressed conditions there was no significant difference in agronomic traits between the control plants, *OsOTS1-OX* and *OsOTS-RNAi* plants whereas rice plants grown under drought conditions showed significant difference in productivity. *OsOTS-RNAi* plants exhibited higher spikelet fertility (~23%) and higher 100-seed weight (~13%) when compared to control plants (Figure 4 and Supplemental Figure 4). These data demonstrate that *OsOTS-RNAi* lines are more tolerant to drought than the control and *OsOTS-OX* plants and OTS1 SUMO protease may be involved in drought protection.

### ***OsOTS1 physically interacts with OsbZIP23***

SUMO proteases reverse SUMOylation conjugation, so called deSUMOylation, leading to the removal of SUMO off its target. DeSUMOylating proteases such as OsOTS1 (Srivastava *et al.*, 2016) cleave the isopeptide bond between the terminal glycine of SUMO and the lysine of the conjugated substrate, releasing free SUMO from the target protein thereby changing the stability of the protein and or interfering with protein-protein interactions which lead to changes in protein functionality. To fully understand the role of OsOTS1 deSUMOylation in rice drought stress responses the identity of its target/s is of paramount importance. To identify a possible SUMOylated target for OsOTS1 we performed a Y2H screen with OsOTS1 as a bait and isolated OsbZIP23 as an interacting prey protein. Yeast strain AH109 co-transformed with OsOTS1 and OsbZIP23 along with the negative controls were grown on respective selective dropout media, 2D (-Leu and - Trp) and 3D (-Leu, -Trp and -His) in the presence of 3-AT. OsOTS1 and OsbZIP23 showed a strong interaction whereas the combinations of OsOTS1 or OsbZIP23 and empty vectors AD or BD did not show any interactions (Figure 5A). The direct interaction of OsbZIP23 with OsOTS1 was further confirmed by GST pulldown assays. The pulldown reactions were performed with recombinant rice proteins, GST tagged-OsOTS1 and Histidine tagged-OsbZIP23. As shown in Figure 5B, His-OsbZIP23 co-purified from the solution with GST-OsOTS1 but not by GST alone, suggesting a physical interaction between OsOTS1 and OsbZIP23.

To validate *in planta* the physical interaction between OsOTS1 and OsbZIP23 co-immunoprecipitation experiments were performed. The full length OsOTS1 was translationally fused with N terminal YFP and full length OsbZIP23 was fused at the N terminus with the HA epitope. Co-immunoprecipitation experiments using *Agrobacterium* mediated transient assays in *N. benthamiana* demonstrated that OsOTS1 interacts with and forms an immunocomplex with OsbZIP23 (Figure 6A). To further confirm *in vivo* physical interaction between OsOTS1 and OsbZIP23 we performed bimolecular fluorescence complementation (BiFC) assays. We detected fluorescence from fully formed YFP only when pYFN43-OsOTS1 and pYFC43-OsbZIP23 constructs were co-expressed but not with either constructs were expressed with the respective vector only constructs, indicating direct interaction between OsOTS1 and OsbZIP23 in plants (Figure 6B).



### ***OsZIP23 proteins are SUMOylated and stabilised in the OsOTS-RNAi lines***

OsZIP23, a bZIP transcription factor, is a major player from the bZIP family in rice for conferring ABA dependant drought tolerance (Xiang *et al.*, 2008). Previously, it was shown to interact with and is phosphorylated by SAPK2 (homologue of SnRK2 protein kinase). Phosphorylation of OsZIP23 promotes the transcriptional activation of its target genes (Zong *et al.*, 2016) indicating that post-translational modification could be a key regulator of this transcription factor. Interestingly we identified high confidence SUMO sites on OsZIP23 (Supplemental Figure 5). Using HA tagged OsSUMO1 constructs we demonstrated that OsZIP23 is SUMOylated by co-immunoprecipitation assays *in planta* in *N. benthamiana* transient assays (Figure 7A).

Previous studies reported that SUMO conjugation regulates target protein stability (Sadanandom *et al.*, 2015). To verify the regulation of OsZIP23 protein stability by SUMO conjugation, we tested the stability of OsZIP23 in *OsOTS1-OX* and *OsOTS-RNAi* transgenic lines through transient transformation in rice (Purkayastha *et al.*, 2010). We observed an enhanced accumulation of OsZIP23 protein in *OsOTS-RNAi* background lines compared to the control and *OsOTS1-OX* lines (Figure 7B). These results show that OsZIP23 SUMOylation is critical for its stability in *OsOTS-RNAi* lines. This enhanced stability of OsZIP23 helps to promote drought tolerance of *OsOTS-RNAi* lines.

### ***OsOTS1 regulates the expression of OsZIP regulated target genes during drought stress***

To gain a deeper understanding of OsOTS1 function through ABA and OsZIP23 activity in drought stress, we determined ABA levels in the *OsOTS-RNAi* and *OsOTS1-OX* lines. Compared to wildtype, *OsOTS-RNAi* lines had at least 3 times more ABA content indicating that depleting OsOTS1 promotes the accumulation of the phytohormone ABA (Figure 8A). Furthermore, we analysed the transcript levels of selected drought responsive genes which are known to be activated by ABA and by OsZIP23 by qRT-PCR in the empty vector control and transgenic lines (Figure 8B-E). The dehydrin Rab21 gene, which encodes a basic glycine-rich protein (Mundy and Chua, 1988) belongs to a group of genes whose expression is regulated by PEG and ABA and is a target gene controlled by OsZIP23 (Zhang *et al.*, 2014). Similarly, a group of hydrophilic proteins encoded by Late Embryogenesis Abundant (LEA) genes accumulate in plants during various stress conditions including drought and salinity suggesting a function in stress protection (Tunnacliffe and

Wise, 2007). Transcript levels are also enhanced by ABA treatment and abiotic stresses (Seo *et al.*, 2011). Interestingly the transcript levels of OsRAB1, OsLEA3/OsLEA5 and OsbHLH148 were all enhanced in *OsOTS*-RNAi lines compared to control and *OsOTS1-OX* lines further supporting our observation that SUMOylation of a key transcript factor, OsbZIP23 directly affects drought responsive transcription which leads to increased tolerance in rice.

## DISCUSSION

A combination of different biotic and abiotic stresses is faced by plants constantly due to their sessile nature and therefore they have evolved sensitive and sophisticated mechanisms that respond rapidly to adapt to a changing environment. Knowledge of these processes will aid in improving the yield potential of rice and other crops. Recently we showed that SUMOylation is a mechanism that functions as a major molecular pathway in governing *Arabidopsis* growth in high salinity by unravelling the function of the SUMO proteases *AtOTS1* and 2 (Conti *et al.*, 2008, Conti *et al.*, 2014). However, till now the role of SUMO in crops plants is largely unknown.

In order to understand the role of SUMO in crops response to stress we revealed the ULP class of SUMO proteases in rice and demonstrated that the SUMO protease activity for the paralogue *OsOTS1* from rice plays a major role in salt stress responses in rice (Srivastava *et al.*, 2016).

As in *Arabidopsis* in comparison to the SUMO E3 ligases the increase in gene numbers of the SUMO proteases in rice betrays the dependence of plants on de-conjugation as a specificity mechanism for regulating signalling pathways. Interestingly as a natural response to drought in rice is to induce the degradation of *OsOTS1*, in reality creating an *OTS1* depleted environment in the cell like in *OsOTS*-RNAi lines. This results in increased drought tolerance in adult *OsOTS*-RNAi rice plants whilst the *OsOTS1-ox* lines are more drought sensitive. This is in contrast to what we observed in rice seedlings during salt stress where *OsOTS1-OX* lines were more tolerant to high salinity. Drought stress at the reproductive stage leads to a significant reduction in rice yield. In *OsOTS1-OX* lines or RNAi lines, there was no difference in yield under well-watered conditions but surprisingly productivity was significantly reduced in drought stress in the *OsOTS1-OX* lines. Significant reduction in all the agronomic attributes contributed to the reduction of *OsOTS1-OX* plant productivity under drought stress. Conversely, in *OsOTS*-RNAi lines we found more panicles were produced and with greater fertility than either the *OsOTS1-OX* or the wild type under drought stress. In

rice, reduction in productivity is mainly due to reduced number of filled spikelets per panicle (Wei *et al.*, 2014) and this is what we observed in *OsOTS1-OX* lines but not in the *OsOTS-RNAi* lines. Indeed, this reduction in performance seems to be linked to increased water loss during drought in *OsOTS1-OX* lines relative to the *OsOTS-RNAi* or wild type lines. Previous studies have demonstrated that the chemical composition of the cuticle is an important factor determining the degree of resistance to water evaporation and also the thickness of wax deposition influences rates of water loss (Riederer and Schreiber, 2001, Oliveira *et al.*, 2003). It will be intriguing to ascertain in the future if there is a role for SUMO in wax deposition and cuticle composition in rice which could explain the increased rates of water loss in *OsOTS1-OX* lines.

Interestingly ABA treatment mimics drought with regards to stimulating *OsOTS1* degradation. Apoplastic pH increases during drought conditions leading to a greater retention of ABA. In addition, local production of ABA in leaves is also induced (Christmann *et al.*, 2007). We've discovered that reducing *OsOTS1* levels in rice promotes the production of ABA which in turn promotes the degradation of *OsOTS1* establishing a positive feedback loop in initiating ABA responses in rice. Degradation of SUMO proteases stimulate the accumulation of SUMO conjugated targets (Conti *et al.*, 2008). SUMOylation modifies the target function in many ways, including stimulating new protein-protein interactions, changing their subcellular localization, stabilizing or marking them for proteasomal degradation (Novatchkova *et al.*, 2004). However, till now there is no information on the targets of SUMO in rice or any mechanistic insight into how SUMOylation can lead to stress tolerance in crops such as rice.

There are 10 subfamilies of bZIP proteins in rice that exhibit distinct gene expression patterns reflect their diverse function in both development and response to the environment (Todaka *et al.*, 2015). *OsbZIP23* gene expression is known to be induced by ABA and this transcription factor has a positive role in mediating drought tolerance in rice (Xiang *et al.*, 2008). In this study we demonstrate that *OsbZIP23* physically interacts with *OsOTS1* in planta via Yeast 2-hybrid, GST-pull down and *in planta* BiFC and immunoprecipitation assays. Crucially *OsbZIP23* is SUMOylated *in planta* and in *OsOTS-RNAi* rice lines the protein accumulates demonstrating that de-SUMOylation is key for protein stability. Previous evidence clearly demonstrates that rice plants that accumulate *OsbZIP23* are more drought tolerant. Here, we provide further evidence of the significance of SUMOylation in mediating *OsbZIP23* accumulation to cope with drought stress. The accumulation of *OsbZIP23* in turn leads to the up-regulation of genes which are thought to provide drought protection (Xiang *et*

*al.*, 2008). This is exemplified by *OsLEA3* whose increased expression has been demonstrated to significantly improve drought tolerance in rice under field conditions (Duan and Cai, 2012). *OsLEA3* gene expression is enhanced in *OsOTS*-RNAi lines but not in the *OsOTS1*-OX lines indicating that OTS1 SUMO proteases through its impact on OsbZIP23 can unravel a new strategy for improving drought tolerance in the field.

Recently, we established a significant role for SUMOylation in stabilising DELLA proteins under stress conditions in *Arabidopsis* (Conti *et al.*, 2014). SUMOylation of DELLA proteins is stimulated during stress resulting in their accumulation to repress growth. *Arabidopsis* OTS1 SUMO protease removes SUMO from DELLA proteins thereby triggering their degradation. *OsOTS1* protease is rapidly destabilised during drought stress in rice suggesting that it may contribute to the hyperSUMOylation and increase in rice DELLA protein, SLR1 to exert helpful growth inhibition during times of stress to conserve energy. SUMOylation may be a key mechanism for fast growth restraint at the onset of stress in crops like rice. The current study demonstrates that *OsOTS1* can in parallel stimulate the expression of stress protectant genes through activating OsbZIP23. Our proposed model is that *OsOTS1* interacts with OsbZIP23 in well-watered conditions but under drought conditions ABA levels increase leading to the degradation of *OsOTS1*. Hence promoting the SUMOylation of OsbZIP23 which results in its accumulation and activation of drought protection gene expression (Figure 9). Consequently, these rice plants exhibit enhanced drought tolerance with increased productivity.

We demonstrate that modulating SUMO conjugation on protein targets has an important impact on rice crop to cope with drought. Our study suggests that the SUMO system may be a conduit for developing drought tolerant rice varieties.

## **MATERIALS AND METHODS**

### ***Plant Material, growth conditions and stress treatments***

Rice (*Oryza sativa* L. cv. Niponbare) seeds were sown in pots (8 x 8 x 10 cm) containing 180 gm of water soaked soil. Plants were grown in white fluorescent light (600 photons m<sup>-2</sup> s<sup>-1</sup>, 14h of light/ 12 h of dark) at 27 ± 1°C/24± 1°C) and 60% relative humidity. In brief, for ABA sensitivity, different genotype plants were germinated and then transplanted to normal MS medium or supplemented with 3µM ABA. The shoot and root growth was observed after about 10 days. For drought stress tolerance testing, plants were grown in pots filled with soil

and drought stress was conducted by withholding water for 10 days followed by recovery for 1 week, and then the survival rates were calculated.

### **Quantification of Water Loss**

Rate of water loss was detected as previously described (Duan and Cai, 2012). Briefly, the leaves of four weeks old control and transgenic plants grown under normal growth conditions were tested for water loss by immediately weighing detached leaves (considered as initial weight). The samples were then retained at room temperature and weighed at different time intervals as an indicator of water loss at dehydrating conditions to calculate rate of water loss as previously described (Duan and Cai, 2012). Twenty plants of each line were used in each experimental replicate and three biological replicates were made. Infrared thermal images were acquired using a FLIR T650SC thermal camera. Stomatal densities were calculated using nail varnish impressions generated from leaf impressions produced using President Plus dental resin (Coltène/Whaledent AG, Switzerland). Four fields of view per leaf of leaves 7 and 8 were counted from 5 plants per line.

### **Total RNA Extraction and Quantitative RT-PCR**

Total RNA was extracted, quantified and cDNA synthesised as previously described (Srivastava et al., 2016). One microgram of total RNA was used for cDNA synthesis and qRT-PCR analysis was performed (Srivastava et al., 2016). Primer sequences are listed in Supplemental Table S1.

### **Recombinant Protein and GST Pull Down Assay**

Recombinant protein expression and production in *E. coli* were previously described (Srivastava et al., 2015). OsOTS1 (GST:OsOTS1), OsbZIP23 (OsbZIP23:His) were expressed in BL21 (DE3) cells and purified using manufacturer's guidelines for GST and Histidine affinity purification tags. For *in vitro* binding experiments, GST and GST-OsOTS1 (2.0 µg) protein were used in pulldown assays as previously described (Srivastava et al., 2016). Elutes was re-suspended in 1x SDS loading buffer, boiled for 5 min and analysed by SDA-PAGE for protein binding. Both input (2%) and pull-down samples were probed with anti-GST and anti-His antibodies.

### **Transformation of Rice Seedlings**

Agrobacterium mediated transient transformation of rice seedlings was performed as previously described (Purkayastha et al., 2010) but with bZIP23 cDNA ORF in pIPKB02 binary vector by using the agrobacterium strain EHA105 in either control *Nipponbare* or transgenic *OsOTS1-OX* and *OsOTS-RNAi* plants to ascertain bZIP23 protein levels in these backgrounds.

### **Bimolecular Fluorescence Complementation (BiFC) Assay**

*N. benthamiana* leaves were co-infiltrated with of *A. tumefaciens* GV3101 cells containing the indicated plasmids or combination of plasmids for the BiFC assays. The BiFC assays were performed as described in Schütze et al., 2009. We have used the vectors pYFN43 and pYFC43 to clone the *OsOTS1* and *OsbZIP23* respectively (Belda-Palazon., 2012). Epidermal cells of transformed leaves of at least 3-4 plants were used for the assays. A minimum of 3 repeats was performed for each construct.

### **Yeast Two Hybrid Assay**

Yeast two-hybrid assays were performed as described in (Srivastava et al., 2016) using the appropriate plasmids containing the indicated genes of interest. To investigate the protein-protein interaction, bZIP23 was individually cloned into the pDEST22 to produce translational fusion proteins with the GAL4 DNA activation domain. However, full-length *OsOTS1* was cloned into the pDEST32 to produce translational fusion proteins with GAL4 DNA binding domain. Yeast strain AH109 was used to test for interactions on triple dropout media lacking Trp, Leu and His with 3-AT (Srivastava et al., 2016).

### **Transient Assays in *N. benthamiana*, Protein Extraction and Western Blot Analysis**

bZIP23 cDNA was cloned in both pEarlygate 104 and pEarlygate 201 so that expressed proteins had a N-terminal GFP or HA tag, and *OsOTS1* was cloned into pEarlygate 104 so that expressed proteins had a N-terminal GFP tag to investigate the interaction between GFP-*OsOTS1* and HA-*OsbZIP23*. SUMO1 was cloned in pEG201 so that expressed proteins had a N-terminal HA tag to investigate the SUMOylation of GFP tagged *OsbZIP23*. Transient gene expression assays in *N. benthamiana* plants was performed with *Agrobacterium*-mediated transformation (Ewan et al., 2011). Protein extraction and analysis was performed as described in Sadanandom et al., 2015) with anti-GFP, SUMO1 or HA antibodies as described in figure legends.

## Statistical Analysis

All the experiments were conducted in randomized design with three to five replicates for each study. Data were statistically analysed using ANOVA and tested for significant differences ( $P < 0.05$ ). All the statistical analysis and significance test has been performed using the Prism 6 Graph Pad software.

## Accession Numbers

The Rice Genome Annotation Project contains all sequences (<http://rice.plantbiology.msu.edu/>) under the following accession numbers: OsOTS1 (LOC\_Os06g29310), OsbZIP23 (LOC\_Os02g52780), LEA3 (LOC\_Os05g46480), LEA5 (LOC\_Os01g21250), bHLH148 (LOC\_Os03g53020) and RAB21 (LOC\_Os11g26570).

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## SHORT SUPPORTING INFORMATION LEGENDS

**Figure S1.** Analysis of germination rates of various rice transgenics treated with ABA.

**Figure S2.** Analysis of stomata densities in the indicated rice transgenics.

**Figure S3.** White light and thermal images of the empty vector control, *OsOTS1-OX* and *OsOTS-RNAi* rice lines subjected to drought stress and subsequently re-watered for 3 days.

**Figure S4.** Comparison of grain and panicle traits from the indicated rice lines in well-watered and drought stressed conditions.

**Figure S5.** Amino acid alignment of homologous bZIP proteins across selected crop plants indicating conservation of SUMO sites.

**Table S1.** List of primers pairs designed for qRT-PCR and thermal cycle programs for cDNA amplification.

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## FIGURE LEGENDS

**Figure 1.** Drought stress promotes degradation of the SUMO protease OsOTS1 in rice. Desiccation mannitol and ABA promotes the degradation of OsOTS1. HA-tagged OsOTS1 protein levels in OsOTS1-OX overexpressing rice plants were determined by immunoblotting using anti-HA antibodies (IB:  $\alpha$ HA). (a) The protein levels of HA-OsOTS1 in control untreated (MS) rice plants. (b) The degradation of HA-OsOTS1 is induced in rice transgenic lines undergoing desiccation stress at 28°C; (c) mannitol (300mM) and (d) ABA (100 $\mu$ M) treatment in the presence of cycloheximide (CHX). The duration of treatments is indicated by numbers of hours. The ponceau stained RbcS protein was used as protein loading control.

**Figure 2.** Rice plants with depleted *OsOTS1* levels are hypersensitive to ABA. White light images of seedlings of *OsOTS1*-OX, *OsOTS*-RNAi and empty vector control (WT) in ABA free medium first two seedlings of WT and three representative seedlings for each of *OsOTS1*-OX and *OsOTS*-RNAi (a) and for medium containing 3 $\mu$ M ABA three representative seedlings for each genotype (b and c). (d and e) Quantification of the relative shoot and root length inhibition by ABA of *OsOTS1*-OX, *OsOTS*-RNAi and empty vector control (WT) plants. Data represent mean  $\pm$  S.E. based on three biological replicates with 25 seedlings in each replicate (\*P <0.01, Two-way ANOVA).

**Figure 3.** Rice plants with depleted levels of OsOTS1 are drought tolerant. Manipulating the SUMO protease OsOTS1 in rice leads to increased drought sensitivity of *OsOTS1*-OX plants. (a) White light images of 4-week old adult plants under well-watered conditions, (b) following recovery after drought for 10 days followed by re-watering for 7 days. (c) Quantification of the survival rate. (d) Quantification of rate of water loss in drought stressed rice transgenics. 5 biological replicates and 25 plants in each line were used for survival rate and water loss analysis in each replicate. Data represent mean  $\pm$  S.E.

**Figure 4.** Comparison of agronomic traits between normal and drought-treated empty vector control, *OsOTS1*-OX and *OsOTS*-RNAi transgenic rice plants. (a) Spider plots showing the agronomic traits in normal well-watered conditions. (b) Spider plots showing the agronomic traits under drought condition during anthesis. Each data point represents a percentage of the mean values (n=25). Mean values of vector control set at 100% as a reference. SB,

secondary branches; CL, culm length; SL, seed length; SW, seed weight /100 seeds; NP, number of panicles; PL, panicle length. (c) Images of adult rice plants of *OsOTS1*-OX and *OsOTS*-RNAi transgenic lines under well-watered and drought conditions. (d) Quantification of relative spikelet fertility of different genotypes under drought conditions. Error bars indicate standard errors based on three biological replicates (\*\*P <0.01, Two way ANOVA)

**Figure 5.** *OsOTS1* and *OsbZIP23* directly interact. (a) Yeast two-hybrid assay to detect interaction of *OsOTS1* with *OsbZIP23* transcription factor. Rice SUMO protease *OsOTS1* was fused to the DNA binding domain and *OsbZIP23* was fused with activation domain in pDEST22 and pDEST32 vectors respectively. Interaction was assessed on SD/-Leu-Trp-His + 25mM 3AT medium. (b) Immunoblot analysis of GST pulls down assay between recombinant His:*OsbZIP23* with GST:*OsOTS1* or GST (IB:  $\alpha$ GST) only to demonstrate that His:*OsOTS1* (IB:  $\square$ His) co-purifies with GST:*OsOTS1* but not with GST.

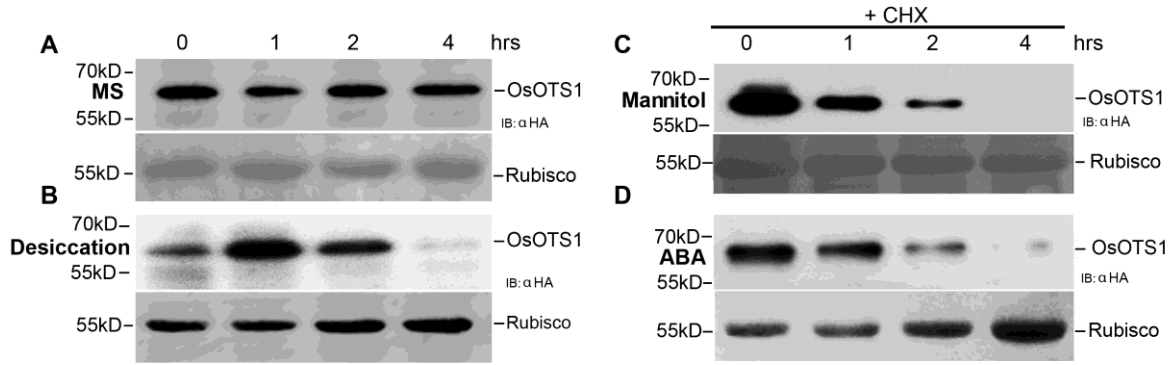
**Figure 6.** *In planta* physical interaction of *OsOTS1* and *OsbZIP23*. (a) Immunoblot analysis of co-immunoprecipitation assays of YFP-*OsOTS1* and YFP proteins with *OsbZIP23*-HA in *N. benthamiana* transient assays. *OsOTS1* fusion protein and YFP were immunoprecipitated using anti-GFP antibody beads and immunoprecipitates were resolved on two different SDS/PAGE gels and blotted on to PVDF membranes to detect for the presence of YFP-*OsOTS1*, YFP (IB:  $\square$  $\alpha$ GFP) and *OsbZIP23*-HA (IB:  $\alpha$ HA). (b) SUMO protease *OsOTS1* physically interacts with *OsbZIP23* in BiFc assays in *N. benthamiana* leaves. Confocal imaging of BiFC assays showing that *OsOTS1*-nYFP and *OsbZIP23*-cYFP interact to form a functional YFP in the nucleus indicated by fluorescence, whereas no fluorescence was detected in leaves co-infiltrated with empty vectors and the respective fusion proteins.

**Figure 7.** SUMOylation is required for the stability of the transcription factor *OsbZIP23* and this is governed by *OsOTS1* SUMO protease in rice. (a) SUMOylation of *OsbZIP23* in *N. benthamiana* leaves. The figure shows immunoblot analysis of immunoprecipitation of GFP-tagged *OsbZIP23*, with HA-tagged SUMO1 and GFP only proteins from *N. benthamiana* transient assays. *OsbZIP23*-GFP fusion proteins were immunoprecipitated using anti-GFP beads (IP: $\alpha$ GFP). Upper panel shows immunoblots probed with anti-HA antibodies to indicate the conjugation of HA-tagged SUMO1 to *OsbZIP23*-GFP (right lane). GFP only control lane (left lane) shows no SUMO banding pattern with anti-HA antibodies (IB:  $\square$  $\alpha$ HA).

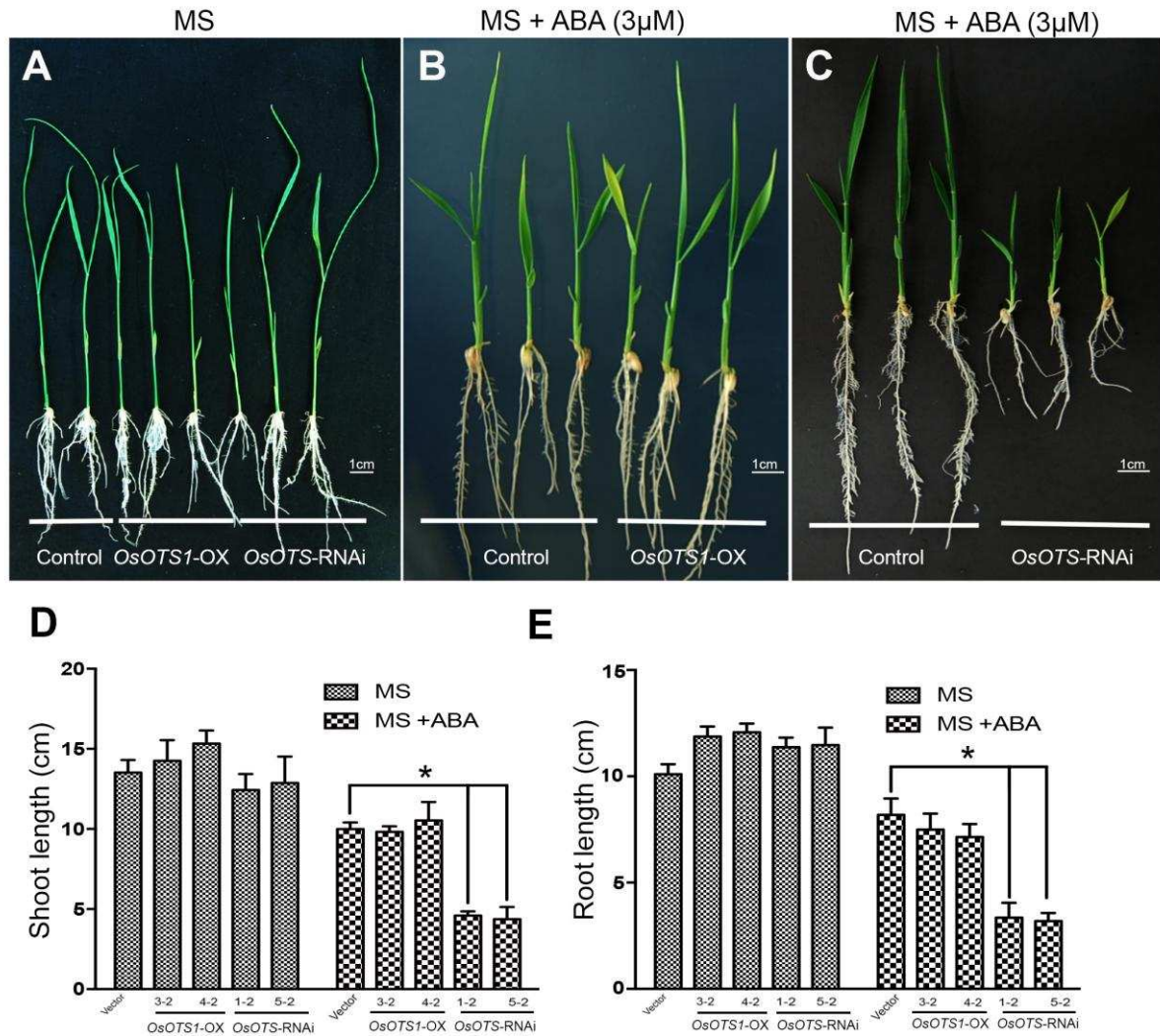
Lower panel shows immunoblots probed with anti-GFP (IB:  $\alpha$ GFP) to indicate GFP only and fusion protein levels. (b) Immunoblot analysis of agrobacterium-mediated transient assays expressing OsbZIP23-GFP fusion protein in the various transgenics as indicated. Immunoblots were probed with anti-GFP (IB: $\alpha$ GFP) to detect OsbZIP23-GFP fusion protein levels. It shows that OsbZIP23-GFP is more abundant in *OsOTS-RNAi* lines. The ponceau stained RbcS protein was used as protein loading control.

**Figure 8.** Analysis of the relative expression levels of ABA and OsbZIP23 mediated drought responsive genes (a) Amounts of ABA in fresh rice leaves determined by PGR LC-MS. 30 days old plant leaves grown in normal conditions were processed and analyzed. (b-e) RNA was extracted from 10 day old seedling of MS grown control, *OsOTS1-OX* and *OsOTS-RNAi* lines to perform q-PCR using gene specific primers. Our data demonstrate that *OsOTS-RNAi* lines have enhanced expression of ABA and OsbZIP23-mediated drought responsive genes. Error bars indicate standard errors based on the three biological replicates. (\*P <0.05, \*\*P<0.01, Two-way ANOVA).

**Figure 9.** ABA-mediated OsOTS1 degradation in drought stress promotes the accumulation of OsbZIP23. A working model illustrates the regulatory role of OsOTS1 on OsbZIP23 stability via deSUMOylation. OsOTS1 interacts with OsbZIP23 in well-watered conditions but under drought conditions ABA levels increase leading to the degradation of OsOTS1. Hence promoting the SUMOylation of OsbZIP23 which results in its accumulation and activation of drought protection gene expression. Consequently, these rice plants exhibit enhanced drought tolerance with increased productivity.

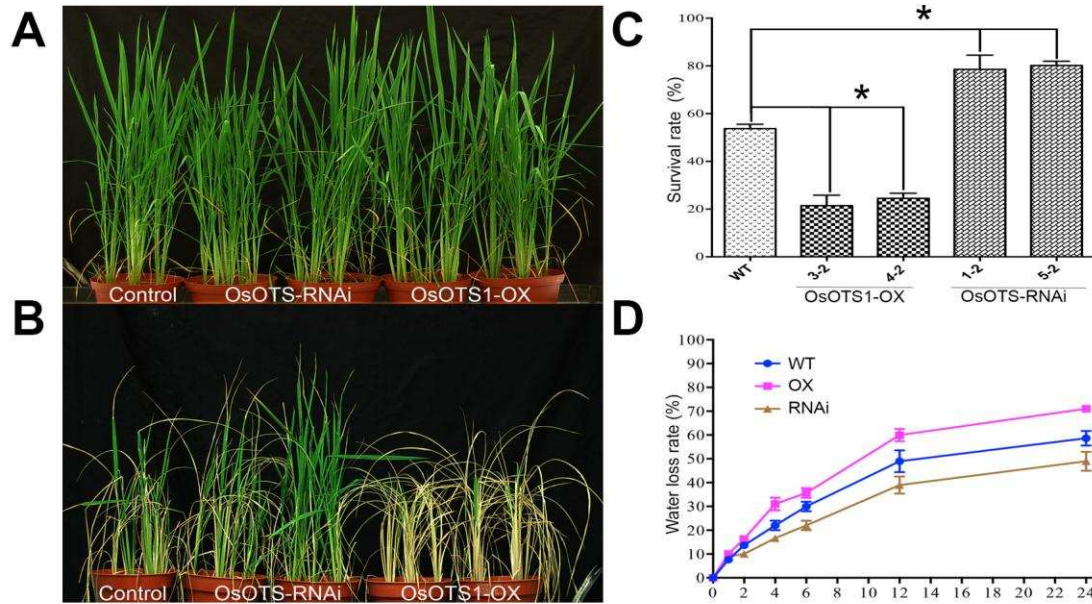


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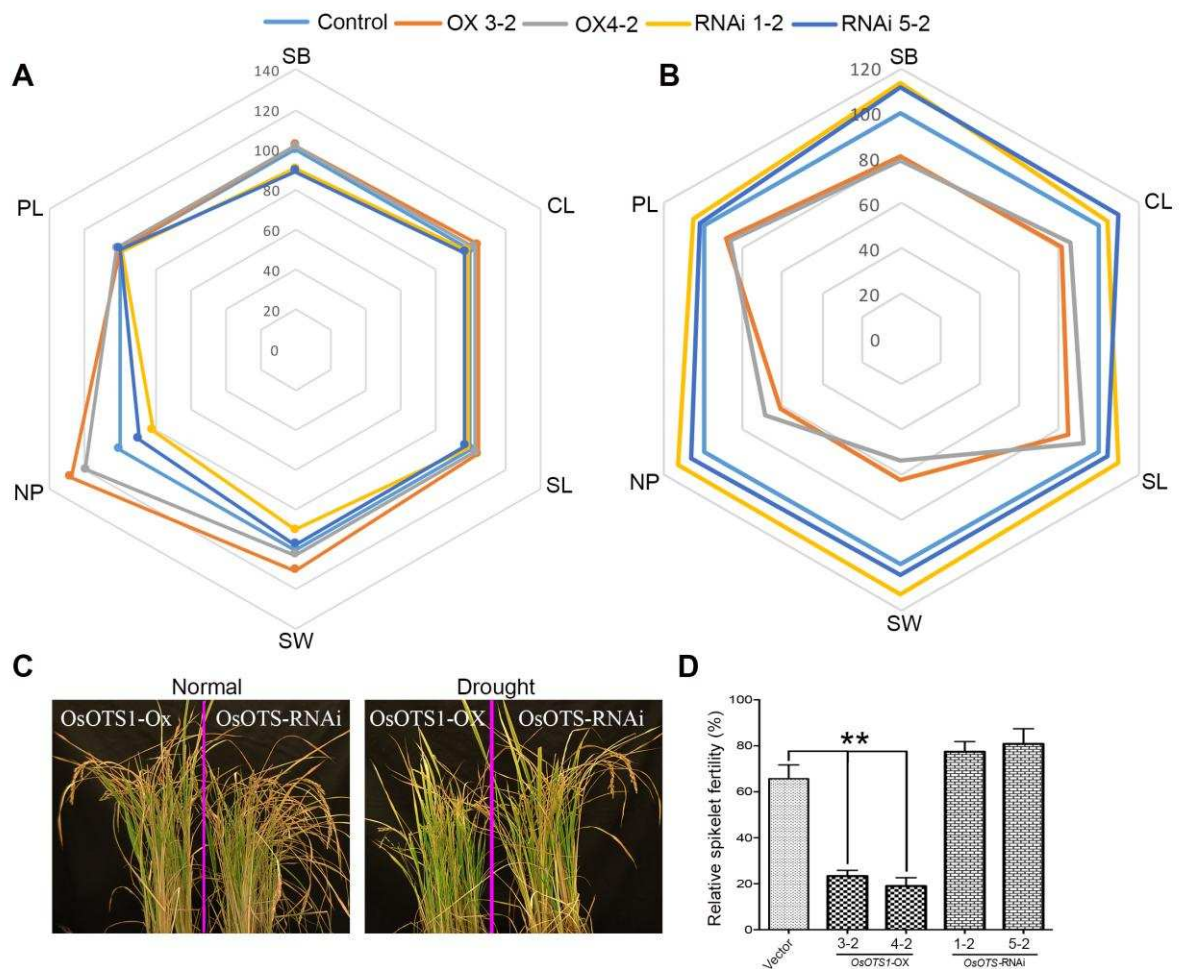
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(C) Quantification of the survival rate from (B).

(D) Quantification of rate of water loss in drought stressed rice transgenics.

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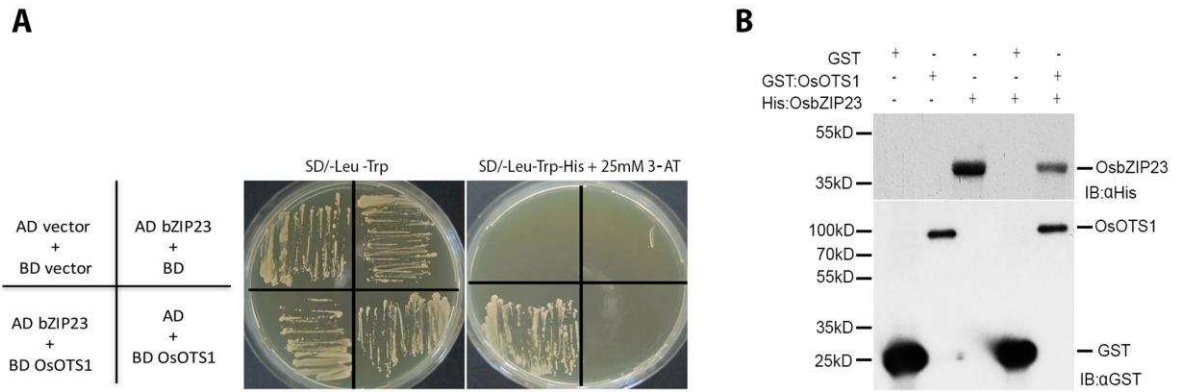
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**(C)** Images of adult rice plants of *OsOTS1-OX* and *OsOTS-RNAi* transgenic lines under well-watered and drought conditions.

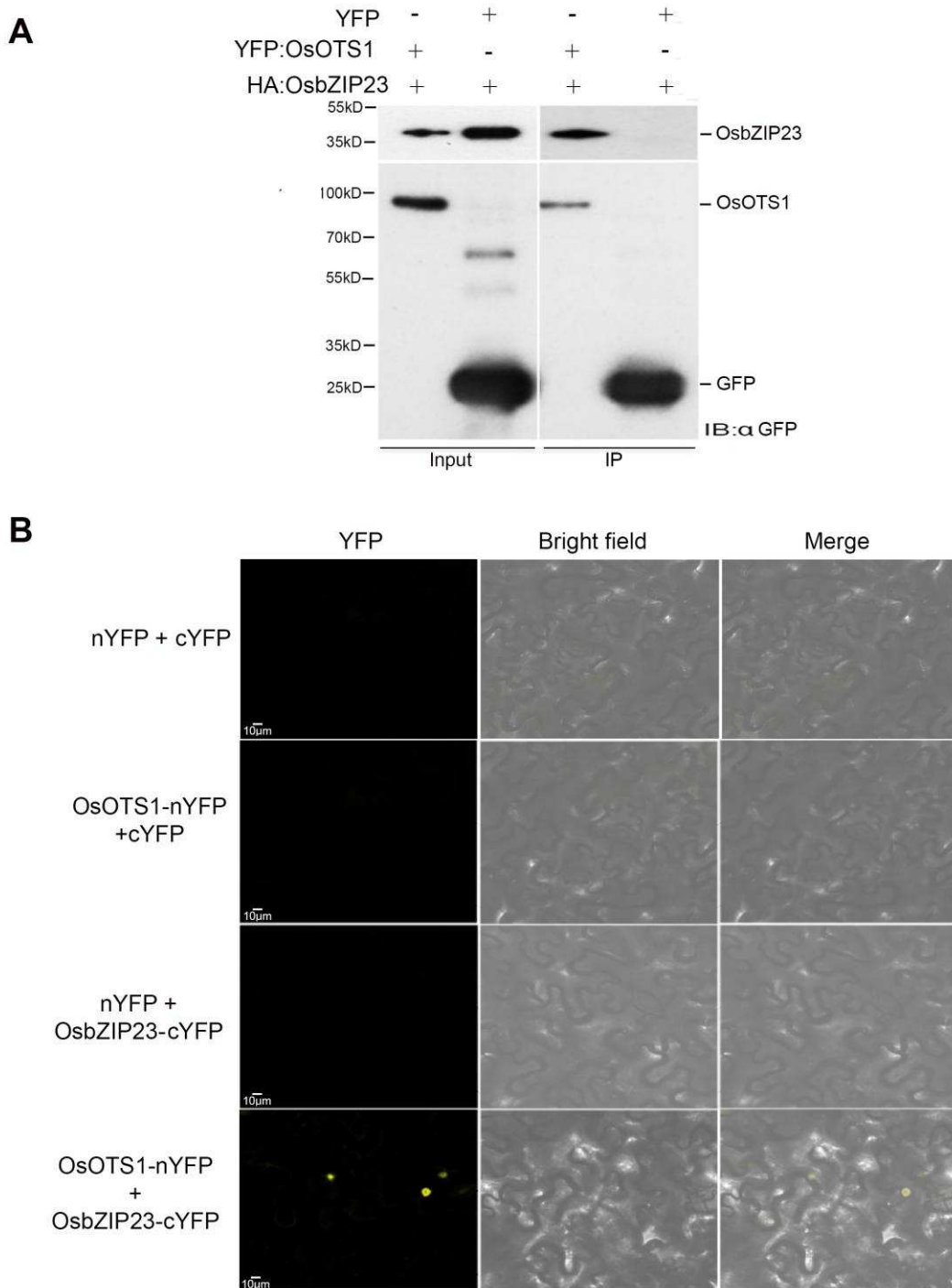
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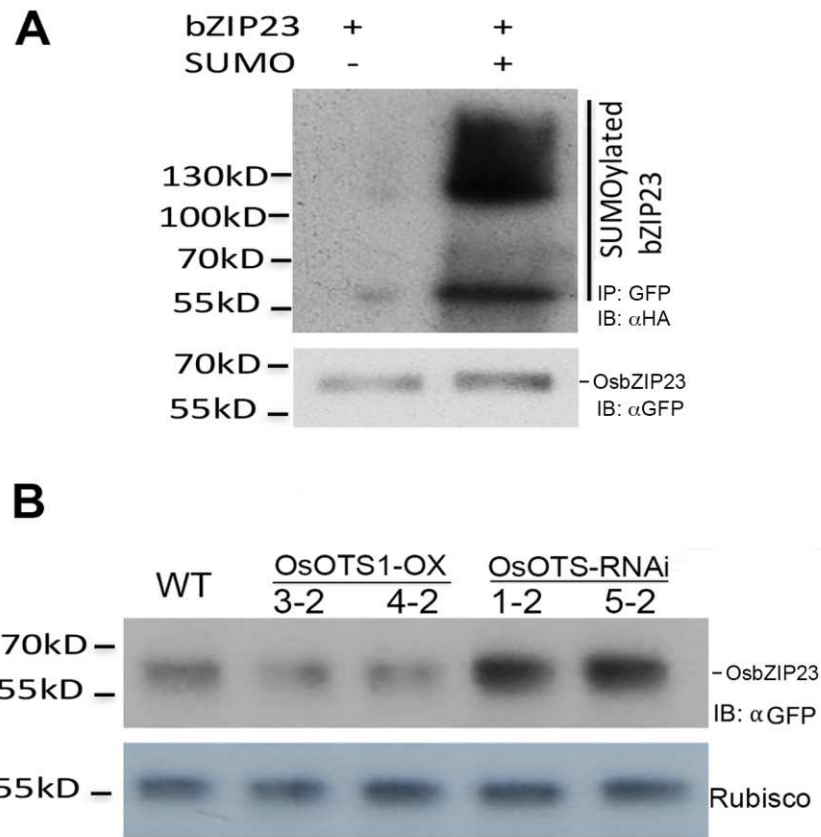
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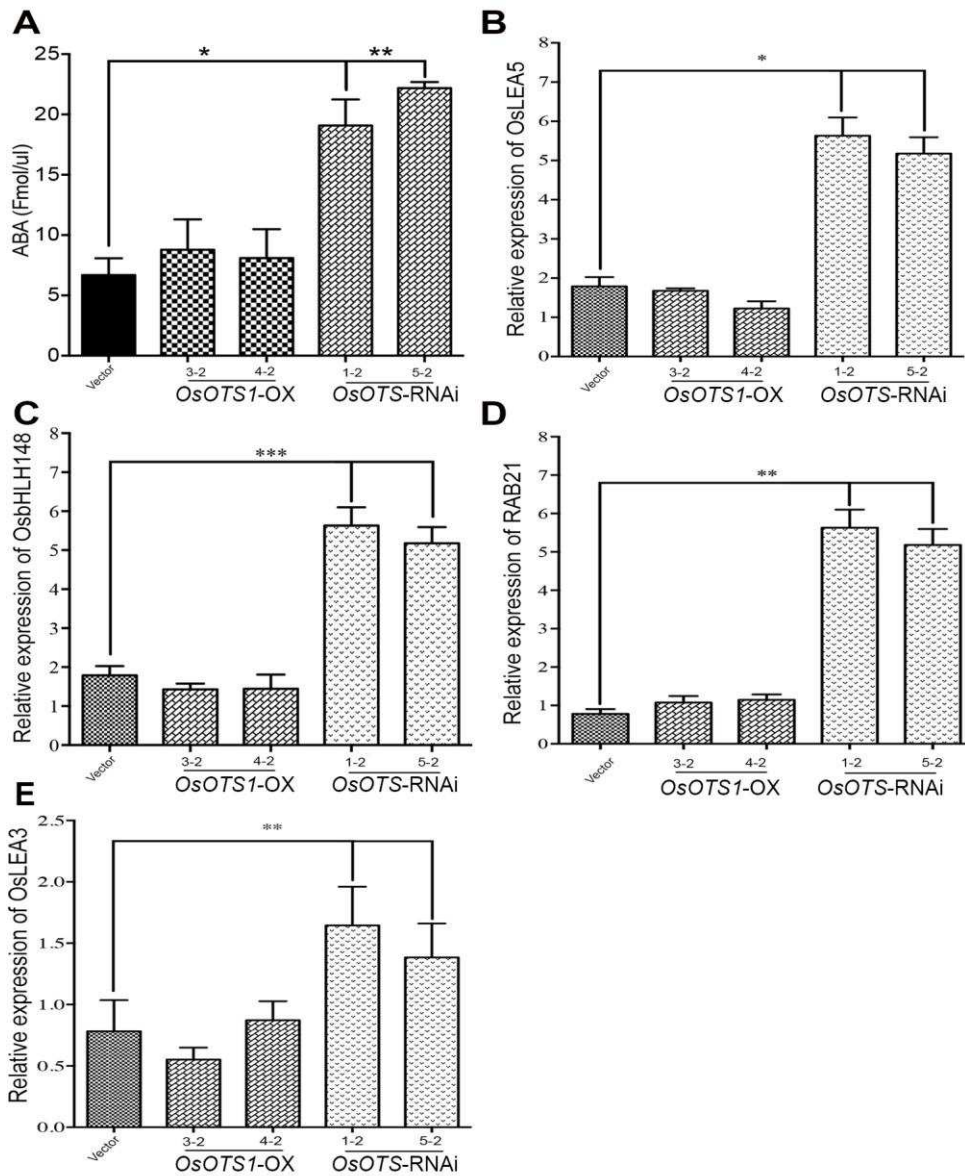
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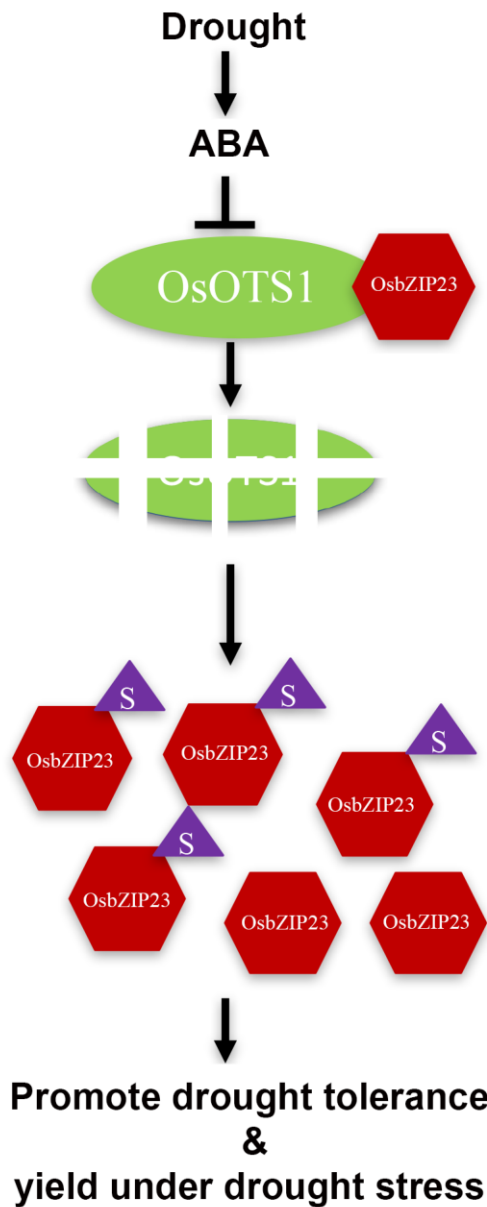
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**(A)** Amounts of ABA in fresh rice leaves determined by PGR LC-MS. 30 days old plant leaves were processed and analyzed.

**(B-E)** RNA was extracted from control, *OsOTS1-OX* and *OsOTS-RNAi* lines to perform q-PCR using gene specific primers. Our data demonstrate that *OsOTS-RNAi* lines have enhanced expression of ABA and OsbZIP23-mediated drought responsive genes. Error bars indicate standard errors based on the three biological replicates. (\* $P < 0.05$ , \*\* $P < 0.01$ , Two-way ANOVA).



**Figure 9.** ABA-mediated OsOTS1 degradation in drought stress promotes the accumulation of OsbZIP23. A working model illustrates the regulatory role of OsOTS1 on OsbZIP23 stability via deSUMOylation. OsOTS1 interacts with OsbZIP23 in well-watered conditions but under drought conditions ABA levels increase leading to the degradation of OsOTS1. Hence promoting the SUMOylation of OsbZIP23 which results in its accumulation and activation of drought protection gene expression. Consequently these rice plants exhibit enhanced drought tolerance with increased productivity.