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OsASR5 enhances drought tolerance through a stomatal closure pathway associated with ABA and H_2O_2 signaling in rice

Jinjie Li[1], Yang Li[1], Zhigang Yin[1], Jihong Jiang[1], Minghui Zhang[1], Xiao Guo[1], Zhujia Ye[1], Yan Zhao[1], Haiyan Xiong[1], Zhanying Zhang[1], Yujie Shao[1], Conghui Jiang[1], Hongliang Zhang[1], Gynheung An[2], Nam-Chon Paek[3], Jauhar Ali[4], Zichao Li[1]*

[1] Key Lab of Crop Heterosis and Utilization of Ministry of Education and Beijing Key Lab of Crop Genetic Improvement, China Agricultural University, Beijing, People's Republic of China, [2]Department of Plant Systems Biotech and Crop Biotech Institute, Kyung Hee University, Yongin 446-701, Korea,[3]Department of Plant Science, Plant Genomics and Breeding Institute, Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea, [4]International Rice Research Institute, Metro Manila, Philippines

Author for correspondence:

Zichao Li

TEL/ FAX: +86-10-62731414

Email: lizichao@cau.edu.cn

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Running title: Overexpression of *OsASR5* enhances drought tolerance by reducing water loss in *Oryza sativa* L. and *Arabidopsis*

Summary

Drought is one of the major abiotic stresses that directly implicate plant growth and crop productivity. Although many genes in response to drought stress have been identified, genetic improvement to drought resistance especially in food crops is showing relatively slow progress worldwide. Here we report the isolation of *Abscisic acid-*, *stress-*, and *ripening* (*ASR*) genes from upland rice variety, IRAT109 (*O. sativa* L. ssp. *japonica*), and demonstrated that overexpression of *OsASR5* enhanced osmotic tolerance in *E.coli*, and drought tolerance in *Arabidopsis* and rice by regulating leaf water status under drought stress conditions. Moreover, overexpression of *OsASR5* in rice increased endogenous ABA level and showed hypersensitive to exogenous ABA treatment at both germination and post germination stages. The production of H_2O_2, a second messenger for the induction of stomatal closure in response to ABA was activated in overexpression plants under drought stress conditions, consequently, increased stomatal closure and decreased stomatal conductance. In contrast, the loss-of-function mutant, *osasr5* showed sensitivity to drought stress with lower relative water content under drought stress conditions. Further studies demonstrated that OsASR5 functioned as chaperone-like protein, and interacted with stress-related HSP40 and 2OG-Fe (II) oxygenase domain containing proteins in yeast and plants. Taken together, we suggest that *OsASR5* plays multiple roles in response to drought stress by regulating ABA biosynthesis, promoting stomatal closure, as well as acting as chaperone-like protein that possibly prevents drought stress-related proteins from inactivation.

Key words: Drought, *Oryza sativa*, *OsASR5*, water loss, ABA, stomata.

Introduction

Drought is a major environmental stress affecting plant growth and reducing crop productivity. Due to the water shortage and inadequate rainfall in the rice-growing season, improving drought resistance becomes especially important for stabilizing rice productivity and production. However, drought resistance is a complex trait involves a series of physiological, morphological, cellular and molecular adaptive pathways (Nguyen et al., 1997; Umezawa et al., 2006; Valliyodan and Nguyen, 2006), resulting in a quite slow progress in the genetic improvement of drought resistance worldwide.

Multiple strategies are adapted by plants in response to drought stress, among them drought avoidance and drought tolerance are the two major mechanisms for improving drought resistance (Luo, 2010; Price et al., 2002). Drought avoidance assists plants maintaining tissue water potential by deep root and reducing water loss, especially through promoting stomatal closure (Hu and Xiong, 2014). Upon drought stress, abscisic acid (ABA), a key plant hormone increases dramatically, which in turn leads to a number of molecular and cellular responses, among which the best known are inducing stress-related genes and triggering stomatal closure (Daszkowska-Golec and Szarejko, 2013; Lee and Luan, 2012; Ye et al., 2012). Significant research findings over the last ten years have shown that ABA stimulates H_2O_2 generation mainly by NADPH oxidase in guard cells, and the generated H_2O_2 plays a vital role as essential signal molecules that mediates ABA-induced stomatal closure by activating plasma membrane calcium channels (Kwak et al., 2003; Mustilli et al., 2002; Pei et al., 2000; Wang and Song, 2008; Zhang et al., 2001). Recently, H_2O_2-induced stomatal closure through ABA independent pathway was reported in rice. A zinc finger transcription factor, *DST*, negatively regulates H_2O_2-induced stomatal closure by direct modulation of genes related to H_2O_2 scavenging (Huang et al., 2009). A rice homologue of SRO, *OsSRO1c,* increased stomatal closure by regulation of H_2O_2 homeostasis possibly

through down regulation of *DST* (You et al., 2013). So far, the genes that regulate stomatal movement through ABA-dependent and H_2O_2 mediated pathway in crops have not been identified, and the mechanism of stomata-regulated drought tolerance in crops, is largely unknown.

In most species, *abscisic acid-*, *stress-* and *ripening* (*ASR*) genes belong to a small gene family that is characterized by the presence of an ABA/WDS domain, and have been identified from monocot to dicot, nevertheless, they do not present in *Arabidopsis* (Gonzalez and Iusem, 2014). *ASR* genes were found to express in various organs and growth stages among different species, and responsive to ABA and various abiotic stresses, including drought, cold and salt stresses (Cakir et al., 2003; Chen et al., 2011; Henry et al., 2011; Hu et al., 2013; Huang et al., 2000; Joo et al., 2013; Kalifa et al., 2004; Maskin et al., 2001; Perez-Diaz et al., 2014; Philippe et al., 2010; Saumonneau et al., 2012). Although these genes were discovered two decades earlier and were reported in response to diverse abiotic stresses, till date we lack the complete understanding of the exact molecular functions and physiological roles under drought stress.

Yeast one-hybrid experiments revealed that the grape (*Vitis vinifera*) ASR ortholog named VvMSA binds to the promoter of a hexose transporter gene *VvHT1* (Cakir et al., 2003). By the yeast two-hybrid approach, a protein partner of VvMSA was isolated and characterized as a DREB transcription factor (Saumonneau et al., 2008). Likewise, tobacco (*Nicotiana tabacum*) ASR ortholog named NtTIP1 interacts with a tobacco bZIP transcription factor *in vivo,* and they possibly function in flower development and stress response (Hwan et al., 2012). Until recently, genome-wide chromatin immuno-precipitation data identified that rice OsASR5 binds to the promoter of the putative targets genes, including an ABC transporter required for Al tolerance (Arenhart et al., 2014). Similarly, the targets of tomato ASR1 were reported to be genes involving in cell wall synthesis and remodeling as well as water transport like aquaporins (Ricardi et al., 2014). Interestingly, tomato, plantain and lily ASR proteins were reported to perform a chaperone-like activity that protects reporter enzymes from denaturation induced by freezing or heat in vitro (Dai et al., 2011; Hsu et al., 2011; Konrad and Bar-Zvi, 2008). Moreover, several studies on the heterologous and

homologous expression of *ASR* genes in plant species were reported for functional characterization of *ASR* genes. Overexpression of the *ASR* gene from plantain (*Musa paradisiaca*; *MpASR*) and lily (*Lilium longiflorum*; *LLA23*) in *Arabidopsis* enhanced osmotic, cold and freezing tolerances possibly by acting as osmo-protectant, respectively (Dai et al., 2011; Hsu et al., 2011).Transgenic tobacco plants overexpressing the *ASR* gene from tomato (*Solanum lycopersicum*; *ASR1*) or *Salicornia brachiata* (*SbASR-1*) exhibited improved tolerance to osmotic stress (Jha et al., 2012; Kalifa et al., 2004), and from wheat (*Triticum aestivum*; *TaASR1*) showed enhanced tolerance to water stress (Hu et al., 2013). The ZmASR1 protein influences branched-chain amino acid biosynthesis and transgenic maize (*Zea mays*) plants overexpression of *ZmASR1* maintained kernel yield under water-limited conditions (Virlouvet et al., 2011). Overexpression of *OsASR1* or *OsASR3* in transgenic rice plants also resulted in enhanced tolerances to cold and drought stresses in terms of photosynthetic efficiency (Joo et al., 2013; Kim et al., 2009). It appears that the exact functions of the ASR proteins are still baffling, as the possible roles of the *ASR* genes could not be simply deduced by sequence homology with other known proteins (Virlouvet et al., 2011).

Upland rice (UR) has been evolved as "drought-resistant type" derived from natural and artificial selection under drought stress conditions, while lowland rice (LR) is "drought-sensitive type" in rice, thus identification and elucidating the function of drought responsive genes from UR will promote our understanding of drought tolerance mechanism in rice. To gain new insight into ASR functions in response to drought stress, we characterized the *ASR* gene family from UR variety, IRAT109 (*O. sativa* L. ssp. *japonica*). Three drought-responsive *ASR* genes, *OsASR3*, *OsASR5* and *OsASR6*, were identified from UR, and using *OsASR5* overexpression plants and the loss-of-function mutant, the function and molecular mechanism of *OsASR5* in drought tolerance were characterized and discussed, respectively.

Results

Expression profile of ASR *genes in UR and LR*

Genes preferentially expressed in UR under drought stress conditions were the probable candidate genes to improve drought tolerance. For that reason, the expression changes of the *ASR* genes in response to drought were analyzed between UR variety, IRAT109 and LR variety, Nipponbare (*O. sativa* L. ssp. *japonica*). Rice contains six *ASR* paralogous genes (Philippe et al., 2010), among which *OsASR3* was up-regulated in IRAT109, *OsASR5* and *OsASR6* were induced and up-regulated by drought in IRAT109 relative to Nipponbare (Supplementary Fig.S1). To further study the functions of the *ASR* genes in response to abiotic stress in rice, we currently focused on characterization of *OsASR5*.

To investigate whether the tissue-specific expression of *OsASR5* is different between the two varieties, the expression patterns of *OsASR5* in various organs during seedling and productive stages were analyzed by Quantitative real-time PCR (qRT-PCR). As shown in Fig.1A, *OsASR5* was expressed in various organs at seedling and reproductive stages, interestingly, highly expressed in the sheath and stem tissues of IRAT109 as compared to Nipponbare during reproductive stage. The temporal and spatial expression pattern of *OsASR5* was further investigated by transforming Nipponbare with a fusion gene of Pro_{OsASR5}:*OsASR5*-GFP. The GFP signal was observed in pistil, stamen, glume, guard cell, leaf, root, sheath, and in vascular bundles (Supplementary Fig.S2).

To speculate the function of *OsASR5,* the transcript levels of *OsASR5* in response to polyethylene glycol (PEG), high salinity, cold, ABA and ethylene were analyzed in the leaf tissues. The *OsASR5* transcript was induced rapidly by PEG, NaCl, cold, ABA and ethylene during 1-3 h after treatments both in IRAT109 and Nipponbare, interestingly, the expression levels of *OsASR5* in IRAT109 were much higher than those in Nipponbare (Fig.1B). For instance, there was a significant increase of the *OsASR5* transcripts in 1-2 h after ABA treatment in both varieties, however, the transcript levels of *OsASR5* showed 1.5-20 folds in IRAT109 as compared with Niponbare. These data suggested that *OsASR5* was responsive to multiple abiotic stresses preferentially in UR variety.

Expression of* OsASR5 *enhances osmotic and drought tolerance in E.coli and Arabidopsis

To examine the potential role of *OsASR5* in protecting cells from osmotic stress, heterologous expression of *OsASR5* in *E.coli* (BL21) was carried out. Cells transformed with the empty vector were used as a control (Fig.2A). The growth of the cells transformed either with empty vector or recombinant plasmid showed non- significant differences on fresh LB media. On solid media containing 0.5 M mannitol, the transformants expressing GST-OsASR5 fusion protein showed higher growth rate than those expressed GST protein only (Fig.2B). On liquid media with 0.5 M mannitol, the growth rate of the transformants expressing GST-OsASR5 fusion protein was 3-fold higher than the control after incubation for 10 h (Fig.2C). These results clearly indicated that the heterologous expression of OsASR5 protein increased *E.coli* tolerance to osmotic stress.

To examine if overexpression of *OsASR5* in *Arabidopsis* would increase the tolerance of transgenic lines to drought stress, ten T_3 transgenic lines were obtained and four of them with highest transcript levels of *OsASR5* were used to verify the function of *OsASR5* (Supplementary Fig.S3). There existed no developmental differences between overexpressed and wild type (WT) plants under the normal conditions. However, under drought stress conditions the transgenic plants gave more green leaves with higher leaf area as compared with WT plants that showed significant inhibited growth. Moreover, all of the transgenic plants showed complete recovery after re-watering, while only half of the WT recovered, and water loss rates of detached leaves from transgenic plants were lower than that from WT. These results indicate that heterologous overexpression of *OsASR5* in *Arabidopsis* enhances drought tolerance, suggesting that *OsASR5* is functional in dicots.

Overexpression of* OsASR5 *significantly enhances osmotic and drought tolerance in rice

To directly investigate the function of *OsASR5* in response to osmotic and drought stress in rice, seven transgenic lines with overexpressing *OsASR5* were obtained. Out of them, two lines (OE-3 and OE-19) with highest transcription levels of *OsASR5* were selected to verify

the function of *OsASR5* (Supplemental Fig.S4). The performances of *OsASR5* overexpression lines under high osmotic stress caused by adding high salinity or mannitol were examined. The growth of the *OsASR5* overexpression seedlings was less inhibited (Fig.3A, B), exhibiting higher relative shoot growth and relative shoot fresh weight than those of the non-transgenic (NT) seedlings (Fig.3C, D). These results indicate that overexpressing *OsASR5* in rice could enhance the tolerance of overexpression lines to osmotic stress.

Three-week-old seedlings grown in liquid medium were treated with PEG to create physiological dehydration stress conditions, after recovery was performed, *OsASR5* overexpression lines showed a stronger growth recovery phenotype than that of the NT plants. Almost 31.6-52.5% of *OsASR5* overexpression plants survived, while only 21.6-22.5% of the NT survived under this treatment (Fig.4A). Furthermore, the *OsASR5* overexpression and NT plants were planted in the soil and well watered at the tillering stage. There were no developmental differences between *OsASR5* overexpression and NT plants when normal irrigation was performed. However, after 1 week of stopping irrigation and 2 weeks of recovery, the *OsASR5* overexpression lines showed a distinct recovery rate from that of the NT plants. Almost 33.3-41.1% of *OsASR5* overexpression plants survived, whereas only 5.5-6.6% of the NT plants survived this treatment (Fig.4B). Therefore, it is evidence that overexpression of *OsASR5* results in increased tolerance to drought stress in rice.

Since relative water content (RWC) is one of the most important traits to detect drought tolerance, the RWC in the leaves of *OsASR5* overexpression and NT plants were measured during drought stress. High RWC in the leaves of *OsASR5* overexpression plants was observed as compared with that of NT (Fig.4C), suggesting that *OsASR5* possibly play an important role in reducing water evaporation especially under drought stress conditions. To further evaluate physiological and biochemical changes in *OsASR5* overexpression plants, the contents of free proline and soluble carbohydrates in *OsASR5* overexpression and NT plants were measured following drought stress. The contents of proline and sugar in both *OsASR5* overexpression and NT plants rose continuously during drought treatment, whereas no significant differences were observed between *OsASR5* overexpression and NT plants

(supplementary Fig.S5), suggesting that overexpression of *OsASR5* does not regulate the accumulation of proline and sugar in transgenic plants.

Overexpression of OsASR5 *increases stomatal closure*

As water loss is mainly occurred through stomatal opening in plants, the reduced water loss in the *OsASR5* overexpression plants prompted us to investigate stomatal aperture. The leaf stomatal apertures of *OsASR5* overexpression and NT plants were observed by using scanning electron microscopy. As shown in Fig.5A, B, the percentages of completely closure, completely open and partially open stomata in the *OsASR5* overexpression plants were not obviously different as compared with the NT plants under normal conditions. However, under drought stress conditions, 62.0% of stomata completely closed in the *OsASR5* overexpression plants, while only 43.1% completely closed in the NT plants, in contrast, only 36.7% partially opened in the *OsASR5* overexpression plants, but 54.4% partially opened in the NT plants, whereas non- significant differences in the percentage of completely open stomata were observed. Non-significant differences were observed for the stomatal density between overexpression and NT plants (Fig.5C). Moreover, the stomatal conductance was obviously decreased in *OsASR5* overexpression plants as compared with the NT plants under drought stress conditions (Fig.5D). These results clearly demonstrate that overexpressing *OsASR5* possibly affect the stomatal movements especially under drought stress conditions.

Overexpression of OsASR5 *increased endogenous ABA level and sensitivity to exogenous ABA*

Since ABA can induce stomatal closure and consequently decrease water loss, the endogenous ABA levels were measured in the leaves of *OsASR5* overexpression and NT plants under normal and drought stress conditions. The result showed that the endogenous ABA levels in both *OsASR5* overexpression and NT seedlings were clearly increased by drought stress, interestingly, the level of ABA was much higher in *OsASR5* overexpression seedlings (1513 ng/g fresh weight) than that in NT seedlings (1120 ng/g fresh weight), whereas no obvious difference was observed under normal conditions (Fig.6A). We

hypothesized that *OsASR5* may be involved in regulating ABA biosynthesis in drought stress. To confirm this, the transcript levels of ABA biosynthesis and responsive genes were analyzed. As shown in Fig.6B, the expression levels of *OsNCED4* and *OsNCED5*, *RAB16A* and *RAB16C* were highly up-regulated by drought stress in *OsASR5* overexpression seedlings as compared with NT seedlings, whereas the expression levels of these genes showed non-significant differences between *OsASR5* overexpression and NT seedlings under normal conditions. These results indicate that *OsASR5* may play an important role in drought induced-ABA biosynthesis through up-regulation of ABA biosynthesis genes, such as *OsNCED4* and *OsNCED5*, and regulating ABA responsive genes, such as *RAB16A* and *RAB16C*.

As the expression of *OsASR5* was induced by ABA, we speculate that *OsASR5* may play a positive role in ABA signaling in rice. To confirm this hypothesis, the exogenous ABA sensitivities of *OsASR5* overexpression lines were investigated at germination and post-germination stages. As shown in Fig.6C, no obvious deference in germination rate was observed between overexpression and NT plants in the normal medium, whereas the germination rate of overexpression lines were significantly lower than those of the NT plants in the medium with 2.5 μM ABA at the end of treatment. Similarly, the relative shoot length and root length of the *OsASR5* overexpression plants were significantly shorter than those of the NT plants, while non-significant differences were observed for the growth rate between *OsASR5* overexpression and NT seedlings in the normal medium at the post-germination stage (Fig.6D). These results demonstrated that overexpressing *OsASR5* increased exogenous ABA sensitivity at both germination and post-germination stages, indicating *OsASR5* may be a positive regulator of ABA signaling in rice.

OsASR5 ***modulates H_2O_2 homeostasis in drought stress***

As ABA induced H_2O_2 generation in *Arabidopsis* (Pei et al., 2000; Zhang et al., 2001), and the accumulation of H_2O_2 resulting in stomatal closure was reported in rice (Huang et al., 2009; You et al., 2013), the H_2O_2 levels in the leaves of *OsASR5* overexpression and NT plants were necessarily to be examined. A higher production of H_2O_2 was detected in the

leaves of *OsASR5* overexpression plants under drought stress conditions (Fig.7A, B), suggesting that accumulation of H_2O_2 may increase stomatal closure in *OsASR5* overexpression plants.

Overexpression of *OsASR5* inducing H_2O_2 accumulation also prompted us to determine whether *OsASR5* is involved in oxidative stress response. Germinated seedlings of the *OsASR5* overexpression and NT plants were sown on 1/2 Murashige and Skoog (MS) and 1/2 MS medium containing 2 μM methyl viologen (MV). The growth rate was markedly reduced, and more H_2O_2 was accumulated in *OsASR5* overexpression seedlings as compared with NT seedlings after oxidative stress treatment, while no obvious changes were observed under normal conditions (Fig.7C, D). These results suggest that overexpression of *OsASR5* is sensitive to oxidative stress.

To understand the mechanism of *OsASR5* in modulating H_2O_2 homeostasis, the activities of H_2O_2 scavenging enzymes were measured in *OsASR5* overexpression and NT plants. The results showed that the activity of *APX* was reduced in *OsASR5* overexpression plants as compared with NT plants under drought stress conditions (Fig.7E). Since *peroxidase 24 precursor* that encodes a peroxidase to scavenge H_2O_2 was regulated by *DST*, a negative regulator of H_2O_2 accumulation (Huang et al., 2009), expression levels of *DST* and *peroxidase 24 precursor* were analyzed in *OsASR5* overexpression plants. The results revealed the expression of *DST* and *peroxidase 24 precursor* were significantly repressed in *OsASR5* overexpression plants as compared with NT plants (Fig.7F). These results demonstrated that *OsASR5* could regulate H_2O_2 homeostasis by affecting the activity of H_2O_2 scavenging enzyme, APX and suppressing *DST* and its downstream gene, *peroxidase 24 precursor*.

The osasr5 *mutant is sensitive to drought stress*

To confirm the function of *OsASR5* in response to drought stress, T-DNA insertion line, *osasr5*, was obtained. The T-DNA was inserted in the promoter region, in 310 bp upstream of ATG (Supplemental Fig.S6A). Real-time PCR analysis showed that almost no *OsASR5*

transcript was detected in the insertion line, indicating that *osasr5* was true loss-of-function mutant line (Fig.S6B). The growth of *osasr5* mutant (Dongjing background) is similar with that of Dongjing (DJ), however, *osasr5* mutant was hypersensitive to drought stress by 15% PEG6000 treatment (Fig.8A). The survival rate of *osasr5* mutant was only 39-44%, while 80-83% of the DJ was recovered (Fig.8B). Low relative water content was observed in the leaves of *osasr5* mutant under drought stress (Fig.8C). Moreover, *osasr5* mutant showed reduced ABA sensitivity as compared with DJ at germination stage (Fig.8D, E), *osasr5* mutant also showed increased growth rate and reduced H_2O_2 accumulation after oxidative stress treatment (Fig.8F). Together, these results reconfirmed the function of *OsASR5* in drought stress tolerance.

OsASR5 ***interacts with stress-related proteins in chloroplast***

In our homologous *in vivo* system with transgenic rice protoplast expressing OsASR5-GFP fusion protein, we verified that the OsASR5 protein was localized in chloroplast and nucleus (Supplemental Fig.S7). As ASR proteins were reported to bind with DNA motif (Arenhart et al., 2014; Ricardi et al., 2014), it is necessary to analyze the transcription activity of OsASR5. However, the expression of BD (GAL4-binding domain) -OsASR5 fusion protein in yeast did not lead to reporter gene expression, and did not form homodimers to function (Supplemental Fig.S8), which indicated that OsASR5 has no transcription activity in yeast. To further elucidate the function of OsASR5, a cDNA library of IRAT109 treated with drought stress was constructed for screening OsASR5-interacting proteins by yeast-two-hybrid (Y2H). Using the full-length OsASR5 as bait, twenty-four positive clones were identified, and seven of them were confirmed to be unique interacting proteins (Fig.9A, Supplemental Table.S1). The interaction of OsASR5 with a heat-shock protein, HSP40 and with a 2OG-Fe (II) oxygenase family protein in the chloroplasts of tobacco epidermal cells and rice protoplast were confirmed by bimolecular fluorescence complementation (BiFC) assay (Fig.9B).

OsASR5 ***functions as chaperone-like protein***

ASR proteins are low molecular weight charged and hydrophilic proteins (Goldgur et al., 2007; Gonzalez and Iusem, 2014), while hydrophilic proteins were shown to possess chaperone-like activity (Garay-Arroyo et al., 2000). OsASR5 was predicted to be a hydrophilic protein (data not shown), and was heat stable, which indicates that OsASR5 is not likely to aggregate during high temperature treatment or boiling (Fig.9C). We also examined whether OsASR5 exhibits chaperone activity to protect protein from inactivation. The activity of LDH in the presence or absence of OsASR5 *in vitro* was detected during cycles of freeze-thaw treatment. The activity of LDH was significantly reduced after four cycles of freeze-thaw, while the enzyme activity was markedly retained in the presence of OsASR5 (Fig.9D). It is worth to note that the effect of enzyme protection by OsASR5 is superior to BSA, a cryo-protectant, after six cycles of freeze-thaw. Thus, these results indicate that OsASR5 can function as chaperone-like protein and stabilize proteins against inactivation.

Discussion

OsASR genes preferentially expressed in UR are probably drought-responsive genes in rice

Previous studies reported comparative expression profiles of UR and LR under drought stress conditions using cDNA microarray technology (Ding et al., 2013;Lenka et al., 2011; Wang et al., 2007). In addition, differently expressed genes in the two genotypes were identified and several of them were currently proved to be involved in drought response. For instance, *SNAC1*, *OsLEA3-1* and *OsMIOX* were strongly induced in UR variety by drought stress as compared with LR variety, and overexpression of these genes separately in LR variety showed significantly improved drought tolerance (Duan et al., 2012; Hu et al., 2006; Xiao et al., 2007). In recent years, the expression patterns of *ASR* genes both in tissue specific and abiotic stresses were characterized by several groups (Joo et al., 2013; Philippe et al., 2010),

however, we firstly analyzed the expression changes of the rice *ASR* gene family between UR variety and LR variety. As described in Supplemental Fig.S1, *OsASR3* was up-regulated in UR variety, *OsASR5* and *OsASR6* were strongly induced by drought stress in UR variety, and the expression levels of these genes were 4.2-89.6 fold higher in UR variety than that in LR variety during drought stress. Based on our findings and the knowledge gathered, we could deduce that *OsASR3*, *OsASR5* and *OsASR6* up-regulated in UR variety were possibly drought stress-responsive genes in rice. In order to prove the speculation, these *ASR* genes were overexpressed into the *japonica* rice, Nipponbare, separately. And the role of *OsASR5* in response to drought stress was identified firstly.

OsASR5 plays a positive role in drought stress response

It is widely accepted that the genes induced by abiotic stresses may play positive roles in abiotic stress tolerances. The transcription of *OsASR5* was strongly induced by dehydration, high salinity, cold, ABA and ethylene treatments. *OsASR5* overexpression lines showed improved growth performance under simulated osmotic stress conditions brought by NaCl or mannitol treatment, and also enhanced survival rate under dehydration conditions created by PEG treatment or dry soil conditions brought by restricting irrigation. Expression of *OsASR5* also enhanced osmotic and drought stress tolerances in *E.coli* and *Arabidopsis*, respectively. Furthermore, overexpression of *OsASR5* showed no obvious changes in morphological phenotype in rice and *Arabidopsis* transgenic lines under normal conditions. These results suggested that *OsASR5* is a positive regulator of the responses to drought, osmotic, and dehydration stresses, implying the usefulness of *OsASR5* in genetic improvement of abiotic stress tolerance in several crop species.

OsASR5 confers tolerance to drought stress through a stomatal closure pathway associated with ABA and H2O2 signaling

Stomata control uptake of CO_2 for photosynthesis and restrict water loss by modulating transpiration, thereby playing crucial roles in abiotic stress tolerance (Hetherington and Woodward, 2003; Schroeder et al., 2001). Due to the essential roles of the stomata for plants, the molecular mechanisms of stomatal movement integrated by phytohormone, environmental signaling and many ion channels have been frequently studied in *Arabidospsis* (Daszkowska-Golec and Szarejko, 2013). So far, a total of seven drought-responsive genes that regulating stomatal movement have been identified in rice (Gao et al., 2011; Hu et al., 2006; Huang et al., 2009; Manavalan et al., 2012; Wei et al., 2014; You et al., 2013; Zhang et al., 2011). Among which, *SNAC1*, *OsSDIR1*, *hrf1*, *SQS* and *OsCPK9* were characterized to be sensitive to ABA, modulating stomatal movement possibly through an ABA-dependent pathway (Gao et al., 2011; Hu et al., 2006; Manavalan et al., 2012; Wei et al., 2014; Zhang et al., 2011a), while *DST* and *OsSRO1* regulated stomatal movement due to the accumulation of H_2O_2 through an ABA-independent pathway (Huang et al., 2009; You et al., 2013). Therefore, knowledge on control of stomatal closure and opening remains fragmented in rice. In this study, *OsASR5* was strongly induced by exogenous ABA treatment, and the endogenous ABA level of *OsASR5* overexpression plants under drought stress conditions was much higher than that of NT. Furthermore, *OsASR5* overexpression plants were more sensitive, and *osasr5* mutant was more insensitive to exogenous ABA treatment than that of their WT, respectively. These results indicated that the *OsASR5* was involved in an ABA-dependent pathway.

H_2O_2 generation was dependent on ABA concentration, and was essential for ABA-induced stomatal closure in plants (Kwak et al., 2003;Wang and Song, 2008; Zhang et al., 2001). We found a higher accumulation of H_2O_2 along with the increased ABA level, and the coincidence of reduced rate of water loss with increased stomatal closure in *OsASR5* overexpression plants under drought stress conditions. To our knowledge, we suggest that

OsASR5 modulates stomatal closure probably due to the H_2O_2 accumulation through ABA–dependent pathway under drought stress conditions.

Possible functions of the OsASR5 protein in chloroplast

ASR proteins were previously reported to be localized in both the cytosol and nucleus (Chen et al., 2011; Ricardi et al., 2012; Takasaki et al., 2008), only in the nucleus (Hu et al., 2013; Hwan et al., 2012), or in multiple cellular compartments such as nucleus, cytoplasm and chloroplasts (Arenhart et al., 2014). However, the precise function for the localization of ASR proteins in these subcellular compartments is not clear. We identified HSP40 and a 2OG-Fe (II) oxygenase family protein that interacted with OsASR5 in the chloroplasts of tobacco epidermal cells and rice protoplasts, separately. This is the first report on the interaction of ASR proteins in the chloroplast. HSPs are stimulated in response to abiotic stress and play an important role in protecting plants against many stresses (Timperio et al., 2008; Wang et al., 2014; Alvim et al., 2001; Cho and Hong, 2006; Sato and Yokoya, 2008). A 2OG-Fe (II) oxygenase family protein in rice affects water transport in leaves by affecting the composition and structure of leaf secondary cell walls (Fang et al., 2012). These evidences imply that OsASR5-interacting proteins, HSP40 and 2OG-Fe (II) oxygenase family protein may be playing important roles in response to water stress.

Abiotic stress may result in protein aggregation and degradation. Plants use a number of mechanisms to protect protein from inactivation, including chaperones and chaperone-like proteins, and low-molecular weight organic molecules (Konrad and Bar-Zvi, 2008). *In vitro* assay with purified OsASR5 protein, we confirmed that OsASR5 could protect LDH from cold-induced inactivation, function as chaperone-like protein. Thereby we could conclude that OsASR5 may function as molecular chaperone for the HSP40 and 2OG-Fe (II) oxygenase family protein in chloroplast, and possibly prevent HSP40 and 2OG-Fe (II) oxygenase family protein from inactivation under drought stress conditions.

Based on our knowledge, we tried to summarize a model to explain the role of OsASR5 in improving drought stress tolerance in plant (Fig.10). In conclusion, *OsASR5* has multiple roles in the regulation of drought stress tolerance by increasing ABA and H_2O_2 accumulation thus leading to stomatal closure and reduce water loss, besides by acting as chaperone-like protein that possibly protects some drought stress-related proteins from inactivation under drought stress conditions. Furthermore, overexpression of *OsASR5* did not alter the morphological phenotype of the transgenic lines. Therefore, through this study we could successfully identify a gene, *OsASR5*, which may be potentially useful for engineering drought tolerance in plant.

Experimental procedures

Plant materials and growth conditions

The UR variety, IRAT109 and LR variety, Nipponbare were used in this study. The *japonica* rice variety, DJ and mutant *osasr5* seeds were obtained from the POSTECH RISD (http://www.postech.ac.kr/life/pfg/risd/). Seeds of IRAT109 and Nipponbare were germinated at 32°C for 2 days, and then grown in Hoagland nutrient solution under controlled conditions with 28±2°C temperature, 200 µmol/m^2s^2 light intensity with 14 h light/10 h dark photoperiod and 80% relative humidity. Four-week-old seedlings were subjected to different treatments with 15% PEG6000 (w/v), 200 mM NaCl, low temperature (4°C), 100 µM ABA, 2 mM ethylene and drought by stopping irrigation. The leaf tissues were harvested at 0, 0.5, 1, 2, 3, 6, 10, 16 and 24 h time points for PEG, low temperature, ABA and ethylene treatments. Likewise, leaf tissues were harvested at 0, 0.5, 1, 2, 3, 6, 10 and 16 h time points for NaCl treatment, and at 0, 4, 8, 11, 14, 17, 20, 23 and 26 d time points for drought treatment. All these harvested leaf samples were then rapidly freezed in liquid nitrogen and stored at -80°C for further expression analysis of *OsASR5* between UR and LR.

Osmotic, drought and oxidative stress treatments

For osmotic stress treatment, the seeds of T_3 transgenic and non-transgenic (NT) lines were germinated on 1/2 MS medium under 14 h light (28℃)/10 h dark (25℃) photoperiod conditions for 5 days, and transplanted to 1/2 MS medium containing 150 mM NaCl and 250 mM mannitol, respectively. The shoot length, root length and fresh weight of transgenic lines and NT plants were measured after 7 days. For dehydration treatment, three-week-old seedlings of *OsASR5* overexpression and NT plants, mutant *osasr5* and DJ plants grown in normal Hoagland solution were treated with 15% PEG6000 solution for 14 days and then recovered in normal Hoagland solution for 7 days. The survival rates of each line were examined. For drought treatment, two-week-old seedlings of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT grown at 10 h light (22℃)/14 h dark (18℃) photoperiod in flowerpots with soil and vermiculite (1:2) were not irrigated. After 3 weeks of stopping irrigation, the seedlings were observed for recovery by re-watering for 4 days. Fresh leaf numbers of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT before and after drought stress were examined. One-month-old seedlings of the *OsASR5* overexpression rice transgenic lines and NT plants grown in flowerpots with soil and vermiculite (1:1) were not irrigated. After 1 week of stopping irrigation, the seedlings were observed for recovery by re-watering for 2 weeks. Seedlings were regarded as survivals if the fresh and green young leaves emerged after water supply. The survival ratio was calculated according to the number of survival plants over the treated plants in each flowerpot. For oxidative treatment, the seeds of *OsASR5* overexpression and NT plants germinated on normal 1/2 MS medium were transplanted to 1/2 MS medium containing 2 μM MV, and the plant length was measured at 5 days after transplanting.

Imaging of stomatal opening and measurement of stomatal conductance

Leaves of one-month-old *OsASR5* overexpression and NT plants with drought treatment (without irrigation for 3 d) or normal growth were detached and directly fixed by 2.5% glutaraldehyde. The stomatal pictures were obtained using a scanning electron microscopy (JSM-6390lv, JEOL, Japan), and the percentages of stomatal completely open, partially open

and completely close were calculated. The second fully expanded leaves, counting from the top of the same plants used for imaging stomata, were applied to measure stomatal conductance with a portable gas analysis system (LI-COR 6400, LI-COR, Inc.).

Endogenous ABA level and exogenous ABA sensitivity assay

Endogenous ABA levels were determined according to the method as described previously (Xiong et al., 2014). To test the ABA sensitivity at germination stage, seeds of *OsASR5* overexpression and NT, *osasr5* and DJ lines were germinated on 1/2 MS medium containing 2.5 μM ABA and the germination rates were calculated at the 5th day after initiation. To test sensitivity at post-germination stage, the seeds of overexpression and NT plants germinated on normal 1/2 MS medium were transplanted to 1/2 MS medium containing 2.5 μM ABA. The shoot length and root length of each seedlings were measured after 7 days of the ABA treatment at 14 h light (28℃)/10 h dark (25℃) photoperiod.

Other methods

Details of the methods for RNA isolation and qRT-PCR analysis, plasmid construction and plant transformation, subcellular localization, physiological and biochemical indexes assay, transactivation, yeast-two-hybrid and BiFC assays are available in supplementary methods at *PBJ* online.

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Figure legends

Fig.1 Expression analysis of the *OsASR5* gene. (A) Real-time PCR analysis of the expression level of *OsASR5* in different tissues of LR variety, Nipponbare and UR variety, IRAT109. (B) Stress-inducible expression of *OsASR5* under PEG, NaCl, Cold, ABA and Ethylene treatments. Error bars indicate standard error (SE) based on three replicates.

Fig.2 Enhanced osmotic and drought tolerance in *E.coli* and *Arabidopsis*. (A) Isopropylb-D-thiogalactopyranoside (IPTG) inducible expression of GST and GST-OsASR5 fusion proteins. GST and GST-OsASR5 were not (-) or were (+) induced by IPTG. Arrows indicate expression proteins. (B) Growth analysis of cells spotted on LB agar plate supplemented with 0.5 M mannitol. (C) Growth analysis of cells cultured in liquid medium supplemented with 0.5 M mannitol (n=3). Cell growth densities were measured at 600nm at the indicated time points. (D) Drought stress tolerance assay of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT by stopping irrigation for 3 weeks and recovery with re-watering for 4 days. (E) Fresh leaf numbers of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT before and after drought stress (n=3, 4 plants in each repeat). (F) Water loss rate in the detached leaves of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT under normal conditions (n=3, 12 leaves in each repeat). Data are mean ±SE. ** indicates significant difference at $P<0.01$ probability.

Fig.3 Increased osmotic tolerance of *OsASR5* overexpression plants. (A, B) Growth performance of *OsASR5* overexpression and NT seedlings under high salinity and mannitol treatments at 7 d after transplanting, respectively (n=3, 5 plants in each repeat). (C, D) The relative plant length and fresh

weight of *OsASR5* overexpression and NT seedlings corresponding to A, B, respectively. Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

Fig.4 Enhanced drought tolerance of *OsASR5* overexpression plants. (A) Physiological dehydration stress tolerance assay of *OsASR5* overexpression and NT plants under 15% PEG6000 treatment. Survival rates of *OsASR5* overexpression and NT plants after dehydration stress were examined (*n*=3, 15 plants in each repeat). (B) Drought stress tolerance assay of *OsASR5* overexpression and NT plants by stopping irrigation for 1 week and recovery with re-watering for 2 weeks. Survival rates of *OsASR5* overexpression and NT plants after drought stress were examined (*n*=3, 9 plants in each repeat). (C) Relative water content of *OsASR5* overexpression and NT plants under 15% PEG6000 treatment at the indicated time points (*n*=3). Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

Fig.5 Overexpression of *OsASR5* increasing stomatal closure. (A) Scanning electron microscopy images of three levels of stomatal apertures. Bar, 5 μm. (B) The percentage of three levels of stomatal apertures in the leaves of *OsASR5* overexpression and NT plants under normal and drought stress conditions (*n*=300 stomata for NT under normal conditions; *n*=248 stomata for OE-19 under normal conditions; *n*=322 stomata for NT under drought stress; *n*=272 stomata for OE-19 under drought stress). (C) Stomatal density of the middle leaves of *OsASR5* overexpression and NT plants (*n*=3).Three random scopes were used in each repeat. (D) Stomatal conductance of *OsASR5* overexpression and NT plants (*n*=3). Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

Fig.6 ABA accumulation and sensitivity of *OsASR5* overexpression plants. (A) ABA contents of *OsASR5* overexpression and NT plants under normal and drought stress conditions (*n*=3). (B) Real-time PCR analysis of the expression of ABA biosynthesis and responsive genes under normal and drought stress conditions (*n*=3). (C) Germination rates of *OsASR5* overexpression and NT seeds under ABA treatment (*n*=3, 30 seeds in each repeat). (D) Growth performance and relative plant length of *OsASR5* overexpression and NT seedlings under ABA treatment (*n*=3, 5 plants in each repeat). Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

Fig.7 H_2O_2 accumulation in *OsASR5* overexpression plants. (A) 3,3 ϕ-diaminobenzidine (DAB) staining for H_2O_2 in the leaves of *OsASR5* overexpression and NT plants under normal and drought stress conditions. (B) Quantitative measurement of H_2O_2 in the leaves of *OsASR5* overexpression and NT plants under normal and drought stress conditions (*n*=3, 3 plants in each repeat). (C) Growth performance and relative plant length of *OsASR5* overexpression and NT plants after MV treatment (*n*=3, 5 plants in each repeat). (D) DAB staining for H_2O_2 in the leaves of *OsASR5* overexpression and NT plants after MV treatment corresponding to C. (E) Activity of APX and CAT in the leaves of *OsASR5* overexpression and NT plants under normal and drought stress conditions (*n*=3). (F) Expression of *DST* and *Peroxidase 24 precursor* in the leaves of *OsASR5* overexpression and NT plants under normal and drought stress conditions. Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

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Fig. 10 A proposed model explaining the function of OsASR5 in regulation of stomatal status and drought stress tolerance. Under drought stress the expression of OsASR5 was up-regulated, resulting in up-regulation of ABA biosynthesis and responsive genes, such as *OsNCED4* and *OsNCED5*, *RAB16A* and *RAB16C*, leading to ABA accumulation and increased sensitivity to exogenous ABA. Up-regulation of OsASR5 also affected the activity of H_2O_2 scavenging enzyme, APX, and suppressed *DST* and its downstream gene, *peroxidase 24 precursor*, leading to H_2O_2 accumulation. ABA and H_2O_2 accumulation promote stomatal closure, resulting in increased water content, and finally enhancing drought tolerance. Furthermore, OsASR5 functioned as molecular chaperone and interacted with HSP40 and 2OG-Fe (II) oxygenase family protein, may prevent those drought-stress related proteins from inactivation under drought stress conditions. However, the function of those interacted proteins for drought stress tolerance remains to be elucidated in future studies.

Supplementary data

Supplementary data are available at *PBJ* online.

Fig.S1. Drought inducible expression of *ASR* genes in UR and LR varieties.

Fig.S2. The temporal and spatial expression pattern of *OsASR5* in the transgenic lines harboring a fusion gene of Pro_{OsASR5}:*OsASR5*-GFP.

Fig.S3. RT-PCR analysis of *OsASR5* transcript levels in different *Arabidopsis* transgenic lines.

Fig.S4. Transcription levels of *OsASR5* in *OsASR5* overexpression rice transgenic lines.

Fig.S5. Free proline and soluble sugar contents of *OsASR5* overexpression and NT plants under 15% PEG6000 treatment.

Fig.S6. Identification of *osasr5* T-DNA insertion mutant.

Fig.S7. Subcellular localization of OsASR5-GFP fusion protein.

Fig.S8. OsASR5 transcriptional activation and homodimerization analysis.

Table S1. OsASR5 interacting proteins identified in yeast two-hybrid screening.

Supplementary methods.

Figures

Fig1.

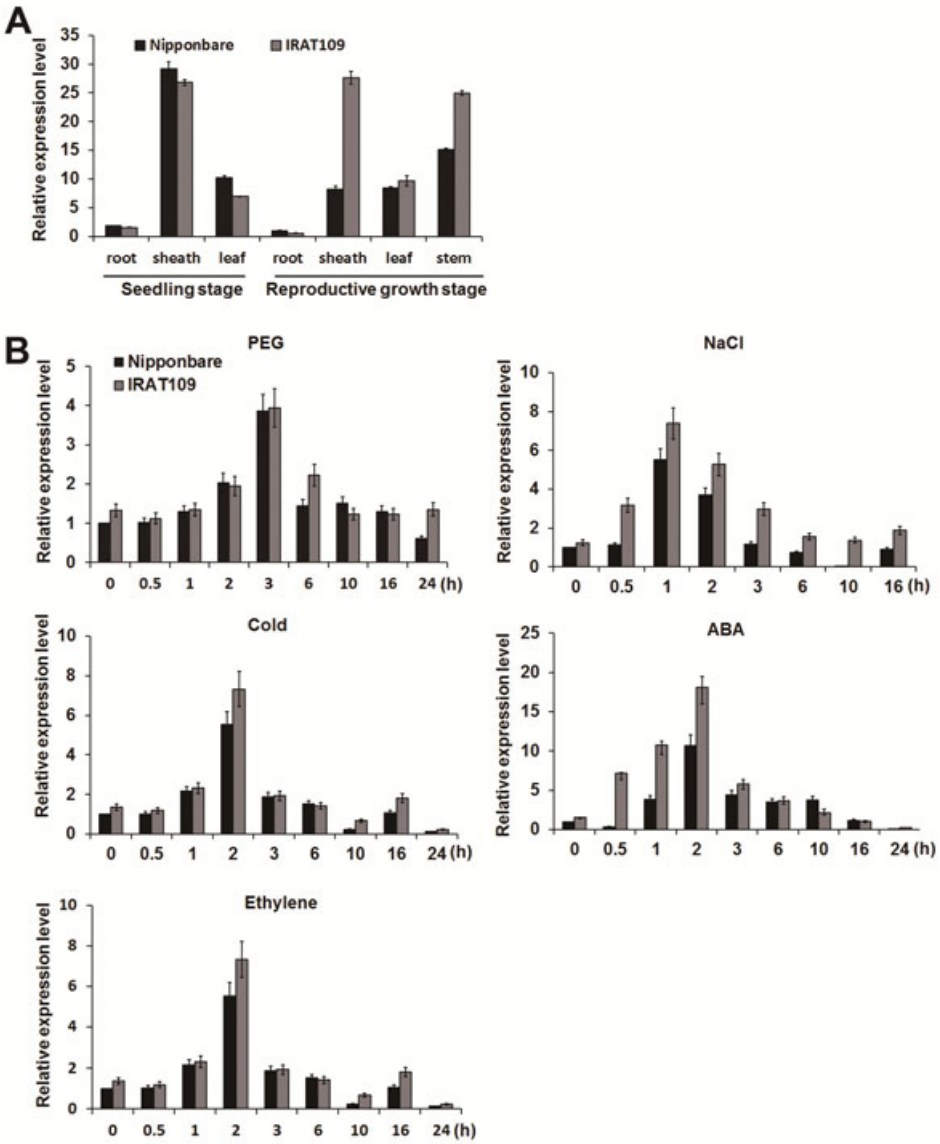

Fig.1 Expression analysis of the *OsASR5* gene. (A) Real-time PCR analysis of the expression level of *OsASR5* in different tissues of LR variety, Nipponbare and UR variety, IRAT109. (B) Stress-inducible expression of *OsASR5* under PEG, NaCl, Cold, ABA and Ethylene treatments. Error bars indicate standard error (SE) based on three replicates.

Fig.2

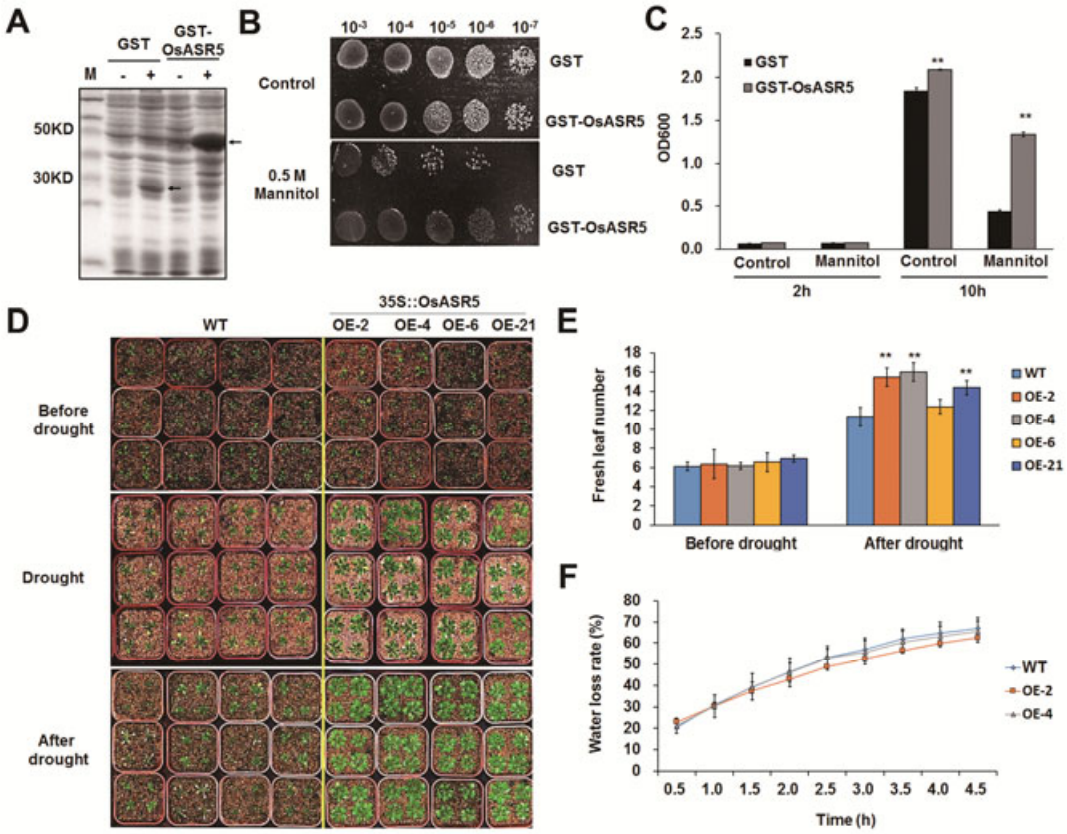

Fig.2 Enhanced osmotic and drought tolerance in *E.coli* and *Arabidopsis*. (A) Isopropylb-D-thiogalactopyranoside (IPTG) inducible expression of GST and GST-OsASR5 fusion proteins. GST and GST-OsASR5 were not (-) or were (+) induced by IPTG. Arrows indicate expression proteins. (B) Growth analysis of cells spotted on LB agar plate supplemented with 0.5 M mannitol. (C) Growth analysis of cells cultured in liquid medium supplemented with 0.5 M mannitol (n=3). Cell growth densities were measured at 600nm at the indicated time points. (D) Drought stress tolerance assay of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT by stopping irrigation for 3 weeks and recovery with re-watering for 4 days. (E) Fresh leaf numbers of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT before and after drought stress (n=3, 4 plants in each repeat). (F) Water loss rate in the detached leaves of *OsASR5* overexpression *Arabidopsis* transgenic lines and WT under normal conditions (n=3, 12 leaves in each repeat). Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

Fig.3

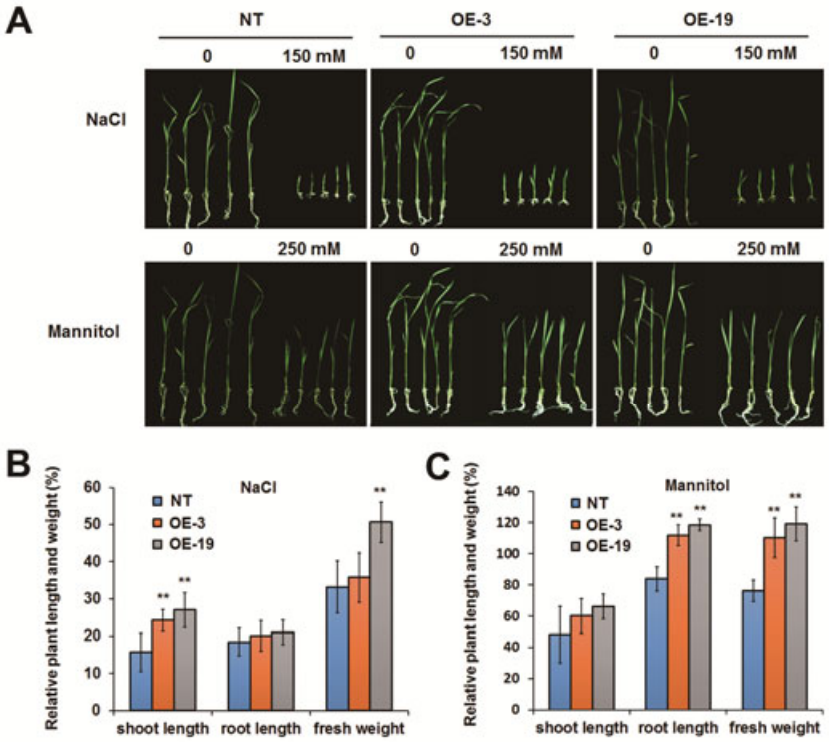

Fig.3 Increased osmotic tolerance of *OsASR5* overexpression plants. (A, B) Growth performance of *OsASR5* overexpression and NT seedlings under high salinity and mannitol treatments at 7 d after transplanting, respectively (*n*=3, 5 plants in each repeat). (C, D) The relative plant length and fresh weight of *OsASR5* overexpression and NT seedlings corresponding to A, B, respectively. Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

Fig.4

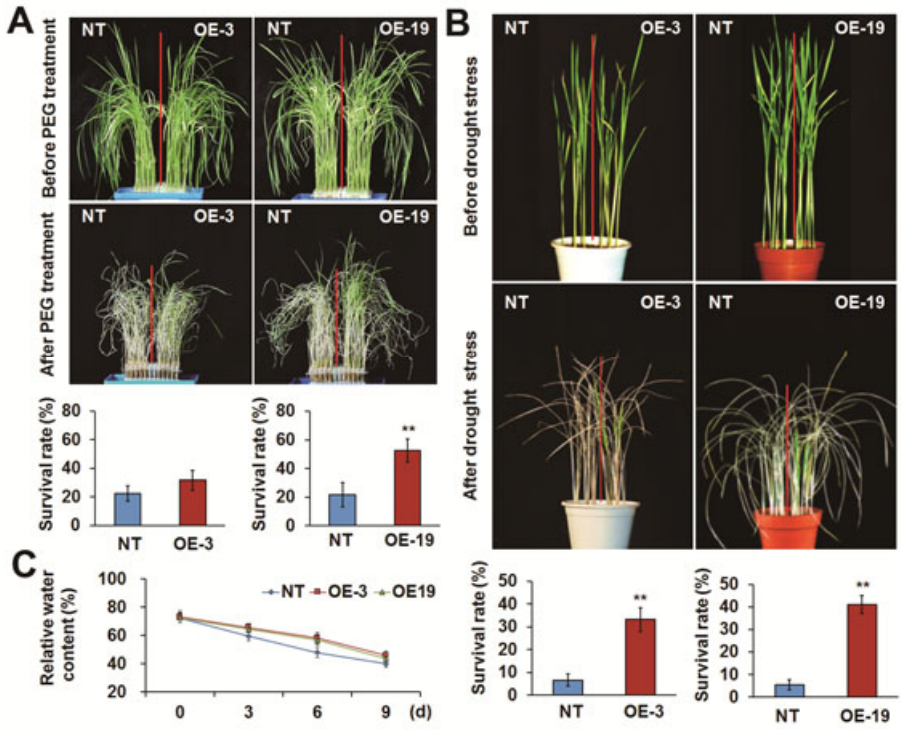

Fig.4 Enhanced drought tolerance of *OsASR5* overexpression plants. (A) Physiological dehydration stress tolerance assay of *OsASR5* overexpression and NT plants under 15% PEG6000 treatment. Survival rates of *OsASR5* overexpression and NT plants after dehydration stress were examined (n=3, 15 plants in each repeat). (B) Drought stress tolerance assay of *OsASR5* overexpression and NT plants by stopping irrigation for 1 week and recovery with re-watering for 2 weeks. Survival rates of *OsASR5* overexpression and NT plants after drought stress were examined (n=3, 9 plants in each repeat). (C) Relative water content of *OsASR5* overexpression and NT plants under 15% PEG6000 treatment at the indicated time points (n=3). Data are mean ±SE. ** indicates significant difference at $P<0.01$ probability.

Fig.5

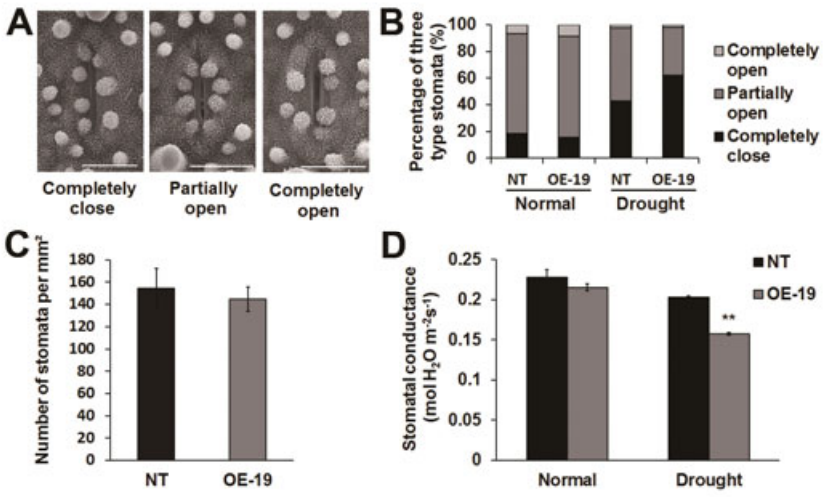

Fig.5 Overexpression of *OsASR5* increasing stomatal closure. (A) Scanning electron microscopy images of three levels of stomatal apertures. Bar, 5 μm. (B) The percentage of three levels of stomatal apertures in the leaves of *OsASR5* overexpression and NT plants under normal and drought stress conditions (*n*=300 stomata for NT under normal conditions; *n*=248 stomata for OE-19 under normal conditions; *n*=322 stomata for NT under drought stress; *n*=272 stomata for OE-19 under drought stress). (C) Stomatal density of the middle leaves of *OsASR5* overexpression and NT plants (*n*=3).Three random scopes were used in each repeat. (D) Stomatal conductance of *OsASR5* overexpression and NT plants (*n*=3). Data are mean ±SE. ** indicates significant difference at P<0.01 probability.

Fig.6

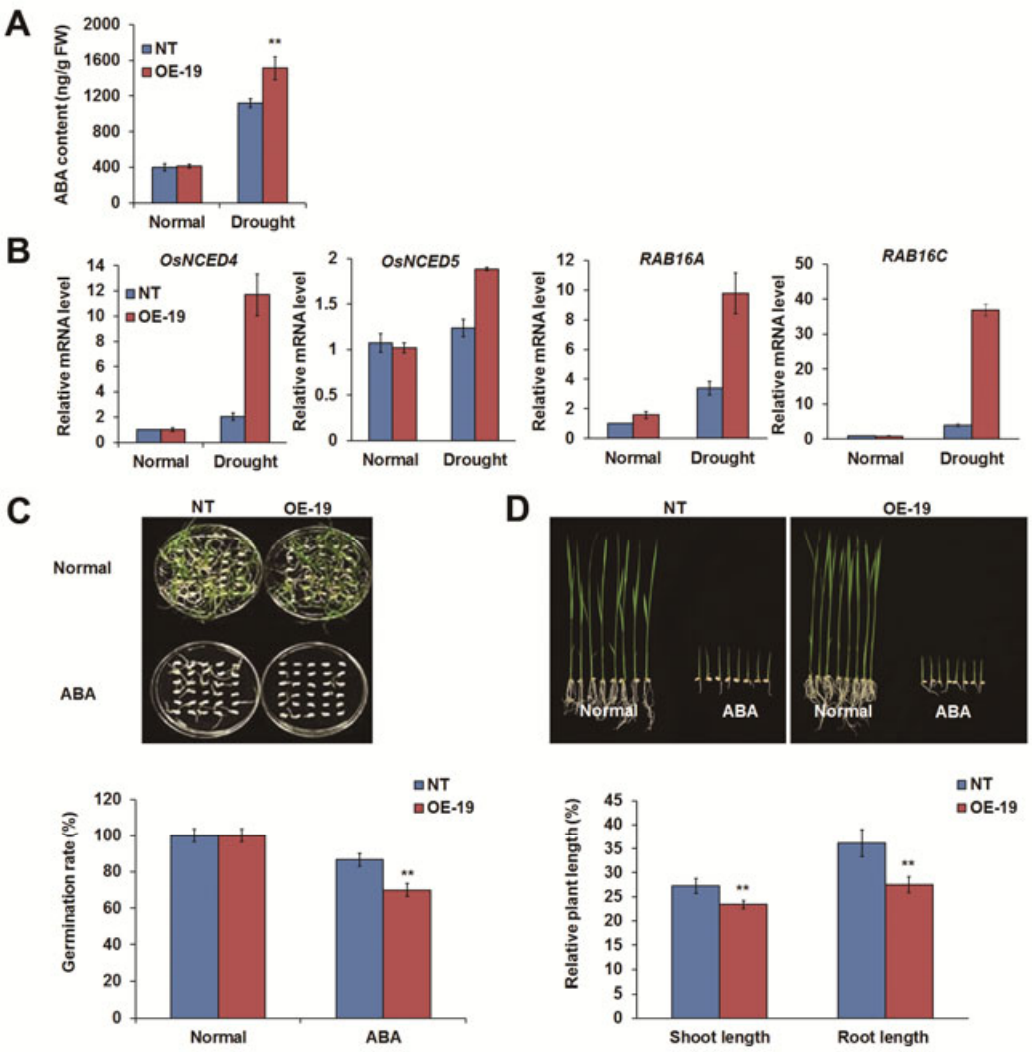

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Fig.7

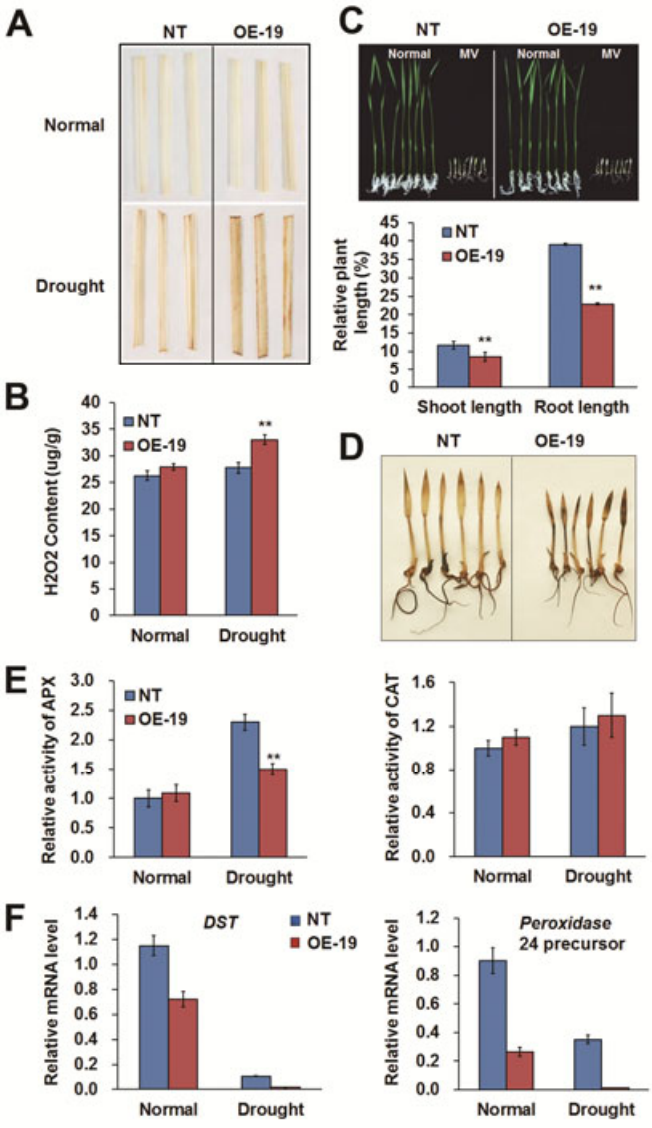

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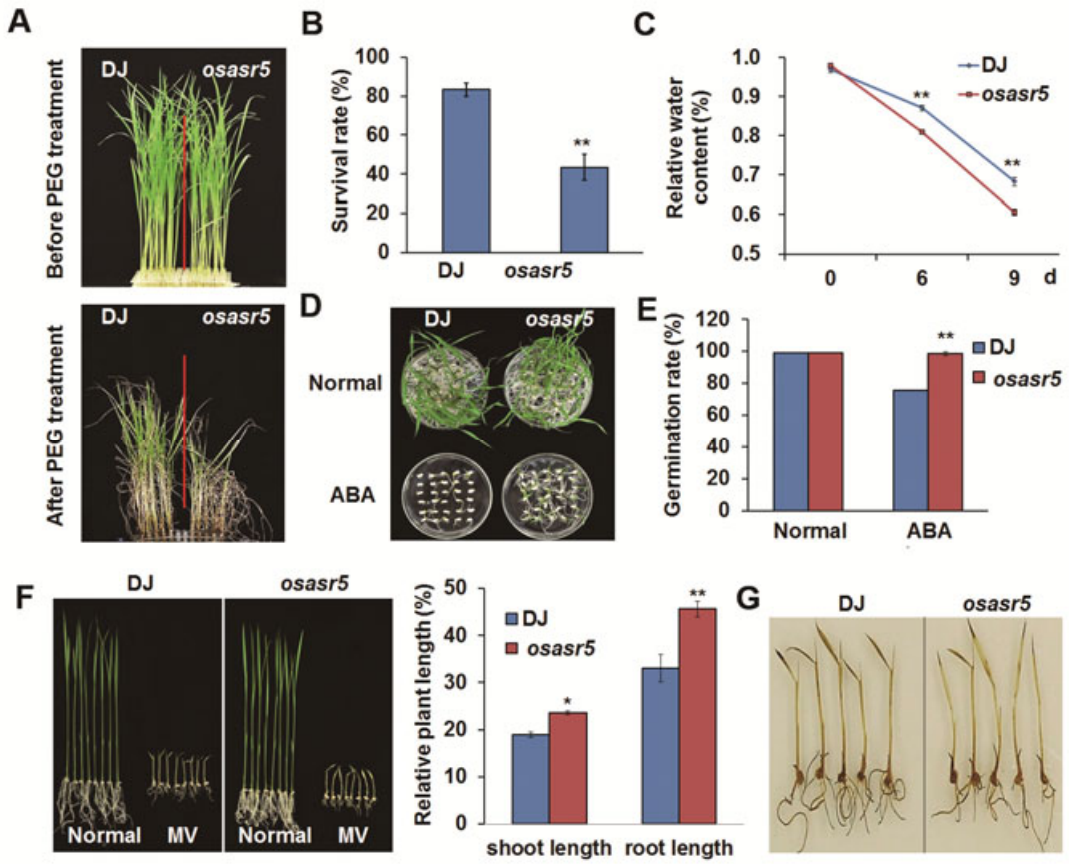
Fig.8



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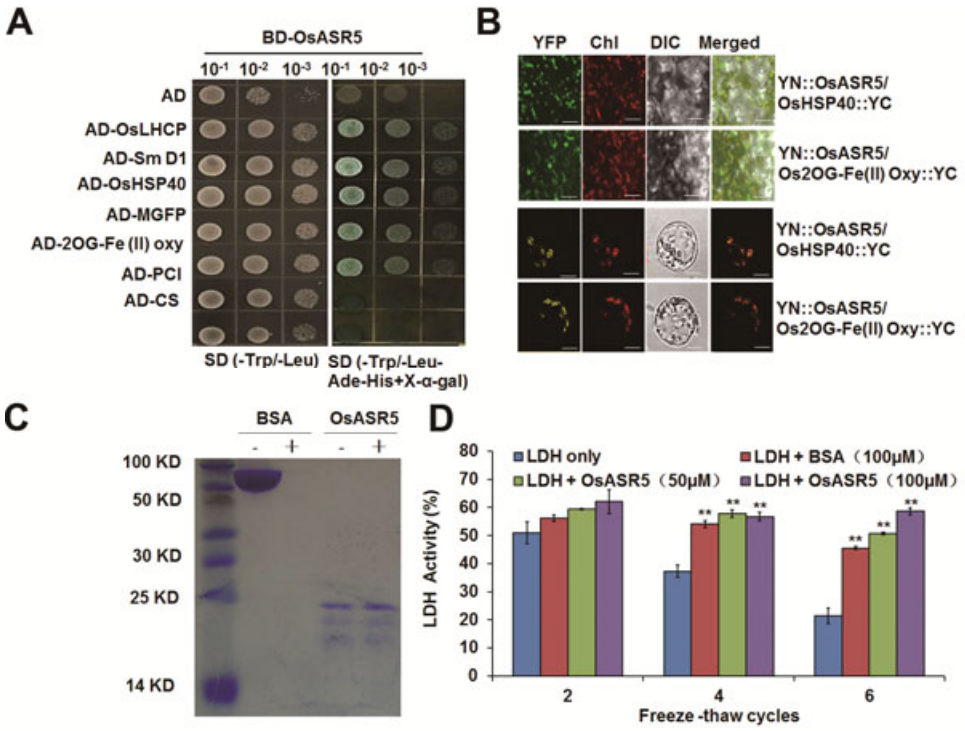

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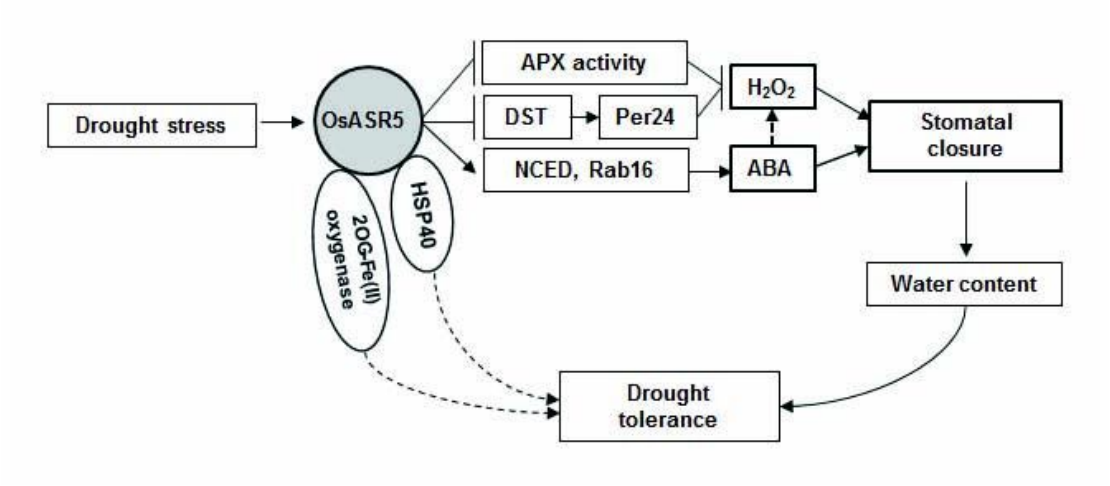
Fig.10



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