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Overexpression of the Rice SUMO E3 Ligase Gene *OsSIZ1* in Cotton Enhances Drought and Heat Tolerance, and Substantially Improves Fiber Yields in the Field under Reduced Irrigation and Rainfed Conditions

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Regular Paper

The Arabidopsis SUMO E3 ligase gene *AtSIZ1* plays important roles in plant response to abiotic stresses as loss of function in *AtSIZ1* leads to increased sensitivity to drought, heat and salt stresses. Overexpression of the *AtSIZ1* rice homolog, *OsSIZ1*, leads to increased heat and drought tolerance in bentgrass, suggesting that the function of the E3 ligase SIZ1 is highly conserved in plants and it plays a critical role in abiotic stress responses. To test the possibility that the SUMO E3 ligase could be used to engineer drought- and heat-tolerant crops, the rice gene *OsSIZ1* was overexpressed in cotton. We report here that overexpression of *OsSIZ1* in cotton results in higher net photosynthesis and better growth than wild-type cotton under drought and thermal stresses in growth chamber and greenhouse conditions. Additionally, this tolerance to abiotic stresses was correlated with higher fiber yield in both controlled-environment and field trials carried out under reduced irrigation and rainfed conditions. These results suggest that *OsSIZ1* is a viable candidate gene to improve crop yields under water-limited and rainfed agricultural production systems.

Keywords: Drought stress • Heat stress • SUMO E3 ligase • Sumoylation • Transgenic cotton.

Abbreviations: APX, ascorbate peroxidase; AtSIZ1, *Arabidopsis thaliana* SUMO E3 ligase 1; AVP1, Arabidopsis pyrophosphate-energized vacuolar membrane proton pump 1; GST; glutathione *S*-transferase; GWC, gravimetric water content; HSP, heat shock protein; OS-1, *OsSIZ1*-overexpressing line 1; OsSIZ1, *Oryza sativa* SUMO E3 ligase 1; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; SIZ1, SAP and Miz domain 1; SNT, segregated non-transgenic; SOD, superoxide dismutase; SUMO, small ubiquitin-like modifier; WT, wild type; WUE, water use efficiency.

Introduction

Abiotic stresses, in particular drought and heat, cause huge losses in crop productivity worldwide (Boyer 1982). Global climate change in terms of the rise in atmospheric temperature and infrequent rainfall patterns will further hinder sustainable agricultural crop production (Viola et al. 2010). Drought and heat stresses often occur simultaneously in nature, and the adverse effects of these two stresses are often more severe than the effects conferred by these stresses alone. In August 2000, loss of >US$4.2 billion revenue occurred in the USA due to the combined effects of heat and drought stresses (Mittler 2006).

Cotton (*Gossypium* sp.) is a very important textile fiber crop, which is produced in 76 countries covering >32 Mha worldwide (Singh et al. 2007). Cotton is also an important source of oil and livestock feed, even as low-protein food for humans in some poor countries (FAO 2005). Following India and China, the USA ranks third in cotton production in the world (USDA, National Agricultural Statistics Service, 2014/2015). However, cotton production in the USA as well as in the rest of the world has declined recently, and one of the main reasons for the decreased cotton production was the increasingly dry and hot environments world-wide. Therefore, there is an urgent need to develop cotton varieties that can not only survive in severe drought and heat conditions, but can also maintain high yields with minimal yield losses.

Recently several approaches have been used to improve drought tolerance in transgenic cotton. For example,

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overexpression of genes encoding heat shock proteins (HSPs; e.g. *GhHSP26*) by Maqbool et al. (2010), proton vacuolar pyrophosphatase (i.e. *AVP1*) by Pasapula et al. (2011), Arabidopsis transcription factors such as AtRAV1/2 and AtABI5 by Mittal et al. (2014) and the rice SNAC1 by Liu et al. (2014) has been shown to improve the performance of cotton plants under water-limiting conditions. Regulatory genes such as *LOS5* (encodes a molybdenum cofactor sulfurase responsible for aldehyde oxidase activity) by Yue et al. (2012) and isopentenyl transferase (a rate-limiting enzyme for cytokinin biosynthesis) by Kuppu et al. (2013) have also been used to improve the performance of cotton plants under water deficit conditions. However, there are no reports on improving heat tolerance in cotton through the genetic engineering approach.

In addition to the above-mentioned approaches, another way of achieving improved tolerance to abiotic stresses is by up-regulation of an important SUMO E3 ligase named OsSIZ1 (Li et al. 2013). This enzyme participates in a sumoylation reaction, a widely conserved post-translational modification in eukaryotes. Sumoylation is very similar to ubiquitination that involves activation of SUMO's C-terminus by the E1 activating enzyme, followed by its transfer through the E2 conjugating enzyme, and finally the ligation of SUMO protein to target protein substrates via the E3 ligase (Johnson 2004). SUMO is attached to the target protein with the help of an isopeptide bond formed between the C-terminal glycine of SUMO and the lysine residue found in the conserved motif of target proteins, i.e. ΨKXE (Ψ is a hydrophobic amino acid, mostly isoleucine or valine, and X can be any residue) (Hilgarth et al. 2004). Attachment of SUMO to target protein substrates in turn is known to modulate protein activity, stability, subcellular localization and a plant's response to various environmental stresses (Xu and Yang 2013).

Of all the components of the sumoylation reaction, the SUMO E3 ligase has been extensively studied in Arabidopsis by forward genetics and reverse genetic approaches (Novatchkova 2012). To date, the SUMO E3 ligase, e.g. SIZ1, has been identified in mammals, yeast and several plant species. In Arabidopsis, there is only one *SIZ1* gene, i.e. *AtSIZ1*. However in rice, two *AtSIZ1* homologs were found, *OsSIZ1* and *OsSIZ2*. The OsSIZ1 and AtSIZ1 proteins share 51% identity at the amino acid level (Park et al. 2010). The SUMO E3 ligase is known to regulate various aspects of plant growth and development such as ABA signaling (Miura et al. 2009), innate immunity through salicylic acid signaling (Miura and Hasegawa 2010), nitrogen assimilation (Park et al. 2011), spikelet fertility in rice (Thangasamy et al. 2011) and flowering (Jin et al. 2008). Besides its roles in regulating plant growth and development, Arabidopsis SIZ1 was shown to play roles in stress tolerance against various environmental stresses such as low temperature stress via sumoylation and stabilization of the ICE1 transcription factor (Miura et al. 2007b), heat stress (Kurepa et al. 2003, Yoo et al. 2006, Saracco et al. 2007), water deficit stress (Catala et al. 2007), phosphate starvation stress (Miura et al. 2005) and metal stress such as copper stress (Chen et al. 2011). Although, *OsSIZ1* has been studied in bentgrass (Li et al. 2013), its roles in protecting other plants, expecially a crop plant like cotton, were not known. To test if *OsSIZ1* could be used to engineer multistress-tolerant crops, *OsSIZ1* was introduced into wild-type (WT) cotton plants and thorough analyses were conducted to study how *OsSIZ1*-transgenic cotton plants would respond to increased water deficit conditions, higher than normal temperature treatment and combined heat stress and drought stress conditions. Furthermore, we analyzed the performance of *OsSIZ1*-transgenic cotton plants and control plants under limited irrigation and rainfed conditions in the field. All of our findings strongly suggest that the SUMO E3 ligase SIZ1 plays a critical role in plant response to environmental stresses, and *OsSIZ1* has a great potential to be used for improving stress tolerance in crops.

Results

Creation and molecular analysis of *OsSIZ1*-transgenic cotton plants

The rice SUMO E3 ligase gene *OsSIZ1* fused to the maize ubiquitin promoter and nopaline synthase terminator was cloned into the pBI121 based binary vector (Li et al. 2013), which was used for cotton transformation. A total of 40 independent transgenic lines were obtained by using the *Agrobacterium*-mediated transformation method (Bayley et al. 1992). DNAs isolated from the T_1 plants were used in the PCR analysis for the presence of the *OsSIZ1* gene. It appeared that all putative transgenic plants contained *OsSIZ1*. An example of the PCR analyses is shown in **Fig. 1A**. The selected PCR-positive lines were used for RNA blot analysis, and again we found that all PCR-positive lines also contained the *OsSIZ1* transcript. One RNA blot is shown in **Fig. 1B**. From the RNA blot analysis data, we identified four 'high expression' lines, i.e. OS-1, OS-3, OS-5 and OS-6, and propagated these four lines to the T_3 and T_4 generations. We also identified a non-transgenic line among the segregated lines of the OS-1 lineage in the T_4 generation, and we named this line SNT (segregated non-transgenic). Then we isolated genomic DNAs from these four *OsSIZ1*-transgenic lines and DNAs from WT and SNT for DNA blot analysis. We found that OS-1, OS-3 and OS-5 are probably single T-DNA insertion lines, OS-6 might be a two T-DNA insertion line, and WT and SNT plants do not contain the *OsSIZ1* transgene (**Fig. 1C**). The homozygous plants from these lines were chosen for physiological analyses in greenhouse, growth chamber and field studies.

OsSIZ1 overexpression in cotton leads to increased heat tolerance

To test the performance of *OsSIZ1*-transgenic plants under high temperature conditions, plants from these four independent transgenic lines, the SNT and the WT were grown in an environmentally controlled growth chamber. Plants were grown under optimal conditions (28/22°C day/night with a 16/8 h light/dark cycle) for 4 weeks, followed by growth to maturity under mid-day heat stress conditions (ramping temperature to 37°C from 12:00 to 16:00 h). Prior to the implementation of the heat stress, no phenotypic differences were observed between control plants (SNT and WT) and *OsSIZ1*-transgenic plants (**Fig. 2A**). After heat treatment for 45 d, transgenic plants

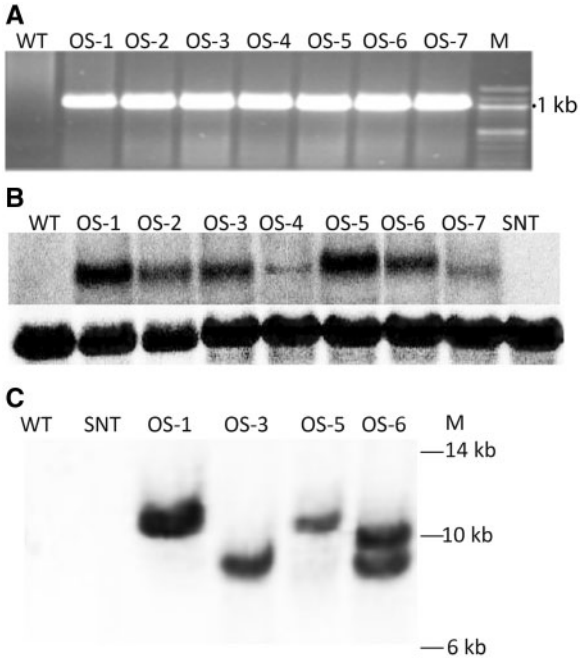

Fig. 1 Molecular analysis of *OsSIZ1*-transgenic cotton. (A) PCR analysis of *OsSIZ1*-transgenic cotton. The *OsSIZ1* gene-specific primers OsSIZ1-F1 and OsSIZ1-B1 were used in the PCR. (B) RNA blot analysis of *OsSIZ1*-transgenic cotton. The transcript of *GhUBQ7* (i.e. cotton ubiquitin gene 7) was used as the loading control (lower lane). (C) DNA blot analysis of four selected *OsSIZ1*-transgenic cotton lines. The restriction enzyme *Eco*RI was used to digest the genomic DNAs of all samples. WT, wild-type; SNT, segregated non-transgenic; OS-1 to OS-7, independent *OsSIZ1*-transgenic cotton lines; M, DNA markers in kilobases.

Fig. 2 Phenotypes of wild-type and *OsSIZ1*-transgenic cotton plants before and after heat treatment. (A) Before heat treatment. Plants were grown under normal conditions for a month. (B) Plants were treated with heat stress (37°C for 4 h d^{-1}) for 45 d before the photograph was taken. WT, wild-type; OS-1 and OS-3, two independent *OsSIZ1*-transgenic lines.

grew taller and larger (**Fig. 2B**), producing on an average 3–4 more nodes than WT plants (Supplementary Tabls S1). Leaf-level gas exchange was measured between 14:00 and 16:00 h after growth for 38 d under elevated afternoon temperatures (**Fig. 3**). Net photosynthesis was at least 16% higher in *OsSIZ1*-transgenic plants compared with WT and SNT plants (**Fig. 3A**). Although net photosynthesis decreased in both transgenic and non-transgenic plants under heat stress, the decline in *OsSIZ1*-transgenic plants was approximately 34% and in non-transgenic plants it was 44%, relative to non-stressed plants grown at 28°C day temperatures (**Fig. 3A**). The increased growth (**Fig. 2B**) and maintenance of net photosynthesis (**Fig. 3A**) were correlated with an increase in boll number (**Fig. 3B**) and fiber yield (**Fig. 3C**) in *OsSIZ1*-transgenic plants compared with non-transgenic plants. No significant differences were observed between transgenic and non-transgenic plants grown under control conditions (**Fig. 3**, black bars).

OsSIZ1 overexpression in cotton leads to increased drought tolerance

To test the performance of *OsSIZ1*-transgenic plants under drought stress conditions, plants from these four independent transgenic lines, the SNT and the WT were grown in an environmentally controlled growth chamber. Plants were grown under optimal conditions (28/22°C day/night with a 16/8 h light/dark cycle) for 4 weeks. Plants were then divided into two groups of 10 plants for each independent transgenic line and non-transgenic line: (i) full irrigation—pots were irrigated to field capacity every other day; and (ii) deficit irrigation—pots were irrigated with 400 ml every other day (~50% soil gravimetric water content; GWC). No phenotypic or physiological differences were observed between *OsSIZ1*-transgenic and non-transgenic plants grown under full irrigation treatment for the duration of the experiment (**Fig. 4**, black bars). However, under deficit irrigation treatment, *OsSIZ1*-transgenic plants displayed at least 34% higher photosynthetic rates than non-transgenic plants (**Fig. 4A**). Deficit irrigation resulted in a 61% mean decline in net photosynthesis in non-transgenic plants compared with a 43% decline in *OsSIZ1*-transgenic plants (**Fig. 4A**). Similar to performance under elevated day temperatures, *OsSIZ1*-transgenic plants produced significantly more bolls and higher fiber yields than non-transgenic plants (**Fig. 4B, C**). On average, deficit irrigation resulted in a 29% decrease in fiber yield compared with full irrigation for *OsSIZ1*-transgenic plants and a 49% decrease in fiber yield compared with full irrigation in non-transgenic plants (**Fig. 4C**). When grown under deficit irrigation, *OsSIZ1*-transgenic plants produced 30% more fiber than non-transgenic plants (**Fig. 4C**). Additionally, *OsSIZ1*-transgenic plants had a significantly higher total root biomass compared with non-transgenic plants grown under deficit irrigation (**Figs. 5A, B**).

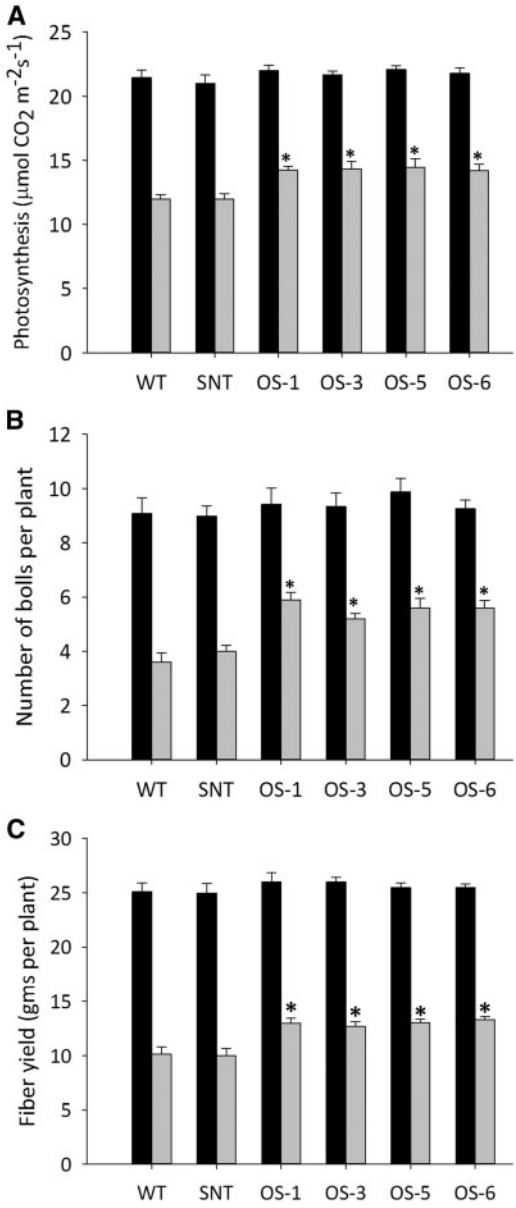

Fig. 3 Performances of control and *OsSIZ1*-transgenic cotton plants under heat stress conditions. (A) Photosynthetic performance of cotton plants under heat stress conditions. WT, wild-type; SNT, segregated non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1*-transgenic lines; black columns, photosynthetic rates under normal growth condition (i.e. 28°C daytime temperature); gray columns, photosynthetic rates under heat stress conditions (i.e. 37°C for 4 h during the daytime). Results shown are the means ± SE ($n = 7$); *, statistically significant at 5%. (B) Boll numbers of control and *OsSIZ1*-transgenic cotton plants after heat stress treatment. Black columns, boll number per plant under normal growth conditions; gray columns, boll number per plant after heat stress treatment. (C) Fiber yields of control and *OsSIZ1*-transgenic cotton plants after heat stress treatment. Black columns, fiber yields per plant under normal growth conditions; gray columns, fiber yield per plant after heat stress treatment.

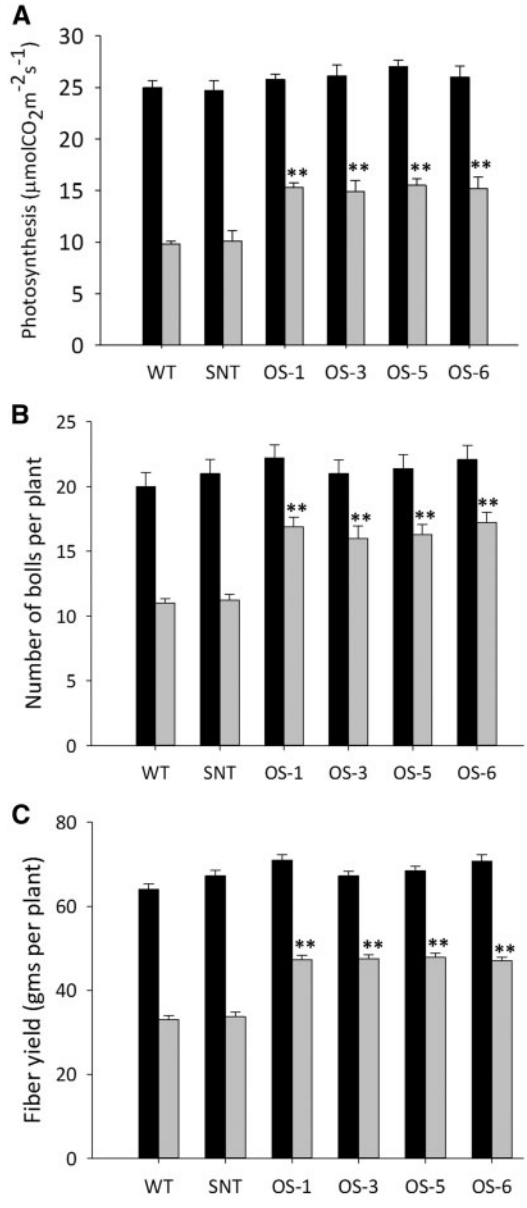

Fig. 4 Performances of control and *OsSIZ1*-transgenic cotton plants under reduced irrigation conditions. (A) Photosynthetic performance of cotton plants under reduced irrigation conditions. WT, wild-type; SNT, segregated non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1*-transgenic lines; black columns, photosynthetic rates under normal irrigation conditions; gray columns, photosynthetic rates under reduced irrigation conditions. Results shown are the means ± SE ($n = 10$); **, statistically significant at 1%. (B) Boll numbers per plant of cotton plants under reduced irrigation conditions. Black columns, boll numbers per plant under normal irrigation conditions; gray columns, boll numbers per plant under reduced irrigation conditions. (C) Fiber yields per plant of cotton plants under reduced irrigation conditions. Black columns, fiber yields per plant under normal irrigation conditions; gray columns, fiber yields per plant under reduced irrigation conditions.

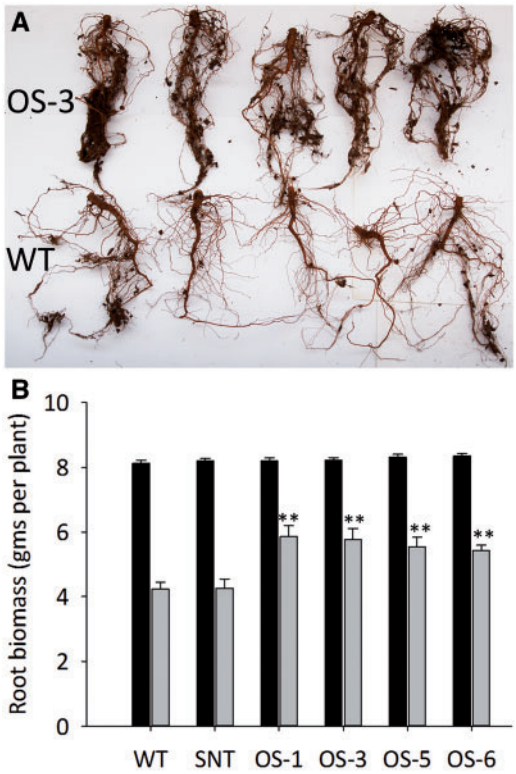

Fig. 5 Root phenotypes and biomasses of control and *OsSIZ1*-transgenic plants after reduced irrigation treatment. (A) Root phenotypes of wild-type and the *OsSIZ1*-transgenic line OS-3 after reduced irrigation treatment. (B) Biomass analyses of control and *OsSIZ1*-transgenic plants after reduced irrigation treatment. WT, wild-type; SNT, segregated non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1*-transgenic lines; black columns, root biomass of plants grown under normal irrigation conditions; gray columns, root biomass of plants grown reduced irrigation conditions Results shown are the means ± SE (n = 10); **, statistically significant at 1%.

OsSIZ1 overexpression in cotton leads to increased water use efficiency

As an additional test for plant water use and drought tolerance, we measured leaf relative water content (RWC) and water use efficiency (WUE) in *OsSIZ1*-transgenic and non-transgenic plants. **Fig. 6A** shows that *OsSIZ1*-transgenic plants maintained a higher leaf RWC compared with non-transgenic plants during the course of the deficit irrigation treatment described above. Further, *OsSIZ1*-transgenic plants showed significant increases in WUE and biomass accumulation under optimal temperature conditions (28/22°C day/night with a 16/8 h light/dark cycle) in the glasshouse (**Figs. 6B, C**). We found that *OsSIZ1*-transgenic plants displayed at least 18% higher WUE compared with non-transgenic plants, and the time to reach permanent wilt was extended to at least 10 d under these growth conditions (**Fig. 6C**).

OsSIZ1 overexpression in cotton leads to improved performance under combined heat stress and deficit irrigation

To test the performance of *OsSIZ1*-transgenic cotton plants under simultaneous exposure to heat stress and deficit

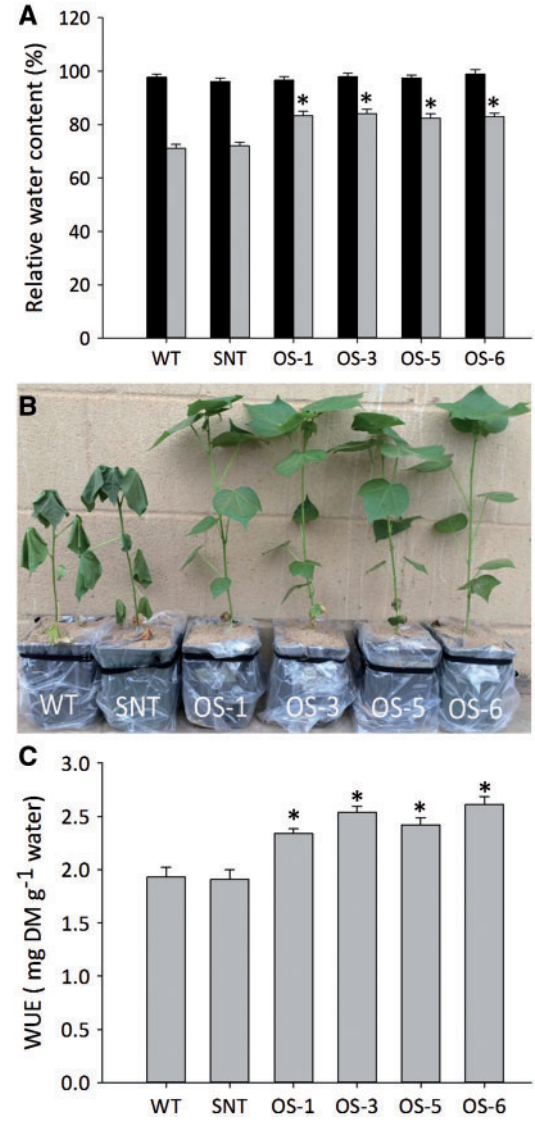

Fig. 6 Relative water contents, phenotypes and water use efficiency of control and *OsSIZ1*-transgenic plants under water deficit conditions. (A) Relative water contents in leaves of control and *OsSIZ1*-transgenic plants grown under normal irrigation and reduced irrigation conditions. (B) Phenotype of control and *OsSIZ1*-transgenic plants 21 d after an equal amount of water was initially used to irrigate plants. WT, wild-type; SNT, segregated non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1*-transgenic lines. (C) Water use efficiency of control and *OsSIZ1*-transgenic plants in amount of biomass (in milligrams) produced per gram of water used. Results shown are the means ± SE (n = 10); *, statistically significant at 5%.

irrigation, we grew plants under optimal growth conditions for 4 weeks (described above), followed by growth to maturity under: (i) heat stress—full irrigation and growth temperature increased to 37°C from 13:00 to 15:00 h; and (ii) heat stress with deficit irrigation—approximately 50% soil GWC and growth temperature increased to 37°C from 13:00 to 15:00 h. Similar to the experiments described previously, we measured net photosynthesis 35 d after stress treatments were started, as well as the number of bolls per plant and total fiber yield

(**Fig. 7**). For growth under heat stress conditions (**Fig. 7**, black bars), *OsSIZ1*-transgenic plants outperformed non-transgenic plants with respect to net photosynthesis, boll production and fiber yield. Growth under combined heat stress and reduced irrigation had a greater impact than heat stress or deficit irrigation alone; however, *OsSIZ1*-transgenic plants showed significantly higher net photosynthesis, boll production and fiber yield than non-transgenic plants (**Fig. 7**, gray bars). These data show that *OsSIZ1*-transgenic plants are significantly more tolerant to heat stress that is likely to occur under drought conditions in the field, compared with non-transgenic plants.

OsSIZ1 overexpression in cotton leads to improved performance under field conditions

To test the performance of *OsSIZ1*-transgenic cotton plants under production conditions, field trials were conducted in 2014 and 2015 at the USDA-ARS Cropping Systems Research Laboratory in Lubbock, Texas. For 2014 field trials, plants were grown under high irrigation (15 mm per week) and low irrigation (7.5 mm per week). Similar to the experiments described above, mid-day net photosynthesis was measured during peak boll setting in the first week of August 2014, between 11:00 and 13:00 h. **Fig. 8A** shows that *OsSIZ1*-transgenic lines displayed at least 29% higher photosynthetic rates than non-transgenic plants under low irrigation conditions and there were no significant differences in net photosynthesis under high irrigation treatment. At the end of the growth season, *OsSIZ1*-transgenic lines produced at least 32% more bolls (**Fig. 8B**) and had 32% higher fiber yields (**Fig. 8C**) than non-transgenic plants under low irrigation. No significant differences were found under high irrigation conditions (**Fig. 8B, C**, black bars).

For the 2015 field trials, the low irrigation treatment was replaced with a rainfed treatment (no applied irrigation). Late-day (14:00–16:00 h) net photosynthesis was measured in the first week of August. Similar to our previous experiments, there were no significant differences in net photosynthesis, boll production or fiber yield under the high irrigation treatment. Overall, the rainfed treatment appeared to have a much larger effect on plant performance for 2015 than previous experiments, but *OsSIZ1*-transgenic plants still outperformed non-transgenic plants under rainfed conditions, showing significantly higher late-day net photosynthesis (**Fig. 9A**, gray bars), 35% higher boll production (**Fig. 9B**, gray bars) and 21% higher fiber yield (**Fig. 9C**, gray bar).

OsSIZ1-transgenic cotton plants display higher photosynthetic capacity and rate of carboxylation at saturating CO_2 concentrations under water deficit conditions

Under rainfed conditions, *OsSIZ1*-transgenic plants performed significantly better than control plants by displaying higher photosynthetic rates under increasing carbon dioxide concentrations until saturation (**Fig. 10A**); however, no significant differences were observed between transgenic and control plants grown under high irrigation conditions (Supplementary Fig.

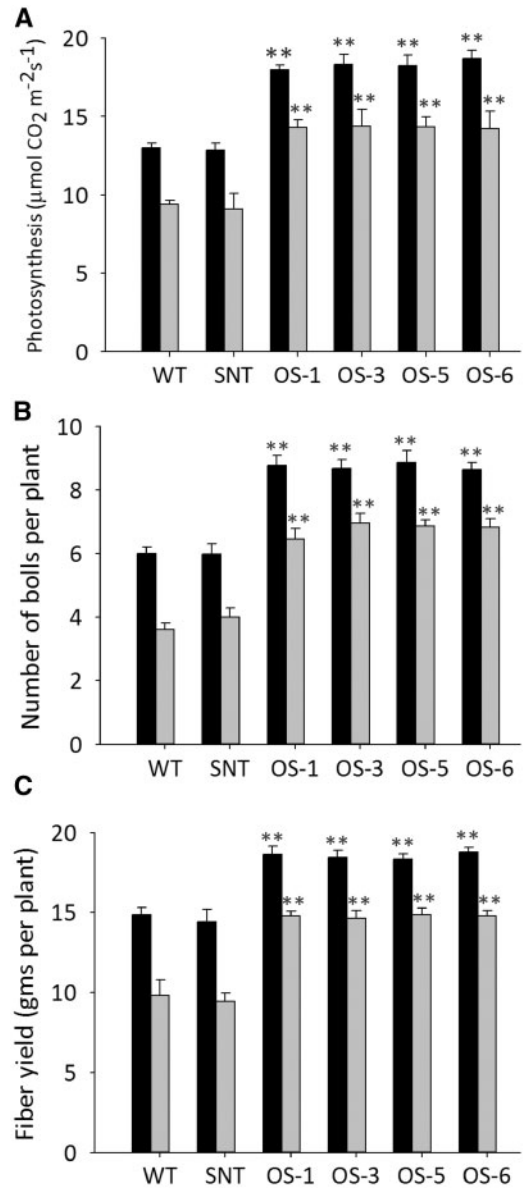

Fig. 7 Performances of control and *OsSIZ1*-transgenic cotton plants under heat and heat plus reduced irrigation conditions. (A) Photosynthetic rates of control and *OsSIZ1*-transgenic cotton plants under heat and heat plus reduced irrigation conditions. WT, wild-type; SNT, segregated non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1*-transgenic lines; black columns, photosynthetic rates of plants grown under heat treatment (i.e. 37°C for 2 h during the day); gray columns, photosynthetic rates of plants grown under heat plus reduced irrigation conditions (i.e. 37°C for 2 h during the day plus reduced irrigation). Results shown are the means ± SE ($n = 7$); **, statistically significant at 1%. (B) Boll numbers per plant of control and *OsSIZ1*-transgenic cotton plants under heat and heat plus reduced irrigation conditions. Black columns, boll numbers of plants grown under heat treatment; gray columns, boll numbers of plants grown under heat plus reduced irrigation conditions. Results shown are the means ± SE ($n = 7$). (C) Fiber yields per plant for control and *OsSIZ1*-transgenic cotton plants under heat and heat plus reduced irrigation conditions. Black columns, fiber yields of plants grown under heat treatment; gray columns, fiber yields of plants grown under heat plus reduced irrigation conditions. Results shown are the means ± SE ($n = 7$).

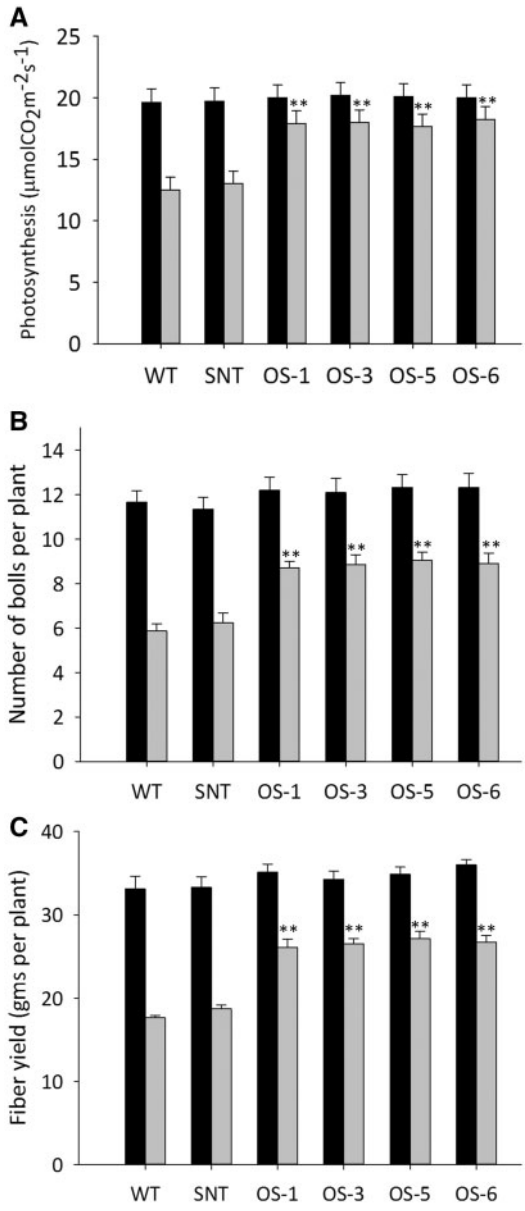

Fig. 8 Performances of *OsSIZ1*-transgenic plants as compared with wild-type plants under field conditions in 2014. (A) Photosynthetic rates of wild-type and *OsSIZ1*-transgenic plants under irrigation and limited irrigation conditions. (B) Number of bolls per plant in wild-type and *OsSIZ1*-transgenic plants under irrigation and limited irrigation conditions. (C) Fiber yields per plant in wild-type and *OsSIZ1*-transgenic plants under irrigation and limited irrigation conditions. Black bars, irrigation condition; gray bars, limited irrigation condition. WT, wild-type; SNT, segregating non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1*-transgenic plants. Results shown are the means ± SE; **, statistically significant at 1%.

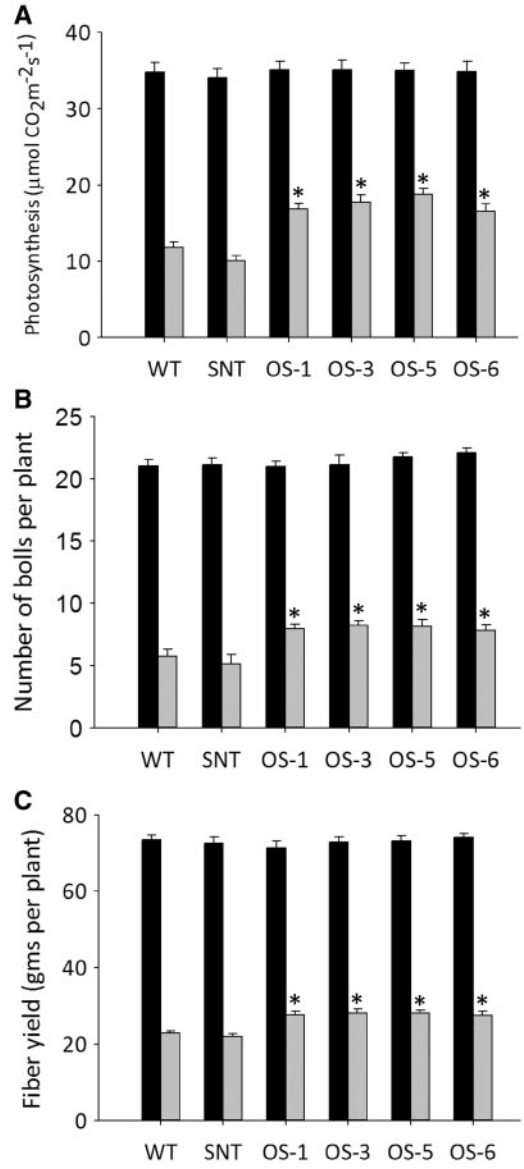

Fig. 9 Performances of *OsSIZ1*-transgenic plants as compared with wild-type plants under field conditions in 2015. (A) Photosynthetic rates of wild-type and *OsSIZ1*-transgenic plants under irrigation and dryland conditions. (B) Number of bolls per plant in wild-type and *OsSIZ1*-transgenic plants under irrigation and dryland conditions. (C) Fiber yields per plant in wild-type and *OsSIZ1*-transgenic plants under irrigation and dryland conditions. Black bars represent the irrigation condition while gray bars represent the dryland condition. WT, wild-type; SNT, segregating non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1* transgenic plants. Results shown are the means ± SE; *, statistically significant at 5%.

S1). Under rainfed conditions, V_{cmax} values for *OsSIZ1*-transgenic plants were not significantly different from those of control plants (**Fig. 10B**), but significant differences were observed between J_{max} values for *OsSIZ1*-transgenic and control plants (**Fig. 10C**). Therefore, it appears that the higher rate of ribulose bisphosphate regeneration, not the higher activity of Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase), might be the reason for better net photosynthesis of *OsSIZ1*-transgenic plants under rainfed conditions.

OsSIZ1 overexpression in cotton leads to increased expression of several stress-related genes

Plant response to dehydration may involve both ABA-dependent pathways and ABA-independent pathways, and therefore may involve the transcription factor DREB2A- or

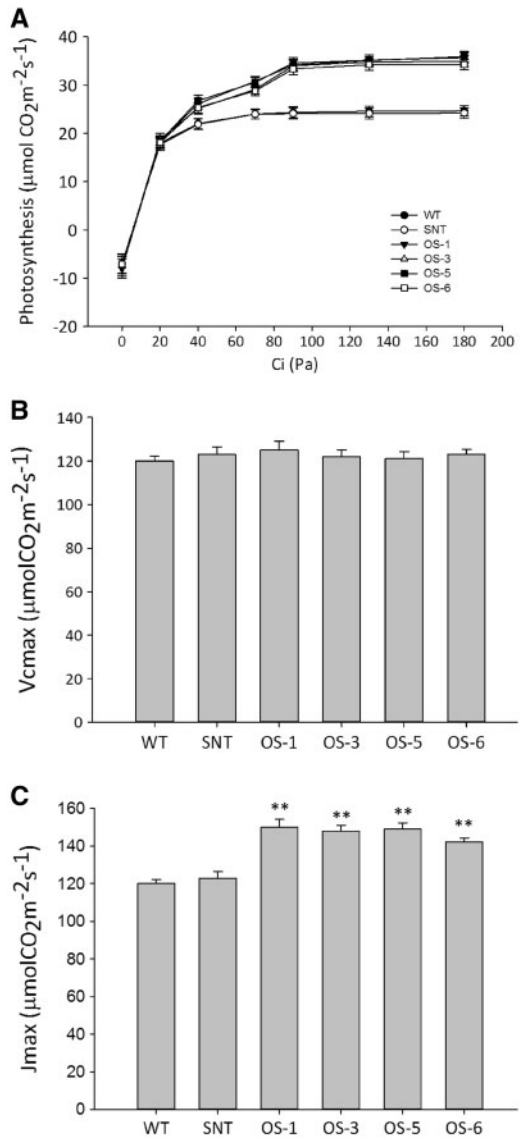

Fig. 10 Photosynthetic rates, V_{cmax} and J_{max} of control and *OsSIZ1*-transgenic plants under rainfed condition in the field. (A) Photosynthetic rates at different CO_2 concentrations (i.e. A/C_i curve). (B) Maximal rates of carboxylation (V_{cmax}) of control and *OsSIZ1*-transgenic plants. (C) Maximal rates of electron transport (J_{max}) of control and *OsSIZ1*-transgenic plants. WT, wild-type; SNT, segregated non-transgenic; OS-1, OS-3, OS-5 and OS-6, four independent *OsSIZ1*-transgenic plants. **, Statistically significant at 1%.

ERD1-mediated gene expression (Shinozaki et al. 2003, Sakuma et al. 2006, Shinozaki and Yamaguchi-Shinozaki 2007). Catala et al. (2007) reported that SIZ1 might regulate drought stress response through the ABA-dependent pathways. Therefore, we analyzed the expression of *GhRD22* that encodes a dehydration-responsive protein and *GhDREB* that encodes a dehydration-responsive element-binding protein under heat, reduced irrigation and combined heat and reduced irrigation conditions using quantitative real-time PCR. We found that the *GhRD22* transcript was significantly induced in both control and *OsSIZ1*-transgenic plants under reduced irrigation and heat plus reduced irrigation conditions. The transcript of *GhRD22* was found to be around 1.5- and 1.6 fold higher in *OsSIZ1*-transgenic plants than in control plants under reduced irrigation and heat plus reduced irrigation conditions, respectively (Supplementary Fig. S2A). The *GhRD22* transcript was not up-regulated under control and heat stress conditions (Supplementary Fig. S2A). To test if the response of these plants to dehydration is mediated through the ABA-independent pathways, *GhDREB* transcript was also analyzed under these conditions. We found that, similarly to *GhRD22*, the *GhDREB* transcript was also induced under reduced irrigation and combined heat and reduced irrigation conditions, but not under normal growth and heat stress conditions (Supplementary Fig. S2B). However, no significant differences were observed in the *GhDREB* transcript between control and *OsSIZ1*-transgenic plants under all stress conditions (Supplementary Fig. S2B). It appears that the *OsSIZ1*-mediated stress response might involve the ABA-dependent pathways, not the ABA-independent pathway.

Plant HSPs are produced in response to heat stress, and their production is critical for plants to acquire heat tolerance (Vierling 1991), as many of those HSPs are molecular chaperones that protect plants against cellular damage caused by heat stress. To evaluate the potential involvement of HSPs in stress tolerance in *OsSIZ1*-transgenic cotton plants, the transcripts of two cotton HSP genes, *GhHSP96* and *GhHSP100*, were analyzed in control and *OsSIZ1*-transgenic plants. We found that the *GhHSP96* transcript was up-regulated in both control and *OsSIZ1*-transgenic plants under heat, reduced irrigation and heat plus reduced irrigation conditions (Supplementary Fig. S3A). The transcript level of *GhHSP96* in *OsSIZ1*-transgenic plants was 1.83-, 2.6- and 2.4-fold higher than in control plants under heat, reduced irrigation and heat plus reduced irrigation conditions, respectively. The combined stresses of heat and reduced irrigation caused the highest level of the *GhHSP96* transcript, a likely reason why *OsSIZ1*-transgenic plants performed the best under combined stresses of heat and reduced irrigation. Although *GhHSP100* transcript was induced by both drought and heat stress (as expected), no significant differences were found between control and *OsSIZ1*-transgenic plants under these stress conditions (Supplementary Fig. S3B). It appears that overexpression of *OsSIZ1* leads to increased transcripts of HSP genes such as *GhHSP96* in transgenic plants, which might contribute to the increased tolerance to heat and heat plus reduced irrigation conditions.

A major consequence of heat stress or water deficit stress is the production of reactive oxygen species (ROS), which are highly toxic to cells, as ROS can adversely react with lipids, DNA and proteins, and thereby damage cellular components (Hossain et al. 2009). Higher plants possess the ability to remove ROS enzymatically through the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione *S*-transferase, (GST) or non-enzymatically via direct reaction of ROS with ascorbate and reduced glutathione (Bowler et al. 1992). We tested if the better performance of *OsSIZ1*-transgenic plants under water deficit and high temperature conditions was partly due to the higher expression levels of their antioxidant genes. Indeed we found that *OsSIZ1*-transgenic plants showed

significantly higher transcript levels of *GhGST* (~1.2-fold higher) and *GhSOD* (~1.5-fold higher) under heat stress conditions (Supplementary Fig. 4A, B). Under water deficit and water deficit plus heat stress conditions, *OsSIZ1*-transgenic plants showed higher transcript levels of *GhGST* (~2.1- and 2.0-fold higher, respectively), *GhSOD* (~1.8- and 1.9-fold higher, respectively) and *GhAPX* (~2.1- and 2.2-fold higher, respectively) than control plants (Supplementary Fig. 4A–C).

Discussion

In this report we demonstrated that overexpression of the rice SUMO E3 ligase gene *OsSIZ1* in cotton significantly increases tolerance to heat stress, deficit irrigation stress and the combined effects of heat stress and water deficit stress in controlled-environment and field trial experiments. The enhanced growth and yield were concurrent with increased root biomass, increased whole-plant WUE and increased expression of key stress-responsive genes in *OsSIZ1*-transgenic cotton plants compared with non-transgenic plants. The molecular mechanism for the increased tolerance to heat stress, reduced irrigation and heat plus reduced irrigation in *OsSIZ1*-transgenic cotton is probably caused by the action of the SUMO E3 ligase activity of OsSIZ1. It was previously shown that the Arabidopsis *siz1* mutant lost water more quickly and displayed lower survival rates than WT plants under severe drought stress conditions (Catala et al. 2007). Yoo et al. (2006) also showed that the germination, seedling development and survival rate of the *siz1* mutant were impaired when *siz1* mutant plants were exposed to high temperatures. In contrast, overexpression of *OsSIZ1*, a homolog of the Arabidopsis *SIZ1*, led to increased tolerance to drought stress and heat stress in bentgrass (Li et al. 2013). The fact that *OsSIZ1* works in both the monocot plant bentgrass and the dicot plant cotton indicated that the *OsSIZ1*-mediated stress tolerance is conserved in plants.

Recently the complete sequence of an Upland cotton (*Gossypium hirsutum* TM-1) was published (Li et al. 2015), from which a homolog of *AtSIZ1* was annotated a year later. We compared the protein sequence of GhSIZ1 with those of AtSIZ1 and OsSIZ1, and we found that GhSIZ1 shares 65% identity with AtSIZ1 and 52% identity with OsSIZ1 (Supplementary Fig. S5). Similar to both AtSIZ1 and OsSIZ1, cotton SIZ1 also possesses five conserved domain structures, i.e. SAP, PHD, PINIT, SP-RING and SXS (Supplementary Fig. S5). Based on the protein sequence alignment and the phylogenetic relationships from selected plant and animal SIZ/PIAS proteins (Supplementary Fig. S6), the SIZ1 protein family appear to be highly conserved proteins in eukaryotes. Although we do not know if GhSIZ1 has similar functions to OsSIZ1, it is tempting to speculate similar functions for GhSIZ1, based on the high sequence similarity found among GhSIZ1, AtSIZ1 and OsSIZ1; in particular, the five structural domains that appear important to the enzymes' activities are well conserved. Validation of the function of *GhSIZ1* in vivo and association of the locus of *GhSIZ1* with potential existing cotton mutants will be needed for cotton biologists in the future. Nevertheless, the effort of introduction of *OsSIZ1* into cotton to improve cotton's heat and drought tolerance 7 years ago was a success for cotton biotechnology.

In order to provide a mechanistic explanation for the higher photosynthetic performance of *OsSIZ1*-transgenic cotton in comparison with control plants under rainfed conditions in the field, photosynthetic rates were measured with increasing carbon dioxide concentrations until saturation. According to V_{cmax} and J_{max} values obtained from A/C_i curve analysis (**Fig. 10**), we conclude that under rainfed conditions, ribulose bisphosphate regeneration, not Rubisco activity, is the main biochemical limitation for reduced photosynthetic rates in control plants. The rate of ribulose bisphosphate regeneration corresponds to the maximum rate of electron transport (J_{max}). Under water deficit stress, transgenic plants expressing the rice *OsSIZ1* gene showed better rates of electron transport. Therefore, we assume that under conditions of water deficit stress, sumoylation by OsSIZ1 somehow protects the electron transport machinery.

Abiotic stresses such as heat shock, low temperatures and drought were reported to trigger a significant increase in SUMO–protein conjugate levels (Kurepa et al. 2003, Murtas et al. 2003, Yoo et al. 2006, Catala et al. 2007, Miura et al. 2007a, Li et al. 2013). An increase in SUMO conjugates might be one of the reasons for improved abiotic stress tolerance in transgenic plants. Plant response to heat, drought and combined heat with drought might involve changes in gene expression of ABA-dependent and ABA-independent pathways (Shinozaki et al. 2003, Sakuma et al. 2006). The *GhRD22* gene is involved in drought stress tolerance via ABA-dependent pathways, but ABA is not essential for its induction. We found that the *GhRD22* transcript was up-regulated in both WT and *OsSIZ1*-transgenic plants under heat and heat plus reduced irrigation conditions, but the up-regulation was approximately 1.5-fold higher in *OsSIZ1*-transgenic plants than in control plants (Supplementary Fig. S2A). In contrast, we did not find significant differences in the *GhDREB* transcript between control and *OsSIZ1*-transgenic plants under all conditions tested (Supplementary Fig. S2B). Therefore, we believe that the *OsSIZ1*-mediated drought tolerance is probably via ABA-dependent pathways.

Another possible reason for the increased heat tolerance in *OsSIZ1*-transgenic plants is the synthesis of molecular chaperone HSPs that help protein folding during translation, protein re-folding after denaturation caused by abiotic stresses, or directing the misfolded proteins for degradation (Vierling 1991). Studies in various plant species showed a correlation between plant heat tolerance and the expression of HSP genes (Malik et al. 1999, Hong and Vierling 2000, Queitsch et al. 2000). Therefore, we tested the expression of two HSP genes from *G. hirsutum*, *GhHSP96* and *GhHSP100*, in control plants and *OsSIZ1*-transgenic plants grown under heat, reduced irrigation and heat plus reduced irrigation conditions. We found that the transcript level of *GhHSP96* was indeed 1.8- to 2.6-fold higher in *OsSIZ1*-transgenic plants than that in control plants under all stress conditions tested (Supplementary Fig. S3A).

The transcript level of *GhHSP100* was also up-regulated significantly under all stress conditions, but no significant differences were found between control and *OsSIZ1*-transgenic plants (Supplementary Fig. S3B). It appears that the *OsSIZ1*-mediated stress tolerance does involve up-regulating certain HSP genes such as *GhHSP96*.

Furthermore, earlier reports suggest that alleviation of oxidative damage and increased tolerance to abiotic stress are often correlated with more efficient antioxidant systems (Aghaei et al. 2009). Our results are consistent with previous studies and it can be said that enhanced performance of *OsSIZ1*-transgenic plants under water deficit conditions might be partly due to its higher efficiency to manage oxidative stress by increased expression of antioxidant genes such as *GhGST*, *GhSOD* and *GhAPX1* (Supplementary Fig. S4A–C).

Drought and heat are two major envrionmental stresses that often go hand in hand in nature, and cause huge crop losses worldwide, including the cotton loss in Texas High Plains. As our data showed that *OsSIZ1*-transgenic plants performed significantly better under heat, reduced irrigation and heat plus reduced irrigation conditions in a growth chamber, and produced 32% higher fiber yield under reduced irrigation conditions in the field and 21% higher fiber yield under dryland/rainfed conditions in the field, we believe that this gene could be used to improve crop yields in dryland agricultural production systems in other crops as well. This gene works in a monocot (bentgrass) and a dicot (cotton); it probably will work in other major crops as well. It will be worthwhile to test if overexpression of *OsSIZ1* in major staple crops such as rice, wheat, corn and sorghum would lead to increased yields, and our prediction would be that it does.

Materials and Methods

Cotton transformation

The *pUbi::OsSIZ1* construct (Li et al. 2013) was cloned into the pBI121-based binary vector and then introduced into cotton (*Gossypium hirsutum* cv. C312) via *Agrobacterium*-mediated transformation (Bayley et al. 1992) with minor modifications (Yan et al. 2004).

Genomic DNA isolation for PCR and DNA blot analyses

A 2 g aliquot of cotton leaves were ground into fine powder in liquid nitrogen, then transferred into 50 ml Falcon tubes. To the Falcon tubes 10 ml of Buffer I [2 ml of 1.75 mM glucose, 1 ml of 1 mM Tris pH 8.0, 200 µl of 0.25 M Na_2-EDTA, 1 ml of 20% PVP40, 0.1% vitamin C, 1 ml of 1% diethyldithiocarbamic acid (DIECA), 1 ml of 1% β-mercaptoethanol and 3.8 ml of H_2O] were added and tubes were vortexed thoroughly and kept at 4°C for 10 min followed by centrifugation for 10 min at 8,000 × *g* at 4°C. The pellet was re-suspended into 10 ml of pre-heated Buffer II [1 ml of 1 mM Tris pH 8.0, 5 ml of 2.8 M NaCl, 800 µl of 0.25 M Na_2-EDTA, 1 ml of 20% PVP40, 1 ml of 20% cetyltrimetylammonium bromide (CTAB), 0.1% ascorbate, 1 ml of 1% DIECA, 100 µl of 1% β-mercaptoethanol, 100 µl of H_2O], mixed thoroughly and kept in a 65°C water bath for 50 min, inverting the tube gently several times at 10 min intervals. Then an equal volume of chloroform : isoamyl alcohol (24 : 1) was added to the tubes and inverted gently 30–50 times followed by centrifugation for 10 min at 14,500 × *g* at 15°C. The DNA extraction step was repeated once again, then DNA in the supernatant was precipitated using 0.6 vol. of isopropanol followed by centrifugation at 10,000 × *g* for 5 min. The DNA pellet was washed twice with 75% ethanol and then dissolved into 1 ml of TE buffer (pH 8.0). To remove RNA, 1 µl of RNase A solution (10 mg ml^{-1}) was added and kept at 37°C for 2 h. Genomic DNA was then purified by phenol/chloroform extraction followed by isopropanol precipitation, and finally dissolved into 100 µl of TE buffer (pH 8.0), and the DNA concentration was measured using the Nanodrop ND-1000 (Thermo Scientific).

PCR was carried out in a thermal cycler with ExTaq DNA polymerase and *OsSIZ1* gene-specific primers OsSIZ1-F1 and OsSIZ1-B1. A 50 µl aliquot of the PCR mix contained 5 µl of 10 × PCR buffer, 2 µl of 50 mM $MgCl_2$, 4 µl of dNTP mix (2.5 mM each), 0.2 µM each of OsSIZ1-F1 and OsSIZ1-B1, 5 U $µl^{-1}$ *Taq* DNA polymerase and 1 µl of DNA template (0.1 µg $µl^{-1}$); the remaining volume was made up with water. The PCR was programmed with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 45 s, extension at 72°C for 90 s and a final extension at 72°C for 10 min. For DNA blot analysis, the NPT-II-specific primers Kan-F and Kan-R were used in a PCR to produce a kanamycin gene-specific probe. The probe was prepared by random priming of the PCR product using the method of Feinberg and Vogelstein (1983). The procedure of DNA hybridization was described by Pasapula et al. (2011).

RNA isolation and RNA blot analysis

Total RNAs were isolated from cotton leaves using the hot borate extraction method of Pang et al. (2011). The concentration of RNA was determined using Nanodrop (Thermo Scientific), and 30 µg of total RNAs were loaded into wells and electrophoresed for 5 h. The resolved RNAs in the gel were blotted onto a Biotrans nylon membrane and cross-linked in a UV cross-linker. After drying for 2 h, the membrane was hybridized to the *OsSIZ1*-specific probe that was made by PCR using primers OsSIZ1-F2 and OsSIZ1-B2, or to the ubiquitin gene- (i.e. *GhUbi7*) specific probe that was made by PCR using primers Ub7-F and Ub7-R. The procedure of RNA hybridization was described by Pasapula et al. (2011).

Determination of leaf relative water content (RWC)

After 4 weeks from germination, greenhouse-grown plants were treated with regular irrigation (control) and water deficit stress (one-third of regular irrigation) conditions for 1 month, and leaf samples were collected to analyze the RWC according to Parida et al. (2007) with some modifications. Leaves alone with petioles were detached from plants and FW was measured immediately. Then the petioles of leaves were immersed in distilled water overnight and turgid weight (TW) was recorded. Finally, DW was recorded after drying the leaf samples in an oven for 48 h at 60°C. Two independent biological and four technical replicates were performed for this experiment. RWC was calculated using the formula: RWC (%) = (FW − DW)/(TW − DW) × 100.

Determination of water use efficiency (WUE)

WUE was determined using the protocol of Xin et al. (2008). Cotton seeds were sown in 500 ml pots, allowed to germinate under greenhouse conditions (28/22°C day/night and natural light) and maintained under full irrigation by regular watering. Twelve days after seedling emergence, all pots were irrigated to field capacity and weighed. Immediately after weighing, the pots were sealed with two Uline poly bags and a small slit was made in the top bag to allow the cotton plant to continue growth. The slit was sealed with clear packing tape and covered with a layer of sand to prevent water loss through the opening in the top bag. The poly bags were tightly fixed onto the pots with an elastic band. Plants were grown under glasshouse conditions until they reached permanent wilt, at which time above-ground biomass was collected and a final pot weight was measured. Biomass samples were put in an oven for 24 h at 60°C. The dry weight was recorded. WUE was calculated as plant dry weight divided by the total water used (initial pot weight − final pot weight).

Plant growth conditions and stress treatments

Drought stress in the greenhouse. Cotton plants sown in 3 gallon pots in commercial potting mix LC-1 (Sun Gro Horticulture Canada Ltd) were allowed to germinate and establish for 4 weeks before the drought stress was started. For optimal/regular irrigation, 1,200 ml of water were added every other

day. For reduced irrigation (drought stress), 400 ml of water were added to each pot every other day. The treatment was continued until boll development and maturation. During the drought stress treatment, photosynthetic rates were measured using a Li-6400 (LI-COR Inc.). Measurements were taken after 1 month from the start of reduced irrigation treatment for the third fully expanded leaves. Net CO_2 assimilation rates were assessed at a CO_2 concentration of 400 μmol mol^{-1}, approximately 50% relative humidity, 28°C block temperature, 500 μmol s^{-1} air flow rate and a photon flux density of 1,800 μmol m^{-2} s^{-1}. Five measurements were taken for each sample. To document the phenotypic differences between controls (WT and SNT) and *OsSIZ1*-transgenic plants, pictures were taken at various times. At the end of the experiment, bolls per plant were counted and fiber yield per plant was analyzed. Fresh root biomasses were also measured. Ten biological replicates for each line were grown. The experiment was repeated three times.

Heat stress in the growth chamber. Cotton plants sown in 3 gallon pots in potting mix LC-1 were allowed to germinate and establish at 28°C during the day (06:00–22:00 h) and at 22°C in the evening (22:00–06:00 h for 4 weeks before the heat stress was applied. When heat stress was applied, the temperature in the growth chamber, originally set at 28°C during the day, was increased to 37°C from 24:00 to 16:00 h. The relative humidity was maintained at 60% and photoperiod was set at 16 h light/8 h darkness. Photosynthetic measurements were taken during heat stress treatment. The treatment continued until boll formation and maturation. At the end of the experiment, boll number and fiber yield per plant were measured. The experiment was repeated twice with seven biological replicates for each line.

Combined stresses of heat and reduced irrigation in the growth chamber. Cotton plants sown in 3 gallon pots in potting mix LC-1 were allowed to germinate and establish for 4 weeks before the combined stresses of heat and drought were applied. For optimal/regular irrigation, 1,200 ml of water were added every other day. For reduced irrigation, 600 ml of water were added to each pot every other day. The heat stress was 37°C for 2 h every day (from 13:00 to 15:00 h). The combined stresses of heat and reduced irrigation continued until boll development and maturation. Photosynthetic measurements were taken during the combined stress treatments. At the end of experiment, boll number and fiber yield per plant were measured. The experiment was repeated twice with seven biological replicates for each line.

Field trial experiments

Field trials with six different genotypes (WT, SNT, and four independent *OsSIZ1*-transgenic lines) were conducted in the USDA-ARS Cropping Systems Research Laboratory in Lubbock, Texas. For 2014 field trials, plants were grown under high irrigation (15 mm per week) and low irrigation (7.5 mm per week). The crop received a total of 456 mm of in-season rainfall (May–September). There were 90 seeds of each genotype sown in each irrigation scheme. Sowing was done in the first week of June in paired rows with a randomized block design. Gas exchange measurements were taken in the first week of August, and all measurements were taken between 11:00 and 13:00 h. At the end of the season, bolls and fiber yield per plant were analyzed. For the 2015 field trials, the low irrigation treatment was replaced with a rainfed treatment (no applied irrigation). Total in-season rainfall for 2015 was 480 mm (May–September). The Lubbock weather conditions in 2014 and 2015 are shown in Supplementary Table S2.

Determination of CO_2 response curves (A/C_i curve)

WT, SNT and four independent *OsSIZ1*-transgenic lines were grown in the field under both high irrigation and rainfed conditions. At 2 months from sowing, photosynthetic capacity of plants grown under both high irrigation and rainfed conditions were measured under increasing carbon dioxide conditions (0–180 Pa). V_{cmax} (maximum carboxylation rate allowed by Rubisco) and J_{max} (maximum rate of photosynthetic electron transport) were estimated by fitting the FvCB model to the CO_2 response curves (Farquhar et al. 1980).

Statistical analysis

Student *t*-test considering one-tailed unequal variance was performed to compare the performance of WT, SNT and *OsSIZ1*-transgenic lines. All *P*-values were from a comparison between controls (WT and SNT) and transgenic plants. Statistical analysis was performed using Microsoft® Office Excel 2007. $*P < 0.05$ and $**P < 0.01$ are two significance levels shown in the data presented in figures.

Oligonucleotide primers

The following primers were used in this study: Kan-F, GATTGAACAAGATGGATTGCACG; KanR, CCCGATCATATTGTCGCTCAGG; OsSIZ1-F1, ATGGCGGACCTGGTTTCCag; OsSIZ1-F2, GCAAAATGGAAATGAACAAA; OsSIZ1-B1, CAATAGATACTGATTCTGAGTAG; OsSIZ1-B2, ATAGTGACAGTGATTTGGAA; Ub7-F, CCTAGCCGCTGTACTTCTACTCCC; and Ub7-R, GGACTCTACTCAATCCCCACCAG.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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