TaASR1-D confers abiotic stress resistance by affecting ROS accumulation and ABA signalling in transgenic wheat

Ding Qiu¹,†, Wei Hu²,†, Yu Zhou¹, Jie Xiao¹, Rui Hu¹, Qihui Wei¹, Yang Zhang¹, Jialu Feng¹, Fusheng Sun¹, Jiutong Sun¹, Guangxiao Yang¹,‡ and Guanyuan He¹,‡

¹The Genetic Engineering International Cooperation Base of Chinese Ministry of Science and Technology, Key Laboratory of Molecular Biophysics of Chinese Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology (HUST), Wuhan, China
²Key Laboratory of Biology and Genetic Resources of Tropical Crops, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, China

Abstract

Cultivating new crop cultivars with multiple abiotic stress tolerances is important for crop production. The abscisic acid-stress-ripening (ASR) protein has been shown to confer abiotic stress tolerance in plants. However, the mechanisms of ASR function under stress condition remain largely unclear. In this study, we characterized all ASR family members in common wheat and constitutively overexpressed TaASR1-D in a commercial hexaploid wheat cultivar Zhengmai 9023. The transgenic wheat plants exhibited increased tolerance to multiple abiotic stresses and increased grain yields under salt stress condition. Overexpression of TaASR1-D conferred enhanced antioxidant capacity and ABA sensitivity in transgenic wheat plants. Further, RNA in situ hybridization results showed that TaASR1-D had higher expression levels in the vascular tissues of leaves and the parenchyma cells around the vascular tissues of roots and stems. Yeast one-hybrid and electrophoretic mobility shift assays revealed that TaASR1-D could directly bind the specific cis-elements in the promoters of TaNced1 and TaGpx1-D. In conclusion, our findings suggest that TaASR1-D can be used to breed new wheat cultivars with increased multiple abiotic stress tolerances, and TaASR1-D enhances abiotic stress tolerances by reinforcing antioxidant capacity and ABA signalling.

Introduction

Abiotic stresses such as salinity and drought are major causes of crop yield reduction (Ben-Ari et al., 2018; Mäkinen et al., 2018; Zhu and Troy, 2018). Therefore, improving the tolerance of crops to abiotic stress is of great importance for increasing crop yield. Abiotic stresses can induce reactive oxygen species (ROS) production in several cellular organelles directly and indirectly, and excessive ROS inhibits the growth and development of crops (Mhamdi and Van Breusegem, 2018; Waszczak et al., 2018). Therefore, enhancing the antioxidant capacity of crops under abiotic stresses greatly contributes to improving crop tolerance.

Abscisic acid (ABA) is a phytohormone and the central regulator enhancing plant tolerance to abiotic stresses (Vishwakarma et al., 2017). Increasing ABA content in vivo by overexpressing ABA synthesis genes or applying exogenous ABA could enhance plant stress tolerance and reduce the damage it causes (Khadri et al., 2006; Park et al., 2008; Qin and Zeevaart, 2002; Wei et al., 2015). ABA-deficient mutants, in general, appeared to be more severely damaged than wild-type (WT) plants under abiotic stress conditions (Etehadnia et al., 2008; Ozfidan et al., 2012). It was previously revealed that the expression of many genes related to enzymatic and non-enzymatic antioxidant systems could be induced by ABA (Ahmad et al., 2010; Ashraf and Foolad, 2007; Ding et al., 2009). In addition to enhancing plant abiotic stress tolerance by regulating antioxidant systems, ABA can also increase the viability of plants in unsuitable environments by regulating the accumulation of osmotic adjustment, ion transport, stomatal closure and inhibition of growth (Vishwakarma et al., 2017). One of the major mechanisms by which ABA exerts the multiple functions mentioned above is to regulate the expression of ABA-induced transcription factors (Yoshida et al., 2019).

The abscisic acid-stress-ripening (ASR) transcription factor can be induced by ABA, and a variety of environmental stress signals, such as drought, high salt, low temperature, heavy metal and constitutive expression of ASRs usually results in plants being more adaptive to adverse external environments (González and Isem, 2014). Several observations suggested that the accumulation of ROS and the activities of antioxidative enzymes under abiotic stress were regulated by ASRs, implying that a critical role of ABA is to regulate ROS homeostasis (Hu et al., 2013; Li et al., 2017a; Wang et al., 2016a). Meanwhile, overexpression or ectopic expression of ASRs in rice (Oryza sativa), groundnut (Arachis hypogaea), foxtail millet (Setaria italica) and maize (Zea mays) enhanced crop endurance to drought, salinity, cold and even maintained maize yields under water deficit conditions (Kim et al., 2009; Li et al., 2017b; Tiwari et al., 2015; Virlouvet et al., 2011). Thus, ASR genes have the potential to modulate crop tolerance to multiple abiotic stresses based on the above
description. However, the mechanisms of ASR improving crop stress tolerance are still poorly understood.

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops, and breeding new wheat cultivars with stronger abiotic stress resistance is of great importance to ensure food security. Previously, we demonstrated that ectopic expression of *TaASR1-D* in tobacco (*Nicotiana tabacum*) enhanced antioxidant capacity, upregulated the expression of some stress-associated genes, and increased drought and osmotic stress tolerance (Hu et al., 2013). In this study, all ASR genes in the wheat genome were identified, and *TaASR1-D* was transformed into the common hexaploid wheat cv. Zhengmai 9023. The transgenic wheat lines exhibited increased tolerance to oxidative, osmotic, drought and salinity stresses than WT plants, and the grain yields under salt condition were also increased. Furthermore, we found that *TaASR1-D* functions by enhancing antioxidant system and ABA signalling.

**Results**

**Genome-wide identification of the ASR gene family in wheat**

Currently, two studies have identified 29 and 33 ASR genes in wheat, respectively (Li et al., 2020; Zan et al., 2020). However, in this study, we identified 36 genes in wheat (Table S1), and all genes except *TaASR8-U* are located on chromosome groups 2, 3, and 4 (Figure S1a). Duplication events occurred in *Tears*, *TaASR7* and *TaASR8* on chromosomes 3A and 3D. In general, the gene structure of ASR contains two exons, and we found that *TaASR3* genes have only one exon, but other family members possessed a conserved gene structure (the wrong predictive coding sequence of *TaASR1-A* containing three exons was artificially corrected). We renamed all *Tears* family members based on their evolutionary relationships and chromosome locations. Motif analysis indicated that there were seven conserved motifs in the *Tears* family, and these results were generally consistent with the results of evolutionary analysis (Figure S1b). The sequences of conserved motifs are shown in Figure S2. Motif 2 was the ABA-WDS domain, and all members contain this domain except *TaASR3-A, TaASR3-B, TaASR3-C* and *TaASR7-B*, which are missing a part of the conserved sequence of the ABA-WDS domain (Figure S3). The phylogenetic analysis implied that *TaASR1* exhibits a close phylogenetic relationship with *ZmASR1* and *OsASR1* (Figure S4). Previous reports showed that overexpression of *ZmASR1* and *OsASR1* led to increased multiple abiotic stress tolerances in maize and rice, respectively (Arenhart et al., 2016; Li et al., 2017a; Li et al., 2018a; Virlouvet et al., 2011). In addition, our previous work had revealed that *TaASR1-D* could improve the antioxidant capacity of transgenic tobacco (Hu et al., 2013). Therefore, it was likely that overexpression of this gene in wheat could increase oxidative stress tolerance.

**Overexpression of *TaASR1-D* in transgenic wheat improves oxidative stress tolerance**

To verify this speculation, we constructed a *TaASR1-D* overexpression vector and transformed it into common wheat by the biolistic bombardment method (Figure S5a), and four transgenic lines were obtained (L1, L2, L23 and L39). The transcript levels of *TaASR1-D* in four homozygous lines ranged from 2.07-fold to 4.21-fold compared with that in WT plants (Figure S5b). Then, the T3 generations of two transgenic lines and WT plants were subjected to treatment by methyl viologen (MV) to identify the function of *TaASR1-D* under oxidative stress (Figure 1a). The relative shoot and root lengths of three-day-old seedlings after 7 days’ treatment by 2 μM MV showed that the overexpression (OE) lines, especially their shoot length, were longer than WT, suggesting that the growths of OE lines were less inhibited by the oxidant (Figure S6a). The relative ion leakage (IL) rates of transgenic plant leaves which immersed in the MV solution were lower than those of WT, which indicated that the plasma membrane of OE lines might be less injured under oxidative stress (Figure S6b). Seven days after H2O2 treatment, the leaves of three-leaf-stage WT seedlings were severely curled and wilted, while the leaves of OE lines were still green (Figure S6c). These results suggested that transgenic wheat plants were less injured by oxidants and exhibited increased oxidative stress tolerance. If the plants show improved antioxidant capacity, they might be less injured by oxidants induced by environmental stresses and have concomitant stronger resistance to stresses. To determine whether the OE lines have stronger antioxidant capacity than WT, we analysed the physiological indices of three-leaf-stage wheat plants under oxidative, osmotic and salt stresses. The H2O2 levels of leaves were detected by 3,3-diaminobenzidine (DAB) staining after 4 days’ stress treatments (Figure S6d). Compared with OE lines, the accumulation of brown precipitate in WT leaves was more severe under stress conditions. The results of the H2O2 contents and O2− contents after 7 days of stress treatments also suggested that the transgenic plant accumulated less ROS under oxidative, osmotic and salt stress conditions (Figure 1b,c). The malondialdehyde (MDA) contents and IL rates of OE lines were lower than those of WT plants, implying that the plasma membrane of transgenic plants suffered less oxidative damage during abiotic stress treatment (Figure 1d,e). In addition, several physiological indices related to antioxidant capacity and stress tolerance were examined. The SOD, CAT and GPx activities of two OE lines were generally higher than those of WT plants under stress treatment conditions (Figure 1f-h), while there was no significant difference in POD activities relative to WT (Figure 1i). The soluble sugar contents of OE plants were higher than those of WT plants under normal and stress conditions (Figure 1j). However, the OE lines contained significantly less proline contents compared with WT plants (Figure 1k). These results indicated that overexpressing *TaASR1-D* in wheat caused decreased oxidative damage and increased antioxidant capacity under abiotic stresses, implying that overexpression of *TaASR1-D* confers transgenic wheat seedlings with resistance to multiple abiotic stresses.

**Overexpression of *TaASR1-D* in transgenic wheat improves osmotic and drought stress tolerances**

To confirm the function of *TaASR1-D* under osmotic and drought stresses, we analysed the tolerances of OE plants to osmotic and drought treatments. The three-day-old seedlings of OE lines and WT were grown in medium containing 300 mM mannitol for 7 days, and their shoot and root lengths were then recorded (Figure 2a). Higher relative shoot and root lengths of OE seedlings indicated that overexpression of *TaASR1-D* improved the growth ability of transgenic wheat seedlings under osmotic stress (Figure 2b). Seven days after mannitol treatment, all three-leaf-stage plants showed injured phenotypes by osmotic stress, but the WT plants displayed more severe wilting and yellowing (Figure S7).

To examine the tolerance of OE lines to water deficiency, WT and OE seedlings planted in the same pot were subjected to drought for 20 days. After stopping watering, severe dehydration...
symptoms of drought-stressed plants could be observed, but the WT plants exhibited an earlier and more severe rolling and wilting phenotype than OE lines during the period of drought treatment (Figure 2c). During the process of rewatering, the OE plants had better recovery ability and recovered earlier to normal leaf morphology compared with WT plants. The average survival rates of the two OE lines were 78% and 80%, which were much higher than that of WT (26%) (Figure 2d). Moreover, water loss rates and relative water contents (RWC) of detached leaves from WT and OE plants at tillering stage indicated that wheat seedlings overexpressing TaASR1-D had greater water retention capacity under dehydration condition (Figure 2e,f). These results

Figure 1 Overexpression of TaASR1-D increased the antioxidant capacity of transgenic plants. (a) Phenotypes of three-day-old WT and OE plants after normal and 2 μM MV treatments for 7 days. H2O2 contents (b), O2− contents (c), MDA contents (d), ion leakage rates (e), SOD activities (f), CAT activities (g), GPx activities (h), POD activities (i), soluble sugar contents (j), and proline contents (k) in the leaves of three-leaf-stage WT and OE plants under normal and stress conditions (n = 3). MV, methyl viologen; FW, fresh weight. All experiments were performed with at least three biological replicates. Data are the mean ± SE, and asterisks indicate significant differences between WT and OE lines using a t-test (*, P < 0.05; **, P < 0.01)
demonstrated that overexpressing TaASR1-D in wheat conferred a positive effect on osmotic and drought stress tolerances.

**Overexpression of TaASR1-D in transgenic wheat improves salt stress tolerance**

We had proven that wheat seedlings overexpressing TaASR1-D had higher antioxidative enzyme activities and lower ROS accumulation under high-salt condition. To evaluate the effect of overexpressing TaASR1-D in wheat exposed to salt stress, we tested the tolerance of three-day-old and three-leaf-stage wheat seedlings to 200 mM NaCl treatment. The relative shoot lengths of OE lines were higher than those of WT plants, which suggested that the shoots of OE plants were less inhibited by high salinity, whereas there was no significant difference in relative root lengths between them (Figure 3a,b). The three-leaf-stage seedlings of OE lines displayed significantly reduced sensitivity to high salinity relative to WT after NaCl treatment for 7 days (Figure S8).

To further explore the mechanism by which TaASR1-D improves salt tolerance of transgenic wheat seedlings, we measured the Na\(^+\) and K\(^+\) contents in the shoots of WT and OE plants (Figure 3c,d). It could be observed that the Na\(^+\) contents were increased while K\(^+\) contents were decreased both in WT and OE plants after salt treatment. No significant difference was found in K\(^+\) concentrations between WT and OE plants under normal or saline conditions; nevertheless, the OE plants had higher Na\(^+\) accumulation and Na\(^+\):K\(^+\) ratios than WT plants after NaCl treatment (Figure 3e).

**Transgenic TaASR1-D wheat plants have higher grain yields under salt stress**

It had been proved that TaASR1-D improved the tolerance of wheat seedlings to abiotic stresses, but it was still unclear whether TaASR1-D had a positive effect on stress tolerance at the later developmental stage and grain yield. Thus, we alternately
planted WT and two OE line plants in large containers to analyse the abiotic stress tolerance during the reproductive stage, as well as the agronomic traits and yield parameters under normal and stress conditions (Figure S9). For abiotic stress tolerance, we analysed the physiological indices of plants at the heading stage under severe drought (water content of soil 34.2%) and high-salt (containing 1% NaCl of soil dry weight) conditions. The H$_2$O$_2$, O$_2^-$ and MDA contents of OE lines were lower than those of WT, and these implied that overexpression of TaASR1-D could reduce the accumulation of ROS and oxidative damage to the plasma membrane during the reproductive stage under stress conditions (Figure S10a-c). However, the antioxidant enzyme activities (except SOD activity) and proline contents were lower in transgenic plants compared with WT plants (Figure S10d-h).

To analyse the agronomic traits and yield parameters under drought and salt conditions, we stopped watering at the beginning of the heading stage until harvest and planted seedlings in soil containing 1% NaCl with normal water content until harvest, respectively. Under control and drought conditions, many agronomic traits and yield parameters of transgenic lines, such as plant height, dry biomass, tiller number, spike number, spikelet number per spike, grain number per spike, grain number per plant and grain yield per plant, were lower than those of WT plants (Figure 4). However, these results of OE lines were always higher than those of WT under salt stress condition. In addition, the grain weights and grain widths of OE lines under salt stress condition were higher than those of WT. It was worth noting that the grain length–width ratios of OE lines were lower than that of WT under normal and stress conditions. These results suggested that overexpression of TaASR1-D conferred transgenic plants with increased stress tolerance during the reproductive stage and higher grain yields under salt stress condition.

**Overexpression of TaASR1-D in transgenic wheat enhances ABA signalling**

Our previous research had shown that the expression of TaASR1-D was upregulated by ABA treatment, and the expression of NtNCED1, which is the rate-limiting enzyme of ABA biosynthesis, was also upregulated in TaASR1-D-overexpressing plants under normal and osmotic stress conditions (Hu et al., 2013). This implied that a relationship exists between TaASR1-D and ABA. To explore the effects of TaASR1-D on ABA, we examined the sensitivity of transgenic wheat seedlings to exogenous ABA. No significant difference in primary root lengths of post-germination seedlings was observed between WT and OE plants in the absence of ABA, whereas the lengths of two OE lines were significantly shorter than those of WT plants under ABA treatment conditions (Figure 5a,b). The growth inhibition by exogenous ABA was also investigated (Figure 5c). The relative shoot lengths and biomasses of the transgenic plants in the presence of ABA appeared to be more sensitive, while the relative root lengths did not (Figure 5d). These results showed that TaASR1-D played a positive role in ABA signalling.

We further analysed the endogenous ABA contents of WT and L39 plants under normal, H$_2$O$_2$, mannitol and NaCl treatment conditions. It could be observed that the ABA contents were increased both in WT and OE plant leaves after 6 h of stress treatments, while the ABA contents of L39 seedlings were always higher than those of WT (Figure 5e). However, the ABA content of L39 was lower when the osmotic stress treatment was
extended to 24 h (Figure S11). It is well known that ABA can induce the production of ROS through the activation of respiratory burst oxidase homologue (RBOH) under stress conditions (Xia et al., 2015). To confirm whether TaASR1-D participates in this process, we compared the contents of endogenous H$_2$O$_2$ in the case of pretreatment with or without fluridone, which is an inhibitor of ABA biosynthesis (Figure 5f). After treatment with 100 mM H$_2$O$_2$ for 24 h, the non-pretreated WT plant leaves accumulated less H$_2$O$_2$ than that of the non-pretreated OE leaves. However, the H$_2$O$_2$ accumulation of pretreated WT plants was higher than that of OE plants, and there was an obvious reduction in the L2 line compared with the non-pretreated OE leaves. These results implied that the OE plants had a stronger capacity to metabolize H$_2$O$_2$ in the case of inhibiting endogenous ABA synthesis, and the improved tolerance of OE plants to oxidative stress might not all be derived from the increased ABA signalling.

**Related gene expression analyses of transgenic wheat**

To further explain the role of TaASR1-D under stress, we analysed the expression of some other genes related to ABA, ROS metabolism and abiotic stress response. Our results revealed that overexpression of TaASR1-D altered the ABA contents in wheat seedlings under normal and stress conditions; therefore, we analysed the expression of two ABA biosynthetic genes, TaNCED1 and TaABA1, and one ABA-induced gene, TaNAC2D (Figure S12a-c). The results showed that these genes had higher expression levels in OE plants, except for H$_2$O$_2$ treatment. To further investigate the ROS metabolism of transgenic plants, we tested the transcript levels of several genes encoding ROS scavengers. It could be observed that the transcript levels of TaSOD1 and TaCAT3 in OE plants were higher in most instances, while the expression of TaPOD-A1 was lower than that of WT under ABA, osmotic and salt treatments (Figure S12d-f). The TaGPx1-D expression levels of OE lines were higher under normal and mannitol treatment conditions, and lower under ABA, H$_2$O$_2$, and NaCl treatments compared with that of WT (Figure S12g). The expression analysis results of those four ROS scavenging enzymes were largely consistent with their enzymatic activities. In addition, the expression patterns of three sodium ion transporter genes, TaSOS1, TaHKT2;1 and TNHX1, were examined (Brini et al., 2007; Ramezani et al., 2013; Schachtman and Schroeder, 1994). Transcripts of TaSOS1 and TaHKT2;1 were downregulated by ABA and H$_2$O$_2$, and their expression levels in OE plants were lower than those in WT plants under salt treatment (Figure S12h,i). TNHX1 expression was increased after ABA and H$_2$O$_2$ treatments, and its transcripts in OE plant leaves were higher than those in WT plants under salt treatment (Figure S12j). Given the importance of sugar to plant abiotic stress tolerance and the increased soluble sugar contents in OE plant leaves, we examined the expression of TaHT and TaSUT. The results showed that the transcripts of TaSUT and TaHT were subdued by ABA and stress treatments, and OE plants exhibited...
more intensive decreases in expression under control and treatment conditions (Figure S12k,l).

Tissue-specific expression of the TaASR1-D

The biological functions of gene depend on their spatiotemporal expression. To further reveal the functions of TaASR1-D, the RNA in situ hybridization was applied to analyse its tissue-specific expression on the cross-sections of root, stem and leaf of wheat cultivar Chinese Spring. A digoxigenin-labelled TaASR1-D anti-sense oligonucleotide was used as the probe to detect the locations of TaASR1-D mRNA, and a sense oligonucleotide was used as native control. It could be observed that the hybridization signal was widely located on the root and stem cross-sections, and no signal was detected on the corresponding control sections (Figure S13a-d). In addition, the parenchyma cells around vascular tissues had stronger hybridization signals than others. However, the transcripts of TaASR1-D were mainly enriched in vascular tissues of wheat leaves compared with the control section (Figure S13e,f).

TaASR1-D binds the promoters of TaNCED1 and TaGPx1-D

The above-mentioned results indicated that TaASR1-D could regulate the endogenous ABA content and GPx activity of transgenic wheat seedlings, and the expression of TaNCED1 and TaGPx1-D was upregulated under normal condition. Thus, TaNCED1 and TaGPx1-D might be the downstream target genes of the TaASR1-D transcription factor. As the orthologous protein of TaGPx1-D in rice was localized in the mitochondrion (Passaia et al., 2013), the higher mitochondrial GPx activities of OE lines compared with WT which were measured in etiolated seedlings further suggested that TaGPx1-D might be directly regulated by TaASR1-D (Figure 6a). In addition, previous studies showed that TaNCED1 and OsGPx3 (orthologous gene of TaGPx1-D in rice) were specifically expressed in vascular tissues and had a similar expression specificity with TaASR1-D (Ji et al., 2011; Passaia et al., 2013). Analysis of cis-elements found that the GT-1 and GAVGAVGA boxes were found in their promoters (Figure S14),

Figure 5 Overexpression of TaASR1-D enhanced ABA signalling. (a) ABA sensitivity assay of primary root growth. (b) Root lengths of WT and OE seedlings under normal and ABA treatment conditions. (c) ABA sensitivity assay of seedling growth. (d) Relative values of shoot lengths, root lengths and biomasses after ABA treatment. (e) Relative ABA contents of WT and L39 under normal and stress treatment conditions. (f) H2O2 contents of WT and OE leaves under normal condition and H2O2, fluridone (Fd) + H2O2 treatment for 24 h. FW, fresh weight. All experiments were performed with at least three biological replicates. Data are means ± SE, and asterisks indicate significant differences between WT and OE lines using a t-test (*, P < 0.05; **, P < 0.01)
and it has been proved that the homologous protein of TaASR1-D in rice could directly bind to these two cis-elements (Li et al., 2018b). The binding of TaASR1-D to these two cis-elements was confirmed by yeast one-hybrid assay. As shown in Figure 6b, the yeast cells transformed with TaASR1-D and GT-1/GAVGAVGA cis-elements had increased growth ability on SD/-Trp/-Leu/-His/3-AT (3-amino-1,2,4-triazole) nutritional media compared with the yeast cells containing TaASR1-D and empty vector. Furthermore, the direct interaction between TaASR1-D and the promoters of TaNCED1 and TaGPx1-D was validated by electrophoretic mobility shift assay (EMSA). The recombinant TaASR1-D-His fusion protein was purified from E. coil, and the promoter fragments of TaNCED1 and TaGPx1-D containing these two cis-elements respectively were used as probes (Figure 6c). It could be observed that the recombinant protein could interact with biotin-labelled promoter fragments, and the combined bands were weakened and disappeared when 40-fold and 80-fold molar excesses of cold competitor probes were added, respectively (Figure 6d,e). However, the complexes displayed a slight weakening after adding 80-fold molar excesses of unlabelled mutant competitor probes. These results indicated that the binding of TaASR1-D to the promoter fragments was element-specific. On the other hand, the two complex bands suggested that TaASR1-D could bind the promoters in the form of monomer and homodimer as reported (Wetzler et al., 2018).

Discussion

Studies have shown that the overexpression of ASRs enhances the antioxidant system and confers abiotic stress tolerance to transgenic plants. Hence, we speculated that ASR has the potential to promote multiple abiotic stress resistance in crops by enhancing antioxidant capacity, and then, we identified all members of the ASR family in common wheat (Table S1). The phylogenetic analysis revealed that ZmASR1 and OsASR1 have close evolutionary relationships with TaASR1 (Figure S4). These two genes have higher transcript levels than other family members and have been demonstrated to increase multiple abiotic stress tolerances of transgenic crops (Pérez-Díaz et al., 2014; Virlouvet et al., 2011). Our previous study had shown that TaASR1-D expression was induced by ABA and H2O2, and overexpressing TaASR1-D could enhance the tolerance of transgenic tobacco to oxidative, osmotic and drought stresses (Hu et al., 2013). Therefore, TaASR1-D was selected to transform the elite hexaploid wheat cv. Zhengmai 9023, and four transgenic lines were obtained (Figure S5).

Figure 6 TaASR1-D bound the promoters of TaNCED1 and TaGPx1-D. (a) Cytoplasmic and mitochondrial GPx activities of two-week-old etiolated WT and OE seedlings. (b) Yeast one-hybrid assay of TaASR1-D interacted with GT-1 and GAVGAVGA cis-elements. (c) The promoter fragments of TaNCED1 (Probe1) and TaGPx1-D (Probe2) were used for EMSA, and the sequences of cis-elements with or without mutations are shown in red font. TaASR1-D directly bound to the specific sequences in TaNCED1 (d) and TaGPx1-D (e) promoters via EMSA. Arrowheads indicate free probes and protein–DNA complexes. EMSA, electrophoretic mobility shift assay; +, presence; -, absence. Experiments were performed with at least three biological replicates. Data are means ± SE, and asterisks indicate significant differences between WT and OE lines using a t-test (*, P < 0.05).
Phenotypes and physiological states under oxidant treatments indicated that overexpression of TaASR1-D conferred oxidative stress tolerance to transgenic wheat plants (Figure 1a and Figure S6). The lower MDA contents and IL rates of OE plants after mannitol and NaCl treatments suggested that the transgenic seedlings were subjected to less oxidative damage compared with WT seedlings (Figure 1d,e), and the results of ROS contents and antioxidant enzyme activities implied that OE plants were more resistant to these two stresses (Figure 1). The phenotypes and higher relative shoot lengths of OE lines after osmotic and salt treatments confirmed that OE plants had pronounced resistance to osmotic and salt stresses. Moreover, the higher survival rates after drought treatment and lower water loss rates of OE lines demonstrated that the overexpression of TaASR1-D conferred tolerance against drought stress in transgenic wheat (Figure 2c-f). These results demonstrated that the transgenic lines had improved tolerance to multiple abiotic stresses by the increased antioxidant system.

The mechanism by which ASRs regulate abiotic stress tolerance remains unclear, and previous studies have attempted to interpret it at the cellular level. In our opinion, the explanation at the molecular or cellular level does not adequately illustrate the role of ASRs in regulating the abiotic stress response. The tissue-specific expression analysis by in situ hybridization in roots, stems and leaves showed that TaASR1-D had higher expression levels in the vascular tissues of leaves and the parenchyma cells of roots and stems (Figure S13). It is known that these tissues were the main locations for ABA synthesis in plants, and several genes of ABA synthesis were specifically expressed there (Endo et al., 2008; Koivai et al., 2004). Our results had shown that the OE wheat seedlings were hypersensitive to ABA relative to WT (Figure 5a-d), and at least two reports indicated that ASR affected the biosynthesis of ABA (Dominguez et al., 2013; Li et al., 2017a). Therefore, we measured the endogenous ABA contents of OE and WT plant leaves under normal and 6-h treatment conditions, and the results suggested that the endogenous ABA contents of OE plants were always higher compared with those of WT (Figure 5e). It could be observed that the transcript levels of ABA upregulated genes were always higher in transgenic lines under normal and treatment conditions, while the expression levels of ABA downregulated genes were lower than those of WT (Figure S12). These results indicated that overexpressing TaASR1-D enhanced ABA signalling in wheat seedlings, and TaASR1-D may be involved in the function of vascular tissue during ABA synthesis and ABA signalling.

We had demonstrated that OE plants had enhanced oxidative stress tolerance and ABA signalling. Intriguingly, it was revealed that ABA could induce the production of ROS by RBOH at the apoplasm and upregulate the antioxidant system by this pathway (Hu et al., 2005; Zhang and Jiang, 2002). Thus, does overexpression of TaASR1-D inhibit the induction of ROS by ABA? Furthermore, does the increased antioxidant capacity of OE plants dependent on the enhancement of ABA signalling? Exogenous H₂O₂ was applied as a stress signal to activate the process of ABA-triggered ROS production, and the results showed that OE plants accumulated more endogenous H₂O₂ contents after 24 h of treatment compared with WT. In contrast, the endogenous H₂O₂ levels of OE plants pretreated by fluridone were reduced and lower than those of WT. It is therefore likely that the stronger antioxidant capacity of OE plants under normal and long-term stress conditions resulted in lower ROS accumulation than that of WT, and the enhanced ABA signalling triggered more ROS production in apoplast during short-term stress. Moreover, the pathway by which TaASR1-D increased the antioxidant system does not wholly depend on the ABA signalling, and thereby the H₂O₂ contents of transgenic lines were lower than that of WT plants in the case of inhibited endogenous ABA biosynthesis.

In plants, ASRs function as molecular chaperones and transcription factors (González and Iusem, 2014). Since we had demonstrated that TaASR1-D was localized in the nucleus and possessed transcriptional activity (Hu et al., 2013), these suggested that TaASR1-D functioned as a transcription factor. In this study, it had been demonstrated that overexpression of TaASR1-D increased ABA sensitivity and regulated the endogenous ABA contents. Many studies had proved that NCED genes were specifically expressed in the vascular bundle like TaASR1-D, and the expression of TaNCED1 was restricted to the parenchyma cells surrounding vascular tissues (Bang et al., 2013; Endo et al., 2008; Ji et al., 2011). Furthermore, the TaNCED1 expression of OE lines was higher than that of WT under normal and stress conditions except for H₂O₂ treatment (Figure S12a). Besides, the GPx genes also had been reported to be specifically expressed in vascular tissues (Milla et al., 2003; Passaia et al., 2013), and the GPx activity and TaGPx1-D expression of OE lines were regulated compared with WT (Figure 1h and Figure S12g). TaGPx1-D was predicted to be located in mitochondria, and the etiolated OE plants had higher mitochondrial GPx activities than that of WT (Figure 6a). These results implied that TaASR1-D could directly regulate the expression of TaNCED1 and TaGPx1-D. Analysis of the TaNCED1 and TaGPx1-D upstream sequences revealed that the GT-1 and GAVGAVGA boxes were found in their promoters, and these two cis-elements could be bound by ASR in rice (Li et al., 2018b). The results of yeast one-hybrid assay and EMSA showed that TaASR1-D could directly bind the promoters of TaNCED1 and TaGPx1-D, and these two cis-elements were required for the binding (Figure 6b,d).

Results of Na⁺ and K⁺ contents in the shoots revealed that the OE plants accumulated more Na⁺ than that of WT plants after NaCl treatment (Figure 3c), and these results might be caused by decreased expression of sodium exclusion genes (Figure S12h,i). However, it was often observed that there was no strong correlation between sodium content and salinity tolerance (Naem et al., 2020), and the transgenic lines displayed stronger growth ability under high-salt conditions compared with WT plants (Figure 3a,b). It is known that high-salinity environment induces osmotic stress, oxidative stress and the over-accumulation of toxic sodium. Thus, the increased salt tolerance of transgenic lines might be due to the enhanced osmotic and oxidative stress tolerance and the Na⁺ compartmentation in vacuoles by increased TNHX1 expression (Figure S12j). The lower expression levels of sugar transporters and higher soluble sugar concentrations also contributed to the increased osmotic stress tolerance of OE lines under salt treatment (Figure 1j and Figure S12k,l). Moreover, the parenchyma cells of root and vascular tissue play vital roles in controlling the uptake and long-distance transport of sodium (Zhang et al., 2010), and TaASR1-D was specifically expressed in these tissues (Figure S13). If TaASR1-D could weaken the efflux of sodium in these tissues, similar as its effect in the overexpressed plant, it might be beneficial to reduce the Na⁺ accumulation at the whole-plant level. However, more experiments are needed to confirm this hypothesis.

To further evaluate the value of the TaASR1-D gene, the physiological indices were examined during the reproductive
stage. The ROS accumulations and MDA contents of transgenic plants were lower than those of WT plants under normal and stress conditions (Figure S10a-c), and these results were consistent with those of seedlings. However, the antioxidant enzyme activities of OE plants were not consistently higher during this development stage compared with WT (Figure S10d-g). This may be due to the differences in endogenous ROS levels between WT and OE plants under relative long-term stress conditions. As the ROS accumulation is induced by ROS (Ben Rejeb et al., 2015), it is reasonable for OE plants to have lower proline contents under stress conditions regardless of three-leaf-stage or heading stage.

The agronomic traits and yield parameters of OE and WT wheat plants were also examined when harvesting. It was interesting that the grain yields of OE lines were lower under drought stress but higher under salt stresses than those of WT (Figure 4). It is known that grain weight, grain number per spike and spike number per plant are the most important traits regarding to the grain yield per plant. Analyses of these yield parameters in this study showed that the decreased grain number per spike and increased grain weight were the main contributors for the grain yield changes of transgenic lines under drought and salt stresses, respectively. The drought treatments were performed at the beginning of heading stage, and drought stress could lead to pollen sterility and decreased grain number at the early reproductive stage (Li et al., 2010). Extensive researches indicated that the increased endogenous ABA content was the reason for the reduced seed setting rate caused by water and temperature stresses (De Storme and Geelen, 2014; Morgan, 1980; Olivier et al., 2007; Saini and Aspinall, 1982), and changing the content and sensitivity of ABA affected the grain number and seed setting rate (Gao et al., 2018; Mphande et al., 2021). In this study, TaASR1-D regulated the expression of TaNAC1 and endogenous ABA content, and the OE plants were hypersensitive to ABA. Thus, it was likely that the higher ABA content of OE lines led to lower grain number per spike and grain yields per plant compared with those of WT under drought treatment. However, there were studies suggesting that exogenous ABA increased grain number under salt stress, and the grain weight and grain yield of salt-stressed rice and wheat were also increased (Aldesuquy and Ibrahim, 2001; Gurmani et al., 2006; Saeedipour et al., 2015), it is reasonable for OE plants to have lower proline contents under drought and salt stresses, respectively. The drought treatments were performed at the beginning of heading stage, and drought stress could lead to pollen sterility and decreased grain number at the early reproductive stage (Li et al., 2010). Extensive researches indicated that the increased endogenous ABA content was the reason for the reduced seed setting rate caused by water and temperature stresses (De Storme and Geelen, 2014; Morgan, 1980; Olivier et al., 2007; Saini and Aspinall, 1982), and changing the content and sensitivity of ABA affected the grain number and seed setting rate (Gao et al., 2018; Mphande et al., 2021). In this study, TaASR1-D regulated the expression of TaNAC1 and endogenous ABA content, and the OE plants were hypersensitive to ABA. Thus, it was likely that the higher ABA content of OE lines led to lower grain number per spike and grain yields per plant compared with those of WT under drought treatment. However, there were studies suggesting that exogenous ABA increased grain number under salt stress, and the grain weight and grain yield of salt-stressed rice and wheat were also increased (Aldesuquy and Ibrahim, 2001; Gurmani et al., 2006; Saeedipour et al., 2015). Therefore, the diversity of ABA functions might lead to different results in the grain yields of transgenic lines under drought and salt stresses, respectively.

Collectively, in this study, we constitutively overexpressed TaASR1-D in wheat cv. Zhengmai 9023 and obtained OE lines with enhanced tolerance to various abiotic stresses. The grain yields of transgenic wheat lines were increased under salt condition compared to WT. Moreover, our results indicated that the mechanism by which overexpression of TaASR1-D increased stress tolerance of transgenic wheat plants is through improving the antioxidant system and ABA signalling. Based on its expression pattern and molecular function, we proposed a new model to explain the role of TaASR1-D in the abiotic stress response (Figure 7). When wheat plant encounters abiotic stresses, the stress signals (ABA/H_{2}O_{2}) induced the expression of TaASR1-D in root and vascular tissues, and TaASR1-D enhanced the antioxidant system and ABA synthesis. The increased endogenous ABA can activate ROS generation in apoplast by RBOH and be transported into the vascular system (Kuromori et al., 2018; Mittler and Blumwald, 2015). Thus, the stress signals were further amplified and the defence system was activated by TaASR1-D.

### Experimental procedures

#### Identification and sequence analysis of the TaASRs

To identify all TaASR genes in wheat, we used the ABA_WDS hidden Markov model (HMM) profile (PF02496) to blast the wheat proteome database (IWGSC Refsequence v1.1) with the HMMER3.0 package. The chromosomal location information was acquired by using Triticeae Multi-omics Center (http://202.194.139.32). The subcellular locations of TaASR proteins were predicted by Cell-Plloc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-Plloc-2). TaASR protein sequence analyses of relative molecular mass, isoelectric point, conserved motifs and phylogenetic relationships were performed using methods described previously (Hu et al., 2018; Xiao et al., 2019). The ASR sequences of seven other species used for phylogenetic analysis were obtained from public publications (Çakir et al., 2003; Golan et al., 2014; Huang et al., 2016; Jha et al., 2012; Joo et al., 2013; Virlouvet et al., 2011; Wang et al., 2016a). The promoter sequences of TaNAC1 and TaGPx1-D were obtained from EnsemblPlants (http://plants.ensembl.org/index.html).

#### Plant materials and treatments

See supplementary Method S1.

#### Plasmid construction and plant transformation

The coding sequence of TaASR1-D was inserted into the pAH25 plasmid driven by the maize ubiquitin promoter. The wheat genetic transformation was performed according to the described method (Li et al., 2017c). The seeds of transgenic wheat were used in experiments from homozygous T_{2} generation.

#### Physiological indices measurements

The contents of H_{2}O_{2}, MDA, soluble sugar, proline, and the activities of SOD, CAT, GPx and POD were evaluated using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The superoxide anion contents were measured using an assay kit (Suzhou Keming Biotechnology Co. Ltd., Suzhou, China). The endogenous ABA contents were assayed according to the instructions of the plant ABA ELISA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The assays of RWC and water loss rate were based on the method described (Zheng et al., 2011). To detect the IL rates under MV treatment, the first leaves of three-leaf-stage seedlings were detached and washed three times with deionized water, and then, the leaves were submerged in 10 ml of water with 5 μM MV. The electrolyte conductivities of these solutions were measured at 0, 6, 12, 24, 36, 48 and 60 h. The whole plants were used to assay the IL rates of the control and 7-day treatment seedlings, and the electrolyte conductivities were determined according to our previous study (Hu et al., 2013).

#### Histochemical staining

To detect the endogenous H_{2}O_{2} levels under normal and stress conditions, three-leaf-stage wheat seedlings were given treatments for 4 days on 1/2 MS medium with or without 100 mM H_{2}O_{2}, 200 mM NaCl and 300 mM mannitol, and subsequently stained by DAB as previously reported (Hu et al., 2013). These results were imaged using a stereomicroscope (Stemi 508, Carl Zeiss, Jena, Germany).

To study the tissue-specific expression of TaASR1-D, the roots, stems and leaves of wheat cv. Chinese Spring plants during the...
heading stage were used for RNA in situ hybridization. The digoxigenin-labelled probes were synthesized by AuGCT DNA-SYN Biotechnology Co., Ltd. (Wuhan, China), and the probe sequences are shown in Table S2. The hybridization experiments were performed based on the publication with some modifications (Wu and Wagner, 2012), and the images were acquired using an upright microscope (BX53, Olympus, Tokyo, Japan).

Determination of Na\textsuperscript{+} and K\textsuperscript{+} contents

To analyse the accumulation of Na\textsuperscript{+} and K\textsuperscript{+} in leaves under normal and high-salinity conditions, the three-leaf-stage seedlings were treated for 7 days with or without 200 mM NaCl. Then, the leaves were collected, and the Na\textsuperscript{+} and K\textsuperscript{+} contents were measured by atomic absorption spectroscopy (TAS-990, Persee, Beijing, China).

Quantitative real-time RT-PCR (qRT-PCR) analysis

The extraction of RNA and synthesis of cDNA was performed according to the kit instructions (Tiangen Biotech, Beijing, China). The qRT-PCR was conducted by using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) and a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). In addition, all relevant genes and primers are listed in Table S2, and some of them were designed based on other publications (Kumar et al., 2013; Wang et al., 2016b). The expression data were calculated via the 2\textsuperscript{-ΔΔCt} method (Livak and Schmittgen, 2001).

Figure 7  Diagrammatic representation of the proposed model for TaASR1-D function under abiotic stress. The expression of TaASR1-D is induced by stress signals (ABA/H\textsubscript{2}O\textsubscript{2}), and then, TaASR1-D triggers the antioxidant system to scavenge the excess ROS in a cell. Meanwhile, TaASR1-D activates the transcription of TaNCED1 and upregulates endogenous ABA synthesis. The enhanced ABA signalling participates in the ROS wave by regulating the activity of RBOHs to produce ROS in the apoplast, and the upregulated ABA can be exported to the extracellular fluid by ABA transporters to amplify the delivered stress signalling by vascular tissue and activate defence system.

Yeast one-hybrid assay

The recombinant vectors of pGADT7-TaASR1-D, pHIS2-GT-1 and pHIS2-GAVGAVGA were transformed into the yeast cell AH109 according to the manufacturer’s protocol (Clontech, USA), and pGADT7-TaASR1-D co-transformed with pHIS2 empty vector was used as the negative control. Transformed strains were selected on double-dropout SD medium (SD/-W/-L) and by PCR, and the binding activities were evaluated according to the growth status of yeast cells on SD/-W/-L/-H (40 mM 3-AT) and SD/-W/-L/-H (80 mM 3-AT) media.

Electrophoretic mobility shift assay

The ORF of TaASR1-D was cloned into pET-28a(+), and then, the recombinant vector was transformed into E. Coli Rosetta (DE3) competent cell (CWbio, Beijing, China). The histidine-tagged TaASR1-D-recombinant protein was purified using Ni\textsuperscript{2+} affinity chromatography (GE Healthcare, Waukesha, USA), and the probes labelled with or without biotin were synthesized by TSINGKE Biological Technology Co., Ltd. (Wuhan, China). The EMSA was performed according to the method described with modifications (Yu et al., 2019). In brief, the binding buffer contained 10 mM Tris-HCl pH 7.5, 2 mM MgCl\textsubscript{2}, 50 μM ZnCl\textsubscript{2}, 50 mM NaCl, 0.2 mM EDTA and 10% (v/v) glycerol, and samples were separated on a 10% native polyacrylamide gel in 0.5 x TBZ buffer (45 mM Tris-H\textsubscript{2}BO\textsubscript{3} pH 8.0, 1 mM ZnCl\textsubscript{2}). Then, the samples were transferred to a positively charged nylon membrane (Millipore, Bedford, MA, USA), and the detection was performed.
using HRP-Streptavidin at a 1:4000 dilution (Beyotime, Shanghai, China).

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Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

DQ, WH, GH and GY conceived the study. DQ, WH and YZ performed most of the experiments. WH conducted wheat transformation. JX, RH, QW, YZ, JF, FZ, FS and JS provided technical assistance. DQ wrote the draft manuscript. DQ and WH contributed equally to this work.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The *in silico* analysis of putative wheat (*Triticum aestivum*) ASR family members.

Figure S2. The predicted conserved motif sequences in TaASRs.

Figure S3. Multiple sequence alignment of all putative TaASR protein sequences.

Figure S4. Phylogenetic relationships of ASR proteins from eight plant species.

Figure S5. Four transgenic wheat lines were acquired.

Figure S6. The transgenic wheat seedlings were less damaged by the oxidants.

Figure S7. Phenotypes of wheat seedlings at the three-leaf stage after 300 mM mannitol treatment for 7 days.

Figure S8. Phenotypes of wheat seedlings at the three-leaf stage after 200 mM NaCl treatment for 7 days.

Figure S9. Schematic diagram of planting pattern for the physiological and yield analysis.

Figure S10. Physiological indices of WT and OE lines during the heading stage under normal and stress conditions.

Figure S11. ABA contents of WT and L39 seedlings under mannitol treatment.

Figure S12. TaASR1-D regulated the expression levels of ABA-related and stress-responsive genes.

Figure S13. Tissue-specific expression of TaASR1-D in root, stem, and leaf.

Figure S14. Locations of GT-1 and GAVGAVGA boxes in the promoters of TaNCED1 and TaGPx1-D.

Table S1. Gene and protein characteristics of the putative ASRs in wheat (*Triticum aestivum*).

Table S2. Primer and probe sequences used in the present study.

Method S1. Plant materials and treatments.