Expression of *Arabidopsis* Ornithine Aminotransferase (AtOAT) Encoded Gene Enhances Multiple Abiotic Stress Tolerances in Wheat

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Abstract

Key Message **The drought and salt tolerances of wheat were enhanced by ectopic expression of the *Arabidopsis* ornithine aminotransferase (AtOAT) encoded gene. The OAT was confirmed to play a role in proline biosynthesis in wheat.**

Abstract Proline (Pro) accumulation is a common response to both abiotic and biotic stresses in plants. Ornithine aminotransferase (OAT) is pyridoxal-5-phosphate dependent enzyme involved in plant proline biosynthesis. During stress condition, proline is synthesized *via* glutamate and ornithine pathways. The OAT is the key enzyme in ornithine pathway. In this study, an OAT gene *AtOAT* from *Arabidopsis* was expressed in wheat for its functional characterization under drought, salinity and heat stress conditions. We found that the expression of *AtOAT* enhanced the drought and salt stress tolerances of wheat by increasing the proline content and peroxidase activity. In addition, it was confirmed that the expression of *AtOAT* also played a partial tolerance to heat stress in the transgenic wheat plants. Moreover, quantitative real-time PCR (qRT-PCR) analysis showed that the transformation of *AtOAT* up-regulated the expression of the proline biosynthesis associated genes *TaOAT*, *TaP5CS*, and *TaP5CR*, and down-regulated that of the proline catabolism related gene *TaP5CDH* in the transgenic plants under stress conditions. Moreover, the genes involved in ornithine pathway (Orn-*OAT*-P5C/GSA-*P5CR*-Pro) were up-regulated along with the up-regulation of those genes involved in glutamate pathway (Glu-*P5CS*-P5C/GSA-*P5CR*-Pro). Therefore, we concluded that the expression of *AtOAT* enhanced wheat abiotic tolerance *via* modifying the proline biosynthesis by up-regulating the expression of the proline biosynthesis associated genes and down-regulating that of the proline catabolic gene under stresses condition.

Introduction

The expansion of the global human population, which is a serious challenge for sustainably social development of the world, requires continuous increase in crop production. Among the cereal crops, wheat (*Triticum aestivum* L.) is ranked the second in terms of total global production but the first in terms of the total cultivation area (FAO 2018). However, wheat productivity is heavily affected by abiotic stresses including drought, salinity, and heat (Abhinandan et al. 2018). To produce more foods, wheat has to be planted in some arid and salinize arable lands. Especially, global warming, drought and heat stresses, which have happened more frequently with increased intensity, impose a serious threat in wheat production (Liu et al. 2015). Thus, it is of importance to develop wheat varieties adapted to these abiotic stress conditions, and identification of more useful genes by genetic engineering approaches is helpful to this breeding objective.

Plants under salinity and drought stresses have evolved a range of physiological and biochemical responses, such as accumulation of compatible osmolytes like proline (Pro) and the increased activity of antioxidant enzymes (Sarker and Oba 2018a, 2018b; Sarker et al. 2018). Proline is the most important

osmolyte in plants as a stress indicator because of its large water-solubility (162.3 g/100 ml H_2O) and strong hydrating ability. It plays a crucial role in osmoregulation in algae, plants and animals by protection of enzyme and cellular structure (Delauney et al. 1993; Kishor et al. 1995; Schobert 1977; Solomon et al. 2006). It also serves as a scavenger of free radicals and energy source to regulate redox potential and maintain the nitrogen content (Alia and Saradhi 1991; Sarker and Oba 2018c, 2020). There exist two proline biosynthesis pathways in plants, namely glutamate (Glu) and ornithine (Orn) pathways, in front of biotic and abiotic stress conditions (Anwar et al. 2018; Delauney et al. 1993; Hare et al. 1999; Roosens et al. 1998; Yoshiba et al. 1997). In the glutamate pathway, glutamate is reduced into pyrroline-5-carboxylate (P5C) by P5C synthase (P5CS). In addition, plants can synthesize proline *via* ornithine pathway, in which ornithine is firstly converted into P5C by OAT in mitochondria, and then P5C is either transformed into glutamate by P5C dehydrogenase (P5CDH) initiating glutamate pathway (Funck et al. 2008), or transferred to cytosol to produce proline by P5C reductase (P5CR) (Fig. S1) (Miller et al. 2009). The coordinated action of P5CDH and P5CR encoding genes determines the final use of P5C under stress condition (Rizzi et al. 2015). Proline catabolism occurs through sequential actions of proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH), which convert P5C to glutamate (Szabados and Savoure 2010). Pyrroline-5-carboxylate is a common intermediate in both proline biosynthesis and catabolism pathways (Rizzi et al. 2015; Anwar et al. 2018). Many studies have clarified that proline accumulation is mainly controlled by up-regulation of the genes involved in proline biosynthesis pathway and down-regulation of the genes involved in proline catabolism pathway, when plants are exposed to different stresses (Yoshiba et al. 1997; Anwar et al. 2018).

The OAT is the key enzyme in ornithine pathway of proline biosynthesis. As a transaminase, this enzyme is involved in the conversion of ornithine into glutamate and glutamyl-5-semi-aldehyde (GSA). The biosynthesis of glutamate from ornithine is speculated as ornithine pathway, but the exact route of the ornithine pathway for proline biosynthesis is still unclear as an unknown exit route of P5C/GSA from mitochondria to cytosol is required (Stránská et al. 2008). The glutamate pathway for proline biosynthesis is well known in plants, but the ornithine pathway is poorly understood. Similar to the glutamate pathway, the ornithine pathway has been found to play a significant role in multiple stress conditions (Hervieu et al. 1995; You et al. 2012; Anwar et al. 2018). Several studies have revealed the correlation between OAT and proline biosynthesis under abiotic stress conditions such as cold (Charest and Ton Phan 1990), salt (Roosens et al. 1998), dehydration (Hervieu et al. 1994), and ABA (Yang et al. 2000). Similar to P5CS, OAT is also a rate-limiting enzyme in proline biosynthesis (Roosens et al. 2002), except that OAT is not feedback inhibited by proline (Roosens et al. 1999). A positive role of OAT was also found in radish (*Raphanus sativus* L.) plants under salt stress (Hervieu et al. 1995). Overexpression of an OAT encoding gene in tobacco (*Nicotiana tabaccum* L.) increased proline content up to 3-fold higher in the transgenic plants than in the wild-type (WT) plants (Roosens et al. 2002). Similarly, overexpression of *Arabidopsis OAT* (*AtOAT*) in rice (*Oryza sativa* L.) was reported to enhance its tolerances to salt and drought stresses by increasing proline content (Wu et al. 2003).

Considering the important roles of *AtOAT* in the tolerance to multiple abiotic stresses in other plants, it is necessary to investigate its functions and molecular mechanism in wheat for the improvement in environmental tolerances. At present, no study has been conducted on the functional characterization of *AtOAT* in wheat. In this study, we expressed *AtOAT* gene in wheat and analysed its effects on tolerance against salt, drought, and heat and also dissected the ornithine pathway of proline biosynthesis in transgenic wheat plants. Our results will be helpful for further developing new wheat varieties with enhanced tolerance to various abiotic stresses.

Materials And Methods

Plant materials

Two spring wheat varieties Fielder and Ningchun 4 used in this study were obtained from the National Crop Germplasm Bank of China at the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (ICS-CAAS). Relatively, Fielder is regarded as a drought sensitive genotype and Ningchun 4 as a moderately drought resistant genotype. Five wheat plants per pot (25 cm in diameter and 18 cm in height) were grown in a growth chamber with 45% humidity at 24°C under a 16/8 h light/dark photoperiod cycle. Immature embryos 15 d post anthesis were collected from the two wheat varieties to be used for *Agrobacterium*-mediated transformation. A maize (*Zea mays* L.) inbred line Zheng 58, which was kindly provided by Dr. Yongjun Liu at ICS-CAAS, was planted in a greenhouse as a pollen donor to produce stable transgenic lines *via* chromosome elimination.

Plasmid construction and wheat transformation

The full-length complementary DNA (cDNA) sequence of *AtOAT* (accession number: At5g46180) was amplified from seedlings of the *Arabidopsis* ecotype Columbia-0 using primers AtOAT-F and AtOAT-R (Table S1). After confirmation by sequencing, it was inserted onto an expression vector *pWMB174* (Fig. S2), which contains two independent T-DNA regions, one carrying *bar* selectable marker under the control of *CaMV35S* promoter and *Agrobacterium* nopaline synthase (NOS) terminator, and the other carrying maize ubiquitin (ubi) promoter, a multiple cloning site (MCS) and the NOS terminator. The *AtOAT* gene was digested with *Sma*I and *Spe*1 and inserted onto the digested *pWMB174* with the same enzymes at the MCS under the control of ubi promoter to form a new recombination vector *pWMB175* (Fig. 1a). The new vector was transformed into *A. tumefaciens* strain C58C1 by triparental mating (Ditta et al. 1980) and further introduced into wheat immature embryos using the methods described previously (Ishida et al. 2015; Wang et al. 2017).

Quickstix strip detection

Leaf segments of the putative transgenic wheat plants generated from *Agrobacterium*-mediated transformation were collected and detected by a QuickStix Kit (EnviroLogix, USA) for the selection marker

existence of *bar* gene as described previously (Wang et al. 2017). All the putative transgenic plants were tested by this method and further confirmed by other methods.

DNA extraction and PCR amplification

Genomic DNA (gDNA) was extracted from the putative transgenic wheat plants using NuClean Plant Genomic DNA Kit (CWbio Inc., Beijing, China). The DNA pellet was dissolved in ddH_2O and quantified to be used in PCR amplification as templates. The presence of the transgene was detected by specific primer AtOAT-F/AtOAT-R (Table S1), and PCR was carried out in a 15 µL reaction buffer containing 6 µl of sterile distilled water, 1 µL of template DNA (100-200 ng μL^{-1}), 0.5 µL of each primer (10 µmol L^{-1}), and 7.5 µL of 2× Taq Master Mix (CWbio Inc., Beijing, China). DNA amplification was run on an ABI Thermal Cycler (ProFlex PCR,Thermofisher Scientific) by the following steps: an initial denature at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min and 30 sec, and a final extension at 72 °C for 8 min. The amplified *AtOAT* product was separated on a 1% agarose gel and visualized under ultraviolet light after Gene color II (Gene-Bio, Beijing, China) staining.

Generation of stable homozygous transgenic wheat plants by chromosome elimination

Twenty spikes from each T_1 transgenic lines derived from Fielder and Ningchun 4, respectively, were emasculated when they were fully emerged out of flag leaves. The emasculated spikes were pollinated with maize pollens and then treated by spraying 100 mg L^{-1} 2,4-D solution once (1 ml for each spike) in the next two consecutive days. The haploid wheat embryos were rescued on a medium (1/2 MS medium, 30 g L^{-1} sucrose and 2.4 g L^{-1} gelrite, pH 5.8) at 21 d day after the pollination. The plantlets with healthy roots were directly treated by adding a freshly prepared colchicine solution containing colchicine 0.45 g L^{-1}, DMSO 20 mL L^{-1}, GA_3 100 mg L^{-1} and Tween 20 0.3 mL L^{-1} on the medium for 30 h. Finally, the treated plantlets were rinsed with water 3 times and transplanted into pots. Positive plants were selected by PCR analysis with the target gene specific primer using the same condition aforementioned.

Chromosome preparation and fluorescence in situ hybridization

Mature grains of the transgenic wheat plants were germinated on filter paper in a 25 °C growth chamber for 2 to 3 d, and roots of 2 cm in length were sampled. Root tips were cut and treated with nitrous oxide (NO) for 2 h prior to immersion in 90% glacial acetic acid solution for 5 min. Chromosome spreading was prepared by a previously described method (Guo et al. 2016). Fluorescence *insitu* hybridization (FISH) was performed using vector *pWMB175* as a probe, which was labelled with fluorescein-12-dUTP. Hybridization was performed following a previous published protocol (Guo et al. 2016), and the

hybridization signals were observed under an Olympus BX-51 microscope with a Photometric SenSys Olympus DP70 CCD camera. Three stable transgenic lines from Fielder and Ningchun 4 each, which were confirmed by PCR and FISH, were selected for further functional investigation.

Treatments of abiotic stresses

For drought stress treatment, seeds from the transgenic and the WT wheat plants were sown in a plastic box (80 cm × 50 cm × 15 cm in dimension) containing substrate peat moss by a completely randomized design, and three replicates were set up. Four-liter water was provided to each box every week after germination. At the tillering stage, irrigation was stopped and volumetric water content (VWC) in the pots was measured. When the VWC is below 5%, the survival rate was calculated following the method described previously (Rong et al. 2014).

To identify the salt tolerance of the transgenic wheat plants, mature embryos of the *AtOAT* expressed wheat and the WT plants were entirely isolated, inoculated onto 1/2 MS medium containing 150 and 200 mM NaCl, and cultured at 25 °C for 30 d under light condition with an optical density of 100 μmol m^{-2} s^{-1} and a photoperiod of 16 h light and 8 h dark. Germination percentage was calculated after 8 d and survival rate and root and shoot lengths were investigated after 30 d.

For heat stress treatment, five plants from each transgenic line and the WT were grown in a plastic box (35 cm × 25 cm × 8 cm in dimension) with three replicates. At the tillering stage, plants were treated in a heat chamber at 42 °C and samples were collected at different time regimes (0 h, 12 h, 24 h, and 36 h) for analyzing relative expression profiles of the target gene and its associated genes.

Measurementsof physiological parameters under abiotic stresses

To measure physiological parameters related to drought stress, plants were grown in a plastic box (80 cm × 50 cm × 15 cm in dimension) containing natural field soils and maintained in a glasshouse with 16 h light at 24 °C and 8 h dark at 16 °C. Four-liter water was provided to each box every week after germination. At the tillering stage, water was withheld for 21 d and leaf samples were collected at different time regimes (0-, 7-, 14- and 21-day) during the stress treatment. Free proline level was measured according to the previously described method (Zhang et al. 2009). Antioxidant enzymes were extracted from 0.1 g leaves with 1.8 ml phosphate buffer (PBS; 0.1 mol L^{-1}, pH 7.4) on ice, and the supernatant after centrifuge at 10,000 *g* for 10 min was directly used to measure peroxidase (POD) and catalase (CAT) activities using corresponding detection kits (A084-1 and A007-1 Jiancheng, China).

For the measurement of physiological parameters related to salt stress, 25 wheat plants were grown in a plastic box (20 cm × 10 cm × 10 cm in dimension) containing natural field soil and maintained in a glasshouse with the conditions aforementioned. At the tillering stage, plants were treated with 250 mM

NaCl solution 5 times in 30 d (one time every six days) until the clear symptoms of stress were observed. Total chlorophyll content was measured by the previously described method (Sarker and Oba 2018c). Free proline content and antioxidant enzymes activities were measured as described aforementioned.

RNA extraction and expression profile

The expression level of the target gene in the stable transgenic wheat plants was determined by semi-quantitative PCR using its specific primer (Table S1). The semi-quantitative PCR amplification was run on an ABI thermal cycler (ProFlex PCR) in a reaction buffer (20 µL) containing 10 µL Taq master mix, 0.5 µM of each primer, and 400 ng of cDNA. The cycling conditions were started from an initial denature at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 20 sec, and a final ending extension at 72 °C for 5 min.

For analyzing the expression of *AtOAT* in the positive wheat seedlings, RNA was extracted from their roots and leaves. To determine the expression profiling of the proline biosynthesis and catabolism associated genes, total RNA was extracted from the leaves in the stressed transgenic wheat plants after NaCl treatment on medium for 30 d and after water-withholding stress for 15-18 d in soil using a TRIZOL reagent (TaKaRa, Japan). The cDNA was further synthesized using a cDNA synthesis kit (CWbio Inc., Beijing China) according to manufacturer's protocol.

To examine the role of *AtOAT* in proline biosynthesis, the expression profiling of the genes involved in proline biosynthesis (*TaOAT*, *TaP5CS*, and *TaP5CR*) and catabolism (*TaP5CDH*) was determined by quantitative real-time PCR (qRT-PCR) with their specific primers (Table S1) in the stress treated transgenic wheat plants and the WT. The primers for *TaOAT* and *TaP5CR* were adopted from previous publications (Anwar et al. 2020, Dudziak et al. 2019). The sequences for *TaP5CS1* and *TaP5CDH* were retrieved from plant ensemble genome database (https://plants.ensembl.org/Triticum_aestivum/Tools/Blast) using *Hordeum vulgare* (accession # AF467539.1) and *Arabidopsis thaliana* (Accession # AY150430.1) as queries. Three alleles for *TaP5CS1* (TraesCS1A02G357900, TraesCS1B02G374500, and TraesCS1D02G362700) and three alleles for *TaP5CDH* (TraesCS1A02G281400, TraesCS1B02G290600, and TraesCS1D02G280700) were retrieved and their specific primers were designed using primer premier 5 (Premier Biosoft, http://www.premierbiosoft.com/).

The qRT-PCR was performed on an Applied Biosystems™ 7500 Real-Time PCR System in a reaction buffer (20 µL) containing 10 µL 2× RealStar Green Fast Mixture with ROXII, 400 ng of cDNA, and 0.4 µM of each primer. The PCR samples were preheated at 95 °C for 10 min, followed by 40 cycles of amplification (95 °C for 15 sec, 60 °C for 30 sec, 72 °C for 30 sec). The relative expression or mRNA level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). A constitutively expressed wheat gene adenosine diphosphate ribosylation factor (*TaADP*) was used for the normalization of gene transcript level (Paolacci et al. 2009). Data were averaged from three biological replicates and statistical significance was calculated by IBM-SPSS statistic 20 at $\alpha < 0.05$ using the Duncan's multiple range test.

Results

Generation of homozygous transgenic wheat plants expressing AtOAT

Haploid embryos from the cross between the transgenic wheat plants and maize were rescued with a frequency of 11% for Fielder and 8% for Ningchun 4 as receptors, respectively (Table S2). After chromosome doubling, 37 homozygous transgenic wheat plants expressing *AtOAT*, 24 for Fielder and 13 for Ningchun 4, were generated (Fig. S3). Seed setting percentages of homozygous plants derived from Fielder and Ningchun 4 were 50% and 40%, respectively. Cytological observation confirmed that the haploid plants induced by maize pollen before doubling had 21 chromosomes and the stable transgenic plants after doubling had 42 chromosomes (Fig. 1b, c). Fluorescence *in situ* hybridization assay displayed that the T-DNA cassette containing *AtOAT* was integrated into the distal regions of one or two pairs of chromosomes in the stable transgenic wheat plants (Fig. 1d). Results also indicated that *AtOAT* was inserted into wheat genome by one or two copies. Three independent lines OE-F1, OE-F2, and OE-F3 from Fielder and the other three independent lines OE-N1, OE-N2, and OE-N3 from Ningchun 4 were selected for the functional characterization of *AtOAT* gene in wheat further. Semi-quantitative PCR revealed that the *AtOAT* transgene was highly expressed in the homozygous transgenic wheat plants (Fig. 1e, f).

Transgenic wheat plants showed an enhanced salt tolerance under controlled condition

On the 200 mM NaCl containing medium, mature embryos of the transgenic wheat plants derived from Ningchun 4 showed a higher germination percentage than those of the WT plants in which only a few mature embryos were germinated (Fig. S4a). Moreover, the transgenic plants derived from Ningchun 4 grew faster than the WT (Fig. 2), and the WT plants were severely affected as compared to the transgenic plants. After being cultured for 30 d on the NaCl containing medium, the survival rate of the WT plants (< 10%) was less than that of the transgenic plants (up to 40%) (Fig. 2a, b).

The transgenic plants derived from Fielder on the aforementioned salt containing medium showed a similar result on mature embryos germination percentage to that from the Ningchun 4 derived transgenic plants under the stress condition. But they did not grow well and displayed a stunted growth status in later growth stages (Data not shown). Thereby, these transgenic plants were tested on the medium containing 150 mM NaCl. Mature embryos of the three transgenic lines showed a higher germination rate in a similar manner as cultured on 200 mM NaCl containing medium (Fig. S4d). It was found that the transgenic plants grew much better than the WT. Especially, a higher survival rate ranging from 40–70% was observed in the transgenic plants compared to the WT (Fig. 2e, f). The transgenic plants derived from both varieties exhibited longer and more secondary roots and longer shoots under the salt stress condition than the WT (Fig. S4b, c, e, f). It was revealed that *AtOAT* was highly expressed in the leaves and roots of the transgenic lines under the salt stress (Fig. 2c, d, g, h). These results indicate that the expression of *AtOAT* enhances wheat tolerance to salt stress.

Physiological analysis discovered that proline content, chlorophyll contents, POD and CAT activities were not significantly changed in the transgenic lines derived from both Fielder and Ningchun 4 and their wild types under normal condition (Fig. 3; Fig. S5, 6). In contrast, proline content was significantly enhanced in the transgenic lines as compared to the two wild types under salt stress condition (Fig. 3b; Fig. S5b). Chlorophyll content was reduced under salt stress condition as compared to normal condition but it was relatively higher in the transgenic plants than that in the wild types (Fig. 3c; Fig. S5c). The POD activity was slightly enhanced in the transgenic plants compared to the wild types under salt stress condition (Fig. 3d; Fig. S5d). No difference in catalase activity between the transgenic lines derived from Ningchun 4 and the WT was observed under normal and salt stress conditions (Fig. S6a), while only one transgenic line derived from Fielder (OE-F2) showed an enhance catalase activity (Fig. S6b).

Transgenic Wheat Plants Showed An Enhanced Drought Tolerance

The transgenic lines and their two wild types of Fielder and Ningchun 4 were subjected to water withholding at the tillering stage (Fig. 4). After water withholding for 15 d, the WT Fielder's growth was severely affected as compared to its corresponding transgenic lines (Fig. 4a); while the WT Ningchun 4 and its transgenic lines were less affected by the drought stress. After water recovery for 10 d, the survival rate was only 40% for Fielder and 77 to 93% for its transgenic lines; but there was no difference observed between the WT Ningchun 4 and its transgenic lines (Fig. 4a, c). Considering that Ningchun 4 is more drought-tolerant than Fielder, a prolonged drought period of 18 d was adapted for Ningchun 4 and its transgenic lines. After water recovery for 10 d the survival rate was 68 to 75 % for the transgenic lines and 48.8% for the WT Ningchun 4 (Fig. 4b, 4e). These results indicate that the incorporation of *AtOAT* enhances the drought tolerance not only in the drought susceptible wheat variety Fielder but also in the drought-tolerant wheat variety Ningchun 4. Furthermore, the expression of *AtOAT* in the transgenic lines was significantly enhanced under drought condition as compared to the normal condition (Fig. 4d, f). These results indicate that the enhancement of drought tolerance in the transgenic wheat plants is attributed to the expression of *AtOAT*.

Under normal condition (0-day) there was no significant difference in proline content among the transgenic plants and their wild types except a slight increase in the two transgenic lines (OE-N1 and OE-N2) from Ningchun 4. Proline content was gradually enhanced with the duration of drought stress in the transgenic lines and their WT. A significant difference in proline content was observed between the transgenic lines and their WT at the three time points (7-, 14- and 21-day after water withholding) during the stress period and this difference was also obvious even after water recovery for a week (Fig. 5a, b). In general, POD activity was increased during the drought stress and decreased after water recovery in the transgenic lines and their two wild types. A significant difference in POD activity was observed during the stress period and no difference was found after recovery between the transgenic plants and their wild types (Fig. 5c, d). For the CAT activity, however, no significant difference was observed in the transgenic lines and their wild type Ningchun 4 at 0- and 7-day after water withholding, while a slight increase was

observed at 14-day of drought stress. Afterwards, CAT activity was declined in all plants and no significant difference was observed at 21-day and after drought stress between the transgenic and the WT Ningchun 4 plants (Fig. S7a). On the other hand, no significant difference was observed in the transgenic lines and their WT Fielder at 0-day, while one transgenic line (OE-F2) showed a slight enhanced CAT activity as compared to the WT Fielder at 7- and 14-day after water stress. Afterwards, a declined CAT activity was observed in all plants and no significant difference was found between the transgenic lines and Fielder at 21-day after water withholding (Fig. S7b). It is indicated that the expression of *AtOAT* not only regulated proline biosynthesis but also triggered antioxidant defense system to combat the water stress condition in wheat.

Transgenic wheat plants showed a slight tolerance to heat shock stress

After heat stress treatment 42°C for 36 h, the WT plants started wilting from 24 to 36 h after the stress, and the transgenic plants grew normally (Fig. 6a, c). This indicates that the expression of *AtOAT* confers wheat with certain level of tolerance against heat stress. The qRT-PCR analysis displayed that the relative expression of *AtOAT* in the transgenic Ningchun 4 lines was significantly induced at 12 and 24 h and then reduced at 36 h after heat stress, and it was very low before heat stress; the relative expression of *AtOAT* in the transgenic Fielder lines was significantly induced at 12 and 24 h and peaked at 36 h after heat stress, and it was in a negligible level before heat stress (Fig. 6b, d). These results indicate that the improvement of heat tolerance in the transgenic wheat plants could arise from the expression of *AtOAT*.

Expression of proline metabolic genes in response to salt, drought and heat stresses

The expression levels of the wheat endogenous genes involved in the proline biosynthesis, including *TaOAT*, *TaP5CS1*, and *TaP5CR*, were enhanced in the Ningchun 4 transgenic lines as compared to the WT under drought and salt stresses. Similarly, the expression of proline catabolic gene *TaP5CDH* was down-regulated in the transgenic lines in comparison with the WT plants under drought and salt stresses (Fig. 7). Similar expression pattern was also observed in the Fielder transgenic lines (Fig. S8). The up-regulated expression of proline biosynthesis genes and down-regulated expression of proline catabolic genes caused an increased proline accumulation under drought and salt stresses in the transgenic wheat plants. Based on the expression results, it is concluded that glutamate pathway involved in the proline biosynthesis was active during drought and salt stresses as the expressions of *TaP5CS1* and *TaP5CR* were up-regulated in the transgenic wheat plants. Meanwhile, the expression of endogenous *TaOAT* was also up-regulated in the transgenic plants, which infers that the ornithine pathway is also involved in proline accumulation due to stress conditions.

Under heat stress, the expression levels of glutamate pathway of proline biosynthesis genes *viz TaP5CS1* and *TaP5CR* were significantly enhanced in the transgenic plants as compared to the WT plants, but the expression of the key gene for ornithine pathway of proline biosynthesis *viz TaOAT* remained unchanged (Fig. 7, S8). Like the situation under drought and salt stresses, the expression of proline catabolic gene *TaP5CDH* was also down-regulated in the transgenic lines derived from Fielder and Ningchun 4 in comparison with their WT plants under heat stress (Fig. 7, S8). Taken together, only the glutamate

pathway for proline biosynthesis was activated during heat stress. In general, it was concluded that the expression of *AtOAT* enhanced the abiotic stress tolerance in the transgenic wheat plants *via* up-regulating proline biosynthesis genes and down-regulating proline catabolism genes, which further leads to the accumulation of proline during stress conditions.

Discussion

It is widely accepted that plants adapt to abiotic stress conditions by accumulating proline (Liang et al. 2013, Szabados and Savoure 2010). Normally, proline functions in protein biosynthesis *via* oxidation, and converts into glutamate for transamination or into ketoglutaric acid for Krebs cycle (Stewart and Hanson 1980). Under environmental stress condition, proline starts accumulation by increasing its biosynthesis and reducing its consumption (Yoshiba et al. 1997). Ornithine aminotransferase is thought to be involved in proline biosynthesis. Several studies have demonstrated the positive correlation of OAT and proline synthesis during stress condition. For example, *AtOAT* showed a positive correlation with salt tolerance and was found to be associated with proline biosynthesis (Roosens et al. 1998). In another study, it was proven that the expression of *AtOAT* provides an additional way for proline biosynthesis in *Nicotiana plumbaginifolia* through increasing OAT activity (Roosens et al. 2002). Similarly, the expression of *AtOAT* in rice increased proline content from 5 to 15-fold compared to the WT plants under salt and drought stress conditions (Wu et al. 2003). It was also demonstrated that the overexpression of *OsOAT* enhanced osmotic tolerance in the transgenic rice (You et al. 2012). Recently, overexpression of *TaOAT-5BL* was found to enhance the drought tolerance in the transgenic wheat by increase in proline content (Anwar et al. 2020). The present study showed that the expression of *AtOAT* enhanced proline content in the transgenic wheat lines under drought and salt stress conditions (Fig. 3b, 5a, b). Based on the current results and previous studies, it is concluded that *AtOAT* expression enhances drought and salt tolerance in the transgenic wheat plants *via* promoting proline accumulation.

Oxidative stress interrupts plant normal cellular metabolism that leads to the production of reactive oxygen species (ROS) including singlet oxygen ($1O_2$), hydroxyl radicals (OH•), hydrogen peroxide (H_2O_2), alkoxyl radical (RO), and superoxide radical (O_2•−), which mainly results in an enhancing electrons leakage (Chaves and Oliveira 2004; Foyer and Noctor 2012). Plants have evolved diverse mechanisms to keep the ROS homeostasis, such as antioxidant enzymes (POD and CAT), and superoxide dismutase (SOD). Hydrogen peroxide is decomposed by different peroxidases, such as ascorbate peroxidase (APX), glutathione peroxidase (GPX), and CAT (Asada 1999; Dat et al. 2000; Sarker and Oba 2018c). The ROS produced in response to biotic or abiotic stress caused oxidative damage to plants. Plants detoxify the negative effect of ROS by initiating antioxidant defense system.

The POD catalyses the reduction of H_2O_2 using various electron donors, such as phenolic compounds, lignin precursors, auxin and secondary metabolites. Several studies reported an enhanced POD activity in response to drought and salinity in *Arabidopsis* (Jung et al. 2004), *Ramonda serbica* (Veljovic-Jovanovic et al. 2006), and *Medicago sativa* (Wang et al. 2009). In addition, an increased POD activity was tested in *Quercus ilex* and *O. sativa* under abiotic stresses by proteomic analysis (Echevarría-Zomeño et al. 2009;

Raorane et al. 2015). Moreover, the expression of *POD* genes was found to be increased in *Tamarix hispida* under drought stress (Gao et al. 2010). In the present study, we found that POD activity was significantly increased in the transgenic wheat lines as compared to each WT under drought and salt stress conditions (Fig. 4d, 6c, d). These results are consistent with one of the previous studies which demonstrated that *OsOAT* overexpression led to higher POD activity in transgenic rice in comparison with the wild type (You et al. 2012).

Generally, in our study the CAT activity was not significantly enhanced in Fielder and Ningchun 4 derived transgenic lines as compared to each WT. But a slight increased CAT activity was observed at 14-day after water withholding in the Ningchun 4 derived transgenic lines as compared to the WT. No change on CAT activity was found in the Fielder derived transgenic lines and the WT and a slight increased CAT activity was only observed in the Ningchun 4 derived transgenic lines under this extend water stress, which might be due to the different drought sensitivity response of the two cultivars. A previous study demonstrated that the antioxidant defense capacity and enzymatic activities of POD, SOD, and CAT during abiotic stresses had genotype dependency (Abedi and Pakniyat 2010). Taking the above results together, we can infer that the increased POD activity might be considered as a key point for the decomposition of H_2O_2, especially under the fact of CAT inactivation. Furthermore, it is concluded that expression of *AtOAT* enhances wheat drought and salt tolerance not only by boosting proline biosynthesis but also by regulating antioxidant defense system.

The stimulation of proline biosynthesis under salt and drought stress was associated with an increase in the expression of *P5CS1* and *P5CR* (Hu et al. 1992; Yoshiba et al. 1995). Several studies have indicated that P5CS is the critical enzyme in proline biosynthesis during abiotic stress conditions (Savouré et al. 1995; Szoke et al. 1992). The proline accumulation *via* P5CS and P5CR is known as glutamate pathway (Szabados and Savoure, 2010). Glutamate pathway of proline biosynthesis is predominantly active during abiotic stress conditions. An increased expression of *P5CS* under osmotic stress was reported in wheat, barley (*Hordeum vulgare* L.), tomato (*Solanum lycopersicum* L.), switchgrass (*Panicum virgatum*), and *Lepidium draba* (Rana et al. 2016; Bandurska et al. 2017; Guan et al. 2019; Lopez-Galiano et al. 2019; Goharrizi et al. 2020). Our present study also reveals the activation of glutamate pathway in wheat under drought, salt and heat stresses as an increased expression of *TaP5CS1* and *TaP5CR* was observed in *AtOAT* expressed transgenic plants. Proline accumulation is regulated by the transcription levels of *P5CS* and *ProDH* (Verbruggen and Hermans 2008). During stress conditions, the expression of *ProDH* and *P5CDH* is suppressed when the transcript of *P5CS* is induced (Hein et al. 2016; Anwar et al. 2018). In this study, expression of *TaP5CDH* is remarkably decreased when the expressions of proline biosynthesis associated genes (*TaP5CS1* and *TaP5CR*) increased under drought, salt and heat stresses. Therefore, we can conclude that proline is accumulated in the stressed transgenic wheat plants through glutamate pathway.

In addition to the glutamate pathway, proline can also be biosynthesized from ornithine through OAT and P5CR (Fig. S1) (Rizzi et al. 2015). This controversial pathway initiates in mitochondria where ornithine is converted to GSA/P5C by the action of OAT enzyme (Liang et al. 2013). Then, P5C, the product of OAT

activity, is converted to glutamate by P5CDH and re-entered into glutamate pathway or is converted to proline by P5CR after transported from mitochondria to cytosol (Funck et al. 2008; Miller et al. 2009). The glutamate pathway for proline biosynthesis is well understood in plants but the ornithine pathway is not dissected clearly. Nerveless, several indirect shreds of evidence have been reported on the existence of ornithine pathway associated with proline accumulation under stress conditions. The positive relation of OAT activity and proline accumulation was revealed in the NaCl treated radish cotyledons and detached rice leaves (Hervieu et al. 1994; Yang and Kao 1999). The transgenic *Arabidopsis* plants overexpressing *AtOAT* showed increased salt and osmotic tolerances as their biomass and germination rates were significantly higher than the WT plants (Roosens et al. 2002). Additionally, it was found that exogenous application of ornithine increased OAT activity and proline accumulation in salt-stressed cashew plants (Da Rocha et al. 2012). Like *Arabidopsis AtOAT*, rice *OsOAT* was also found to have a key tolerant role to drought and other oxidative stresses as the overexpression of *OsOAT* enhanced the osmotic stress tolerance of the transgenic rice plants (You et al. 2012).

Our study provides a direct evidence for activation of ornithine pathway as an increased expression level of *TaOAT* was detected in the transgenic wheat lines, which led to the conversion of ornithine into P5C. However, a reduced expression level of *TaP5CDH* indicated that P5C was not converted into glutamate. Instead, it was transported to cytosol from mitochondria and then reduced into proline by the action of P5CR (Fig. 7, S8). Here, the important challenge is that how OAT-derived P5C is transported from mitochondria to cytosol (Fig. S1). There is an indirect evidence for the transportation of OAT-derived P5C from mitochondria to cytosol without any experimental confirmation (Da Rocha et al. 2012; Funck et al. 2010; Miller et al. 2009). The results obtained in our study are consistent with the findings by Da Roha et al. (2012) who demonstrated that the decreased expression of *P5CDH* and increased expression of *OAT* caused the accumulation of P5C in mitochondria and this OAT-derived P5C was moved to cytosol from mitochondria due to toxicity. In our present study, the increased expression of *TaOAT* and *TaP5CR* along with the decreased expression of *TaP5CH* in the transgenic wheat plants during drought and salt stress provided a clear evidence of activation of ornithine pathway. The unchanged expression of *TaOAT* indicated that ornithine pathway was not activated in the transgenic wheat plants under heat stress. We speculated that the ornithine pathway is also activated along with the glutamate pathway and plays a significant role in tolerance to drought and salt stresses in the transgenic wheat plants under the stress conditions. You et al. (2012) reported that the expression of *OsOAT* was highly induced in response to heat stress. In our study, the transgenic wheat plants just displayed a partial tolerance to heat stress. It might be the reason that only the glutamate pathway was activated in the transgenic wheat plants in front of heat stress.

Homozygosity is tremendously required in transgenic wheat study that guarantees the stable incorporation and inheritance of transgene. Normally, it takes 2–3 years to produce homozygous transgenic seeds for further investigation (Passricha et al. 2016). Double haploid (DH) technology ensures the production of complete homozygous wheat lines in a single year making the selection process effective in the breeding (Hussain et al. 2013). Thus, doubled haploid technology may significantly reduce the time for producing homozygous transgenic plants. There are two major methods

for producing wheat doubled haploids, which are known as androgenesis (anther culture and microspore culture) and gynogenesis (using wheat–maize hybridization). The chromosome elimination through wheat–maize hybridization is the most effective and widely used technique (Santra et al. 2017). In the present study, gynogenesis method was used to produce the homozygous transgenic lines. Thus, this method could help us to accelerate our transgenic wheat research by generating homozygous transgenic lines in a single generation.

Conclusions

The expression of *Arabidopsis AtOAT* encoding gene in wheat enhanced the tolerance of the transgenic plants to drought, salinity, and heat. The enhanced tolerance to the abiotic stresses in the transgenic plants was contributed to the increased proline accumulation and peroxidase activity. Further study indicated that the expression of the main genes associated with proline biosynthesis or catabolism. In addition to the main pathway of proline biosynthesis (e.g., *P5CS* and *P5CR*-Glu pathway), the alternative pathway, such as *OAT* and *P5CR*-ornithine pathway, played an important role in proline accumulation under drought and salinity stresses. However, the ornithine pathway is not activated during heat stress. We speculated that OAT-induced P5C was transported to cytosol from mitochondria and then reduced to proline by P5CR in the transgenic plants under the stress conditions.

Declarations

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Conflicts of interest/Competing interests

N/A

Availability of data and material

N/A

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Author contributions

AA performed most of the experiments and drafted the manuscript. KW and LD conducted wheat transformation. JW detected the transgenic wheat plants by fluorescence *insitu* hybridization. LS did part of abiotic stress tolerance test of the transgenic wheat plants. XY conceived the study and revised the manuscript.

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Figures

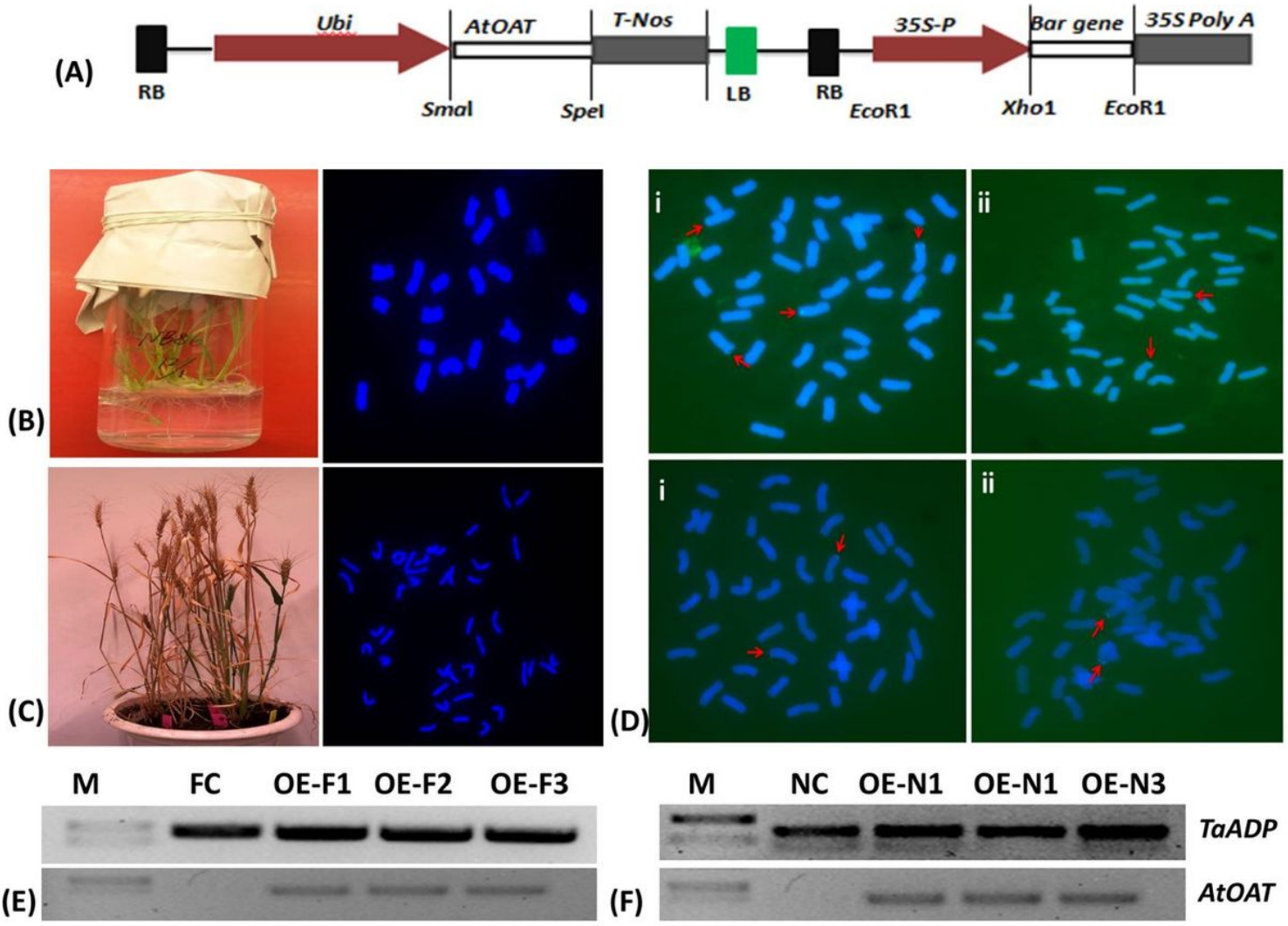

Figure 1

Generation and identification of stable transgenic wheat lines expressing AtOAT. a Schematic representation of expression vector pWMB175 containing AtOAT gene. b Haploid plants with 21 chromosomes. c Doubled haploid plants with 42 chromosomes. d Detection of the AtOAT gene integrated into wheat genome by fluorescent in situ hybridization (FISH). I and II are the transgenic lines derived from Fielder (FC), and III and IV are the transgenic lines from Ningchun 4 (NC). Two hybridization signals indicated that AtOAT are integrated into wheat genome by one copy, and four hybridization signals indicated that AtOAT are integrated into wheat genome by two copies. The presence of the transgene is indicated by arrows. e Semi-quantitative PCR analysis of the transgenic lines (OE-F1, OE-F2, and OE-F3) from FC. f Semi-quantitative PCR analysis of the transgenic lines (OE-N1, OE-N2, and OE-N3) from NC.

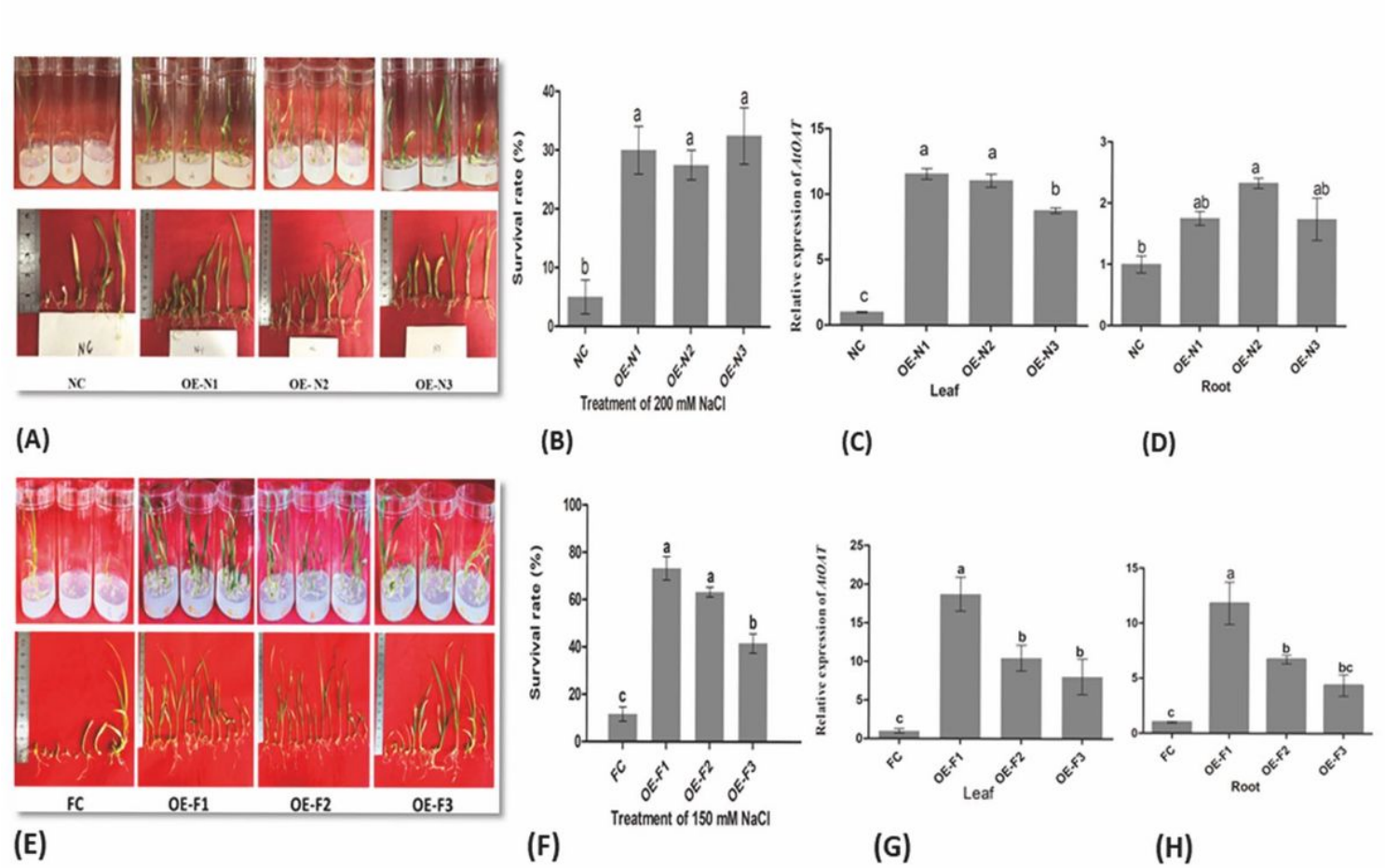

Figure 2

Characterization of salt tolerance in the transgenic wheat lines expressing AtOAT in in vitro salts stress condition. a Salt tolerance test of the transgenic lines expressing AtOAT (OE-N1, OE-N2, and OE-N3) derived from Ningchun 4 (NC). b Survival rate of the transgenic lines expressing AtOAT derived from NC after salts stress. c Relative expression of AtOAT in the leaves of transgenic lines derived from NC under salt stress. d Relative expression of AtOAT in the roots of transgenic lines derived from NC under salt stress. e Salt tolerance test of the transgenic lines expressing AtOAT (OE-F1, OE-F2, and OE-F3) derived from Fielder (FC). f Survival rate of the transgenic lines expressing AtOAT derived from FC. g Relative expression of AtOAT in the leaves of the transgenic lines derived from FC. h Relative expression of AtOAT in the leaves of the transgenic lines derived from FC. For relative expression analysis, data from three biological replicates were averaged and analyzed with IBM-SPSS statistic 20. Letters in bold represent significant differences between groups at α = 0.05. Bars indicate the standard errors of the means.

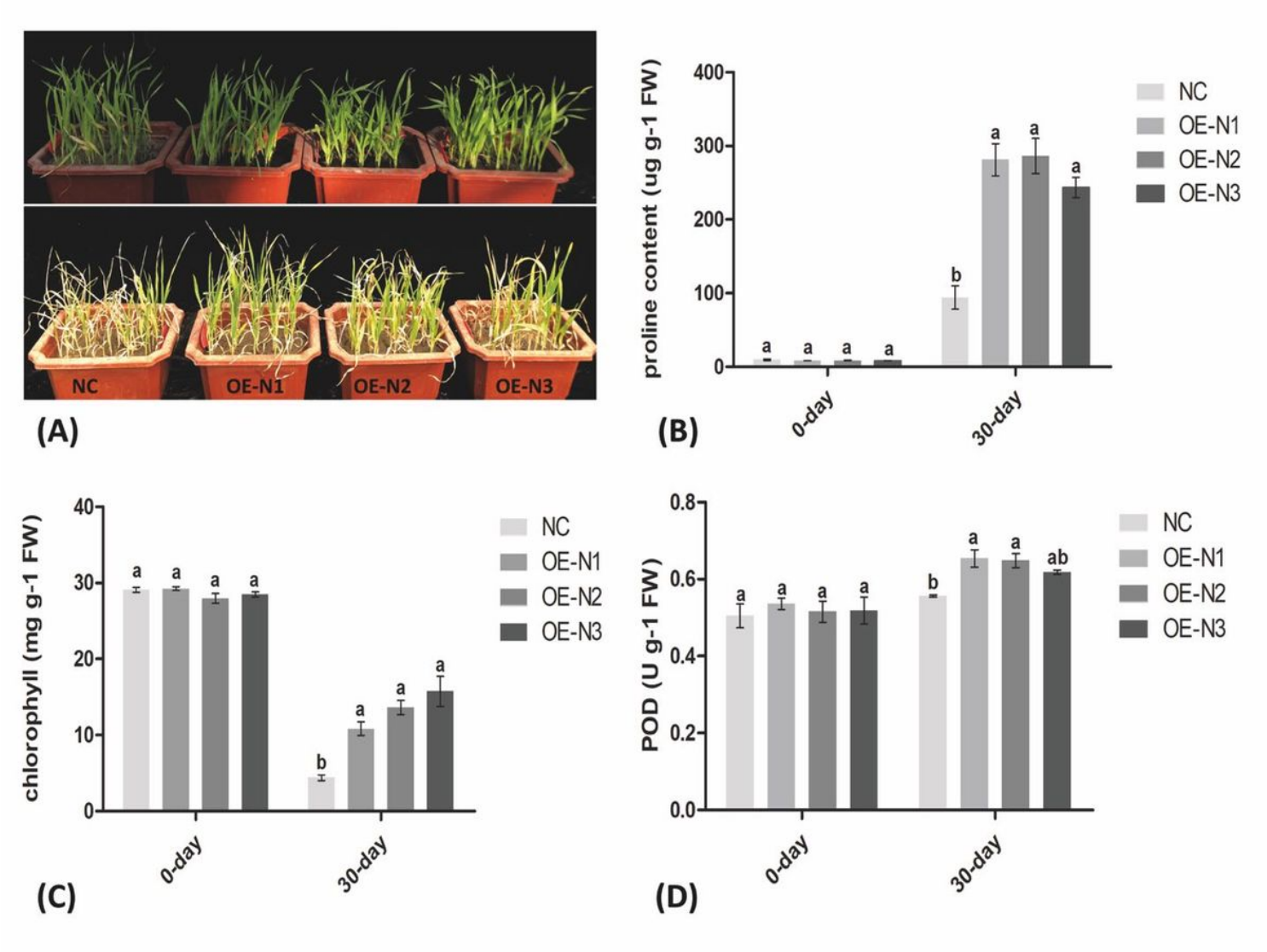

Figure 3

Determination of related physiological parameters in the transgenic wheat lines expressing AtOAT under salt stress. a Salt tolerant performance of the transgenic plants and their wild types after irrigating 250 mM salt solution for 5 times. b Free proline content of the transgenic lines (OE-N1, OE-N2, and OE-N3) and their WT Ningchun 4 (NC) after the salt stress. c Chlorophyll content of the transgenic lines and their wild types after the salt stress. d Peroxidase (POD) activity in the transgenic lines and their wild types after the salt stress. The data were averaged from three replicates and analyzed with IBM-SPSS statistic 20. Letters in bold represent significant differences between groups at α = 0.05. Bars indicate the standard errors of the means.

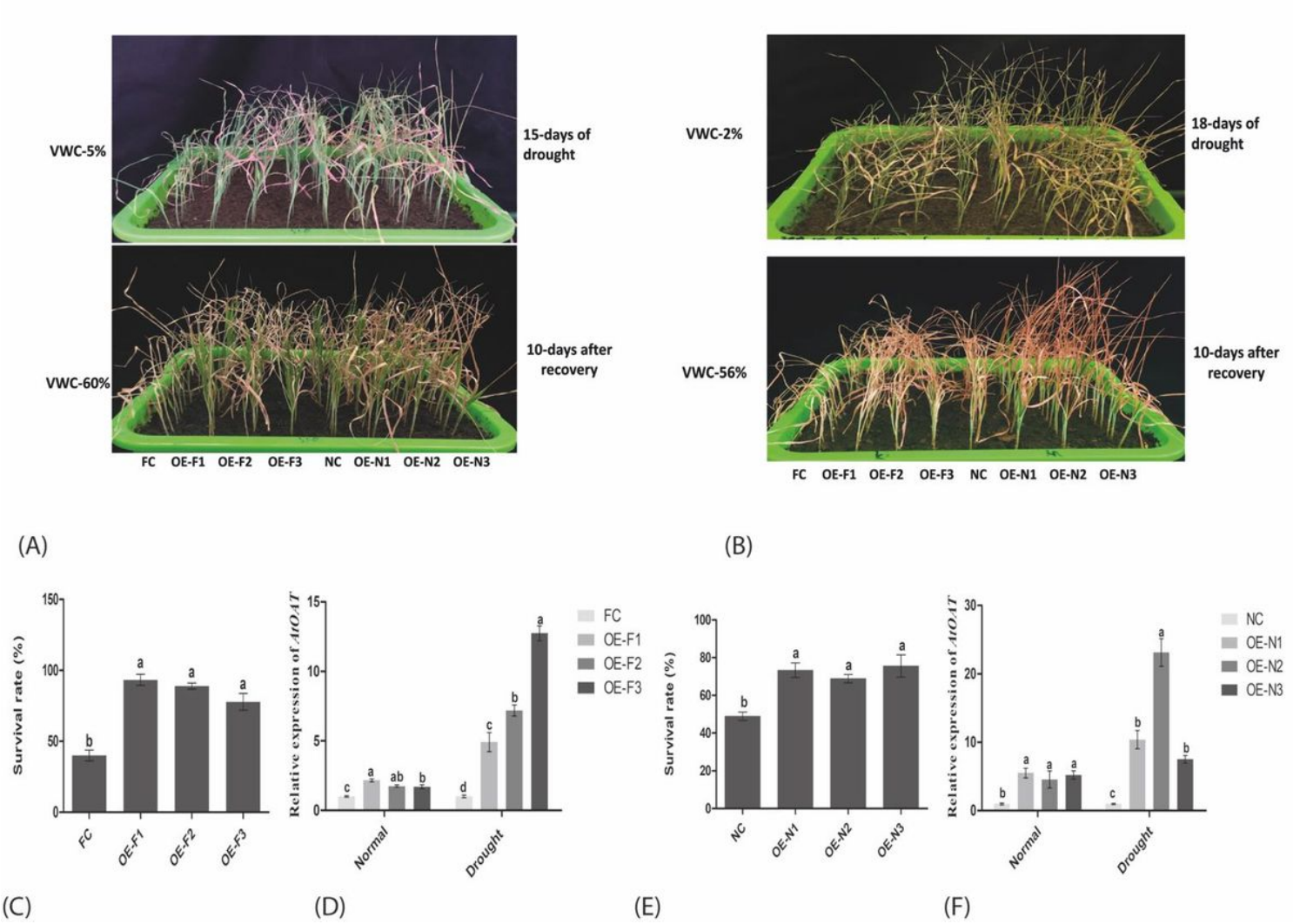

Figure 4

Characterization of drought tolerance in the transgenic wheat lines. a Drought tolerance test of the transgenic lines and their WT Fielder after water withholding for 15 d and water recovery for 10 d. b Drought tolerance test of the transgenic lines and their WT Ningchun 4 after water withholding for 18 d and water recovery for 10 d. c Survival rate of the transgenic lines and their WT Fielder after water stress for 15 d. d Relative expression of AtOAT in the transgenic lines and their WT Fielder under normal and drought stress conditions. e Survival rate of the transgenic lines and their WT Ningchun 4 after water stress for 18 d. f Relative expression of AtOAT in the transgenic lines and their WT Ningchun 4 under normal and drought conditions. The VMC represents the volumetric water content of the soil. Data of the qRT-PCR were averaged from three biological replicates and analyzed with IBM-SPSS statistic 20. Letters in bold represent significant differences between groups at α = 0.05. Bars indicate the standard errors of the means.

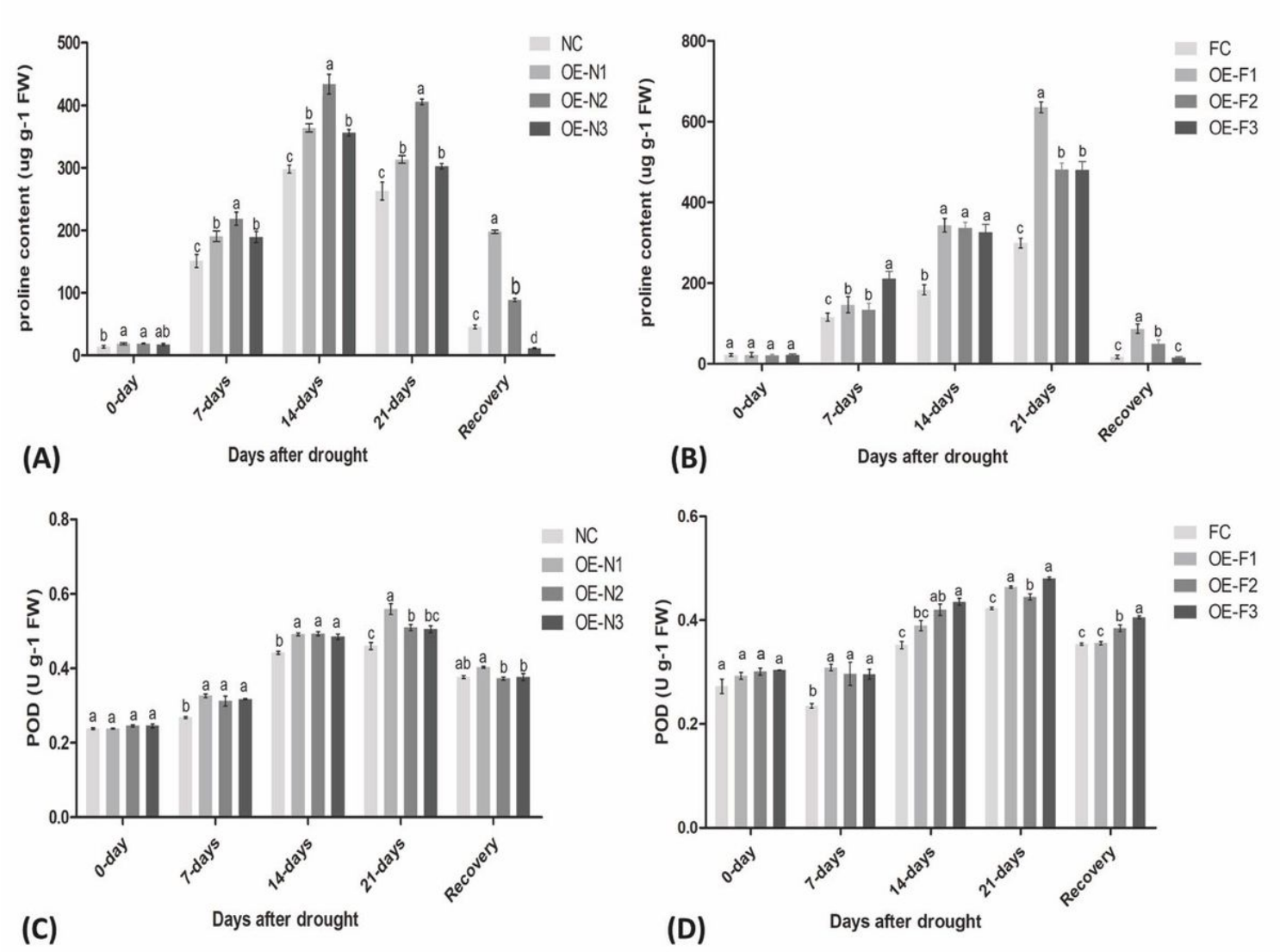

Figure 5

Determination of related physiological parameters in the AtOAT transgenic wheat lines in response to drought stress. a Free proline content in the transgenic lines (OE-N1, OE-N2, and OE-N3) and their WT Ningchun 4 (NC) under water stress for different time. b Free proline content in the transgenic lines (OE-F1, OE-F2, and OE-F3) and their WT Fielder (FC) under water stress for different time. c POD activity in the transgenic lines and their WT NC under water stress for different time. d POD activity in the transgenic lines and their WT FC under water stress for different time. Five plants per replicate were analyzed and in total there were three replicates. A significance test was applied using IBM-SPSS statistic 20. Letters in bold represent significant differences between groups at α = 0.05. Bars indicate the standard errors of the means.

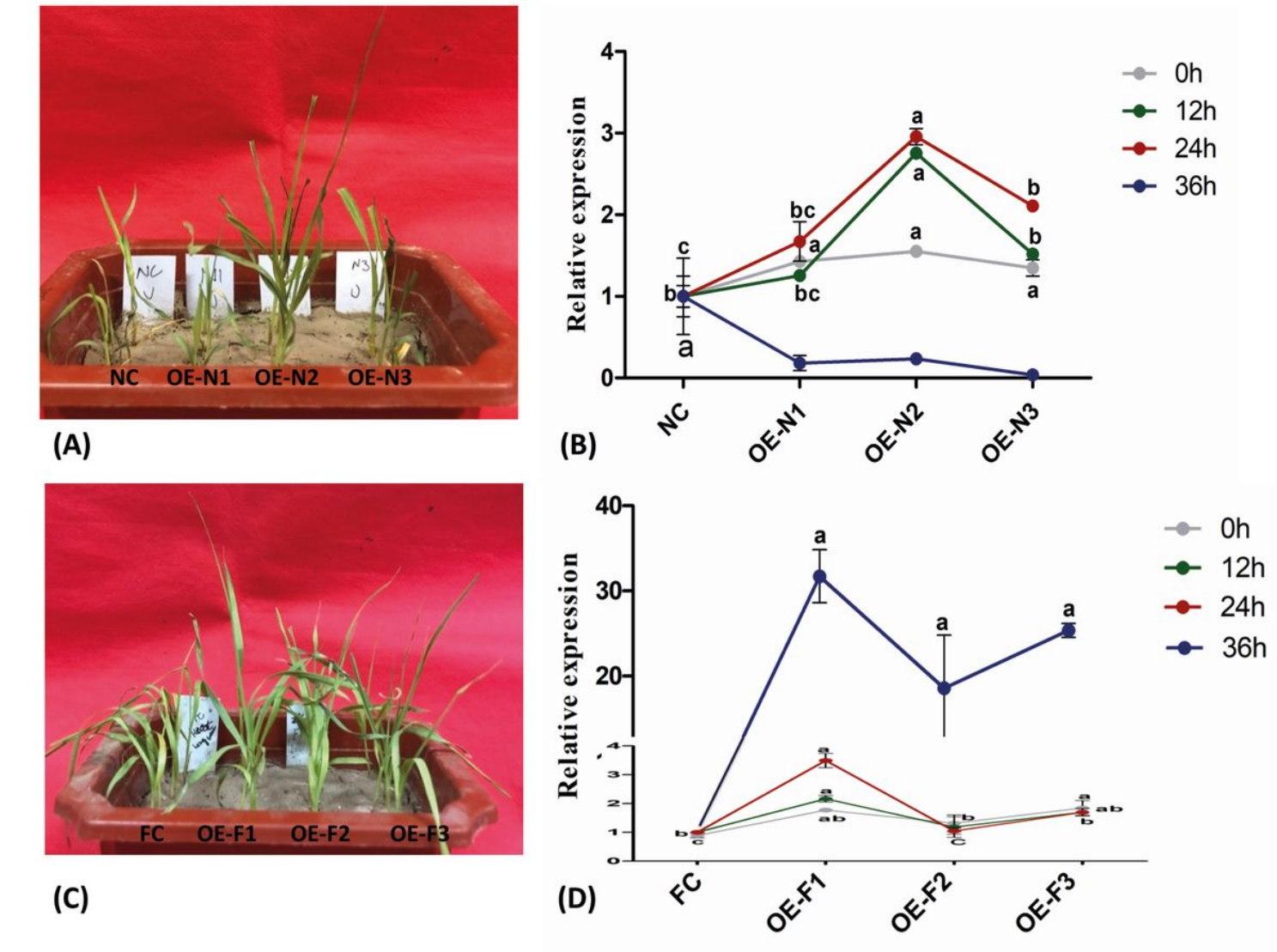

Figure 6

Characterization of heat stress tolerance in the transgenic wheat lines expressing AtOAT. a, c Heat tolerance test of the transgenic wheat lines and their WT Ningchun 4 (NC) and Fielder (FC). b, d Relative expression of AtOAT in the transgenic lines (OE-N1, OE-N2, OE-N3, OE-F1, OE-F2, and OE-F3) and their WT NC and FC under heat stress for different time. Data of the qRT-PCR from three biological replicates were averaged and analyzed with IBM-SPSS statistic 20. Letters in bold represent significant differences between groups at α = 0.05. Bars indicate the standard errors of the means.

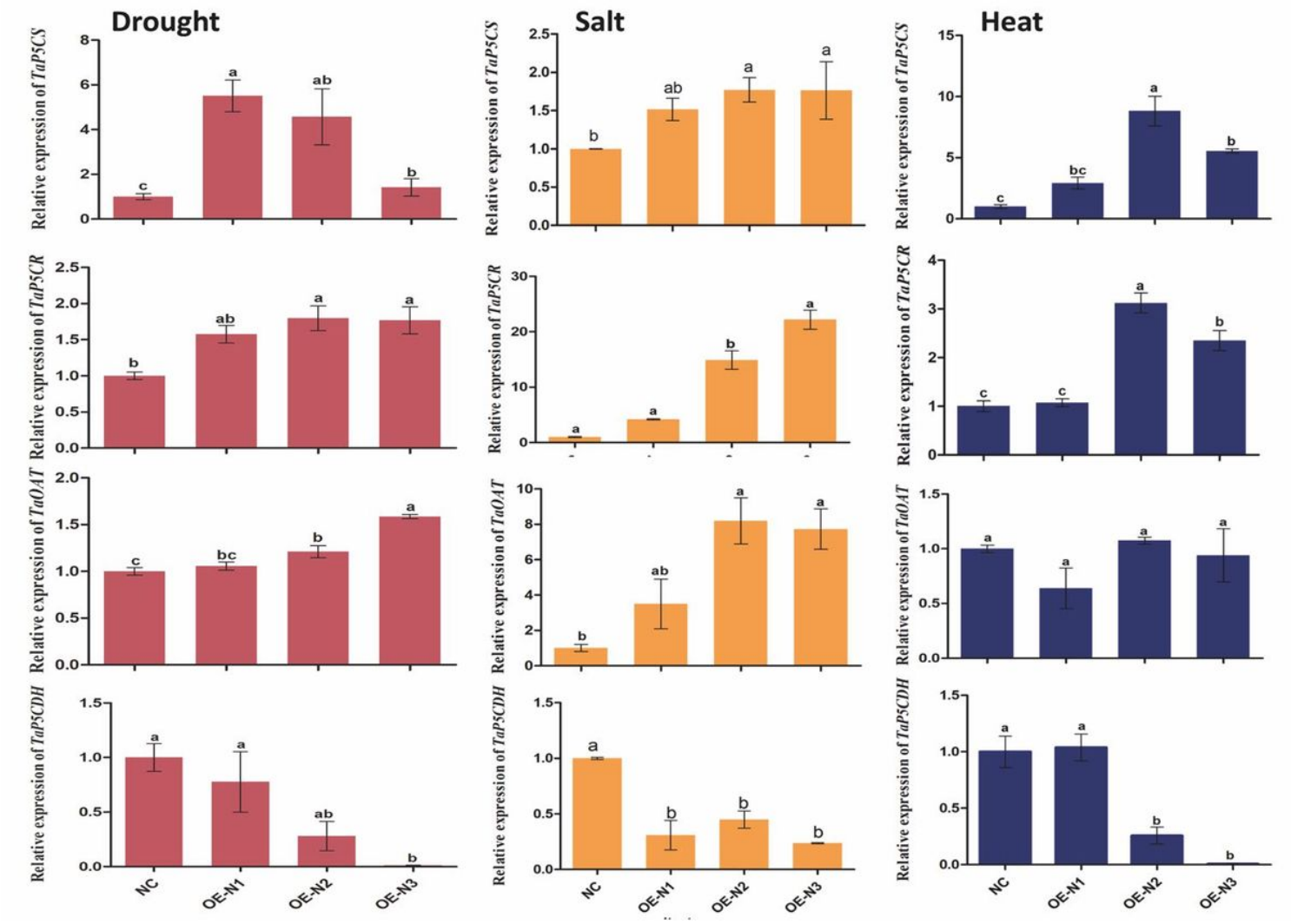

Figure 7

Relative expression profiling of related gene to proline biosynthesis and catabolism in the transgenic wheat lines expressing AtOAT under abiotic stress conditions. The red column represents the expression profiles of the proline biosynthesis related genes TaP5CS, TaP5CR, and TaOAT, and the proline catabolism related gene TaP5CDH under drought stress condition. The orange column represents the expression profiles of the proline biosynthesis and catabolism related genes under salt stress condition. Blue column represents the expression profiles of the proline biosynthesis and catabolism related genes under heat stress condition. Data represent the average of three biological replicates. Statistical significance was calculated by IBM-SPSS statistic 20. Letters in bold represent significant differences between groups at α = 0.05. Bars indicate the standard errors of the means. NC represents the WT Ningchun 4. OE-N1, OE-N2, and OE-N3 are three transgenic lines derived from NC.

Supplementary Files

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- SupplementaryfilePCR128.docx