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OVEREXPRESSION OF *KVP5CS1* INCREASES SALT TOLERANCE IN TRANSGENIC TOBACCO

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Abstract

Proline is a generally accumulated osmolytes in plants under salt stress. Overexpressing *P5CS* gene for more proline production is considered as an effective strategy to improve plant salt tolerance. In this study, *KvP5CS1* was cloned from a halophyte *Kosteletzkya virginica* and transferred to the genome of tobacco (NC89) by *Agrobacterium*-mediated method. Under normal condition, there is no obvious difference in the growth, chlorophyll and proline content, malondialdehyde (MDA) content, superoxide dismutase (SOD) and peroxidase (POD) activity between wild-type (WT) and transgenic seedlings, except that catalase (CAT) activity in wild type was lower than that in transgenic seedlings. However, after treatment by 200 mM NaCl stress for 14 days, transgenic seedlings grew better and showed higher levels of chlorophyll and proline, stronger antioxidant enzyme activities, lower level of malondialdehyde (MDA) than wild type seedlings. These results suggested that overexpression of *KvP5CS1* resulted in proline accumulation, stronger antioxidant capacity and increased salt tolerance in transgenic tobacco, indicating *KvP5CS1* was involved in plant salt tolerance and might be a promising gene to breed salt-tolerant crops growing in salinized farmlands and coastal lands.

Key words: Proline accumulation; Salt tolerance; *KvP5CS1*; Transgenic tobacco; *Kosteletzkya virginia*.

Introduction

Along with climate change and anthropogenic activities, soil salinization is becoming a worldwide problem that severely destroy the fertility of soil and crop productivity. At present, more than 1/3 of the world's irrigated lands are subjected to different levels of salinization, resulting in huge agricultural production loss (Fahad *et al.*, 2015). Furthermore, the area of salinized soil is increasing at a rate of 10% yearly because of climate change and intensive anthropogenic activities. It is predicted that the salinized area will exceed 50% of the world's cultivated area (Jamil *et al.*, 2011). Under this context, breeding new salt-tolerant crops is of vital importance to feed the burgeoning population (Yamaguchi & Blumwald, 2005; Shabala, 2013). And now genetic transformation of salt-tolerant genes especially from halophytes is used as a solution to improve plant salt tolerance, and it has attracted extensive research in recent years (Rajalakshmi & Parida, 2012; Himabindu *et al.*, 2016; Mishra & Tanna, 2017).

Halophytes, surviving in saline environments, have developed complex strategies to cope with high salinity and their salt tolerance mechanisms generally include accumulating inorganic ions and organic solutes for osmotic regulation, keeping ion balance by reducing Na^+ intake and increasing Na^+ efflux as well as cellular or tissue compartmentalization, detoxification of ROS by non-enzymatic and enzymatic antioxidant systems (Shabala, 2013; Flowers & Colmer, 2015). Among these strategies, proline accumulation is an universal defense mechanism in halophytes trapped in salt stress, indicating its importance in plant salt tolerance (Ashraf & Foolad, 2007; Bueno *et al*., 2017). Proline can not only act as an organic osmolyte but also scavenge free radicals, stabilize cytoarchitecture, supply energy and activate stress responses as a signal substance (Szabados & Savouré, 2010; Sharma & Verslues, 2011). In recent years, many attempts have been made to breed salt-tolerant plants by overexpressing the genes associated with proline synthesis for more proline production (Kishor *et al.*, 1995; Zhang *et al*., 2015; Signorelli & Monza, 2017).

Proline is synthesized from glutamate or ornithine in plants and the Δ^1-pyrroline-5-carboxylate synthetase (P5CS) has been identified as the key enzyme for proline synthesis via glutamate pathway (Hu *et al*., 1992; Trovato *et al*., 2008). Numerous studies have shown that transgenic plants overexpressing *P5CS* genes can significantly increase proline production and salt tolerance, which have been confirmed on *Arabidopsis thaliana*, tobacco, rice and many other plants (Khan *et al.*, 2015; Per *et al.*, 2017). These transgenic studies reveal the importance of *P5CS* genes in plant salt tolerance and also encourge us to investigate *P5CS* homologous genes in halophytes (Song & Wang, 2015).

Kosteletzkya virginica is a halophytic plant, natively distributing in coastal areas (Zhou *et al.*, 2010). In our previous studies, we have cloned several proline metabolism-related genes including *KvP5CS1* and analyzed their expression under salt stress. The results indicated a positive relationship between the up-regulated expression of *KvP5CS1* gene and proline accumulation in the leaves of *Kosteletzkya virginica* seedlings under salt stress (Wang *et al.*, 2015a; Wang *et al.*, 2015b). Based on this, the aim of the present study was to generate transgenic tobacco by overexpressing *KvP5CS1* gene controlled by CaMV35S promoter and to evaluate their

salt tolerance versus non-transgenic tobacco through various physiological parameters including proline content, chlorophyll content, MDA, SOD, POD and CAT. This helped to elucidate the mechanism of increased salt tolerance in transgenic plants overexpressing *P5CS* genes for proline overproduction.

Materials and Methods

Plant materials and culture conditions: *Kosteletzkya virginica* seeds were sown in plastic pots containing sand and irrigated by 1/2 Hoagland's solution every 3 days. Two weeks old seedlings were stressed by 200mM NaCl for 12h and used to extract total RNA. Wild-type tobacco seeds (NC89) were sterilized and grown on MS solid medium in order to obtain sterile tobacco leaves, whereas transgenic tobacco seeds were sown on solid MS medium containing kanamycin (50 mg/L) to select transgenic plants. The transgenic seedlings were then transferred to plastic pots containing sand and irrigated by 1/2 Hoagland's solution for further analysis. All the above plant materials were cultured in an artificial greenhouse at 25℃ and 65% relative humidity with 14h light /10h dark.

Tobacco transformation and identification: Total RNA extraction of *Kosteletzkya virginica* and reverse transcription were respectively according to the instructions of RNAiso Plus kit (TaKaRa, Japan) and cDNA synthesis kit (Transgen AT321-01, China). The open reading frame (ORF) of *KvP5CS1* gene (GenBank accession KR029088) (Wang *et al.*, 2015b) was amplified by PCR and the restriction site SmaI and SalI were respectively added to the forward primer P5CS-ORF-F and the reverse primer P5CS-ORF-R (Table 1). The amplified fragments were ligated into the *pEASY*^{TM}-Blunt Zero cloning vector (Transgen CB501-02, China) and sequenced. The ORF of *KvP5CS1* gene was digested from the sequenced cloning vector with *SmaI/SalI* and then ligated into the *SmaI/SalI* digested binary vector *pBI121*. The recombinant plasmid *pBI121-KvP5CS1* was then introduced to *Agrobacterium* strain GV1301 and used for tobacco transformation. Transgenic tobacco plants were produced by *Agrobacterium*-mediated method and selected on MS medium containing 50 mg/L kanamycin during *In vitro* regeneration. The kanamycin-resistant seedlings were further analyzed by PCR and RT-PCR with the specific primers (P5CS-ORF-F and P5CS-ORF-R).

Table. 1 Primers used in the present study.

Names of primer	Primer sequence (5'---3')
P5CS-ORF-F	CCCGGGCGACCCCATGGATCCTTCACG
P5CS-ORF-R	GTCGACAATGCCACACGCAAGTTATG
P5CS-qRT-F	CATCAACACGGAAGTTCTCACA
P5CS-qRT-R	ACTTATTCCAACCTCAGCACCC
NtEF-α-qRT-F	GCAATGGGTGCTTCAGCTTTAC
NtEF-α-qRT-R	GGGCTCTTTCGCAATCTCCT

Letters with underline indicate restriction enzyme sites

Expression analysis of *KvP5CS1* in transgenic tobacco: The expression level of *KvP5CS1* in putative transgenic lines was analyzed by qRT-PCR method in the study (Wang *et al.*, 2015b). The tobacco *NtEF-α* gene was used as a reference gene and all the primer sequences for qRT-PCR were provided in Table 1. The transgenic tobacco lines with high expression level of *KvP5CS1* were selected to produce T1 generation seeds by self-pollination for further salt tolerance analysis.

Salt tolerance analysis in transgenic tobacco: The surface-sterilized T1 seeds of transgenic lines (P2 and P5) were planted onto solid MS medium containing 50 mg/L kanamycin and wild-type (WT) seeds were planted onto only solid MS medium. Two weeks old seedlings of kanamycin-resistant P2 and P5 lines were confirmed by genomic PCR detection using the specific primers of *KvP5CS1*. Then these positive transgenic T1 seedlings (P2 and P5 lines) and WT seedlings were transferred to plastic pots containing sand and were irrigated with 1/2 Hoagland's solution. All the tobacco seedlings were cultured in the same artificial greenhouse mentioned above. At the stage of 5-6 leaves, these seedlings from different lines were respectively assigned to two groups: one group was watered by 1/2 Hoagland's solution with 200mM NaCl and the other was watered by only 1/2 Hoagland's solution used as controls. After treatment for 14 days, their physiological and biochemical parameters were evaluated including chlorophyll content, proline content, MDA content and the activities of antioxidant enzymes (SOD, POD and CAT) according to the method described in our previous study (Wang *et al.*, 2015a).

Statistical analysis: All the experiments were carried out with three replications and all the data was the mean of three replications. The standard deviation (SD) and the significant differences among the means were analyzed using analysis of variance (ANOVA) followed by least significant difference tests (LSD).

Results

Generation and identification of transgenic tobacco: The recombinant vector *pBI121- KvP5CS1* was constructed and introduced to tobacco plants. After 30-day screening *In vitro* regeneration, a few kanamycin-resistant buds were induced from transgenic leaf discs on differentiation medium with 50 mg/L kanamycin. Then these positive buds were transferred to rooting medium with 50 mg/L kanamycin and finally 8 robust transgenic plants (named as P1-P8) were obtained. Genomic PCR detection using the specific primers of *KvP5CS1* gene showed that 2289-bp bands were found in P1-P8 seedlings except WT (Fig. 1A). RT-PCR analysis showed that the same bands were found in P1, P2, P4, P5 and P8, but not in P3, P6, P7 and WT (Fig. 1B), implying that *KvP5CS1* was integrated and expressed in transgenic lines (P1, P2, P4, P5 and P8). Furthermore, qRT-PCR analysis indicated that the expression level of *KvP5CS1* in P2 and P5 was higher than that in P1, P4 and P8 (Fig. 2). Therefore, P2 and P5 transgenic lines were selected to produce T1 seeds for further study.

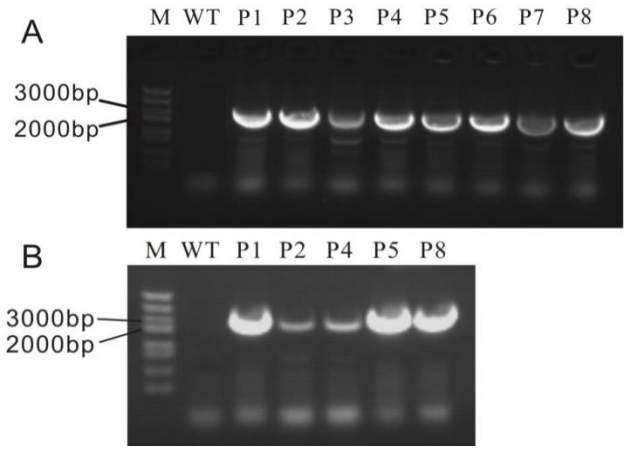


Fig. 1. DNA-PCR (A) and RT-PCR (B) detection for transgenic lines. M, DNA Marker; WT, wild type; P1-P8, different transgenic lines.

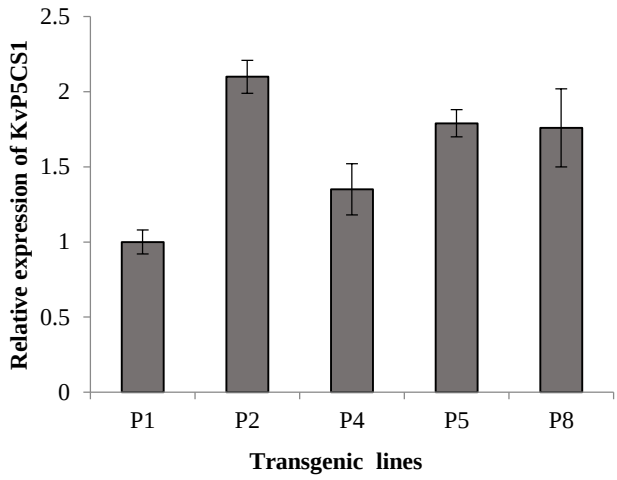
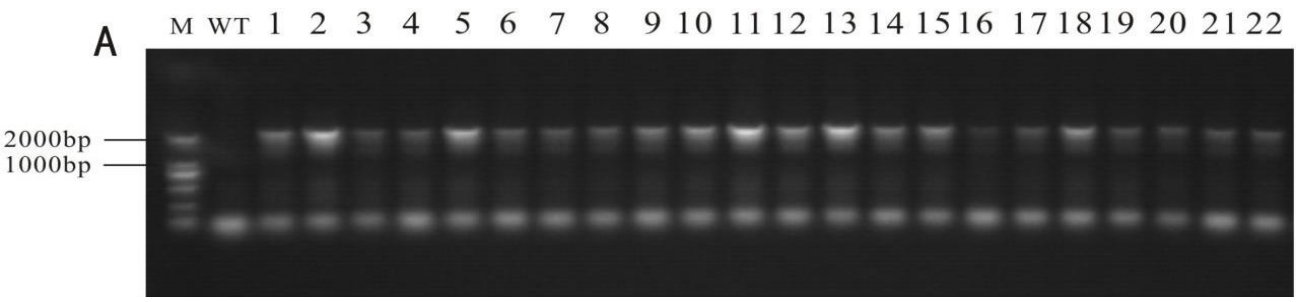
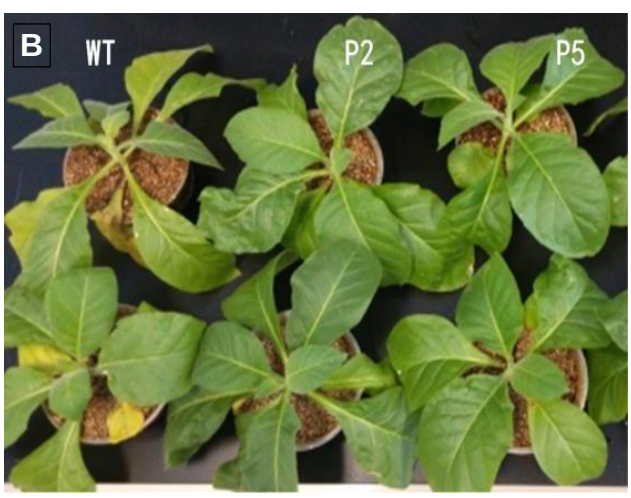


Fig. 2. qRT-PCR detection for T_0-generation transgenic lines. P1, P2, P4, P5 and P8, different transgenic lines.





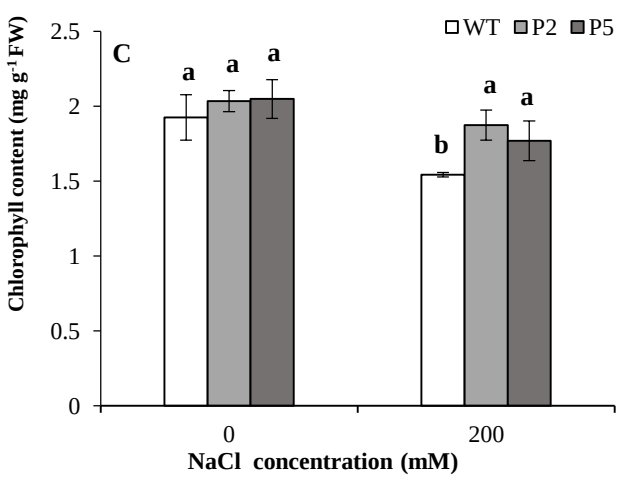


Fig. 3. DNA-PCR detection (A) , growth (B) and chlorophyll content (C) in WT and transgenic lines. M, DNA Marker; WT, wild type; 1-11,P2; 12-22, P5.

Salt tolerance analysis of transgenic tobacco: The kanamycin-resistant T1 seedlings from transgenic P2 and P5 lines were positively confirmed by genomic PCR detection using specific primers of *KvP5CS1* (Fig. 3A). Under normal condition, no obvious difference was found in the growth and chlorophyll content between wild-type and transgenic seedlings, but under 200 mM NaCl stress for 14 days, the transgenic seedlings grew better and had a higher chlorophyll content compared to wild-type seedlings with yellow and wilting leaves (Fig. 3B and Fig. 3C), indicating that the overexpression of *KvP5CS1* conferred salt tolerance to the transgenic tobacco lines.

Increased proline production in transgenic tobacco: Under normal condition, the proline content was similar between wild-type and transgenic seedlings in leaves, while under salt stress the proline content increased significantly in all seedlings. In addition, the increment of proline in both P2 and P8 lines was greater than in wild type (Fig. 4). The proline content in wild type was 3.78 times of that under non-salt stress, while the proline content in P2 and P5 was 7.61 and 6.23 times of that under non-salt stress, respectively. The results suggested that overexpression of *KvP5CS1* promoted the synthesis of proline in transgenic tobacco, especially under salt stress.

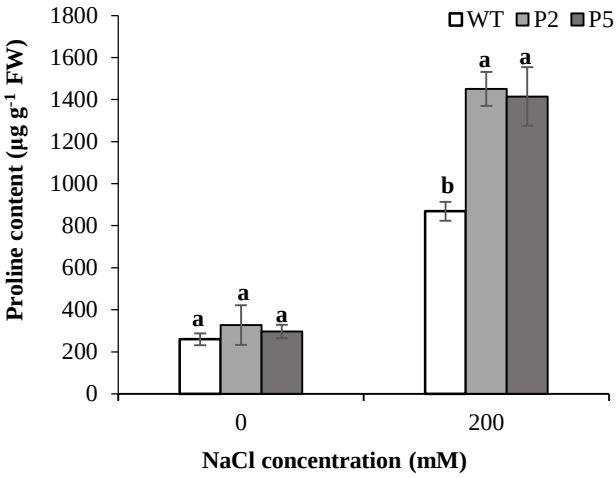


Fig. 4. Proline content in WT and transgenic lines.

Reduced MDA accumulation and increased antioxidant ability in transgenic tobacco: Under normal condition, the MDA content, SOD activity and POD activity in transgenic tobacco were similar to those in wild type, except that the CAT activity in wild type was lower than that in transgenic tobacco (Fig. 5). By contrast, under salt stress the MDA content in wild type was higher than that in transgenic tobacco, whereas the activities of SOD, POD and CAT in transgenic tobacco were all higher than those in wild type. The results suggested that overexpression of *KvP5CS1* increased the activities of antioxidant enzymes (SOD, POD and CAT) in transgenic tobacco which reduced oxidative damage caused by salinity.

Disscussion

Proline accumulation has been discovered in plants subjected to salt and drought stresses (Delaney & Verma, 1993; Hayat *et al*., 2012; Perveen & Nazir, 2018) and proline metabolism engineering by overexpressing synthetic genes or suppressing catabolic genes has also been explored for many years (Kavi-Kishor & Sreenivasulu, 2014). A correlation between transcript level of *P5CS* gene and proline content was shown in a number of plants. In addition, the overexpression of *P5*CS in transgenic plants could increase proline content and salt tolerance. For instance, overexpression of *P5CS* from *Vigna aconitifolia* in transgenic rice induced proline accumulation and increased biomass under salinity and waterlogging conditions (Roosens *et al.,* 1998). Overexpression of *AtP5*CS increased proline content as well as salt tolerance in transgenic tobacco and potato (Zhang *et al.*, 2014). Overexpression of *VaP5*CS produced a higher proline concentration, lower MDA and Na^+ accumulation in leaves and maintained a stable photochemical efficiency of PSII in transgenic sugarcane than in controls (Guerzoni *et al.*, 2014).Transgenic *sorghum bicolor* overexpressing *P5CSF129A* gene from *Vigna aconitifolia* got higher yields under salt stress than controls (Surender *et al.*, 2015). Many other studies overexpressing *P5CS* genes were also reported in transgenic wheat, carrot, petunias and pigeon pea (Sawahel & Hassan, 2002; Yamada *et al.*, 2005; Surekha *et al.*, 2014). All these transgenic plants grew better and had more proline and chlorophyll, less lipid peroxidation than non-transgenic controls under salinity. Although the explicit role of proline is still ambiguous in transgenic plants, at least these findings suggest that *P5CS* gene plays an important role in abiotic stress tolerance of plants. In the present study, we also obtained similar results by overexpressing *KvP5CS1* in tobacco. Under salt stress for 14 days, the transgenic tobacco seedlings grew better than wild tobacco. The proline content was similar between wild tobacco and two transgenic lines under non-stress condition, but under salt stress two transgenic lines produced more proline than control. Furthermore, the transgenic lines had more chlorophyll content, less lipid peroxidation and stronger antioxidant ability than wild tobacco, suggesting that the overexpression of *KvP5CS1* gene resulting in proline accumulation in association with the antioxidant enzymes (SOD, POD and CAT) increased salt tolerance in transgenic tobacco. So overexpressing *KvP5CS1* gene may provide an alternative strategy to breed new salt-tolerant plants in future.

However, the relationship between salt tolerance and proline accumulation in plants is still a contentious issue because proline accumulation is not always consistent with the salt tolerance of plants, and sometimes it even causes detrimental effects in some species or cultivars (Hester *et al.*, 2001; Widodo *et al.*, 2009; Mahmoudi *et al.*, 2011; Zhao *et al.*, 2014). Recently the function of proline in saline conditions and the relevance between proline and salt tolerance have been reviewed in detail and the authors enumerated lots of positive and negative examples about the relevance between proline and salt tolerance. They proposed that proline is important for salt tolerance in some plants but has nothing to do with salt tolerance in others (Verbruggen & Hermans, 2008; Mansour & Alithe, 2017). Obviously, there is still no consistent conclusion about the role of proline in plants and there is a long way to go before the verification of proline function in salt tolerance. As for us, it will take great efforts to confirm the funciton of *KvP5CS1* in the salt tolerance of transgenic tobacco throughout the growth period and to try its transformation on crop plants. In the near future, *KvP5CS1* might be a promising gene to breed new salt-tolerant crops which can grow in salt-affected farmland and also exploit marginal lands such as coastal region.

Conclusion

Based on the close relationship between proline accumulation and high expression of *KvP5CS1* induced by salinity in our previous study, *KvP5CS1* was firstly transferred to the genome of tobacco by *Agrobacterium*-mediated method. The transgenic tobacco overexpressing *KvP5CS1* exhibited both proline accumulation and improved salt tolerance compared to non-transgenic tobacco, indicating *KvP5CS1* was involved in plant responses to salinity but its exact function in plant salt tolerance still needs further study.

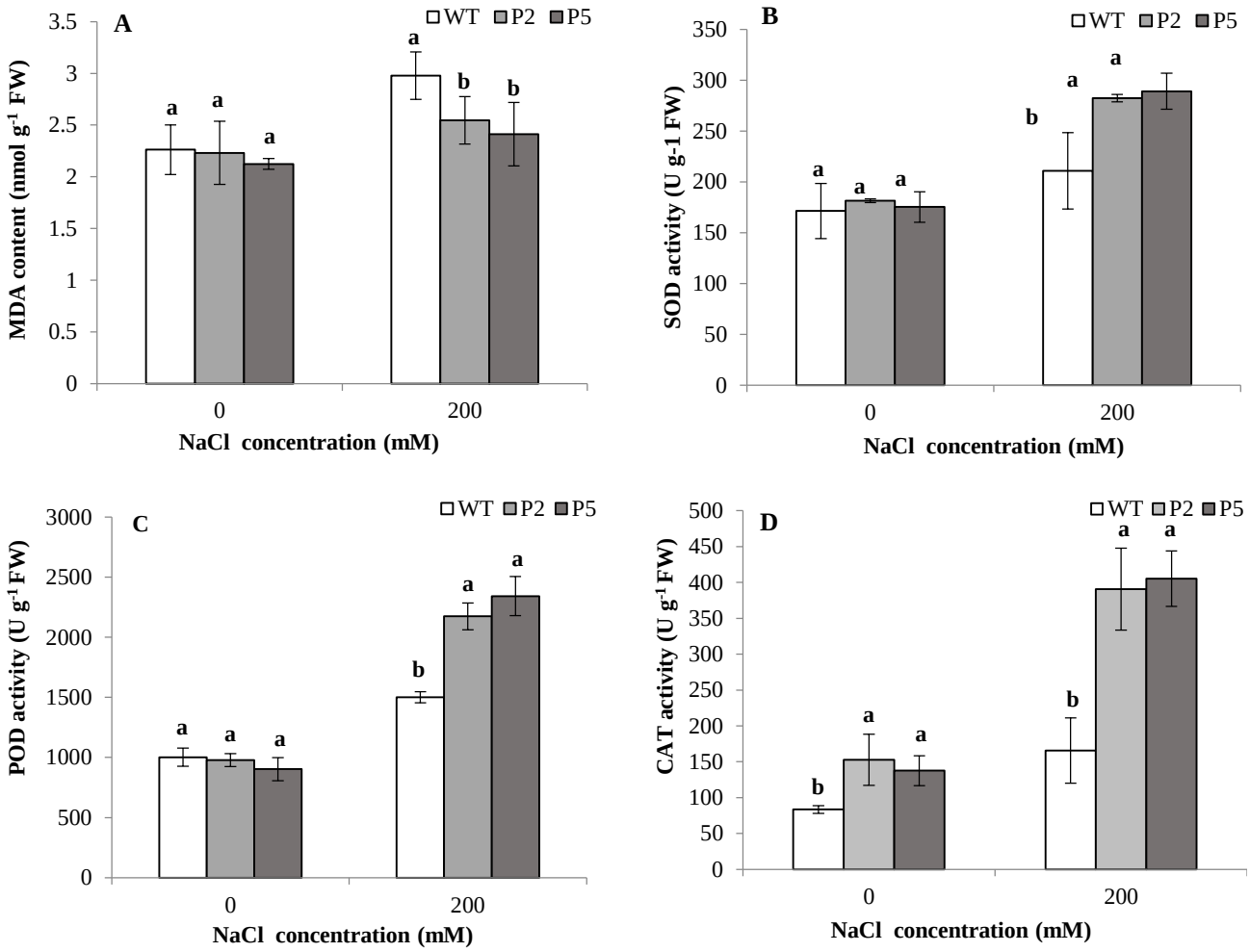


Fig. 5. MDA content (A), SOD activity(B), POD (C) and CAT (D) in WT and transgenic lines.

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