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A novel aspartic protease inhibitor inhibits the enzymatic browning of potatoes

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ABSTRACT

Enzymatic browning greatly affects the quality of fresh-cut potatoes (*Solanum tuberosum* L.). The transcript level of a novel aspartic protease inhibitor gene, *StASPI*, was found significantly higher in browning-less potatoes than in browning-susceptible potatoes, and exogenous aspartic protease inhibitor Pepstatin A inhibited the browning of potato pulp. Therefore, we speculated that *StASPI* plays important roles in browning-resistance of potatoes. The effects of overexpressed-*StASPI* on browning, total free amino acids (FAAs), antioxidant enzyme activity and ROS accumulation in potatoes were investigated in this study. Results showed that overexpression of *StASPI* effectively reduced enzymatic browning after cutting, significantly decreased protease activity, and reduced the accumulation of total FAAs in potatoes. Browning degrees of transgenic potato mash were increased by supplementing exogenous FAAs, but the degrees were still significantly lower than that of wild-type (WT) mash. Furthermore, overexpression of *StASPI* decreased PPO activity, enhanced the activities of antioxidant enzymes SOD and CAT, and reduced H_2O_2 and O_2^- contents. These results indicated that overexpression of *StASPI* inhibited the enzymatic browning of potatoes, decreasing FAAs accumulation, reducing PPO activity, and enhancing activity of antioxidant enzymes. This study provides a new perspective on the strategies for inhibiting enzymatic browning of potatoes.

1. Introduction

Potato (*Solanum tuberosum* L.) is an economically important staple crop (Zaheer and Akhtar, 2016). In recent decades, fresh-cut products including processed potato have been increasing in popularity (Toivonen and Brummell, 2008; Francis et al., 2012). However, processed potato products are prone to enzymatic browning, causing a considerable loss in quality and value (Ma et al., 2017). Although many technologies to inhibit browning have been investigated in the processed produce industry (Xiao et al., 2011; López-Gálvez et al., 2015; Rizzo et al., 2018), it is still a challenge to better solve this problem.

Enzymatic browning is mainly caused by the oxidation of phenolic substrates under the catalysis of polyphenol oxidase (PPO). However, there are abundant of free amino acids (FAAs) and soluble proteins in potato tubers, which could combine with o-quinone to form brown quinones or colorless substance, thus promoting or inhibiting browning (Ali et al., 2016, Gacche et al., 2006). Wang et al. (2015) showed that postharvest curing treatment (PCT) alleviated potato browning after cutting, though the treatment increased the phenolic content and PPO activity. Cantos et al. (2002) also reported that there were no significant correlations between browning and PPO activity or phenolic compounds. Membrane integrity plays an important role in browning-resistance of processed products for the compartmentalization of browning-related enzymes and substrates (Toivonen and Brummell, 2008). The stability of the cell membrane is highly influenced by reactive oxygen species (ROS). However, ROS homeostasis is

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maintained by antioxidants such as polyphenols, ascorbic acid as well as antioxidant enzymes including super oxide dismutase (SOD) and catalase (CAT) (Ahmad et al., 2014; Dunnill et al., 2017). SOD and CAT are important and indispensable antioxidant enzymes in many defense strategies in plants (Ighodaro and Akinloye, 2018). SOD catalyzes the dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), which can be decomposed by CAT, thus reducing ROS content (Ighodaro and Akinloye, 2018). Furthermore, proteases are also associated with enzymatic browning of potatoes (Cantos et al., 2002).

Proteases can be activated by various stresses, such as low temperature and physical injury, leading to the hydrolysis of endogenous proteins and the increase of free amino acids (Montero et al., 1995; Ré et al., 2012; Yoon et al., 2017). Additionally, proteases can promote ROS accumulation (Kuo et al., 2006; Lim et al., 2006). Previous studies found that aspartic proteases (APs) induced H_2O_2 generation in fungal spores (Mendieta et al., 2006). Therefore, regulating proteases might be an important way to control enzymatic browning. Protease inhibitors, which are abundant in potato tubers and plant seeds (Bauw et al., 2006; Kunert et al., 2015), act as safety valves to regulate activities of proteases (Grosse-Holz et al., 2018; Velasco-Arroyo et al., 2018; Stael et al., 2019). Protease inhibitors play crucial roles in many biological processes such as programmed cell death (Boex-Fontvieille et al., 2015), sprouting (Munger et al., 2015), and resistance to biotic and abiotic stresses (Vyver et al., 2003; Zhang et al., 2008a,b). However, there is little information about the effects of protease inhibitors on enzymatic browning of potatoes.

In our previous studies, we found potatoes from a cultivar "*Kexin #4*" [bred from hybridization of '*Pulsatilla*' (female) and '*Katahdin*' (male)] are much more prone to browning than those from the cultivar "*Kexin #13*" (self-bred progeny of '*Mira*'). And a technical treatment, postharvest curing treatment (PCT), effectively inhibited potato browning after fresh-cut, though PCT increased PPO activity and phenolic compounds (Wang et al., 2015). To study the molecular mechanism underlying potatoes with different browning-susceptibilities, RNA-seq analysis was performed using these materials (Dong et al., 2020). Through transcriptomic analysis, the differentially expressed genes and the enriched differential terms were found mainly involved with protease inhibitors (Dong et al., 2020). Among these protease inhibitors, a novel aspartic protease inhibitor gene (PGSC0003DMG400009511) according to the website of Potato Genomics Research (http://solanaceae.plantbiology.msu.edu/index.shtml) and National Center for Biotechnology Information (NCBI) (XM_006362605.2), named as *StASPI*, was identified in this study. The purpose of this study was to investigate the effects of *StASPI* on browning and its mechanisms affecting browning via overexpressing *StASPI*.

2. Materials and treatment

2.1. Materials and treatment

Two potato cultivars with significant difference in browning-susceptibility (browning-less "*Kexin #13*" and browning-susceptible "*Kexin #4*"), harvested from Keshan Potato Research Centre, Heilongjiang, China, were used for the discoloration verification and *StASPI* expression analysis. Twelve potatoes each from "*Kexin #4*" and "*Kexin #13*" were washed, peeled and cut into slices, respectively. Twelve slices from each group were randomly selected, diced, frozen in liquid nitrogen and then stored at −80 °C for quantitative real-time PCR (qRT-PCR) analysis immediately after cutting. The remaining slices were stored at 3 ± 1 °C and used for browning observation.

For PCT, potatoes (*Netherlands #15*) were harvested at a local potato farm (Tai'an, Shandong, China), transported to the laboratory immediately and subjected to PCT. For PCT, potatoes were placed in a thermostat-controlled cabinet at 16 ± 1 °C for 10 d and control potatoes were stored at 3 ± 1 °C for the same duration of time according to the procedure of Dong et al. (2020). After PCT, potatoes were subjected to fresh-cut process according to the method of Dong et al. (2020). Samples were taken at 10 d during PCT for *StASPI* expression analysis and the discoloration of potato slices was verified during the storage after fresh-cut.

For warming treatment, potatoes (*Netherlands #15*) were purchased from Laiwu Liujie Cold Storage Factory, transported to the laboratory and then stored at cold storage (3 ± 1 °C) until use (about three months). Non-damaged and non-defective tubers (three groups with 36 tubers of each) were placed at 25 ± 1 °C for 12 h as treatment and the same number of potatoes were stored at 3 ± 1 °C for the same duration of time, served as the control. Samples were carried out at pre-and after-treatment described above. The left potatoes were subjected to fresh-cut process described by Dong et al. (2015). Samples were taken at 0, 3 and 6 h after cutting for *StASPI* expression analysis.

2.2. RNA extraction and quantitative real-time PCR analysis

For qRT-PCR analysis, Primer Premier 5.0 (Premier Biosoft International, Palo Alto, California) was used for generating primer pairs of *StASPI* (Forward: GATGCCCCTTGTCCAGATGG; Reverse: CTATGGTTCCTCCCGTCTCC). Total RNA was extracted using the OmniPlant RNA Kit (DNase I) (CWBIO Inc., Beijing, China) according to the instruction and was reverse transcribed into cDNA using the HiFi-Script cDNA Synthesis Kit (CWBIO Inc., Beijing, China). The transcript level of *StASPI* was measured by qRT-PCR using the Power UltraSYBR Mixture (CWBIO Inc., Beijing, China) and the CFX96^{TM} Real-Time System (Bio-Rad). Relative expression of *StASPI* was normalized to the reference gene beta-actin (Hemavathi et al., 2009). The levels of gene expression were calculated using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001).

2.3. Browning measurement

The surface browning of potato slices was measured using the colorimeter (CR-400, Minolta Co., Osaka, Japan), calibrated by a standard white tile (L* = 97.06, a* = 0.04, b* = 2.01). L* value (lightness) was used for browning evaluation. The higher the value, the lower the browning is. The browning degree of potato mash was analyzed using spectrophotometric method at 410 nm according to Jiang (2000).

2.4. Evaluation of aspartic protease inhibitor Pepstatin A on browning

Potatoes (*Netherlands #15*) were washed, peeled, cut into about 3 × 3 × 3-mm cubes, and then mixed. Five grams of cubes was weighed into two centrifuge tubes, 10 mL of 0.1 mol L^{-1} phosphate buffer solution (PBS, pH = 6.8) and containing either 0 (control) and 0.1 g L^{-1} Pepstatin A (Yuanye, Bio-Technology Co., Ltd, Shanghai, China) and incubated at 20 °C for 10 min. To make Pepstatin A dissolve completely, 0.01 g of Pepstatin A was first dissolved in 0.1 mL DMSO and then diluted with PBS to 100 mL. PBS from the control was added with the same amount of DMSO. After incubation, mixtures were then homogenized using an IKA T10 basic homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) for 10 s. The color changes of the mixtures were recorded photographically at 15 and 30 min and the browning degree of each sample was measured as described above.

2.5. Plasmid construction, genetic transformation and plant cultivation

The Methods of plasmid construction, genetic transformation and plant cultivation were performed according to Dong et al. (2020) with minor modification. Plasmid construction: the open reading frame (ORF) of *StASPI* was cloned using the forward primer with a *BamHI* site (TCTAGAATGATGAAGTGTTTATTTTTGTTA) and the reverse primer with a *HindIII* site (CCCGGGCTAAACTTCCTGGAATAAGACATC). The purified ORF fragment was ligated into the expression vector *PBI121*. The recombinant plasmid was introduced into *Agrobacterium*

tumefaciens. One positive colony was cultivated in YEP liquid medium and used for genetic transformation. Genetic transformation: Potato seedlings (*Desiree*) were cultivated for about 25 d in plantlet bottle. Internodes explants measuring 3 to 5 mm or leaf explants were used agrobacterium-mediated transformation. The uninfected wild-type (WT) explants served as controls. Plant cultivation: The positive plants and the control were subjected to RT-PCR and qRT-PCR analysis. Three lines of the positive plants with high expression levels of *StASPI*, as well as control plants, were cultivated the greenhouse (16 h light, 20–22 °C; 8 h dark, 15–18 °C). When the potatoes reached commercial maturity (the leaves changed from green to yellow), tubers were harvested and stored at 3 ± 1 °C until used for cutting process for browning evaluation. Samples were taken immediately after cutting for the physiological and biochemical analysis.

2.6. Measurement of aspartic protease inhibitor content

Frozen samples were ground into powder with a nitrogen grinding apparatus (IKA A11 basic; IKA Werke GmbH & Co., KG, Staufen, Germany). The protein concentration of StASPI in tubers was determined using Plant aspartic proteinase inhibitor (SPI) ELISA Kit (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). 0.1 g potato powder was mixed with 1 mL of PBS (pH = 7.4) and sonicated at 4 °C. Afterwards, the mixture was centrifuged for 20 min at 2,500 × *g*, and the supernatant was used for measurement according to the manufacture's instruction. The concentration (g kg^{-1}) of aspartic protease inhibitor was calculated based on a standard curve.

2.7. Free amino acid (FAA) analysis

FAAs were extracted and measured by high-performance liquid chromatography following the method of Dong et al. (2020). For FAAs extraction, 0.4 g of frozen potato powder was mixed with 10 mL of 80 % methanol, sonicated and centrifuged for 10 min at 15,000 × *g*. The supernatant (1 mL) was mixed with 0.5 mL of 1.2 % phenylisothiocyanate (acetonitrile, HPLC grade, v/v) and 0.5 mL of 14 % triethylamine (acetonitrile, HPLC grade, v/v) and placed at 25 °C (1 h) for derivatization. Acetic acid (0.1 mL, ≥ 99.0 % purity) and *N*-hexane (2 mL) was added into the mixture and allowed to stand for purifying. The supernatant was aspirated and the solution underlayers was filtered through 0.25-μm-pore-size nylon membrane for HPLC analysis. The contents of FAAs were calculated according to calibration curves of corresponding amino acid standards, expressed as g kg^{-1} fresh weight of potato powder.

2.8. Supplementation experiment of exogenous amino acids

The difference of each FAA content in transgenic and WT potato tubers was calculated. For each transgenic line, we created exogenous amino acid solution containing all the decreased amino acids in transgenic tuber compared to WT. To conduct the experiment *in vitro*, seven centrifuge tubes were prepared: one for WT and six for the three transgenic lines (two tubes per transgenic line). Two grams of WT and transgenic frozen potato powder was weighed into the corresponding tubes respectively. Then, 10 mL of deionized water was added into the tube containing WT sample and three tubes containing transgenic sample (one tube per line) respectively. The amino acid solution (10 mL) was then added into the other three tubes (one tube per line) respectively, making the final concentrations of each FAA in transgenic tubes equal to that in WT tube. Afterwards, the mixtures were homogenized for 5 s and stand for 1 h, to record their browning by photograph. Then, the mixture was centrifuged at 12,000 × g for 10 min at 4 °C and the absorbance of supernatant was measured at 410 nm. The browning degree of these potato mash was indicated by A_{410} according to the method described by Jiang (2000).

2.9. Analysis of PPO activity and antioxidation related indices

Polyphenol oxidase (PPO), catalase (CAT) and superoxide dismutase (SOD) activity and H_2O_2 content were all determined according to the methods described by Dong et al. (2015) with minor modifications. 0.5 g potato powder was mixed with 0.25 g insoluble polyvinylpolypyrrolidone (PVPP) (if needed) and 5.0 mL of phosphate buffer with corresponding pH and centrifuged at 11,140 × *g* for 20 min at 4 °C, and the supernatant was used for analysis.

Superoxide anion radical (O_2^-) content was measured according to Yang et al. (2016) with minor modifications. 2.0 g potato powder was mixed with 5 mL of phosphate buffer (65 mmol L^{-1}, pH = 8.0) and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was mixed with 0.5 mL of 65 mmol L^{-1} PBS and 0.1 mL of 10 mmol L^{-1} hydroxylammonium chloride and heated at 25 °C for 20 min. After adding 1 mL of 58 mmol L^{-1} sulfanilamide and 1 mL of 8 mmol L^{-1} α-naphthylamine, the mixture was incubated at 25 °C for 20 min. Absorbance was measured at 560 nm.

2.10. Protease activity measurement

Protease activity was measured according to the method described by Dong et al. (2020). 1.0 g of frozen potato powder, mixed with 5 mL of Tris-HCl buffer (0.05 mol L^{-1}, pH = 6.2), was centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was used to measure protease activity. Absorbance of the mixture containing 1.0 mL of the supernatant and 1.5 mL of 0.1 mmol L^{-1} sodium benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA, dissolved in 0.05 mol L^{-1} Tris-HCl buffer) was monitored by spectrophotometer at 395 *nm* for 10 min at 37 °C immediately. One unit of protease activity was expressed as the increase of 0.01 absorbance per hour per kilogram fresh weight (U kg^{-1} h^{-1}).

2.11. Statistical analysis

All experiments were conducted at least in triplicate. Analysis of variance (ANOVA) was conducted by Duncan's multiple range test or Student's *t*-test using the SPSS 17.0 Statistical Software Program (SPSS Inc. Chicago, IL, USA).

3. Results

3.1. StASPI is highly expressed in browning-less potato tubers

Two different potato varieties, *Kexin #4* and *Kexin #13*, had significant difference in browning-susceptibilities, and postharvest curing treatment (PCT) effectively delayed fresh-cut browning of potatoes (Wang et al., 2015). RNA-seq analysis showed most of the common up-regulated genes in browning-less potatoes are protease inhibitors compared with browning-susceptible potatoes, including a novel aspartic protease inhibitor gene (PGSC0003DMG400009511) according to Potato Genomics Research (http://solanaceae.plantbiology.msu.edu/index.shtml) and National Center for Biotechnology Information (NCBI) (XM_006362605.2) (Dong et al., 2020), named as *StASPI* in this study. Here, the differences in browning-susceptibility and *StASPI* transcript levels between potatoes from *Kexin #4* and *Kexin #13* and between potatoes with and without PCT were verified (Fig. S. 1). Results showed that the relative expression of *StASPI* gene was significant higher in browning-less potatoes (*Kexin #13* and control potatoes without PCT) than that in browning-susceptible potatoes (*Kexin #4* and potatoes with PCT) (Fig. S. 1), which were consistent with the study of Dong et al. (2020). Besides, warming treatment alleviated potato browning effectively and potatoes with warming treatment displayed much lower visual browning than control on day 1 after fresh-cut (Fig. 1A). In addition, the relative expression of *StASPI* in warming-treated potatoes was increased almost 7.5-fold after warming treatment compared with control, and still maintained higher transcript

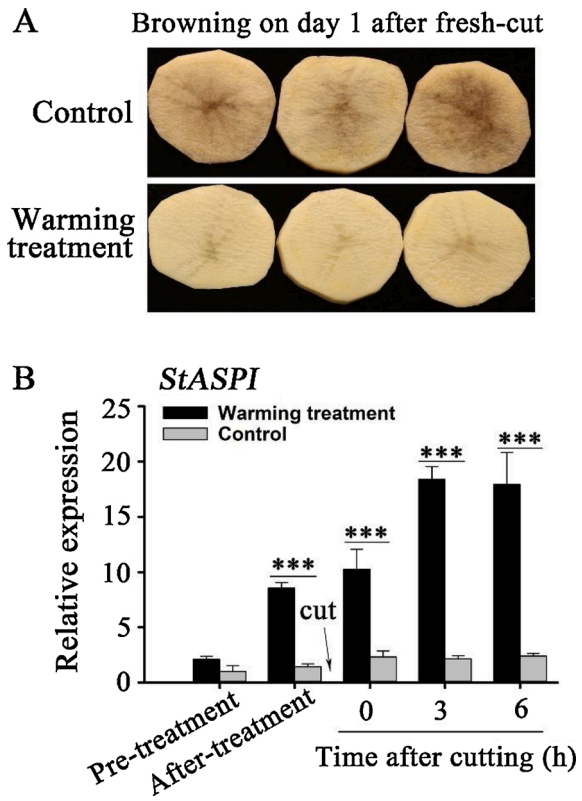

Fig. 1. Visual browning (A) and relative expression of *StASPI* (B) in potatoes with and without warming treatment. Asterisks indicate a significant difference between two samples (***p < 0.001). Error bars represent SD (n = 9).

level after fresh-cut (Fig. 1B). Therefore, we speculated that *StASPI* should be associated with the browning-resistance of potatoes.

3.2. Pepstatin A inhibited browning of potato pulp

To test the effect of aspartic protease inhibitor on browning, cubed potatoes were treated with or without exogenous aspartic protease inhibitor Pepstatin A *in vitro*, and their browning degree were compared after homogenizing into potato pulp. As is shown in Fig. 2, potato pulp treated with Pepstatin A hardly exhibited brown at 15 min after homogenization, whereas obvious browning was found in control pulp (Fig. 2A-i). As time going on, the browning-inhibition of Pepstatin A became more significant. At 30 min, browning of control pulp became severer while browning of Pepstatin A-treated pulp changed very little (Fig. 2A-ii). The browning degrees of these pulp were measured by spectrophotometry, reflecting the browning differences of potato pulp treated with and without Pepstatin A (Fig. 2B). The result demonstrated that Pepstatin A significantly inhibited the browning of potato pulp, suggesting that aspartic protease inhibitor might play an important role in inhibiting the enzymatic browning of potatoes.

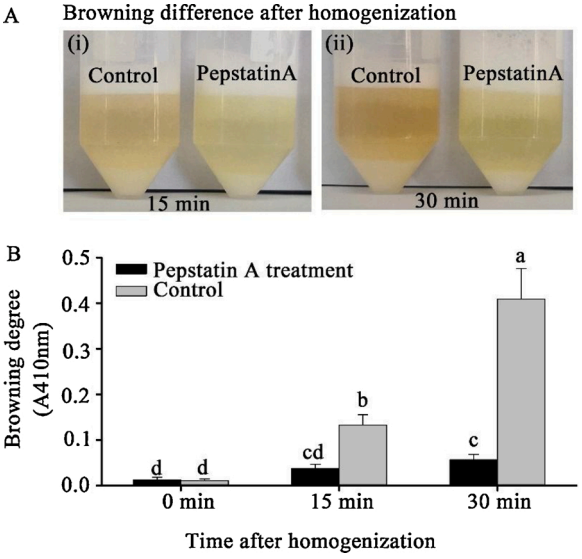

Fig. 2. Effect of Pepstatin A on visual browning (A) and browning degree (B) of potato mash at different time points after homogenization. Different letters indicate statistically significant differences (**p < 0.01). Error bars represent SD (n = 3).

3.3. Overexpressed-StASPI decreased enzymatic browning of potatoes

To further investigate the role of *StASPI* on enzymatic browning of fresh-cut potato, *StASPI* gene was cloned from potato tuber and overexpressed in potato plants. Through callus culture, a total of 17 independently transformed plants were identified, and the transcript levels of *StASPI* in the transgenic and WT potato plants were analyzed by qRT-PCR. Three transgenic lines with highest transcript levels (*StASPI*-6, *StASPI*-11 and *StASPI*-12) as well as WT plants were selected for cultivation. After reaching commercial maturity, potato tubers were harvested and used for enzymatic browning evaluation. Enzymatic browning of transgenic and WT potatoes was compared at 3 h after cutting into slices (Fig. 3A). Potato slices from three transgenic lines all showed less browning than that from WT (Fig. 3A). The higher L* value presents the lower browning degree. L* values of potato slices in three transgenic lines were all significantly higher (**p < 0.01) than that in WT (Fig. 3B), suggesting the lower browning of transgenic slices. Moreover, the relative expression levels of *StASPI* (Fig. 3C) and aspartic protease inhibitor content (Fig. 3D) in transgenic tubers were all significantly higher (**p < 0.01) than those in WT tubers, indicating that *StASPI* was overexpressed at both transcript and protein levels in transgenic tubers and played important roles in accumulation of aspartic protease inhibitor. These results indicated that overexpression of *StASPI* significantly inhibited enzymatic browning of potatoes, which proved our hypothesis.

3.4. Influence of StASPI on FAAs, protease and PPO activities in potato tubers

Aspartic protease inhibitors could inhibit both the aspartic protease cathepsin D and the serine protease trypsin (Guo et al., 2015). Protease can influence the accumulation of FAAs through hydrolyzing endogenous proteins under biotic or abiotic stress (Ré et al., 2012; Yoon et al., 2017) and FAAs accumulation can promote potato browning (Dong et al., 2020). Therefore, FAAs content and protease activity of the transgenic and WT potato tubers were measured. The result showed that most of each FAA exhibited lower contents in transgenic tubers compared with WT (Fig. 4). Consequently, the content of total FAAs in each transgenic line was significantly lower than that in WT tubers (**p < 0.01) (Fig. 5A). In addition, protease activity in transgenic tubers was much lower (**p < 0.01) than that in WT (Fig. 5B). These results indicated that overexpression of *StASPI* might decrease FAAs accumulation through reducing protease activities, which might contribute to the browning-resistance of potatoes.

To investigate the contribution of the decreased FAAs accumulation to browning-resistance, the decreased FAAs in transgenic potato were exogenously supplemented into the transgenic potato mashes, adjusting their concentrations to the levels of WT. Then, the browning degrees of transgenic and WT mashes were compared (Fig. 5C, D). As shown in Fig. 5C, D, the transgenic potato mash supplemented with exogenous FAAs browned more severely than those without supplementing any differential amino acids (**p < 0.01), which demonstrated that

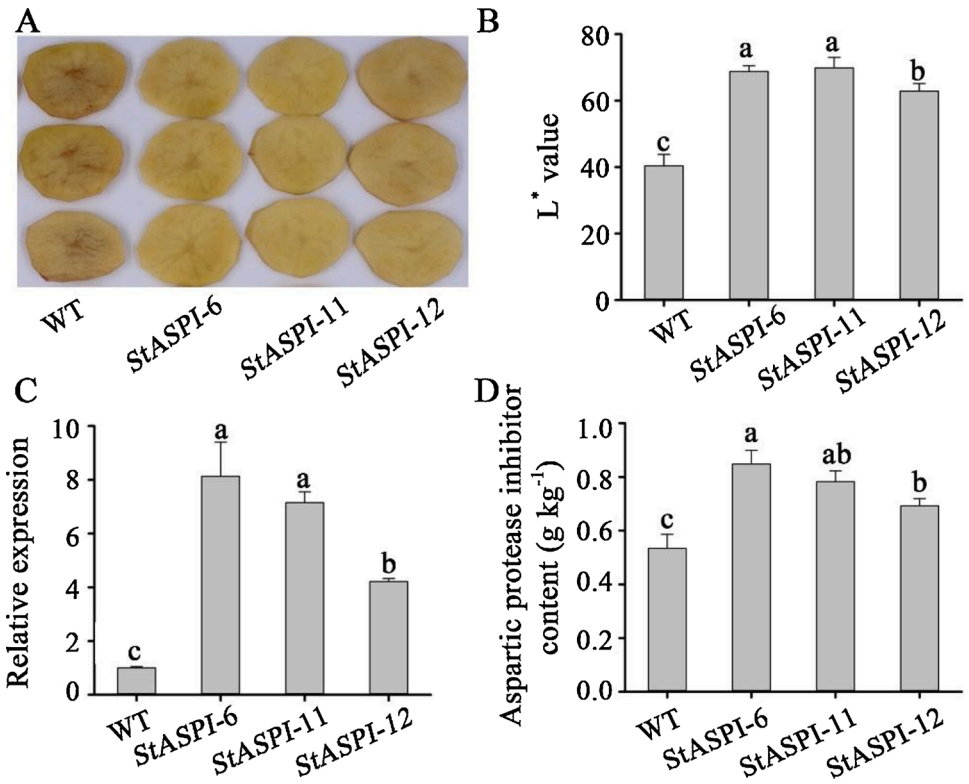

Fig. 3. Visual browning (A) and L* value (B) at 3 h after cutting, relative expression of *StASPI* (C) and aspartic protease inhibitor content (D) in transgenic and WT potatoes. Different letters indicate statistically significant differences (**p < 0.01) and error bars represent SD (n = 3). *StASPI-6*, *StASPI-11* and *StASPI-12* are three transgenic lines; WT, wild-type.

increasing FAAs accumulation accelerated the browning of potato mash, according with the study of Dong et al. (2020). However, the browning degrees of the transgenic mashes supplemented with FAAs were still much less than that of WT (**p < 0.01) (Fig. 5C, D). These results indicated that overexpression of *StASPI* could inhibit potato browning through decreasing the accumulation of total FAAs, but the browning-inhibition by overexpressed *StASPI* was not only through decreasing FAAs, but also through other pathways. Therefore, PPO activity was measured. The result showed that PPO activity declined by 27.18 %, 32.13 % and 29.32 % in three transgenic lines respectively, significantly lower (**p < 0.01) than that in WT tubers (Fig. 5E). This result suggested that overexpression of *StASPI* decreased PPO activity, which also contributed to the browning-inhibition of potatoes.

3.5. Effects of overexpressed-StASPI on antioxidant activity and ROS accumulation in potato tuber

The effects of overexpressed-*StASPI* on $O_2^{\cdot-}$, H_2O_2 content, SOD and CAT activity in transgenic and WT potato tuber were evaluated. Contents of both H_2O_2 and $O_2^{\cdot-}$ in tubers of three transgenic lines were significantly lower (**p < 0.01) than those in WT tubers (Fig. 6A, B). ROS is generated in cells under normal as well as wide range of stressful conditions, which can be scavenged by both SOD and CAT (Bienert et al., 2006). Therefore, relative activities of SOD and CAT in tubers were measured and shown in Fig. 6C, D. Activities of SOD and CAT in transgenic tubers were all significantly higher than those in WT (**p < 0.01) (Fig. 6C, D). CAT activity increased much more significantly, approximately 70 %, 85 % and 60 % in three transgenic lines respectively, compared with WT (Fig. 6D). These results indicated that overexpression of *StASPI* enhanced the activities of antioxidant enzymes CAT and SOD to improve the ability of scavenging ROS, such as decreasing the accumulation of H_2O_2 and $O_2^{\cdot-}$. Thus, this contributed to browning-inhibition of fresh-cut potatoes.

4. Discussion

Potato browning caused by mechanical injury leads to severe losses in the potato processing industry. In a previous study, we found that varieties of "*Kexin #13*" and "*Kexin #4*" had significant differences in browning-susceptibility (Dong et al., 2020). Also, PCT effectively inhibited browning of potatoes (Wang et al., 2015). The transcript levels of aspartic protease inhibitor *StASPI* was highly up-regulated (***p < 0.001) in browning-less variety "*Kexin #13*" and PCT-treated potatoes compared with browning-susceptible variety "*Kexin #4*" and potatoes without PCT (Fig. S. 1). Moreover, intermittent warming treatment could enhance cold tolerance and reduce browning induced by chilling injury in many fruits and vegetables (Wang et al., 2018). In this study, we found warming treatment at 25 °C for 12 h effectively alleviated the browning of cold-stored potatoes after fresh-cut, and interestingly the treatment significantly increased *StASPI* expression level in potatoes (Fig. 1). Therefore, these results suggested that *StASPI* might play important roles in resistance to enzymatic browning of potatoes. The hypothesis is supported by evidences that exogenous aspartic protease inhibitor Pepstatin A treatment delayed browning of potato mash *in vitro* (Fig. 2) and overexpressed-*StASPI* inhibited the fresh-cut browning of potato effectively (Fig. 3).

4.1. StASPI inhibited the enzymatic browning of potatoes through decreasing FAAs levels and PPO activity

In potato tubers, tyrosine is the main substrates of browning, and other FAAs are also involved with browning, accelerating or inhibiting browning (Ali et al., 2016; Goyer and Pellé, 2018). Previous studies showed tyrosine is highly correlated with browning and its level in potatoes mainly resulted from the protein hydrolysis related to endogenous protease, rather than tyrosine synthesis (Dean et al., 1992; Sabba and Dean, 1994). Pool of FAAs can be increased because of the protein

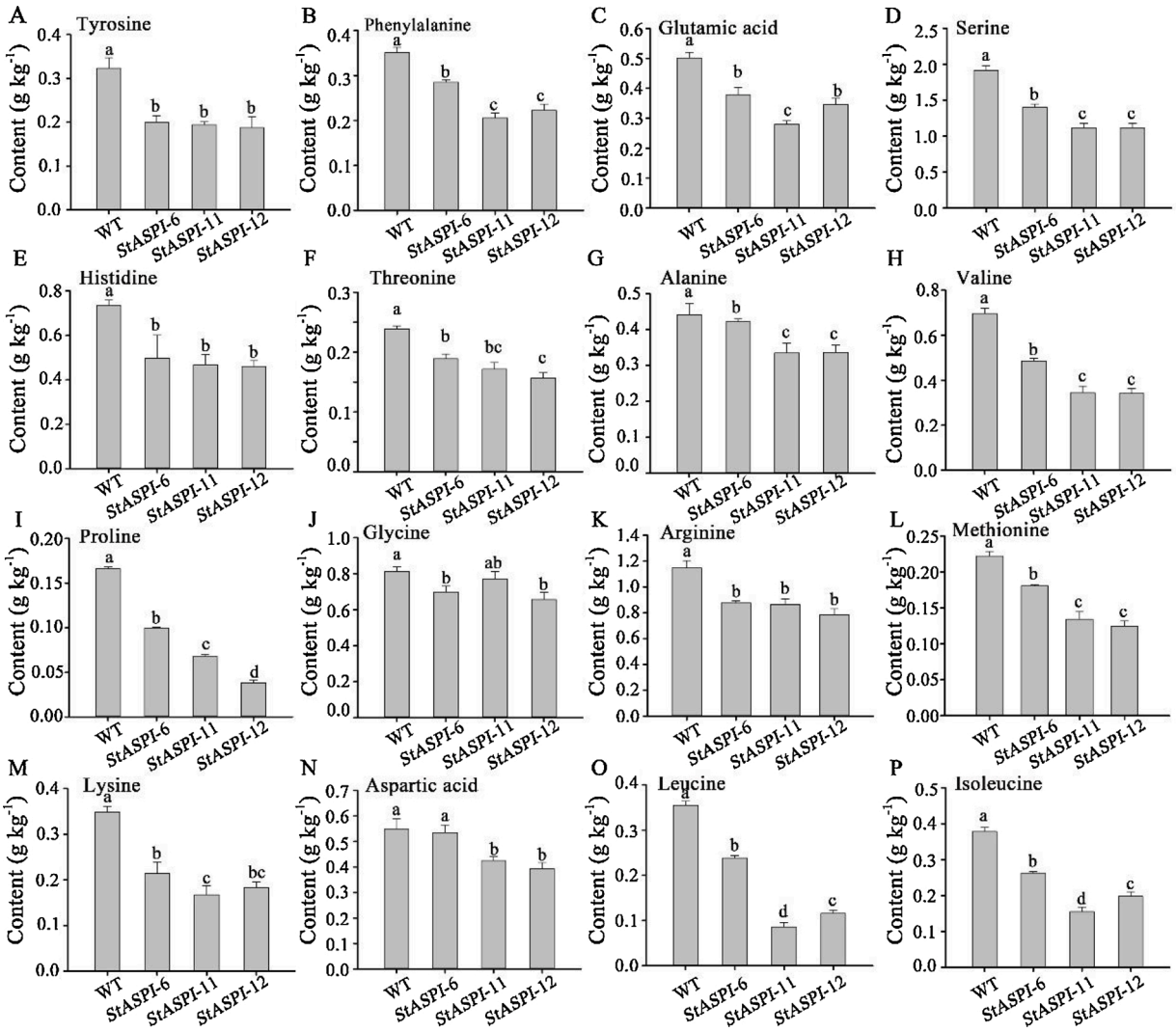

Fig. 4. Contents of free amino acids in transgenic and WT tubers. Different letters indicate statistically significant differences (*p < 0.05) and error bars represent SD (n = 3). *StASPI*-6, *StASPI*-11 and *StASPI*-12 are three transgenic lines; WT, wild-type.

proteolysis, caused by various proteases during various stresses or senescence (Hildebrandt et al., 2015). And, previous studies reported that biotic and abiotic stresses during cultivation or storage enhanced proteolytic activity, and thus increased total FAAs levels (Montero et al., 1995; Dionne et al., 2001; Yoon et al., 2017). However, protease inhibitors negatively regulate protease activities (Stael et al., 2019), which might influence the accumulation of total free amino acids in plants. Munger et al. (2015) reported that two overexpressed cereal cysteine protease inhibitors K52 and K53 individually reduced accumulation of free amino acids through decreasing protein hydrolysis. StASPI, as an aspartic protease inhibitor, can inhibit both aspartic protease cathepsin D and serine protease trypsin (Guo et al., 2015). In this study, overexpression of *StASPI* decreased the activity of proteases and reduced FAA accumulation in potato tubers (Figs. 4 and 5A, B). The increase of FAAs by supplementing exogenous FAA promoted browning of potato mash *in vitro* (Fig. 5C, D). These results suggested that overexpressed *StASPI* decreased the accumulation of FAAs through modulating protease activity, thus inhibiting potato browning after cutting.

Furthermore, overexpression of *StASPI* also significantly inhibited PPO activity (**p < 0.01) (Fig. 5E). Little information is available in previous research about the effect of aspartic protease inhibitor on PPO activity. Aspartic protease inhibitor Pepstatin A effectively alleviated browning of potato mash (Fig. 2), and PPO activity in Pepstatin A-treated potato mash was significantly lower (**p < 0.01) than that in control (Fig. S. 2A). However, when the commercial purified PPO enzyme was treated with Pepstatin A, there were no differences in the activities between PPO enzymes treated with and without Pepstatin A (Fig. S. 2B). These results indicated that the process of aspartic protease inhibitor inhibiting PPO activity was not a direct action. Previous studies showed proteases can act upstream of PPO and cleavage the latent PPO into active PPO (Kanost and Jiang, 2015; Nakhleh et al., 2017). *StASPI* inhibited protease activities (Fig. 5B). Hence, *StASPI* might decrease PPO activity through inhibiting protease activities. However, there are hundreds of proteases in plants and functions of proteases are mostly unknown (van der Hoorn, 2008). Therefore, the identification of the protease that may associate with PPO and influence its activity is a future research direction to study the mechanism of *StASPI* reducing PPO activity.

4.2. *StASPI inhibited potato browning through reducing ROS accumulation*

Besides phenolic substrate and associated enzymes, membrane stability is also an important contributor to enzymatic browning of fresh-cut fruits and vegetables for the separation of substrate and enzymes (Toivonen and Brummell, 2008). The stability of cell membranes is influenced by ROS accumulation and antioxidative defense (Ahmad et al., 2008). Various stresses, such as cold storage and cutting injury,

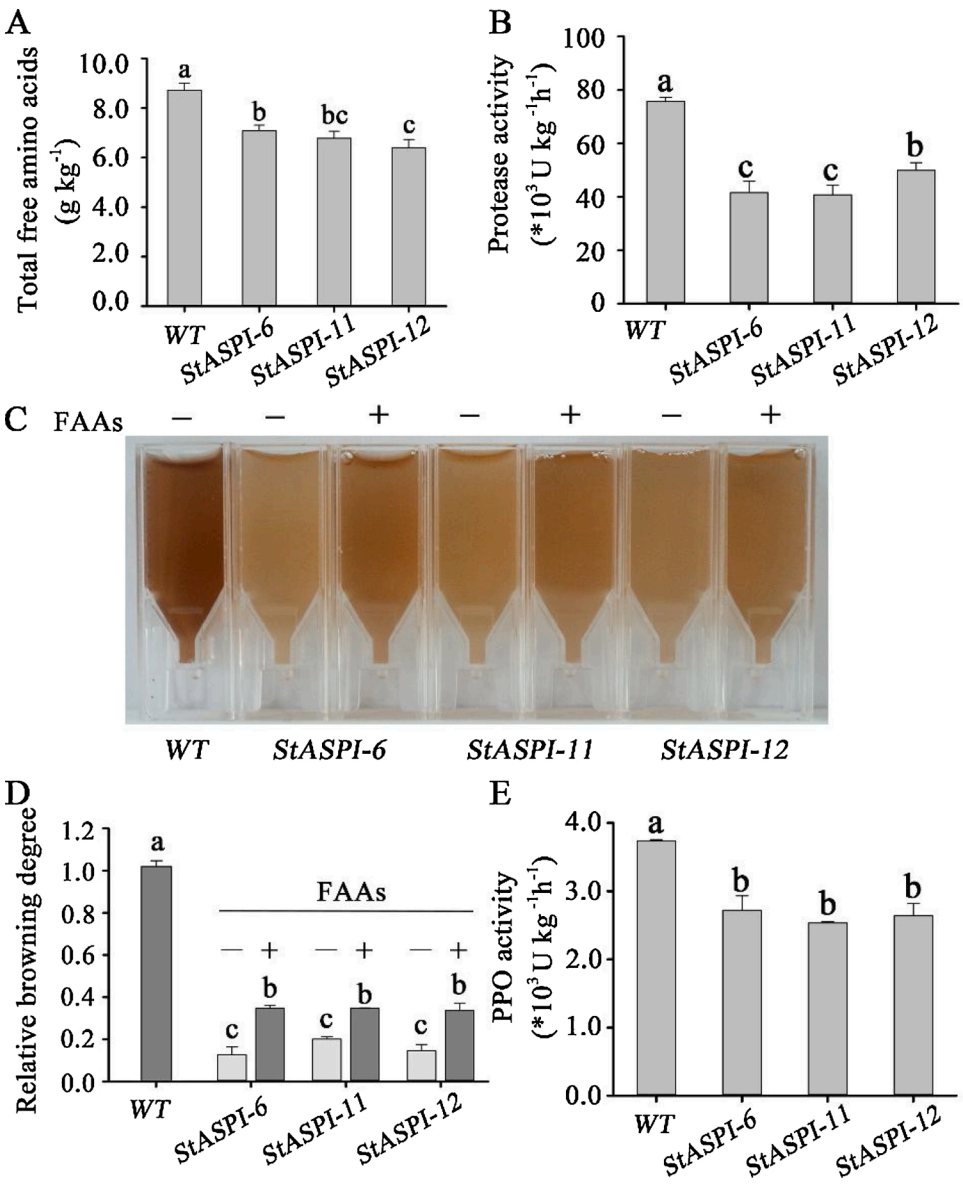

Fig. 5. Effect of overexpressed *StASPI* on total FAA content (A), protease activity (B) and PPO activity (E) in potato tubers and browning comparation of WT and transgenic mash supplemented with and without exogenous differential FAAs (C, D). Different letters indicate statistically significant differences (**p < 0.01) and error bars represent SD (n = 3). *StASPI*-6, *StASPI*-11 and *StASPI*-12 are three transgenic lines; WT, wild-type; FAA, free amino acids; "+" and "–" mean that transgenic mash was added with (+) and without (–) exogenous differential FAAs.

induce more ROS generation in tissues (Lukatkin, 2002; Zhang et al., 2008a,b). Excessive ROS causes oxidative stress and damages the cellular integrity (Ahmad et al., 2008). In addition, abundant ROS could induce the transcription factors of proteolytic degradation and active proteolytic enzymes (Pillai et al., 2005), which would in turn induce generation of ROS and further aggravate membrane breakdown (Lim et al., 2006). This process increases the risk of tissue deterioration and serious browning in fresh-cut products. Therefore, high level of protective enzymes such as SOD and CAT is essential for counteracting the toxic effects of excessive ROS (Ahmad et al., 2008; Zhang et al., 2008a,b) and controlling the homeostasis of ROS levels in tissues and cells (Ahmad et al., 2014; Dunnill et al., 2017) to protect plants cells from oxidative stress. Previous studies showed that protease inhibitors played multiple roles in enhancing the resistance to various biotic or abiotic stresses (Zhang et al., 2008a,b). Overexpression of cysteine protease inhibitor conferred drought tolerance and protected against oxidative stress through decreasing ROS content in *Arabidopsis* (Tan et al., 2015). Serine protease inhibitor significantly inhibited ROS generation in lung tissues (Sakamoto et al., 2017), and protease inhibitor increased SOD and CAT activities of liver in rat (Jung et al., 1999). However, there is little knowledge about the effects of aspartic protease inhibitor on ROS accumulation and antioxidant enzymes in plants. Antioxidant system including antioxidant enzymes, CAT and SOD, is responsible for controlling the homeostatic mechanism of ROS levels in tissues and cells (Ahmad et al., 2014; Dunnill et al., 2017), and over-expressed protease inhibitor could un-regulate the stress-responsive proteins (Munger et al., 2012). Thus, over-expressed *StASPI* might inhibit the generation of ROS content through regulating the activities of proteases and antioxidant enzymes. Our results showed that overexpression of *StASPI* decreased H_2O_2 and O_2^- accumulation (Fig. 6A, B), and increased the activities of SOD and CAT in potato tubers (Fig. 6C, D). These findings suggested that overexpression of *StASPI* enhanced the activities of antioxidant enzymes CAT and SOD, improving the ability of scavenging ROS, and thus decreasing the accumulation of H_2O_2 and O_2^-, which made a contribution to inhibiting potato browning after cutting.

5. Conclusion

In this study we demonstrated that *StASPI* played an important role in resistance to enzymatic browning of potatoes. Overexpression of *StASPI* could effectively alleviate enzymatic browning of potatoes through decreasing protease activity to reduce total FAA accumulation

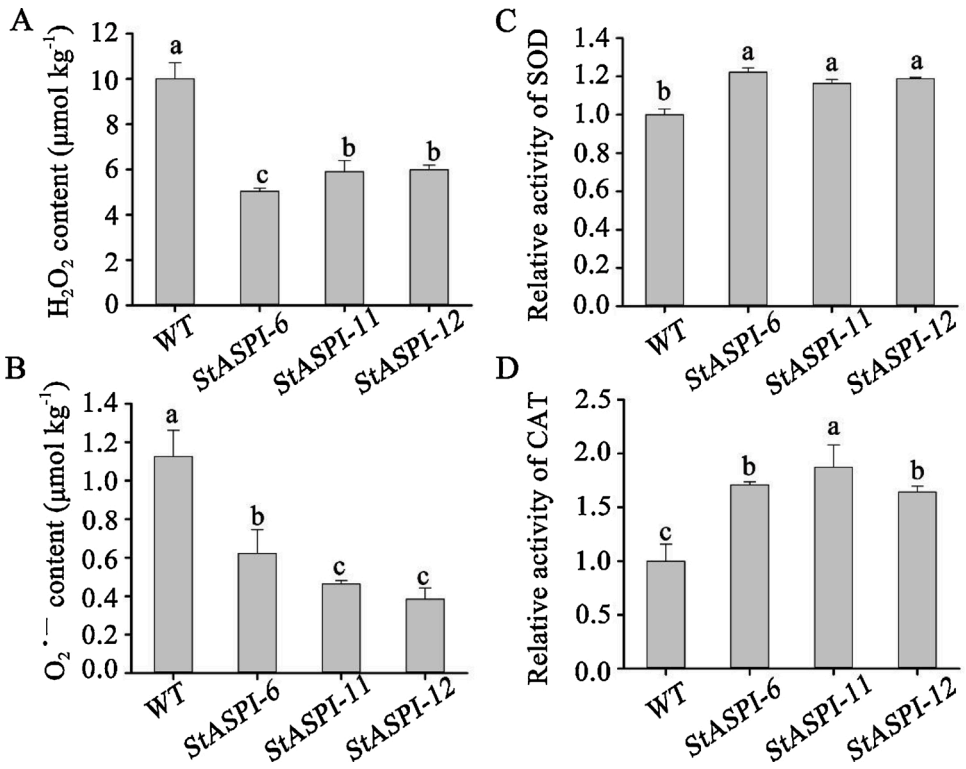

Fig. 6. Effect of overexpressed *StASPI* on H_2O_2 content (A), $O_2^{\bullet-}$ content (B), relative activity of SOD (C) and CAT (D). Different letters indicate statistically significant differences (**p < 0.01) and error bars represent SD (n = 3). *StASPI*-6, *StASPI*-11 and *StASPI*-12 are three transgenic lines; WT, wild-type.

in potato tubers. Moreover, overexpression of *StASPI* lowered PPO activity, enhanced activities of antioxidant enzyme SOD and CAT, and decreased H_2O_2 and $O_2^{\bullet-}$ contents in potato tubers, which also contributed to browning-resistance of potatoes after fresh-cut. This study provides a new perspective on the strategies for inhibiting enzymatic browning of potatoes.

Author statement

QW and CJ designed the research; TD, YC and ZZ performed the experiment; GL and SZ provided the technical support; QW and TD analyzed the data and wrote the manuscript; CJ revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2020.111353.

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