Food Chemistry 237 (2017) 205–213

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Changes in ROS production and antioxidant capacity during tuber sprouting in potato

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ARTICLE INFO

Article history:
Received 31 March 2017
Received in revised form 16 May 2017
Accepted 19 May 2017
Available online 20 May 2017

Keywords:
Solanum tuberosum L.
Tuber
Dormancy
NADPH oxidase
Gene expression

ABSTRACT

Potato dormancy is a complex process with an extensive release phase. This study investigated involvement of reactive oxygen species during tuber dormancy release. We found that tuber sprouting was delayed by treatment with diphenylene iodonium chloride, an NADPH oxidase inhibitor; NADPH oxidase catalyze the production of ROS. In situ ROS localization and ROS content estimation revealed that dormancy release was associated with an accumulation of superoxide anion and hydrogen peroxide in tuber buds. The antioxidant compounds and enzymes display important changes during the progression of dormancy. The application of Ca^{2+} induced superoxide anion production and promoted *in vitro* tuber bud sprouting. Among the seven homologues of NADPH oxidases in potato, the expression of *StrbohA* and *StrbohB* were detected in particular when dormancy break. In addition, the expression of key genes that function in the potato dormancy release are discussed in relation to ROS metabolism in other plants.

1. Introduction

The potato (*Solanum tuberosum* L.) tuber is an underground stem that is formed by the stoppage of longitudinal growth and the subsequent swelling. The meristem activity of apical regions and lateral buds of developing tubers is repressed during tuber swelling. Tuber dormancy is established at the start of tuber formation. This dormancy state lasts for a finite period of time following tuber harvest, even if the tubers are placed under optional conditions for sprouting (15–20 °C, darkness, relative humidity about 90%). The length of the dormancy period is dependent on the genotype as well as on both pre- and post-harvest conditions (Sonnewald, 2000). Following a period of storage, dormancy is released and apical buds start to grow. The length of dormancy is a very important consideration in the management of tuber storage, processing, and planting. Tuber dormancy release is a complex process that is known to involve several physiological and biochemical changes. Carbohydrate metabolism and hormonal regulation have been reported to be associated with the dormancy release process in potato, very few studies have focused on the potential role of reactive oxygen species (ROS) and antioxidant metabolism during this important physiological process.

It is well known that exogenous ROS application can induce the breaking of dormancy, this has been demonstrated both in plant seeds and in vegetative buds. *In planta*, ROS produced by NADPH oxidase has been shown to play various important roles in plant development and growth (Foreman et al., 2003). An increasing number of studies have provided evidence that ROS are involved in plant seed germination and dormancy alleviation (Bailly, El-Maarouf-Bouteau, & Corbineau, 2008; Ishibashi et al., 2015; Leymarie et al., 2012; Sarath, Hou, Baird, & Mitchell, 2007). In potato tubers, the application both of hydrogen peroxide (H_2O_2) and inhibitors of catalase (CAT) results in a reduction of the dormancy period and in rapid sprouting (Bajji, Hamdi, Gastiny, Rojas-Beltran, & du Jardin, 2007). H_2O_2 can easily cross cell membranes, thus it has been proposed as a dormancy breaking signal. The enhanced gene expression of redox regulation has been observed during the release of dormancy in tubers (Liu et al., 2015).

NADPH oxidase, which are also known as respiratory burst oxidase homologues (rboh), are named owing to their homology with the gp91phox domain of the animal respiratory burst oxidase (Keller et al., 1998). Rboh enzymes are localized to the plasma membrane, where they can transfer electrons from cytosolic NADPH or NADH to apoplastic oxygen, leading to the production of apoplastic superoxide (Sagi et al., 2004). It has been

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http://dx.doi.org/10.1016/j.foodchem.2017.05.107
0308-8146/

demonstrated that rbohB (respiratory burst oxidase homolog B), a typical membrane-bound rboh enzyme that produces superoxide, plays a role in seed germination in Arabidopsis (Müller, Carstens, Linkies, Torres, & Leubner-Metzger, 2009). Superoxide anions ($O_2^{\cdot-}$) produced by NADPH oxidase were reported to regulate seed germination and seedling growth in barley (Ishibashi, Tawaratsumida, Zheng, Yuasa, & Iwaya-Inoue, 2010). NADPH oxidases have effect on α-amylase activity during seed germination (Ishibashi et al., 2010). NADPH-dependent $O_2^{\cdot-}$-generating activities in plasma membrane fractions were diphenylene iodonium (DPI) sensitive (Kobayashi, Kawakita, Maeshima, Doke, & Yoshioka, 2006; Pakizeh, Kuliev, Mammadov, Ardjmand, & Hasani, 2011). The NADPH oxidase inhibitor DPI retarded germination, stunted root growth, and partially inhibited NADPH oxidase activity in switchgrass seeds (Sarath et al., 2007).

In order to contribute to understanding of the role of ROS in the release of tuber dormancy, ROS production was measured, and the effect of NADPH oxidase inhibitor DPI application on tuber sprouting behavior was evaluated in this study. As the maintenance of the cellular ROS homeostasis requires a fine-tuned balance between ROS generation and scavenging, the activity of the NADPH oxidase, the antioxidant enzymes and compounds, were investigated during the release of dormancy in tubers. Additionally, we examined the expression of *Strboh* genes during tuber dormancy release process. Finally, we evaluated the expression of key genes that are known to regulate the release of dormancy in tubers, and we discussed the potential relationships among gene expression and ROS metabolism involved in dormancy release in relation to what we have known in other plant species.

2. Materials and methods

2.1. Plant Materials

Potato (*S. tuberosum* L. cv. Russet Burbank and cv. QR01) tubers used in the present work were field grown in the experimental farm of Yuling potato breeding station in late April 2015 in the Shaanxi Province of China. Plants of Russet Burbank and QR01 were managed under standard agronomic practices without irrigation and were harvested in late September. Once harvested, healthy and uniform tubers (dia. 35–45 mm) were manually selected and stored under darkness conditions around 15 °C for fully maturity and wound healing for a period of a month. After that, tubers of 'Russet Burbank' were placed to release dormancy in the darkness conditions (approximately 22 ± 2 °C, approximately 60% RH). Tubers of 'QR01' were stored at 4 °C in the dark until further use. 'Russet Burbank' tubers were thoroughly washed with tap water and then rinsed twice with distilled water prior to sampling. For sample collection, the top 1 cm bud end (with major eye of a potato tuber) tissue were excised and flash frozen at day 0 (start storage), day 30, day 45, day 60 (dormancy break) and day 67 (sprouting) of storage tuber respectively. Three biological replicates were assayed for biochemical studies and RNA extraction. The collected tuber tissues were further crushed in a powder under liquid nitrogen, then stored at −80 °C before analysis.

2.2. In vitro tuber sprouting assay

To evaluate the roles of NADPH oxidase in dormancy processes in tubers, *in vitro* tuber meristem complexes isolated from QR01 tubers after five months storage at 4 °C were treated with inhibitor of NADPH oxidase DPI. Discs (complex of apical bud meristem) of around 1 cm height containing one bud eye were excised from tubers (Dormant 'QR01' potato) using a 8 mm diameter cork borer. Discs were washed three times for 15 min in sterile buffer (20 mM MES, 300 mM D-mannitol, and 5 mM ascorbic acid, pH 6.5). Discs were treated by 0, 2.5, 10 and 20 μM DPI for 10 min respectively and subsequently placed in petri dishes (16–18 discs per dish and two replicates) lined with moist filter paper. Petri dishes were sealed and stored in darkness under tissue culture conditions for two weeks. The filter paper was regularly moistened by adding sterile water. Sprouting of tuber discs were considered as ≥3 mm sprouts occurrence.

2.3. Measurements of $O_2^{\cdot-}$ production rate and H_2O_2 content

$O_2^{\cdot-}$ was measured by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\cdot-}$ according to Elstner and Heupel (1976) described. Briefly, 0.5 g of tuber powder was homogenized with 5.0 mL of 50 mM potassium phosphate buffer (pH 7.8) on ice. After centrifugation at 12 000 rpm for 20 min at 4 °C, 1.0 mL of the supernatant was incubated with 1.0 mL of 50 mM phosphate buffer and 1.0 mL of 1 mM hydroxylamine hydrochloride at 25 °C for 60 min, then 1.0 mL of 17 mM sulfanilamide and 7 mM naphthylamine was added to the incubation mixture. After reaction at 25 °C for 20 min, the absorbance was read at 530 nm. A standard curve was developed to calculate the production rate of $O_2^{\cdot-}$ from the chemical reaction of KNO_2 and hydroxylamine.

H_2O_2 was determined as Sergiev, Alexieva, and Karanov (1997) described with some modifications. 0.5 g of tuber powder was homogenized with 5.0 mL 0.1% (w/v) trichloroacetic acid (TCA) on ice. After centrifugation at 12000 rpm for 20 min, 0.5 mL of the supernatant was incubated with 0.5 mL of 0.1 mM sodium phosphate buffer (pH 7.0) and 2.0 mL of 1 M potassium iodide (KI). The absorbance of incubation mixture was read at 390 nm. The content of H_2O_2 was given on a standard curve.

2.4. Staining of superoxide ion and hydrogen peroxide

For $O_2^{\cdot-}$ and H_2O_2 detection, around 1 cm length × 8 mm width (1 mm thickness) of hand-cut longitudinal sections of tuber apical bud complex were incubated in 0.1 mg·mL^{-1} nitroblue tetrazolium (NBT) (in 10 mM Tris-HCl buffer, pH 7.4) and 0.1 mg·mL^{-1}diaminobenzidine (DAB) (in 50 mM Tris-Ac buffer, pH 5.0) at room temperature for 30 min in darkness respectively. The $O_2^{\cdot-}$ and H_2O_2 were visualized as either a blue color at the site of NBT precipitation or a brown color at the site of DAB polymerization. Stained tuber samples were rinsed by 70% ethanol and sterile water, and photographed under natural light. NBT stained sections of apical bud complex were further fixed with 5% formalin, embedded with paraffin, and sectioned to visualize subcellular deposits of superoxide anion by light microscope.

2.5. Total antioxidant capacity assay

The total antioxidant capacity was evaluated by a commercial kit (Cat. No. S0119, Beyotime) following the instructions, 1.0 g of tuber powder was homogenized with 50 mM phosphate buffer and centrifuged at 12000 rpm for 10 min at 4 °C. Appropriately diluted supernates or standard solution (10 μL) were mixed with $ABTS^+$ working solution (170 μL) and reacted for about 6 min before measuring the absorbance at 405 nm by a microplate reader. The results were calculated as mmol·g^{-1}TE.

2.6. Ascorbate and glutathione analysis

Both ascorbate (AsA) and dehydroascorbate (DHA) was measured as described by Wang and Jiao (2001). 0.5 g of tuber powder was homogenized with 0.3 M TCA and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was detected by reading