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NADPH oxidase inhibitor diphenyleneiodonium and reduced glutathione mitigate ethephon-mediated leaf senescence, H_2O_2 elevation and senescence-associated gene expression in sweet potato (*Ipomoea batatas*)

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ABSTRACT

Ethephon, an ethylene releasing compound, promoted leaf senescence, H_2O_2 elevation, and senescence-associated gene expression in sweet potato. It also affected the glutathione and ascorbate levels, which in turn perturbed H_2O_2 homeostasis. The decrease of reduced glutathione and the accumulation of dehydroascorbate correlated with leaf senescence and H_2O_2 elevation at 72 h in ethephon-treated leaves. Exogenous application of reduced glutathione caused quicker and significant increase of its intracellular level and resulted in the attenuation of leaf senescence and H_2O_2 elevation. A small H_2O_2 peak produced within the first 4 h after ethephon application was also eliminated by reduced glutathione. Diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, delayed leaf senescence and H_2O_2 elevation at 72 h, and its influence was effective only within the first 4 h after ethephon treatment. Ethephon-induced senescence-associated gene expression was repressed by DPI and reduced glutathione at 72 h in pretreated leaves. Leaves treated with L-buthionine sulfoximine, an endogenous glutathione synthetase inhibitor, did enhance senescence-associated gene expression, and the activation was strongly repressed by reduced glutathione. In conclusion, ethephon-mediated leaf senescence, H_2O_2 elevation and senescence-associated gene expression are all alleviated by reduced glutathione and NADPH oxidase inhibitor DPI. The speed and the amount of intracellular reduced glutathione accumulation influence its effectiveness of protection against ethephon-mediated effects. Reactive oxygen species generated from NADPH oxidase likely serves as an oxidative stress signal and participates in ethephon signaling. The possible roles of NADPH oxidase and reduced glutathione in the regulation of oxidative stress signal in ethephon are discussed.

Introduction

Leaf senescence is the final stage of leaf development (Buchanan-Wollaston, 1997; Lim et al., 2007) and is influenced by endogenous and exogenous factors, including plant growth regulators, starvation, dark, cold, drought, salt, wounding, insect attack and pathogen infection (Yoshida, 2003; Chen et al., 2012a). Leaf cells undergo highly coordinated changes in structure, metabolism, and gene expression during senescence in a defined order, including breakdown of chloroplast and organelles, catabolism of chlorophyll and macromolecules, elevation of reactive oxygen species, and activation of senescence-associated gene expression (Lim et al., 2007; Chen et al., 2012b).

Ethylene plays a key role in leaf senescence. Previous reports demonstrate that elevated oxidative stresses caused by developmental cues and environmental stimuli, including seed germination (Ishibashi et al., 2013), pathogen infection (Hyodo et al., 2003), ozone (Ranieri et al., 2003), UV-B (Wang et al., 2002), salt stress (Ghanem et al., 2008), wounding (Boller and Kende, 1980) and ectopic Cu/Zn superoxide dismutase gene expression (Kim et al., 2008) enhanced ethylene production. In tomato plant, salt

Abbreviations: CCI, chlorophyll content index; DAB, diaminobenzidine; DPI, diphenyleneiodonium; MES, 2-(N-morpholino)-ethane sulphonic acid; RT-PCR, reverse transcription-polymerase chain reaction.

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stress enhanced ethylene biosynthesis and promoted leaf senescence (Ghanem et al., 2008). The NaCl-induced cell death was also due to ethylene-mediated reactive oxygen species production and could be attenuated by exogenous catalase in tomato cell suspension (Poór et al., 2013). In *Arabidopsis*, ozone-induced increase of reactive oxygen species and leaf damage in ethylene-insensitive mutant *ein2* was mitigated by artificial elevation of reduced glutathione content (Yoshida et al., 2009). In sweet potato, ethephon-promoted leaf senescence and H_2O_2 elevation were correlated with the expression level of a leaf-type catalase (Chen et al., 2011) and attenuated by exogenous catalase SPCAT1 fusion protein (Chen et al., 2012b). These data suggest that oxidative stress may play important roles in ethylene biosynthesis, ethylene signaling, and ethylene-mediated effects.

Production of reactive oxygen species is governed by the photosynthesis rate, photorespiration, respiration, NADPH oxidases and other producing systems (Foyer and Noctor, 2003). Among these systems, the plasma membrane NADPH oxidase plays a key role. Reactive oxygen species produced by NADPH oxidases participates in cellular signaling associated with plant development and stress response, including elongation of root hair (Foreman et al., 2003), seed germination (Ishibashi et al., 2010), plant defense to pathogens (Torres et al., 2002), hypersensitive response in incompatible interactions (Yoshioka et al., 2003), salt stress tolerance (Poór et al., 2013) and wounding response (Jih et al., 2003). It transfers electrons from cytoplasmic NADPH to oxygen, and serves as one of the major source of superoxide anion (O_2^-), which is subsequently transformed by superoxide dismutase into H_2O_2 (Cross and Segal, 2004). Plant NADPH oxidase complexes have been identified and characterized in several species, including *Arabidopsis* (Keller et al., 1998; Torres et al., 1998), barley (Trujillo et al., 2006; Lightfoot et al., 2008), potato (Yoshioka et al., 2001), rice (Groom et al., 1996; Yoshie et al., 2005), tobacco (Yoshioka et al., 2003), and tomato (Amicucci et al., 1999), which suggest the role of NADPH oxidase as a common signal component among various plant species.

In plants, glutathione and ascorbate are the main antioxidants for the removal of reactive oxygen species and play important roles in the metabolic functions and redox signaling (Shao et al., 2008; Foyer and Noctor, 2011). For ascorbate, the small antioxidant molecule L-ascorbic acid serves as a cofactor for many enzymes (Arrigoni and De Tullio, 2000) and contributes to the detoxification of reactive oxygen species (Conklin and Barth, 2004). The endogenous level of ascorbic acid is determined by both de novo biosynthesis and recycling of the oxidized forms (dehydroascorbate and monodehydroascorbate) via corresponding reductases (Noctor and Foyer, 1998; Conklin and Barth, 2004). The endogenous ascorbic acid level has recently been suggested to be associated with the biosynthesis of ethylene (Arrigoni and De Tullio, 2000), the regulation of senescence and senescence-associated gene expression (Barth et al., 2004, 2006).

For glutathione, it serves as the most abundant low-molecular weight thiol in the cellular redox system, and is used for detoxification of reactive oxygen species and transmission of redox signals (Noctor and Foyer, 1998; Meyer, 2008). The endogenous level of reduced glutathione is determined by both de novo biosynthesis and recycling of the oxidized form, GSSG. Enzymes such as γ-glutamylcysteine synthetase and glutathione synthetase are involved in the two ATP-dependent steps of glutathione biosynthesis. Glutathione reductase is associated with the reduction of oxidized GSSG form to reduced GSH form (Noctor and Foyer, 1998; Noctor et al., 2012). Reactive oxygen species production and elevated oxidative stress from environmental stresses, including cold, ozone and pathogens can modify whole leaf glutathione redox state (Sen Gupta et al., 1991; Vanacker et al., 2000; Gomez et al., 2004). Therefore, the cytosolic glutathione redox potential has been shown to increase in response to wounding (Meyer et al., 2007). However, plants deficient in H_2O_2-metabolizing enzymes, catalase, exhibited remarkably change in the ratio of reduced GSH to oxidized GSSG forms of glutathione (Smith et al., 1984; Queval et al., 2009). Deviation of redox potential due to either depletion of reduced glutathione/ascorbic acid or increase of their oxidized forms can be used for fine tuning the activity of targeted proteins and gene expression. Therefore, the homeostasis of glutathione–ascorbate cycle and redox signaling can be integrated together (Meyer, 2008; Foyer and Noctor, 2011).

In sweet potato, ethephon promoted leaf senescence, H_2O_2 elevation and senescence-associated gene expression (Chen et al., 2000, 2004, 2006, 2009, 2010a). Several ethephon-inducible senescence-associated cysteine proteases, including *SPAE*, *SPCP2* and *SPCP3* have been ectopically expressed in transgenic *Arabidopsis* plants, which caused altered developmental characteristics (Chen et al., 2008, 2010a, 2013) and salt and drought stress responses (Chen et al., 2010a, 2013). Ethephon signaling required external Ca^{2+} ion (Chen et al., 2010b) and calmodulin *SPCAM* (Chen et al., 2012c). Leaf senescence and H_2O_2 elevation caused by ethephon were negatively correlated with a major leaf-type catalase activity (Chen et al., 2011), and also attenuated by exogenous catalase SPCAT1 fusion protein (Chen et al., 2012b). In this manuscript we show for the first time that ethephon alters the balance of intracellular glutathione and ascorbate contents, which in turn interfere the H_2O_2 homeostasis. The NADPH oxidase and reduced glutathione are likely components associated with the regulation of oxidative stress signal in ethephon leading to leaf senescence, H_2O_2 elevation and senescence-associated gene expression in sweet potato.

Materials and methods

Plant materials

The storage roots of sweet potato (*Ipomoea batatas* (L.) Lam.) were grown in a growth chamber at 28 °C/16 h and 23 °C/8 h night cycle. Plantlets sprouted from the storage roots provided detached mature leaves for treatments with (1) ethephon, (2) reduced glutathione, (3) NADPH oxidase inhibitor diphenyleneiodonium (DPI), and (4) L-buthionine sulfoximide, respectively. Mature leaves between the 3rd and the 7th positions counted downward from the shoot apex were collected for experiments. There were at least three replicates for each treatment. Each experiment was performed at least three times. Samples from treatments mentioned above were individually collected and used for analysis as described below.

Ethephon and L-buthionine sulfoximide treatments

Detached mature leaves were placed on a wet paper towel containing 3 mM 2-(N-morpholino)ethanesulphonic acid (MES) buffer (pH 5.8) plus 1 mM ethephon or different concentrations of L-buthionine sulfoximide (1, 2 and 5 mM, respectively) and kept at 28 °C/16 h and 23 °C/8 h cycle in the dark. For ethephon, samples were collected individually at (1) 0, 24, 48 and 72 h or (2) 0, 2, 4, 6, 24 and 72 h after treatment for analysis, including leaf morphology, chlorophyll content, Fv/Fm, diaminobenzidine (DAB) staining, H_2O_2 measurement, glutathione and ascorbate determination, and senescence-associated gene expression. For L-buthionine sulfoximide, samples were harvested at 72 h after treatment for assay of senescence-associated gene expression.

Effector treatment

Influence of effectors such as reduced glutathione and NADPH oxidase inhibitor DPI on leaf senescence, H_2O_2 elevation, and