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ORIGINAL ARTICLE

Influence of salicylic acid on rubisco and rubisco activase in tobacco plant grown under sodium chloride *in vitro*

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Abstract The present study was designed to evaluate the influence of salicylic acid (SA) on the growth of salt stress (sodium chloride) induced in tobacco plants. In addition, quantification of rubisco and rubisco activase contents of the plants was also determined in treatments with the control, 10^{-4} mM SA, 50 mM NaCl, 100 mM NaCl, 150 mM NaCl, SA + 50 mM NaCl, SA + 100 mM NaCl and SA + 150 mM NaCl, respectively after *in vitro* culture for 5 weeks. The growth of the tobacco plant decreased in 50 mM and 100 mM NaCl when not treated with SA. However, the growth was accelerated by SA, and the growth retardation caused by NaCl was improved by SA. The content of rubisco was improved by SA only in plants treated with 50 mM NaCl, and the activity of rubisco was increased by SA resulting in the decreased effect of NaCl, but only in 50 mM NaCl treated plants. The content of rubisco activase decreased due to NaCl, and SA did not improve the effect caused by NaCl. The activity of rubisco activase was increased by SA resulting in decreased activity caused by NaCl, but increased effect by SA was not recovered to the level of NaCl untreated plants. The activity of rubisco and rubisco activase, which decreased due to denaturing agents, did not demonstrate significant improvement when compared to the control.

1. Introduction

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) occupying about more than 50% of water-soluble protein existing in leaves of higher plants consists of two subunits and the larger subunit is coded in plastid genome, and is synthesized in the plastid ribosome of stroma. This constitutes the entire structure in eight subunits in total by chaperone. Small subunits are coded in the nuclear genome, and are synthesized on cytoplasmic ribosome, constituting the entire structure in 8 subunits in total (Gatenby and Ellis, 1990). In general, rubisco like this has a high affinity with CO_2 for the carboxylation among dark reactions to proceed smoothly depending on low atmospheric concentration of CO_2 (Roh et al., 1996). In addition, the activation of rusbisco *in vivo* is catalyzed by rubisco

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activase which is a water-soluble chloroplast enzyme (Salvucci et al., 1985).

Rubisco activase expedites the dissociation of ribulose-1,5-bisphosphate (RuBP) by binding to deactivated rubisco-RuBP complex, thereby increases the activation of rubisco. ATP is required at this time of binding to rubisco. Rubisco activase similar to this started to be refined in chloroplasts of spinach, and is also found in higher plants and green algae (Wang and Portis, 1992).

Salicylic acid (SA) belongs to secondary metabolites found in plants, and serves as a plant growth regulator in phenolic compounds, which is biosynthesized through a phenylpropanoid pathway (Durner et al., 1997). SA is an elicitor to create resistive materials against plant pathogens, and contributes to systemic acquired resistance (Yalpani et al., 1993). In tobacco plants, SA induces the gene expression of pathogenesis-related, accumulating the Pathogenesis related (PR) proteins (Klessig and Malamy, 1994). SA also controls the biosynthesis of ethylene and K^+ absorption in plants (Leslie and Romani, 1986), stimulates the chemical parameters for interaction among organisms (Raskin, 1992), and activates nitrogen reduction (Jain and Stivastra, 2006). At the time of blooming of arisaema like a taro, it also works as an endogenous regulator for heat generation, and secretion of insect pheromones (Raskin et al., 1987). It also affects the closing of stomata, production of fruits and seed germination (Cutt and Klessig, 1992).

Plants on the globe are affected by biotic stresses like fungi, bacteria, and viruses, and by abiotic stresses like moisture, temperature, ions, and salts (Sticher et al., 1997). SA induces the protective action against loss due to environmental stresses like these (Horvath et al., 2007), and also induces the stress-resistance unique to the species (Kogel and Langen, 2005).

In general, plants are affected by salt stress, but cotton, barley, and spinach are known to show a relatively strong salt tolerance (Greenway and Munns, 1980). Salt stress is caused simply by occurrence of metabolic disorders due to deterioration of moisture content in the soil (Poljakoff-Mayber and Lerner, 1994) or due to excessive accumulation of Na^+ and Cl^- in plants (Flowers et al., 1977). Salt stress does damage to the photosynthesis of plants at a variety of levels, such as in plastids, gas exchange in stomata, structure and function of the thylakoid membrane, and the electron transport system (Sudhira and Murthy, 2004).

Currently, there are lots of reports stating that salt stress affects the moisture content (Binzel et al., 1985) and the content of proline, glycinebetaine in plants (Meloni et al., 2004), and that it also affects the activation of antioxidative enzymes like catalase, peroxidase and superoxide dismutase, nitrate reductase, and carbonic anhydrase (Mittler, 2002; Meloni et al., 2004; Yusuf et al., 2008).

Reports showed that SA improved the growth, photosynthesis, and content of chlorophyll affected by salt stress through researches on seedlings of maize (Khodary, 2004), barley (El-Tayeb, 2005), *Brassica juncea* (Yusuf et al., 2008) and wheat seedlings (Kang et al., 2012). In addition, there is a report stating that in seedlings of rice affected by salt stress, the activation of SA photosynthesis enzymes was induced, causing the inner level of SA to increase (Sawada et al., 2006). Likewise, the study with regard to growth and enzymes related to salt stress and SA in plants has been reported independently, and the study on rubisco and rubisco activase related to NaCl and SA is not yet known. Influence of SA on the effect of NaCl affecting growth, rubisco and rubisco activase was studied in tobacco plants.

2. Materials and methods

2.1. Chemicals and apparatus

Murashige and Skoog (MS) media used in this study was obtained from Duchefa Biochemie (Haarlem, Netherland). SA, sodium chloride, enzymes, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). For isolation and determination of content and activity of enzymes, a refrigerator centrifuge (Kontron T-324), a fraction collector (Bio-Rad 2110), a UV–VIS spectrophotometer (GeneQuant 100), and a ELISA microplate reader (Bio-Rad 680) were used.

2.2. Growth of tobacco plant

Seeds of tobacco (*Nicotiana tabacum* L.) were germinated and grown aseptically in a cell culture vessel containing MS agar medium (Murashige and Skoog, 1962). Shoots were cut into 3 cm segments and used as explants. 2 explants were placed on an induction MS medium of 8 groups, respectively. Explants were separated in 8 groups of control (not treated with SA and NaCl), SA, 50 mM NaCl, 100 mM NaCl, 150 mM NaCl, SA + 50 mM NaCl, SA + 100 mM NaCl, and SA + 150 mM NaCl. These explants were maintained on these media at 27 ± 2 °C under a 16-h light (800 μM/m^2/s PFD) and 8-h dark photoperiod (Roh et al., 1996). Plant growth of each experiment was measured by total fresh weight and leaves weight, and then compared. Fully expanded leaves from 5 week old mature plants were used as material for rubisco and rubisco activase studies. All experiments were independently triplicated.

2.3. Isolation of rubisco

Rubisco was isolated from tobacco leaves using a modified method of Wang et al. (1992). Leaf tissue was ground to a fine powder with a pre-cooled mortar and a pestle in liquid nitrogen and then extracted in the extraction buffer containing 50 mM 1,3-bis(tris(hydroxymethyl) methylamino)propane (BTP) (pH 7.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM benzamidine, 0.01 mM leupeptin, 1.5% PVPP and 3 mM mercaptobenzothiazole (MBT). The leaf slurry was filtered through four layers of cheesecloth and one layer of Miracloth. Filtered solution was centrifuged at 30,000*g* for 40 min. $(NH_4)_2SO_4$ powder was slowly added into the supernatant to 35% saturation and stirred for 30 min. The supernatant and pellet were collected by centrifugation at 8000*g* for 10 min. The supernatant contains rubisco and the resuspended pellet contains rubisco activase. The supernatant collected was brought to 55% saturation of $(NH_4)_2SO_4$ by the addition of powder. The pellet collected by centrifugation at 8000*g* for 10 min was resuspended in 5 ml of 20 mM BTP (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$ and 2 mM MBT (buffer A), and 50% PEG-10 K was added to a final concentration of 18%. The resulting precipitate was collected by centrifugation at 8000*g* for 10 min and resuspended in buffer