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Interplay between GST and nitric oxide in the early response of soybean (*Glycine max* L.) plants to salinity stress

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ABSTRACT

Glutathione-*s*-transferases (GSTs) and nitric oxide (NO) have both been implicated in the response of plants to salinity stress. However, their interplay and underlying mechanisms are relatively unknown. The present study attempts to provide new insight into the time course effects of NO application on GST biosynthesis regulation in *Glycine max* L. leaves under salt stress. A 150 μM concentration of sodium nitroprusside (SNP), a widely used NO donor, was sprayed on soybean seedlings for two days at 24 h intervals, followed by application of 200 mM NaCl. The relative water content (RWC), total chlorophyll content (CHL), stomatal conductance (g_s), ABA content, malondialdehyde (MDA), hydrogen peroxide content (H_2O_2), along with GST enzyme and isoenzyme activities and *GST1* and *GST4* transcript levels were determined at 0 h, 6 h and 12 h after stress imposition. The results indicated that salt treatment alone did not alter MDA, H_2O_2 or ABA content and stomatal conductance in soybean leaves, most likely due to short-term (6 h and 12 h) application, although lower RWC and CHL were recorded. SNP treatment alone increased ABA content and reduced stomatal conductance, but did not change RWC, CHL, MDA (except at 12 h) and H_2O_2. However, exogenous SNP application protected soybean leaves from salt stress by increasing RWC, CHL and ABA content, as well as by lowering stomatal conductance in order to maintain water balance. A significant increase in GST activity was recorded under salt stress alone at 6 h. Conversely, SNP application lowered GST activity in soybean leaves at 0 h and 12 h, while it increased at 6 h, supported by GST isoenzyme activities. Thus, it could be suggested that exogenous NO application induced GST activity in an ABA-dependent manner, while GST activity could also be induced by salt stress independent of ABA. In addition, SNP pre-treatment in salt-stressed seedlings lowered GST activity at 6 h and 12 h, in line with the GST isoenzyme expression profile. Finally, *GST1* and *GST4* transcript levels were significantly induced in both salt-stressed and SNP pre-treated and subsequently stressed samples at 6 h and 12 h, while a more variable regulation pattern was observed in plants treated only with SNP. Overall, our findings suggest that both NO and salt stress act as potent regulators of GST gene and enzyme expression through both ABA-dependent and independent pathways.

Introduction

Glutathione-*s*-transferases (GSTs) were discovered in animals in the 1960s as a result of their importance in the metabolism and detoxification of drugs (Wilce and Parker, 1994). In plants, it was determined first in 1970 that GSTs were effective for the detoxification of herbicides in maize (Dixon et al., 2010). GSTs are mostly cytoplasmic proteins, but microsomal plastidic, nuclear and apoplastic isoforms are also documented (Frova, 2003). They form large enzyme superfamilies subdivided into distinct classes based on several criteria (Sheehan et al., 2001). The two largest classes (phi and tau) are plant-specific, whereas the theta and zeta classes are also found in mammals and other organisms. In soybean, GH2/4, also known as Gmhsp26-A, was cloned independently as a heat shock protein (Czarnecka et al., 1988) and an auxin-induced protein (Hagen et al., 1984).

In recent years, it was determined that GSTs (EC 2.5.1.18) catalyze the nucleophilic attack of the thiol group of the tripeptide glutathione (GSH) to various electrophilic molecules. They can also protect against oxidative stress and play a role as non-enzymatic carrier proteins for ligands (Sheehan et al., 2001). GSTs can also catalyze GSH-dependent peroxidase reactions that scavenge toxic organic hydroperoxides and protect from oxidative damage. In addition to this role, plant GSTs play a pivotal role in response to

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biotic and abiotic stress, hormones and during growth and development (Dixon et al., 2002; Moons, 2005). They have been shown to bind hormones such as auxin and cytokinin (Zettl et al., 1994) and can be induced by a wide variety of phytohormones including ethylene, auxin, methyl jasmonate, salisylic acid and ABA (Wagner et al., 2002; Moons, 2003).

Over the past years, nitric oxide (NO) has emerged as a signal molecule in many important physiological processes in higher plants, mediating a variety of physiological functions including growth and development (Neill et al., 2003), biochemical interactions, and defense responses against plant abiotic and biotic stresses including salinity (Mata and Lamattina, 2001). Previous reports have suggested NO as a stress-inducing agent (Antoniou et al., 2014), whereas others have assigned it as a protective molecule (Tanou et al., 2012), functioning as an antioxidant by scavenging reactive oxygen species (ROS) (Laspina et al., 2005). Interestingly, the existence of a cross-talk between ROS and NO is well documented and has been recently reviewed by Molassiotis and Fotopoulos (2011).

Salt stress leads to secondary oxidative stress through an induction of ROS such as superoxide radicals, hydrogen peroxide and hydroxyl radicals, which unbalances the cellular redox state and causes lipid peroxidation (Shi et al., 2007). It is well known that NO plays an important role in this physiological process, directly protecting the cells from destruction through the formation of peroxynitrite ($ONOO^-$) as well as acting as a key signaling molecule to induce tolerance to salt stress (Molassiotis and Fotopoulos, 2011). Furthermore, NO has been found to increase ABA content and induce stomatal close for maintaining water balance (Xing et al., 2004). Many experiments using NO donors such as sodium nitroprusside (SNP) have shown that NO acts as a signal in inducing salt tolerance by increasing Na/K ratio. In previous reports, exogenous NO stimulated plasma membrane H^+-ATPase activity in plants under salt stress, thus likely improving ion transport levels (Liu et al., 2014), while Uchida et al. (2002) reported that NO induces ROS scavenging enzyme activities in rice under salt stress.

This report attempts to provide new insight into the early response effects of NO pre-treatment on the regulation of GST gene and enzyme levels in soybean seedlings grown under salt stress conditions, employing a wide array of physiological (relative water content (RWC), stomatal conductance, chlorophyll content), biochemical (malondialdehyde (MDA) content, hydrogen peroxide, ABA content) and molecular (qRT-PCR) analyses.

Materials and methods

Experimental design and plant materials

Soybean (*Glycine max* L. Merr.) seeds were obtained from a commercial provider (May, Bursa, TR). The seeds were sterilized in 5% hypochloride solution for 10 min, rinsed three times with distilled sterile water, and then sown in plastic trays (10 cm × 14 cm) filled with soil under dark conditions. After germination, seedlings were taken into a growth chamber at 25 °C with 16 h/8 h day/night photoperiod and light intensity of 500 $\mu mol\ m^{-2}\ s^{-1}$ with half-strength Hoagland solution for 3 weeks.

Seedlings were pre-treated with a 150 μM concentration of sodium nitroprusside (SNP) (nitric oxide (NO) donor) by spraying for two days at 24 h interval. On the third day, leaves that were exposed to SNP and controls were treated with 200 mM NaCl. In total, there were four different groups: SNP, SNP + NaCl, NaCl and Controls. After stress imposition, leaves were harvested at 0 h, 6 h and 12 h and stored at −80 °C.

Stomatal conductance

Stomatal conductance was measured at 0 h, 6 h and 12 h of salt stress treatment using a portable steady-state porometer according to the manufacturer's instructions (SC-1). The data were collected from six sample leaves per replicate.

Total chlorophyll content

The chlorophyll content of leaves was measured in accordance with the method specified by Lichtenthaler and Wellburn (1983). The pigments of 0.1 g fresh leaves were extracted in 80% (v/v) acetone.

Relative water content

Fresh weights (FW) of seedlings were initially determined. The samples were subsequently oven-dried at 70 °C for 72 h and then dry weights (DW) were determined. The relative water content (RWC) was calculated in accordance with Smart and Bingham (1974).

Malondialdehyde content

The level of lipid peroxidation, as an indicator of cellular damage, was measured in terms of malondialdehyde (MDA) content according to Heath and Packer (1968). Leaf samples (~0.1 g) were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant (0.5 ml) was mixed with 1.5 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid (TBA). The mixtures were heated at 95 °C for 30 min and then quickly cooled in an ice bath. The mixtures were centrifuged at 10,000 × *g* for 5 min at 4 °C and their absorbance was measured at 532 nm. The value of non-specific absorption at 600 nm was subtracted from the 532 nm reading. The MDA content was calculated using the Lambert-Beer law, with extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ and expressed as nmol MDA per g FW.

Hydrogen peroxide content

Leaf hydrogen peroxide content was assayed as described by Loreto and Velikova (2001). Frozen leaf material (~0.1 g) was homogenized on ice with 0.1% (w/v) TCA. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the assay mixture was read at 390 nm and the content of H_2O_2 was calculated based on a standard curve of known concentrations of H_2O_2.

ABA content

ABA content levels were determined in accordance with Flores et al. (2011), with the use of (UHPLC–MS/MS; AGILENT 6064, Belgium). Stock standard solutions of individual compounds (with concentrations ranging from 200 to 300 mg/L) were prepared by exact weighing of the powder and dissolution in methanol (HPLC-grade, Sigma, USA).

Total GST enzyme activity

Glutathione-*s*-transferases (GST) activity was determined by the method of Habig et al. (1974) by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using reduced glutathione