2004 BELTWIDE COTTON CONFERENCES, SAN ANTONIO, TX – JANUARY 5-9

NITRIC OXIDE PRODUCTION IN COTTON CALLUS

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Abstract

The production of nitric oxide (NO) in cotton callus tissue derived from Coker 312 has been examined in relation to salt stress and the activity of four antioxidant enzymes. A series of experiments was undertaken in which hermetically sealed jars containing cotton callus were linked together by glass tubing. The results of these experiments reveal the activity of a gaseous agent that up-regulates the activity of four antioxidant enzymes which allows salt-sensitive callus tissue to survive on media amended with 150 mM NaCl. A second series of experiments were carried out to measure the amount of NO produced by cotton callus under salt stress conditions. The results of these experiments show NO production to be higher in salt sensitive callus growing on media containing 0mM NaCl and also in salt tolerant callus acclimated to grow on media containing 150mM NaCl and subsequently transferred to media containing 250mM NaCl. NO production was reduced to about half of these values in salt sensitive callus transferred from media containing 0 mM NaCl to media containing 150mM NaCl.

Introduction

Nitric Oxide (NO), an inorganic free radical, is synthesized from the biological conversion of L-arginine to L-citrulline by Nitric Oxide Synthase (NOS). Once produced, NO can be converted into nitrate and nitrite in the presence of oxygen and water. It is soluble in lipids and in water and is therefore capable of diffusing freely in the cell (Ninnemann *et al*, 1996). The conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline assay indicates the production of nitric oxide as being involved in an adaptive mechanism of plants under NaCl induced physiological stress (Virgen *et al.* 2002). Additional evidence also suggests that NO is a novel effector in plant growth, development, and defense responses (Cueto *et al,* 1996). This study further examines the possible role of NO in the signal transduction pathway induced by salt stress. Experiments were conducted in which callus tissue was grown for 10-14 days in hermetically sealed jars containing growth media amended with either 0, 150 or 250 mM NaCl and the concentration of NO measured using an NO analyzer. The callus transferred onto this media had been grown on media containing either 0mM NaCl (salt sensitive callus), or 150mM NaCl (salt-tolerant callus). A second series of experiments was carried out to investigate the possible role of gasses in inducing the antioxidant enzyme response in cotton callus. Jars containing callus were linked together by hermetically sealed glass tubes.

Materials and Methods

Callus Tissue

Callus tissue was generated from sterile hypocotyls of Coker 312 by the method described by Trolinder and Goodin (1987). The callus tissue was maintained on T1 media containing MS salts (Murashige and Skoog, 1962) supplemented with Gamborg's vitamins (Gamborg, 1978), 0.75 mg/L $MgCl_2$, 0.1 mg/L 2,4-D, 0.1 mg/L kinetin, 2 g/L phytogel, and 30 g/L glucose, adjusted to a pH of 5.8 (Trolinder and Goodin, 1987). Media described as 150mM and 250mM media was amended with NaCl to a final concentration of 150mM or 250 respectivly. For studies with a NaCl-tolerant callus, a NaCl-tolerant cell line was selected as described by Gossett *et al.* (1996) and maintained on T1 media amended with 150 mM NaCl.

Measurement of Nitric Oxide

NO was measured using a Dasibi™ Environmental Corporation Model 2108 Chemiluminescent Nitrogen Oxides Analyzer. Measurements of NO were carried out two weeks after callus was transferred to the sealed jars.

Nitric Oxide Production

Approximately one gram of callus was transferred to 46mm diameter x 42mm high glass jars with a hermetically sealing lid. The media occupied approximately 18cm^3, leaving 58cm^3 of space in which the callus was incubated. For the nitric oxide experiments, two glass tubes (3mm I.D. x 12cm) were inserted into the lid and the join hermetically sealed with epoxy putty. Tygon tubing (4mm I.D.x 6mm) was placed onto the end of the glass tubes and the tygon tubing clamped off during the experiment. NO measurements were made by connecting one of the tygon tubes to the NO analyzer and releasing the clamps. For the linked jar experiments, glass tubing of the same type was used to connect the two jars.

Enzyme Extraction and Analysis

Samples were prepared for antioxidant enzyme analyses by the methods previously described by Anderson et al., (1992) as modified by Gossett et al., (1994b), Samples were prepared for the following analyses: ascorbate peroxidase (APX); catalase, and glutathione reductase (GR). Enzymes were extracted by homogenizing approximately one gram of frozen callus tissue, 0.25 gram of insoluble PVP, and one drop of antifoam A emulsion in 3.0 ml of ice cold PIPES buffer (pH 6.8). The PIPES buffer contained 6mM cysteine hydrochloride, 10mM D-isoascorbate, 1mM EDTA, 1% PVP-10, and 0.3% Triton X-10. The homogenate was centrifuged for 20 minutes at 4°C at 15,000 rpm. After centrifugation and the recording of volumes of supernatant, 1 ml of the supernatant was desalted and centrifuged through a 10 ml bed of Sephadex G-25 according to the method outlined by Anderson et al., (1992). The eluate volume was measured and immediately analyzed for catalase activity. The remaining eluate was stored in microfuge tubes at -70°C for subsequent analysis of ascorbate peroxidase (APX), glutathione reductase (GR), and peroxidase activities. Catalase activity was determined by measuring the disappearance of H_2O_2 at 240 nm in a reaction mixture containing 1.9 ml H_2O, 1.0m1 0.059 M H_2O_2 in potassium phosphate buffer (pH 7.0) with 0.1 ml extract (Beers and Sizer, 1952). APX activity was assayed at 265 nm by measuring the ascorbic acid-dependent reduction of H_2O_2 in a reaction mixture containing 100ul each of 1.5mM Na ascorbate, 1mM EDTA, 1mM H_2O_2, 500 µl of 166 mM HEPES-KOH (pH 7.0), and 100 µl of sample (Anderson et al, 1992). Peroxidase activity was determined at 675nm by measuring the H_2O_2-dependent oxidation of reduced 2,3',6–trichloroindophenol in a reaction mixture with 950ul of 17mM $Na_2S_2O_3$, 120mM H_2O_2, and 0.3mM 2,3', 6–trichloroindophenol in 40mM $NaPO_4$ buffer (pH 6.0), and 50µl of sample (Nickel and Cunningham, 1969). Glutathione reductase activity was measured at 340nm and was determined by monitoring glutathione–dependent oxidation of NADPH. The reaction mixture consisted of 950µl of 0.5 nM GSSG, 0.15mM NADPH, and 3mM $MgCl_2$ in 50mM Tris (pH 7.5) and 50µl of sample (Schaedle and Bassham, 1977). One unit of catalase and peroxidase activity was defined as the amount necessary to decompose 1 mM of substrate/min at 25°C. One unit of ascorbate peroxidase and glutathione reductase activity was defined as the amount of enzyme necessary to reduce 1µmole of substrate/min at 25°C (Gossett et al., 1994). All data were subjected to a one-way analysis of variance and significance was determined at a 95% confidence limit. Data points were derived from a mean of a minimum of three replicates.

Results and Discussion

When cotton callus is grown upon Murashige and Skoog media (Murashige and Skoog 1962) with no additional NaCl and is subsequently transferred to media containing 150mM NaCl the tissue dies within approximately two weeks. However the results of our experiments show that death of callus tissue exposed to 150mM NaCl did not occur when the jar in which it was growing was connected via a glass tube to a jar containing callus acclimated to grow on media amended with 150mM NaCl (*i.e.* NaCl-tolerant callus). In addition, the salt-sensitive callus showed a significant up-regulation of the activity of catalase, glutathione reductase, peroxidase and ascorbate peroxidase under these conditions (Table 1). Measurements of nitric oxide in unlinked jars showed that NO is produced by cotton callus. When salt-sensitive callus is grown on media containing 0mM NaCl, significantly more NO is detected than when salt-sensitive callus grown on media containing 0mM NaCl is transferred to media containing 150mM NaCl, a situation which leads to the death of callus tissue within two weeks. However, when salt-tolerant callus is transferred from media containing 150mM NaCl to media containing 250mM NaCl, NO is detected at similar levels to those recorded for salt sensitive callus that has not been exposed to NaCl (Figure 1). Our future experiments to further examine to the role of nitric oxide in plant stress physiology will include the use of NO and ethylene scrubbers placed between the glass jars.

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Table 1. Fresh weights and activity of antioxidant enzymes of salt-sensitive (SS) and salt-tolerant (ST) callus grown on media containing 0mM, 150mM or 250mM NaCl.

Sample	Fresh Weight	Catalase	Glutathione Reductase	Peroxidase	Ascorbate Peroxidase
0 callus (SS) control	6.45±0.90	41.5±3.8	32.1±1.6	173±6	478±15
0 callus (SS) on 150 mM NaCl media	1.39±0.77	-	-	-	-
150 callus (ST) control	7.69±0.56	38.1±3.2	51.6±1.7	324±2	890±35
0 callus (SS) on 0 media linked to 0 callus on 0 media	5.65±0.48	44.5±2.7	55.5±2.2	231±6	841±42
0 callus (SS) on 150mM NaCl media linked to 150mM NaCl callus on 150mM media	7.43±0.43*	147.3±7.5*	60.4±2.1*	237±3*	919±24*

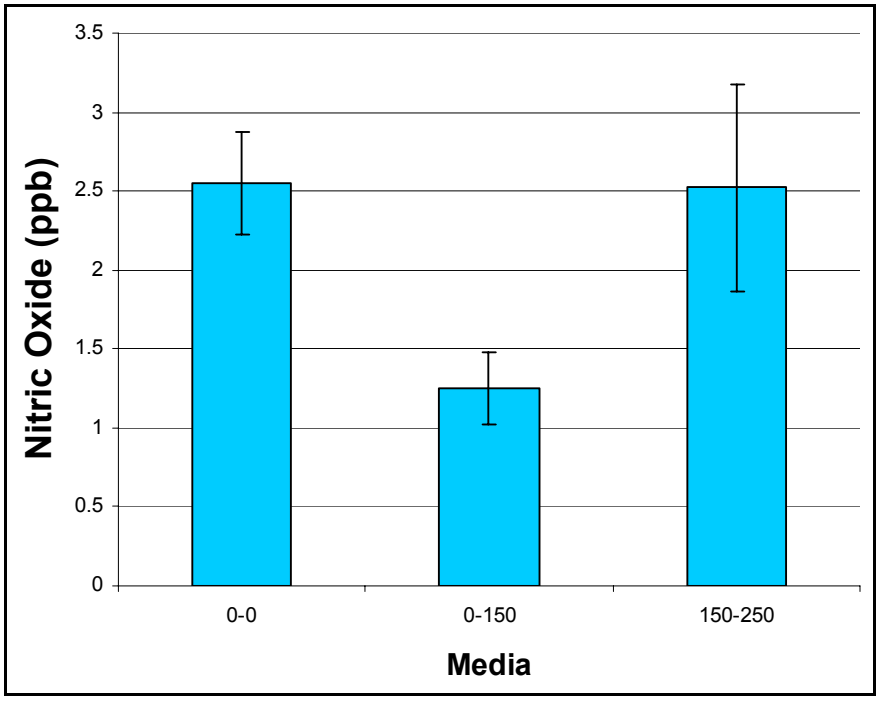

Figure 1. Nitric oxide deteced two weeks after transfer of salt sensitive callus from media amended with 0 NaCl to media amended with 0mM NaCl (0-0) and to media amended with 150mM NaCl (0-150), and salt tolerant callus transfered from 150mM media to 250 mM media.