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Research Paper

Ethylene triggers salt tolerance in maize genotypes by modulating polyamine catabolism enzymes associated with H_2O_2 production

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ABSTRACT

The current study was undertaken to investigate if there is a relationship between metabolisms of ethylene and polyamines in the processes of salinity acclimation of salt-tolerant and salt-sensitive maize genotypes. Biphasic ethylene production (at 5.5 and 12.5 h) was registered only in salt-sensitive plants during NaCl exposure. In the salt-tolerant genotype, the unique ethylene peak at 5.5 h was closely related to increased polyamine accumulation (a polyamine-dependent H_2O_2 signalling process), whereas the same did not occur in the salt-sensitive genotype. The absence of H_2O_2 signalling at 5.5 h in the salt-sensitive genotype was related to a burst in ethylene production at 12.5 h, known as 'stress ethylene', as well as a concomitant decrease in total polyamine content by salinity. The lack of stress ethylene synthesis in the salt-tolerant genotype was attributed to down-regulation of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) activity and *ZmACO5b* gene expression. Our findings suggest that ethylene is intimately involved in salt stress acclimation through activation of a complex pathway of signalling by H_2O_2 that is polyamine catabolism-dependent.

1. Introduction

Separately, ethylene and polyamines can act as regulators during plant growth and development as well as inducers of resistance against environmental stresses (Gupta et al., 2013; Lasanajak et al., 2014; Müller and Munné-Bosch, 2015). Ethylene and polyamines are biochemically related, since they share a common precursor in biosynthetic pathways, suggesting that alteration in ethylene production can affect polyamine homeostasis (Grzesiak et al., 2013).

Ethylene (C_2H_4) is a gaseous hormone that regulates diverse aspects of plant growth and development; it is considered a senescence hormone (Van de Poel and Van Der Straeten, 2014; Van de Poel et al., 2015). Recent evidence has shown that ethylene plays an important role in plant responses to abiotic stress, such as oxidative, ozone, salt and drought stress (Moeder et al., 2002; Wi et al., 2010; Habben et al., 2014). Contribution of ethylene to salt acclimation processes can vary with respect to type of response, including enhanced ethylene production and/or improved expression of ethylene receptors (Wu et al., 2008; Jiang et al., 2013; Zhai et al., 2013; Shi et al., 2015). However, how ethylene signalling and production are involved in the plant response to salinity is poorly understood.

Deletion of the *soil salinity tolerant1* (*sst1*) allele improved soil salinity tolerance of *Arabidopsis thaliana* via loss of ethylene overproducer1 (ETO1, whose normal function is to reduce ethylene production by degrading ethylene biosynthetic enzymes) function and enhancement of ethylene production (Jiang et al., 2013). In a different way, overexpression of the ethylene response factor (ERF) protein in tobacco transcriptionally regulates several genes responsive to osmotic and oxidative stress by a reactive oxygen species (ROS)-mediated regulatory pathway, alleviating harmful salt effects on photosynthetic carbon

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; ADC, arginine decarboxylase; DAO, diamine oxidase; MACC, 1-malonylaminocyclopropane-1-carboxylic acid; PAO, polyamine oxidase; Put, putrescine; ROS, reactive oxygen species; Spd, spermidine; Spm, spermine

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assimilation/metabolism (Wu et al., 2008). Also, tobacco plants over-expressing ERF7 from *Glycine max* displayed enhanced salt tolerance by low oxidative damage and increased levels of both chlorophyll and soluble carbohydrates (Zhai et al., 2013).

Although increased ethylene synthesis has been cited as a determinant for salt tolerance, other findings have shown that the reduction in ethylene production can display a positive role in salt tolerance of plants. Transgenic tobacco plants with poor ethylene biosynthesis showed elevated salt tolerance, which seems to occur through decreased ROS accumulation by up-regulating gene expression and activity of ROS-detoxifying enzymes (Wi et al., 2010). In the same way, an *A. thaliana ACS7*-knockdown mutant displayed reduced ethylene synthesis and improved salinity tolerance (Dong et al., 2011).

Some reports have suggested that ethylene and H_2O_2 may act as synergistically and self-amplifying signalling molecules in feed-forward loop regulation. Salt-tolerant *A. thaliana* callus and plants exhibited a close relationship between ethylene and H_2O_2 by inducing a cyanide-resistant alternative pathway (alternative oxidase), highlighting that ethylene could act mutually with H_2O_2 (Wang et al., 2010). In concordance with *A. thaliana* mutants, ethylene alleviated the harmful salt effects on germination by modulating the endogenous concentration of H_2O_2 in germinating seeds (Lin et al., 2012).

Polyamine catabolism-generated H_2O_2 also has been proposed to play an important role in plant defence against abiotic stress, functioning as a signalling molecule in crosstalk regulation (Campestre et al., 2011; Guo et al., 2014). In polyamine metabolism, H_2O_2 is originated from a reaction catalysed by diamine oxidase (DAO) and polyamine oxidase (PAO) localized mainly in the apoplast (Groppa and Benavides, 2008; Alcázar et al., 2010; Tavladoraki et al., 2012). In addition, others studies has shown that PAO can be found in vacuole, cytoplasm and peroxisomes (Cervelli et al., 2004; Tavladoraki et al., 2006; Moschou et al., 2008c). *Nicotiana tabacum* plants overexpressing the *pao* gene from *Zea mays* displayed a decreased polyamine content associated with H_2O_2 generation, which, in turn, up-regulated the antioxidant genes necessary for cell survival/adaptation (Moschou et al., 2008a). Nevertheless, the same transgenic plants showed ROS accumulation and oxidative damage higher than those of wild-type plants when subjected to abiotic stress. This response was attributed to polyamine secretion from cells to the apoplast and, after catabolism by PAO, higher ROS levels leaded to programmed cell death. Yet, in salt-stressed tobacco plants over-expressing (*S-pao*) or under-expressing (*A-pao*) PAO, Moschou et al. (2008b) reported an improved abundance of all transcripts involved in polyamine biosynthetic pathway in *S-pao* plants and a delay in up-regulation of the respective genes in *A-pao* plants. The authors suggested that the apoplastic ROS generation may induce either the expression of stress-responsive genes or programmed cell death syndrome, depending on the specific threshold.

Polyamine metabolism does not only increase H_2O_2 production to signalling levels but also provides a polyamine pool for defence purposes. Gong et al. (2014) demonstrated that tomato plants over-expressing S-adenosyl-L-methionine synthetase (*SlSAMS1*) displayed enhanced tolerance to alkali stress due to spermidine (Spd) and spermine (Spm) accumulation, ROS scavenging and greater photosynthetic performance compared to wild-type lines. In a later study, the authors found that the PAO-originated H_2O_2 is a downstream signal of *SlSAMS1*-conferred alkali stress tolerance (Gong et al., 2016).

Despite such clear demonstration of a crucial role of polyamines, ethylene and H_2O_2 in plant growth, development and stress responses, crosstalk between polyamines and ethylene under salt stress as well as ethylene-mediated signalling in salinity tolerance are poorly understood. In this study, we explored the ethylene and polyamine metabolisms in two salt-contrasting maize genotypes to investigate their relationship in salinity tolerance of plants. We found that the ethylene production acts as a signal of salt tolerance/sensitivity in maize plants. Our data point to a role of polyamine catabolism-dependent H_2O_2 as a link between ethylene and polyamine metabolism in plant salt responses.

2. Materials and methods

2.1. Plant material and growth conditions

Zea mays L. seeds of salt-sensitive (BR5011) and salt-tolerant (BR5033) genotypes (Azevedo Neto et al., 2004) were germinated in distilled water-moistened filter paper in a growth chamber with a 12-h photoperiod (25 ± 3 °C night/day) for 7 days. Thereafter, ten uniform seedlings were transferred to 10-L trays containing half-strength Hoagland nutrient solutions (Hoagland and Arnon, 1950). After thirteen days, the plants were transferred to 5-L plastic pots (one plant per pot) and subjected to saline treatment with NaCl at 0 (control) and 80 mM NaCl (salt stress). Fresh leaf and root (for ethylene measurement) tissues were harvested for analysis in different times (from 0 to 72 h) depending on assay. The experiment was carried out in a greenhouse and the environmental conditions were as follows: a mean air temperature of 28 ± 3 °C, a mean air relative humidity of 65 ± 5%, an average midday photosynthetic photon flux density of 1200 μmol m^{-2} s^{-1}.

2.2. Ethylene measurement

Ethylene production was determined by gas chromatography coupled to mass spectrometer (GCMS) (QP2010, Shimadzu, Tokyo, Japan). Leaf or root samples of control and salt-treated plants were enclosed in vials containing water-moistened filter papers for 1 h. Thereafter, 1.0 mL of headspace gas was injected in split mode (1:500), using helium as a carrier gas at a constant flow of 0.86 mL min^{-1} on an RTX-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Restec, Bellefonte, USA). The oven temperature was overall constant at 60 °C for 3 min, and the temperatures of the injector, interface and ion source were, respectively, 150, 200 and 200 °C. Each sample was run at least three times by GCMS, and the ethylene concentrations were quantified with reference to a standard curve.

2.3. Ethylene metabolites analyses

Total and free 1-aminocyclopropane-1-carboxylic acid (ACC) contents were extracted from fresh leaves and quantified by GCMS according to the Lizada and Yang (1979) procedure, which was recently updated by Bulens et al. (2011). The amount of conjugated ACC (MACC) was calculated by subtracting the amount of free ACC from that of the total ACC.

2.4. Ethylene biosynthesis enzyme activities

ACC synthase (ACS) and ACC oxidase (ACO) activities in leaves were measured using GCMS as described by Bulens et al. (2011). Incubation time, optimum temperature, optimum pH and substrate concentration were optimised to provide linear rates. Enzyme activities were expressed as nanomoles of ethylene per hour per milligramme of protein. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.5. Measurement of free, conjugated and bound polyamines

For extraction of polyamines, samples from fresh leaves were homogenised in 5% (v/v) perchloric acid (PCA) at 4 °C on an orbital shaker for 1 h and then centrifuged at 27,000 × *g* at 4 °C for 30 min. The same procedure was repeated twice. Free polyamines were derived with benzoyl chloride and directly measured in the homogenate by high performance liquid chromatography (HPLC) (Shimadzu, Tokyo, Japan). In parallel, the homogenate and pellet were individually hydrolysed with 12 M hydrochloric acid (HCl) and heated at 110 °C for 18 h in