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Research article

Nitrosative responses in citrus plants exposed to six abiotic stress conditions

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ABSTRACT

Nitrosative status has emerged as a key component in plant response to abiotic stress; however, knowledge on its regulation by different environmental conditions remains unclear. The current study focused on nitrosative responses in citrus plants exposed to various abiotic stresses, including continuous light, continuous dark, heat, cold, drought and salinity. Morphological observations and physiological analysis showed that abiotic stress treatments were sensed by citrus plants. Furthermore, it was revealed that nitrosative networks are activated by environmental stress factors in citrus leaves as evidenced by increased nitrite (NO) content along with the release of NO and superoxide anion ($O_2^{\cdot-}$) in the vascular tissues. The expression of genes potentially involved in NO production, such as *NR*, *AOX*, *NADHox*, *NADHde*, *PAO* and *DAO*, was affected by the abiotic stress treatments demonstrating that NO-derived nitrosative responses could be regulated by various pathways. In addition, *S*-nitrosoglutathione reductase (GSNOR) and nitrate reductase (NR) gene expression and enzymatic activity displayed significant changes in response to adverse environmental conditions, particularly cold stress. Peroxynitrite ($ONOO^-$) scavenging ability of citrus plants was elicited by continuous light, dark or drought but was suppressed by salinity. In contrast, nitration levels were elevated by salinity and suppressed by continuous light or dark. Finally, *S*-nitrosylation patterns were enhanced by heat, cold or drought but were suppressed by dark or salinity. These results suggest that the nitrosative response of citrus plants is differentially regulated depending on the stress type and underscore the importance of nitrosative status in plant stress physiology.

1. Introduction

Climatic factors, such as extreme temperatures (heat, cold), drought and salinity are major abiotic environmental stressors that limit plant growth and development, and consequently agronomical yield [1]. At cellular level, adverse abiotic conditions disrupt cellular homeostasis and induce excess levels of reactive oxygen species (ROS) leading to oxidative stress [2]. More recently, the term nitrosative stress was introduced to describe the excess of reactive nitrogen species (RNS) in plants, particularly under adverse environmental conditions [3]. Among the RNS molecules, nitric oxide (NO) possesses a key role in controlling a number of physiological functions and signaling pathways [4]. There are several mechanisms via which NO is produced in plants [5,6], and once produced, NO readily reacts with various targets, such as thiols and the metallic centers of proteins [7]. In the group of RNS, apart from NO, other NO-related molecules, such as *S*-nitrosothiols, *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^-$) are known to play key signaling roles in plant cells [3]. GSNO is an *S*-nitrosothiol which is formed by the reaction of NO with reduced glutathione and is thought to function as a mobile reservoir of bioactive NO in plants [8]. Peroxynitrite is formed via the reaction of NO with the superoxide anion ($O_2^{\cdot-}$), with this scavenging effect of $O_2^{\cdot-}$ by NO being considered its antioxidant *modus operandi* [9].

Abbreviations: AOX, alternative oxidase; BSA, bovine serum albumin; CLSM, confocal laser scanning microscope; c-PTIO, 2-(4-carboxyl-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-2DA, 4,5-diaminofluorescin diacetate; DAO, diamine oxidase; DHE, dihydroethidium; DTPA, di-ethylene-triamine-penta-acetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GAE, gallic acid equivalence; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; NADH, nicotinamide adenine dinucleotide reduced form; NADHde, NADH dehydrogenase; NADHox, NADH oxidase; NC, nicked circular forms of DNA; NiR, nitrite reductase; NL, nicked linear forms of DNA; NO, nitric oxide; NR, nitrate reductase; $ONOO^-$, peroxynitrite; PAO, polyamine oxidase; PMSF, phenylmethanesulfonyl fluoride; PTMs, post-translational protein modifications; RNS, reactive nitrogen species; ROS, reactive oxygen species; SC, closed circular double stranded supercoiled DNA; SNOs, *S*-nitrosothiols; SOD, superoxide dismutase.

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Overwhelming evidence suggests that RNS signaling is largely governed by post-translational protein modifications (PTMs), including tyrosine (Tyr) nitration and *S*-nitrosylation [4]. Protein nitration results from a chemical reaction, in which $ONOO^-$ adds a nitro-group to the *ortho* position of the aromatic ring of Tyr residues [4]. *S*-Nitrosylation refers to the covalent attachment of a NO moiety to the thiol side chain of cysteine (Cys) [10]. In plants, these RNS-derived PTMs appear to be physiologically relevant during stressful conditions [11]; however, its regulation by various environmental factors is not well understood.

In recent years, oxidative stress responses have been described as a major physiological switch during abiotic stress conditions [2]; however, knowledge regarding the RNS-derived impact on abiotic stress physiology is still limited [12]. Recently, we showed that citrus plants exhibited remarkable nitrosative changes in response to salinity stress [11]. Therefore, the current study investigated the impact of various stressful environmental conditions (continuous light, continuous dark, heat, cold, drought, salinity) on the nitrosative status of citrus plants. The results document the function of a robust nitrosative network in stressed citrus plants and elucidate specific nitrosative hallmarks involved in plant responses to different abiotic stress conditions.

2. Results

2.1. Abiotic stress conditions impose nitrosative syndromes in citrus plants

In the present study, specific nitrosative responses were analyzed in citrus plants exposed to six different abiotic stress conditions. As an initial step toward achieving this goal, it was necessary to investigate if abiotic stress treatments were effectively imposed to citrus plants. In this context, phenotypical and physiological features were evaluated. Dark treatment and especially light, heat and drought led to a significant decrease in chlorophyll content index (CCI) compared with unstressed control plants, whereas citrus exposed to cold and salinity showed a further decrease in CCI level (Fig. 1B). Light and dark treatments increased electrolyte leakage compared with control; however, drought resulted in further increased electrolyte leakage, whereas an additional increase was observed by cold and especially by heat (Fig. 1C). Meanwhile, salt-treated citrus evidently displayed the highest level of electrolyte leakage level compared with the rest of the abiotic treatments (Fig. 1C). Fig. 1A shows the effects of abiotic treatments on plant phenotype as evidenced by typical visual injury symptoms of the corresponding abiotic stress imposed.

A significant increase in nitrite (NO) content via Griess reaction was especially observed under heat and continuous light, as well as under continuous dark, drought and salinity treatments (Fig. 1). On the contrary, cold treatment did not cause significant differences in nitrite (NO) content (Fig. 1). The distribution of NO in cross-sections of primary veins using DAF-2DA fluorescence and subsequent CLSM analysis showed that the spatial pattern of NO accumulation was mainly localized in the vascular tissue (xylem and phloem) (Fig. 2C). By attributing a value of 1 to the relative fluorescence of the control plants, it was evidenced that NO fluorescence was increased in response to all abiotic stress treatments (Fig. 2A), indicating that citrus plants could undergo NO-originated nitrosative syndromes under various environmental stimuli. Similarly, the impact of abiotic treatments on $O_2^{\bullet-}$ production was evaluated in leaf tissues by the fluorescent probe DHE. A red fluorescence (in web version) attributable to $O_2^{\bullet-}$ accumulation was also detected in vascular tissue (Fig. 2D). Meanwhile, treatments with continuous light, continuous dark, heat, cold and salinity induced $O_2^{\bullet-}$ production (Fig. 2B). As a negative control (NC), DAF-2DA or DHE fluorescence was reduced by c-PTIO (Fig. 2C) or by SOD (Fig. 2D), suggesting that these fluorescent probes exert their effect through the release of NO and $O_2^{\bullet-}$, respectively.

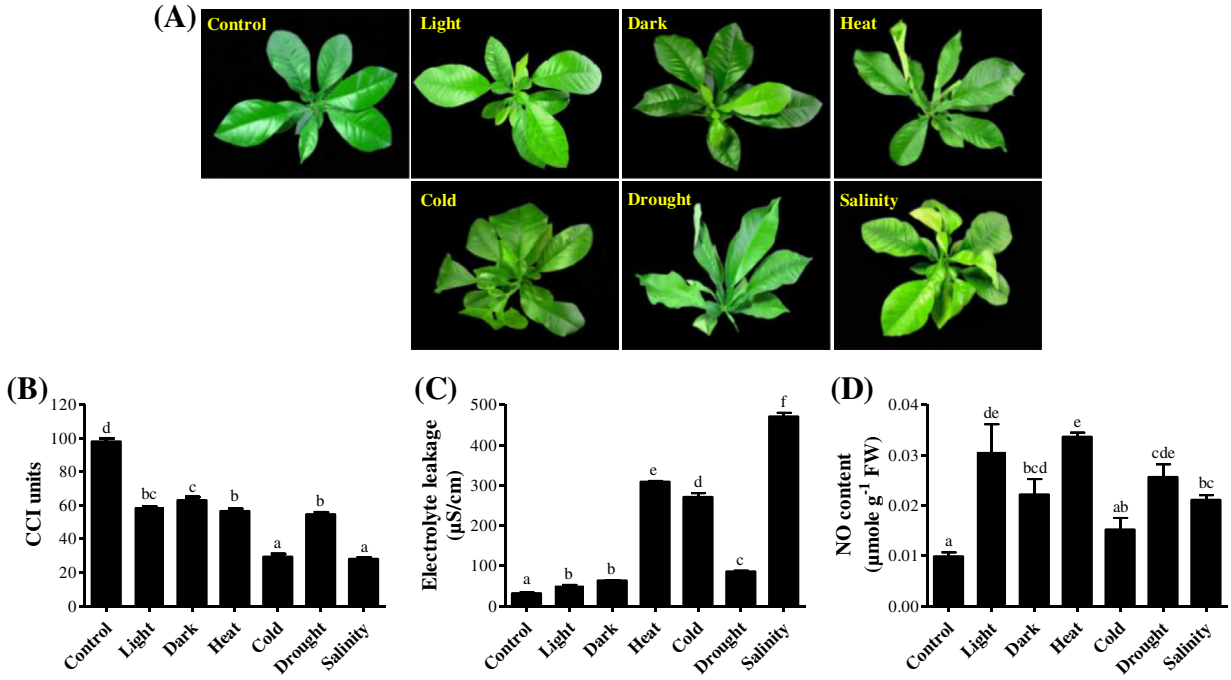

Fig. 1. Phenotypes of citrus plants subjected to continuous light, continuous dark, heat, cold, drought and salinity (A). Chlorophyll content index (CCI) (B), electrolyte leakage (C) and nitrite (NO) (D) content in leaves of citrus exposed to abiotic stress treatments. Bars marked by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$). Results are means ± SE from six (B and C) or three replications (D).