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Tipburn in salt-affected lettuce (*Lactuca sativa* L.) plants results from local oxidative stress

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ABSTRACT

Tipburn in lettuce is a physiological disorder expressed as a necrosis in the margins of young developing leaves and is commonly observed under saline conditions. Tipburn is usually attributed to Ca^{2+} deficiencies, and there has very limited research on other mechanisms that may contribute to tipburn development. This work examines whether symptoms are mediated by increased reactive oxygen species (ROS) production.

Two butter lettuce (*Lactuca sativa* L.) varieties, Sunstar (Su) and Pontina (Po), with contrasting tipburn susceptibility were grown in hydroponics with low Ca^{2+} (0.5 mM), and with or without 50 mM NaCl. Tipburn symptoms were observed only in Su, and only in the saline treatment. Tipburn incidence in response to topical treatments with Ca^{2+} scavengers, Ca^{2+} transport inhibitors, and antioxidants was assessed. All treatments were applied before symptom expression, and evaluated later, when symptoms were expected to occur. Superoxide presence in tissues was determined with nitro blue tetrazolium (NBT) and oxidative damage as malondialdehyde (MDA) content. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities were assayed.

Under control and saline conditions, tipburn could be induced in both varieties by topical treatments with a Ca^{2+} scavenger (EGTA) and Ca^{2+} transport inhibitors (verapamil, $LaCl_3$) and reduced by supplying Ca^{2+} along with a ionophore (A 23187). Tipburn symptoms were associated with locally produced ROS. $O_2^{\bullet-}$ and oxidative damage significantly increased in leaf margins before symptom expression, while topical antioxidant applications (Tiron, DPI) reduced symptoms in treated leaves, but not in the rest of the plant. Antioxidant enzyme activity was higher in Po, and increased more in response to EGTA treatments, and may contribute to mitigating oxidative damage and tipburn expression in this variety.

Introduction

Tipburn in lettuce (*Lactuca sativa* L.) is a significant source of economic losses. It is a physiological disorder expressed as a necrosis in the margins of young developing leaves and it is usually attributed to Ca^{2+} deficiencies (Collier and Tibbitts, 1982; Saure, 1998). Calcium is a xylem-mobile element, and deficiency symptoms are observed in young expanding leaves in leaf vegetables such as lettuce, in enclosed tissues in head-forming vegetables and in celery, and in developing fruits depending on phloem rather than xylem water supply (White and Broadley, 2003). Localized Ca^{2+} deficiencies in leaf blades can cause tipburn in lettuce (Barta and Tibbitts, 2000) and blossom end rot (BER) in fruits of tomato and pepper (Ho and White, 2005).

Calcium is an essential plant macronutrient with key structural and signaling roles. Calcium ions act as osmotic agents within vacuoles, as a membrane stabilizing elements, as strengthening agents in cell walls; and as secondary messengers for a multitude of signals, in the regulation of enzyme synthesis (Gilliham et al., 2011) and is required for normal cell growth. Nevertheless, and in contrast to observations that sustain a link between vigorous growth and tipburn incidence (White and Broadley, 2003), tipburn and BER (Adams and Ho, 1992) are also promoted under adverse growing conditions such as salinity or water stress (Saure, 1998). Calcium imbalance is a common consequence of substrate salinity (Lauchli, 1990). Salinity restricts water uptake and transpiration and was

Abbreviations: APX, ascorbate peroxidase; BER, blossom end rot; CAT, catalase; DPI, diphenyleneiodonium; EGTA, ethylene glycol-bis-(2-aminoethyl)-N,N′,N′-tetraacetic acid; MDA, malondialdehyde; NBT, nitro blue tetrazolium; PI, plastochron index; Po, Pontina; ROS, reactive oxygen species; SOD, superoxide dismutase; Su, Sunstar; TCA, trichloroacetic acid.

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shown to reduce Ca^{2+} uptake and transport to young lettuce leaves (Lazof and Bernstein, 1999), which may partially explain tipburn stimulation by salinity.

In some cases, no relation between improvement in leaf Ca^{2+} and decrease in tipburn incidence has been observed (Cresswell, 1991; Saure, 1998), yet there has very limited research on other mechanisms contributing to tipburn development. BER developing in fruits of pepper and tomato grown under saline conditions has been recently associated with the production of reactive oxygen species (ROS) in the affected zone (Aktas et al., 2005; Ho and White, 2005). This is a novel view in tipburn etiology and it has not been investigated before whether tipburn in lettuce is also related to ROS.

ROS are highly reactive oxygen derivatives and include singlet oxygen, superoxide radical, hydrogen peroxide, and hydroxyl radical. ROS production generally increases under biotic and abiotic stress conditions, as reviewed by Miller et al. (2010). On the other hand, controlled ROS production appears to be a general characteristic of expanding plant cells and organs (Rodríguez et al., 2002). While excess ROS exert adverse effects (oxidative damage) stemming from their interaction with macromolecules: lipids, proteins, nucleic acids and carbohydrates, low ROS levels participate in signaling events (Dat et al., 2000; Mittler et al., 2011) that regulate ion channel activity and gene expression.

ROS production occurs in almost all cell compartments (Mittler et al., 2004). ROS balance is controlled by diverse antioxidant mechanisms (Dat et al., 2000), including enzymatic and non-enzymatic components (Foyer and Noctor, 2005) acting also in various cell compartments. Antioxidant activity during hypersensitive response has been characterized in lettuce (Bestwick et al., 2001). Salt stress commonly results in ROS production (Hernández et al., 2001) and activation of the antioxidant system (Mhadhbi et al., 2011). The contribution of increased antioxidant activity to salt tolerance has been repeatedly reported in the literature (Munns and Tester, 2008), but the association between antioxidant activity and tipburn susceptibility has not been evaluated.

In this work we examined whether reduced apoplastic Ca^{2+} stimulates tipburn expression in lettuce leaves, and whether symptoms are mediated by increased ROS production. To this end, tipburn incidence in response to topical treatments with Ca^{2+} scavengers, Ca^{2+} transport inhibitors and antioxidants were compared in two lettuce varieties differing in tipburn susceptibility, grown under saline conditions. Antioxidant enzyme activity was measured in leaf zones where tipburn symptoms were later expected to either develop or not.

Materials and methods

Plant material and growth conditions

This study was performed in butter lettuce (*Lactuca sativa* L.) genotypes Sunstar (Su) and Pontina (Po). In preliminary experiments (Carassay et al., unpublished) these varieties had shown contrasting responses to tipburn predisposing conditions: Su was susceptible to this physiogenic disease and Po was not.

Seeds were sown in germination trays according to ISTA recommendations (ISTA, 1996) and kept at in a growth chamber at 20 °C under a 12 h photoperiod. Thirty days later, seedlings were transplanted to 7-L black trays containing modified Hoagland solution (Fernández and Johnston, 1986) with a reduced Ca^{2+} level (0.5 mM), which, according to the preliminary experiments stimulated symptom expression. There were 8 plants per tray and the nutrient solution was changed weekly. Electrical conductivity, pH and temperature were monitored to insure constant conditions, light intensity was 350 $\mu mol\, m^{-2}\, s^{-1}$ with a 16 h photoperiod.

Table 1
Topical treatments to modify Ca^{2+} concentration and oxidative stress.

Topical treatment	Effect
EGTA (50, 25 and 10 mM, pH 7.8)	Chelator of apoplastic Ca^{2+}
Verapamil (10 mM)	Ca^{2+} ion blocker
$LaCl_3$ (10 mM)	Ca^{2+} ion blocker
Ionophore (A 23187) (5 μM + Ca^{2+} 1 mM)	Promotes Ca^{2+} entry through plant membranes
Tiron (50 mM)	Antioxidant, ROS scavenger
DPI (50 μM)	Inhibitor of plasma membrane NADPH oxidase and peroxidases

Temperature was initially set at 22 °C, daytime temperature was raised to 25 °C at the onset of salt treatments and gradually increased till the end of the trial when temperatures were 36 °C and 27 °C by day and night, respectively. This temperature change scheme was previously observed to increase symptom expression. Seven days after transplantation, salt treatments were given to half of the plants by providing NaCl at gradually increasing concentrations (10, 25 and 50 mM) with the nutrient solution. Since salinity imposes growth restrictions, plants were compared at similar development stages by using the plastochron index (PI) developed by Erickson and Michelini (1957), and under these experimental conditions, tipburn expression under salinity occurred at PI 22–23.5, according to preliminary experiments.

Tipburn assessment

Combined tipburn incidence and severity (tipburn index, TI) was evaluated according to Frantz et al. (2004) as TI: $\{[(S\cdot 5)+(M\cdot 3)+(L\cdot 1)]\cdot 100\}/P\cdot 5$, where S = number of plants with severe tipburn; M is the number of plants with medium tipburn; L is the number of plants with light tipburn and P is the total number of plants. The proportion of leaf area affected by tipburn was estimated in digital photographs by measuring necrotic areas and total leaf area with the image processing software ImageJ (http://rsbweb.nih.gov/ij).

Modulation of tipburn expression

The purpose of the following treatments was to assess the association between tipburn symptoms, local Ca^{2+} concentrations and oxidative stress. Briefly, Ca^{2+} concentration was locally modified by painting the edge of leaves with a Ca^{2+} chelator (EGTA), Ca^{2+} channel blockers $LaCl_3$ and verapamil, and by adding 1 mM Ca^{2+} along with a Ca^{2+} ionophore (A 23187). Oxidative stress development was modified by supplying Tiron (sodium 4,5-dihydroxybenzene-1,3-disulfonate), a ROS scavenger and diphenyleneiodonium (DPI). DPI is a suicide inhibitor of the phagocytic NADPH oxidase and also an inhibitor of NADH-dependent H_2O_2 production by peroxidase (Frahry and Schopfer, 1998) and has been used to reduce ROS production in plant systems. Treatments are detailed in Table 1.

All treatments were applied to leaves 17 or 18, the first ones to show symptoms in previous assays. Leaves were treated at PI 20, before symptom expression, and tipburn was evaluated at expected occurrence PI.

Oxidative stress determination and superoxide localization in leaf tissues

Malondialdehyde (MDA) content is taken as a parameter for oxidative damage in plant tissues. MDA was determined in leaf 17, at PI 21.5, according to the method described by Heath and Parker (1968). Briefly, 200 mg of tissue from the leaf margins were