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Salt-induced delay in cotyledonary globulin mobilization is abolished by induction of proteases and leaf growth sink strength at late seedling establishment in cashew

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ABSTRACT

Seedling establishment in saline conditions is crucial for plant survival and productivity. This study was performed to elucidate the biochemical and physiological mechanisms involved with the recovery and establishment of cashew seedlings subjected to salinity. The changes in the Na+ levels and K/Na ratios, associated with relative water content, indicated that osmotic effects were more important than salt toxicity in the inhibition of seedling growth and cotyledonary protein mobilization. Salinity (50 mM NaCl) induced a strong delay in protein breakdown and amino acid accumulation in cotyledons, and this effect was closely related to azocaseinolytic and protease activities. In parallel, proline and free amino acids accumulated in the leaves whereas the protein content decreased. Assays with specific inhibitors indicated that the most important proteases in cotyledons were of serine, cysteine and aspartic types. Proteomic analysis revealed that most of the cashew reserve proteins are 11S globulin-type and that these proteins were similarly degraded under salinity. In the late establishment phase, the salt-treated seedlings displayed an unexpected recovery in terms of leaf growth and N mobilization from cotyledon to leaves. This recovery coordinately involved a great leaf expansion, decreased amino acid content and increased protein synthesis in leaves. This response occurred in parallel with a prominent induction in the cotyledon proteolytic activity. Altogether, these data suggest that a source–sink mechanism involving leaf growth and protein synthesis may have acted as an important sink for reserve mobilization contributing to the seedling establishment under salinity. The amino acids that accumulated in the leaves may have exerted negative feedback to act as a signal for the induction of protease activity in the cotyledon. Overall, these mechanisms employed by cashew seedlings may be part of an adaptive process for the efficient rescue of cotyledonary proteins, as the cashew species originates from an environment with N-poor soil and high salinity.

Introduction

Seedling establishment is a physiological process that is essential to plant survival, especially under adverse conditions such as salinity and low availability of mineral nutrients in soils. Under these conditions, germination and cotyledonary reserve mobilization are delayed, and seedling growth is strongly diminished (Voigt et al., 2009). Salinity may disturb these processes by osmotic and ionic effects, but the seed water absorption affected by these processes is crucial for germination (Wierzbicka and Obidzińska, 1998; Thomas et al., 2010; Marques et al., 2013). The ionic or osmotic effects of salinity on germination and reserve mobilization are

Abbreviations: 2-DE, two dimensional electrophoresis; DAS, days after sowing; E-64, 10 μM trans-epoxysuccinyl-L-leucylamide (4-guanidine) butane; ESI, electrospray ionization; PEPS, pepstatin; PMSF, phenylmethylsulfonyl fluoride; Q-TOF, quadrupole time-of-flight; RWC, relative water content; SSP, seed storage protein; UA, unity of activity; Ψs, osmotic potential.

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dependent on the salt concentrations, exposure time and seedling sensitivity (Maia et al., 2010). Although genetic and hormonal factors largely control the mechanisms of germination, seedling establishment is substantially affected by adverse environmental conditions (Thomas et al., 2010). The biochemical mechanisms involved with the salt effects on protein mobilization during seedling establishment are still not well known (Ashraf et al., 2003; Nieves et al., 2011; Shahzad et al., 2012).

Seed reserve proteins are essential for seedling establishment, and their mobilization under salinity is closely related to plant growth and survival (Voigt et al., 2009). Although the activity of seed proteases is essential for protein mobilization, the effects of salinity on these proteins and on protein mobilization are poorly understood (Marques et al., 2013). In dicots, there are large accumulations of 11S and 7S globulins, and 2S albumins are found in small amounts (Shewry et al., 1995; Tan-Wilson and Wilson, 2012). These SSPs (seed storage proteins) are synthesized and processed by a secretory pathway and accumulate within protein storage vacuoles and/or other cellular compartments (Herman and Larkins, 1999; Tan-Wilson and Wilson, 2012). In general, proteases accomplish two major functions: a regulatory function, which involves the activation or inactivation of specific proteins, and a general proteolytic function, which is a less specific process resulting in the bulk breakdown of reserve proteins and elimination of denatured proteins (Tan-Wilson and Wilson, 2012).

Mobilization of SSP requires the orchestrated action of multiple proteases that are stored in mature seeds and are *de novo*-synthesized during germination (Müntz et al., 2001). Endopeptidases or proteases catalyze the hydrolysis of peptide bonds, whereas exopeptidases or peptidases promote the cleavage of peptide bonds at either the N- or C-terminus of the polypeptide chain (Müntz et al., 2001). Based on the mechanism of catalysis, proteolytic enzymes are categorized as serine-, cysteine-, aspartic-, or metalloproteases (Schaller, 2004). A previous work demonstrated that SSP mobilization in the cotyledons of cashew seedlings was strongly delayed by mild NaCl salinity (Voigt et al., 2009). Because the salt-induced delay of seedling growth is associated with the accumulation of amino acids in cotyledons, it had been proposed that salinity might induce a delay in SSP mobilization through the source–sink mechanisms involving amino acids as possible signal molecules (Voigt et al., 2009).

The overall effects of salt stress on plant growth and metabolism have been intensely studied, and the literature on this issue has increased exponentially in recent years. Salinity effects on germination and seedling establishment are also widely studied (Yang et al., 2009), but there have been few studies of the biochemical and molecular mechanisms involved in the different phases from germination to seedling establishment. Moreover, studies involving the late phase of seedling establishment, which is critical for plant establishment in agronomical and ecological terms, especially under extreme environment conditions must be more widely studied. Cashew is a native species originated from the Brazilian coast regions, and it is widely distributed and cultivated in areas subject to salinity and with soils very poor in nutrient mineral (Ferreira-Silva et al., 2010). However, this species is capable of germinating, surviving and displaying high yield under these adverse conditions, utilizing very efficient physiological mechanisms that are still not completely understood (Silveira et al., 2003).

Cashew seeds were chosen as a model in this study because of their singular capacity to cope with adverse environmental conditions in terms of seedling establishment and survival. Moreover, this species is a native species tolerant to salinity and other abiotic stresses that remain poorly studied (Ferreira-Silva et al., 2011, 2012). Additionally, most published studies involving the effects of salinity concentrate on the phases of germination and early seedling development (Prisco and Vieira, 1976; Kayani et al., 1990; Marques et al., 2013). Moreover, the physiological mechanisms that regulate the salt-induced delay in cotyledonary protein mobilization and recovery during the late stage of seedling establishment are scarcely studied. Also is important understand these mechanisms for elucidating of physiological processes involved with improvement of crop yield and plant survival under stressful conditions.

This work was performed to test the hypothesis that the salt-induced delay in the mobilization of cotyledonary proteins during the late phase of seedling establishment in cashew is associated with an inhibition of proteolytic activity. The delay in the protein mobilization was primarily associated with negative osmotic effects on seed imbibition and was related to a decrease in the leaf growth sink strength at late seedling establishment. In this phase, the seedlings displayed a strong leaf growth recovery that coincided with a prominent stimulation in the protease activity and 11S globulin breakdown. The proteases exhibited a singular mechanism, displaying a strong stimulation in enzymatic activity after seedling establishment. The physiological significance of this singular regulation mechanism in salt-treated seeds is discussed.

Materials and methods

Plant material and seedling growth conditions

Cashew nuts (*Anacardium occidentale* L.) from the CCP 76 clone were provided by EMBRAPA-CNPAT, Brazil. The nuts were disinfected with 0.2% NaClO (w/v) for 10 min, rinsed with running tap water and imbibed in distilled water for 24 h. The imbibed nuts were sown in 0.8 L pots filled with vermiculite moistened to 70% of field capacity with distilled water (control) or with 50 mM NaCl. Once a week, the vermiculite was deeply leached with distilled water to avoid salt accumulation. The seedlings were maintained in a growth chamber under the following conditions: $27 \pm 2\,^{\circ}C$, $70\% \pm 10$ relative humidity, 12-h photoperiod and a photosynthetic photon flux density of 200 $\mu mol\,m^{-2}\,s^{-1}$.

Cotyledons from the control and salt treatment were harvested at each developmental stage: full epicotyl emission with the onset of primary leaf expansion (16 days after sowing – DAS), full expansion of the four primary leaves (20 DAS) and expansion of the four secondary leaves (24 DAS) – complete seedling establishment. Salt-treated seedlings exhibited a delay of approximately 4 days compared with control. Cotyledons, roots and leaves were rapidly harvested, frozen in liquid N_2 and stored at $-80\,^{\circ}C$ until the biochemical determinations.

In vitro protease activity and protease inhibitor assays

Cashew cotyledon samples (1 g) were powdered with liquid N_2 and extracted with 100 mM Tris–HCl buffer pH 8.0 containing 2 mM DTT and 3% PEG 6000 (w/v). The mixture was centrifuged at $12{,}000 \times g$ for 20 min at $4\,^{\circ}C$. The supernatant was used as the crude extract for determining the soluble protein by the Bradford method, using BSA as a standard. The azocaseinolytic and proteolytic activities of cashew cotyledons were evaluated *in vitro* according to Ainouz and Freitas (1991). The protein extract was incubated with reaction medium containing 50 mM sodium acetate, pH 5.0, with 5 mM L-cysteine, 0.1% Triton X-100 (v/v) and 1% azocasein (w/v) for 1 h at $37\,^{\circ}C$, and the reaction was stopped by the addition of 150 μL of 200 mM TCA. Protease activity was quantified spectrophotometrically at 440 nm and expressed as a unit of activity (UA) mg^{-1} protein h^{-1}, where one UA was equivalent to 0.01 units of absorbance. The activity of serine, cysteine, and aspartic proteases were specifically inhibited using 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM trans-epoxysuccinyl-L-leucylamide