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Exogenous ornithine is an effective precursor and the δ-ornithine amino transferase pathway contributes to proline accumulation under high N recycling in salt-stressed cashew leaves

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ABSTRACT

The role of the δ-ornithine amino transferase (OAT) pathway in proline synthesis is still controversial and was assessed in leaves of cashew plants subjected to salinity. The activities of enzymes and the concentrations of metabolites involved in proline synthesis were examined in parallel with the capacity of exogenous ornithine and glutamate to induce proline accumulation. Proline accumulation was best correlated with OAT activity, which increased 4-fold and was paralleled by NADH oxidation coupled to the activities of OAT and Δ^1-pyrroline-5-carboxylate reductase (P5CR), demonstrating the potential of proline synthesis via OAT/P5C. Overall, the activities of GS, GOGAT and aminating GDH remained practically unchanged under salinity. The activity of P5CR did not respond to NaCl whereas Δ^1-pyrroline-5-carboxylate dehydrogenase was sharply repressed by salinity. We suggest that if the export of P5C from the mitochondria to the cytosol is possible, its subsequent conversion to proline by P5CR may be important. In a time-course experiment, proline accumulation was associated with disturbances in amino acid metabolism as indicated by large increases in the concentrations of ammonia, free amino acids, glutamine, arginine and ornithine. Conversely, glutamate concentrations increased moderately and only within the first 24 h. Exogenous feeding of ornithine as a precursor was very effective in inducing proline accumulation in intact plants and leaf discs, in which proline concentrations were several times higher than glutamate-fed or salt-treated plants. Our data suggest that proline accumulation might be a consequence of salt-induced increase in N recycling, resulting in increased levels of ornithine and other metabolites involved with proline synthesis and OAT activity. Under these metabolic circumstances the OAT pathway might contribute significantly to proline accumulation in salt-stressed cashew leaves.

Introduction

Proline accumulation is one of the most common responses to water and salt stress in plants and is often considered to be involved in stress resistance mechanisms (Lutts et al., 1999; Szabados and Savoure, 2010). However, there is controversy over several aspects of proline accumulation. It is still dubious whether this stress-induced accumulation is a useful adaptive trait or simply an injury-induced physiological response (Delauney and Verma, 1993; Silveira et al., 2003). For instance, stress-induced proline accumulation is not always rapid, beginning only when tissue injury is evident (Hare et al., 1999). Another unclear aspect regards the biochemical triggers involved with proline biosynthesis and accumulation in response to a wide range of abiotic stresses or, for that matter, how these processes operate in a specific plant tissue or organ (Hare et al., 1999).

It is generally acknowledged that most proline accumulation induced by stress arises from increased synthesis from glutamate. Indeed, in plants proline can be synthesized from glutamate via Δ^1-pyrroline-5-carboxylate (P5C) by two successive

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Abbreviations: AA, total amino acids; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; OAT, δ-ornithine amino transferase; P5C, Δ^1-pyrroline-5-carboxylate; P5CDH, Δ^1-pyrroline-5-carboxylate dehydrogenase; P5CR, Δ^1-pyrroline-5-carboxylate reductase.

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reductions, which are catalyzed by P5C synthetase and P5C reductase (Hare et al., 1999). Alternatively, proline might also be synthesized from ornithine through δ-ornithine amino transferase (OAT; EC 2.6.1.13) yielding glutamic γ-semialdehyde (Roosens et al., 1998, 2002), a common intermediate originated from both glutamate and ornithine precursors. However, proline accumulation can also result from a decrease in its oxidation (Hare et al., 1999; Szabados and Savoure, 2010) and lower demand for protein synthesis (Silveira et al., 2003). OAT is a nuclear-encoded mitochondrial enzyme (Funck et al., 2008; Stránská et al., 2008) requiring pyridoxal phosphate as a cofactor while Δ^1-pyrroline-5-carboxylate reductase (P5CR) and Δ^1-pyrroline-5-carboxylate synthetase are localized probably in chloroplast/cytosol (Szabados and Savoure, 2010).

Glutamate is a key compound in amino acid metabolism and can be synthesized by several pathways. The most important one involves glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.7.1), the GS/GOGAT cycle, which is widely distributed in all plant tissues (Lea and Azevedo, 2007). In addition, under conditions of high ammonia concentrations, such as those found in salt stressed plants, Glu might also be intensively synthesized by glutamate dehydrogenase (GDH) (EC 1.5.1.2) in its aminating mode (Lutts et al., 1999). Thus, in stressed tissues, if GS, GOGAT and GDH activities are maintained at sufficient levels in order to avoid NH_3 toxicity, high amounts of glutamate and glutamine could be produced (Viégas and Silveira, 1999; Lin and Kao, 2001).

Ornithine, another important proline precursor, can be synthesized from glutamate by amino transferases with the formation of acetylated derivatives and also by the ornithine cycle where glutamine is the initiator substrate, supplying carbamoyl phosphate and glutamate for ornithine synthesis. Arginine can also act as an ornithine precursor following hydrolysis by action of arginase in the urea cycle (Thompson, 1980). On the other hand, exogenous ornithine appears to be preferentially catabolized to glutamate by an ornithine amino transferase in mitochondria. However, the extent to which this reaction occurs under physiological conditions is little known (Thompson, 1980). Thus, the metabolism of Glu and Orn is very complex, displaying several interconnections and regulatory mechanisms that are still largely unknown, especially under stress conditions.

Usually, under water and salt stress, amino acid metabolism is largely altered, protein synthesis is impaired and proteolysis increased (Lutts et al., 1999). As a consequence, the concentrations of several metabolites involved directly or indirectly with proline synthesis are increased, especially amino acids and ammonia (Silveira et al., 2003). Thus, proline synthesis and accumulation might result from a generalized disturbance in nitrogen and amino acid metabolism associated with increased protein catabolism and stress-induced senescence (Viégas and Silveira, 1999). This would be consistent with the fact that proline buildup has a smaller effect on cellular metabolism, since it is a terminal product of a relatively short and highly regulated pathway (Hare and Cress, 1997).

We have previously verified that proline accumulation in cashew (*Anacardium occidentale*) leaves under water and salt stress is apparently more a symptom of injury caused by disturbances in amino acid metabolism than an adaptive response (Viégas and Silveira, 1999; Silveira et al., 2003; Ferreira-Silva et al., 2008). It is important that these observations were made in a plant species that displays adaptive traits to salinity and drought conditions. Otherwise, such responses could be understood to be a consequence of a lack of adaptation. Indeed, cashew plants, which are widely cultivated in Asia and Brazil, have developed several genetic and physiological adaptation mechanisms to overcome environmental stress (Ferreira-Silva et al., 2008).

There are several gaps in the literature regarding the metabolic mechanisms of proline accumulation in plant tissues. For example, what is the importance of secondary precursors such as ammonia, arginine and glutamine on proline synthesis and accumulation under stress conditions? Could an increase in the endogenous pools of these metabolites contribute to increase the availability of glutamate and ornithine precursors and consequently proline accumulation? The importance of the δ-ornithine amino transferase pathway for proline accumulation in plants subjected to abiotic stresses is still debated. Díaz et al. (2010) have suggested that the OAT pathway may have a role only under particular conditions or developmental stages. Instead of a single cause and a biochemical pathway, there appears to be an emerging view that the origin and mechanisms of proline accumulation are multiple and complex. Therefore, different metabolic and physiological circumstances would determine the reason and manner in which proline is accumulated, and therefore the understanding of these circumstances is important.

This work examines relationships between proline accumulation in salt-stressed cashew leaves and metabolic alterations involving important enzymes and metabolites involved with proline synthesis. In parallel, assays were performed on intact plants and leaf discs to examine the relative effectiveness of exogenous glutamate or ornithine to induce proline accumulation. Our results indicate that increased OAT activity and ornithine concentration can impact the supply of substrates for proline synthesis.

Materials and methods

Plant material and growth conditions

Seeds of cashew (*Anacardium occidentale* L.), clone CP 06 (from EMBRAPA-CNPAT, Brazil), were surface sterilized for 10 min in 5% (v/v) sodium hypochlorite and thoroughly rinsed with distilled water. The seeds were germinated in vermiculite saturated with 0.1 mM $CaSO_4$. Thirty-five days after germination, seedlings with similar height (24 ± 2 cm) were selected and transferred to 1-L plastic pots containing a 5-fold diluted Hoagland and Arnon (1950) nutrient solution. The plants were kept in a growth chamber with a day/night temperature of 27/24 °C, 80% relative humidity, a 12-h photoperiod and photosynthetically active radiation density of 250 μmol m^{-2} s^{-1}.

Dose-dependent and time-course experiments in whole plants

In the dose–response experiment, 45-day-old cashew plants were transferred to nutrient solutions containing 0 (control), 100, 200, 300 and 400 mM NaCl applied in a single step, since cashew seedlings do not suffer osmotic shock with these NaCl concentrations (Silveira et al., 2003), and treated for 48 h. In the time-course experiment, another set of 45-day-old plants were transferred to nutrient solutions containing 0 (control) or 200 mM NaCl and were treated for 0, 12, 24, 48 and 72 h. In another time-course experiment, a set of plants with same age were exposed to 200 mM NaCl over five days in order to evaluate salt-induced toxicity and proline accumulation. Following each treatment, fully expanded leaves were harvested, frozen in liquid N_2 and maintained at −85 °C until use for biochemical determinations. The experiment was carried out in a growth chamber, as described above.

Experiments with exogenous precursors using whole plants or leaf discs

In the experiments using glutamate and ornithine as exogenous precursors, 45-day-old cashew plants were first subjected to the absence (control) or presence of 200 mM NaCl for 48 h in a growth chamber. The leaves were then fully sprayed with 10 mM glutamate or ornithine dissolved in 10 mM Hepes buffer pH 6.0 and kept