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Exogenous ABA induces salt tolerance in indica rice (*Oryza sativa* L.): The role of *OsP5CS1* and *OsP5CR* gene expression during salt stress

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ABSTRACT

Abscisic acid (ABA) applied exogenously at 100 μM prior to and during the salt-stress period induced salt tolerance in both the salt-susceptible (LPT123) and the genetically related salt-resistant (LPT123-TC171) rice lines, enhanced the survival rate by 20%, and triggered proline (Pro) accumulation earlier than that by salt-stress alone, supporting a role for Pro as an osmoprotectant. In both rice lines, salt-stress induced *OsP5CS1* gene expression, suggesting that proline accumulation occurs via *OsP5CS1* gene expression during salt stress. An increase in the endogenous ABA level was required for the induction of *OsP5CS1* gene expression by salt stress. Under salt stress, topical ABA application-induced *OsP5CS1* gene expression only in the salt-resistant line but up-regulated *OsP5CR* gene expression in both rice lines, suggesting that the increased proline accumulation and salt resistance induced by topical ABA application may result from the up-regulation of *OsP5CR* and not, directly at least, from *OsP5CS1*. Moreover, exogenous ABA application up-regulates *OsCam1-1* (the salt-stress-responsive calmodulin) gene expression, and calmodulin was shown to play a role in the signal transduction cascade in proline accumulation during salt stress. These data suggest the role of the calmodulin signaling cascade and the induction of *OsP5CR* gene expression in proline accumulation by exogenous ABA application.

1. Introduction

Green plants are some of the most specious eukaryotes on terrestrial earth after insects and nematodes, with some 500,000 species, and probably diversified from a single land colonization. However, terrestrial plants are subject to often extreme and hostile changes in their environment, but, in contrast to many animals and prokaryotes, as sessile organisms they are essentially unable to move away from rapidly changing adverse environmental conditions. Therefore, they have evolved a remarkable ability to cope with highly variable environmental stresses, including cold, drought and soils with changing salt and nutrient concentrations, as part of their successful colonization of the ever changing terrestrial habitats. With respect to domesticated plants, the inability to respond to, or the trade-off costs of such stress responses to, these changing environmental stresses together represent the primary cause of crop losses across the world (Boyer, 1982), reducing average yields for most major crop plants by more than 50% (Bray et al., 2000). In contrast, this can be compared to the estimated yield loss caused by pathogens, which although well known and researched as a problem, actually account for typically only around 10–20% loss (Kreps et al., 2002). In addition to competitive economic issues, the ever increasing demand for both food and material crops, including green fuels, against the decreasing availability of new land for agriculture, means that increased efficiency of existing agricultural practices is essential. Therefore, the reduction of crop yield loss through stress is one important prime target along with pathogen and disease reduction. A number of attempts have been pursued to significantly reduce the effects of environmental stress, such as screening and selective breeding for new plant cultivars with desired stress-tolerant characters, and the investigation of stress-tolerant plants as model systems to evaluate the stress-tolerant mechanisms.

Exposure to desiccation, salt stress and low temperature are generally accompanied by an increase in endogenous abscisic acid (ABA) levels prior to activation of a number of water- and

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salt-stress-induced genes (Chandler and Robertson, 1994), the products of which are thought to be involved in protection of the cell or in recovery from the stress-mediated physiological insult. Indeed, ABA is an important signal for triggering plant responses to adverse environmental conditions during vegetative growth (Leung and Giraudat, 1998; Nambara and Marion-Poll, 2005), and ABA coordinates many of these stress responses, including the immediate stomatal closure, osmolyte accumulation and the induction of the synthesis of stress-related proteins, such as late embryogenesis abundant and heat shock proteins, reactive oxygen scavengers, etc. However, whilst many abiotic-stress-inducible genes are controlled by ABA, some are not, which indicates that both ABA-dependent and ABA-independent regulatory systems are involved in stress-responsive gene expression (Bray et al., 2000; Zhu, 2002).

In support of this, there are observations that the application of exogenous ABA to both whole plants, and in tissue culture explants or protoplasts, facilitated the adaptation to subsequent increased salinity (salt stress) in several phylogenetically diverse plants. ABA treatment prior to an increased salinity insult was reported to improve the growth of the common bean (*Phaseolus vulgaris*) (Khadri et al., 2007), to reduce leaf abscission and increase salt tolerance in citrus plants (Gómez-Cardenas et al., 2003), and to induce salt adaptation in jojoba (*Simmondsia chinensis*) shoots grown *in vitro* (Mills et al., 2001).

Moreover, exogenous ABA application was shown to reduce the sodium concentration or its translocation to shoots, resulting in a salt-tolerant adaptation in both the cereal grass *Sorghum* (Amzallag et al., 1990) and the common bean (Khadri et al., 2007), whilst the increase in the K^+/Na^+ ratio in rice following exogenous ABA application correlated with the increased salinity tolerance (Bohra et al., 1995).

Adaptation by plants to stress involves morphological, physiological and biochemical changes, including the accumulation of osmolytes such as proline (McCue and Hanson, 1990; Demiral and Türkan, 2006), which are thought to play an adaptive role during osmotic stress (for review, see Delauney and Verma, 1993; Türkan and Demiral, 2009). Under salt-stress conditions, the elevated ABA levels inhibit Na^+ and Cl^- transport to shoots in intact bean seedlings (Karmoker and Van Steveninck, 1979), and it has been suggested that the resultant changes in ion concentrations together with the increase in endogenous ABA levels generate the signal for the induction of stress-induced genes. If correct, ABA may play an important role in the tolerance of plants to increasing salinity.

Proline is a dominant organic molecule that accumulates in many organisms (Sawahel and Hassan, 2002) following exposure to environmental stresses, and especially under salt-stress conditions (Delauney and Verma, 1993; Jiménez-Bremont et al., 2006; Tripathi et al., 2007; Xue et al., 2009). However, the role of proline accumulation in salt tolerance is contradictory and unresolved. Some researchers have suggested that proline accumulation may be related to the degree of salt (Igarashi et al., 1997; Demiral and Türkan, 2006) or osmotic (Hien et al., 2003) tolerance in rice whilst, in contrast, others have suggested that it is a symptom of salt-stress injury rather than an indicator for resistance (Lutts et al., 1996; Bangyeekhun et al., 2004). Nevertheless, within this controversy, Hien et al. (2003) suggested that for rice cultivars proline accumulation is a possible indicator of osmotic tolerance.

Some support for a potentially general protective rather than symptomatic role for proline accumulation and salt-tolerance comes from the observations that the exogenous topical application of ABA to non-stressed plants triggers proline accumulation in the cereals barley (Pesci, 1987), maize (Ober and Sharp, 1994) and rice (Chou et al., 1991), as well as *Brassica* and *Arabidopsis* (Finkelstein and Somerville, 1990). In addition, under salt-stressed conditions, a low level of ABA (1 μM) increased proline accumulation in the shoot of the common bean which also correlated with enhanced salt tolerance (Khadri et al., 2007).

Proline is synthesized from glutamate via two intermediates, glutamic γ-semialdehyde and Δ′-pyrroline-5-carboxylate. The first step is catalyzed by Δ′-pyrroline-5-carboxylate synthetase (P5CS) and is rate-limiting (Delauney and Verma, 1993; Hu et al., 1992; Kavi Kishor et al., 1995; Yoshiba et al., 1995; Igarashi et al., 1997; Hong et al., 2000).

Homologous *P5CS* genes have been isolated from various plant species, including two *P5CS* gene homologues in rice (*Oryza sativa* L.), *OsP5CS1* and *OsP5CS2* (Igarashi et al., 1997; Hien et al., 2003). However, their partial characterization to date reveals different transcript expression patterns between them. Thus, *OsP5CS1* transcripts are up-regulated by NaCl, osmotic, dehydration and cold shock (Igarashi et al., 1997), whilst *OsP5CS2* transcripts are additionally up-regulated by mannitol (Hien et al., 2003). In addition, although both genes are up-regulated by exogenous ABA under normal conditions, the kinetics differ with a faster and stronger up-regulation of *OsP5CS1* transcripts compared to that for *OsP5CS2* in whole indica rice seedlings, and differential expression levels in different tissues (Hur et al., 2004). This is in good agreement with the differential expression patterns of the two homologous genes in *Arabidopsis*, *AtP5CS1* and *AtP5CS2*, in terms of tissue tropism and the kinetics and levels of transcript expression (Strizhov et al., 1997; Yoshiba et al., 1999; Abraham et al., 2003).

The data summarized above raise the issue of whether *OsP5CS1* gene expression is related directly to salt stress and ABA function in *O. sativa* L., since it remains unknown if *OsP5CS1* acts via an ABA-dependent pathway under salt stress. Thus, as a required prelude to evaluate such, the objective of the present investigation was to determine the appropriate concentration of exogenous ABA application that can induce salt tolerance and also trigger proline accumulation and, additionally, to establish the relationship between *OsP5CS1* gene expression levels and ABA function. Based on previous reports, where exogenous ABA treatment increased *P5CS* transcript levels in *Arabidopsis thaliana* (Strizhov et al., 1997; Yoshiba et al., 1999; Abraham et al., 2003), we considered the possibility that *OsP5CS* might be regulated by ABA in rice. If that is the case, we speculate that exogenous ABA application at a dose that induces salt tolerance and proline accumulation should induce *OsP5CS1* transcript levels. However, if not, other genes in the proline biosynthesis pathway should be investigated.

The proline synthesis pathway with glutamate as a precursor requires two enzymes, pyrroline-5-carboxylate synthase (P5CS), and pyrroline-5-carboxylate reductase (P5CR). In *Arabidopsis*, during osmotic stress, only *P5CS*, but not *P5CR*, gene expression was well correlated with proline content (Yoshiba et al., 1995; Savouré et al., 1997). In maize and wheat the activity of P5CR during water stress was increased in correlation with the accumulation of proline, whilst increased Ca^{2+} levels also led to the elevated accumulation of proline in both plant species (Nayyar, 2003). The calmodulin antagonist, trifluoperazine, decreased P5CR activity and proline content, suggesting the involvement of Ca^{2+} and calmodulin in P5CR function (Nayyar, 2003).

Genome-wide identification and analyses of the rice calmodulin and related potential calcium sensor proteins indicated that there are 243 proteins in the rice genome that possibly have an 'EF-hand' motif. These include five *O. sativa* L. *Cam* (*OsCam*) genes and 32 *O. sativa* L. *Cam*-like (*OsCML*) genes (Boonburapong and Buaboocha, 2007). The *OsCam1-1* gene expression level was shown to be rapidly (1 h after stress) and highly increased in response to salt stress (0.15 M) (Phean-o-pas et al., 2005, 2008), and then slowly decreased 2 and 4 h after the treatment. This result indicates that