Salt stress induces transgenerational tolerance by allantoin accumulation in Arabidopsis seeds

Carolina Martini[1], Ignacio Lescano López[2], Joaquín Suárez[1], Tomás M Tessi[1], Marcelo Desimone[1,3]

[1]Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba (Argentina)

[2]Unidad de Estudios Agropecuarios (UDEA) - Centro de Investigaciones Agropecuarias (CIAP) - CONICET – INTA, Córdoba (Argentina)

[3]Instituto Multidisciplinario de Biología Vegetal (IMBIV – CONICET)

Corresponding author: marchelodesimone@gmail.com, +54 9 351 3155932

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Main conclusion

Plants under salt stress generate seeds with higher allantoin content and increased germination rates in saline environment representing a novel mechanism of transgenerational tolerance.

Abstract

Seed germination is a critical phase in plant life cycle and is strongly influenced by environmental factors. High salinity in soils impairs seed germination limiting further seedling establishment and plant growth. Allantoin, a metabolite generated in the purine degradation pathway, was previously shown to accumulate and confer salt stress tolerance in seedlings. The aim of this work was to know if allantoin accumulation in seeds influences germination rate under salt stress conditions. The germination capacity under salt conditions was improved by increasing allantoin content in Arabidopsis seeds. Moreover, we found that allantoin levels rise during seed maturation when mother plants were irrigated with salt during the flowering stage. The germination rate in saline media was improved in seeds developed under salt conditions compared to controls, indicating that allantoin accumulation in seeds mediates a transgenerational tolerance to salt stress.

Introduction

The development of sustainable agriculture production systems requires a better understanding of plant adaptation to unfavorable conditions. One critical limiting factor for crop production is the capacity of seed germination and seedling establishment in soils with elevated salt content (Arif et al., 2020). Salinity affects plant development by lowering soil water potential and therefore limiting its availability for growth, as well as causing ion toxicity on biological structures. To alleviate salt stress several physiological adaptations have been described including the accumulation of compatible osmolytes to decrease intracellular water potential, ion exclusion or compartmentalization and induction of cellular detoxification and repair systems (Arif et al., 2020). Although it is well established that salinity delays or impedes seed germination in the majority of crops, the subjacent mechanisms conferring stress alleviation are scarcely investigated.

In a previous work, we showed that allantoin accumulation in Arabidopsis seedlings is a mechanism that confers salt stress tolerance (Lescano et al., 2016). Allantoin is a degradation product of purines that serves in tropical legumes for long distance transport of organic nitrogen. In addition, this compound accumulates

in other plant species as a consequence of different stress situations. Osmotic, drought, salt and cadmium stress particularly repress allantoinase expression triggering allantoin accumulation (Irani and Todd, 2018, Lescano et al., 2016, Nourimand and Todd, 2019, Watanabe et al., 2014). Interestingly, it has been reported that the expression of genes related to abscisic acid (ABA) metabolism are affected by allantoin accumulation in seedlings suggesting a signaling link with this hormone (Watanabe et al., 2014). ABA is a main regulator of seed dormancy and germination. Therefore, it is reasonable that allantoin could potentially be involved in this process. Supporting this idea, DNA methylation of allantoinase participates in maternal and environmental control of seed dormancy (Iwasaki et al., 2019).

These facts open the question of whether allantoin can influence seed germination under stress conditions. Furthermore, as was previously reported for seedlings, stress could potentially trigger allantoin accumulation in developing seeds and this could regulate germination under stress conditions. Here we addressed these questions by studying the effect of allantoin by its exogenous supplementation or endogenous accumulation on Arabidopsis seed germination under salt stress conditions.

Results

To study a potential role of allantoin in salt stress alleviation in seeds, the germination rate of Arabidopsis seeds was determined in agar plates containing selected compounds. In agreement with previous reports (Claeys et al., 2014), a delay in germination time after imbibition of Arabidopsis seeds was observed if 100 mM NaCl was included in the media indicating toxicity caused by salinity on seedling development (Fig.1). Nevertheless, just all seeds were able to germinate under the selected conditions. To determine a possible role of allantoin in germination, this compound was added to the agar plates at a concentration of 100 mM. Without salt, a lower germination rate was observed at the first day in media containing allantoin, but 100 % of seeds germinated after 48 hrs. of light exposition. On the contrary, seeds under salt conditions showed a significant increase in germination rate if allantoin was included in the growth medium (Fig.1a). To know if the effect of allantoin was specific, structurally or presumable functionally related compounds, such as allantoate and proline, were tested instead using the same concentrations. Both compounds showed equivalent inhibitory effects at the first day of germination as observed for allantoin under control conditions (Fig.1a). However, the effect under salt conditions was clearly dissimilar: whereas proline, a known compatible osmolite, was unable to cause changes in the germination rate compared with the control, allantoate showed an inhibitory effect (Fig.1a). These findings indicate that allantoin itself and not its

degradation product is responsible for promoting the protective effect and suggest a function different from osmoregulation. This set of experiments was a first indication that allantoin could be a promoting metabolite for seed germination in saline soils.

The findings using exogenously applied allantoin, prompted us to study seed germination of mutants with constitutively altered allantoin content. Since the expression of allantoinase has been shown to be a key regulation step for allantoin accumulation and it is upregulated during seed imbibition (Narsai et al., 2011) (Online Resource 3), two opposite genetic backgrounds were compared with WT (wild-type) seeds: an allantoinase knockout (*aln-1*) previously described (Lescano et al., 2016) and transgenic lines with constitutive expression of allantoinase (pUBI10-ALN) (Online Resource 1). The allantoin content of *aln-1* seeds was ~8-fold higher than WT seeds, whereas pUBI10-ALN seeds contained around one half of allantoin concentration compared with the WT (Fig.2a). All genotypes showed no significant differences in germination rates under control conditions (Fig.2 b, c). When 100 mM NaCl was added to the growth media, no significant differences were observed in the germination rates of pUBI10-ALN and WT, but the germination of *aln-1* seeds was significantly faster compared to the WT (Fig.2b, d). This finding suggests that allantoin accumulation in seeds could be a natural mechanism to permit or accelerate seed germination in saline soils.

To evaluate this hypothesis, we studied the allantoin content and the germination rate of seeds developed from plants that were irrigated or not with 75 mM NaCl at the bolting stage (Fig 3). In all cases, allantoin content positively correlated with the developmental stage of the siliques and was higher in isolated mature seeds (Fig 3a). Moreover, plants subjected to salt stress during flowering stages presented siliques and seeds with significantly higher allantoin content compared with control plants. In accordance, allantoin accumulation has been previously observed in vegetative organs, particularly in the roots of plants exposed to salt stress (Lescano et al., 2016, Lescano et al., 2020). The germination rate in media without NaCl was similar for seeds formed with or without salt stress (Fig.3b). In contrast, the addition of 100 mM NaCl to the germination media showed a significantly higher germination rate of seeds obtained from plants that were exposed to salt stress compared to those obtained from control plants (Fig.3c). This result indicates that allantoin accumulation in seeds developed during salt stress improves germination in saline medium. We also measured germination rates using other NaCl concentrations. Already at 150 mM NaCl, the differences between seeds formed with or without stress were slighter, suggesting that allantoin can improve germination rate only at lower salt concentrations (Online Resource 2a, Fig 3c). Since NaCl can

cause stress by two possible ways, namely water sequestration (osmotic) or by ion toxicity, we also tested the germination rate by using sorbitol as an osmotic compound that did not cause toxicity. By the presence of 200 or 300 mM sorbitol that is osmotically comparable with 100 or 150 mM NaCl, respectively, the germination rates of seeds formed with or without salt stress were quite similar (Fig.3d, Online Resource 2b). This suggests that the improvement of germination rates observed in seeds formed under salt stress could be more related to ion toxicity than osmolarity.

Discussion

The results presented here indicate that allantoin accumulation in Arabidopsis seeds improves their germination rate under salt stress conditions. This conclusion is supported by increased germination rates of (i) WT seeds in the presence of exogenously applied allantoin (Fig.1) (ii) *aln-1* seeds with constitutively high allantoin content (Fig.2) and (iii) WT seeds with elevated allantoin content obtained from plants subjected to salt stress (Fig 3). This beneficial effect on seed germination is in agreement with previous investigations showing that allantoin improves seed germination under stress conditions e.g. cadmium toxicity (Nourimand and Todd, 2019) or high temperature (Ying et al., 2022). In contrast, seed dormancy is negatively regulated by allantoinase expression, indicating that allantoin levels are important to impose dormancy (Iwasaki et al., 2019). It has been shown that inhibition of allantoinase expression by DNA methylation is part of a maternal inheritance and is enhanced by cold temperatures during seed maturation. Thus, allantoin levels can promote seed dormancy or germination in different developmental stages and environmental contexts. We used for our germination experiments seeds after ripening in which dormancy has been already broken. Different allantoin levels were measured in dry seeds developed from plants grown under salt stress or control conditions (Fig.3a) of mother plants suggesting that allantoin plays different roles in germination and dormancy.

In a previous work, we found that allantoin accumulated in seedlings subjected to salt stress mainly in the roots due to allantoinase downregulation, but allocation to the leaves is necessary for stress alleviation, as the knockout deficient in the allantoin transporter AtUPS5 shows a more susceptible phenotype than WT (Lescano et al., 2016). We found here that seeds formed under salt stress accumulate allantoin (Fig.3a) suggesting that this compound is also transported from the roots, but we cannot exclude that allantoin is synthesized in the seeds. Allantoinase expression is present in siliques in the maternal tissues, but not in dry seeds (Online Resource 3-4). Moreover, we did not observe any change in the expression pattern even

under salt stress. Interestingly, another putative allantoin transporter (AtUPS4), which shows a high homology degree with AtUPS5, is expressed exclusively in the last phase of seed development (Schmidt et al., 2006). The role of this transporter in the process described here remains part of ongoing investigations. The mechanism of action of allantoin in stress alleviation via ABA remains controversial. This compound clearly influences the ABA metabolism (Watanabe et al., 2014). The expression of AtNCED3, a key enzyme in ABA biosynthesis, is induced in seedlings exposed to drought stress or in *aln* mutants. Increased levels of allantoin also cause the activation of AtBG1u18, the ABA deconjugation enzyme. Both effects of allantoin cause increased ABA levels. However, it has also been observed that the expression of ABA degradation enzymes (AtCYP707A1, AtCYP707A2) is increased in *aln* mutants. Interestingly, the expression of these two genes is limited to seed maturation and early events of seed germination (Okamoto et al., 2006). Therefore, it is possible that allantoin acts as an inductor of ABA synthesis in the context of seedling response to drought or salt stress, whereas it induces ABA catabolism during germination.

In summary, we inform here a potential novel mechanism for germination in saline environments mediated by allantoin accumulation in the seeds. Further investigations should point out the regulatory factors implied and to which extent this mechanism occurs in different groups of seed plants.

Figure captions

Fig.1 Germination of WT seeds in allantoin-supplemented media. Seeds were sowed on 0.5X MS plates with 0 or 100 mM NaCl supplemented with 10 mM allantoin, allantoate or proline. After stratification at 4ºC for 2 days, plates were placed in a growth chamber (day 0) and germination percent was registered daily. **a** Germination percent of WT plants treated with 0 or 100 mM NaCl. The averages ± standard deviations of 3-4 biological replicates are shown. Asterisks indicate significant differences using WT as control (*$p<0.033$, **$p<0.002$, ***$p<0.001$, Bonferroni's multiple comparisons test). **b** Representative images of germinating seeds on day 2; scale bar = 1 cm.

Fig.2 Germination of transgenic lines with different capacities of allantoin accumulation. Seeds of WT, *aln-1* and pUBI10:ALN were sowed on 0.5X MS plates with 0 or 100 mM NaCl. After stratification at 4ºC for 2 days, plates were placed in a growth chamber (day 0) and germination percent was registered daily. **a** Allantoin concentration of dry seeds of the genotypes used in this experiment. **b** Germination percent. **c** Representative images of germinating seeds on day 2; scale bar = 1 cm. The averages ± standard deviations

of 3 (a) or 4 (b) biological replicates are shown. Asterisks indicate significant differences using WT as control (*p<0.033, **p<0.002, ***p<0.001, Bonferroni's multiple comparisons test).

Fig.3 Response of seeds developed from Arabidopsis plants exposed to salt stress. Plants were irrigated with water (mock) or 75 mM NaCl after bolting. Seeds of different developmental stages were collected and stored for allantoin quantification. 2 months after harvesting, seeds were sowed on 0.5X MS plates without additives, or adding NaCl or sorbitol. After stratification at 4ºC for 2 days, plates were placed in a growth chamber (day 0) and germination percent was registered daily. **a** Allantoin concentration in seeds during different seed developmental stages. Numbers of siliques stages correspond to Mizzotti et al. (2018). Germination percent under **b** control, **c** salt and **d** osmotic stress conditions. The averages ± standard deviations of 3 (a) or 4 (b-d) biological replicates are shown. Asterisks indicate significant differences with mock (*p<0.033, **p<0.002, ***p<0.001, Bonferroni's multiple comparisons test).

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana WT and *aln-1* mutant line (Salk_146783) were in the Col-0 background. For replication, seeds were sowed on trays with soil: vermiculite (2:1) mix and stratified for 2 days at 4°C. Then, trays were placed in a growth chamber under an 8hrs. light/16hrs. dark photoperiod at 22°C and a light intensity of 120 μE/m^2s for 4 weeks. Plants were transferred to a growth chamber under a 16h light/8h dark photoperiod for 3-4 additional weeks. Seeds for further experiments were collected after two month of drying.

Construction of pUBI10:ALN transgenic lines

The binary vector pCambia 1380 with the coding region of AtALN (pCALN) (Lescano et al., 2016) was used for cloning. The promoter region of AtUBI10 (pUBI10) comprising the 643 bp genomic DNA before the start codon was amplified by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) with primers 5'-GAATTCTACCCGACGAGTCAG-3' and 5'-AGGATCCTCGAGCTGTTAATCAG-3', cloned into pJet1.2 using CloneJET PCR Cloning Kit (Thermo Scientific) and sequenced (Macrogen Inc., Korea). The pUBI10 sequence was excised with EcoRI/BamHI and introduced into pCALN to obtain the

pUBI10:ALN construct. Restriction enzymes and T4 DNA ligase for cloning were supplied by Thermo Scientific.

pUBI10:ALN construct was introduced into *Agrobacterium* strain C58 for transformation of 8-10 weeks *Arabidopsis* WT plants using the floral dip method (Clough and Bent, 1998) and transgenic plants (T1 to T3 generation) were selected using hygromycin B (InvivoGen). Genomic DNA was extracted as described above, and T-DNA insertion was confirmed using vector reverse primer 5'-GACCGGCAACAGGATTCA ATC-3' and forward primer 5'-CCCAAAGAGCTCTTCTTCTTCAC-3'. Four pUBI10:ALN T3 lines were selected for further experiments. AtALN expression level of these transgenic lines was analyzed as previously described (Lescano et al., 2016).

Germination assays and stress experiments

For germination experiments, seeds were sowed on 0.5X MS agar plates and stratified for 2 days at 4°C. Then, plates were placed in a growth chamber under an 8hrs. light/16hrs. dark photoperiod at 22°C and a light intensity of 120 $\mu E/m^2s$ (day 0 for experimental treatments). Germination percent was registered daily. For stress experiments, NaCl or sorbitol (Sigma-Aldrich) at the desired concentration were added to media prior autoclaving. Allantoin, allantoate or proline (Sigma-Aldrich) were filtered with 0,22 μm filter (Whatman) and then added to the sterile MS media before solidification.

Plants were grown on soil:vermiculite (2:1) mix and irrigated with distilled water before bolting (6 weeks approximately). After bolting, plants were irrigated with 100 ml of distilled water or 75 mM NaCl solution. Treatments were repeated after one week. Seeds of different developmental stages were collected and stored at -20ºC for further allantoin determination as previously described (Lescano, 2020). Seeds for germination assays were harvested after two months of drying.

Data analysis and Statistics

Graph Pad Prism 7 was used for graphing and statistical analysis. The normality was tested by Shapiro-Wilk's test with Infostat v2018 (Di Rienzo et al., 2011). Pair-wise comparison t-tests with Bonferroni's P value correction were applied to identify significant differences between treatments.

Author contribution statement

CM did the stress and germination assays, and assisted in manuscript writing. ILL did the cloning work and generated the transgenic lines. Ureide determination was performed by CM and ILL. JS assisted in assays and the manuscript writing. TMT carried out microscopy of GUS stained material. Project conception and the writing of the manuscript was performed by ILL and MD.

Conflict of interest

The authors declare that they have no conflict of interest.

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