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Short communication

High-quality forage production under salinity by using a salt-tolerant AtNXH1-expressing transgenic alfalfa combined with a natural stress-resistant nitrogen-fixing bacterium

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ABSTRACT

Alfalfa, usually known as the "Queen of Forages", is the main source of vegetable protein to meat and milk production systems worldwide. This legume is extremely rich in proteins due to its highly efficient symbiotic association with nitrogen-fixing strains. In the last years, alfalfa culture has been displaced to saline environments by other important crops, including major cereals, a fact that has reduced its biomass production and symbiotic nitrogen fixation. In this short communication, we report the high forage production and nutrient quality of alfalfa under saline conditions by alfalfa transformation with the AtNHX1 Na^+/H^+ antiporter and inoculation with the stress-resistant nitrogen-fixing strain *Sinorhizobium meliloti* B401. Therefore, the incorporation of transgenic traits into salt-sensitive legumes in association with the inoculation with natural stress-resistant isolates could be a robust approach to improve the productivity and quality of these important nitrogen-fixing crops.

Since the pioneer characterization of the vacuolar Na + /H + antiporter AtNHX1 in *Arabidopsis thaliana* by Blumwald's team at University of Toronto in Canada (Apse et al., 1999), several articles have described AtNHX1-homologous genes from different plant species. In addition to the understanding of the direct and indirect roles of these antiporters in abiotic stress adaptation, the massive expansion of the study of AtNHX1 and AtNHX1-related genes has enabled the experimental corroboration of the high efficiency of their heterologous expression to improve salt tolerance in several crops (Chen et al., 2008; He et al., 2005; Li et al., 2010; Li et al., 2011; Sahoo et al., 2016; Zhang and Blumwald, 2001). However, there are some technical and biological factors that have limited the commercial use of this technology. These include the need to produce events with specifically high transgene expression and the possibility to maintain the interactions of transgenic crops with beneficial microbes under saline conditions. In alfalfa, these two constraints are exceptionally hard, because it is necessary to produce a vast number of events to find one showing high-level transgene expression (Rogan and Fitzpatrick, 2004), and because, under saline conditions, the symbiotic alfalfa-*Sinorhizobium* interaction for nitrogen fixation is impaired (Palma et al., 2013). To bypass the first constraint, we have previously developed a highly efficient process for the transformation of the highly regenerative alfalfa clone C23 and for the rapid and inexpensive production of transgenic alfalfa libraries by using the binary vector pPZP200BAR (Jozefkowicz et al., 2016). Using this framework, we were able to produce a transgenic alfalfa event (alfalfa-*AtNHX1*) that combines high-level and ubiquitous expression of the *AtNHX1* gene (Fig. 1a). To bypass the second constraint, we used the nitrogen-fixing bacterium *Sinorhizobium meliloti* B401, a natural stress-resistant strain isolated from alfalfa monoculture under water-deficit conditions by the National Instituteof AgriculturalTechnology from Argentina (http://inta.gob.ar). Contrary to alfalfa plants inoculated with the stress-sensitive phenotype of the model strain *Sinorhizobium meliloti* 1021 (Galibert et al., 2001), those inoculated with the stress-resistant strain B401 show high nitrogen fixation rates in semiarid environments (Jozefkowicz et al., 2017), suggesting that this natural stress-resistant strain is an adequate inoculant for alfalfa production under stress conditions.

In concordance with the general classification of legumes as salt-sensitive crop species (Läuchli, 1984), moderate saline soils displaying Electrical Conductivity (EC) of 10 dS/m, mainly due to NaCl, reduce alfalfa yield by about 50% and almost completely inhibit alfalfa nodulation under field conditions (Smith, 1994). In this study, we

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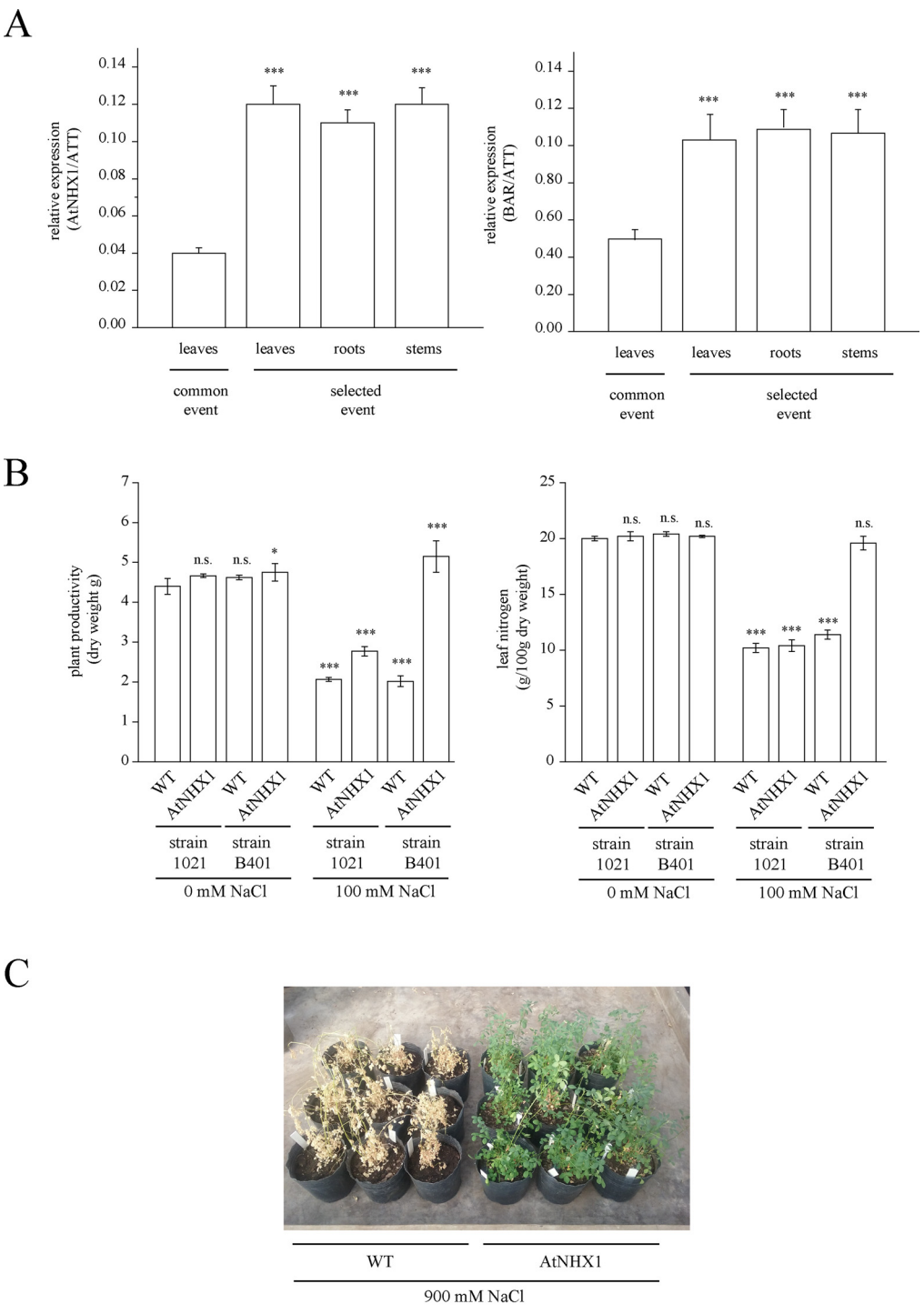

Phenotypic Segregation Data for salt-tolerant transgenic trait

Generation	Number tested	Number tolerant	AtNHX1 % tolerant	AtNHX1 % expected	chi-square significance test
T1	138	67	48.5	50	n.s.
T2	401	194	48.3	50	n.s.
T3	325	162	49.8	50	n.s.

n.s. = not significant

Fig. 1. Combination of salt-tolerant transgenic alfalfa germplasm with natural stress-resistant nitrogen-fixing bacteria to maximize high-quality legume forage production under physiological saline stress conditions. (a) We screened two transgenic libraries containing 2000 putative independent events of alfalfa transformed with the binary vector pPZP200BAR-AtNHX1 (Additional File 1) by *Agrobacterium tumefaciens* (Jozefkowicz et al., 2016) for glufosinate and salinity tolerance. We selected an event showing both herbicide tolerance (10 mg/L glufosinate) and salinity tolerance (1.2 M NaCl). A representative RT-qPCR assay shows the high-level and ubiquitous expression of the *AtNHX1* and BAR genes in the selected event compared to a random-selected event. The expression of the herbicide tolerance in these events was confirmed by glufosinate tolerance assays under greenhouse conditions (Additional File 2). (b) The selected transgenic event showing high and ubiquitous expression of the *AtNHX1* gene was crossed manually with the unrelated wild-type alfalfa clone 19-17 by using transgenic parental plants as the pollen donor. The progeny from this cross was discriminated between wild-type (WT) and transgenic (AtNHX1) plants by PCR assays against the *AtNHX1* gene (Additional File 3). Biomass production and nitrogen content in 4-month-old WT and AtNHX1 plants inoculated with the model strain 1021 or the stress-resistant bacterium B401 were quantified as previously described (Fox et al., 2016; Jozefkowicz et al., 2017) by using 1-L pots containing a mixture of soil:vermiculite (1:1) and irrigated with tap water supplemented with 100 mM NaCl to induce nitrogen deficit and saline stress, respectively. Biomass production, nitrogen content and nodule number were also analyzed in 8-month-old plants, supporting that the alfalfa-*Sinorhizobium* saline-sensitive phenotype can be completely suppressed by AtNHX1 plants inoculated with strain B401 (Additional File 4). Stable isotope dilution analysis (Fox et al., 2016) and the quantification of the levels of leghemoglobin, free oxygen, ATP and NADPH in nodules (Soto et al., 2013) confirms the high levels of nitrogen fixation in AtNHX1 plants inoculated with strain B401 under saline conditions (Additional file 5) and that nodules from salt-exposed B401-treated AtNHX1 plants can provide an optimal microenvironment for nitrogenase activity (Additional File 6), respectively. Each experiment contains 40 individual plants per treatment. All values are mean ± SEM, n = 3 and n = 5 in panels (a) and (b), respectively. Asterisks represent statistically significant differences (*p < 0.05, ***p < 0.001) according to the ANOVA followed Dunnett's contrast test. (c) Rapid and inexpensive analysis of the salt-tolerant transgenic trait associated with the *AtNHX1* gene in transgenic alfalfa progenies. The selected transgenic event showing high and ubiquitous expression of the *AtNHX1* gene was crossed manually with a mix of wild-type alfalfa cultivars by using transgenic parental plants as the pollen donor through three backcrossing generations (T1, T2 and T3). The progeny from this cross was molecularly discriminated between wild-type (WT) and transgenic (AtNHX1) plants as described above. The expression (up) and the Mendelian inheritability (down) of the transgenic trait was analyzed in 5-L pots containing a mixture of soil:vermiculite (3:1) and irrigated with tap water supplemented with 900 mM NaCl to induce lethal stress in the nontransgenic plants. Statistical significance for the segregation data was determined using Chi square analysis.

mimicked this suboptimal but physiological levels of salinity by using non-saline soil (EC: 1.8 dS/m) irrigated either with tap water alone (EC: 1.3 dS/m) or with tap water supplemented with 100 mM NaCl (EC: 10 dS/m) under growth chamber conditions. Wild-type alfalfa (WT) plants inoculated with the model strain 1021 and the stress-resistant strain B401 showed a dramatic decrease in plant productivity (53 and 56%) and nitrogen content (49% and 44%) under salinity with respect to WT plants under optimal conditions (Fig. 1b). On the other hand, *AtNHX1*-expressing transgenic (AtNHX1) plants inoculated with 1021 showed an increase in biomass accumulation (38%) but not an improvement in nitrogen content compared with WT plants under saline stress (Fig. 1b). This is consistent with a general improvement of plant growth under saline conditions by the heterologous expression of the Arabidopsis Na+/H + antiporter and the absence of red nodules in these roots (data not shown). Importantly, the traditional alfalfa-*Sinorhizobium* saline-sensitive phenotype was completely suppressed by AtNHX1 plants inoculated with strain B401 in terms of biomass production and nitrogen content (Fig. 1b). In fact, salt-exposed B401-treated transgenic plants showed higher biomass accumulation (14%) than wild-type plants under optimal conditions (Fig. 1b).

Since cultivated alfalfa expresses self-incompatibility and inbreeding depression, a commercial variety of this important legume forage crop comprises an extensive number of heterozygous parent plants (Busbice et al., 1972; Rumbaugh et al., 1988). Therefore, to avoid inbreeding depression, introgression of transgenic traits into elite alfalfa germplasm needs several backcrossing generations (Rogan and Fitzpatrick, 2004). Then, previous to the introgression process, and to maximize its feasibility and minimize its cost, in alfalfa, it is particularly important to use a rapid and inexpensive method for the selection of transgenic plants expressing the transgenic trait in backcrossing generations. In this context, *AtNHX1*-expressing transgenic plants were crossed with heterogeneous populations composed of a mix of traditional cultivars, and the T1, T2 and T3 progenies were selected under nonphysiological conditions consisting in irrigation of putative transgenic plants with tap water supplemented with 900 mM NaCl (Fig. 1c). Under this extreme stress condition, transgenic but not wild-type segregant plants survived, showing a survival rate of 48.3–49.8% (Fig. 1c). This conforms to a Mendelian inheritance of the salt-tolerant transgenic trait (50%) and suggests that the *AtNHX1* gene can be used as a non-molecular selectable marker for both the introgression process and tolerance to saline environments. Although this proposal is focused on alfalfa due to its exceptional self-incompatibility and inbreeding depression, the use of the *AtNHX1* gene as a non-molecular selectable marker to reduce the cost of the introgression process can be extended to other valuable crops. This is because the production of almost all commercial transgenic cultivars begins with the transformation of a highly regenerative non-elite genotype, and then needs to introgress the selected event into elite germplasm, regardless of the reproductive behavior of the transformed plant species.

1. Conclusion and perspectives

Alfalfa, native to Asia and probably introduced in western regions by Persian invasions, has been cultivated as a forage crop since the beginning of documented history and the most widely used forage legume for animal and milk productions in the last two centuries. Due to its ability to establish efficient symbiosis with nitrogen-fixing rhizobial strains, the production of animal proteins from alfalfa culture does not require the use of nitrogen fertilizers derived from fossil fuel, mitigating our dependence on non-renewable energy sources and reducing the emission of greenhouse gases. This ancestral and sustainable system for animal production is currently vulnerable by the displacement of alfalfa culture to marginal saline lands by other important plant species, including major cereal crops. These salt stress conditions affect several physiological and biochemical processes, including nitrogen fixation, which considerably reduces the productivity and quality of alfalfa under field conditions (Orloff, 2007). Although conventional and transgenic varieties of alfalfa with varying degrees of tolerance to salinity have been developed (Kang et al., 2016; Sandhu et al., 2017), no reported approaches have yet been able to maintain optimum levels of biomass production and nitrogen content under physiological saline conditions in alfalfa or other legume crops. This can be attributed, at least in part, to the inability of these stress-tolerant germplasms to maintain beneficial plant-microbe interactions during stress conditions. Although there is a strong legal framework for the deregulation of transgenic plants and there are hundreds of commercial transgenic cultivars worldwide (http://www.cera-gmc.org), almost no country envisages the release of genetically modified microorganisms (GMMs) into agroecosystems, and consequently, there are no GMMs such as salt- or acid-resistant recombinant inoculants in the market. Therefore, the beneficial microorganisms associated with commercial transgenic plants should be natural isolates without genetic manipulations, at least in the next years or decades. In this context, here we demonstrated that the combination of salt-tolerant transgenic plants and natural stress-resistant nitrogen-fixing bacteria can be considered as an efficient approach for optimal high-quality biomass production under saline conditions. To our knowledge, this is the first report of high-quality legume forage production under saline conditions.

Author contribution statement

Conceived and designed the experiments: GS. Performed the experiments: MS PE CG NA. Analyzed the data: MS PE NA GS. Wrote the paper: GS.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.04.013.

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