Transformation and Expression of Na⁺/H⁺ Antiporter Vacuolar (*AgNHX1*) Gene in Potato (*Solanum Tuberosum* L.) Plants Under Salt Stress

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Research Article

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Abstract

Salinity is the main influencing factor in the productivity of most agricultural crops. Salinity has a negative effect on potato plants and yield. Previous research work shows that the transfer of the Na^+/H^+ antiporter gene to field crops improves the tolerance of plants to high salt concentrations. In this study, a vacuolar-type Na^+/H^+ antiporter gene from a halophytic plant, *Atriplex gmelinii* (*AgNHX1*) was transferred into potato via *Agrobacterium*. Leaf explants induced callus on Murashige & Skoog's (MS) medium accompanied by NAA (2.00 mg l⁻¹) and BAP (0.25 mg l⁻¹). The *in-vitro* leaf generated the callus which was transferred to the regeneration medium containing ZR (2.00 mg l⁻¹) and NAA (0.25 mg l⁻¹). The stable integration of the *AgNHX1* gene in genetically modified potato plants was confirmed by PCR analysis. RT-PCR and northern blot hybridization indicated that *AgNHX1* expressed highly in transgenic potato plants. Our results indicated that the expression of *AgNHX1* enhanced the salt tolerance in transgenic potato plants with good growth. This happened because, under salt stress, the accumulation of Na^+ content and proline was more in shoots as compared to control. It showed that the expression of *AgNHX1* activity in genetically modified potato plants was four-fold higher than in unmodified potato plants. Thus, it can be said that *AgNHX1* gene expression could increase salt tolerance in potato plants.

Key Message

Potato is an important crop, but it is moderately tolerant to salinity, which limits the expansion of its cultivation on the newly reclaimed lands. The aim of the research manuscript is to genetically modify the characteristic potato cv. Spunta to increase its salt tolerance. The research manuscript was first mentioned with the transfer of the a vacuolar-type Na^+/H^+ antiporter gene from a halophytic plant, *Atriplex gmelini* (*AgNHX1*) was transferred into potato via *Agrobacterium* and results demonstrated that the plants containing the *AgNHX1* gene tolerate salinity of up to 250mMNaCl.

Introduction

Potato (*Solanum tuberosum* L.) is a member of the *Solanaceae* family and is cultivated throughout the world (Munns, 2002). The most consumed variety of potatoes is moderately sensitive to salinity stress. The leaves of the potato plant are the most sensitive to salt (Maas, 1985; Katerji et al. 2000). Salt stress is one of the most important abiotic stresses that limit the expansion of field crops cultivation due to its effect on the growth and production of field crops (Lopez et al. 2002). It affects the morphology, metabolism, and most of the biochemical reactions of plants (Hilal et al. 1998). Salinity-tolerant crops are suitable for many lands that must be expanded in the coming years to meet the increased demand for food and solve the problem of water scarcity occurring in most of the countries of the world (Sajal and Nasrin 2020). This could be achieved either through traditional methods, such as selection and breeding, or through biotechnology and modern molecular biology approaches. Traditional breeding does not show the required results as the important crops demonstrate very low genetic variation in the gene pool and the breeding programs will need many years for the development of the new variety. Therefore, the

genetic modification of important plants and their conversion from salt-sensitive to salt-tolerant is of interest to plant scientists around the world to improve salt tolerance and expand the cultivation of crops (Borsani et al. 2003). Modern technologies in plant tissue culture and genetic engineering have made it possible to incorporate good traits into many economically important crop plants, including potatoes. In some studies, transformed potato plants were produced via *Agrobacterium*-mediated genetic transformation with abiotic stress genes (Horsch et al. 1985; Bayat et al. 2010; Borna et al. 2010; Krivosheeva et al. 2014; Szalonek et al. 2015). It is followed by *in vitro* regeneration by either direct or indirect somatic embryogenic development. The production of transgenic plants may fail due to failure in the regeneration process. Production of salt-tolerant potato varieties depends on genetic engineering tools, *in vitro* regeneration system, and high regeneration frequency of plants from cells (Hansen et al. 1999; Fiegert et al. 2000; Mutasim et al. 2010). In most cases, cytokinin helps to enhance shoot induction, but their response to *in vitro* regeneration varies from one cultivar to another (Shirin et al. 2007; Tara et al. 2017; Rodriguez et al. 2000). Zeatine riboside (ZR) is an important cytokinin for shoot induction from potato cells when performing genetic transformation using *Agrobacterium* (Yadav and Sticklen 1995; Wendt et al. 2001; Molla et al. 2011). It decreases calluses formation and promotes shoot induction (Beaujean et al. 1998). However, 6-Benzylaminopurine (BAP) and thidiazoron (TDZ) are also used as plant hormones for *in vitro* regeneration (Ehsanpour and Jones, 2000). TDZ is a substituted N-phenyl urea which is an important regulator that promotes the maturation of somatic embryogenesis and increases the shoot induction of callus in many *in vitro* regeneration systems of plants (Afrasiab et al. 2017). Na^+/H^+ antiporter genes are important for improving the plant's tolerance to salinity using genetic engineering. The role of Na^+/H^+ antiporter genes during exposure to various stresses was recently elucidated in cell homeostasis along with their role in the development of salt and drought tolerance in plants. The movement of Na^+/H^+ ions across the plasma membrane and tonoplast is achieved by ion exchangers or antiporters that transport Na^+ ions out of the cell or sequester them into the vacuole in exchange for H^+ ions (Shi et al. 2003). The characterization of tonoplast ion pumps that function in Na^+ influx within the vacuoles is accomplished in various plant species (Fukuda et al. 1999; Khan et al. 2015). Transport mechanisms help in moving the ions in vacuoles through the tonoplast and remove the harmful ions from the cytosol (Aharon et al. 2003). Barley *HvNHX2* and *HvNHX3* genes which are Na^+/H^+ antiporter were transferred under 35S promoter into potato plants which conferred improved tolerance to NaCl (Bayat et al. 2010; Krivosheeva et al. 2014). The objective of the study is to obtain transgenic potato plants cv. Spunta by introducing the *AgNHX1* gene encoding a Na^+/H^+ antiporter from *Atriplex gmelinii* to eliminate the problems related to the soil salinity.

Material And Methods

In vitro potato culture and callus formation

Tubers were grown at 27°C in a greenhouse within 30-40 days. The nodal stem segments were cut from greenhouse grown plants and explants were sterilized. Explants were transferred to MS (Murashige and Skoog, 1962) mineral salts containing myo-inositol (0.1 g L⁻¹), sucrose (30 g L⁻¹), indole-3-butyric acid

(0.05 mg l⁻¹), 6-benzylaminopurine (0.5 mg l⁻¹), paclobutrazol (1.0 mg l⁻¹) and phytigel (2.5 g l⁻¹). The samples were incubated in a culture chamber and kept at 25±2°C in an incubator by providing light for 16 hrs (3000 lux). Young leaves of potato (*Solanum tuberosum*) cv. Spunta were removed from 3 to 4 weeks in-vitro-grown plantlets and then grown on MS media composed of different concentrations of NAA (0.50-3.00 mg l⁻¹) combined with BAP (0.25-1.00 mg l⁻¹) for callus formation. The samples were incubated in growth room at 25±2°C in the dark. The explants were sub-cultured every one month on a new callus stimulation medium and multiplication for three months. Callus induction frequency (%) [The number of explants inducing callus/the total explants X 100], fresh weight of callus/explant and relative water content (%) [(fresh weight of callus- dry weight of callus)/ (dry weight of callus) × 100] were estimated after one month.

Regeneration of in- vitro potato shoots

The calluses were picked and transferred onto the regeneration media. MS medium contains ZR (0.50-3.00 mg l⁻¹) and TDZ (1.00-4.00 mg l⁻¹) alone or combined with NAA at 0.25 and 0.5 mg l⁻¹ (Sigma-Aldrich, USA) for shoot induction after 4-6 weeks when calluses were kept at 25 ± 2°C in an incubator by providing light for 16 hrs. Data were collected on in-vitro regeneration (%), mean number of shoots/ callus and mean of shoot length/callus (cm) after five weeks. The new small shoot induction was transferred and transplanted onto rooting medium which contained IBA (0.05 mg l⁻¹), BAP (0.50 mg l⁻¹), and PBZ (1.0 mg l⁻¹). For in vitro production of microtubers, developmental shoots were transferred to the same media in addition to sucrose (80 g l⁻¹) and then the plantlets were kept without light for two months at 20°C for two months. The plantlets were planting in plastic pots have soil that is a mixture of peat and sand (1:1), then incubated under 28±2°C in a greenhouse after covering with transparent bags. After two weeks, the cover of the polyethylene bags is gradually opened.

Construction and *A. tumefaciens* culture

The plant expression vector pBI121 in strain LBA4404 of *A. tumefaciens* was used for potato transformation. The binary plasmid is containing the AgNHX1 gene which was cloned from a halophytic plant, *Atriplex gmelini* encoding a Na⁺/H⁺ antiporter, under the control of CaMV 35S promoter and NOS terminator (Fig. 1), and the selective kanamycin resistance gene (nptII) under the control of NOS promoter. The binary plasmid pBI121- AgNHX1 was harbored into bacteria (LBA4404); an efficient and direct transformation procedure described by Soliman (2018a) with some modifications in the transformation procedures. The bacteria single colony of *A. tumefaciens* strain LBA4404 incubated on LB solid medium which consisted of Bacto-trypton (10 g l⁻¹), Yeast extract (5 g l⁻¹) NaCl (10 g l⁻¹) and agar (15 g l⁻¹) contained kanamycin (kan) and 50 mg l⁻¹ streptomycin (strep) were picked and grown on liquid LB medium containing kanamycin (100 mg l⁻¹) and streptomycin (50 mg l⁻¹) on a shaker at 28°C and 200 rpm for 48 h.

Agrobacterium-mediated transformation of potato (*Solanum tuberosum*).

Agrobacterium preparation

An initial of *Agrobacterium* was prepared from single colony from streaked plate (LB agar), in 10 ml of sterile LB broth containing streptomycin (10 mg l⁻¹) and kanamycin (50 mg l⁻¹). The single-colony *agrobacterium* was incubated in a shaker at 180 rpm in dark at 28°C over night and then 50 ml bacteria culture incubated a 250 ml flask which contained 50 ml of LB medium supplemented with same antibiotics previously mentioned under the same conditions. When the optical density (OD₆₀₀) of bacterial reaches 1.0, centrifugation is done at 6000 rpm for 10 min 4°C to collect the bacterial cells. Then it is placed in liquid MS medium and the optical density was adjusted to 0.8-1.0. Thus, it suitable to be used incubates with explants.

Potato transformation

Explants were placed in suspension of *A. tumefaciens* for 10 min with gentle agitation, to increase the transformation rate; the explants were frequently dipped into the *Agrobacterium* culture during the cutting of the explants. The explants were removed and dried on sterile filter paper to remove the excess bacteria. The excised leaf pieces were grown on the best chosen callus medium from the callus induction experiments and incubated in the growth chamber for 2-3 days without light. After two incubation days, explants were again rinsed in sterile distilled water, then dried with sterile paper for two min. and cultured on callus medium which contained kanamycin (100 mg l⁻¹) and cefotaxime (500 mg l⁻¹). The samples were incubated in a culture chamber without light at 25±2°C and then continuously subcultured on the same medium every one month.

Molecular analysis of putative AgNHX1 transgenic gene

Transgenic potato plants were confirmed by the PCR, RT-PCR analyzes and northern blot hybridization.

DNA isolation and PCR analysis

Young leaves of genetically modified and unmodified potato plants are used to extract genomic DNA according to (Hemaid 2018a) and then analyzed by PCR machine (Applied Biosystems™ Cat. No; A24811; Thermo Fisher Scientific, USA). Specific primers (forward primer, '5-ATG GCG GTT CCACAT CGT AG-3'; reverse primer, '5-TCA CTT CCA CTGAAC GTT GT-3') were used for the detection of the AgNHX1 gene by PCR. The PCR reaction was heated to 95°C/2mi., and then 35 cycles are at 95°C/30sec. for denaturation, 59°C/ 30sec. for annealing, 72°C/60sec. for extension and then 72°C/ 10 min. as the final extension step. A PCR product when migrated in 1% agarose gel with ethidium bromide was shown. A 1 kb ladder DNA (Invitrogen TrackIt 1 Kb Plus DNA Ladder) was used as molecular weight size marker which covers a range of DNA fragment size between 10000 bp and 250 bp. A gel documentation system was used to visualize and photograph the DNA bands. The transformation efficiency was calculated using PCR result by calculating the number of potato plants containing the AgNHX1 gene compared to total number tested.

RT-PCR expression analysis

Reverse transcription-PCR technique was performed to determine the expression of the AgNHX1 gene in genetically modified potato plants. Total RNA was isolated from genetically modified potato plants and unmodified potato plants as control by RNA Purification Mini Kit (Thermo Fisher Scientific Baltics UAB, Lithuania). The reverse transcription reaction procedure is based using cDNA synthesis kit (Thermo Fisher Scientific, Lithuania). RT-PCR was reacted with approximately 1.0 µg RNA as a template for synthesis of 0.1 µg cDNA. Following, RT-PCR was performed with specific designed primers forward (‘5-GAAATACAACCATAACTACGGAACC-3’) as primer forward and (‘5-TAACTAAAACCTCACCAACCAAGAC-3’) as primer reversed with PCR. PCR program was on 94oC/5 min., and then 30 cycles was at 94oC/ 30 s for denaturation, 60oC /30 s for annealing and 72oC/45s for extension and then 72oC/7 min as the final extension step and then the products of PCR appeared when migrated into a 1% agarose gel by electrophoresis.

Northern blot analysis

Total RNA from potato leaf were isolated on 1% denaturing agarose/formaldehyde gel and could be transferred by capillarity to nylon N+ membrane as described (Sambrook and Russel, 1989). The RT-PCR fragment (448bp) was amplified with specific AgNHX1 primer and labeled with [α -32P] as probes and then hybridized with [α -32P]dCTP-labeled cDNA and exposed to X-ray films. RT-PCR analysis were used RNA free – DNA (1.0 µg) with 0.5 µg oligo dT12-18, 200 µM dNTPs, RT-buffer, DTT (0.01 M) and SuperScript II (200 units) (SuperScript™ II Reverse Transcriptase, Life Technologies Cat.# 18064-014). Amplification PCR conditions were on 48°C/45 min., and then 30 cycles was at 94°C/ two minutes for denaturation, 65°C/ thirty seconds for annealing and 72°C/two minutes for extension and then 72oC/five minutes as the final extension step. The products of RT-PCR appeared when migrated into a 1% agarose gel by electrophoresis.

Measurement of shoot fresh, dry weight, microtuber weight/plantlet and Na⁺ content

Fresh weight of shoots (FW) and dry weight of shoots (DW) (g plant⁻¹), microtuber weight /plantlet (gm) and Na⁺ content in potato (*Solanum tuberosum*) cv. Spunta were evaluated under different NaCl treatments. Shoots were dried at 80 °C in in an oven set at 65 ° C temperature for about 48 hours to calculate the dry weight of the callus. To determine Na⁺ ion concentration in plant, 0.5 g of dried different parts of plant were digested a concentrated mixture of HNO₃, H₂SO₄ and HClO₄ in a ration of 5:1:2 (Imamul Huq and Alam 2005). The tubes containing plant samples and a mixture of acids were covered overnight then, the tubes were placed on a hot plate and digestion continued until the white fumes appeared and the sample became colorless. Na⁺ content in the digestion solution was measured using a Flame Photometer (Corning, 410). Microtuber weight/plantlet (gram) was calculated after two months.

Determination of proline content

Measurement of the content of free proline derived from the leaves of potato (*Solanum tuberosum*) plants with sodium chloride by measuring the amount of color obtained from the reaction of proline with ninhydric acid (McMannus et al. 2000) was performed in this study. A SmartSpec™ 3000 Bio-Rad

spectrophotometer was operated at 520 nm and a standard curve was used to determine the proline concentration based on the fresh weight of the plants.

Statistical analysis

All the experimental design was statistically analyzed by formulating a randomized design with three replicates. Statistical analysis using the technique of variance analysis (ANOVA) by multiple range tests is employed for evaluating the recorded facts (Steel et al. 1997). The means significance was compared by applying the L.S.D. and the test is done at a 5% probability level.

Results And Discussion

Callus induction and in vitro shoot regeneration

In the present study, the complete regeneration was successfully achieved by using in vitro leaf explants from the cultured stem node section of the potato (*Solanum tuberosum*) cv. Spunta through callus culture. Leaf explants formed callus on MS medium contain NAA (0.50-3.00 mg l⁻¹) with BAP (0.25-1.00 mg l⁻¹) as shown in Table 1. The result showed that the highest callus induction frequency (92.5±0.72) was obtained on MS medium accompanied by NAA (2.0 mg l⁻¹) and BAP (0.25 mg l⁻¹) as compared to the other treatments. While the fresh weight of callus per explant (4.65±0.73 gram) was significantly higher when cultured on MS medium containing NAA (0.50 mg l⁻¹) and BAP (1.0 mg l⁻¹) than other treatments. The results also showed that the relative water content (81.35±0.89) was significantly higher in callus induction when produced from MS medium complemented with NAA (2.00 mg l⁻¹) and BAP (0.5 mg l⁻¹) as shown in Figure 2 A. On the other hand, the lowest callus induction frequency (32.5±0.66) was obtained on Murashige & Skoog's media complemented with NAA (0.50 mg l⁻¹) and BAP (1.00 mg l⁻¹). The fresh weight of callus (2.05±0.38) per explants and relative water content (42.39±0.27) was less when treated with NAA (3.0 mg l⁻¹) and BAP (0.25 mg l⁻¹). The in vitro regeneration of callus, when treated with MS medium accompanied by NAA (2.0 mg l⁻¹) and BAP (0.25 or 0.5 mg l⁻¹), was found to be excellent as compared to the other treatments. It should be noted that the percentage of callus formation and growth of shoot regeneration does not occur when NAA and BAP were used alone. Callus was sub-cultured monthly and maintained on the same medium. These results are in agreement with Abeuova et al. (2020). They reported that the success in finding a protocol for the in vitro regeneration of potato plants by using leaf explants depends on a variety of factors, the most important of which are cultivars and combinations of PGRs. Although, many previous studies have demonstrated that 2, 4-D is an important plant hormone for the induction of potato callus (Arinaitwe et al. 2018; Serine et al. 2020). However, in the current study, NAA with BAP was used in callus induction of potato and the obtained results are in agreement with Jayasree et al. (2001); Ahmet and Sezai (2015).

The calli formed embryo-like structure, produced from different concentrations of NAA and BAP were transferred to media accompanied by the ZR at 0.50-3.00 mg l⁻¹ and TDZ at 1.00-4.00 mg l⁻¹ alone or combined with NAA at 0.25 and 0.50 mg l⁻¹ in order to reach the best concentration for inducing shoot formation. The best concentrations of growth regulators to know the percentage of in vitro regeneration,

number of shoots/explants, and length of shoot/explant are shown in Table 2. The maximum in vitro regeneration ($98.6 \pm 0.93\%$), number of shoots/explant (9.65 ± 0.78), and mean shoot length/explant (3.95 ± 0.78) were occurred on Murashige & Skoog's medium accompanied by ZR (2.0 mg l⁻¹) and NAA (0.25 mg l⁻¹) as compared to the other treatments (Fig. 2 B). Also, different concentrations of TDZ with NAA were evaluated for the development of maturation of somatic embryogenesis from calli and shoot induction. TDZ at a concentration of 4.00 mg l⁻¹ with 0.25 mg l⁻¹ NAA were able to produce of best in vitro regeneration percentage ($95.8 \pm 0.79\%$), the number of shoots/explant (8.25 ± 0.62), and the length of shoot /explant (2.28 ± 0.57) than other TDZ concentrations in combination with NAA (as shown in Figures 2 C and D). The other treatments of ZR and TDZ had a lower ability to undergo callus germination to form somatic embryos and then differentiating into plantlets. These results agreed with those obtained by Soliman (2018b), who reported that TDZ in combination with NAA was the best PGRs to produce the highest shoot length/explant. Also, in different studies, TDZ was considered one of the best growth regulators to obtain a large number of potato plantlets and shoot induction of callus under stress conditions as compared with the other growth regulators (Khalafalla et al. 2010). Some previous studies also indicated the efficiency of TDZ to enhance Agrobacterium-mediated transformation in Petunia hybrid (Thirukkumaran et al. 2009).

Transformation of potato (*Solanum tuberosum*)

To improve the salt tolerance of potato (*Solanum tuberosum*) cultivar Spunta, the AgNHX1 gene encoding a Na⁺/H⁺ antiporter responsible for defense against salt stress (Metwali et al. 2015; Soliman 2018a) was synthesized and cloned in the plant expression vector pBI121DNA. The binary plasmid pBI121- AgNHX1 was introduced into *A. tumefaciens* strain LBA4404 using direct transformation. This study, for the first time, reports the development of transgenic potato plants harboring the AgNHX1 gene. Leaf explants of potato cultivar Spunta were wounded and incubated with a single cell culture of *A. tumefaciens* strain LBA4404 carrying the AgNHX1 and nptII genes in the pBI121 plasmid vector for 10 minutes. The calluses derived from in vitro leaf explants were selected on Murashige & Skoog's medium complemented with NAA (2.0 mg l⁻¹), BAP (0.25 mg l⁻¹), and Kanamycin (100 mg l⁻¹) as a selective agent. Shoots were regenerated from these calluses on Murashige & Skoog's medium complemented with ZR (2.0 mg l⁻¹) and NAA (0.25 mg l⁻¹) (Fig. 3A). Putative potato shoots were rooted on Murashige & Skoog's medium accompanied by IBA (0.05 mg l⁻¹), BAP (0.5 mg l⁻¹), and PBZ (1.0 mg l⁻¹) after 35 days of in vitro incubation (Fig. 3B) which started producing microtubers using the same media in addition to 80 g l⁻¹ sucrose at $26 \pm 2^\circ\text{C}$ in complete darkness for two months (Fig. 3C). Acclimatization was successfully performed and the highest percentage of surviving transformed potato plants was 98% as shown in Figure 3D.

PCR detection of transformed potato plants

The PCR results of the transgenic potato plants showed a DNA band of 2600 bp when using AgNHX1 primers as shown in Fig. 4. This indicates that the purified DNA from the regenerated potato shoots carries the AgNHX1 that was transformed from the vector and the transgenic shoots. In addition, no

amplification product appeared in the non-transformed shoots. The frequency of transformation in potato plants was 44% which means that from 150 tested plants only 66 were transformed.

AgNHX1-expression in transgenic potato plants

Total RNA was purified from genetically modified potato and unmodified plants as a negative control using GeneJET Plant RNA Purification. The first-strand cDNA was synthesized by reverse transcription (cDNA) kit (Thermo Fisher Scientific Baltics UAB, Lithuania). AgNHX1 specific primers were used with cDNA (template) for amplification by PCR. The specific AgNHX1 primers successfully amplified fragments of 448 bp in genetically modified potatoes compared to non-transformed plants (used as negative control) where no band was detected (Fig. 5). The RT-PCR results indicated that AgNHX1 expression was in all transgenic potato plants. The expression of the AgNHX1 gene in the transformed potato plants was confirmed by mRNA detection. In examining the mRNA for the AgNHX1 gene was observed by northern blot analysis. The presence of the AgNHX1 gene amplified about 448 bp from cDNA in putative transgenic potato plants and showed a higher level of gene expression at the transcriptional level as compared to non-transgenic potato plants (Fig. 6).

These results are in agreement with Metwali et al. (2015) who carried out the RT-PCR analysis that showed the expression of AgNHX1 in *Ficus carica* L. cultivar (Black Mission Fig). The AgNHX1 gene expression was increased by salt stress and the amount of mRNA also increased 7 times as compared to control plants. The RT-PCR results of Soliman (2018a) also revealed the AgNX1 gene expression in grape tissues.

Evaluating AgNHX1- expressing potato plants under salinity level

In the current study, the growth of AgNHX1-expressing potato plantlets was most tolerated at a concentration of 250 mM of NaCl (Fig.7). The AgNHX1-expressing potato plantlets were significantly vigorous than non-transgenic plants under different NaCl treatments after one month. Effects of salt stress on the shoot fresh weight (FW) and dry weight (DW) (g plant⁻¹), microtuber weight/plantlet (gm), and Na⁺ content (mg–1 DW) in potato (*Solanum tuberosum*) cultivar Spunta with different concentrations of NaCl were showed in Fig. 8 and 9. The growth of non-transgenic potato plants was inhibited by increasing NaCl treatment, while in vitro shoot growth of AgNHX1 expressing potato plants was completely stopped and died at 300 mM NaCl concentration. This may be due to the compartmentation of Na⁺ ions into vacuoles provide an efficient mechanism to avert the toxic effects of Na ion in the cytosol (Li et al. 2010). Increasing salinity is also accompanied by significant reductions in the fresh and dry weight of shoots. The results showed that the Na⁺ content was increased in AgNHX1 transgenic plants when compared with non-transgenic plants. The accumulated Na⁺ was increased in the transgenic plants with increased NaCl levels. The Na⁺ content was 38.5 mg-1 DW with 150 mM NaCl, 43.9 mg-1 DW with 200 mM NaCl which increased to 67.3 mg-1DW with 300mM NaCl. The effect of different NaCl concentrations on the microtuber weight per plantlet (g) of potato cultivar Spunta cultured on MS medium supplemented with 0.05 mg l⁻¹ IBA, 0.5 mg l⁻¹ 6 BAP, 1.0 mg l⁻¹ PBZ, and 80 g l⁻¹ sucrose was evaluated. The results indicated that the fresh weight of microtuber per plantlet (g) was decreased

significantly with an increase in saline levels. These results pointed out the potential of the AgNHX1 gene that can enhance the growth of potato plants under salt stress conditions. The results of the present study are in agreement with those of Krivosheeva et al. (2014) who reported the expression of HvNHX3 in transgenic potato plants cv. Yubiley, Zhukova and Skoroplodny7 under salt stress. The results also showed that the proline content in transformed fresh weight (FW) of the shoot was higher than in non-transformed fresh weight (FW) of the shoot. The transformed potato plants tolerated 250 mM NaCl and showed a more than fourfold increase in proline content (76.2µg g⁻¹ FW) as compared to non-transformed plants. Enhanced proline synthesis in stressed plants was detected in several studies (Rabie and Almadini 2005; Naseem and Bano 2014; Fan et al. 2015; Mosaad et al. 2020). The hypothesis claim that the increase in the proline content under abiotic stress may refer to the effect of amino acid proline in maintaining the cell water status; thereby, helping the plant to cope and decrease the osmotic potential with the salinity stress (Al-Mansoori et al. 2006; Mousavi et al. 2020). Soliman (2018a) reported that the physiological mechanisms responsible for enhanced tolerance to salt stress can be accounted for by the enhanced accumulation of proline, inhibition of Na⁺ accumulation, and retention of K⁺ via up-regulating of the Na⁺/H⁺ antiporter gene (AgNHX1).

Conclusion

Salinity is a serious problem worldwide that causes the reduction of most field crops. It is necessary to improve the tolerance of plants to salinity in order to expand the cultivation of saline soils. A large number of vacuolar Na⁺/H⁺ antiporter genes have been identified and isolated in many plants and their expression into plant cells is important for plants tolerance of high soil salinity. The results of our study showed the transfer of a vacuolar-type Na⁺/H⁺ antiporter gene from a halophytic plant, *Atriplex gmelinii* (AgNHX1) into potato (*Solanum tuberosum*) cultivar Spunta via *Agrobacterium*-mediated transformation and overexpression under high salinity. Also, the study confirmed that transgenic potato plants contain the AgNHX1 gene that tolerated the high concentrations of salinity and grew under 250 mM NaCl concentration. This study indicates that the vacuolar Na⁺/H⁺ antiporter (AgNHX1) gene is of promising importance in the development of the field to enhance salt tolerance by genetic engineering and increase their cultivation in newly reclaimed areas.

Abbreviations

BAP: 6-Benzylaminopurine; IBA: Indole-3-butyric acid; Kn: kinetin, TDZ: Thiadiazuron (*N-phenyl-N-1,2,3-thiadiazol-5-ylurea*); ZR: Zeatin riboside; MS: Murashige and Skoog's medium; NAA: α -Naphthalene Acetic Acid; LB: Luria broth medium; PBZ: paclobutrazol; *npII*: Neomycin phosphotransferase; PCR: Polymerase chain reaction; RT-PCR: Reverse transcriptase polymerase chain reaction.

Declarations

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Author contributions

Hemaid I A Soliman planned the whole work, performed the tissue culture and molecular experiments, conducted provided lab facilities, contribution in the statistical analysis and conceived the research, performed molecular analysis. **Naif M S Kadasa** helped in write up and designed the figures and tables, analyzed data, reviewed several draft of the manuscript MS.

References

1. Abeuova LS, Kali BR, Rakhimzhanova AO, Bekkuzhina SS, Manabayeva SA (2020) High frequency direct shoot regeneration from Kazakh commercial potato cultivars. Peer J 8:p.e 9447. <https://doi.org/10.7717/peerj.9447>.
2. Afrasiab H, Rashid N, Akram M (2017) An efficient method for direct shoot regeneration from leaf explants of *solanum nigrum* induced by Thidiazuron. Int J Agric Biol 19:348–354
3. Aharon GS, Apse MP, Duan S, Hua X, Blumwald E (2003) Characterization of a family of vacuolar Na⁺/H⁺ antiporters in *Arabidopsis thaliana*. Plant Soil 253:245–256
4. Ahmet MK, Sezai E (2015) Callus induction, shoot proliferation and root regeneration of potato (*Solanum tuberosum* L.) stem node and leaf explants under long-day conditions. Biotechnology & Biotechnological Equipment Vol 29 No 6 1075-1084
5. Al-Mansoori TA, Alaa El-Deen MN, Caligari PD (2006) Evaluation of in vitro screening techniques for salt tolerance in date palm. In: III International Date Palm Conference Vol 736: 301–307
6. Arinaitwe AB, Magembe E, Barekye A, Rob S. (2018) In vitro somatic embryogenesis and regeneration potential of two potato varieties in Uganda. African Journal of Biotechnology Vol 17(15) 515-520
7. Bayat F, Shiran B, Belyaev DV, Yur'eva N, Sobol'kova GI (2010) Potato plants bearing a vacuolar Na⁺/H⁺ antiporter HvNHX2 from barley are characterized by improved salt tolerance. Russ J Plant Physiol 57: 696-706
8. Beaujean A, Sangwan RS, Lecardonnel A, Sangwan-Norreel BS (1998) Agrobacterium-mediated transformation of three economically important potato cultivars using sliced internodal explants: An efficient protocol of transformation. J Exp Bot 49:1589–95
9. Borna RS, Hoque MI, Sarker RH (2010) Agrobacterium mediated genetic transformation for local cultivars of potato (*Solanum tuberosum* L.) using marker genes. Plant Tiss Cult Biotechnol 20: 145-155
10. Borsani O, Valpuesta V, Botella MA (2003) Developing salt tolerant plants in a new century: a molecular biology approach. - Plant Cell Tissue Organ Culture 73: 101-115

11. Ehsanpour AA, Jones MGR (2000) Evaluation of direct shoot regeneration from stem explants of potato (*Solanum tuberosum* L.) cv. Delaware by thidiazuron TDZ. *J Sci Tech Agric Natl Res* 4: 47-54
12. Fan X, Hu H, Huang G, Huang F, Li Y, Palta J (2015) Soil inoculation with *Burkholderia* sp. LD-11 has positive effect on water-use efficiency in inbred lines of maize. *Plant Soil* 390:337-349
13. Fiegert AK, Mix-Wagner G, Vorlop KD (2000) Regeneration of *Solanum tuberosum* L. cv. Tomensa: Induction of somatic embryogenesis in liquid culture for the production of artificial seed. *Landbauforschung Volkenrode* 50: 199-202
14. Fukuda A, Nakamura A, Tanaka Y (1999) Molecular cloning and expression of the Na⁺/H⁺ exchanger gene in *Oryza sativa*. *Biochim Biophys Acta*. 1446: 149–155
15. Hansen J, Nielsen B, Nielsen SVS (1999) In vitro shoot regeneration of *solanum tuberosum* cultivars: Interactions of medium composition and leaf, leaflet and explant position. *J Natl Sci Found Sri-Lanka* 27: 17-28
16. Hilal M, Zenoff AM, Ponessa G, Moreno H, Massa ED (1998) Saline stress alters the temporal patterns of xylem differentiation and alternative oxidative expression in developing soybean roots. *Plant Physiol* 11: 695-701
17. Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Sci* 227:1229-1231
18. Imamul Huq SM, Alam MD (2005) A hand book on analyses of soil, plant and water. Bacer-Du, University of Dhaka Bangladesh 246p
19. Jayasree T, Pavan U, Ramesh M, Rao AV, Reddy KJM, Sadanandam A. (2001) Somatic embryogenesis from leaf cultures of potato. *Plant Cell Tissue Organ Culture* 64: 13-17
20. Katerji N, Van-Hoorn J, Hamdy A, Mastrorilli M (2000) Salt tolerance classification of crops according to soil salinity and to water stress day index. *Agric. Water Manage* 43:99–109
21. Khalafalla MM, AbdElaleem KG, Modawi RS (2010) Callus formation and organogenesis of potato (*Solanum tuberosum*L.) cultivar Almera. *J Phytol* 2:40–46.
22. Khan MS, Ahmad D, Khan MA (2015) Trends in genetic engineering of plants with (Na⁺/H⁺) antiporters for salt stress tolerance. *Biotechnol Biotechnol Equip* 29:815–825
23. Krivosheeva A, Varlamova T, Yurieva N, Sobol'kova G, Kholodova V, Belyaev D (2014) Potato transformation with the HvNHX3 gene and the improvement of transformant salt tolerance. - *Russ J Plant Physiol* 61: 792-800
24. Li Y, Zhang Y, Feng F, Liang D, Cheng L, Ma F, Shi S (2010) Overexpression of a *Malus vacuolar* Na⁺/H⁺ antiporter gene (MdNHX1) in apple rootstock M.26 and its influence on salt tolerance. *Plant Cell Tissue Organ Culture* 102:337–345
25. Lopez CML, Takahashi H, Yamazaki S (2002). Plant–water relations of kidney bean plants treated with NaCl and foliarly applied glycinebetaine. *J Agron Crop Sci* 188:73–80
26. Maas EV (1985) Crop tolerance to saline sprinkling water. *Plant Soil* 89:273–284

27. Mc Mannus MT, Bielesek RL, Caradus JR, Barker DJ (2000) Pinitol accumulation in mature leaves of white clover in response to water deficit. *Environ Exp Bot* 43: 11-18
28. Metwali EM, Soliman HI, Fuller MP, Al-Zahrani H S and Howladar S M (2015) Molecular cloning and expression of a vacuolar Na⁺/H⁺ antiporter gene (AgNHX1) in fig (*Ficus carica* L.) under salt stress. *Plant Cell Tissue Organ Culture* 123: 377–387
29. Molla MM, Nasiruddin KM. Al-Amin M, Haque MS, Khanam D (2011) In vitro regeneration of potato (*Solanum tuberosum* L.). *Bangladesh J Plant Breed Genet* 24: 07-14
30. Mosaad IS, Serag AH, Moustafa-Farag M, Seadh AK (2020) Effect of exogenous proline application on maize yield and the optimum rate of mineral nitrogen under salinity stress. *J Plant Nutr* 43:354-370
31. Mousavi SN, Ebadi M, Khorshidi M, Hokmabadi H, Masoudian N (2020) Effect of salinity on photosynthetic and enzymatic activities and tuberization yield in the genotype of potato cultivar Agria under in vitro conditions. *J Neotropical Agric* 7:8-19
32. Munns R (2002) Comparative physiology of salt and water stress. - *Plant Cell Environ* 25: 239-250
33. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473-479
34. Mutasim MK, Khadiga GAE, Rasheid SM (2010) Callus formation and organogenesis of potato (*Solanum tuberosum* L.) cultivar Almera. *J Phytology* 2:40–46
35. Naseem H, Bano A (2014) Role of plant growth-promoting rhizobacteria and their exopolysaccharide in drought tolerance of maize. *J Plant Interact* 9:689-701
36. Rabie G, Almadini A (2005) Role of bioinoculants in development of salt tolerance of *Vicia faba* plants under salinity stress. *African Journal of Biotechnology* 4:210-222
37. Rodriguez E, Trujillo C, Orduz S, Jaramillo S, Hoyos R, Arango R (2000) Standardization of an appropriate culture medium for the regeneration of leaf explants using two Colombian potato varieties (*Solanum tuberosum* L.). *Revista Facul National de Agron Msdellin* 53: 887-899
38. Sajal R, Nasrin C (2020) Effects of leaching on the reclamation of saline soils as affected by different organic and inorganic amendments. *J Environ Sci Sustain Develop* 3: 329-354
39. Sambrook J, Russel D (1989) *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbour Laboratory Press, 215 p. ISBN 0-87-969577-3
40. Serine S, Hasan T, Chowdhury H , Islam A , Hossain B (2020) Callus induction and virus free potato mini-tuber production through meristem culture. *European Journal of Agriculture and Food Sciences* 2(1)
41. Shi H, ha Lee B, Jye Wu S, Zhu JK (2003) Overexpression of a plasma membrane Na⁺/H⁺ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nat Biotechnol* 21:81–85
42. Shirin F, M Hossain, MF Kabir, M Roy, SR Sarker (2007) Callus induction and plant regeneration from internodal and leaf explants of four potato cultivar. *World J Agril Sci* 3: 01-06

43. Soliman HI (2018a) Production of genetically modified grape (*Vitis vinifera* L.) plants. *International Journal of Horticulture, Agric Food Sci* 2:111-120
44. Soliman HI (2018b). Molecular cloning of sucrose isomerase gene and *Agrobacterium*-mediated genetic transformation of potato (*Solanum tuberosum* L.) plants. *International Journal of Environment Agriculture and Biotechnology* 3 (3): 864-874
45. Steel RGD, Torrie JH, Dicky DA (1997) *Principles and Procedures of Statistics, A Biometrical Approach*. 3rd Edition, McGraw Hill, Inc. Book Co., New York 352-358
46. Szalonek M, Sierpien B, Rymaszewski W, Gieczewska K, Garstka M (2015) Potato annexin STANN1 promotes drought tolerance and mitigates light stress in transgenic *Solanum tuberosum* L plants. *PLoS ONE* 10: 1-38
47. Tara SR, Krishnaprasad B, Veena SA 2017 Direct and indirect regeneration of potato cultivar kufriyoti. *J of Biotechnol Biochemi* 3:31–34
48. Thirukkumaran G, Ntui VO, Khan RS, Mii M (2009) Thidiazuron: an efficient plant growth regulator for enhancing *Agrobacterium*-mediated transformation in *Petunia hybrida*. *Plant Cell Tissue Organ culture* 99:109-115
49. Wendt SN, Peters JA, Oliveira AC, Bobrowski VL, Costa FLC, Madruga CS, Vighi IL (2001) Plant regeneration and molecular characterization of potato cultivar Macaca obtained from gamma irradiated explants. *J New Seeds* 3: 17-37
50. Yadav NR, Sticklen MB (1995) Direct and efficient plant regeneration from leaf explants of *Solanum tuberosum* L. cv. Bintje. *Plant Cell Tissue Organ Culture* 14: 645-647

Tables

Table 1. Influence of plant growth regulators concentrations on callus induction frequency percentage, callus fresh weight/explant and relative water content (%) of the potato (*Solanum tuberosum*) cv. Spunta via leaf explants after 4 weeks.

Growth regulator (mg l ⁻¹)		Frequency (%) of callus induction	callus fresh weight/explant (g)	Relative water content (RWC)	Shoot regeneration
NAA	BAP				
0.5	0.25	±0.59g57.5	2.85±0.66h	±0.66h46.36	++
1.0	0.25	69.2±0.54f	2.57±0.59i	±0.59g52.45	++
2.0	0.25	92.5±0.72a	2.25±0.43j	±0.43b79.90	+++
3.0	0.25	79.8±0.61c	±0.38k2.05	42.39±0.27i	++
0.5	0.5	±0.27j48.0	3.88±0.69d	±0.65f62.80	+
1.0	0.5	±0.48h55.0	3.68±0.57e	±0.71e65.20	++
2.0	0.5	±0.53b89.2	3.52±0.55f	±0.89a81.35	+++
3.0	0.5	±0.44e71.5	3.37±0.62g	±0.60c72.25	+
0.5	1.0	±0.66k32.5	4.65±0.73a	55.40±0.49g	+
1.0	1.0	±0.39i51.0	±0.65b4.33	62.70±0.55f	+
2.0	1.0	±0.58d77.8	±0.44c4.20	±0.64c73.25	++
3.0	1.0	±0.49f69.3	±0.39d3.87	69.75±0.78d	++

Values are means ± standard error of three replicates from two experiments. For each experiment, bars with the same letters are not significantly different at $P \leq 0.05$ level. Shoot regeneration are visually estimated as No regeneration= -, Poor= +, Good= ++, very good= +++

Table 2. Influence of plant growth regulators concentrations; Zeatine riboside (ZR), thidiazoron (TDZ) and α -Naphthalene Acetic Acid (NAA) on regeneration percentage, number of shoots/explant and shoot length/ explant of the potato (*Solanum tuberosum*) cv. Spunta after 35 days.

Growth regulators (mg l ⁻¹)			Regeneration (%)	No. shoots/explant	Shoot length/explant (cm)
ZR	TDZ	NAA			
0.5	0.0	0.25	78.5±0.67g	5.85±0.35f	2.00±0.55e
1.0	0.0	0.25	91.8±0.62e	7.05±0.42d	2.80±0.62d
2.0	0.0	0.25	a±0.9398.6	9.65±0.78a	3.95±0.78a
3.0	0.0	0.25	97.5±0.89b	8.52±0.65b	3.60±0.69b
0.0	1.0	0.0	68.4±0.17i	4.35±0.28h	0.85±0.32i
0.0	2.0	0.0	77.2±0.69h	5.71±0.32f	1.05±0.41h
0.0	3.0	0.0	89.7±0.61f	6.45±0.54e	1.55±0.38g
0.0	4.0	0.0	±0.89d92.5	±0.61c7.98	±0.49f1.79
0.5	0.0	0.0	74.5±0.58h	4.65±0.25h	1.82±0.52f
1.0	0.0	0.0	88.7±0.39f	5.75±0.38f	2.25±0.53e
2.0	0.0	0.0	±0.60e91.5	5.40±0.30g	3.32±0.74c
3.0	0.0	0.0	90.8±0.52e	6.25±0.40e	3.15±0.67c
0.0	1.0	0.25	77.5±0.33h	5.30±0.29g	1.78±0.50f
0.0	2.0	0.25	82.8±0.41g	6.50±0.45e	1.87±0.51f
0.0	3.0	0.25	91.9±0.63e	6.97±0.47d	1.94±0.64f
0.0	4.0	0.25	95.8±0.79c	8.25±0.62c	2.28±0.57e
0.5	0.0	0.5	76.4±0.68h	5.45±0.36g	1.90±0.63f
1.0	0.0	0.5	88.4±0.71f	6.28±0.41e	2.45±0.49d
2.0	0.0	0.5	b±0.8897.3	8.75±0.71b	3.30±0.72c
3.0	0.0	0.5	95.5±0.82c	8.14±0.58c	3.25±0.82c
0.0	1.0	0.5	72.3±0.25h	5.25±0.28g	1.60±0.40g
0.0	2.0	0.5	80.0±0.49g	6.05±0.39e	1.75±0.48f
0.0	3.0	0.5	87.8±0.65f	7.15±0.52d	1.83±0.56f
0.0	4.0	0.5	92.8±0.70d	7.36±0.57d	2.05±0.59e

Values are means ± standard error of three replicates from two experiments. For each experiment, bars with the same letters are not significantly different at $P \leq 0.05$ level.

Figures

Figure 1

Diagram of T-DNA of the expression binary vector pBI121 containing the *AgNHX1* gene under the control of cauliflower mosaic virus 35S (P35S) promoter cauliflower mosaic virus 35S promoter and nopaline synthase (**NOS**) terminator with selective kanamycin resistance (*nptII*) gene selectable marker gene driven by NOS promoter. **RB**: Right border, **LB**: left border.

Figure 2

Callus induction and shoot regeneration of potato (*Solanum tuberosum*) cultivar Spunta. Callus formation was obtained on MS medium supplemented with 2.0 mg l⁻¹ NAA and 0.25 mg l⁻¹ BAP after 4 weeks **(A)**. In vitro shoot formed when cultured callus on MS medium supplemented with 2.0 mg l⁻¹ ZR and 0.25 mg l⁻¹ NAA **(B)**. Shoot induction obtained on MS medium supplemented with 4.0 mg l⁻¹ TDZ in combination with 0.25 mg l⁻¹ NAA **(C)**. In vitro shoot obtained on MS medium containing 0.5 mg l⁻¹ BAP, 1.0 mg l⁻¹ PBZ after four weeks **(D)**.

Figure 3

Production of potato (*Solanum tuberosum*) cultivar Spunta carrying the *AgNHX1* gene. Putative potato shoots formed when cultured callus on MS medium supplemented with 2.0 mg l⁻¹ ZR and 0.25 mg l⁻¹ NAA (A), in vitro rooting (B), *in vitro* microtubers were obtained on MS medium containing 0.5 mg l⁻¹ BAP, 1.0 mg l⁻¹ PBZ and 80 g l⁻¹ sucrose at 26±2°C in complete dark for two months (C) and acclimatization of potato plantlets to ex vitro conditions (D).

Figure 4

PCR analysis of DNA with *AgNHX1* gene in potato (*Solanum tuberosum*). Lanes T1-T7 PCR detection of *AgNHX1* gene from putative transgenic potato plants amplifying about 2.6 kb. Lane P: DNA amplified from pBI121- *AgNHX1* (**positive control**). Lane C: DNA from non-transformed plants (negative control). Lane M: TrackIt™ 1 Kb DNA Ladder.

Figure 5

RT-PCR-amplified fragment (448bp) with a primer set designed for *AgNHX1* gene in potato (*Solanum tuberosum*) cv. Spunta. PCR products are electrophoresed on 1 % agarose gel and stained with ethidium bromide. Lanes R1-R6 transformed potato plants. Lane C. Non-transformed plant. Lane M: TrackIt™ 1 Kb DNA Ladder.

Figure 6

Northern blot analysis for the confirmation of transformed potato plants. Total RNA (10 µg) was supplied for northern blot analysis for *AgNHX1* gene (A). *AgNHX1* mRNA was hybridized with the α -³²P labeled cDNA (B). Lane C: non-transformed plant and Lanes R1- R6 transformed potato plants.

Figure 7

Evaluating transgenic potato plants carrying *AgNHX1* gene under different NaCl treatments. In vitro regenerated shoots of potato (*Solanum tuberosum*) after 4 weeks from culturing on medium supplemented with 250 mM NaCl (A). In vitro rooted shoots of potato after 4 weeks from culturing on different NaCl treatments (B).

Figure 8

Shoot fresh weight (FW)/plantlet, dry weight (DW)/ plantlet (gm), microtuber weight /plantlet (gm) and Na⁺ content (mg–1 DW) for *AgNHX1*-expressing potato plantlets cv. Spunta and non-transgenic plantlets as control under different NaCl treatments.

Figure 9

Effects of different levels of NaCl on proline content of in vitro shoot fresh weight (FW)/ plant ⁻¹ of potato (*Solanum tuberosum*) cultivar Spunta of AgNHX1 expressing transgenic plants compared with non-transgenic plants