Salinity stress response of non transformed and *AtCKX* transgenic centaury (*Centaurium erythraea* Rafn.) shoots and roots grown *in vitro*

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Summary

Common centaury (*Centaurium erythraea* Rafn.) is a plant species that can inhabit saline soils. It is known as plant with high spontaneous regeneration potential *in vitro*. In present work we evaluated shoots and roots salinity tolerance of non transformed and three *AtCKX* transgenic centaury lines to graded NaCl concentrations (0, 50, 100, 150, 200 mM) *in vitro*. Overexpression of *AtCKX* genes in transgenic centaury plants resulted in an altered cytokinins (CKs) profile leading to a decline of bioactive CK levels and, at the same time, increased contents of storage CK forms, inactive CK forms and/or CK nucleotides. Significant increment of fresh shoot weight was obtained in shoots of non transformed and *AtCKX1* transgenic line only on medium supplemented with 50 mM NaCl. However two analyzed *AtCKX2* transgenic lines reduced shoot growth at all NaCl concentrations. In general, centaury roots showed higher tolerance to salinity than shoots. Non transformed and *AtCKX1* transgenic lines tolerated up to 100 mM NaCl without change in frequency of regeneration and number of regenerated plants. Roots of two analyzed *AtCKX2* transgenic lines showed different regeneration potential under salt stress. Regeneration of transgenic *AtCKX2*-26 shoots even at 200 mM NaCl was recorded. Salinity stress response of centaury shoots and roots was also evaluated at biochemical level. Free proline, malondialdehyde and hydrogen peroxide content as well as antioxidative enzymes activities were investigated in shoots and roots after 1, 2, 4 and 8 weeks. In general, adition of NaCl in culture medium elevated all biochemical parametars in centaury shoots and in roots. Considering that all analyzed *AtCKX* transgenic centaury lines showed altered salt tolerance to graded NaCl concentrations *in vitro* it can be assumed that CKs might be involved in plant defence to salt stress conditions.

Key words: centaury, regeneration *in vitro*, *AtCKX*, NaCl, oxidative stress, reactive oxygen species, cytokinins

Introduction

Stress represents the specific response of the biological system to negative factors that altered previously existing homeostasis. Stress is reflected on changes in the physiological state of organism, which lead to endangerment of regular life processes in plants (Gaspar et al. 2002). Plants are sedentary organisms and they are constantly exposed to numerous stressful environmental conditions. The stress factors can be abiotic, which are basically physical-chemical origin, and biotic with biological nature. Both types of stress adversely have effect on plant growth and development. In natural ecosystems, stress factors are closely related and their physiological effects are often interleave (Krasensky and Jonak 2012). On the other hand, in controlled laboratory conditions, it is possible to investigate the effect of only one selected stress factor such as investigation of salinity stress by growing of plants on graded NaCl concentrations.

Salinity represents one of the major abiotic stresses limiting yield and productivity of crops all over the world (Ashraf et al. 2008). The high concentration of salt in the soil in two ways endangered the plant growth and development (Sairam and Tyagi 2004). Firstly, low osmotic potential of the substrate disabled plant to absorb enough water and maintain a balance between transpiration and water absorption. In this first phase, known as osmotic stress, rapid growth reduction of young leaves and shoots is noticed. In the second phase, known as ionic stress, plants could not prevent passive salinization and accumulation of Na^+ and Cl^- ions primarily in older leaves. In most plant species, toxic concentrations of Na^+ ions are much higher than in the case of Cl^- ions. As a result of osmotic and ionic stress, salinity usually leads to oxidative stress featured by the appearance and accumulation of reactive oxygen species (ROS) and free radicals (Ashraf 2009). These compounds can damage plant cell membrane and impair functions of proteins, enzymes and nucleic acids function. The effect of salinity is similar as the effect of water deficiency and the most plant species exposed to salinity respond in the same way as plants exposed to water deficit (Acosta-Motos et al. 2017). In the case of water deficiency there is no physically accessible water for absorbtion. Toward this, in osmotic stress water is present often in large quantities, but the plants since the low water potential of the substrate can not absorb enough water.

Plant species acclimated to salinity in arid and semiarid regions all over the world are able to grow and complete their life cycles normally. The ability for acclimation on salinity depends on plant species and vary of interactions between salinity and environmental factors such as soil, water and climatic conditions (Rai et al. 2011). Most of plant species hardly survive or even die on 100 mM NaCl (Wang et al. 2019). In order to accommodate to salinity, the plants have developed numerous strategies, including modifications in various physiological and biochemical processes to survive under unfavorable conditions. Biochemical pathways leading to improved plant salt tolerance are connected with employment of a number of antioxidant compounds such as ascorbate, glutathione, proline, carbohydrates, carotenoids, and antioxidative enzymes such as superoxide dismutase, catalase, peroxidase and enzymes of ascorbate-glutatione cycle (Ashraf et al. 2010). Numerous studies have shown increased proline content in plants grown under different environmental conditions. Proline accumulation has been reported during drought, high salinity stress, UV irradiation, heavy metals, oxidative stressand also in response to biotic stress (Szabados and Savouré 2010). Metabolism and proline accumulation were mostly studied in plants exposed to osmotic stress (Verbruggen and Hermans 2008). It was shown that free proline content varies from species to species but in stress conditions the content can increase even 100 times compared to control. It was also noticed that accumulation of proline is higher in stress tolerant genotypes than in sensitive genotypes. Proline is an important component in the protection of the cell membrane against

oxidative stress induced by salt stress (Okuma et al. 2000). In addition, proline stabilize the protein structure and increased accumulation of proline affects the redox balance in the cell (Maggio et al. 2002). Also there are literature data describing that exogenously applied proline contributes to osmoregulation, plant growth and increased resistance of plants to stress factors (Ashraf and Foolad 2007).

Lipid peroxidation products such as malondialdehyde (MDA) is a decomposition product of polyunsaturated fatty acids of biomembranes and considered as useful and reliable indicator of oxidative damage of membranes caused by ROS (Jiang and Zhang 2001). Measurements of thiobarbituric acid reactive substances (TBARS) have been made last fifty years, often using the method of Heath and Packer (1968). Stress-induced toxic oxygen species, such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) serve as important signaling molecules in plants grown under various stress conditions (Ślesak et al. 2007). During oxidative stress H_2O_2 is produced in chloroplasts and mitochondria through electron transport (Lu et al. 2009). In this process, firstly oxygen is reduced to superoxide and further to H_2O_2 spontaneously. It is known that H_2O_2 production increased as plants response to abiotic stress (Bat'ková et al. 2008). Because of high toxicity, the endogenous H_2O_2 level is strictly controlled by two major enzymatic systems. Conversion of oxygen to superoxide and further to H_2O_2, is also catalyzed by H_2O_2 generating enzyme known as superoxide dismutase (SODs; EC 1.15.1.1) (Møller, 2001) while removing of peroxide is enabled by H_2O_2 scavenging enzymes such as catalase (CAT, EC 1.11.1.6.) and peroxidase (POX, EC 1.11.1.x) (Sofo et al. 2015).

Centaurium erythraea Rafn., known as common centaury, belongs to the Gentianaceae family and represent the most investigated plant species among genus *Centaurium*. Centaury is an annual or biennial plant species, distributed in northern hemisphere, Great Britain, Scandinavian and Mediterranean countries, Southwest Asia and North Africa (Chevallier 2000). Numerous plant species of genus *Centaurium* can also be found in North America, California and Mexico (Jäger 1978). Centaury is well-known medicinal plant species and has been used for centuries to cure febrile conditions, regulate blood sugar, treat anaemia, jaundice and gout, and increase appetite and stimulate digestion. Centaury also represent the cosmopolitan plant species that inhabits mountain slopes, dry grasslands and scrublands, as well as saline soils. There are few reports about salinity stress response of centaury species grown *in vitro* (summarized in Petropoulos et al. 2018) but only one report about common centaury (Šiler et al. 2007). It was reported that root culture of common centaury is tolerant to salinity (up to 200 mM NaCl) without change in shoot and root growth and biomass production (Šiler et al. 2007).

Plant growth regulators play important physiological role in plant responses to environmental conditions including salinity tolerance of plants (Atia et al. 2018, Llanes et al. 2018). Besides investigations considering the effect of exogenous plant growth regulators on salinity stress tolerance, important information may be obtained with genetically modified plant species with altered level of endogenous plant hormones. There are literature data describing both positive and negative influences of cytokinins (CKs) on stress tolerance (Veselov et al. 2017). Cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) is the only known catabolic enzyme that specifically catalyses cytokinin degradation in plant tissues (Mok and Mok 2001, Schmülling et al. 2003). Overexpression of the *AtCKX* genes in transgenic centaury plants resulted in an altered CKs profile leading to a decline of bioactive CK levels and, at the same time, increased contents of storage CK forms, inactive CK forms and/or CK nucleotides (Trifunović et al.

2015). The regeneration of transgenic *AtCKX1* and *AtCKX2* centaury plants were also described recently (Trifunović et al. 2013).
The aim of present work was to evaluate shoots and roots salinity tolerance response of non transformed and three transgenic centaury *AtCKX* lines in regard to salinity caused by increased NaCl concentrations *in vitro*. The main focus was to investigate the difference in regeneration potential as well as accumulation of free proline and oxidative stress markers (MDA and H_2O_2) and antioxidant enzyme systems in centaury shoots and roots.

Materials and methods

Plant material

Non transformed and transgenic *AtCKX1* and *AtCKX2 C. erythraea* plants was initiated and maintained as described previously by Trifunović et al. (2013). Mother stock cultures of non transformed and one *AtCKX1* (line 29) and two *AtCKX2* (lines 17 and 26) transgenic centaury lines were selected as a primary plant material.

Growth media and culture conditions

Shoot and root explants of non transformed and transgenic *AtCKX1* and *AtCKX2* lines (≈ 10 mm long) were separately cultured *in vitro*, on half-strength MS medium (½MS, Murashige and Skoog 1962) solidified with 0.7% agar and supplemented with 3% sucrose, 100 mg l^{-1} *myo*-inositol. All explants were cultured on ½MS hormone free or ½MS medium supplemented with graded concentrations of NaCl (0, 50, 100, 150 and 200 mM). The medium was adjusted to pH 5.8 with NaOH/HCl and autoclaved at 121 °C for 25 min. Shoot explants (10) were cultured in 1l bottles with 100 ml of culture media, while root explants (20-25) were grown in petri dishes (∅ 10 cm) filled with 25 ml of culture media. All *in vitro* cultures were grown at of 25±2 °C and a 16/8h light/dark photoperiod ("Tesla" white fluorescent lamps, 65W, 4500K; light flux of 47 μmol s^{-1} m^{-2}).

Effect of NaCl on *in vitro* regeneration in shoot and root culture

In shoot culture the change of fresh shoot weight was recorded after eight weeks. In root culture, the frequency of shoot regeneration and the average number of regenerated shoots per root explant of non transformed and transgenic *AtCKX1* and *AtCKX2* lines were evaluated also after eight weeks. All *in vitro* culture experiments were repeated three times, with 90 explants per each treatment.

Analysis of biochemical parametars in shoot and root cultures during growth under salt stress

All biochemical parametars (free proline, MDA, H_2O_2 and activity of antioxidative enzymes) were determined at the beginning of shoot and root culture and after one, two, four and eight weeks of growth on ½MS medium supplemented with graded concentrations of NaCl.

Determination of free proline

Free proline content was determined by ninhydrin reaction described by Bates et al. (1973). The plant material (250 mg) was homogenized in liquid nitrogen. Extraction of free amino acids was carried out in HPLC-grade methanol. The cell debris was spun down and supernatant was

mixed with chloroform and HPLC-grade water. The aqueous phase was reextracted with chloroform, separated and evaporated to dryness. The samples were resuspended in water and plant sample (or proline standard) was mixed with ninhydrin reagent. The samples were further incubated in water bath at 100 °C for 4 min, cooled and diluted in ethanol. The proline reacts with ninhydrin (2,2-dihydroxyindane-1,3-dione) and produce a yellow compound (Friedman 2004). The absorbance of the yellow reaction product was measured at 350 nm and corrected for background absorbance using reactions with ethanol instead of ninhydrin, prepared in parallel for each sample, as blanks. The proline content was determined from the standard curve. The results are presented as μM/g of fresh sample weight.

Lipid peroxidation

The level of lipid peroxidation was measured as malondialdehyde (MDA) content by the procedure described by Heath and Packer (1968). The plant material (100 mg) was homogenized in liquid nitrogen and extracted with 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 4 °C for 10 min and the supernatant was mixed with 20% TCA and 0.5% 2-thiobarbituric acid (TBA). The reaction mixture was heated at 95 °C for 30 min in water bath and then cooled on ice. After centrifugation at 4 °C for 10 min MDA was determined spectrophotometrically. The absorbance of the supernatant was measured at 532 and 600 nm. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of H_2O_2

Content of H_2O_2 was determined as described by Velikova et al. (2000). In breaf, plant material (150 mg) was homogenized in liquid nitrogen and extracted with 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 4 °C for 15 min and the supernatant was further mixed with 10 mM potassium phosphate buffer (pH 7.0) and 1M KI. H_2O_2 content was determined spectrophotometrically by measuring the absorbance at 390 nm. The concentration of H_2O_2 was calculated using its extinction coefficient of 0.28 mM^{-1} cm^{-1}.

Enzyme extraction

Plant material (500 mg) was homogenized in 0.1 M potassium phosphate extraction buffer (pH 7), containing 1.5% insoluble polyvinylpyrrolidon, 10 mM dithiothreitol (DTT) and 1 mM phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 4 °C for 5 min and protein content was further determined from supernatant according to Bradford (1976). Total protein content was determined from the standard curve made using serum albumin solution. Quantification of SOD, CAT and POX was further performed.

Quantification of SOD activity

SOD activity was determined spectrophotometrically by a modified method of Beyer and Fridowich (1987). The reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM ethylenediaminetetraacetic acid, 260 mM methionine, 1.5 mM nitroblue tetrazolium chloride (NBT) and 0.04 mM riboflavin. For each sample, six dilutions were prepared (sample volume: 0, 5, 10, 15, 20 and 25 μl; buffer volume: 800, 795, 790, 785, 780 and 775 μl) and placed in a microtitar plate. The reaction mixture was further illuminated for 30 min at 25 °C and then the absorbance was measured at 540 nm. One unit of SOD activity is the amount of sample required for 50% inhibition of NBT photoreduction and is presented as specific activity (U/mg). All measurements were repeated three times.

Quantification of CAT activity

Catalase activity was determined spectrophotometrically by the method of Aebi (1984). The method is based on monitoring the kinetics of disappearance of H_2O_2 which can be detected by measuring the decrease in absorbance (at 240 nm) of reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0), 20 mM H_2O_2 and enzyme extract. Catalase activity was measured every 20 s for 3 min at a temperature of 20 °C. Unit of catalase activity is defined as the amount of enzyme that degrades 1 µmol of H_2O_2 in 1 min and is indicated as µmol min^{-1} mg^{-1} (U/mg).

Quantification of POX activity

Activity of POX was determined spectrophotometrically by the method of Kukavica and Veljović-Jovanović (2004). The reaction mixture contained 0.05 M potassium phosphate buffer (pH 6.5) and 1M pirogallol as enzyme substrate. The reaction was started by addition of 30% H_2O_2 after the first 20s. The POX catalyzed oxidation of pyrogallol with H_2O_2 to purpurogallin which was monitored by absorbance determination at 430 nm. Enzyme activity is indicated as µmol min^{-1} mg^{-1} (U/mg).

Statistical analysis

The effect of graded NaCl concentrations (0-200 mM) on plant growth and regeneration potential of non transformed and transgenic *AtCKX* lines plants were evaluated using standard two-factor analysis of variance (ANOVA). The same method was also used for analyzis of biochemical parameters during 8 weeks of culture for each line separately. The results are presented as mean ± SE. All percentage data were subjected to angular transformation and number data to square root transformation prior to analysis. After analysis, data were subjected to inverse transformation for presentation. The comparisons between the mean values were made using a Fisher LSD (the least significant difference) post-hoc test calculated at a confidence level of $p \leq 0.05$.

Results

The effect of NaCl on *in vitro* regeneration in shoot culture of *C. erythraea*

Centaury shoots succesfully grown eight weeks under NaCl-induced stress conditions. Under control conditions, on medium without NaCl, *AtCKX*-altered transgenic centaury shoots showed enchanced growth in comparison to non transformed shoots (**Fig 1**). Non transformed and *AtCKX1* transgenic centaury shoots cultured on graded NaCl concentrations showed increased fresh shoot weight only on medium supplemented with 50 mM NaCl compared to other NaCl concentration and corresponding control. The addition of NaCl in culture medium significantly reduced growth of both analyzed *AtCKX2* transgenic shoots on all graded NaCl concentration. Regardless to reduced growth and changed morphology, non transformed and *AtCKX* transgenic shoots tolerated and succesfully survived on medium supplemented with 200 mM NaCl (**Fig 2**). It was also noticed that non transformed and all transgenic *AtCKX* centaury shoots spontaneously rooted on NaCl free medium while root development was inhibited on medium supplemented with NaCl.

The effect of NaCl on *in vitro* regeneration in root culture of *C. erythraea*

Contrary to shoot culture, the addition of graded concentration of NaCl into the culture medium had lower negative effect on *in vitro* shoot induction in root culture of *C. erythraea*. Non transformed and *AtCKX1* transgenic roots tolerated more salinity in culture medium than *AtCKX2* transgenic roots (**Fig 3**). The frequency of shoot regeneration remained stable on root explants of non transformed and *AtCKX1* transgenic line cultured on ½MS hormone free medium up to 100 mM of NaCl (**Fig 3a**). Significant increment in number of regenerated shoots was noticed in culture of non transformed roots grown on medium supplemented with 100 mM of NaCl (**Fig 3b**). Regeneration potential of root explants of two analyzed *AtCKX2* transgenic lines varied greatly. The lowest average number of regenerated shoots per root explant was recorded in transgenic centaury line *AtCKX2*-17. In all analyzed *AtCKX2* centaury lines, decreased frequency of shoot regeneration noticed when NaCl concentration was increased. Statistically significant decrement of the average number of regenerated shoots of non transformed and all analyzed transgenic centaury lines noticed on media supplemented with 150 and 200 mM NaCl. It was also noticed that in culture of non transformed and two *AtCKX* transgenic centaury lines (*AtCKX1*-29 and *AtCKX2*-17) there were no shoot induction on root explants grown on medium supplemented with 200 mM NaCl. It was interestingly to note that *AtCKX2*-26 transgenic centaury shoots regenerated in root culture even at 200 mM NaCl. All centaury shoots, regenerated in root culture on ½MS media supplemented with different concentrations of NaCl, developed typical rosette form and also showed a similar morphology as shoots regenerated in control root cultures grown without addition of NaCl (**Fig 4**).

Analyzis of biochemical parametars during salt stress in shoot and root of *C. erythreae*

Proline, MDA, H_2O_2 content as well as the activity of antioxidative enzymes was determined at the beginning of *in vitro* culture and after 1, 2, 4 and 8 weeks of shoot and root culture *in vitro* on ½MS hormone free medium supplemented with graded NaCl concentrations. The content of different biochemical parametars in shoots during this period of time is presented above axis line while the results considering roots are presented under axis line.

The shoots of non transformed and *AtCKX* transgenic lines showed the lowest proline content immediately after excision of explants (**Fig 5**). The increment in proline content was observed after cultivation on all nutrient media, control without addition of NaCl as well as on media supplemented with graded NaCl concentrations. The proline content in shoots was gradually increased with enhancement of NaCl in culture media. Contrary to shoots, significant increment of proline content was recorded at all culture media in non transformed roots already after one week. After this first increment of proline content in roots, further decrease was noticed. The highest reduction of proline content was determined after eight weeks of growth on NaCl free medium. Compared to all analyzed samples the highest proline content was recorded in *AtCKX1*-29 roots immidiately after excision. In this transgenic line, after one week of cultivation on NaCl free medium significant reduction of proline content was observed. Proline content remains low during further eight weeks of cultivation. High NaCl concentrations in nutrient medium (>100 mM) notably increased proline content in *AtCKX1*-29 roots. The same pattern of the reduction of proline content was observed in *AtCKX2*-17 transgenic roots cultivated on NaCl free medium. In this transgenic line increased proline content recorded after two weeks on medium supplemented with high NaCl concentration (>100 mM). Continuous increment of proline content was recorded in transgenic *AtCKX2*-26 roots after prolonged cultivation. Also, in this transgenic line, continious increment of proline content in roots was determined during cultivation on NaCl. In general, we can conclude that free proline content, under salt stress, was lower in all transgenic than in non transformed roots.

Salt stress induced a significant changes of MDA accumulation caused by cell membrane lipid peroxidation in all analyzed centaury lines (**Fig 6**). In all analyzed centaury shoots, in comparison to roots, increased MDA content was determined. It was interesting to note that after one week in non transformed shoots grown on NaCl free medium increased MDA content was recorded. Toward this, in the same experimental conditions in transgenic centaury shoots decreased MDA content was detected. Higher NaCl concentrations (>100 mM) induced significant MDA accumulation in non transformed shoots during *in vitro* culture. The similar pattern was observed in shoots of two transgenic lines, *AtCKX1*-29 and *AtCKX2*-26. Also, in shoots of only one transgenic line, *AtCKX2*-17, significantly pronounced MDA content after two weeks of culture on medium supplemented with 200 mM NaCl was recorded. In non transformed centaury roots grown on NaCl free medium during four weeks significantly decreased MDA content was determined. Low MDA content was also recorded in non transformed centaury roots grown on all tested NaCl concentrations. Significantly higher MDA content was observed in roots grown on 150 and 200 mM NaCl after four weeks as well as on 100, 150 and 200 mM NaCl after eight weeks of treatment. Unlike non transformed in all analyzed transgenic *AtCKX* centaury roots grown on media supplemented with all NaCl concentrations increased MDA content was observed.

Changed H_2O_2 content in shoots and roots of non transformed and *AtCKX* transgenic centaury lines are shown in **Fig 7.** Increased H_2O_2 content was recorded in non transfromed centaury shoots during cultivation on higher concentrations of NaCl (>100 mM) after all treatment periods. In transgenic *AtCKX1*-29 shoots increased H_2O_2 content was determined on 150 and 200 mM NaCl after one week of treatment. Increased H_2O_2 content was also recorded in shoots grown on NaCl (>50 mM) after two and four weeks of treatment while after eight weeks increased H_2O_2 content was detected on all applied NaCl concentrations. On the other hand the similar pattern was noticed in shoots of *AtCKX2* transgenic lines. Increased H_2O_2 production was detected in transgenic *AtCKX2*-17 shoots after culturing on NaCl free as well as on media supplemented with graded NaCl concentrations. Shoots of transgenic line *AtCKX2*-26 showed elevated H_2O_2 production after culturing on media supplemented with graded NaCl concentrations after all treatment periods.. Generally, non transformed and *AtCKX* transgenic centaury roots produced more H_2O_2 than centaury shoots. In centaury roots the same pattern like in shoots was observed. After two weeks decreased H_2O_2 content was recorded in non transformed roots while significant increment of H_2O_2 content was detected only at >100 mM NaCl. In culture of non transformed and transgenic line *AtCKX1*-29 significantly higher H_2O_2 content was recorded on media supplemented with graded NaCl concentrations. In roots of both *AtCKX2* transgenic lines increased H_2O_2 production was recorded after two weeks of culture. It was interesting to note that in these transgenic roots H_2O_2 production was increased when NaCl concentrations was also increased on nutrition media. Only exception was *AtCKX2*-17 transgenic roots where after treatment period of two weeks H_2O_2 content was similar in all root samples regardless on applied NaCl concentrations.

The effects of various NaCl concentrations on antioxidant enzyme activities such as SOD, CAT and POX are presented on **Figs 8-10**, respectively. Generally, as the production of H_2O_2 was higher in roots than in shoots, the activity of SOD, which converts superoxide radical to H_2O_2, was higher in centaury roots than in shoots (**Fig 8**). The highest SOD activity was determined in roots grown on media supplemented with higher NaCl concentrations (>100 mM) during four weeks. However after eight weeks of culturing, SOD activity in centaury roots was similar in all roots regardless to NaCl concentration in medium. Interestingly, in non transformed as well as in transgenic centaury shoots increased SOD activity was detected on higher NaCl

concentrations (>100 mM). The highest SOD activity was recorded in shoots of both *AtCKX2* transgenic lines grown on medium supplemented with 200 mM NaCl after first (line *AtCKX2*-26) or two weeks (line *AtCKX2*-17). On the other hand, in non transformed and shoots of transgenic line *AtCKX1*-29 the highest SOD activity was observed after prolonged (8 weeks) culturing on medium supplemented with 200 mM NaCl.

In non transformed shoots increased CAT activity was detected already after one week of culture on graded NaCl concentrations. There were no change of CAT activity during prolongated culture in non transformed shoots. Contrary, during culturing at different NaCl concentrations increased CAT activity in all analyzed transgenic shoots was observed. The highest CAT activity was detected in shoots of all *AtCKX* transgenic lines after four weeks on medium supplemented with 200 mM NaCl. In non transformed and all *AtCKX* transgenic centaury roots increased CAT activity was observed already after one week on NaCl free as well as on all media supplemented with graded NaCl concentrations (**Fig 9**). Also, in all analyzed centaury roots the highest CAT activity was recorded after two weeks on medium with 200 mM NaCl.

POX activity was higher in non transformed shoots than in roots. In this line increased POX activity in shoots was recorded after one week of culture even on control NaCl free medium and media supplemented with graded NaCl concentrations (**Fig 10**). After two weeks of culturing POX activity in *AtCKX* transgenic centaury shoots decreased even at high NaCl concentrations. In transgenic centaury lines POX activity remains high only when shoots were grown at >100 mM NaCl during the whole period of cultivation. In non transformed roots increased POX activity was recorded on media supplemented with graded NaCl concentrations. In roots of transgenic line *AtCKX1*-29 significant increment of POX activity was observed only after two weeks and further on medium supplemented with 200 mM NaCl. In roots of both analyzed *AtCKX2* transgenic lines increased POX activity was recorded after one week on medium supplemented with higher NaCl concentrations (>100 mM). POX activity in shoots and roots of two *AtCKX2* transgenic lines varied. The least POX activity was observed in shoots and roots of transgenic line *AtCKX2*-26.

Discussion

There are numerous literature data describing the effect of salinity on physiological processes such as germination capacity, shoot or root length, fresh and dry weight, protein and sugar content etc. (Ali et al. 2004, Bahmani et al. 2012, Talukdar 2012, Bezirğanoğlu 2017, Yadav and Vamadevaiah 2017). In this experiment the effect of NaCl-induced stress conditions on regeneration potential of centaury shoots and roots was evaluated. Non transformed and three transgenic *AtCKX* lines, with reduced content of CK active forms, was used.
Plant CKs have significant role in plant growth and represent one of the main regulators during plant growth and development (Wani et al. 2016). Transgenic plants with altered activity of cytokinin catabolic enzymes were regenerated for several model systems such as *A. thaliana* (Nishiyama et al. 2011) or tobacco (Mýtinova et al. 2010). It is very well documented that CKs play an important role to salinity stress response (Veselov et al. 2017). For instance, CK-deficient transgenic arabidopsis plants has greater ability to survive at 200 mM NaCl. Also, tobacco *AtCKX2* transgenic plants with reduced content of CKs are more tolerant to salinity and implicate significant role of antioxidative enzymes in this process (Bielach et al. 2017). Non transformed centaury shoots and roots showed significantly different salinity tolerance to graded NaCl concentrations. In addition, we found different salinity response among two investigated *AtCKX2* transgenic lines. In shoot culture of non transformed and *AtCKX1*

transgenic line significant increment of fresh weight was observed when shoots were grown at 50 mM NaCl while higher NaCl concentrations in culture medium influenced on great decrement in shoot growth of all analyzed centaury lines (**Fig 1**). The same result considering fresh shoot weight decrement of two Turkish tobacco varieties on NaCl concentration greater than 200 and 300 mM was reported by Çelik and Atak (2012). Also, fresh shoot weight of *Jatropha curcas* decreased on higher NaCl concentration (Gao et al. 2008). Khalil et al. (2016) showed that NaCl influenced on remarkable reduction for all of the considered growth parameters such as dry weight, root and shoot length of *Medicago ciliaris* and *M. polymorpha*. Generaly, centaury roots showed higher salinity tolerance than shoots. Roots of non transformed and *AtCKX1* transgenic line survived on medium supplemented with 100 mM NaCl without reduction in morphogenetic potential (**Fig 3**). Furthermore there was a significant increment in average number of regenerated shoots in non transformed root culture on medium with 100 mM NaCl (**Fig 3b**). Our results showed that all centaury plants, regenerated on ½MS media supplemented with different NaCl concentrations, developed typical rosette form and also showed a similar morphology as plants regenerated in control root cultures grown on NaCl free media. These findings are in concordance with early reported by Šiler et al. (2007) which showed that centaury keeps the rosette form under salt stress. The authors showed that elevated NaCl concentrations in culture medium had no inhibitory effect on shoot growth. Also, the authors highlighted that salinity affected more on centaury root than shoot development. Šiler et al. (2007) reported that 400 mM NaCl concentration was the most efficient for bud regeneration. In this experimental work spontaneous root development in centaury shoot culture was more inhibited on NaCl-rich medium than shoot development in root culture (**Fig 2** and **Fig 4**). Compared with our results, these variation in centaury salt tolerance could be explained by differences among genotypes of the same plant species. These results certainly support recent report by Petropoulos et al. (2018) describing centaury as common salt tolerant medicinal herb from Mediterranean region.

AtCKX transgenic centaury lines showed different salinity tolerance response than non transformed line implicating the possible involvement of CKs in this process. While non transformed and *AtCKX1* transgenic line showed the same trend of shoot regeneration potencial under salt stress, both *AtCKX2* transgenic lines showed gradually decreased regeneration potential as NaCl concentrations increased in medium (**Fig 3**). Significant decrement in frequency of regeneration and the average number of regenerated shoots in root culture of both *AtCKX2* transgenic lines was also noticed. Both *AtCKX2* transgenic lines showed decreased fresh shoot weight under salt stress conditions (**Fig 2**). It was interesting to note that these transgenic lines showed different regeneration capacity (**Fig 3**). Among all analyzed centaury lines, we have pointed out the higher regeneration potential of *AtCKX2*-26 transgenic line than other lines under NaCl stress conditions. Roots of this transgenic line showed certain regeneration potential even on medium supplemented with 200 mM NaCl which was not recorded in other investigated lines.

Numerous studies have shown that proline content in salt tolerant plant species increased under salt stress compared to salt sensitive plant species (Ashraf and Harris 2004, Tripathi et al. 2007). Aghaei et al. (2009) showed that accumulation of proline is usual response to salt stress. Increased proline content was also observed in soybean, *Physalis peruviana*, *Medicago ciliaris* and *M. polymorpha* under salinity stress (Amirjani 2010, Miranda et al. 2014, Khalil et al. 2016). In non transformed and all analyzed *AtCKX* transgenic centaury lines grown at media supplemented with graded NaCl concentrations increased proline content was detected (**Fig 5**). Previously it was shown that proline protects and stabilize ROS scavenging enzymes and activate alternative detoxification pathways. In salt-stressed tobacco cells, proline increased the

activities of antioxidative enzymes, enhanced POX, CAT, SOD and glutathione-S-transferase activities, and increased the glutathione redox state (Hoque et al. 2008, Islam et al. 2009). Also, in the desert plant species *Pancratium maritimum*, increased proline content improved salt tolerance by stabilizing antioxidative enzymes catalase and peroxidase (Khedr et al. 2003). Although proline accumulation correlate with environmental stress there are literature data describing that some halophyte plant species like *Thellungiella halophila*, *Lepidium crassifolium*, elevated proline content under unstressed conditions even higher than *Arabidopsis* plants grown at high salt concentrations (Murakeozy et al. 2003, Taji et al. 2004). On the other hand, halophyte plant species like *Camphorosma annua* or *Limonium* spp, do not have high proline content. Instead of proline, these plant species accumulated carbohydrate or betain-derived osmolytes (Murakeozy et al. 2003, Gagneul et al. 2007). The capacity for proline accumulation depends on plant species and varies on interactions between salinity and environmental conditions. It can be concluded that proline is certainly one of the factors contributing stress tolerance, but not the absolute requirement for adaptation to extreme environmental conditions.

Due to the fact that MDA, common product of lipid peroxidation, is a sensitive diagnostic index of oxidative injury, evaluation of MDA content during salt stress can provide further evidence of oxidative stress tolerance of centaury plants grown *in vitro*. During oxidative damage of biomembranes in addition to MDA, H_2O_2 is also produced (Lu et al. 2009). Hydrogen peroxide is small molecule crossing the cellular membrane and diffuse over short distances and thus acts as a signaling molecule of stress conditions (Pitzschke and Hirt 2006). In the present study, MDA and H_2O_2 content was increased in centaury plants grown at graded NaCl concentrations (**Figs 6-7**). It was interesting to note that the lowest MDA and H_2O_2 contents were observed in roots of transgenic line *AtCKX2*-26 after eight weeks of culture under salt stress. In this respect increased lipid peroxidation and H_2O_2 content were also reported in many plants under salt stress conditions. Different NaCl concentrations caused significant increase of MDA content in Jerusalem artichoke cultivars (Xue and Liu 2008), *Salicornia persica* and *S. europaea* (Aghaleh et al. 2009), in transgenic and wild potato clones (Mohamed et al. 2010), *Sesuvium portulacastrum* (Lokhande et al. 2011) and *Medicago sativa* (Palma et al. 2014). Salt stress also resulted in enhancement of H_2O_2 content in salt tolerant and salt-susceptible cultivars of cotton (Kumari et al. 2013) and in salt treated grapevine plantlets (Ikbal et al. 2014).

It was not easy to conclude how antioxidative enzymes in centaury are affected by salt stresses. Obviously, CK-deficient centaury plants exhibit imbalance of ROS homeostasis which had an effect on the activity of these enzymes. The SOD family is composed of metalloproteins that catalyze the conversion of the superoxide anion to H_2O_2 (Møller, 2001). Our results showed increased SOD activity in all analyzed centaury plants grown on graded NaCl concentrations (**Fig 8**). It is very important to note that SOD activity was higher in all analyzed centaury roots than in shoots grown *in vitro*. Contrary, Mýtinova et al. (2010) showed lower SOD activity in roots than in shoots of wild type and transgenic *AtCKX2* tobacco plants under control conditions as well as upon salt stress under *ex vitro* conditions. Unlike shoots, the same research also showed increased SOD activity in roots of transgenic *AtCKX2* tobacco plants than wild type tobacco roots grown under control and salt stress conditions. After prolonged growth under salt stress conditions higher SOD activity was detected in non transformed centaury roots than in shoots. There are numerous reports describing induced SOD level like in barley (Khosravinejad et al. 2008), rice (Yamane et al. 2009, Thamodharan and Pillai 2014), *Jatropha curcas* L. (Gao et al. 2008), *Sorghum bicolor* (Omari and Nhiri 2015), *Hyssopus officinalis* L. (Jahantigh et al. 2016), *Leymus chinensis* (Zhou et al. 2017) under salt stress. On the other hand several studies have also reported induced SOD activity in salt tolerant cultivars of sugar beet (Bor et al. 2003)

and salt tolerant plant species *Catharanthus roseus* (Elkahoui et al. 2005). Contrary, there are literature data describing decreased SOD activity in five salt sensitive wheat varieties and at the same time unchanged or little bit higher SOD activity in five salt tolerant wheat varieties (Rao et al. 2013). It can be concluded that increased level of antioxidant enzymes was not necessary for tolerance to salt stress.

The intracellular level of H_2O_2 is regulated by a wide range of enzymes and the most important are CAT and POX (Sofo et al. 2015). Plant catalases are one of the most efficient catalytic enzymes with high capacity for detoxification of H_2O_2. Other important plant enzymes, the peroxidases, also function in this mode. In addition, in defense against active oxygen compounds peroxidases have other important cellular roles. In our research CAT and POX activities showed the same trends as SOD activities. Increased CAT and POX activities in non transformed as well as in three *AtCKX* transgenic centaury lines on all applied NaCl concentrations were recorded (**Figs 9-10**). Elevated CAT activity was also detected in salt stressed *AtCKX2* tobacco plants while decreased CAT activity was determined in leaves of wild type tobacco plants (Mýtinova et al. 2010). The results presented in this work clearly indicate that increased SOD activity accompanied by increased CAT and POX activities in non transformed as well as in all *AtCKX* transgenic centaury lines, demonstrated a crucial role of these antioxidant enzymes in protecting plant tissues from toxic effects of highly damaging reactive oxygen species such as superoxide radical and hydrogen peroxide. It was interesting to note that both analyzed transgenic *AtCKX2* centaury lines showed different POX and CAT activity after eight weeks of growth under salt stress. Higher POX activity was observed in *AtCKX2-17* transgenic line while, at the same time, CAT activity was relatively constant under salt stress conditions. Numerous comparative studies using salt tolerant and salt sensitive genotypes have correlated salt tolerance to increased activities of antioxidant enzymes (Bor et al. 2002, Mittova et al. 2003, Koca et al. 2006, Sekmen et al. 2007, Xue and Liu 2008). Furthermore, overexpression of some antioxidant enzymes improved salt tolerance in some plant species (Rossatto et al. 2017, Soleimani et al. 2017, Wei et al. 2017). The higher activity of antioxidant enzymes of plants can be associated with an improved tolerance to environmental stress conditions.

Ultimately, we can summarize that salinity stress caused morphological, physiological and biochemical changes in centaury tissues. It is well known that hipersaline conditions induce osmotic and toxic effects, reduce plant growth and also reduce water movement through the root (Acosta-Motos et al. 2017). An increased level of NaCl ions in roots has been followed up by increased oxidative stress markers such as MDA and H_2O_2 (Ghosh et al. 2015). High salinity also increase the ROS levels in various plant cells and tissues as a result of irregularities in the electron transport chain (Sharma et al. 2012). It is considered that increased levels of antioxidants usually help to increase plant tolerance (Gill and Tuteja, 2010). Accordingly, in present work, the elevated MDA and H_2O_2 contents as well as increased antioxidative enzymes activities clearly indicated that all of these factors operated together in order to overcome salt stress conditions for better growth and development of centaury plants.

Conclusions

The results of this work showed that in general, centaury roots showed higher salinity tolerance compared to shoots. Significant difference in salt tolerance of three *AtCKX* transgenic centaury lines was also observed. Morphogenic potential of *AtCKX1* transgenic line was similar to non transformed line under increased NaCl concentrations *in vitro*. On the other hand, there were

significant difference in morphogenic response of two investigated *AtCKX2* transgenic lines, specially in root culture. The roots of transgenic line *AtCKX2*-26 were more tolerant to NaCl-induced stress compared to all other analyzed transgenic centaury lines. Biochemical analyzes of this transgenic line showed lower free proline, MDA content and H_2O_2 production as well as lower SOD and POX activity under prolonged salinity conditions *in vitro* in comparison to all other analyzed centaury lines. We san assume that in transgenic line *AtCKX2*-26 changes in endogenous CKs content and altered CK homeostasis possibly influenced on different salinity response. Accordingly, further investigation of endogenous plant hormones content during NaCl-induced stress might be useful tool for better understanding of salinity tolerance of non transformed and CK-deficient transgenic centaury plants grown *in vitro*.

Author contribution

M. Trifunović-Momčilov and D. Paunović contributed to all *in vitro* experiments. M. Trifunović-Momčilov contributed in data analyzes and manuscript preparation. S. Milošević and M. Petrić contributed to all experimental work considering biochemical analyzes. S. Jevremović and I.Č. Dragićević contributed to data analyzes and obtained results interpretation. A. Subotić supervised the whole study and also contributed in preparing the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Fig 1 The effect of graded NaCl concentrations on fresh shoot weight increment of *Centaurium erythraea* shoots after 8 weeks of culture. Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

Fig 2 Non transformed and *AtCKX* transgenic *Centaurium erythraea* shoots after 8 weeks of culture on ½MS hormone free medium supplemented with 0, 100 and 200 mM NaCl

Fig 3 The effect of graded NaCl concentrations on shoot regeneration in solid root culture of non transformed and *AtCKX* transgenic *Centaurium erythraea* lines. The frequency of shoot regeneration (a) and the average number of regenerated centaury shoots (b). Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

Fig 4 Induction of *in vitro* morphogenesis of non tra nsformed and *AtCKX* transgenic *Centaurium erythraea* lines after 8 weeks in solid root culture. Regeneration of shoots and roots on ½MS hormone free medium supplemented with 0, 100 and 200 mM NaCl

Fig 5 Proline content in shoots and roots of non transformed line (a), transgenic line *AtCKX1*-29 (b), *AtCKX2*-17 (c) and *AtCKX2*-26 (d) at the beginning of *in vitro* culture and after 1, 2, 4 and 8 weeks of growth on ½MS medium supplemented with graded concentrations of NaCl. Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

Fig 6 Malondialdehyde (MDA) content in shoots and roots of non transformed line (a), transgenic line *AtCKX1*-29 (b), *AtCKX2*-17 (c) and *AtCKX2*-26 (d) at the beginning of *in vitro* culture and after 1, 2, 4 and 8 weeks *in vitro* on ½MS medium supplemented with graded concentrations of NaCl. Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

Fig 7 Hydrogen peroxide (H_2O_2) content in shoots and roots of nontransformed line (a), transgenic line *AtCKX1*-29 (b), *AtCKX2*-17 (c) and *AtCKX2*-26 (d) at the beginning of *in vitro* cultureand after 1, 2, 4 and 8weeks*in vitro* on ½MS medium supplemented with graded concentrations of NaCl. Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

Fig 8 The activity of superoxide dismutase (SOD) in shoots and roots of nontransformed line (a), transgenic line *AtCKX1*-29 (b), *AtCKX2*-17 (c) and *AtCKX2*-26 (d) at the beginning of *in vitro* culture and after 1, 2, 4 and 8 weeks *in vitro* on ½MS medium supplemented with graded NaCl concentrations. Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

Fig 9 The activity of catalase (CAT) in shoots and roots of non transformed line (a), transgenic line *AtCKX1*-29 (b), *AtCKX2*-17 (c) and *AtCKX2*-26 (d) at the beginning of *in vitro* culture and after 1, 2, 4 and 8 weeks *in vitro* on ½MS medium supplemented with graded NaCl concentrations. Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

Fig 10 The activity of peroxidase (POX) in shoots and roots of non transformed line (a), transgenic line *AtCKX1*-29 (b), *AtCKX2*-17 (c) and *AtCKX2*-26 (d) at the beginning of *in vitro* culture and after 1, 2, 4 and 8 weeks *in vitro* on ½MS medium supplemented with graded concentrations of NaCl. Data represent mean ± standard error. Means marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$)

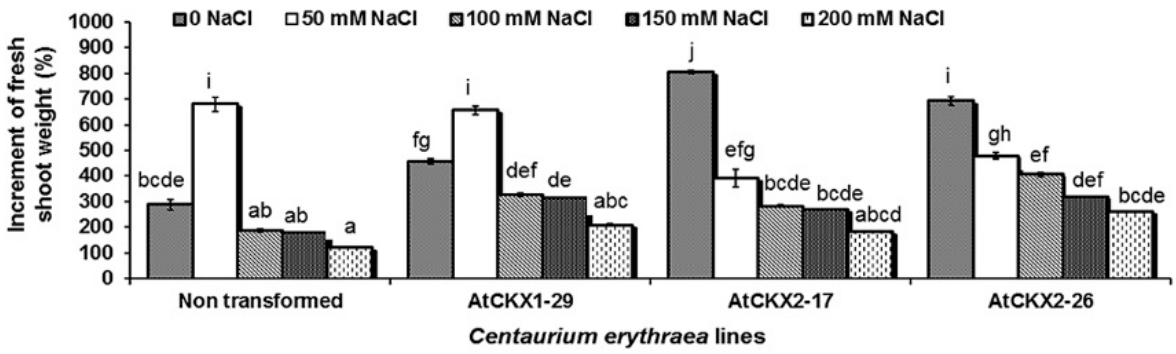
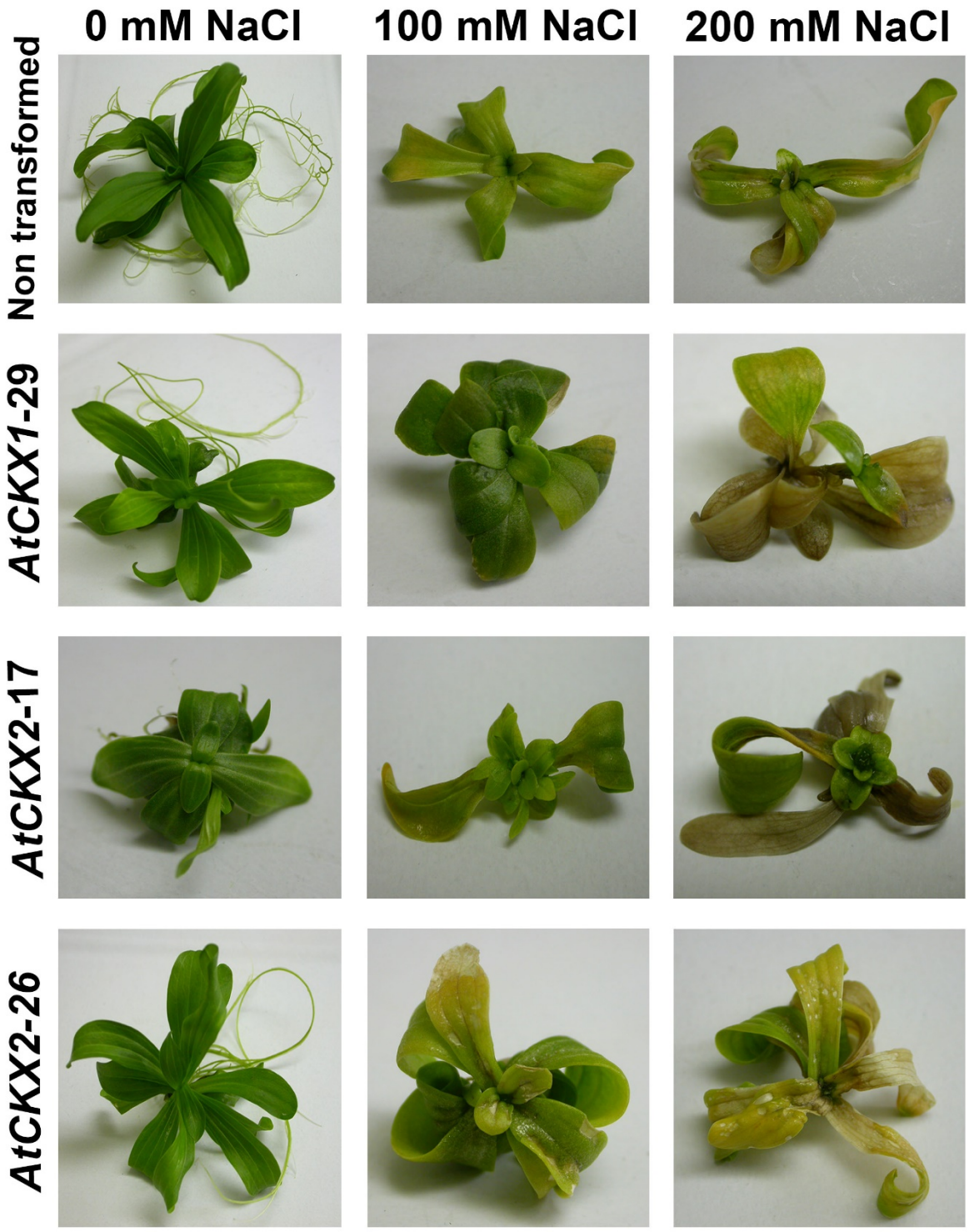

0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
Increment of fresh shoot weight (%)
1000
900
800
700
600
500
400
300
200
100
0
bcde
i
ab
ab
a
fg
i
def
de
abc
j
efg
bcde
bcde
abcd
i
gh
ef
def
bcde
Non transformed
AtCKX1-29
AtCKX2-17
AtCKX2-26
Centaurium erythraea lines

Fig. 1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100 mM NaCl
200 mM NaCl
Non transformed
AtCKX1-29
AtCKX2-17
AtCKX2-26

Fig. 2

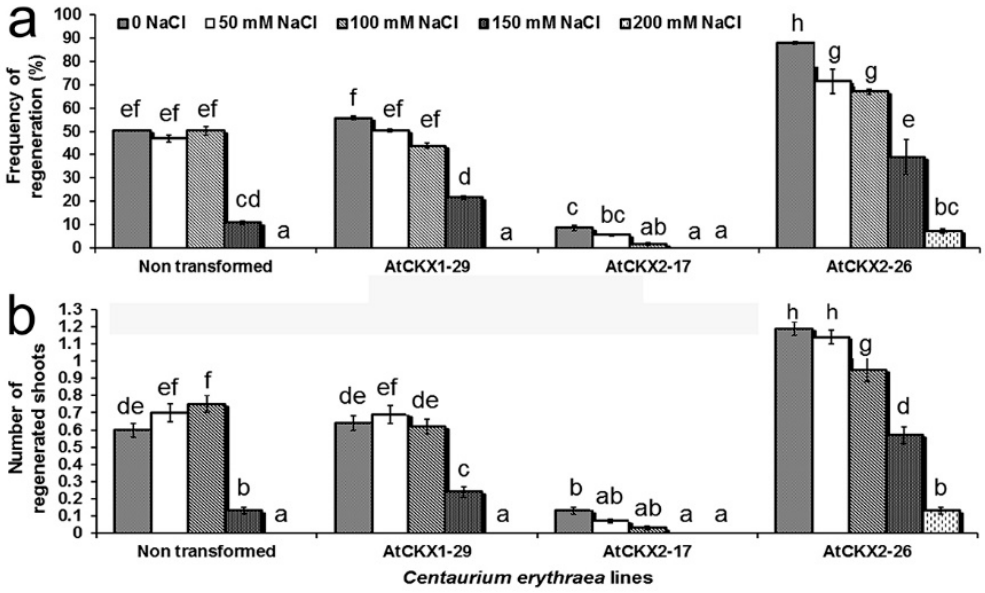
a
0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
Frequency of regeneration (%)
ef ef ef cd a
f ef ef d a
c bc ab a a
h g g e bc
Non transformed
AtCKX1-29
AtCKX2-17
AtCKX2-26
b
Number of regenerated shoots
de ef f b a
de ef de c a
b ab ab a a
h h g d b
Non transformed
AtCKX1-29
AtCKX2-17
AtCKX2-26
Centaurium erythraea lines

Fig. 3

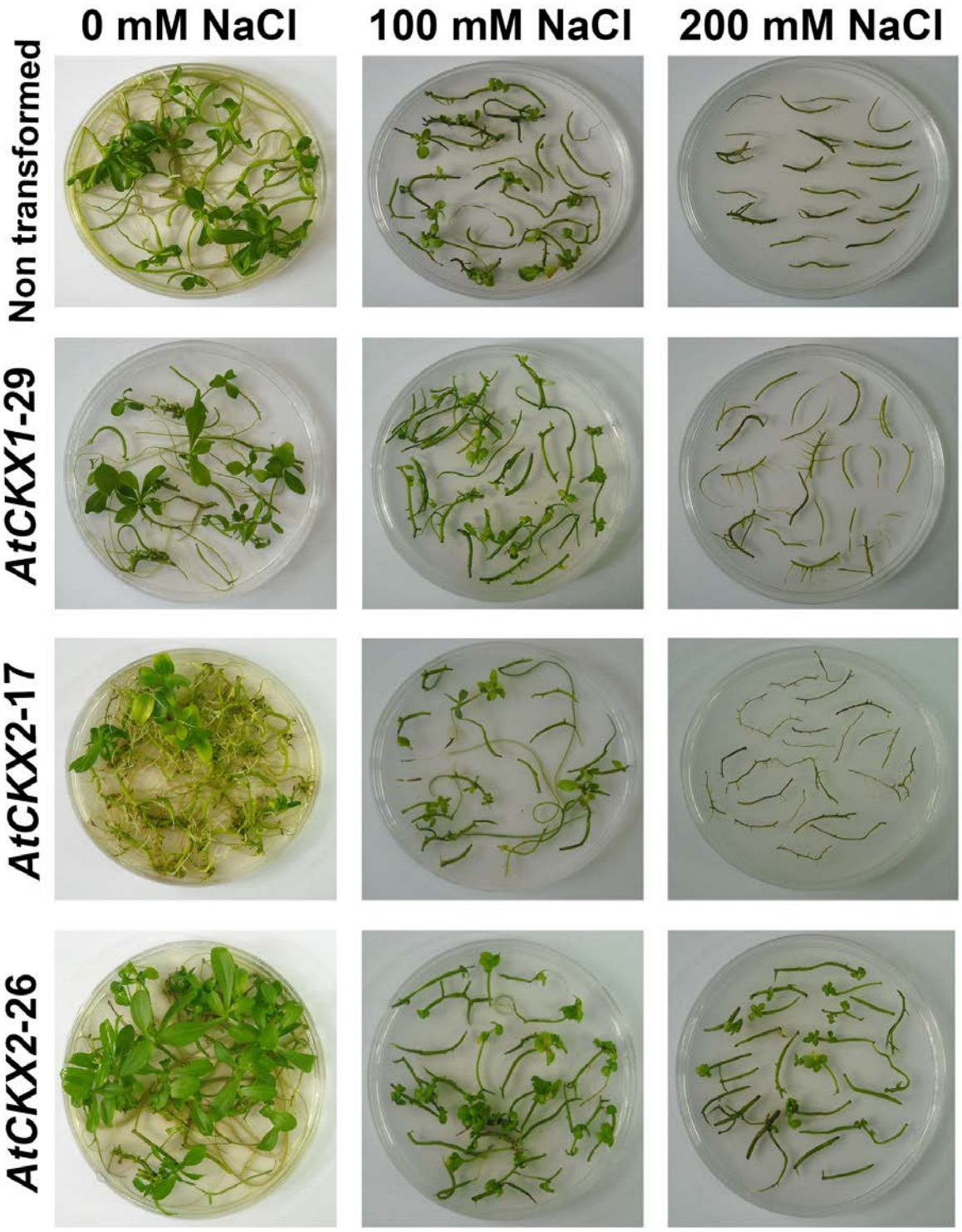
0 mM NaCl
100 mM NaCl
200 mM NaCl
Non transformed
AtCKX1-29
AtCKX2-17
AtCKX2-26

Fig. 4

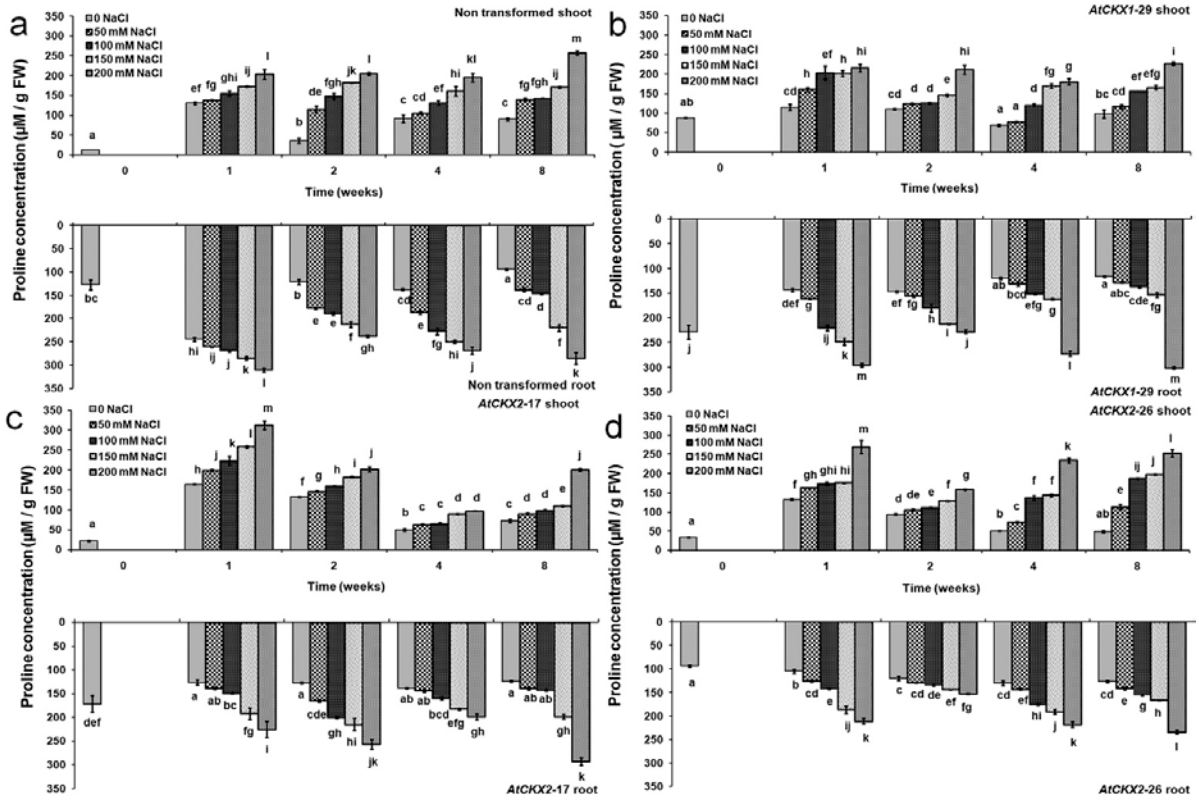
a
Non transformed shoot
0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
Proline concentration (µM / g FW)
Time (weeks)
Non transformed root
b
AtCKX1-29 shoot
AtCKX1-29 root
c
AtCKX2-17 shoot
AtCKX2-17 root
d
AtCKX2-26 shoot
AtCKX2-26 root

Fig. 5

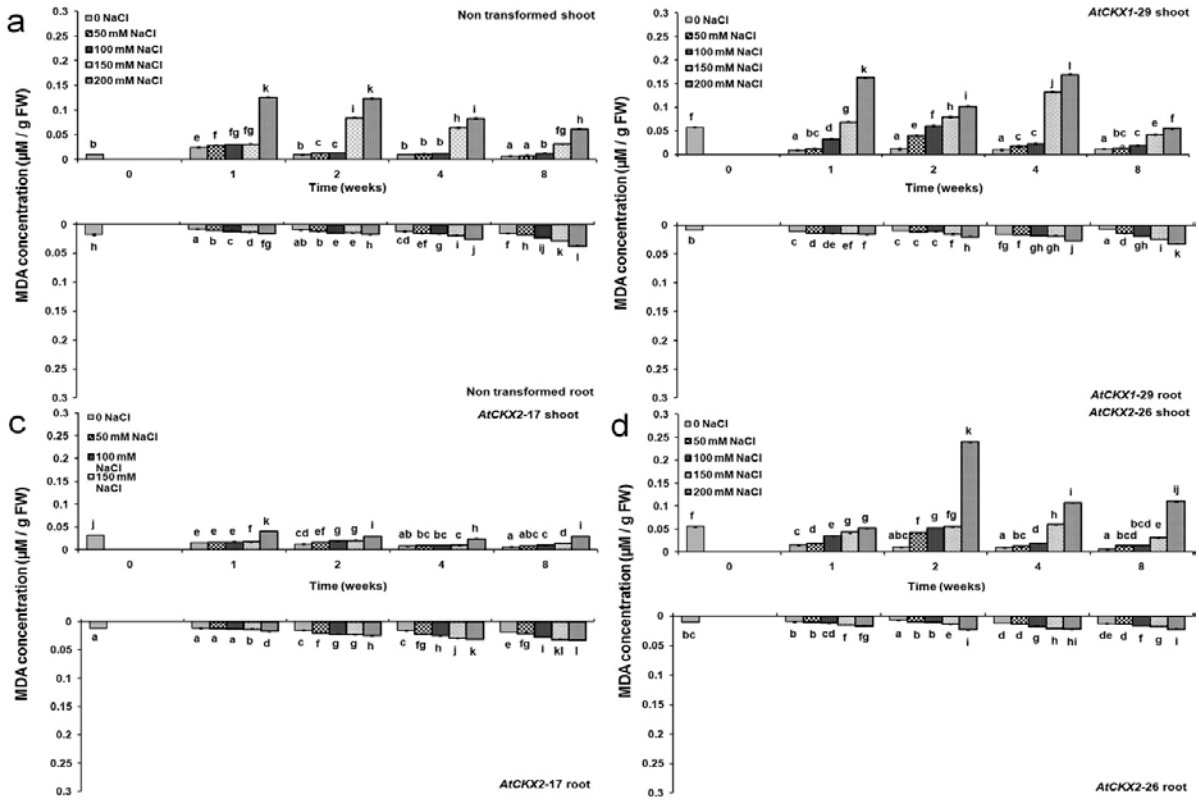
a
Non transformed shoot
0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
MDA concentration (µM / g FW)
Time (weeks)
Non transformed root
AtCKX1-29 shoot
AtCKX1-29 root
c
AtCKX2-17 shoot
AtCKX2-17 root
d
AtCKX2-26 shoot
AtCKX2-26 root

Fig. 6

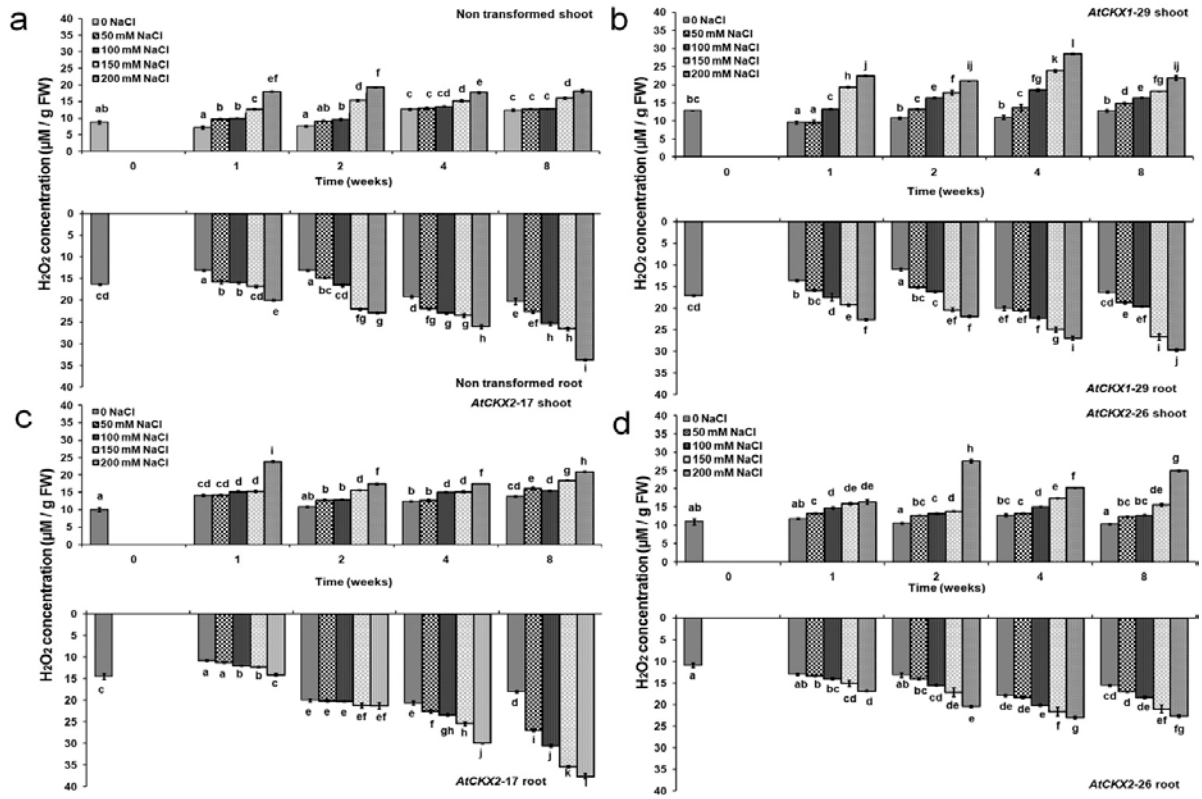
a
Non transformed shoot
0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
H2O2 concentration (µM / g FW)
Time (weeks)
Non transformed root
b
AtCKX1-29 shoot
AtCKX1-29 root
c
AtCKX2-17 shoot
AtCKX2-17 root
d
AtCKX2-26 shoot
AtCKX2-26 root

Fig. 7

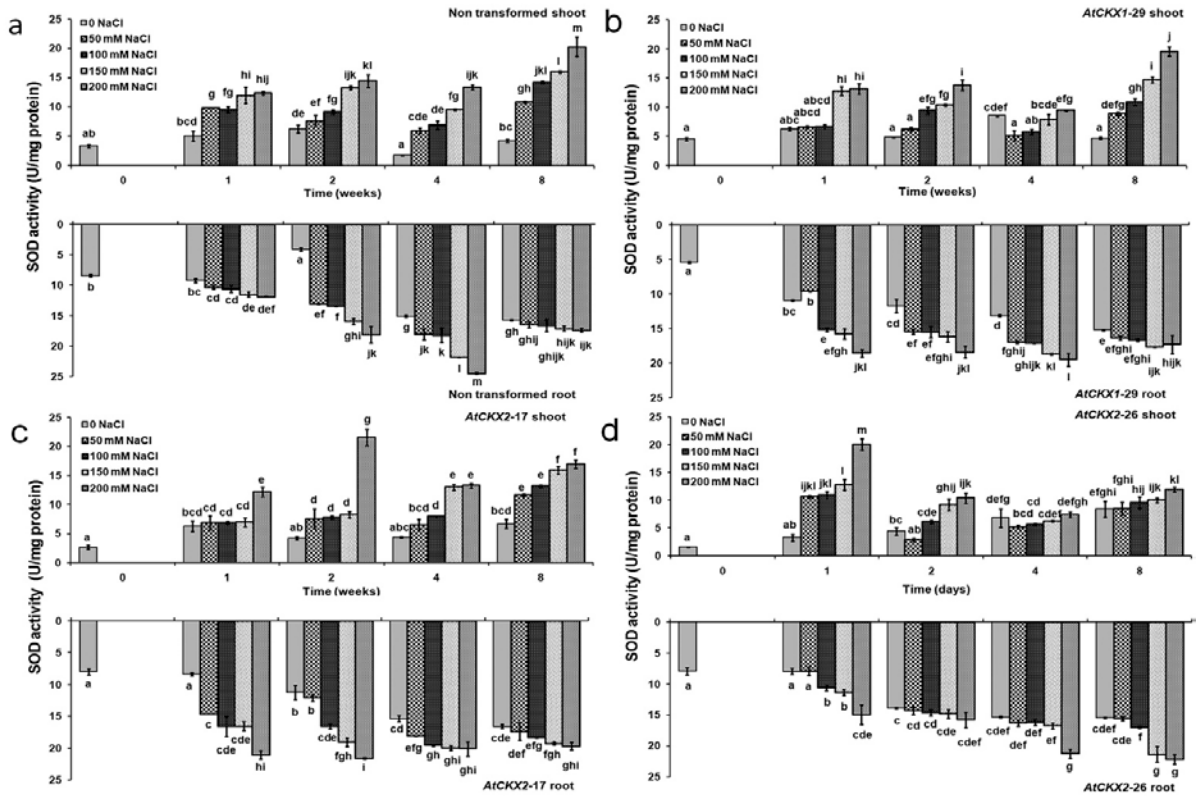
a
Non transformed shoot
0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
SOD activity (U/mg protein)
Time (weeks)
Non transformed root
b
AtCKX1-29 shoot
AtCKX1-29 root
c
AtCKX2-17 shoot
AtCKX2-17 root
d
AtCKX2-26 shoot
AtCKX2-26 root
Time (days)

Fig. 8

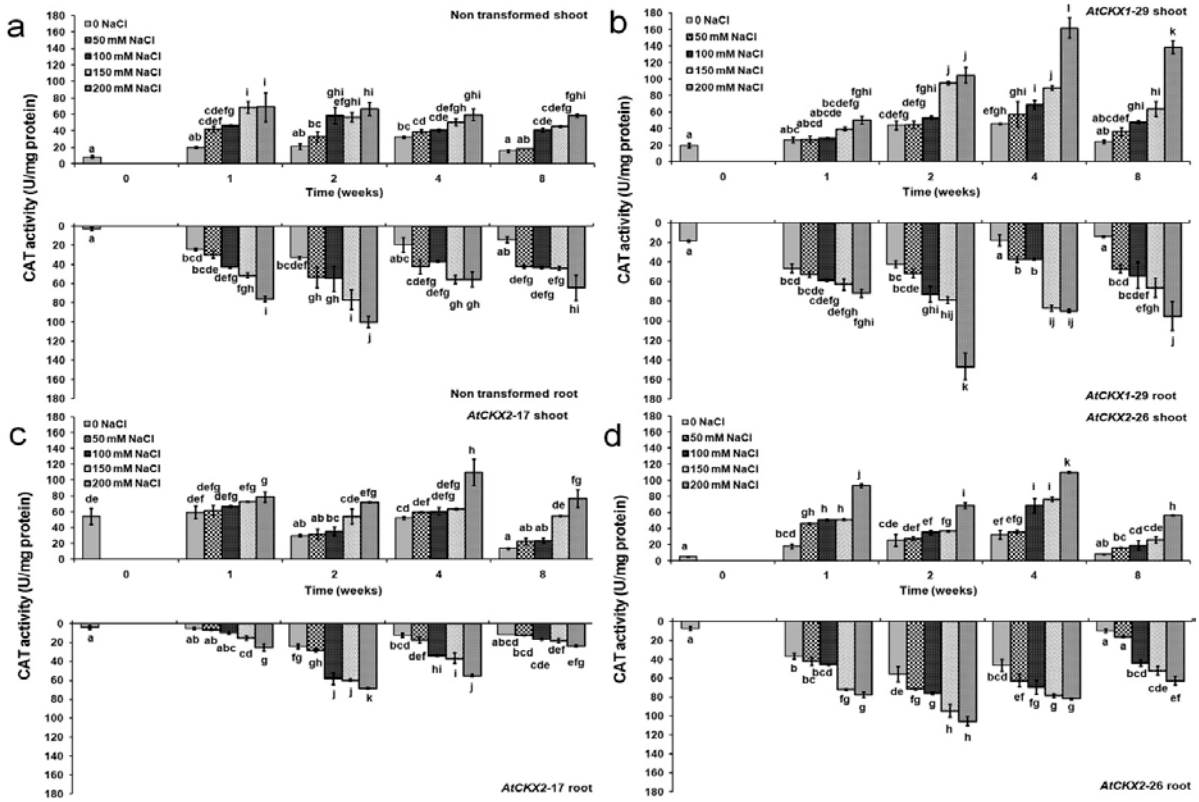
a
Non transformed shoot
Non transformed root
b
AtCKX1-29 shoot
AtCKX1-29 root
c
AtCKX2-17 shoot
AtCKX2-17 root
d
AtCKX2-26 shoot
AtCKX2-26 root
0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
CAT activity (U/mg protein)
Time (weeks)

Fig. 9

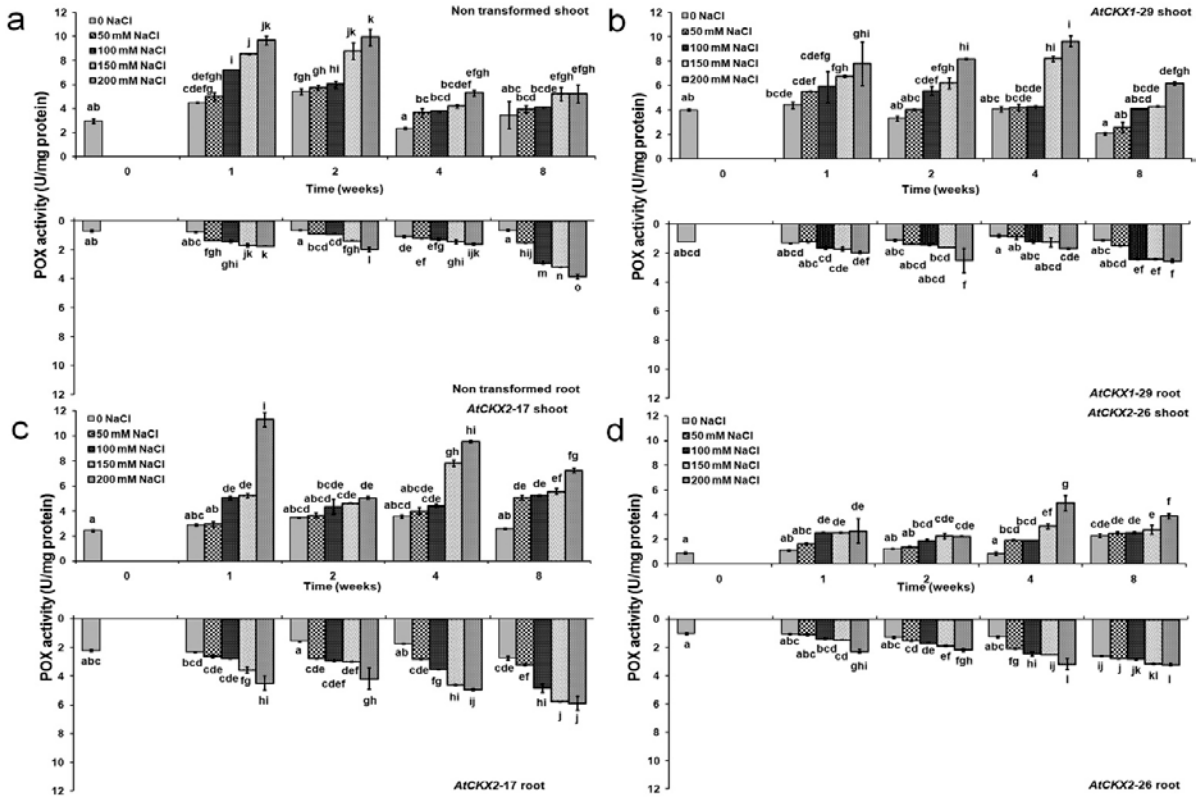
a
Non transformed shoot
0 NaCl
50 mM NaCl
100 mM NaCl
150 mM NaCl
200 mM NaCl
POX activity (U/mg protein)
Time (weeks)
b
AtCKX1-29 shoot
AtCKX1-29 root
c
Non transformed root
AtCKX2-17 shoot
AtCKX2-17 root
d
AtCKX2-26 shoot
AtCKX2-26 root

Fig. 10