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Changes in Photosynthetic Pigments Content in Non-Transformed and *AtCKX* Transgenic Centaury (*Centaurium erythraea* Rafn) Shoots Grown under Salt Stress In Vitro

Milana Trifunović-Momčilov [1,*], Snežana Milošević [1], Marija Marković [1], Marija Đurić [1], Slađana Jevremović [1], Ivana Č. Dragićević [2] and Angelina R. Subotić [1]

[1] Department for Plant Physiology, Institute for Biological Research "Siniša Stanković"—National Institute of Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia; snezana@ibiss.bg.ac.rs (S.M.); marija.nikolic@ibiss.bg.ac.rs (M.M.); marija.djuric@ibiss.bg.ac.rs (M.Đ.); sladja@ibiss.bg.ac.rs (S.J.); heroina@ibiss.bg.ac.rs (A.R.S.)

[2] Faculty of Biology, University of Belgrade, Studentski Trg 16, 11000 Belgrade, Serbia; dragicev@bio.bg.ac.rs

* Correspondence: milanag@ibiss.bg.ac.rs

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Abstract: The effects of graded sodium chloride (NaCl) concentrations (0-, 50-, 100-, 150-, and 200-mM) on photosynthetic pigment contents in in vitro grown shoots of important medicinal plant species (*Centaurium erythraea* Rafn) were investigated. Non-transformed, one *AtCKX1* and two *AtCKX2* transgenic centaury lines, with altered cytokinin profiles, were used in this study. The chlorophyll (*Chl*) and carotenoid contents differed in the non-transformed and transgenic lines. In general, salinity significantly reduced the *Chl a* and *Chl b* contents in comparison to the NaCl-free medium. The lowest *Chl* content was observed in *AtCKX2* transgenic shoots grown on all the culture media. The total carotenoid content was increased in shoots of non-transformed and both *AtCKX2* transgenic lines grown in 50-mM NaCl. On the other hand, in concentrations >50-mM NaCl, the total carotenoid content was decreased in all analysed centaury shoots. The *Chl a*/*Chl b* ratio in all the shoots increased progressively in the graded NaCl concentrations. Contrarily, the addition of NaCl in the culture medium reduced the *Chl*/carotenoid ratio in centaury shoots. Taken together, the results of this study partly explained the mode of centaury plant adaptations to salt stress in vitro. Thus, the results on centaury shoots confirmed that the determination of the photosynthetic pigment contents can be a very useful non-destructive screening method in order to discriminate susceptible and resistant plant species/lines to salt stress conditions.

Keywords: centaury; salinity; abiotic stress; *AtCKX* genes; chlorophyll; cytokinins

1. Introduction

Common centaury (*Centaurium erythraea* Rafn) belongs to the Gentianaceae family and represents an important medicinal plant species distributed throughout the Northern Hemisphere. In traditional medicine, centaury has for centuries been used to cure numerous health issues such as febrile conditions, blood sugar, anaemia, jaundice, gout, etc. [1]. Centaury is a cosmopolitan plant species that inhabits mountain slopes, dry grasslands, and scrublands. In addition, centaury can also be found in saline soils.

Salinity is one of the most significant environmental stresses limiting cell metabolism and plant productivity [2]. The detrimental salinity effects on plants are the consequence of Na^+ and Cl^- ion toxicity and, also, a water deficit that results in osmotic and ionic stress, which usually leads to numerous physiological and biochemical changes that inhibit plant

growth, development, and protein synthesis [3]. Salinity stress induces remarkable alternations in physiological, biochemical, and molecular processes [4]. Photosynthesis is an important parameter used to monitor plant responses to abiotic stress. Reduced photosynthesis under salinity is not only attributed to stomata closure and reduced intercellular CO_2 concentrations but, also, photosynthetic pigment photooxidation and photosynthetic enzymes activity, as well as chlorophyll (*Chl*) and carotenoid contents due to oxidative stress [5,6]. Salt stress reduces the activity of enzymes involved in *Chl* synthesis and, at the same time, elevates the chlorophyllase activity, enhancing the *Chl* disorganisation [7,8]. It is known that *Chl* has an essential function in photosynthesis [9]. Recently, it has also been reported that salt stress has direct and indirect effects on the *Chl* content and photosynthetic efficiency [10]. The direct effects are referred to as the regulation of activity and expression levels of enzymes involved in *Chl* biosynthesis and photosynthesis, while the indirect effects are referred to as specific regulating pathways such as antioxidant enzymes.

During the time plants generally succeed in adapting to salinity by adjusting their *Chl* levels. The rate of change in the *Chl* content varies among different plant species. Even forty years ago, it was postulated that NaCl increased the activity of the *Chl*-degrading enzyme chlorophyllase, which resulted in a decreased total *Chl* content [11]. Later, it was also shown that, during salinity, the accumulation of ions affected the biosynthesis of different pigment fractions and, consequently, the reduced *Chl* content [12,13]. During the last decade, there has been a lot of literature data describing that salt stress caused the reduction of *Chl* content in numerous plant species, such as corn [14], pumpkin [15], walnut [16], chili pepper [17], bean [18], melon [19], and Moldavian balm [20]. Accordingly, the decrement of the *Chl* content as a response to salt stress conditions can be considered as a general phenomenon that leads to the destruction of the chloroplast structure and instability of the pigment–protein complex and, finally, chlorosis in plants [21].

Besides the regulation of numerous developmental processes in plants, cytokinins (CKs) play an important role in the structural differentiation and biogenesis of chloroplasts and also induce the genes involved in chloroplast development [22–25]. Leaf senescence is a developmental process representing the final stage in the leaf life cycle or can also be initiated as a response to environmental stress conditions. Leaf senescence is characterised by the loss of *Chl*; leaf yellowing; and the degradation of proteins, membrane lipids, and RNA [26]. Determination of the reduced *Chl* amount in leaves can be very useful in the early detection of senescence.

Genetic transformation using specific *CKX* genes enabled the production of plants with an increased overexpression of transgenes and reduced levels of endogenous CKs [27–33]. The decreased amount of CKs is directly related to the increased activity of the catabolic enzyme cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12), the only known enzyme involved in the catabolism of specific CKs to date. The individual members of the *AtCKX* gene family are expressed differentially [28]. In transgenic Arabidopsis plants, *AtCKX1* expression was detected in the shoot apex, young flowers, and in the roots. On the other hand, the expression of the *AtCKX2* promoter was detected in the shoot apex while no *AtCKX2* expression was observed in Arabidopsis roots [28]. It is also known that AtCKX proteins have different subcellular localisations. AtCKX1 is a mitochondrial protein, and the final destination of AtCKX1 is the vacuole. AtCKX2 represents the peptide for targeting the endoplasmic reticulum and subsequent transport to the extracellular region [28]. Plants with 'cytokinin deficiency syndrome' showed decreased apical dominance and shoot growth, reduced leaf size, delayed flowering, and also, a reduced number of flowers. Considering that the senescence-delaying effect of CKs is well-known, it was suggested that these plant hormones are key components in the regulation of leaf senescence [34]. Accordingly, it can be assumed that specific transformants with reduced endogenous CK levels could represent very useful tools in the investigation of photosynthetic pigment contents and senescence, ultimately.

The salinity stress response of centaury species grown in vitro is described in a few literature data summarized in Reference [35]. Considering common centaury, there have been only two reports about the salinity stress response. Šiler et al. [36] described the effects of salinity on the in vitro growth and photosynthesis of centaury shoots. Recently, Trifunović-Momčilov et al. [37] showed the physiological and biochemical aspects of the salinity stress response of non-transformed and *AtCKX* transgenic centaury shoots and roots grown in vitro. It was shown that centaury roots showed a higher salinity tolerance compared to shoots. Considering the fact that maintaining the *Chl* content contributes to a salt tolerance, in this study, we investigated the influence of salinity in vitro on the photosynthetic pigments of non-transformed and *AtCKX* transgenic centaury shoots with a reduced amount of bioactive CKs.

2. Materials and Methods

2.1. Plant Material, Growth Media, and Culture Conditions

Non-transformed and transgenic *AtCKX* centaury plants were obtained as previously described [38]. One *AtCKX1* (line 29) and two *AtCKX2* (lines 17 and 26) transgenic centaury lines were selected as the starting plant materials. The selection of these three transgenic lines was based on the previously described overexpression of *AtCKX* genes in centaury shoots and roots, which resulted in an altered CKs profile, leading to a decline of the bioactive CK levels and, at the same time, increased contents of storage CK forms, inactive CK forms, and/or CK nucleotides [32]. The shoots of non-transformed and transgenic *AtCKX* lines were grown in vitro on half-strength plant growth regulator-free MS medium (½MS [39]) solidified with 0.7% agar (Torlak, Belgrade, Serbia) and supplemented with 3% sucrose and 100-mg l^{-1} *myo*-inositol (Sigma-Aldrich, St. Louis, MO, USA). Four weeks after the last subculture, isolated centaury shoots of the non-transformed and all the transgenic *AtCKX* lines were grown on new ½MS plant growth regulator-free medium or ½MS medium supplemented with a range of NaCl (Lach-Ner, Neratovice, Czech Republic) concentrations (50-, 100-, 150-, and 200-mM) during the next eight weeks. The medium was adjusted to pH 5.8 with NaOH/HCl and autoclaved at 121 °C for 25 min. All in vitro cultured plants were grown at 25 ± 2 °C and at a 16-h/8-h photoperiod ('Tesla' white fluorescent lamps, 65 W, 4500 K; light flux of 47 μmols^{-1} m^{-2}). The experiments were repeated three times, with 90 explants per each treatment.

2.2. Quantification of Photosynthetic Pigments

The isolation of the *Chl* and carotenoids was accomplished from the leaves of non-transformed and *AtCKX* transgenic centaury shoots after eight weeks of cultivation. Pigment extraction was done from leaves collected in the bottom part of the rosette. The total *Chl* and carotenoid contents were extracted using 96% ethanol. The plant material was heated in a water bath at 70 °C. The absorbance of the pigments was further measured with a UV–visible spectrophotometer (Agilent 8453, Life Sciences, Santa Clara, CA, USA) at 470, 648, and 664 nm. The concentrations of *Chl a*, *Chl b*, and the carotenoids were calculated using the equations proposed by Lichtenthaler [40]:

$$Chl\ a\ (\mu g/g) = 13.36 \times A_{664} - 5.19 \times A_{648}/FW$$

$$Chl\ b\ (\mu g/g) = 27.43 \times A_{648} - 8.12 \times A_{664}/FW$$

$$\text{Carotenoids}_{(x+c)}\ (\mu g/g) = (1000 \times A_{470} - 2.13 \times C_a - 97.64 \times C_b)/209/FW$$

The *Chl a*/*Chl b* ratio was calculated from the quotient of *Chl a* and *Chl b* (C_a/C_b), while the *Chl*/carotenoids ratio was calculated from the quotient of their total values ($C_{(a+b)}/C_{(x+c)}$). Determination of the photosynthetic pigments was accomplished in three biological samples per those non-transformed and each of the transgenic lines. Additionally, the absorbance of the supernatant was measured three times for each sample. The concentrations of

all the analysed photosynthetic pigments were presented as μg/g of fresh sample weight (FW).

2.3. Statistical Analysis

The effect of a range of NaCl concentrations (0–200 mM) on the photosynthetic pigment contents of 8-week-old non-transformed and transgenic *AtCKX* shoots were evaluated using a standard two-factor analysis of variance (ANOVA). The results were presented as the mean ± SE. The comparisons between the mean values were made using Fisher's LSD (least significant difference) post-hoc test calculated at a confidence level of $p \leq 0.05$.

3. Results

3.1. Effect of NaCl on Photosynthetic Pigments Content of Centaury Shoots Grown In Vitro

The shoots of non-transformed and three transgenic *AtCKX* lines successfully survived eight weeks on ½MS medium supplemented with a range of NaCl concentrations (0-, 50-, 100-, 150-, and 200-mM). The morphological appearance of the shoots in the non-transformed and *AtCKX* lines grown on NaCl-free medium showed normal rosette morphology and oval leaves with dark green colour. However, salt stress deteriorated the phenotypic appearances in all the salt-treated centaury shoots (Figure 1). Cultivation of centaury shoots in increased salt-enriched mediums caused the rolling and burning of the leaf tips and yellowing of the whole plant. With an increase of the NaCl concentrations in the culture media, the profiles of the leaves evolved from green to lighter tones. In addition, most yellow leaves with altered morphology were observed on shoots grown in culture media supplemented with 150- and 200-mM NaCl. The chlorosis of leaves is one of the most common symptoms of salt stress in plants due to the decreasing amounts of photosynthetic pigments. In the common centaury cultures, chlorosis reached higher levels with the increased NaCl concentrations. The highest level of chlorosis at the highest salinity rate (200-mM) in both the non-transformed and transgenic lines was observed. Visible leaf damage was detected in all the centaury lines, but the leaves of the *ATCKX2*-26 line exhibited drastic senescence symptoms, as indicated by the intense necrotic areas in the leaves (Figure 1). Moreover, it was interesting to note that the non-transformed and all the *AtCKX* transgenic centaury shoots spontaneously rooted in NaCl-free and the medium supplemented only with 50-mM NaCl after eight weeks.

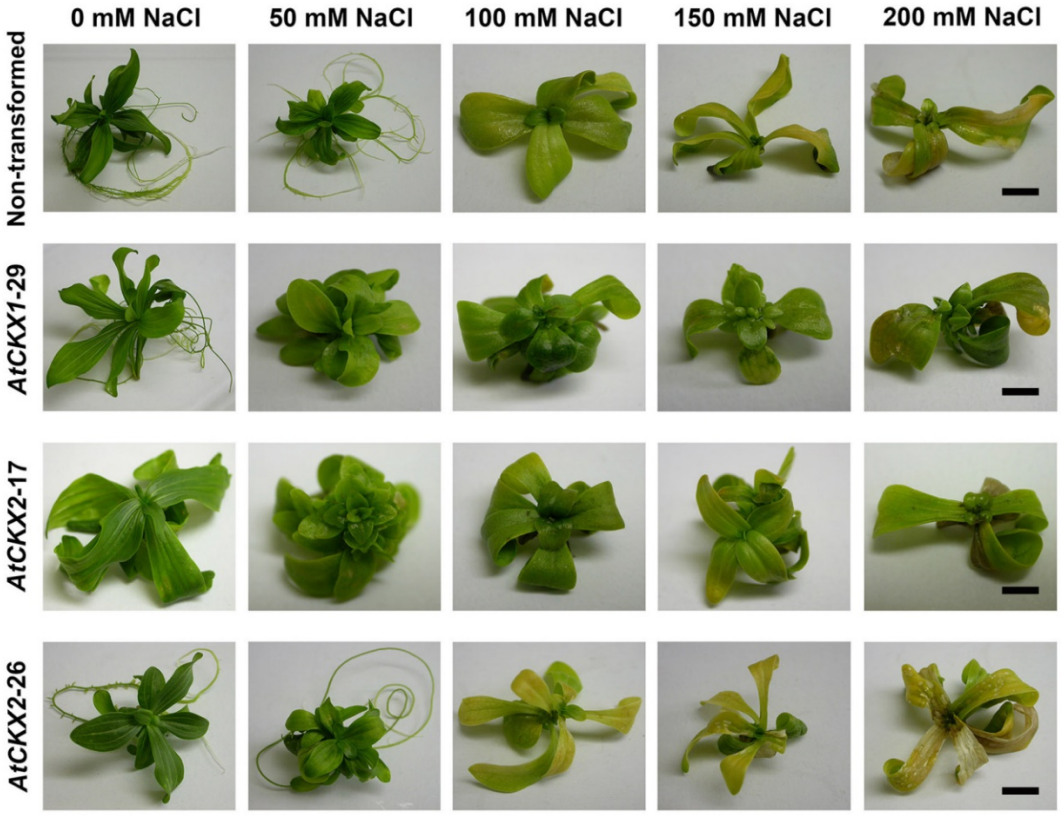

Figure 1. Eight-week-old non-transformed and *AtCKX* transgenic *Centaurium erythraea* shoots grown in ½MS medium supplemented with a range of NaCl concentrations (0-mM, 50-mM, 100-mM, 150-mM, and 200-mM). Bars indicate 10 mm.

The total *Chl* (*Chl a* + *Chl b*) content is shown in Figure 2a. It was evident that the biosynthesis of *Chl* in common centaury was altered and affected by the salinity stress. When the shoots were grown in NaCl-free medium, significant differences in the total *Chl* content between the non-transformed and transgenic *AtCKX* lines were observed. In the non-transformed and transgenic line *AtCKX1*-29, the total *Chl* content was similar in the shoots grown in NaCl-free medium, while the total *Chl* contents in both *AtCKX2* transgenic lines varied greatly. One transgenic line *AtCKX2*-26 had a significantly higher total *Chl* content in comparison to the non-transformed shoots and transgenic line *AtCKX1*-29. A higher total *Chl* content in this transgenic line was the consequence of a higher content of *Chl b*, which was detected in this line in comparison to all the other shoots (Figure 2c). Contrarily, the other *AtCKX2* transgenic line (17) showed a several times lower total *Chl* content when the shoots were grown in the NaCl-free medium. Regarding the response to salinity, all the analysed centaury shoots showed different patterns. In the non-transformed shoots and transgenic lines *AtCKX1*-29 and *AtCKX2*-26, a decreased total *Chl* content started with a slight salinity application (50-mM NaCl). Contrarily, in transgenic line *AtCKX2*-17, a significant decrement of the total *Chl* content was observed in shoots grown in a two-times higher NaCl concentration (100-mM). Although the same trend of *Chl* degradation was noticed in all the analysed centaury lines, significant differences were detected. The total *Chl* content was decreased in all the *AtCKX* transgenic lines in 150- and 200-mM NaCl in comparison to the non-transformed shoots.

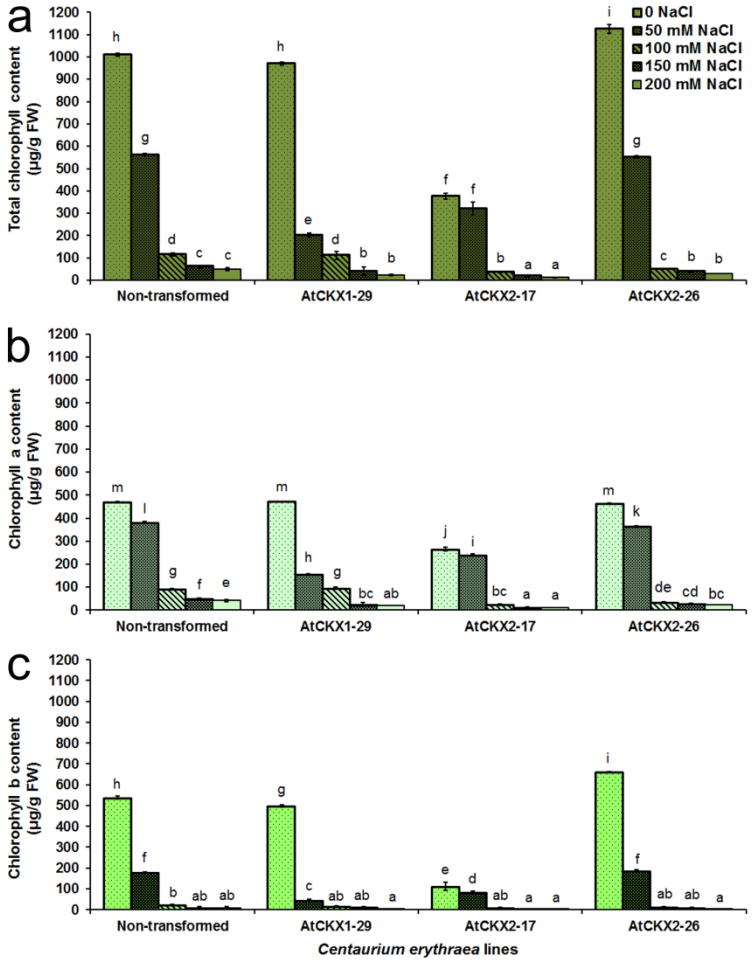

Figure 2. Total *Chl* (**a**), *Chl* a (**b**), and *Chl* b (**c**) contents in the shoots of non-transformed and transgenic centaury lines (*AtCKX1*-29, *AtCKX2*-17, and *AtCKX2*-26) after 8 weeks of growth on ½MS medium supplemented with 0-, 50-, 100-, 150-, and 200-mM NaCl. The data represent the mean ± standard error. Means marked with a different letter are significantly different from the control, according to the LSD test ($p \leq 0.05$).

The effect of salinity on *Chl a* and *Chl b* was separately analysed (Figure 2b,c). Different patterns in the decrement of *Chl a* in the non-transformed and transgenic *ACKX* shoots were observed. A rapid decrement in the *Chl a* content was observed at concentrations >100-mM in the shoots of non-transformed and both *AtCKX2* transgenic lines, while, in the shoots of the *AtCKX1*-29 transgenic line, a significant decrement of the *Chl a* content was detected even in 50-mM NaCl. Regardless of the initial values, the *Chl a* content continued to decrease until 200-mM NaCl in all the analysed centaury shoots. It was interesting to note that, as in the case of the total *Chl* content, the *Chl a* content was decreased in all the *AtCKX* transgenic lines in 150- and 200-mM NaCl in comparison to the non-transformed shoots. The alteration of the *Chl b* content was also noticed in centaury shoots under salinity conditions. When the NaCl concentration in the medium increased, the *Ch b* content in the shoots was accordingly decreased. The content of *Chl b* in the shoots of the non-transformed and transgenic lines greatly varied. In the non-transformed and *AtCKX2*-26 transgenic line, the similar *Chl b* contents in the shoots grown at 50-mM of NaCl were determined, while, in the other concentrations, a significantly rapid decrement was observed. The smallest *Chl b* content was detected in 200-mM NaCl in all the centaury shoots.

The total carotenoid content was evaluated in all the centaury shoots (Figure 3). It is evident that the total carotenoid content was different in the transgenic lines in

comparison to the non-transgenic line when they were grown in the NaCl medium. When the non-transformed and *AtCKX2* transgenic lines were grown in NaCl-free medium, a similar total carotenoid content was observed, while, in the *AtCKX1* transgenic line, the carotenoid content was significantly lower. Additionally, the same pattern was recorded in slightly saline conditions. In 50-mM NaCl, the total carotenoid content was increased in the non-transformed shoots and *AtCKX2* transgenic lines in comparison to the NaCl-free medium. In the *AtCKX1* transgenic shoots, the total carotenoid content remained unchanged. Further, in 100-mM NaCl, the total carotenoid content continued to decrease slowly in the non-transformed and *AtCKX1* shoots, while, in the *AtCKX2* transgenic shoots, a more rapid decrease in the total carotenoid content was observed. In comparison to the non-transformed shoots, in all the *AtCKX* transgenic lines, the most decreased total carotenoid content was detected in 150- and 200-mM NaCl.

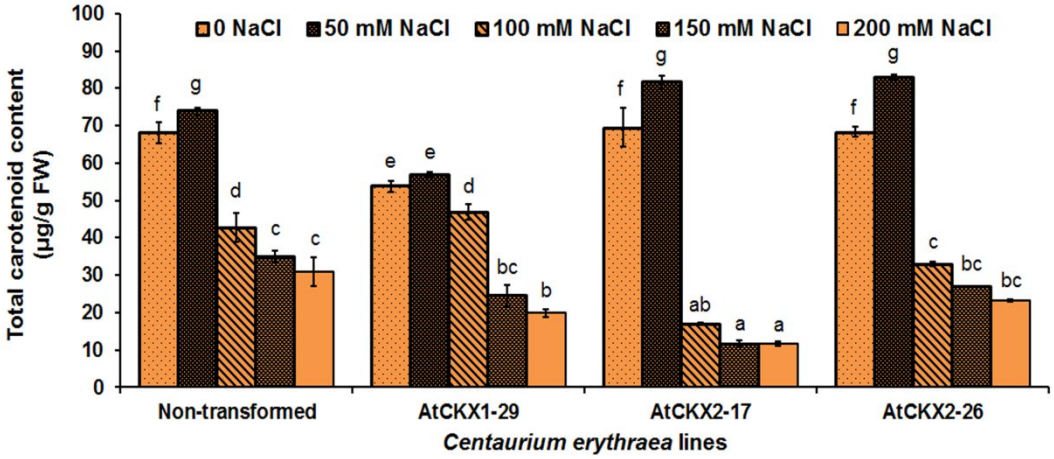

Figure 3. The total carotenoid content in the shoots of non-transformed and transgenic centaury lines (*AtCKX1*-29, *AtCKX2*-17, and *AtCKX2*-26) after 8 weeks of growth on ½MS medium supplemented with 0-, 50-, 100-, 150-, and 200-mM NaCl. The data represent the mean ± standard error. Means marked with a different letter are significantly different from the control, according to the LSD test ($p \leq 0.05$).

3.2. Effect of NaCl on Photosynthetic Pigments Ratio

The evaluation of the *Chl a*/*Chl b* ratio of the common century non-transformed and *AtCKX* transgenic lines is shown in Figure 4. The non-transformed and transgenic lines showed different *Chl a*/*Chl b* ratios in the shoots grown, even those grown in the NaCl-free medium. The non-transformed and two transgenic centaury lines (*AtCKX1*-29 and *AtCKX2*-26) showed similar *Chl a*/*Chl b* ratios, while, in transgenic line *AtCKX2*-17, an almost doubled *Chl a*/*Chl b* ratio was noticed when the shoots were grown in NaCl-free medium. Regardless of the NaCl, an incremental increase in the nutrition medium in the *Chl a*/*Chl b* ratios in all the analysed centaury lines was observed. In shoots grown in 200-mM NaCl, the highest *Chl a*/*Chl b* ratio was determined in the non-transformed and *AtCKX1*-17 and *AtCKX2*-29 transgening lines. The lowest change in the *Chl a*/*Chl b* ratio under salinity stress was achieved in the *AtCKX2*-26 transgenic line.

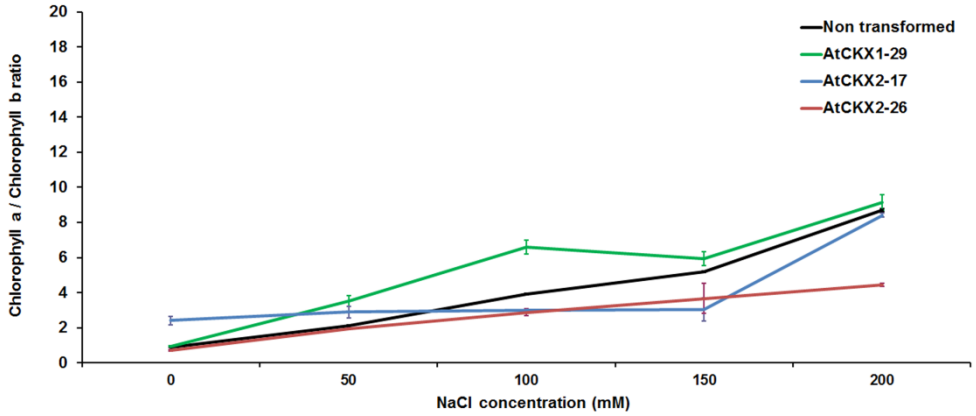

Figure 4. *Chl a* and *Chll b* ratio in the shoots of non-transformed and transgenic centaury lines (*AtCKX1*-29, *AtCKX2*-17, and *AtCKX2*-26) after 8 weeks of growth in ½MS medium supplemented with 0-, 50-, 100-, 150-, and 200-mM NaCl. The data represent the mean ± standard error. Means marked with a different letter significantly different from the control, according to the LSD test ($p \leq 0.05$), are presented in Supplementary Materials.

The ratio of the total *Chl* and total carotenoids determines the level of plant senescence. The obtained results in our study showed a different total *Chl*/carotenoid ratio in the non-transformed and transgenic lines (Figure 5). As in the case of the *Chl a*/*Chl b* ratio, when the non-transformed and two transgenic centaury lines (*AtCKX1*-29 and *AtCKX2*-26) are grown in a NaCl-free medium, a similar total *Chl*/total carotenoids ratio was detected. In the same medium, transgenic line *AtCKX2*-17 showed about a three times lower initial total *Chl*/total carotenoids ratio than the non-transformed and other transgenic lines. The addition, the 50-mM NaCl medium remarkably reduced the total *Chl*/total carotenoids ratio in the non-transformed (14.84 → 7.61), *AtCKX1*-29 (18.05 → 3.56), and *AtCKX2*-26 (16.49 → 6.66) transgenic shoots, while, in the shoots of *AtCKX2*-17, the reduction was lower (5.59 → 3.94). It is obvious that a significant reduction of the *Chl*/total carotenoids ratio was detected in the transgenic centaury lines with regards to the non-transformed line. The reduction of the *Chl*/total carotenoid ratio was further continued in 100-mM NaCl and maintained at a similar level in 150- and 200-mM NaCl in all the centaury lines.

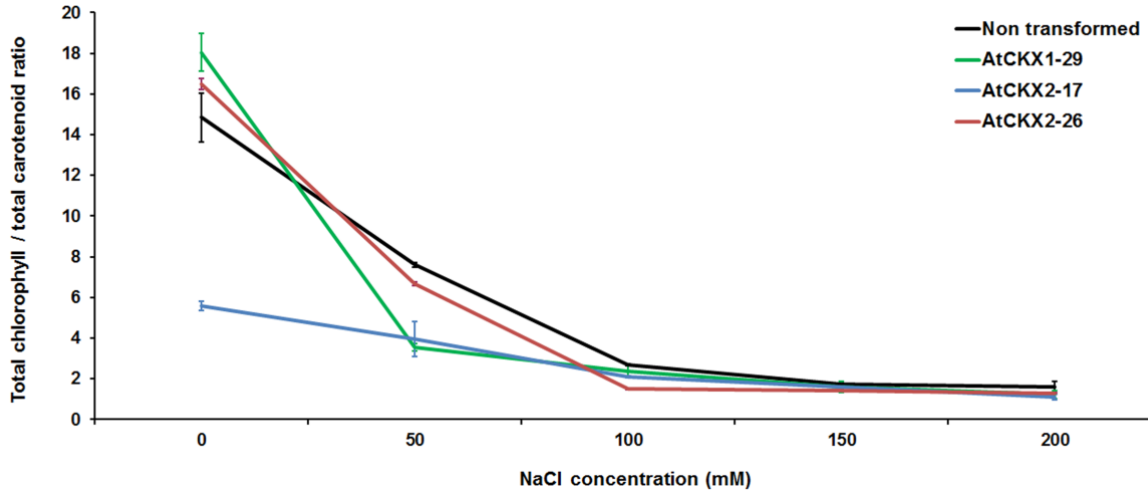

Figure 5. The total *Chl* and total carotenoid ratio in the shoots of non-transformed and transgenic centaury lines (*AtCKX1*-29, *AtCKX2*-17, and *AtCKX2*-26) after 8 weeks of growth in ½MS medium supplemented with 0-, 50-, 100-, 150-, and 200-mM NaCl. The data represent the mean ± standard error. Means marked with a different letter significantly different from the control, according to the LSD test ($p \leq 0.05$), are presented in Supplementary Materials.

4. Discussion

In this study, we postulated that a reduced amount of bioactive CKs in *AtCKX* transgenic centaury plants could alter the photosynthetic pigment contents in shoots grown under salt stress in vitro. There is a lot of evidence in the literature that the phenomenon of senescence is also regulated by CKs [41] and reducing the *Chl* content represents one of the significant senescence markers [42–45]. It is also known that salt stress causes progressive *Chl* degradation, which leads to a reduced light absorption in plant leaves [5,46–48]. The results of this work showed that, in the transgenic *AtCKX2*-17 shoots, an almost three times lower total *Chl* content was observed in comparison to the non-transformed shoots. Thus, it can be assumed that a reduced amount of cytokinin speeds up senescence. On the other hand, a total *Chl* content similar to that in the non-transformed shoots was determined in the transgenic line *AtCKX1*-29, while a significantly higher total *Chl* content was observed in *AtCKX2*-26. An increased total *Chl* content was also obtained in the transgenic *AtCKX2* tobacco line, where the leaves remained green longer than the control leaves, which turned yellow faster. In addition, it was observed that the amount of total *Chl* in the transgenic tobacco leaves decreased during the time [49]. Despite our theoretical expectations, the transgenic *AtCKX* leaves of *Arabidopsis thaliana* also did not show accelerated senescence [28]. Some previous investigations also showed that an increased CKX activity in senescing barley [50] and a high expression of the CKX genes throughout the senescence in *Trifolium repens* [51] support our findings that *CKX* genes in fact do not induce senescence but facilitate the progression of senescence. Ciobanu and Sumalan [52] reported that the tomato *Chl* content decreased with an increasing salinity, and the changes in the *Chl* content of the leaves occurred barely after a prolonged growth of six weeks. The modulation of the photosynthetic performance in *Pennisetum* leaves under long-term moderate salinity stress also involved the inhibition of leaf pigment synthesis [53].

Soil salinity and saline habits, as well as arid climates, greatly affect the synthesis of plant pigment components [54]. In general, the decrement of photosynthetic pigment contents under salt stress is considered to be a result of the slow synthesis or fast breakdown of pigments [55]. In all the centaury shoots, decreased *Chl a* and *Chl b*, as well as the total *Chl* content in increased NaCl concentrations in the culture medium, were recorded. These results are in accordance with a previous work, which reported that salt stress induced with NaCl caused a decrement in the total *Chl* content in *Phaseolus vulgaris* [56], *Catharanthus roseus* [57], *Vigna unguiculata* [58], *Vigna subterranean* [59], *Ocimum basilicum* [60], and *Solanum lycopersicum* [61]. Recent research showed that salinity significantly decreased the *Chl* content and photosynthetic efficiency of sweet sorghum [62] and peppermint plants [63]. It was also reported that *Chl a*, *Chl b*, and the total *Chl* content in centaury leaves decreased with the increment of the salt concentration [36]. The reduction of *Chl* under salt stress could be attributed to the increased activity of photosynthetic enzymes and instability of the pigment–protein complex [5,64–66]. Khan [67] also reported that saline stress slowed the production of the photosynthetic pigments. The reduction of the total carotenoid content was observed in all the centaury shoots cultured in mediums supplemented with >50-mM NaCl. The obtained results agreed with those reported in *Hordeum vulgare* [68], *Salvinia molesta* [69], *Cornus sericea* [70], and melons [19]. Salt stress induced the degradation of β-carotene, which further resulted in a decreased carotenoid content [71]. Carotenoids are integrated constituents of thylakoid membranes, and they absorb and transfer light to *Chl* and, thus, protect *Chl* from photooxidation [72]. Accordingly, the degradation of carotenoids might cause *Chl* degradation as well. The degradation of photosynthetic pigments under salinity is probably the consequence of increased toxic ions, enhanced chlorophyllase enzyme activity, and the damaging of the photosynthetic apparatus [20]. Recent research showed that a high level of toxic ions originating from different salts in wastewater induced the loss of photosynthetic pigments, including *Chl a*, *Chl b*, and carotenoids in wheat [73]. Considering that salinity caused an altered

photosynthetic pigment metabolism, it is clear that the *Chl* content is strictly regulated under abiotic stress [7].

Chl a is present in the reaction centres of photosystem I and II, while *Chl b* is present exclusively in the pigment antenna system [74]. The weight of the *Chl a*/*Chl b* ratio is an important indicator of the photosynthetic apparatus adaptation to the light conditions [75]. The *Chl a*/*Chl b* ratio in dicotyledonous plants is usually 3:1 [76]. In the present study, there was a tendency toward an increased *Chl a*/*Chl b* ratio with the increased salinity level in all the analysed centaury shoots. In the non-transformed shoots, in the range of the NaCl concentrations, the *Chl a*/*Chl b* ratio ranged between 0.88 and 8.72 μg/g FW; in transgenic line *AtCKX1*-29, the detected ratio was 0.95–9.14; in *AtCKX2*-17, 2.41–8.40; and in *AtCKX2*-26, 0.71–4.45. An increased *Chl a*/*Chl b* ratio was also observed in the control and transgenic *AtCKX2* leaves of tobacco [49]. Additionally, salt stress increased the *Chl a*/*Chl b* ratio in pepper [77]. During the process of *Chl* degradation, *Chl b* is converted to *Chl a*, and this might explain the increment of the *Chl a*/*Chl b* ratio [78]. Traditionally, the sun-shaded tolerance in plants has been defined based on the *Chl a*/*Chl b* ratio [79,80]. The *Chl* a/*Chl b* ratio has variable values, and when the ratio is ~3, it is clear that plants are adapting to the light conditions. The plants species living in the shadow showed *Chl a*/*Chl b* ratios lower than in the sun-exposed plants [40]. Thus, a decrease in the *Chl a*/*Chl b* ratio might be interpreted as an enlargement of photosystem II. Considering that *Chl a* is placed in photosystem I and II, it can be supposed that *Chl a* is probably primarily degraded during senescence. A stronger decrement of the *Chl a* content in comparison to *Chl b* and, accordingly, a reduced *Chl a*/*Chl b* ratio was also observed during leaf senescence in *Hordeum vulgare* leaves [81] and during dark-induced senescence in *Cucurbita pepo* cotyledons [82]. Previous investigations have also demonstrated darkness-induced cotyledon senescence in Arabidopsis seedlings [83]. Furthermore, it was indicated that CKs could serve as a regulatory mechanism coordinating senescence in individually darkened cotyledons [84]. Additionally, the in vitro culture conditions, although constantly controlled, enable light flux alterations in comparison to nature, and plants need to adapt to lowered light conditions. Thus, the ability of the whole plant to control the senescence progression underlies the coordination of senescence progression in individual leaves.

The weight ratio of the total *Chl* and total carotenoids is an indicator of the greenness of plants [40]. In a natural habitat, the *Chl*/carotenoids ratio is normally between 4.2 and 5 in leaves of sun-exposed plants and between 5.5 and 7.0 in leaves of shade-exposed plants. Lower values of the *Chl*/carotenoids ratio represent an indicator of the damage of the plant photosynthetic apparatus, which is expressed by a faster breakdown of *Chl* than carotenoids. Leaves with a *Chl*/carotenoids ratio between 2.5 and 3.5 are usually a yellow-green colour, and these leaves are experiencing senescence progress [85]. All the centaury plants used in this study were grown in vitro. The addition of NaCl in the medium reduced the *Chl*/carotenoids ratio in the centaury shoots. In the non-transformed shoots, in the range of the NaCl concentrations, the *Chl*/carotenoids ratio ranged between 14.84 and 1.61; in the transgenic line *AtCKX1*-29, the detected ratio was 18.05–1.22; in *AtCKX2*-17, 5.59–1.10; and in *AtCKX2*-26, 16.49–1.30. Based on the value of the *Chl*/carotenoids ratio in the non-transformed and *AtCKX* centaury plants grown in the NaCl-free medium, it can be assumed that the transgenic plants had a delayed senescence. Previous investigations have shown that the transgenic *AtCKX* leaves of *A. thaliana* and tobacco remained green even longer than the leaves of the non-transformed plants and, consequently, did not show an accelerated but, contrary to expectations, showed a delayed senescence [28,49]. Obviously, the decrement of CKs is certainly one of the preconditions, but not the initial signal, for the beginning of senescence. On the other hand, a decreased value of the *Chl*/carotenoids ratio in the non-transformed, as well as in the transgenic *AtCKX*, centaury shoots grown in a range of NaCl concentrations could be an indicator of accelerating the senescence process in the salt stress conditions. The increased values of the *Chl a*/*Chl b* ratio and, at the same time, decreased value of the weight *Chl*/carotenoids ratio are caused by the high

irradiance adaptation response of the photosynthetic pigment apparatus of sun leaves [86].

5. Conclusions

Non-transformed and transgenic *AtCKX* centaury lines have different photosynthetic pigment contents as a response to salt stress conditions. Contrary to the theoretical expectations, the transgenic *AtCKX* centaury leaves did not show accelerated senescence, although the photosynthetic pigment contents decreased with the increasing salinity in the culture media. According to our results, it is clear that a low content of CKs in transgenic *AtCKX* centaury lines has a very important role in the progression of salt-induced senescence, but it is not a major signal definitively triggering its onset. However, the effect of salinity on photosynthetic pigments is highly specific and certainly requires additional investigation in order to provide an improved understanding of the pigment content variations among transgenic centaury plants.

Supplementary Materials: The following are available online et www.mdpi.com/article/10.3390/agronomy11102056/s1, Table S1: Photosythetic pigments ratios in the shoots of non-transformed and *AtCKX* transgenic centaury lines after 8 weeks of growth in ½MS medium supplemented with graded NaCl concentration. The data represent the mean ± standard error. Means marked with a different letter significantly different from the control, according to the LSD test ($p \leq 0.05$).

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