Chapter

Transgenic *AtCKX* Centaury Plants Grown *In Vitro*

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Abstract

The production and breeding of plants with desired properties are possible by a fundamental biotechnological technique, genetic engineering. Applying and developing of genetic engineering procedures also enable preservation and improvement of plant species endangered in nature, including medicinal plant common centaury (*Centaurium erythraea* Rafn.). Numerous developmental processes in plants are controlled by cytokinins (CKs). The only so far known enzyme involved in CK catabolism is cytokinin oxidase/dehydrogenase (CKX). Genes coding for two *Arabidopsis* CKX isoforms, *AtCKX1* and *AtCKX2*, were successfully introduced into centaury root explants. Subsequently, the contents of endogenous CKs in *AtCKX*-overexpressing centaury plants grown *in vitro* were investigated. Simultaneous secondary metabolite analyses showed antibacterial and antifungal activity of transgenic centaury plants and suggested their use as potential producers of anticancer compounds. Considering that centaury can inhabit saline soils in natural habitats, following investigations included evaluation of salinity tolerance *in vitro*. All obtained and summarized results indicated that transgenic *AtCKX* centaury plants could serve as a suitable model for studies of numerous physiological and developmental processes under endogenous phytohormonal control.

Keywords: centaury, *Centaurium erythraea* Rafn., *in vitro* propagation, *AtCKX* genes, cytokinins, salinity

1. Introduction

A fundamental biotechnological method, genetic engineering, enables the production of plants with desired properties. Further breeding of obtained plants is possible by another biotechnological tool of great importance such as *in vitro* culture techniques. Applying of tissue culture procedures enables regeneration and multiplication of desired plants in controlled conditions in short time periods. Developing of plant tissue cultures also allows preservation and improvement of plant species endangered in nature, especially medicinal plant species such as e.g. common centaury (*Centaurium erythraea* Rafn.).

1.1 Genetic transformations of plants

Genetic engineering of plants represents a unique field of biotechnology where genes and other DNA sequences are manipulated to obtain plants with certain desired properties. The necessary steps in genetic engineering are the isolation and modification of the gene of interest, the vector construction, transformation and

the final selection of transgenic plants. Genetic transformation of plants implies stable incorporation of foreign genes into the plant genome [1]. Genetic transformation of plants can be very successfully achieved in two ways:

1. Techniques of direct gene transfer (microbombardment - biolistic method, microinjection, electroporation, polyethylene glycol mediated transformation, protoplast fusion)

2. Gene transfer techniques through biological vectors (viruses or bacteria - *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*)

Direct gene transfer techniques are continually developing and improving over the years. However, a considerable variation in the stability, integration and expression of the introduced gene represent their significant deficiencies. On the other hand, techniques of gene transfer through bacteria of the genus *Agrobacterium* have proven to be very efficient and straightforward. These techniques allow a more precise and stable integration of the desired gene into the plant genome [2].

1.1.1 Plasmids of bacteria of the genus Agrobacterium

The *Agrobacterium* genus is a group of Gram-negative soil bacteria. Unlike the wild type, laboratory *Agrobacterium* strains are commonly used for genetic transformations of numerous plant species [3]. Different plant species show different susceptibility to infection by *Agrobacterium* bacteria [4]. In general, dicotyledonous plants are more susceptible to infection by *Agrobacterium* bacteria than monocotyledons. Bacteria of the genus *Agrobacterium* can be present in the vascular tissue of plants (xylem and phloem) but without showing disease symptoms. It indicates that the interaction of bacteria and plant cells does not always occur. Infection and pathological changes of plant tissue occuronly after injury of the plant cells when released phenolic compounds causea positive chemotoxic reaction in *Agrobacterium*. These phenolic compounds further induce genes, located on chromosomal and plasmid DNA, responsible for bacterial virulence. During genetic transformations, the plasmids in bacterial cells serve as vectors. Bacteria of the genus *Agrobacterium* have large conjugated plasmids (200–250 kb), parts of which have been successfully integrated into the plant genome during transformation processes for almost half a century [5]. In these bacteria, two basic types of plasmids are identified by the kind of disease they cause: Ti-plasmid (tumor-inducing) in *A. tumefaciens* and Ri-plasmid (root-inducing) in *A. rhizogenes*.

Both types of plasmids contain three genetic regions that are necessary for the genetic transformation process:

1. T-DNA (transferred DNA) – a part of plasmid DNA, size 10–30 Kbp. The transport of T-DNA into a plant cell allows expression of bacterium genes in the plant itself [6].

2. *Vir* (virulence) region, size 35 Kbp, which consists of several large loci (*vir*A, *virB*, *virC*, *virD*, *virE*, *virG*, *virH* and *virJ*). *Vir* genes encode the synthesis of vir proteins, which are responsible for the process of recognizing specific compounds synthesized by a plant cell after injury and transferring T-DNA to the host cell.

3. The bacterial chromosome region, which is made up of *chv* (chromosomal virulence) genes that regulate bacterial chemotaxis and bacterial binding to an

injured plant cell [7]. According to Simonović [8], the *chvA* and *chvB* genes are responsible for the production and secretion of cyclic 1,2-glycans while *chvE* product forms a transmembrane receptor complex together with the plasmid *virA* product.

The development of plant tumor tissue occurs after a part of the plasmid DNA (T-DNA) is transported and incorporated into the DNA of the plant. T-DNA of Ti- and Ri-plasmids carries oncogenes, the genes responsible for tumor formation and encoding enzymes that catalyze the synthesis of auxins and cytokinins. Transcription of these genes leads to an increased amount of these plant hormones, which stimulates uncontrolled cell division, tumor development and mass proliferation of transformed cells [9].

1.1.2 Mechanism of genetic transformation of a plant cell using Agrobacterium tumefaciens

Successful genetic transformations of plants using *Agrobacterium* require a bacterial strain with appropriate density, explant tissue culture, determination of the substrate and time for cocultivation, as well as temperature and selection regime [10]. In wild strains of A*grobacterium*, the Ti- and Ri- plasmids have a T-DNA region divided into two sequences (T_L and T_R) between which is a T_C sequence that is not transmitted to the plant genome [11]. Oncogenes are located on the T_L sequence while genes for opine synthesis are located on the T_R sequence. Opines accumulate in transformed plant cells thus representing metabolic markers for genetic transformations. They seem useless for the plant as no appropriate enzymes for their degradation have been found in plant tissues. Pathogenic bacteria use released opines as a source of carbon and nitrogen. The genes on the plasmid itself encode the enzymes for catabolism of opines whereby the opine, whose synthesis is induced in transformed tissue, is degraded [12]. In laboratory strains of *Agrobacterium*, natural T-DNA can be replaced by a gene of interest that should be introduced into the plant genome by genetic transformation.

Genetic transformation of plants by *Agrobacterium* is a process that, in general, consists of three steps:

1. *Recognition and "attack" of a plant cell by a bacterium*

   Injured plant cells begin to synthesize specific compounds (amino acids, sugars and organic acids). In dicotyledons these are phenolic derivatives syringone and acetosyringone [13] while in monocotyledons it is ethyl ferrulate [14]. These compounds cause a positive chemotoxic reaction that leads to interaction of plant cells and bacteria. Binding of a bacterium to the plant cell represents the first step in the mechanism of genetic transformation.

2. *Transfer of T-DNA into a plant cell*

   VirD and VirE proteins play a significant role in the formation of single-stranded T-DNA, its cutting and transfer to the plant cell. The products of the VirD region (VirD1 and VirD2 proteins) are part of an endonuclease complex catalyzing specific single-stranded breaks that release a single-stranded T-DNA segment from the Ti plasmid. After excision, the T-chain shifts polarly and covalently binds the VirD2 protein. This protein protects the T-chain from destruction and remains there during transport to the plant cell. The transport of VirD2/T-DNK complex (immature T-complex) into the cytoplasm of the host cell, together with several other vir proteins (VirE2, VirF and VirE3), is performed through a specialized

membrane transport system. In cytoplasm of the plant cell, a mature T-complex is formed, which is protected from the action of nucleases during transport to the nucleus [15].

3. *T-DNA gene incorporation and expression*

The last, and probably the most critical step of genetic transformation, is the integration of T-DNA into the host genome. Integration into the plant genome occurs by non-homologous recombination in any part of the genome, taking preferentially place in regions with a higher percentage of adenine-thymine (AT) bonds [16]. Transgene expression depends on numerous factors such as e.g. the number of copies of an introduced gene and the site of transgene integration [17–19]. Stably integrated T-DNA is transferred to the next generation [20].

The mechanism of genetic transformation today involves the use of so-called "disarmed" *A. tumefaciens* strains, in which oncogenes are removed from the plasmids. The system of binary vectors usually used in genetic transformation processes divides the original Ti-plasmid into two plasmid vectors: an artificial (recombinant) Ti-plasmid carrying the gene of interest and an auxiliary plasmid [21]. In each binary plasmid, additionally to a foreign gene of interest the marker genes are also inserted and divided into selective and reporter genes [22]. The selective marker genes are most often prokaryotic genes that encode resistance to some antibiotics (kanamycin, hygromycin etc.). This negative selection implies using a selective medium which allows the distinction of transformed plant cells from non-transformed ones. Reporter genes are common but not necessary parts of the binary vector. These genes encode products which can be involved in the monitoring of transformation process (growth of transformed cells, regeneration efficiency and growth of transgenic plants). Visual reporter genes have been increasingly used because they do not require special artificial substrates for detection, and their expression is easily visually detected in tissue without destroying.

1.2 Cytokinins

Cytokinins (CKs) are plant hormones that individually or in combination with other hormones regulate numerous developmental and physiological processes in plants [23]. The name cytokinins comes from their originally discovered function, which is the stimulation of cell division – cytokinesis [24]. Cytokinins are divided into two major groups: natural and synthetic.

Natural CKs by chemical structure represent adenine derivatives. Depending on the component that binds to the adenine ring, natural CKs are further categorized into two groups:

1. *Isoprenoid CKs* – appear when one isoprenoid C_5 unit binds to the N^6 atom of adenine [25]. Natural isoprenoid CKs include N^6-(Δ^2-isopentenyl)adenine (iP), *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z) and dihydrozeatin (DHZ) and their derivatives (ribosides, *N*- and *O*-glucosides, ribotides) differing in their biological functions. The chemical structures of isoprenoid cytokinins are shown in **Figure 1**. The contents and distribution of individual isoprenoid CKs in plants vary depending on the plant species, particular plant tissue, developmental stage, etc.

2. *Aromatic CKs* – appear when an aromatic ring (either unsubstituted or substituted by a hydroxy or methoxy group) is attached to the N^6 atom of adenine

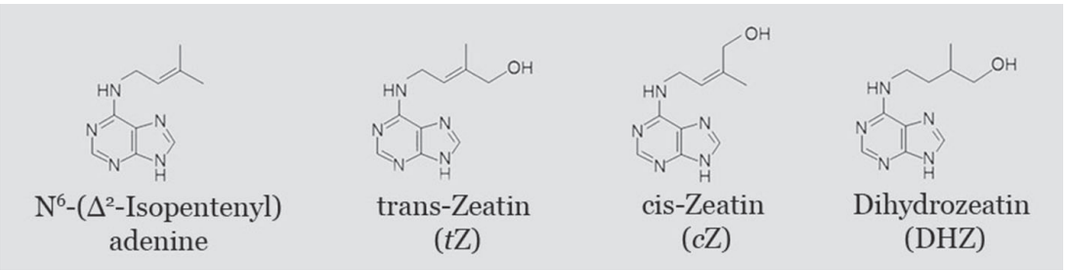

Figure 1.
Chemical structures of natural isoprenoid cytokinins.

[26]. Substitution of the aromatic ring with a hydroxy group produces hydroxy derivatives such as *ortho*-topolin (oT) and *meta*-topolin (mT), while substitution with a methoxy group creates methoxy derivatives such as *ortho*-methoxytopolin (meoT) and *meta*-methoxytopolin (memT). Hydroxylation of the aromatic ring in *meta* position is a prerequisite for high CK activity [27, 28] and *meta*-topolin shows the most considerable effects in plants of all derivatives. The chemical structures of natural aromatic cytokinins are shown in **Figure 2**. Despite the isolation of the first naturally occurring aromatic CK as early as in the early 1970s [29], the distribution and function of aromatic CKs in the plant kingdom remain still unclear [30].

Synthetic CKs by chemical structure represent phenylurea derivatives. These substances show CK effects, however, to date they have not been discovered to be synthesized in plant tissues by normal metabolic pathways. The first identified synthetic CK was diphenylurea (DFU), discovered in 1955 [31]. The unexpected discovery of this compound stimulated the synthesis of numerous analogues such as CPPU (N-phenyl-N′- (2-chloro-4-pyridyl) urea) and TDZ (thidiazuron). These synthetic substances, presented in **Figure 3**, are highly stable and have stronger CK activity compared to zeatin [32–35].

1.2.1 Cytokinin catabolism

During plant development the levels of endogenous CKs are tightly metabolically regulated in plant tissues. 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). This enzyme irreversibly degrades CKs by removing the N^6-substituted side-chain to form adenine and the unsaturated aldehyde 3-methyl-2-butenal [36] (**Figure 4**). To date, CKX is the only known enzyme involved in the catabolism of specific CKs, and thus it represents a crucial factor in the control of CK levels in plant tissues. It is known that especially iP, *t*Z and their ribosides are sensitive to the CKX action. On the other hand, *c*Z is less sensitive [37] even

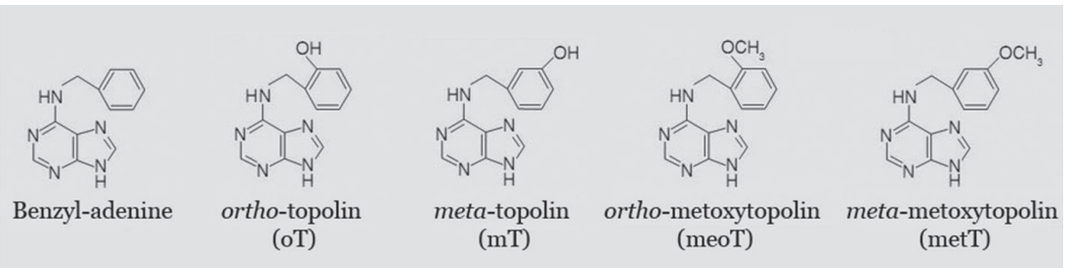

Figure 2.
Chemical structures of natural aromatic cytokinins.

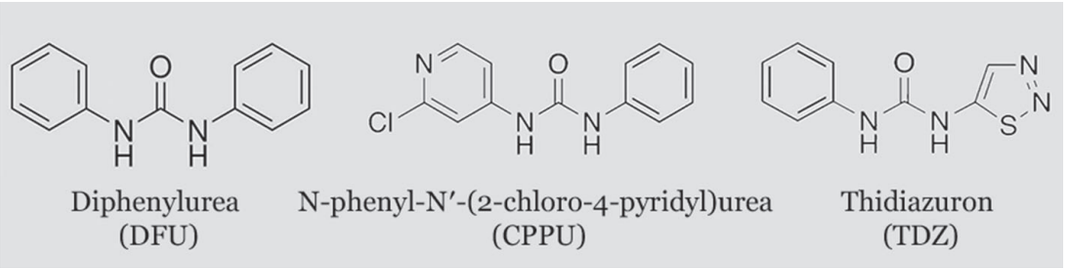

Figure 3.
Chemical structures of synthetic cytokinins.

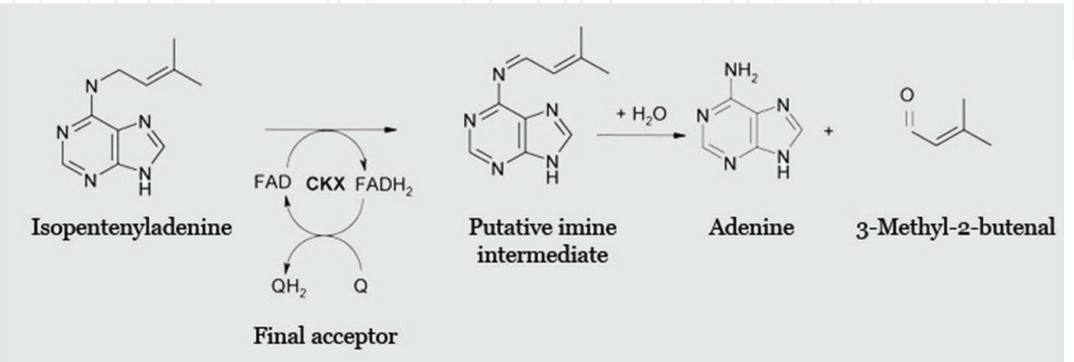

Figure 4.
Chemical reaction of irreversible cytokine degradation by CKX enzyme.

though some CKX isoforms exhibit high affinity for the *c*Z isomer as well [38]. CK *O*-glucosides, DHZ, aromatic CKs and their derivatives are believed to be the non-substrates for CKX [39–41]. By contrast, *Arabidopsis thaliana*, barley and maize CKX isoforms showed ability to degrade CK *N*9-glucosides [37, 42, 43].

According to the chemical structure, CKX enzyme is a flavoprotein with the flavin adenosine dinucleotide (FAD) bound domain [44, 45]. The activity of CKX in plant tissues is induced by exogenously applied CKs, both CKX substrates and non-substrates [46–48]. The CKX activity was first discovered in tobacco tissue [49], and subsequently this enzyme has been shown widely distributed in numerous plant tissues such as e.g. maize [50], *Vinca rosea* tumor [36], *Phaseolus vulgaris* callus [51, 52], wheat [53], and tobacco and poplar calli [47, 54]. The CKX enzyme is encoded by a small family of genes whose number varies from species to species. In *Arabidopsis thaliana*, seven genes responsible for the synthesis of CKX enzyme (*AtCKX1–7*) have been identified. These genes are expressed variously in different plant tissues [55, 56]. Individual *AtCKX* genes are expressed in the same tissues where CKs are synthesized (axillary buds, trichomes and vascular tissues) or in the nearby of these tissues. Genes encoding the CKX enzyme have been cloned also from numerous plant species (a detailed literature review is presented in **Table 1**).

1.3 Genetic transformation of plants using *AtCKX* genes

Genetic transformations of plants with specific genes encoding the CKX enzyme are beneficial for investigations of CK homeostasis. Thus, plants exhibiting increased overexpression of the *CKX* genes and a reduced amount of endogenous CKs can be obtained. Genetically engineered CKX plants can serve as a sound and valuable model system for studying physiological and morphological processes that are under control of CKs. To date, the impact of overexpression of different *CKX* genes on CK metabolism has been investigated in several plant species, as summarized in **Table 2**. The overexpression of *CKX* genes in tobacco and *A. thaliana* plants

Plant species	*CKX* genes	References
Arabidopsis thaliana	*AtCKX1–7*	[55, 56]
Zea mays	*ZmCKX1-5*	[57]
	ZmCKX10	[41, 58–60]
Dendrobium sp	*DSCKX1*	[61, 62]
	DhCKX	[63]
Hordeum vulgare	*HvCKX1*	[64]
Oryza sativa	*OsCKX1–11*	[65]
Pisum sativum	*PsCKX*	[66, 67]
Setaria italica	*SiCKX1–11*	[68]
Triticum aestivum	*TaCKX1–6*	[69–73]
Brassica napus	*BnCKX1–7*	[74]
Gossypium hirsutum	*GhCKX*	[75]
Glycine max	*GmCKX1–17*	[76]
Jatropha curcas	*JcCKX1–11*	[77]
Medicago sativa	*MsCKX*	[78]

Table 1.
The list of CKX genes cloned and identified in different plant species.

Plant species	Initial explants	Genes used for transformation	References
Nicotiana tabacum L cv. Samsun	Leaf explants	*AtCKX1-AtCKX4*	[79]
Arabidopsis thaliana	In planta transformation	*AtCKX1-AtCKX6*	[55]
Physcomitrella patens	Protoplast from liquid culture	*AtCKX2*	[80]
Solanum tuberosum cv. Solara	Leaf explants	*AtCKX1*	[81]
Solanum tuberosum cv. Désirée	Leaf explants	*AtCKX2*	[82]
		AtCKX1	[83]
Centaurium erythraea Rafn.	Root explants	*AtCKX1, AtCKX2*	[84]

Table 2.
The list of plant species genetically transformed using CKX genes isolated from Arabidopsis thaliana.

was demonstrated to affect significantly the phenotype of transformed plants, causing the *cytokinin deficiency syndrome*. In transgenic plants with this syndrome, apical dominance was decreased, shoots grew slowly, leaf size was reduced, the plants bloomed later and had a smaller number of flowers. Increased root growth size and activity of the root apical meristem (RAM) were observed. At the same time, decreased size and the shoot apical meristem (SAM) activity were also found. These symptoms were more evident in plants overexpressing *AtCKX1* transgene than in those with *AtCKX2* gene [55, 79]. As most of existing knowledge on regulation of CK levels and effects in plants have been obtained from studies of consequences of exogenously applied CKs, the specific CK-deficient transformants are expected to be more informative and to help in elucidation of the processes controlled by this group of phytohormones.

In plant shoots, CKs stimulate function of apical meristem and cell divisions. The stimulatory role of CKs on cytokinesis was discovered a long time ago [85]. Recently, it has been confirmed by analyses of transgenic *AtCKX A. thaliana* plants with reduced endogenous CK levels. Ultrastructural analysis of SAM cells showed clear cytological changes indicating detention of cell division and accelerated cell differentiation in transgenic CK-deficient *AtCKX A. thaliana* plants [86]. The development of vascular elements is also regulated by CKs. The optimal concentration of CKs in combination with auxins affects the formation of phloem [87]. In the shoots of transgenic *AtCKX A. thaliana* plants, xylem and phloem were reduced [55]. In addition, CKs control leaf formation and growth. Overexpression of the *CKX* transgenes in *AtCKX A. thaliana* plants significantly reduced leaf surface, which is undoubtedly related to cell divisions as previously described [55]. It has been shown that CKs are not able to completely prevent, but can significantly delay the senescence process [88]. Thus, it can be assumed that a reduced amount of endogenous CKs in CK-deficient plants accelerates senescence. Despite these expectations, the leaves of transgenic *AtCKX A. thaliana* plants did not show accelerated senescence [55]. The role of CKs during the plant reproductive development was approved by transgenic *AtCKX A. thaliana* plants, which formed a smaller number of flowers [55]. Enlarged embryos have also been observed in these plants, indicating a role of CKs in cell division during embryogenesis. The enlargement of embryos could be explained on the basis of *CKX* genes overexpression, occurring only in the later stages of embryogenesis [89].

Unlike the shoots, CKs inhibit the function of the RAM. Using the transgenic *A. thaliana AtCKX* plants, it has been confirmed that reduced CK levels strengthen the root system. The primary root growth is directly related to intense cell divisions in the root meristem of transgenic *A. thaliana AtCKX* plants [55]. The results obtained in these transformants are consistent with those by [90] demonstrating that exogenously applied CKs inhibited root elongation of untransformed plants by reducing the RAM size as well as reducing cell divisions. One would expect that the plants transformed with *CKX* genes have inhibited root branching. However, CK-deficient transgenic *AtCKX A. thaliana* plants surprisingly developed numerous lateral roots that elongated rapidly [55].

1.4 *Centaurium erythraea* Rafn.

Common centaury, *Centaurium erythraea* Rafn. (syn. *C. umbellatum* Gillib and *C. minus* Moench), is the most known species from Gentianaceae family. Thanks to numerous pharmacological activities, centaury is listed as a medicinal plant species. In traditional medicine, centaury is used to treat febrile conditions, anaemia, jaundice, gout and to regulate blood sugar [91]. Bitter centaury juices have also been used for an increment of appetite, digestion stimulation and treatment of gastrointestinal tract diseases [92].

Nowadays, *C. erythraea* is rapidly disappearing from natural habitat and it is marked as an endangered plant species. Fortunately, even almost twenty years ago centaury showed vigorous regenerative potential *in vitro* [93–97]. The most interesting and essential information is that spontaneous morphogenesis of centaury is possible on nutrition medium without addition of any plant growth regulator [98]. In addition, during the years, centaury has also been used for studies of numerous developmental processes [99–101]. On the other hand, only few literature data exist describing genetic transformation of centaury. To date, most of Gentianaceae species, including *C. erythraea*, were usually genetically transformed only with *A. rhizogenes* [93, 102]. These investigations have mostly based and described the efficiency of plant regeneration and selection of transformed plant tissues. All of these previous investigations also encouraged us to use *A. tumefaciens* in genetic

transformation of centaury for the first time [84]. The successful production of transgenic *AtCKX* centaury plants allowed to use them as a suitable model for studies of numerous physiological and developmental processes under endogenous phytohormonal control.

2. Methodology

All of the *in vitro* culture experiments were performed with plant material originated from *C. erythraea* Rafn. seeds, obtained from Jelitto Staudensamen GmbH, Schwarmstedt, Germany. The plant tissue culture methods were used to establish a solid centaury root culture further utilizable in the genetic transformation process. In all *in vitro* culture experiments, half-strength MS hormone-free medium (½MS) [103] solidified with 0.7% agar and supplemented with 3% sucrose and 100 mg l^{-1} *myo*-inositol was used. The *A. tumefaciens* strain GV3101 harbouring the pBinHTX plasmid with either the *AtCKX1* or *AtCKX2* gene under the control of the 35S promoter with Triple X enhancer [79] was applied for the transformation of *C. erythraea* roots. Usual molecular biology analyses, including PCR and qPCR reactions, were performed to confirm the insertion of *AtCKX* transgenes into centaury genome. The CKX activity was determined by *in vitro* assays based on the conversion of [2-^{3}H]iP (prepared by the Isotope Laboratory, Institute of Experimental Botany AS CR, Prague, Czech Republic) to [^{3}H] adenine. Detection and quantification of endogenous phytohormones were performed using HPLC/MS system with TSQ Quantum Ultra AM triple-quadrupole high-resolution mass spectrometer

Plant material	Methods	References
C. erythraea Rafn. seeds	Establishment of a solid root culture by plant tissue culture methods	[84]
Centaury root tips (≈10 mm long)	Genetic transformation of cut-edge root explants by immersing into *AtCKX1* and *AtCKX2* bacterial suspensions	
Non-transformed and transgenic *AtCKX* centaury shoots and roots	Molecular biology analyses including PCR and qPCR reactions to confirm insertion of *AtCKX* transgenes into centaury genome	
	Quantification of the CKX activity by *in vitro* assays	
	Detection and quantification of endogenous phytohormones by HPLC/MS	[104]
Non-transformed and transgenic *AtCKX1* centaury methanol extracts	Analyses of secondary metabolites by HPLC	[105]
	Determination of the nhibitory capacity of *in vitro* growth of two human colorectal carcinoma cell lines by sulforhodamine B assay	
Non-transformed and transgenic *AtCKX* centaury methanol extracts and pure secoiridod and xanthone compounds	Investigation of antibacterial and antifungal activity on Gram-positive, Gram-negative bacteria and microfungi by microdilution method	[106]
Non-transformed and transgenic *AtCKX* centaury shoots and roots	Evaluation of the shoots and roots salinity tolerance to graded NaCl concentrations by plant tissue culture methods	[107]

Table 3.
The list of all plant materials and methods used during the investigation of transgenic AtCKX centaury plants grown in vitro.

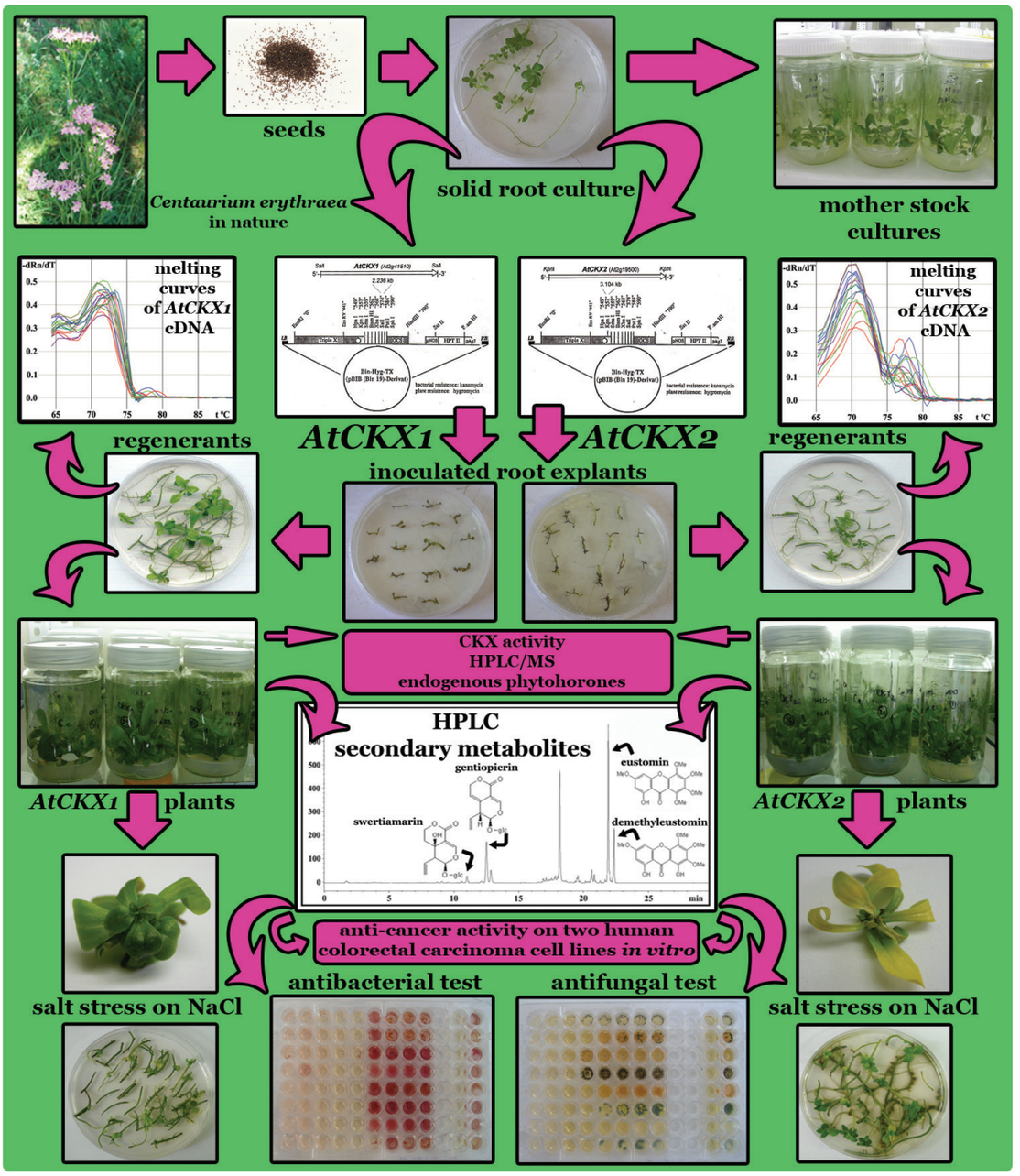

Figure 5.
Illustrative presentation of transgenic AtCKX centaury plants production in vitro and all subsequent investigations conducted to date.

(Thermo Electron Corp., San Jose, CA, U.S.A.) operated in the positive-SRM mode. Secondary metabolites including secoiridoids (swertiamarin and gentiopicrin) and xanthones (eustomin and demethyleustomin) were analysed on an Agilent series 1100 HPLC instrument with DAD detector and a reverse phase Zorbax SB-C18 analytical column. The secondary metabolite profile of transgenic centaury plants was determined to as certain whether these plants might serve as potential producers of anti-cancer compounds. The effect of non-transformed and transgenic *AtCKX* centaury methanol extracts on antibacterial and antifungal activity on bacteria and microfungi was also investigated. The salinity tolerance of non-transformed and *AtCKX* transgenic centaury shoots and roots to graded NaCl concentrations were tested separately *in vitro*. All plant material and methods used in investigations are presented in **Table 3**. The illustrative presentation of transgenic *AtCKX* centaury plants production *in vitro* and all further applied analyses are shown in **Figure 5**.

3. Molecular and morphological characterisation of transgenic *AtCKX* centaury plants

The possibility of direct regeneration of centaury shoots and/or somatic embryos makes root cultures a good model for studying *in vitro* morphogenesis of this plant species. Root cultures, in addition to easy manipulation, are characterised by other advantages such as small differences in physiological responses as well as high growth potential and metabolic activity. During the time, it has been shown that root cultures are suitable for production of genetically, biochemically and phenotypically stable plant material without somaclonal variation. The plant cultures, including a callus phase, theoretically promote a higher mutation rate [108]. The first literature data describing centaury root culture appeared almost twenty years ago, when the adventitious buds regeneration was shown to be induced with callus formation on initial root explants [109]. Spontaneous regeneration of adventitious buds in solid root culture was described, for the first time, by Subotić et al. [94]. These results motivated us to use root tips growing on½MS hormone-free medium as initial explants for further spontaneous regeneration and propagation of centaury plants. In our investigations, a solid root culture has been originally proved to be also a suitable and grateful model for genetic transformation of this medicinal plant species using *A. tumefaciens*.

Because of prosperous selection efficiency and adequate regenerative capacity of the transformants, the selection of suitable explants for initial infection with *A. tumefaciens* represents the most crucial step for a successful process of genetic transformation [110]. The potentially transformed centaury shoots formed directly on the root explants, without callus induction and thus minimized possible somaclonal variation [84]. Genomic PCR with specific primers confirmed the successful integration of the *AtCKX1* and *AtCKX2* transgenes into centaury genome in 30% and 28.2% of the analysed hygromycin-resistant lines, respectively. The quantification of transgene expression was confirmed by highly sensitive and precise method, qRT-PCR. As transgenic *AtCKX1* lines showed low expression and unspecific amplification of cDNA, optimization of PCR and qPCR protocols was necessary. The optimized protocol used for real-time quantification of the transgene expression included combining a gradient of annealing temperatures with the application of seven different PCR enhancers: formamide, DMSO, glycerol, ethylene glycol, trehalose, BSA and Tween-20 [111]. The expression of *AtCKX1* and *AtCKX2* transgenes was detected and quantified in 50% and 63.6% transgenic lines, respectively. The *AtCKX1* roots showed slightly higher expression than shoots whereas the *AtCKX2* transgene was better expressed in shoots compared to roots. Evidently, quantitative differences in transgene expression after incorporation into genome of different centaury lines depended on the transgene copy number and positional variations of transgenes.

All of the *AtCKX* transgenic centaury lines showed decreased regeneration capacity in root culture on the solid hormone-free ½MS medium in comparison to control. However, regenerated shoots spontaneously rooted on the same medium. On the other hand, fresh weight of shoots in most of analysed transgenic centaury lines showed no significant difference in comparison to control. In analogy, the root culture of *Dendrobium* orchid overexpressing *DSCKX1* transgene proved reduced shoot regeneration with decreased biomass compared to the non-transformed controls [112]. Similarly, the increased CKX expression affected on shortened internodes and reduced leaf surface of transgenic *Arabidopsis* and tobacco shoots [44].

4. CKX activity and endogenous phytohormone content in transgenic *AtCKX* centaury plants

All centaury transgenic *AtCKX1* and *AtCKX2* shoots and roots showed increased CKX activity. Two to ten times higher CKX activity was detected in roots compared to shoots, which corresponds with previously demonstrated data in other plants such as tobacco [113], maize and barley [41, 64, 114]. In analogy to *AtCKX*-overexpressing *Arabidopsis* plants [55] the higher CKX activity was detected in shoots and roots of *AtCKX2* than *AtCKX1* centaury lines.

As the CKX represents a valuable regulatory enzyme in CK metabolism, CKX activity directly affects the CK amount in plant tissues [115, 116], the contents and profile of endogenous CKs in centaury non-transformed and *AtCKX* transgenic plants have been determined. In general, centaury shoots grown *in vitro* produced higher content of CKs than roots [104]. Considering production and dominant location of CKs in the roots this distribution of endogenous CKs may be a consequence of *in vitro* conditions itself. The roots of centaury plants overexpressing *AtCKX* transgenes showed higher content of total CKs in comparison to non-transformed ones [84]. Previous investigations on *AtCKX* overexpressing *Arabidopsis* and tobacco plants revealed that increased CKX activity affected on the reduction of different endogenous CK metabolites [44, 55, 79]. Theoretically, increased CKX activity should cause a decline in the total CKs amount. Regardless of this, overexpression of *AtCKX* genes did not contribute to lowered total CKs content in transgenic centaury plants but rather to an alteration in the spectrum of particular CK types. In analogy, transgenic potato plants overexpressing *AtCKX* genes were not found to show decreased total endogenous CK contents while bioactive CK forms were predominantly reduced [82, 83]. Accordingly, although enhanced CKX activity results in more substantial CK degradation in plant tissues it might simultaneously serve as an indirect signal for the plant cell to intensify the biosynthesis of CKs. It may lead to higher accumulation of total endogenous CKs and seems to be a mechanism of re-establishment and maintenance of CK homeostasis in plants [117].

Beside the total CKs contents, an altered amounts of individual endogenous CKs groups were specific for*AtCKX* transgenic centaury plants [104]. The reported considerable differences in endogenous CK spectra might reflect the distinct position and/or the number of transgene copies. The most specific characteristic of transgenic centaury plants was a remarkable reduction of bioactive CK forms, including free bases and their corresponding ribosides. Similarly, the *AtCKX* transgenic tobacco and potato plants also showed decreased contents of bioactive CKs [79, 82, 83, 118, 119]. In transgenic centaury plants, the reduction of bioactive CKs was organ-specific. Thus, considerably declined levels of bioactive CK forms were found in the *AtCKX* shoots although their CKX activity was not enhanced. On the other hand, a significant reduction of bioactive CKs levels was shown in *AtCKX* roots together with an increased CKX activity. A possible explanation is that CKs are probably more degraded in roots, the main location of biosynthesis, and accordingly, a smaller content of bioactive CKs is further transported to centaury shoots. Another one attribute of the *AtCKX* transgenic centaury plants was represented by increased amounts of CK storage forms (*O*-glucosides) and/or of irreversibly inactive (or weakly active)CK *N*-glucosides [104]. This is in accordance with recent literature data demonstrating the same CK pattern in *AtCKX1* transgenic potato plants [83]. Simultaneously, the concentrations of CK precursors in transgenic centaury plants were higher than those of bioactive CKs, which also corresponds with the potato plants [82].

To summarize, the *AtCKX* transgenic centaury plants are characterized by altered CK profiles with reduced levels of bioactive CK forms and, at the same time, increased amounts of storage forms (CK *O*-glucosides), inactive (or weakly active)

forms (CK *N*-glucosides) andCK nucleotides. Previous investigations showed that overexpression of *AtCKX* transgene increased the production of storage CK derivatives in transgenic potato plants [81, 82]. It can be assumed that introduction of *AtCKX* transgenes into centaury genome altered CK metabolism in a way leading to the increased production of endogenous CK conjugates. Simultaneously, the level of bioactive CK forms seems to be controlled, besides the CKX activity, by additional regulatory mechanisms involved in CK metabolism and transport throughout the plant tissues. Considering that bioactive CKs are the preferred substrates of CKX, it is also possible that plant tissues activate new CK biosynthetic pathways and further initiate conversion to storage compounds, deactivation forms and/or CK nucleotides.

Numerous physiological and developmental processes in plants are regulated by the cooperation of CKs and auxins [120–123]. Overexpression of *AtCKX* transgenes in centaury plants altered not only the CK homeostasis, but at the same time it affected metabolism of auxins. In transgenic centaury plants, the auxin indole-3-acetic acid (IAA) concentration was significantly decreased compared to the control. In analogy, previous literature data uncovered that an enhancement of the CKX activity led to the decline of endogenous CK levels and simultaneously to the reduction of endogenous IAA content in *A. thaliana* [79, 122]. In contrast, increased IAA concentration was reported in the *AtCKX1*-overexpressing potato plants [83]. These findings support the fact that the balance between CKs and auxins represents the most crucial factor in the shoot and root development in plants [55, 79].

In transgenic *AtCKX* centaury roots, the IAA/bioactive CKs ratio was lowered in comparison to control. It might be a consequence of a reduced regeneration capacity of transgenic centaury roots [84]. Considering that endogenous IAA content was lowered in the roots, it is presumable that *AtCKX*-overexpression weakened the regeneration of shoots in solid roots culture. In transgenic centaury shoots, the IAA/bioactive CKs ratios varied considerably. Contrary to the centaury plants, endogenous IAA content was significantly increased in the shoots and roots of transgenic *AtCKX1* potato [83].

An altered CK metabolism resulting from the overexpression of *AtCKX* transgenes definitely affected IAA content and IAA/bioactive CKs ratio in transformed centaury plants. Different biosynthetic capacity for CK and IAA production could be a reason for the organ-specific differences among *AtCKX* transgenic plants.

5. Secondary metabolites in transgenic *AtCKX* centaury plants

It is known that *in vitro* cultivation, although under controlled temperature and light conditions, stimulates increased secondary metabolites accumulation [124]. The previous investigations also showed that genetic transformation of centaury roots using *A. rhizogenes* affected the production of secondary metabolites [102, 125]. Considering that centaury plants were transformed using *A. tumefaciens* carrying *AtCKX* genes for the first time, it was interesting to reveal the effects of altered levels of endogenous CKs on secondary metabolites production of these transgenic centaury plants. The results of our analyses indicated modifications of the secondary metabolites production in this valuable medicinal plant species as a consequence of the *AtCKX* transgenes overexpression [105]. In methanol extracts of *AtCKX* transgenic centaury plants, the presence of bitter secoiridoids (swertiamarin and gentiopicrin) and xanthones (eustomin and demethyleustomin) was detected. The fact that *AtCKX* transgenic centaury plants produced the same compounds as non-transformed ones confirmed that there were no qualitative differences in specific secondary metabolites resulting from the *AtCKX* overexpression. On the other hand, quantitative changes in the secondary metabolite contents were

found. Considering that gentiopicrin originates from swertiamarin in the metabolic pathway of iridoids, it is possible that increased activity of the swertiamarin → gentiopicrin converting enzyme stimulated gentiopicrin production. In all *AtCKX* transgenic centaury shoots, secoiridoid swertiamarin represented the predominant component. Still, in almost all centaury *AtCKX* shoots the content of swertiamarin was lowered in comparison to control shoots from *in vitro* cultures as well as from natural habitat. On the other hand, the production of the second bitter secoiridoid, gentiopicrin, was increased in the majority of *AtCKX* transgenic centaury shoots compared to control ones, grown *in vitro* and collected in natural habitat.

Unlike shoots, the *AtCKX* transgenic centaury roots produced decreased content of both secoiridoids, swertiamarin and gentiopicrin, in comparison to control roots grown *in vitro* and from natural habitat. It was shown previously that swertiamarin dominated in centaury shoots and roots from natural habitat while gentiopicrin prevailed in centaury shoots and roots grown *in vitro* [126–129].

Beside secoiridoids, the differences in xanthone content were also detected in *AtCKX* transgenic centaury plants. Most of *AtCKX* transgenic shoots and roots produced more eustomin and demethyleustomin than shoots from natural habitat but less than control *in vitro* shoots. The shoots and roots of only one transgenic centaury line, *AtCKX1*–29, produced significantly increased amount of both xanthones in comparison to both controls, shoots grown *in vitro* and those collected from natural habitat. It is important to note that shoots and roots of this transgenic line were characterised by a reduced level of bioactive forms of CKs [104].

5.1 Transgenic *AtCKX* centaury plants as potential producers of anti-cancer compounds and antimicrobials

The methanol extracts of *in vitro* cultured *AtCKX1*–29 transgenic centaury plants were also tested for their antimicrobial effects [106]. Four Gram-positive, four Gram-negative bacteria and eight species of microfungi were selected for these *in vitro* investigations. The methanol extracts of non-transformed centaury plants, as well as pure secoiridoids (swertiamarin and gentiopicrin) and xanthones (eustomin and demethyleustomin), were used as a control. In general, the extracts of all tested centaury shoots and roots showed an adequate antibacterial activity on all tested bacteria. Methanol extracts of non-transformed and *AtCKX1*–29 transgenic centaury roots and pure gentiopicrin had a low antibacterial activity on *Micrococcus flavus, Escherichia coli* and *Enterobacter cloacae. Staphylococcus aureus* was found the most sensitive bacterial species in the *in vitro* assays. It was also shown that the extract of non-transformed centaury shoots was more effective on *S. aureus* than antibiotics, streptomycin and ampicillin. Interestingly, the pure compounds such as swertiamarin, gentiopicrin, eustomin and demethyleustomin exhibited higher antibacterial activity on all tested bacteria than centaury methanol extracts and commercial antibiotics (streptomycin and ampicillin) used as a positive control. High antimicrobial activity could be ascribed to bitter secoiridoid glycosides, which corresponds with previous literature data [130, 131]. Beside antibacterial impact, all tested methanol extracts of centaury shoots and roots also showed antifungal effects. Most of the applied compounds proved high antifungal activities. All pure secoiridoids and xanthones were more effective than methanol extracts against all fungi. In general, xanthone eustomin showed even 100 times higher antifungal potential than both of the applied mycotics (ketoconazole and bifonazole) used as a positive control. These results are significant as they represent the first report describing centaury xanthones as potential antimicrobials. In general, methanol extracts of non-transformed and transgenic *AtCKX1* centaury shoots and roots showed better antibacterial activity, while pure secoiridoids and xanthones were more active against fungi.

Antioxidant properties of centaury plants and pure xanthones were reported almost twenty years ago [132, 133]. Considering that transgenic *AtCKX1–29* roots produced increased amounts of xanthones, their potential antioxidant activity was evaluated [106]. The transgenic *AtCKX1* centaury shoots and roots were found to exert higher antioxidant activity compared to non-transformed plants. The root extracts were two to five times more effective than those from the shoots. Transgenic *AtCKX1–29* roots, containing the highest amounts of total phenolics, were the most effective the scavenging of the DPPH radicals. The antioxidant effects of transgenic centaury methanol extracts could be assigned to elevated levels of xanthone compounds. Accordingly, centaury plants with increased content of secondary metabolites could be of practical importance in developing novel drugs with a potential use in agronomy, veterinary, medicine and food industry.

6. Transgenic *AtCKX* centaury plants under salinity stress *in vitro*

Taking into account that centaury plants can inhabit saline soils in natural habitats and because it is known that CKs play an essential role in the salinity stress response, the effect of NaCl-induced stress on regeneration potential of centaury shoots and roots was evaluated. The investigated non-transformed as well as *AtCKX1* and *AtCKX2* transgenic lines showed different salinity tolerance to graded NaCl concentrations. In general, a higher salinity tolerance was found for roots compared to shoots. Furthermore, elevated NaCl concentrations in the culture medium had no inhibitory effect on centaury shoot growth. All centaury shoots, regenerated on media supplemented with different NaCl concentrations, showed similar morphology as shoots regenerated in solid root cultures grown on NaCl-free media. Similarly, Šiler et al. [134] demonstrated that centaury plants keep the rosette forms under salt stress *in vitro*. Variations in the salt tolerance could be explained by differences among centaury genotypes, which is related to the recent report describing this species as a common salt-tolerant medicinal herb from Mediterranean region [135]. Whereas the non-transformed and *AtCKX1* transgenic line showed the same trend of shoot regeneration potential under salt stress conditions, the *AtCKX2* transgenic lines differed exhibiting a gradually decreased frequency of regeneration, the average number of regenerated shoots and fresh shoot weight with increased NaCl in the medium. The *AtCKX* transgenic centaury lines differed in their salinity tolerance from the non-transformed control indicating possible involvement of CKs in this process.

Beside morphological traits, biochemical characterisation of *AtCKX* transgenic centaury plants was determined. Amount of endogenous proline is considered one of the factors involved in the plant stress tolerance. Accordingly, an increase in the proline content was found in all *AtCKX* centaury shoots and roots grown on graded NaCl concentrations. Evaluation of malondialdehyde (MDA) level and hydrogen peroxide (H_2O_2) in centaury plants grown *in vitro* during salt stress also provided evidence of their oxidative stress tolerance. In all centaury *AtCKX* shoots and roots, MDA and H_2O_2 contents were increased at graded NaCl concentrations. These findings correspond well with literature data demonstrating an enhancement of MDA and H_2O_2 contents under salt stress [136, 137].

On the other hand, *AtCKX* transgenic centaury plants showed altered reactive oxygen species (ROS) homeostasis. It is rather difficult to summarize and conclude how salt stress affected on antioxidative enzymes activity. An increase of the superoxide dismutase (SOD) activity was detected in all centaury plants grown on graded NaCl concentrations. Interestingly, the SOD activity was always higher in roots than in shoots. Enhanced catalase (CAT) and peroxidase (POX) activities were detected in centaury plants grown on graded NaCl concentrations as well.

Similarly to centaury, transgenic *AtCKX2* tobacco plants showed increased SOD and CAT activity in comparison to wild type while lower SOD activity was found in roots than in shoots under salt stress *ex vitro* [118]. Increased SOD, CAT and POX activities in salt-stressed non-transformed and *AtCKX* transgenic centaury plants indicates a crucial role of these antioxidant enzymes in protecting plant tissues from superoxide radical and hydrogen peroxide and suggests their association with improved tolerance of plants to environmental stress conditions.

Evidently, the salinity stress caused morphological, physiological and biochemical changes in both non-transformed and *AtCKX* transgenic centaury plants. Elevated proline, MDA and H_2O_2 contents as well as increased antioxidative enzymes activities confirmed that centaury plants are trying to overcome salt stress conditions to maintain or re-establish their normal growth and development.

7. Conclusion and future perspectives

This chapter presents a survey of obtaining and developing transgenic *AtCKX* centaury plants grown *in vitro* and is intended to provide a synthesis of the existing literature data. In addition, recent achievements in the characterisation of transgenic centaury plants and determination as well as application of their secondary metabolite content is reported in details.

Centaury is a medicinal plant species endangered in natural habitat. A successful protocol for its genetic transformation using *A. tumefaciens* to introduce foreign, *AtCKX*, genes is described here. This protocol favours centaury root tips as primary infection explants that can directly regenerate shoots without callus phase. As the *in vitro* regeneration of centaury is possible without the addition of exogenous plant growth regulator(s), *in vitro* culture represents an optimal model for studying metabolic pathways of endogenous phytohormones including CKs without any interferences from other exogenous hormones present in nutrition medium. The first step of a successful genetic transformation of centaury was the establishment of stable transformants with *AtCKX* overexpression and enhanced CKX activity. Molecular, morphometric and developmental characterisation of obtained *AtCKX*-transgenic plants as well as chemical profiling of CK metabolites represented the following steps. The subsequent secondary metabolite analyses showed antibacterial and antifungal activity of transgenic centaury plants that can also be used as potential producers of anti-cancer compounds. Considering that centaury can inhabit saline soils in natural habitats, salinity tolerance of transgenic *AtCKX* centaury plants was investigated as well.

All obtained results were summarized and indicated that transgenic *AtCKX* centaury plants can serve as a suitable material for investigations of numerous physiological and developmental processes that are under endogenous phytohormonal control. Also, further studies of endogenous plant hormone regulations during NaCl-induced stress might represent an useful tool for better understanding the salinity tolerance of non-transformed and CK-deficient transgenic centaury plants grown *in vitro*. Future research will certainly be focused on the identification of centaury candidate genes specifically expressed during the salt stress to understand complex regulatory pathways in response of centaury plants to salinity *in vitro*.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, contract number 451-03-68/2020-2114/200007, and by the Czech Science Foundation, project number 19-12262S.

Conflict of interest

The authors declare that they have no conflict of interest.

Abbreviations

CAT	catalase
CKs	cytokinins
CKX	cytokinin oxidase/dehydrogenase
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
HPLC	high-performance liquid chromatography
MDA	malondialdehyde
MS	Murashige and Skoog medium
MS spectrometry	mass spectrometry
POX	peroxidase
ROS	reactive oxygen species
SOD	superoxide dismutase

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