Univerzita Karlova v Praze
Přírodovědecká fakulta
Katedra fyziologie rostlin

Effect of cytokinin deficiency on oxidative damage and antioxidant defence during ageing and abiotic stress

Zuzana Mýtinová

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Prohlašuji, že jsem tuto disertační práci vypracovala samostatně, s použitím citované literatury a že jsem tuto práci ani její podstatnou část nepředložila k získání jiného nebo stejného akademického titulu.

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podpis

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1 Content

2 Abbreviations:

A - antheraxanthin
ABA - abscisic acid
AHK - *Arabidopsis* sensor histidine kinase
AOE - antioxidative enzymes
APX - ascorbate peroxidase
ARR - *Arabidopsis* response regulator
Asc - ascorbate
AtCKX - Arabidopsis CKX
BR - brassinosteroid
CAT - catalase
Chl - chlorophyll
CK - cytokinin
CKX - cytokinin oxidase/dehydrogenase
*c*Z - *cis*-zeatin
DEPS - deepoxidation state
DHA - dehydroascorbate
DNPH - 2,4-dinitophenylhydrazine
DTT - dithiotreitol
DZ - dihydrozeatin
EDTA - ethylenediaminetetraacetic acid
F_0 - minimum Chl fluorescence yield in the dark-adapted state
F_m - maximum Chl fluorescence yield in the dark-adapted state
F_m'-maximum Chl fluorescence yield in the light-adapted state
F_v - variable Chl fluorescence yield in the dark-adapted state
F_v/F_0 - maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2
F_v/F_m - maximum photochemical efficiency
FW - fresh weight
GR - glutathione reductase
GSH - glutathione
GSSG - oxidized glutathione

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

iP - N^6- (Δ^2-isopentenyl)-adenine

IPT - isopentenyl transferase

JA - jasmonic acid

L - lutein

M - metal

MDA - monodehydroascorbate

N - neoxanthin

NBT - nitro blue tetrazolium chloride

NPQ - non-photochemical quenching

PAM - pulse amplitude modulation

PAR - photosynthetically active radiation

PCD - programmed cell death

PS - photosystem

PUFA - polyunsaturated fatty acid

ROS - reactive oxygen species

Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase

SAG - senescence-associated gene

SOD - superoxid dismutase

TAPS - 3-{[tris(hydroxy- methyl)methyl]amino}propanesulfonic acid

TEMED - N,N,N',N'-tetramethylethylendiamine

*t*Z - *trans*-zeatin

V - violaxanthin

WT - wild type

XO - xanthine oxidase

XTT - sodium 3,3´-{-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate

Z - zeaxanthin

Φ_2 - effective quantum yield of photochemical energy conversion in PS2

3 Preface

Ageing is inherent feature of the development of all living organisms, among them also of plants. At the very last stage of development senescence is initiated. Senescence is programmed process with an important function of coordinated degradation and retrieval of cellular components. The nutrients are remobilised from senescing into developing parts of a plant. Naturally there are a lot of physiological, biochemical and molecular studies focused on understanding the triggering and progression of this senescence. However, many questions remain still unanswered. Initiation of senescence is regulated by number of plant external and internal factors. First of all it depends on physiological age of a plant and/or a plant organ. Among internal factors, phytohormones are substantial regulators of senescence. Cytokinins (CKs), which prevent senescence, play the crucial role. The adverse external conditions can promote senescence phase. At the present time the most accepted theory of ageing is based on reactivity of free radicals and oxidative stress. Oxidative damage could contribute to activation of senescence programme.

In this study we tried to find relation between level of phytohormones CKs on the one hand and oxidative damage and antioxidant defence on the other hand during leaf ageing and senescence. As a model plant we chose transgenic tobacco with lowered level of CKs. The existing knowledge of CK effects on activities of antioxidant enzymes (AOE) has been achieved by studying consequences of externally applied CKs or experimentally enhanced endogenous CK contents. However, we used a reverse approach in our work by studying how reduced levels of CKs affect activities of AOE during the life span of tobacco. For this purpose we exploited transgenic tobacco plants overexpressing *Arabidopsis thaliana* gene for cytokininoxidase/dehydrogenase (CKX). Young seedlings of these plants were previously characterized and reported to exhibit increased CKX activity, significantly reduced amounts of endogenous CKs and pronounced phenotypical alterations

Because the level of phytohormones and antioxidants are related to the tolerance against abiotic stresses, we tested differences in susceptibility of control and transgenic tobacco plants subjected to several types of abiotic stress together with difference in their antioxidant capacity.

Studying ageing, leaf senescence and abiotic stress do not only contribute to our knowledge about these fundamental developmental processes, but may also help us to manipulate plant senescence for agricultural applications.

4 Introduction

4.1 Senescence and ageing

Ageing proceeds during the entire life span of plants. It could be divided into several developmental stages. These phases are: embryonic, vegetative and generative phase. The final stage, leading to death, is senescence. Senescence has been defined as the endogenous deteriorative process that is a natural cause of death of cells, tissues, organs and organisms.

Senescence in plants is illustrated clearly by the dramatic colour changes that occur in the autumn. Green leaves on trees and other perennial plants turn to yellow, orange and red before they eventually get brown, die and are defoliated. Senescence is controlled by the remaining parts of the plant, e.g. flower induction and seed development stimulate the senescence of leaves, buds and roots (Hillman *et al*., 1994). Senescence can be regulated not only by an array of internal but also by external factors. Many environmental stresses (such as extreme temperatures, drought, nutrient deficiency, high light, insufficient light/shade or total darkness) and biological insults can induce senescence. Internal factors influencing senescence include age, levels of plant hormones and developmental processes such as reproductive growth. These internal and external factors may act individually or, more probably, in combination (Gan, 2004).

4.1.1 Leaf senescence

During plant life span, a leaf undergoes at least three phases of development. Initially, it is expanding rapidly, importing carbon and nitrogen and undergoing rapid protein synthesis until its full capacity for photosynthesis is reached. Then the mature leaf becomes beneficial to the plant, contributing to the supply of carbon, during which time protein turnover is at a consistently low level. This continues until internal or external conditions initiate the onset of senescence (Buchanan-Wollaston, 1997).

It is generally accepted that leaf senescence is genetically controlled process, which is internally programmed and similar in all mesophyll cells. Many physiological, biochemical and molecular studies show that during leaf senescence cells undergo highly co-ordinated changes in cell structure, metabolism and gene expression. It is obvious, that leaf senescence is not simply a degenerative process, but is also a recycling process in which nutrients are

translocated from the senescing cells to the new buds, young leaves, developing fruits and seeds, or storage tissues. Mostly vascular system, required for nutrient export, is the last to senesce (Gan and Amasino, 1997). This regulation of leaf senescence might have an adaptive value, allowing the plant to complete its life cycle even under stressful condition (Ono *et al*., 2001).

4.1.2 Senescence processes at the cellular level

Leaf cells undergo defined subcellular changes during senescence. The loss of chloroplast integrity occurs first, whereas the breakdown of the nucleus is a relatively late event.

4.1.2.1 Chloroplasts

Chloroplasts are organelles in which the first symptoms of senescence are visible. Following an ordered sequence of events, the chloroplast dismantling begins with swelling, unstacking, and degradation of thylakoids (first those of the lamellae, then the grana) (Peñarrubia and Moreno, 2002). Thylakoid membranes provide an abundant source of carbon that can be mobilised for use as an energy source during senescence. The contact between grana stacks is loosened and, finally the membranes disappear in connection with an increase in the number and size of plastoglobuli (Smart, 1994). Concomitant with the disappearance of the membrane system is the degradation of membrane constituents, such as lipids, proteins and chlorophyll (Chl). (Hörtensteiner and Feller, 2002). With the decrease of chloroplast count the photosynthetic capacity declines as well.

4.1.2.2 Mitochondria and peroxisomes

When the photosynthetic capacity of the leaves is lost, the energy required for the remobilisation of nutrients must be provided by mitochondrial respiration, consequently, nuclear genes coding mitochondrial transport chain proteins are not down-regulated during senescence, in contrast to genes coding components of the photosynthetic apparatus (Buchanan-Wollaston, 1997; Anderson *et al*., 2004). As well as respiratory activities of the

mitochondria the catabolic activities of peroxisomes increase or remain steady during senescence (Smart 1994).

4.1.2.3 Endoplasmatic reticulum

The endoplasmatic reticulum appears at least partially to be unstable during senescence, and has been early degradated (Peñarrubia and Moreno, 2002).

4.1.2.4 Vacuoles

The vacuole plays several roles in the development of senescing cells. The vacuole is the destination of the final products of Chl catabolism (Matile *et al*., 1999; Thomas *et al*., 2001). There is evidence that the isolation of pigments and catabolites in the vacuole represents an adaptation for direct or indirect protection from photodamage, because the free Chl and its derivates are able to produce highly reactive singlet oxygen in the presence of light and oxygen (Matile *et al*., 1999; Feild *et al*., 2001). This organelle terminates regulated senescence by becoming autolytic and facilitating true cell death (Thomas *et al*., 2003).

4.1.2.5 Membranes

One of the most characteristic changes during senescence is the progressive loss of membrane integrity. Common membrane alterations during senescence include a decrease in the total phospholipid and protein content, an increase in neutral lipids, and generalised oxidation. Sterols also decline with physiological ageing. The decrease in the proportion of unsaturated fatty acids leads to a decline in membrane fluidity (Zimmermann and Zentgraf, 2005). As a consequence of all these symptoms, the physicochemical properties of the membranes are progressively altered during senescence and lead to a generalised failure of membrane functions (Peñarrubia and Moreno, 2002).

4.1.2.6 Nucleus

Degradation of the nucleus is one of the latest events during senescence, because all disintegrative and other processes are under the control of nucleus. At the nucleus, senescence

causes a progressive condensation of the chromatin. Condensation begins at early stages but the endonucleolytic fragmentation, that is typical of apoptotic processes, is a relatively late event (Peñarrubia and Moreno, 2002).

4.1.3 Senescence and the biochemical changes

4.1.3.1 Chlorophyll

Chl degradation related to chloroplast breakdown is the first visible symptom of senescence, especially during autumn, when leaves change colour from green to yellow and/or red. The different colours of leaves arise not only from the preferential degradation of Chl over caroteniods but also from the synthesis of red-coloured pigments like anthocyanins (Goodwin, 1958; Lichtenthaler, 1987). Feild *et al.* (2001) suggested that optical masking of Chl by anthocyanins, prefered in some plant species, reduces risk of photooxidative damage to leaf cells as they senesce, which otherwise may lower the efficiency of nutrient retrieval from senescing leaves.

The pathway for Chl degradation has been elucidated in the last few years (Matile *et al.*, 1999) and some of the genes in the pathway have been cloned. The catabolic pathway of Chl during senescence over unstable red Chl catabolites leads to the accumulation of colourless breakdown products (nonfluorescent Chl catabolites) (Tommasini *et al*, 1998; Hörtensteiner, 2004). Interestingly, nitrogen present in Chl is not exported from senescing leaves, but remains within the cells in the form of linear tetrapyrrole catabolites that accumulate in the vacuole (Hörtensteiner and Feller, 2002). The transfer of catabolites from senescent chloroplasts (gerontoplasts) to the vacuole is mediated by ATP-dependent transport systems (Hörtensteiner, 2006).

Chl breakdown is tightly connected with the dismantling of pigment - protein complexes and degradation of Chl-binding proteins (Hörtensteiner, 2006). The degradation pathway for Chls and chloroplast proteins are partially interconnected (Hörtensteiner and Feller, 2002).

Jongebloed *et al.* (2004) studied the development of castor bean leaves from bud break to abscission and they speculated from their results that the phloem blockage preceded Chl degradation in the course of natural ageing of leaves and leaf senescence.

4.1.3.2 Proteins

Numerous biochemical characterisations of increased proteolytic activity during senescence have been presented. In leaves, the vast majority of protease activity detectable with model substrates appears to localise in the large central vacuole (Beers, 1997). During soybean leaf senescence approximately 90% of soluble proteins are lost from the leaves (Wittenbach *et al*., 1980). On the other hand there are a lot of proteins with enzymatic disintegrative activity including protease, peptidases, RNases, peroxidases, chlorophyllases, esterases, cellulases, acid phosphatases, ß-1-3-glucan hydrolases that increase during senescence (Hillman *et al*., 1994).

Up to 75% of leaf proteins are located within the chloroplasts (Buchanan-Wollaston *et al*., 2003). Many protease genes, that show induced expression during senescence, appear to encode enzymes localised in the vacuole and therefore they are not in contact with chloroplast proteins until the membranes disrupt later during senescence. There have been a number of reports indicating that degradation of stromal proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) can be initiated non-enzymatically by reactive oxygen species (ROS) when chloroplasts are incubated in photo-oxidative stress condition (Ishida *et al*., 2000). However, it is not clear whether increased ROS could initiate the early degradation of Rubisco during senescence *in vivo*. Although ROS levels do increase during senescence they are likely the result of macromolecule degradation processes and thus occur after protein and lipid degradation is initiated (Buchanan-Wollaston *et al*., 2003).

4.1.3.3 Lipids

During senescence there is a decline in the structural and functional integrity of cellular membranes, which is the result of the accelerated catabolism of membrane lipids (Thompson *et al*., 1998). During senescence, lipoxygenases are activated. These are enzymes that oxidize free polyunsaturated fatty acids (PUFAs). Lipoxygenases transform PUFAs to lipid hydroperoxides in a reaction called lipid peroxidation. The latter are unstable and are decomposed to a great variety of products. PUFAs are conjugated with phospho- and galactolipids in membranes. Any change in membrane structure apparently activates membrane-bound phospholipases, which release the PUFAs and they become the substrate for lipoxygenases. The connection of senescence with lipid peroxidation is corroborated by an

increase in lipid peroxidation products and ROS with age (Spiteller, 2003). Senescence-enhanced expression of genes encoding enzymes required for β-oxidation and the glyoxylate pathway (Graham and Eastmond, 2002) indicates the importance of these pathways in lipid degradation and remobilization.

4.1.3.4 Nucleic acids

Nucleic acids, especially RNA, form a valuable source of phosphorus in mature leaf. Total RNA levels fall rapidly with the progress of senescence. Nuclear DNA is maintained to allow gene expression to continue, until late stage of the progress (Buchanan-Wollaston *et al*., 2003).

4.1.3.5 Metal ions and nutrients

Other valuable leaf constituents include metal ions such as K, Mo, Cu and Fe, and it is likely that much of these are also mobilized from leaves during senescence. Levels of many compounds measured (Mo, Cr, S, Fe, Cu and Zn) in *Arabidopsis* leaves were reduced by over 50% in senescent leaves when compared to green ones.

Metallothioneins and ferritin act as metal binding proteins. Senescence-enhanced expression of metallothionein-like genes has been detected in several plants (Coupe *et al*., 1995; Hsieh *et al*., 1995; Buchanan-Wollaston and Ainsworth, 1997). In *Brassica napus* the expression of a gene encoding ferritin has been found to increase during senescence (Buchanan-Wollaston and Ainsworth, 1997).

Levels of valuable nutrients N, P and K were reduced by at least 80% (Himmelblau and Amasino, 2001). It is seemed that N status influences the onset of senescence. For instance, alders (*Alnus* subsp.) shed their leaves while they are still green and it is known that N-rich fertilization should be avoided since it delays senescence (Kaskitalo *et al*., 2005).

4.1.4 The molecular biology of leaf senescence

Analysis of the *in vitro* translated proteins revealed that an abundance of majority of leaf mRNAs significantly declined during the progression of senescence, whereas some

mRNAs increased during senescence. The expression of the vast majority of genes is down-regulated, moreover, the expression of many genes is switched off (Lohman *et al*., 1994), whereas the expression of other genes is up-regulated during senescence (Gan and Amasino, 1997). For example, the abundance of transcripts encoding proteins involved in photosynthesis decreases sharply during senescence (e.g. Chl *a/b* binding protein or Rubisco small subunit), in contrast, the expression of subset of genes is up-regulated during leaf senescence, these genes have been named senescence-associated genes (*SAGs*) (Gan, 2004).

Recently many cDNA clones representing *SAGs* have been identified from a range of different plants. The identification of genes that are expressed specifically during senescence is the best evidence that *de novo* transcription is required for senescence to proceed (Buchanan-Wollaston, 1997). It is not surprising that among senescence associated genes are those encoding degradation enzymes such as RNases (Taylor *et al*., 1993), proteinases (Drake *et al*., 1996), lipases (Ryu and Wang, 1995), and genes with products involved in nutrient translocation processes. Some of the *SAGs* are already expressed at a low level before the onset of senescence, but their expression is elevated in senescing cell (Gan, 2004).

4.1.5 Programmed cell death and leaf senescence

To date, numerous examples illustrate the occurrence of programmed cell death (PCD) in plant development, such as petal senescence (Orzaéz and Granell, 1997), xylogenesis (Fukuda, 2000), aleurone deletion (Wang *et al*., 1996) etc. PCD is defined by a morphologically characteristic pattern of cell demise (nuclear condensation, chromatin fragmentation and blebbing of the cytoplasm) (Jacobson *et al*., 1997).

In some cases, the loss of cytoplasmic streaming has been identified as an early indication of PCD that occurs before the point of no return is reached, as defined by the loss of cellular compartmentation. Although cytoplasmic streaming and loss of compartmentalisation are the most widely used markers of plant cell commitment to die, the sequence of ultrastructural changes occurring within a number of systems indicates that, although characteristic for each type of PCD, they differ significantly from each other. Thus whereas loss of the nucleus is one of the early changes of the differentiating xylem, this effect on the nucleus is quite late in senescent leaves. In leaves generally, plastids are the first organelles affected and mitochondria the last; this may reflect the ATP requirements of the process. In some plant organs there is an early dismantling of the organelles involved in

protein synthesis (from nucleus to polysomes, membrane bound ribosomes), in others the early event is the rupture of the tonoplast (Granell, 1999).

The leaf senescence has been recognised for many years as a type of PCD (reviewed by Greenberg, 1996; Beers, 1997; Havel and Durzan, 1999; Cao *et al*., 2003). Yen and Yang (1998) reported the detection of PCD in senescent leaf tissue in five different plant species (*Philodendron hastatum*, *Epipremnum aurum*, *Bauhinia purpurea*, *Delonix regia* and *Butea monosperma*). They detected so-called DNA ladders on agarose gel after electrophoresis, resulting from the cleavage of nuclear DNA into oligonucleosomal fragments in cells of senescent leaves. Condensation of chromatin and damage to nuclear DNA has been also confirmed by Simeonova *et al.* (2000) *via* comet assay in *Ornithogalum virens* and *Nicotiana tabacum.*

Although it is clear that leaf senescence comprises PCD, there is very little information showing how and when PCD occurs within the leaf cells during natural leaf senescence. Cao *et al*. (2003) examined the leaf cells of *Eucommia ulmoides* and have demonstrated the asynchronous progress of PCD during leaf senescence. They presume that asynchronous PCD of cells in the leaf might be beneficial for efficient photosynthesis and nutrient remobilisation in naturally senescent leaves.

4.2 Regulation of leaf senescence

Leaf senescence has been regulated by many environmental and internal factors. The environmental factors include stresses such as extremes of temperature, drought, ozone, nutrient deficiency, pathogen infection, wounding, high light, and shading, whereas the intrinsic factors include age or phytohormone level.

In one general model aimed at the triggers of the senescence program, the leaf senescence is initiated when the photosynthetic rate drops below a certain level. That threshold may be at or near the compensation point at which the leaf no longer contributes the fixed carbon to the rest of the plant (Gan and Amasino, 1997).

4.2.1 Sugar signalling

Because leaves are specialised organs for performance of photosynthesis, the life span of leaves is optimised for the efficient production of photoassimilates, that is, of sugars.

Sugars (especially sucrose, glucose and fructose) are known to act as signalling molecules during various stages of plant development, including leaf senescence (Rolland *et al*., 2002). The expressions of several *SAGs* are induced by sugar starvation and suppressed by sugars (Yoshida, 2003). On the other hand some physiological and genetic analyses favour a hypothesis, that increased sugar levels or sugar signals induce leaf senescence. Sugars accumulate in tobacco leaves at the onset of senescence, and increased sugar levels have been reported in senescing leaves of several plant species (Noodén *et al*., 1997; Stessman *et al*., 2002; Quirino *et al*., 2001).

Transgenic plants over-expressing hexokinase, the sugar sensor that mediates both the sugar signalling and hexose phosphorylation (Rolland *et al*., 2002), exhibit premature senescence, indicating that this protein may have role in controlling senescence (Xiao *et al*., 2000).

It is still unclear how sugar affects the induction of leaf senescence under natural condition but it is suggested that enhanced sugar signal can induce premature senescence, most like *via* a hexokinase function. Increase in sugar level and/or signals repress photosynthetic activity by negative feedback regulation, and this system might be involved in inducing of leaf senescence (Yoshida, 2003).

4.2.2 Hormonal control of senescence

Complex interactions involving the perception of specific signals and the induction of cascades of gene expression must occur to regulate this process. Expression of the genes coding for senescence-related proteins is likely to be regulated *via* common activator proteins that are, in turn, activated directly or indirectly by hormonal signals (Buchanan-Wollaston, 1997). In general, ethylen, abscisic acid (ABA), jasmonic acid (JA) and its derivative methyl jasmonate, and brassinosteroids (BRs) promote senescence. In contrast, CKs, auxins, gibberellins and polyamines are retardants of senescence. While each particular plant growth substance may regulate leaf senescence independently, these regulators more likely act in various combinations to control the leaf senescence processes and interact with each other *via* some cross-talking network (Gan, 2004).

4.2.2.1 Ethylene

It is believed that ethylene is responsible for a climacteric-like respiratory surge (similarly to the ones in fruits and flowers) observed in senescent leaves of several plant species (barley, oat or tobacco). A lot of experiments show that application of ethylene can promote changes characteristic of leaf senescence in many plant species, including degradation of Chl, proteins and increase of catabolic enzymatic activities. Ethylene promoted senescence only in leaves that are old enough and it is less effective in promoting senescence in a leaf compared to fruit or flower (Gan, 2004).

4.2.2.2 Abscisic acid

The external application of ABA promotes senescence in detached leaves, but is much less effective in whole plant. Levels of ABA increase at the onset of senescence in leaves, but decrease at the late stage of senescence. However recent molecular genetic studies of many mutants deficient in either ABA biosynthesis or perception do not reveal specific roles for ABA in promoting leaf senescence (Gan, 2004).

4.2.2.3 Jasmonic acid

JA has also been implicated in senescence. It was shown that exogenous treatment of barley leaves with JA or methyl jasmonate led to a loss of Chl, indicating that senescence was induced (Parthier, 1990). Recently, the role of the JA pathway in senescence has been investigated in *Arabidopsis* (He *et al*., 2002). These authors showed that treatment of *Arabidopsis* with JA resulted in typical premature senescence symptoms. In addition, JA levels were shown to increase during senescence and several enzymes involved in JA biosynthesis showed senescence-enhanced expression. However, a promoting role for JA in leaf senescence has not been without controversy. For example, transgenic potato plants (constitutively expressing a flax allene oxide synthase gene (*ASO*)) overproduce JA, but no visible early senescence is observed (Harms *et al*., 1995), while *Arabidopsis* mutants with suppressed JA synthesis might not display a significantly retarded leaf senescence phenotype. It is possible that other factor may induce leaf senescence in absence of JA (Gan, 2004).

4.2.2.4 Brassinosteroids

BRs play an essential role in diverse developmental programs including senescence. ROS may mediate the BR-induced senescence because after BR treatment the level of malondialdehyde increases markedly while the activities of superoxide dismutase (SOD) and catalase (CAT) are inhibited (Gan, 2004).

4.2.2.5 Cytokinins

The signal that initiates the onset of developmental senescence appears to involve CKs. It has been known for many years that treatment with CKs can delay leaf senescence. Tobacco plants that express a CK biosynthesis gene (the *Agrobacterium ipt* gene; IPT-isopentenyl transferase) under control of senescence-enhancement promoter (*SAG12*) were shown to remain green and non-senescent for an extended period of time (Gan and Amasino, 1995). All aspects of senescence are postponed in the leaves of such transgenic plants, including prevented Chl and protein degradation, and loss of photosynthetic status (Wingler *et al*., 1998). This indicates that the senescence process is delayed at an early stage.

4.2.2.6 Auxin

A retardant role for auxin in leaf senescence has been supported primarily by early studies involving the external application of auxin and an inverse correlation between auxin and leaf senescence. In general, application of either synthetic or natural auxins delays Chl loss and protein degradation in detached leaves of various plant species, and the levels of endogenous auxins decrease at the onset of and during senescence in leaves. However there are some exceptions when auxin treatment has no effect or it even accelerates senescence. It is known that auxin can promote ethylene production, which in turn promotes senescence (Gan, 2004). It is still not clear what role auxins have in regulating leaf senescence.

4.2.2.7 Gibberellins

Most of the early work show that application of gibberellins can effectively inhibit degradation of Chl, proteins and/or nucleic acid in leaves. An inverse correlation between

gibberellins activity and leaf senescence has been observed in several plant species such as lettuce (Gan, 2004).

4.2.2.8 Polyamines

Polyamines, including putrescine, spermidine and spermine, are ubiquitous cellular components that play an important role in cell growth. The exogenous application of polyamines can retard leaf senescence by preventing from Chl loss and membrane peroxidation and by inhibiting RNase and protease activities in many plant species and experimental systems, although there are few negative examples. Polyamine levels are also higher in green than in senescing leaves (Evans and Malmberg, 1989)

4.2.3 Other ways of senescence regulation

When the senescence programme is initiated the cellular components are degraded and nutrients are transported. Various proteolytic processes are known to be activated during senescence (Noodén *et al*., 1997). Increased expression of polyubiquitin-conjugation activity has been reported (Belknap and Garbarino, 1996), and the activity of the 20S proteasome is retained during leaf senescence (Roberts *et al*., 2002). Ubiquitin-dependent proteolysis is likely to be involved not only in degradation processes during senescence but also in regulatory mechanisms (Yoshida, 2003).

Another recent analysis suggests that also lipid degradation may have a regulatory role in the progression of age-dependent senescence (Yoshida, 2003).

4.3 Cytokinins in more details

All the major plant hormones have been implicated in the senescence process, but only ethylene and in particular CKs have been shown definitively to have an antagonistic role in the regulation of senescence (Smart, 1994).

4.3.1 Structure, types and activity of CKs

A number of compounds with CK activity has been identified. Naturally occurring CKs are adenine derivates carrying either an isoprene-derived side chain or an aromatic ring at the N^6 terminus, respectively (Sakakibara, 2006).

Common natural isoprenoid CKs are N^6-(Δ^2-isopentenyl)-adenine (iP), *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), and dihydrozeatin (DZ) and *t*Z and iP sugar derivates, but there is a lot of variation depending on plant species, tissue, and developmental stages. For instance, *t*Z- and iP-type CKs are the major forms in *Arabidopsis*, whereas substantial amounts of *c*Z-type CKs are found in maize (Veach *et al*., 2003), rice (Izumi *et al*., 1988) and chickpea (Emery *et al*., 1998). The aromatic CKs, *ortho*-topolin, *meta*-topolin or benzyladenine are only found in some plant species (Strnad, 1997).

The side chain of zeatin-type CK occurs in ether *cis* or *trans* configuration, depending on which of the two methyl groups of the side chain is hydroxylated. These configurations differed in their activity. The activity of *t*Z was much higher than that of *c*Z (Kakimoto, 2003). Further, iP generally exhibited higher activities compared to *c*Z (Sakakibara, 2006), iP-type CKs are considered to have low biological activity in higher plant system (Van Staden and Drewers 1991) and are susceptible to CKX degradation (see below) (Mok *et al*., 2000), therefore it is *t*Z which is considered to be more active form then iP.

4.3.2 Biosynthesis of CKs

Generally, CK biosynthesis and homeostasis are controlled by internal and external factors such as other phytohormones and inorganic nitrogen sources. The previously widely accepted idea that CKs are synthesized only in root tips, shoot apices and immature seeds (Letham, 1994; Emery *et al*., 2000) is now overcome. It seems that they are synthetized and act at various sites in a plant organism (Sakakibara, 2005).

The CK biosynthesis pathway begins with the transfer of an isopentenyl group from isopentenyl pyrophosphate to adenosine 5´-phosphate producing isopentenyladenine ribotide, which is subsequently converted into various CK forms. The first step is catalysed by an IPT and appears to be the rate-limiting step because overexpression of *ipt* almost always leads to the overproduction of CKs in transgenic plants (Gan, 2004).

For many years, tRNA as a source of CKs has been the subject of debate. Measurement of tRNA breakdown indicated that this process could contribute up to 50 % of

the free CKs. An often argument against tRNA being a considerable source of CK is the fact that the *c*Z is the major CK in tRNA (Mok and Mok, 2001). The biosynthetic pathway leading to aromatic CKs is entirely unknown. To date, no enzymes mediating the conjugation of benzyl ring to adenine have been identified. (Mok and Mok, 2001). A precursor suggested for topolin is the amino acid phenylalanine (Zažímalová *et al*., 1999).

4.3.3 Inactivation of CKs

CKs are irreversibly inactivated by certain modifications in the purine ring structure or by cleavage of the N^6 side chain.

There are two types of conjugations with adenine in purine ring structure in CKs, with glucose or amino acid residues. Glucose conjugates at the N^7 or N^9 position in the adenine ring are formed readily in zeatin or DZ. N^3-glucosides are also formed but N^3-glucosylation is reversible, N^3-glucosides are biologically active and also appear to be less common (Srivastava, 2002). N-glucosylation is often initiated by exogenous application of CK or by an enhanced CK synthesis in *IPT* transgenic plants (Werner *et al*., 2003a). Amino acid conjugates with alanine at the N^9 position are also formed. N-glucosylated or amino acid conjugates are stable over long periods and they seem to be irreversibly inactivated products (Srivastava, 2002).

CKX (EC 1.5.99.12) is the enzyme that catalyses the cleavage of the N^6 side chain from adenine. The enzyme requires the double bond in the side chain for its activity. Hence, their natural substrates are iP and zeatin and their ribosylated derivates. CKX acts by removing the N^6-substituted isoprene chain of CKs or their ribonucleosides to produce adenine and the corresponding aldehyde. In case of iP, the degradation products are adenine and 3-methyl-2-butenal. CKX is inactive against O-glycosylated conjugates, where the side chains have a glucosyl or xylosyl residue, and against synthetic CKs, where the side chains have an aromatic ring (Amstrong, 1994).

CKX has been classified until recently as a copper-containing amine oxidase, using molecular oxygen as the oxidant (Amstrong, 1994; Hare and van Staden, 1994). At present, two entries about CKX exist in the IUBMB Enzyme Nomenclature database, the first propose to integrate the CKX under the recommended name "cytokinin oxidase" to the group of flavin-containing amine oxidase, the other suggested to classify the enzyme as "cytokinin dehydrogenase" that catalysed oxidation of CKs in the absence of oxygen without producing hydrogen peroxide during the catalytic reaction (Galuszka *et al*., 2001). Thus, the name of the

enzyme is now suggested to be cytokinin dehydrogenase or oxidase/ dehydrogenase rather than only oxidase.

CKX from different plant species exhibits the same substrate specificity, however they differ in their molecular weights, pH optima, kinetic constants, and the degree to which their activities are stimulated by copper-imidazole complex *in vitro* (Kamínek, 1992; Amstrong, 1994). These differences might be partially due to a different degree of protein glycosylation, which may affect compartmentalization of the enzyme in plant cell and, access to the substrate (Motyka *et al*., 1996)

Exogenous application of CKs has previously been shown to stimulate CKX activity in cultured tobacco cells and in callus culture of several plants (Motyka *et al*., 2003). Induction of CKX by its own substrate provides an elegant example of a negative feedback control over the endogenous levels of bioactive hormones (Srivastava, 2002).

Activities of CKX have been observed in a number of different plant species including wheat, maize, poplar, soybean, bean, and callus tissue from several plants (Amstrong, 1994). Genes encoding enzymes that catalyse CK breakdown have been isolated e.g. from maize (Morris *et al*., 1999), *Arabidopsis* (Bilyeu *et al*., 2001; Werner *et al*., 2001), and orchids (Yang *et al*., 2003). The *Arabidopsis CKX* gene family currently comprises seven members: *AtCKX1* - *AtCKX7*. Four AtCKXs contain N-terminal signal peptides that possibly direct the proteins to the secretory pathway; two signal peptides predict transport to mitochondria; and a single gene family member has a predict cytosolic location (Bilyeu *et al*., 2001; Werner *et al*., 2001; Schmülling, 2002).

4.3.4 Action of CKs

CKs are defined as compounds that promote cell division in callus and tissue culture. They regulate the ratio of shoot bud number and root extension in tissue culture and in stem cutting. In intact plants, they regulate apical dominance and lateral root initiation. It was shown that exogenous application of CKs inhibits the degradation of Chl and proteins of photosynthetic apparatus (Badenoch-Jones *et al*., 1996; Hudák *et al*., 1996). CKs are known to modulate the activity of genes for light-harvesting Chl-binding proteins (Kulaeva *et al*., 1996) and to promote the reversal of senescence by stimulating the expression of genes for the redifferentiation of senescent plastids - gerontoplasts back into chloroplasts (Zavalenta-Mancera *et al*., 1999). He and Jin (1999) suggested that leaf senescence in maize might follow

a sequence leading from changes in endogenous hormone levels to transmembrane Ca^{2+} fluxes, which then cause lipid peroxidation and subsequent degradation of Chl and protein.

Development of a strategy based on autoregulated CK production exploiting tobacco plants transformed with chimeric gene P_{SAG12}-*ipt* has indicated that maintaining CK contents in leaves above a certain threshold level inhibits transcriptional regulation of senescence-related genes and prevents the onset of senescence in the entire plants (Gan and Amasino, 1995). Following these results it has been reported that CKs delay leaf senescence (Gan and Amasino, 1996). Kim *et al*. (2006) found that AHK3 (AHK - *Arabidopsis* sensor histidine kinase), one of the three CK receptors in *Arabidopsis*, plays a major role in controlling CK-mediated leaf longevity through a specific phosphorylation of a response regulator, ARR2 (ARR - *Arabidopsis* response regulator).

The action of CKs is often masked by interaction with other hormones or substances. For example, CKs can interact with auxins either synergistically or antagonistically and induce the production of ethylene; therefore phenotypic changes are not easily identifiable as directly related to CKs (Mok and Mok, 2001). Accumulation of sugars during senescence, due to the breakdown of accumulated starch or the preferential export of N from the leaf, can block the effect of CKs, especially in low light (Wingler *et al*., 1998).

4.3.5 CK regulation of senescence

Majority, if not all, of our previous knowledge about the role of CKs in plant senescence was derived from experiments that involved the exogenously addition of CKs (Richmond and Lang, 1957; Noodén *et al*., 1979; Singh *et al*., 1992; Noodén and Letham, 1993). The other source is endogenously enhancing of CK synthesis in transgenic tobacco with chimeric gene P_{SAG12}-IPT (Gan and Amasino, 1995). Additional CK might affect processes that are normally not under CK control. For example, the inhibitory effect of exogenous CK on root elongation does not mean necessarily that CKs have a physiological role in controlling root elongation (Schmülling, 2002).

Plants with reduced CK content are expected to be more informative, because the lack of CK might cause a loss-of-function phenotype for physiological and developmental traits in which CKs are limiting (Werner *et al*., 2003b). Werner *et al*. (2001) genetically engineered CKX expression in transgenic tobacco plants to reduce their endogenous CK content. Transgenic tobacco plants expressing any of four *AtCKX* genes showed distinct developmental alterations of the shoot and root. AtCKX2 transgenic plants were dwarfed with

shorter internodes, they flowered later, produced fewer flowers, and had a reduced leaf surface with a smaller vasculature. Histological analyses showed that the shoot apical meristem was smaller, but cell size was not altered. The formation of new leaf primordia (plastochrone) and of new leaf cells were slowed down significantly. In total, only about 5 % of leaf cells of wide-type plants were formed in *AtCKX*-expressing plants indicating a stringent requirement of CKs for leaf cell formation (Werner *et al*., 2001).

It has been suggested before that a reduction in the CK concentration below a threshold level could serve as a signal to trigger leaf senescence (Noodén *et al*., 1990). However, visual leaf senescence did not occur earlier in *AtCKX* overexpressing transformants. In contrast, older leaves remained green (with the exception of intercostal regions) leading to a prolonged life span of individual leaves (Werner *et al*., 2001; Schmülling, 2002).

Further, Werner *et al*. (2003b) have engineered transgenic *Arabidopsis* plants that overexpressed individually six different members of the CKX gene family (*AtCKX*) and have undertaken a detailed phenotypic analysis. Transgenic plants had increased CK breakdown (30 % - 45 % of wild type CK content). The leaves of such CK-deficient *Arabidopsis* plants display signs of delayed rather than accelerated senescence. In their study, Chl was retained longer in CK-deficient compared to wild type leaves, particularly in regions adjacent to major veins. The authors argued against the hypothesis that CKs act as triggers of leaf senescence if their concentration decreased below a threshold level, and suggested instead that altered sink-source relationships in the transgenic plant might interfere with the normal mechanism of senescence. They concluded that decreasing the CK content in leaves is necessary for the progression of senescence but is not a signal that triggers its onset.

Also there is evidence that the enhanced level of CK can induce PCD. The CK 6-benzylaminopurine, if added at high dosage to plants and cultivated cell suspensions of *Arabidopsis thaliana* and *Daucus carota*, they induced PCD by accelerating senescence (Carimi *et al*., 2004).

The delay of senescence in transgenic tobacco (*Nicotiana tabacum*) plants with autoregulated CK production correlates with an elevated extracellular invertase activity. The observation that an increase in extracellular invertase is sufficient to delay leaf senescence was further verified by a complementing functional approach. Transgenic plants were generated that allowed inhibition of extracellular invertase in the presence of CKs. For this purpose, an invertase inhibitor was expressed under control of a CK-inducible promoter. It has been shown that senescence is not any more delayed by CK when the expression of the

invertase inhibitor is elevated. This finding demonstrates that extracellular invertase is required for the delay of senescence by CKs (Lara *et al*., 2004).

Riefler *et al*. (2006) studied mutants with loss-of-function AHK2 and AHK3 receptors of CKs. The earlier leaf senescence has not been noted in CK receptors mutants or CK-deficient plants, and the loss of Chl in detached leaves was not faster in the absence of exogenous CK in loss-of-function mutant compared to the wild type. This is consistent with the idea that a low CK content or reduced CK signalling are not triggering factors for the onset of the senescence process but that a low CK status is license for senescence to occur (Werner *et al*., 2003b).

4.4 Senescence as an oxidative process

As mentioned above the senescence of leaves is an active process that allows redistribution of their nutrients as a plant reaches final stage of its development. On the other hand senescence involves an oxidative process due to the overproduction of ROS (Piquery *et al*., 2000; Scebba *et al*., 2001).

The excessive formation of ROS is induced by several processes occurring during senescence, such as the activation of peroxidases in the peroxisomes, of membrane-bound lipoxygenases and the misleading of electrons in the electron transport chains (Zimmermann and Zentgarf, 2005). Cellular damage caused by ROS, *via* lipid peroxidation, is generally considered to be a major contributor to the senescence syndrome. In addition, ROS may be also involved in triggering senescence. In accordance, several *SAGs* are induced by ROS (Krupinska *et al*., 2003). However, the damage might be reduced or protected by enzymatic and non-enzymatic antioxidant defence system (Foyer *et al*., 1994). In plant system, there is evidence that increased radical levels during senescence are not only caused by the elevated production of radicals, but also by the loss of the compensatory function of AOE (Zimmermann and Zentgarf, 2005).

4.4.1 ROS in general

During the successive reduction of O_2 to water the ROS such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) can be formed (Fig. 1).

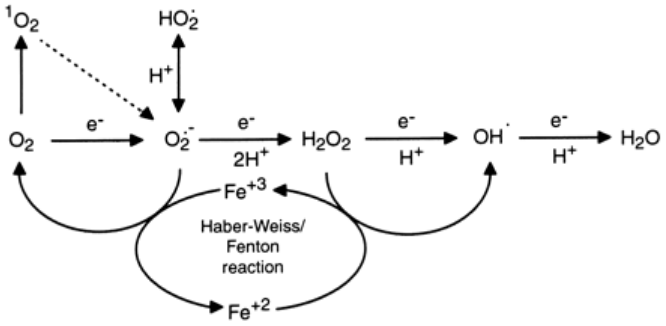

Figure 1.: Conversion of reactive oxygen species (ROS) derived from O_2 (from Vranová *et al*., 2002).

exothermic and can occur spontaneously, either catalysed or not. Reception of excess energy by O_2 can additionally lead to the formation of other type of ROS, the singlet oxygen ($^1O_2$), a highly reactive molecule. $^1O_2$ can live for nearly 4 μs in water and 100 μs in a non-polar environment. $O_2^{\cdot-}$ is a moderately reactive, short-lived molecule ROS with a half-life of approximately 2-4 μs. Therefore, $O_2^{\cdot-}$ does not cross biological membranes and is disproportionated readily to H_2O_2. Hydroperoxyl radicals ($HO^{\cdot}_2$) that are formed from $O_2^{\cdot-}$ by protonation in aqueous solution can cross biological membranes. H_2O_2 is moderately reactive and is relatively long-lived molecule (half-life of 1 ms) that can diffuse some distance from its production site. The most reactive of all ROS is the $OH^{\cdot}$ that is formed from H_2O_2 by the so-called Fenton or Haber-Weiss reactions by using metal catalysts (M^{n+}: Fe^{2+}, Cu^+):

$H_2O_2 + M^{n+} \longrightarrow OH^- + OH^{\cdot} + M^{(n+1)+}$ (Fenton reaction)

Oxidised metal can be er-reduced by $O_2^{\cdot-}$:

$O_2^{\cdot-} + M^{(n+1)+} \longrightarrow M^{n+} + O_2$

These reactions together create so-called Haber-Weiss reaction

$H_2O_2 + O_2^{\cdot-} \longrightarrow OH^- + OH^{\cdot} + O_2$ (Feierabend, 2005).

$OH^{\cdot}$ can potentially react with all biological molecules, and because cells have no mechanism to eliminate this highly reactive ROS, its excess production leads ultimately to death (Vranová *et al*., 2002).

Under normal growth condition, the production of ROS in cells is low (240 μM $O_2^{\bullet -}$ s^{-1} and a steady state level of H_2O_2 is 0,5 μM in chloroplasts). During the oxidative stress the cells enhance the production of ROS (240-720 μM $O_2^{\bullet -}s^{-1}$ and a steady state level of H_2O_2 is 5-15 μM in chloroplasts) (Mittler, 2002).

ROS have a double role in plant cells:

1. as signal molecules
2. as toxic by-products of aerobic metabolism they cause injury or even destruction of the cell components.

4.4.2 ROS as signal molecules

Plants use ROS as signalling molecules especially in response to various stresses or threats to the plant integrity, as pathogen attacks, or non-optimal environmental conditions. These molecules can act as messengers to trigger protein de/activation or induce gene transcription. It has been shown recently that both $^1O_2$ and H_2O_2 induced the transcription of specific sets of genes in plant cells (Navrot *et al*., 2007).

In contrast to $^1O_2$, which is very short-lived molecule, H_2O_2 is relatively stable. It is thus easier to imagine a role for this molecule in simple signal transduction circuits compared to $O_2^{\bullet -}$ or $^1O_2$ (Foyer and Noctor, 2005).

For example, H_2O_2 generation during the oxidative burst is one of the earliest cellular responses to potential pathogenes and elicitor molecules (Lamb and Dixon, 1997). H_2O_2 induces the expression of defence-related gene encoding glutathione-S-transferase and gene encoding phenylalanine ammonia lyase (Desikan *et al*., 1998; Grant *et al*., 2000). H_2O_2 also activates mitogen-activated protein kinase, conserved signalling kinases that modulate gene expression and transduce cellular response to extracellular stimuli (Desikan *et al*., 1999; Grant *et al*., 2000). Furthermore, several studies indicate that H_2O_2 is a key factor mediating PCD in response to pathogene, elicitors and hormones (Desikan *et al*., 1998; Bethke and Jones, 2001). It is already known that H_2O_2 can induce the expression of genes involved in antioxidant defence (Desikan *et al*, 2001).

4.4.3 ROS as toxic molecules

These active molecular species can oxidize chloroplast components at the sites where they are produced because of their high reactivity at nearly diffusion-controlled rates. The diffusion distance from their generation sites and life times of $OH^{\cdot}$ and $^1O_2$ have been estimated to be short. Proteins, lipids, pigments, DNA and all other chloroplasts components would be indiscriminately oxidized by active oxygen, especially by $OH^{\cdot}$ and $^1O_2$, if they are not properly scavenged. The reaction centres of both PSI and PSII and the enzymes of CO_2-fixation cycle in stroma seem to be their primary target (Asada, 1996).

High concentration of ROS can result in non-controlled oxidation of variety of other cellular structures, including DNA, proteins and membrane lipids that may result in the disruption of metabolism and destruction of cellular structures.

4.4.4 Role of ROS during senescence

ROS are generated in plants in many ways in several cellular compartments. These include non-enzymatic mechanisms such as electron transfer to molecular oxygen during photosynthesis and respiration in chloroplasts and mitochondria, and also as metabolic by-products of various enzymes such as photorespiratory glycolate oxidase in peroxisomes, amine oxidase and oxalate oxidase in the apoplast and xanthine oxidase (XO) and enzymes of fatty acid oxidation in peroxisomes. It is also clear that plant cell contains enzymes for which the specific function appears to be ROS generation. These include the NADPH oxidase and cell wall peroxidase (Desikan *et al*., 2005).

The chloroplasts are the main source of ROS in plant cells. During photosynthesis, light energy is absorbed by pigments and transferred to the reaction centres of the photosystems (PSs). Here, the charge separation takes place and the electrons are transferred to CO_2. Since only in some plants the rate of CO_2 fixation is high enough to convert more that 50 % of the absorbed light energy (Baker, 1991), alternative electron acceptors like molecular oxygen are exploited, leading to the formation of $O_2^{\cdot -}$.

In addition, the chloroplasts can form significant amount of $^1O_2$: Chl is the main source of this ROS. Chl absorbs light wavelengths that are among the most intensive that arrive at the Earth's surface from Sun. Unlike other pigments, such as carotenoids, Chl does

not readily undergo thermal de-excitation. Therefore, Chl can be maintained in the excited state long enough and triplet Chl is produced that can transfer its energy to triplet molecular oxygen in the reaction centre of PS II and in the antenna system (Logan, 2005). In the antenna, triplet Chl is produced directly by excited singlet Chl, while in the reaction centre it is formed *via* charge recombination of the light-induced charge pair (Arora *et al*., 2002; Krieger-Liszkay, 2004).

As a part of the photorespiratory pathway, H_2O_2 is formed in the peroxisomes, where it can be also produced during the catabolism of lipids as a by-product of β-oxidation of fatty acids (Vranová *et al*., 2002). Catabolism of purines probably takes place in the peroxisomes as well (del Río *et al*., 1998). The first reaction of this catabolic chain, the oxidation of xanthine to uric acid by XO, generates $O_2^{\bullet -}$, whereas uric acid is oxidized to allantoin, yielding H_2O_2 (Vranová *et al*., 2002).

The mitochondrial respiratory chain is a source of $O_2^{\bullet -}$ formation, particularly when the normal electron flow through the cytochrome pathway is impaired or in non-green tissues. H_2O_2 is formed from $O_2^{\bullet -}$ by a mitochondrial Mn-SOD (Feierabend, 2005).

Another source of ROS in plants that has received relatively little attention is the detoxification reactions catalysed by the cytochromes, in particular cytochrome P450 in the cytoplasm and the endoplasmic reticulum. During these reactions, electron leakage to oxygen and the decomposition of the intermediate oxygenated cytochrom P450 can form $O_2^{\bullet -}$ (Urban *et al*., 1997).

Moreover, ROS are generated in plants at the plasma membrane level or extracellularly in the apoplast. NADPH oxidase in plasma membrane has recently been regarded as a source of ROS for the oxidative burst, which is typical of plant pathogen incompatible reactions. In addition to NADPH oxidase, pH dependent cell wall peroxidases has been proposed as sources of H_2O_2 in the apoplast (Mittler, 2002; Vranová *et al*., 2002; Feierabend, 2005).

4.4.5 Non-enzymatic antioxidants

The most important non-enzymatic or low molecular weight antioxidants in plants are ascorbate (Asc), glutathione (GSH), tocopherols and carotenoids.

4.4.5.1 Ascorbate

Asc is generally the most abundant small antioxidant molecule in plants - particularly in leaves, being about 5-10 times more concentrated than GSH (Smirnoff, 2005). In plants, Asc can accumulate to millimolar concentrations in both photosynthetic and non-photosynthetic tissues (Noctor and Foyer, 1998). However, Asc contents vary considerably among tissues and depend on the physiological status of a plant as well as on environmental factors. Photosynthetic tissues as well as fruits and other storage organs have high concentration of Asc (Horemans *et al*., 2000).

The final step of Asc biosynthesis occurs in mitochondria. Then, it is transported into the chloroplast stroma through the envelope membrane *via* carrier mediated facilitated diffusion (Foyer, 2004). However, Asc is widely utilized in all cell organelles, a complete set of its redox enzymes being present in mitochondria, microbodies, chloroplasts and cytosol of almost all analysed tissues. The cell wall, endoplasmic reticulum, plasma membrane and nucleus also contain Asc (De Gara, 2003).

Asc occurs in almost all plant tissues, with the exception of dry seeds and it tends to be more concentrated in leaves that in roots (De Gara *et al*., 1997). It also occurs in all subcellular compartments including the cell wall, but its concentration in vacuoles is rather low (Davey *et al*., 2000). Asc in chloroplasts can represent from 12 to 30 % of the total Asc content of the leaves. The cytosolic Asc has been evaluated to be approximately 20 mM (Horemans *et al*., 2000).

At physiological pH, it is predominantly present as the Asc anion. It readily loses an electron to produce the monodehydroascorbate (MDA) radical. Further oxidation results in dehydroascorbate (DHA).

The ability of Asc to donate an electron and the relatively low reactivity of the resulting MDA radical is the basis of its biologically useful antioxidant and free radical scavenging activity (Smirnoff, 2005). The Asc pool in healthy leaves is usually about 90 % reduced, with most of the rest present as DHA. MDA does not accumulate to high level (Noctor and Foyer, 1998). The three distinct roles for Asc have been identified. The first is the role of H_2O_2 scavenging *via* ascorbate peroxidase (APX), the second is the scavenging of $^1O_2$ escaping from the thylakoid membrane and may also directly regenerate tocopheryl radicals formed by tocopherol oxidation and can reduce the carotenoid radicals (Smirnoff, 2005). Its best known role is the detoxification of ROS. Asc is also involved in cell division, it is

required for G1 to S transition, and that depletion of Asc contributes to reduced mitotic activity of the quiescent centre, the cells of which have a prolonged G phase (De Gara, 2003).

MDA is quite untypical radical: it does not react with oxygen, indeed it is not a potential trigger of the peroxidative cascade reactions that are typical for radicals. In, contrast, MDA very efficiently undergoes spontaneous disproportionation giving from two molecules of MDA one molecule of Asc and one of DHA (De Gara and Tommasi, 1999).

The cell wall and, probably, the vacuole, are the only cellular compartments in which, under physiological conditions, DHA concentration is higher than that of Asc (De Gara and Tommasi, 1999).

4.4.5.2 Glutathione

GSH is a non-protein sulphur-containing tripeptide, it acts as a storage and transport form of reduced sulphur (Tausz *et al*., 2004). A tripeptide GSH has been found practically in all compartments: cytosol, endoplasmatic reticulum, vacuole and mitochondria, where GSH executes multiple functions. GSH is also present in high concentration in nuclei (Müller *et al*., 2002). Together with its oxidized form (GSSG) maintains a redox balance in the cellular compartments. GSH scavenges cytotoxic H_2O_2, and reacts non-enzymatically with other ROS: $^1O_2$, superoxide radical and hydroxyl radical. The central role of GSH in the antioxidative defence is due to its ability to regenerate another powerful water-soluble antioxidant, Asc, *via* the ascorbate-glutathione cycle (Blokhina *et al*., 2003).

GSH is an abundant metabolite in plants that has many diverse and important functions, including signal transduction. In many reactions involving GSH, the cysteine thiol group is oxidized to yield a disulphide bond in GSSG, and the reverse reaction is catalysed by glutathione reductase (GR) using NADPH. The highly reduced GSH pool maintained by GR is necessary for active protein function and avoids unspecific formation of mixed disulphide bonds that cause protein inactivation or aggregation (Foyer and Noctor, 2005). GSH is generally accepted as the principal electron donor for DHA reduction (Noctor and Foyer, 1998).

GSH is also related to the sequestration of xenobiotics and heavy metals (Tausz *et al*., 2004).

Asc and GSH are closely related, as both are constituents of the antioxidative ascorbate-glutathione cycle, which detoxifies H_2O_2 in the chloroplasts and mitochondria (Noctor and Foyer, 1998).

4.4.5.3 Tocopherols

Tocopherols and tocotrienols are essential components of biological membrane where they have both antioxidant and non-antioxidant function. There are four tocopherol and tocotrienol isomers (α-, β-, γ-, δ-) which structurally consist of chroman head group and a phytyl side chain giving these compounds amphipathic character. Relative antioxidant activity of the tocopherol isomers *in vivo* is α > β > γ > δ, which is due to the methylation pattern and the number of methyl groups attached to the phenolic ring of polar head structure. Hence, α-tocopherol with its three methyl-substituents has the highest antioxidant activity of tocopherols (Kamal-Eldin and Appelqvist, 1996).

Chloroplast membranes of higher plants contain α-tocopherol as the predominant tocopherol isomer, and are hence well protected against photooxidative damage (Fryer, 1992).

Tocopherol is able to dispose oxidizing radicals directly, preventing the chain propagation step during lipid autooxidation. It reacts with alkoxy radical ($LO^{\cdot}$), lipid peroxyl radicals ($LOO^{\cdot}$) and with alkyl radicals ($L^{\cdot}$), derived from PUFA oxidation. In addition to antioxidant functions tocopherol has several non-antioxidant functions in membrane. Tocopherols have been suggested to stabilize membrane structures (Blokhina *et al*., 2003).

4.4.5.4 Caroteniods

Carotenoids, *i.e.* the β-carotene, the xanthophylls lutein (L) and neoxanthin (N) as well as those participating in the xanthophyll cycle, are main lipophilic isoprenoids that are synthetized and localised in plastids. They are involved in the photosynthetic light-harvesting complex and also have photoprotective and antioxidant roles (Smirnoff, 2005). The carotenoids have essential roles in photosynthesis, they contribute to the light capturing. The xanthophylls are also associated with the PSII reaction centre (Robert *et al*., 2004). It is also well established that they contribute to photoprotection by quenching triplet Chl and $^1O_2$ (Havaux and Niyogi, 1999). They are further able to react directly with superoxide and other

free radicals (Krinsky and Yeum, 2003). Carotenoids could prevent lipid peroxidation by the reaction with lipid peroxyl radicals. Carotenoids are more effective than tocopherols as chloroplast antioxidant; they can compensate for tocopherol deficiency in transformed plants (Smirnoff, 2005).

The dangerous triplet state of Chl can be quenched directly by carotenoids in their close proximity. This possibility is given in the antenna system, but not in the reaction centre, because of too large distance between carotenes and triplet Chl (Telfer, 2002).

The presence function of two β-carotene molecules in the PSII reaction centre seems well established, but they do not quench the triplet state of the primary electron-donor Chls, which are known as P680. The β-carotene cannot be close enough to P680 for triplet quenching because that would also allow extremely fast electron transfer from β-carotene to $P^{+}680$, preventing the oxidation of water. The primary function of the β-carotene is probably the quenching of $^{1}O_2$ produced after charge recombination to triplet state of P680 and provide significant protection against oxidative damage (Telfer, 2002).

Xanthophyll cycle is localised in thylakoid membranes and involves deepoxidation of violaxanthin (V) creating zeaxanthin (Z) with antheraxanthin (A) as an intermediate. Along with these xanthophylls also L and N are components of light-harvesting complex II (Croce et al., 1999). L has a direct role in non-photochemical quenching (NPQ) through thermal dissipation of absorbed excess energy (Inoue, 2004) and together with Z is necessary for photoprotection due to ability of quenching of both ^{1}Chl and $^{1}O_2$. In this way they inhibit lipid peroxidation. This capability is impaired in the absence of both Z and L (Niyogi, 1999). Z is involved in NPQ in PSII in which excess energy in PSII is transferred to Z and re-radiated as heat (Smirnoff, 2005). Among these xanthophylls, L occurs in the highest concentration usually comprising between 30 – 60 % of total xanthophylls. N represents a smaller proportion of the total xanthophyll pool but its concentration is remarkably uniform across a wide range of contrasting species and growth environments, where is accounts for between 9-14 % of total xanthophylls (Bungard *et al*., 1999).

4.4.6 Enzymatic antioxidant system

In order to control the level of ROS and to protect cells under stress condition, plant tissues contain also several enzymes scavenging ROS, the most important are SOD, CAT and enzymes of ascorbate-glutathione cycle.

4.4.6.1 Superoxide dismutase

Within a cell, the SODs (EC 1.15.1.1) constitute the first line of defence aginst ROS. Superoxide radical is produced in any location where an electron transport chain operates, and hence O_2 activation may occur in different compartments of the cell, including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplast and the cytosol. It is not surprising that SODs are present in all these subcellular locations (Alscher *et al*., 2002).

SOD catalyses the disproportionation of $O_2^{\cdot-}$ through the redox cycle of the prosthetic metal ions. The enzyme first oxidizes $O_2^{\cdot-}$ to O_2 and resulting reduced enzyme (SOD-$M^{(n-1)+}$) reduces $O_2^{\cdot-}$ to H_2O_2 from the proton from the histidine residue ligated to the prosthetic metal ions:

$$SOD\text{-}M^{n+} + O_2^{\cdot-} \rightarrow SOD\text{-}M^{(n-1)+} + O_2$$

$$SOD\text{-}M^{(n-1)+} + O_2^{\cdot-} + 2H^+ \rightarrow SOD\text{-}M^{n+} + H_2O_2$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2$$ (Asada, 1996).

Based on the metal co-factor present in the enzymes, SODs are classified into three groups: Fe-SOD, Mn-SOD, Cu,Zn-SOD and these SODs are located in different compartments of the cell. Mn-SODs in the mitochondria and peroxisomes and Cu,Zn-SODs in the chloroplasts, the cytosol, and possible the extracellular space (Alscher *et al*., 2002). Fe-SOD, if present, is localized in the chloroplast stroma. The expression of Fe-SOD in generally suppressed but in some species (e.g. *Nuphar luteum*) it is the major chloroplastic SOD (Asada, 1996).

Individual isoenzymes could be distinguished by their different sensitivity to inhibitors H_2O_2 and KCN: Fe-SOD is inactivated by H_2O_2 and is resistant to KCN, Mn-SOD is not inhibited by KCN nor H_2O_2. Cu,Zn-SOD is inactivated by both inhibitors (Alscher *et al.*, 2002).

4.4.6.2 Catalase

In higher plants, CATs (EC 1.11.1.6) are predominately located in the peroxisomes and are the most important enzymes scavenging H_2O_2 produced during photorespiration

(Foyer and Noctor, 2000). All types of plant peroxisomes, *i.e.* leaf peroxisomes, glyoxysomes and non-specialized peroxisomes contain CAT (Feieraband, 2005).

CAT cleaves H_2O_2 to form oxygen and water.

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Maize contains three distinct nuclear CAT genes, *cat1*, *cat2* and *cat3*. The homotetrameric CAT1, CAT2 and CAT3 differ in their molecular and biochemical properties (Scandalios, 1994). They also differ in their cell or organ-specific and temporal patterns of expression and are differentially affected by environmental factors. CAT1 is the only isoenzyme expressed in mature pollen and tissues of the immature developing seed. During germination CAT1 activity declines and CAT2 activity increases. Both CAT1 and CAT2 occur in glyoxysomes (Feieraband, 2005). The maize CAT isoenzyme CAT3, was reported to be associated with mitochondria (Scandalios *et al*., 1980). Two or three CAT isoenzyme genes were also identified in other plants such rice (Iwamoto *et al*., 2000), barley (Skadsen *et al*., 1995), rye (Schmidt *et al*., 2002), tobacco (Willekens *et al*., 1994) or sunflower (Feieraband, 2005).

Relative to the alternative H_2O_2-scavenging systems, CAT is distinguished by very high turnover numbers but rather low affinity toward H_2O_2. A major advantage is that CAT does not depend on any additional reductant for the scavenging of H_2O_2 (Feieraband, 2005).

4.4.6.3 Ascorbate-glutathione cycle

A further method of removal of H_2O_2 is through reaction with Asc catalysed by APX (EC 1.11.1.11) with formation of DHA. In contrast to CAT enzyme APX appears to be a unique enzyme found mainly in plants and algae (Shigeoka *et al*., 2002). It has a high affinity for Asc a as reducing substrate and catalysing the reaction:

$2\ Asc + H_2O_2 \longrightarrow 2\ MDA + 2\ H_2O.$

MDA is directly reduced by photoreduced ferredoxin (red Fd) in thylakoids.

$MDA + redFd \longrightarrow Asc + Fd$

Other way of MDA reduction is with chloroplastic or cytosolic MDA reductase.

$2\ MDA + NAD(P)H + H^+ \longrightarrow 2\ Asc + NAD(P)^+$ (Asada, 1996; 1999)

When MDA radicals fail to be reduced directly to AsA by either the photoreduced Fd or by MDA reductase, MDA disproportionates spontaneously yielding DHA and AsA.

2 MDA $\rightarrow$ Asc + DHA (Asada, 1996; 1999)

APX is found in almost every compartment of a plant cell and it participates in the removal of H_2O_2 as part of the ascorbate-glutathione cycle.

In chloroplasts, APX is found at least in three different forms: thylakoid APX that is bound in thylakoids membrane, and stromal and lumen APXs that are presented as soluble isoforms (Asada, 1999; Shigeoka *et al*., 2002; Mittler *et al*., 2004). In some plants (spinach, pumpkin, tobacco etc.) the thylakoid and stromal isoforms are produced from the same gene by alternative splicing, whereas in others (*A. thaliana*), they are encoded by two distinct genes (Shigeoka *et al*., 2002). In addition to stromal APX, two other stromal enzymes of the ascorbate - glutathione cycle are dually targeted to the chloroplast and mitochondria. These stromal enzymes are MDA reductase and GR (Chew *et al*., 2003). In addition to the chloroplast and mitochondria, enzymes of the ascorbate-glutathione cycle are also present in peroxisomes, glycosomes and the apoplats, suggesting that the ascorbate-glutathione pathway is a key H_2O_2-removal pathway in plants (Mittler and Poulos, 2005).

APX isoenzymes are distributed at least in four distinct cellular compartments: stromal APX, and thylakoid membrane-bound APX in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX, and cytosolic APX. A fifth APX isoenzyme occurs in a mitochondrial membrane-bound form (Shigeoka *et al*., 2002).

APX activities generally increase along with activities of other AOE in response to various environmental stress factors, suggesting that the components of antioxidative systems are co-regulated (Shigeoka *et al*., 2002). Gene expression studies in plants subjected to biotic and abiotic stress revealed that cytosolic APX iosenzymes are the most stress-responsive among the different members of the APX gene family. APX1 has a relatively high level of expression in the absence of stress; however, its expression is dramatically enhanced in response to almost all biotic and abiotic stress studied. In contrast, APX2, the second cytosolic APX, is not expressed under non-stressful controlled condition and its expression is significantly elevated in response to very high light stress (Shigeoka *et al.*, 2002; Mittler and Poulos, 2005).

One of the most characteristic properties of APX is its instability in the absence of Asc. Under conditions where the concentration of Asc is lower than 20 μM, APX activity is rapidly lost. The instability of APX seems to be one reason that APX was not found for a long time in photosynthetic organisms (Shigeoka *et al.*, 2002)

To maintain a certain level of Asc, it has to be regenerated by DHA reductase, which reduces DHA to Asc by oxidizing reduced GSH. GR (EC 1.6.4.2) regenerates GSH *via* the reduction of GSSG using NADPH + H^+ (Zimmermann and Zentgraf, 2005).

$$DHA + 2\ GSH \rightarrow Asc + GSSG$$

$$GSSG + NADPH + H^+ \rightarrow NADP^+ + 2\ GSH$$

GR is localized mainly in the chloroplast stroma, but it is also found in the mitochondria and cytosol (Foyer *et al*., 1991)

Chloroplasts are the major subcelular site for all of the enzymes involved in scavenging H_2O_2 in the cell, namely APX (79 % in chloroplasts), DHA reductase (65 % in chloroplasts) and GR (65 % in chloroplasts) (Strother, 1988).

In unstressed non-senescent tissues GR is very efficient in maintaining GSH in its reduced form, 90 % of GSH in leaf and root is in the reduced form in *Pisum sativum* (Strother, 1988).

4.5 Antioxidants, ageing and onset of the senescence

4.5.1 Ascorbate and glutathione

The amount of Asc decreases significantly from growing shoot meristem to the oldest leaves (Foyer, 2004). The onset of senescence triggers loss of tissue Asc while Asc decline is retarded in stay-green mutants (Horemans *et al*., 2000). The decrease of total Asc content during the ageing was noticed also in *Pistacia lentiscus* L. (Munné-Bosch and Peñuelas, 2003).

On the other hand, the total Asc and GSH content increased during ageing in two different cultivars of rice (Yang *et al*., 2001). Similarly, the total Asc in senescent bean cotyledons increased but the total GSH decreased (Procházková and Wilhelmová, 2007B). Enhancement of total Asc in bean cotyledon is because of increasing level of its oxidised form - DHA. It is known that the redox status of Asc (*i.e.* the relative content of reduced to oxidised Asc forms) depends on the age of the tissue, the degree of stress to which a plant is subjected and appears also to be species-dependent (Horemans *et al*., 2000).

The degree of a decrease in Asc is dependent on cell compartment. Takahama *et al*. (1999) reported that Asc content decreased during ageing in tobacco leaves until it was no

longer detectable in the apoplast, while symplastic Asc remained although the level decreased on ageing.

Asc signalling could have profound effects on gene expression in each of the individual leaf of a plant since the amount of Asc decreases significantly from growing shoot meristem to the oldest leaves (Foyer, 2004). Asc deficiency in *vtc1* (*vitamin c-1*) *Arabidopsis* mutant results in the induction of some *SAGs* (Barth *et al*., 2004). It was reported that low level of Asc promotes senescence, whereas high level delays senescence (Conklin and Barth, 2004).

4.5.2 α-tocopherol

The α-tocopherol content of leaves increases during ageing. In barley flag leaves the α-tocopherol content increases earlier then the decline in Chls suggesting that this change is associated with ageing rather than with onset of senescence (Krupinska *et al*., 2003).

4.5.3 Carotenoids

Munné-Bosch and Peñuelas (2003) reported that content of β-carotene and xanthophyll pigments decreased and that deepoxidation state (DEPS) increased during ageing of *Pistacia lentiscus* L. grown under the field conditions. The same was demonstrated even in senescent bean cotyledons (Procházková and Wilhelmová, 2004; 2007B). Contrariwise the relative content of xanthophyll cycle pigments remained almost constant during ageing in two rice cultivars (Yang *et al.*, 2001). During leaf senescence, N and β-carotene decreased concomitantly with Chl, whereas L and xanthophyll cycle pigments were less affected (Lu *et al*., 2001). More then twice increase of level of β-carotene during natural ageing in bean cotyledons and then the decrease at the end of the life span was reported (Procházková and Wilhelmová, 2004; 2007B).

4.5.4 Antioxidant enzymes

Data concerning dynamics of AOE activities during the plant life span are rather contradictory. It was reported that activities of AOE reach their maxima at the beginning of the leaf development (Dertinger *et al*., 2003). However, their increasing activities during the leaf age with the peak at the stage of fully developed leaves were also presented (Procházková

et al., 2001). In sunflower leaves the SOD, its isoenzyme, APX and CAT activities were highest in the middle-age leaves and declined in subsequent older leaves (Feng *et al*., 2003; Sairam *et al*., 2004).

Decrease in CAT activity seemed to be consistent feature of leaf senescence. Also SOD appeared to decrease during leaf senescence. These changes suggest that free radical levels are likely to be higher in senescing tissues (Strother, 1988). The CAT of pea peroxisomes were depressed during senescence as well (Pastori and del Río, 1994). However, Kumar and Knowles (1993) reported that CAT increased during senescence of potato seed-tubers. Zimmermann *et al.* (2006) analysed activity of CAT during senescence of *Arabidopsis thaliana* and they found out different courses of CAT isoenzymes during senescence. In very late stage, low activity of the seed-specific CAT1 became detectable in leaves. CAT2 activity decreased before loss of Chl could be measured. CAT3 isoform was activated along with a decline of the H_2O_2 content. Differences were also in gene level. *CAT2* expression is down-regulated during leaf senescence, while *CAT3* expression is induced with age and corresponds to an accumulation of H_2O_2 in the vascular bundles.

During senescence new types of isoenzymes could be induced as the two new types of Cu,Zn-SOD in pea peroxisomes were found (Pastori and del Río, 1997). There could be also differences between two cell compartments. Del Río *et al*. (2003) studied the effect of leaf senescence on the peroxisomal and mitochondrial Mn-SOD in detached leaves from pea plants. It was shown that the total level of mitochondrial Mn-SOD increased during the senescence but the peroxisomal one did not changed. It shows that the expression of the peroxisomal and mitochondrial Mn-SODs is regulated differently and that the expression of mitochondrial Mn-SOD is induced during senescence, whereas peroxisomal Mn-SOD could be post-translationally activated. Takahama *et al*. (1999) presented different activity of SOD in apoplast and symplast during senescence. The activity of SOD in the apoplast increased in tobacco leaves during ageing, while that in the symplast decreased.

Moreover, differences in regulation of AOE were observed during ageing between two cultivars of apricot. In the first cultivar the activity of SOD decreased while the activity of CAT and APX increased during the senescence. On the other hand, in the other cultivar the SOD activity increased and CAT and APX declined (Scebba *et al*., 2001).

Evidence is presented that the high levels of two enzymes implicated in antioxidative defence, SOD and CAT are involved in delaying the senescence process during the storage stage of *Cucumis melo L.* (Lacan and Baccou, 1998). The overexpression of SOD or CAT alone had no effect, whereas the combined overexpression of both enzymes led to a clear

prolonged lifespan (Orr and Sohal, 1994). He *et al.* (2005) reported that the increased level of CAT and SOD activity in maize leaves before senescence onset in stay-green mutants (P3845) compared with earlier senescent maize (Hokkou 55) suggested that these enzymes play a role in delaying of senescence. On the other hand, Dertinger *et al.* (2003) conclude from their results that obviously, in tobacco plants, the capacity of antioxidative system to scavenge radicals is sufficiently balanced with metabolism, and its decline with increasing age does not cause senescence and ageing.

4.6 Relationship between the antioxidative system and cytokinins

It was reported that CKs could be assumed as non-enzymatic antioxidants. The CK zeatin riboside was found to act as a scavenger of superoxide anions that might help to maintain seed viability by detoxifying ROS (Gidrol *et al*., 1994). CKs react with superoxide in organic solvents to produce the corresponding amides (Leshem *et al*., 1979). CKs could further retard leaf senescence partly by scavenging active oxygen through lowering endogenous lipoxygenase activity or by inhibiting lipid peroxidation (Swamy and Suguna, 1992).

The influence of exogenously applied CKs on activities of AOE in plants has been reported. Benzyladenine application increased activities of SOD and CAT in maize under waterlogged condition (Liu *et al*., 1996). Liu and Hunag (2002) found out that exogenous application of zeatin riboside prevented a decrease in the activity of SOD and CAT during heat stress. Petit-Paly *et al*. (1999) demonstrated the effect of CKs on CAT activity in tobacco cultivated *in vitro*. The CAT activity observed in suspension-cultured cells decreased slightly during the first hour of CK treatment and increased thereafter to double level detected in untreated cells. In contrast to these results, CAT activity was inhibited in shoot cultures in which the endogenous levels of CK were elevated by the introduction of the *ipt* gene or by an exogenous application of CK to the cultures (Petit-Paly *et al*., 1999). Dhindsa *et al*. (1982) observed that activity of CAT decreased and the activity of SOD was changed only little during *in vitro* senescence of oat. Kinetin treatment of oat leaf segments inhibited decline in the CAT and SOD activities during senescence. Beans treated with DZ before mosaic potexvirus inoculation showed elevated CAT and GR activity (Clarke *et al*., 2002). Moreover, Toyama *et al*. (1995) suggested that CAT activity could be regulated by CKs.

Dertinger *et al*. (2003) studied a response of AOE activities to enhanced CK production stimulated by the onset of leaf senescence of transgenic plants. The activities of AOE were reduced in senescing leaves of control plants, whereas in transgenic plants the decline was slowed down by CKs. Similarly, the overproduction of CKs in transgenic P*ssu-ipt* tobacco plants overexpressing CK biosynthetic gene was found to stimulate activities of AOE throughout the plant ontogeny (Synková *et al*., 2006).

4.7 Senescence and abiotic stress

4.7.1 The role of stress response pathways in senescence

Premature senescence in a plant can be induced by a number of different environmental stresses such as pathogen infection, nutrient or water stress or oxidative stresses induced by ozone or UV-B. In general, the biochemical changes associated with stress-induced senescence are almost identical to those of natural senescence (Peñarrubia and Moreno 2002). For example premature senescence induced by drought stress in the pea was shown to follow a pattern similar to that seen in developmental senescence (Pic *et al*., 2002).

Various stress responses have indicated that they have considerable cross-talk with senescence related gene expression. Gene expression patterns are frequently coincident (Quirino *et al*., 1999), or differ at the relative levels of isoenzyme activities (Khanna-Chopra *et al*., 1999), but some particular genes may display specific expression in senescence processes induced by different factors.

It was reviewed that drought-induced leaf senescence contributes to plant survival under drought stress in several species, since it allows a) an early diversion of resources from vegetative to reproductive development, thus contributing to the completion of plant life-cycle in monocarpic species even under stressful condition, b) remobilisation of nutrients from senescing leaves to young leaves, thus contributing to plant survival in perennials and c) reduction in water loss at the whole-plant, especially when it is accompanied by leaf abscission (Munné-Bosch and Alegre, 2004).

4.7.2 The role of CKs in stress

A general view has emerged that during stress a reduction of CKs supply from the root alters gene expression in the shoot and thereby elicits appropriate responses to ameliorate the effects of stress. CKs levels themselves may also be directly affected by salt stress. Roots are the first tissues exposed to salinity stress, and also the primary site of CK synthesis. Drought and salt stress could also affect the transport of CKs from the root to the leaves, thereby directly influencing CK-induced gene expression (Hare *et al*., 1997).

4.7.3 Activity of AOE during abiotic stress

The abiotic stresses, similarly to ageing, are characterised as forms of oxidative stress and also are accompanied by changes in antioxidant levels. It is difficult to generalise how the activities of AOE are affected by stress. This study is aimed at salinity, drought and Zn-induced stress.

In most cases, the activities of AOE were observed to increase during enhanced concentration of NaCl (Hernandéz *et al*., 1999; Arbona *et al*., 2003). However, there were some exceptions to this generalization, e.g. the activity of SOD was found to decrease in salinity stressed plants (Dionisio-Sese and Tobita, 1998; Savouré *et al*., 1999; Rios-Gondalez *et al*., 2002; Zhu *et al*., 2004). In so far reported cases of zinc-induced stress the activities of AOE increased (Tripathi and Gaur, 2004; Bonnet *et al*., 2000). Responses of AOE activities in plants subjected to drought-induced stress are the most differing. Moran *et al*. (1994) observed that activities of APX, GR and CAT were reduced whereas activity of SOD increased in pea plants stressed by drought. While Ünyayar *et al*. (2005) reported that the activity of APX decreased and GR, CAT and SOD activities were enhanced in tomato in the same stress condition.

It is obvious that the response of individual AOE activities depends on plant species, level of stress, and level of tolerance to stress factors. It is expectable that more tolerant plants will respond to a stress impact by enhancing of AOE activities. Bor *et al*. (2003) compared the effects of salt stress on AOE of two beet species, the sugar beet (*Beta vulgaris L.*) and its wild salt tolerant relative *Beta maritim* L. Already under control conditions the activities of AOE were higher in wild beet and during stress they increased even more. High capacities of the antioxidative scavenging system seemed to correlate with increased salt tolerance. Salt-

tolerant cultivars of rice (Vaidyanathan *et al*., 2003) contained higher levels of CAT activity. The high salt tolerance of wild tomato (*Lycopersicon pennellii*) correlated with high activities of CAT (Shalata and Tal, 1998). APX seems to be a key enzyme in determining salt tolerance in *Citrus* (Gueta-Dahan *et al*, 1997).

Also plants possessing high levels of constitutive or induced AOE activities show increased resistance to stress. The ongoing elucidation of the molecular control mechanisms of abiotic stress tolerance, which may result in the use of molecular tools for engineering more tolerant plants, is based among others on the expression of genes of free-radical scavengers (Wang *et al.*, 2003).

5 Aims of this study

- Characterization of ageing and senescence of transgenic tobacco (AtCKX2) overexpressing *Arabidopsis thaliana* CKX gene, and thus have reduced level of CKs.
- Investigation of relation between reduced level of CKs and oxidative damage during ageing.
- Studying how reduced level of CKs to the capacity of antioxidant system (enzymatic and non-enzymatic) during the plant life span.
- Study of activities of AOE during abiotic stresses (salinity, drought and enhanced concentration of zinc) in the leaves and roots of AtCKX2 tobacco.

6 Material and methods

6.1 Plant material

The control (wild type - WT) and transgenic tobacco (*Nicotiana tabacum* L. cv. Samsun NN) were used as experimental plants. The transgenic plants (clone AtCKX2-38) overexpressing CKX had inserted gene *AtCKX2* from *Arabidopsis thaliana* positioned under the control of a constitutive 35S promoter. The transformed plants were marked AtCKX2. The vector construction was described in Werner *et al*. (2001). The seeds were kind gift of Prof. Thomas Schmülling from Freie Universität Berlin, Germany.

Figure 2.: The tobacco plants 9 weeks old overexpressing gene *AtCKX2*

The seeds were grown *in vitro* at cultivation medium by Murashige and Shoog (1962) in Petri dishes, relative humidity 60/80 %, day/night temperature 25°C/20°C, 200 μmol quantum (PAR-photosynthetic active radiation) $m^{-2}s^{-1}$ and 16 h/8 h light/dark period.

After four weeks the plantlets were replanted to soil for two weeks and plants intended for studying senescence were moved to glasshouse for next seven weeks (25/18°C day/night

temperature, 60 % relative humidity). Day irradiance [overall integrated mid-values were ca. 500 μmol quantum (PAR) m^{-2} s^{-1}] was prolonged by the additional irradiation [photon flux density ca. 200 μmol quantum (PAR) m^{-2} s^{-1}] to 16 hours. Leaves were detached from 12 week-old-plants and than divided into 3 groups, *i.e.* young (from the uppermost part of the plant), mature (from the middle of the plant) and old (from the lower part of the plant). Except for flurescence measurements, the leaves were collected and frozen in liquid nitrogen and stored at -70 ºC until analyses.

For studying of abiotic stress the 42-days-old plants were replanted after *in vitro* pre-cultivation into pearlite and cultivated in a growth chamber (SANYO MLR 350H) at a 16 h photoperiod (130 μmol m^{-2} s^{-1}), day/night temperature 22/18ºC and relative humidity ca. 80 %. After 10 days, the plants were subjected to water deficit stress (stop of watering), salinity (100 mM NaCl) or increased zinc concentration (5 mM $ZnSO_4$). The stress conditions were chosen on the basis of our previous experience obtained with tobacco as well as other plant species (Gaudinová *et al*., 2004, Mýtinová *et al*., 2005). Following 4 days of stress treatment, all leaves and roots of plants were collected and frozen in liquid nitrogen and stored at -70ºC until analyses.

6.2 Measurement of chlorophyll fluorescence parameters

Chl fluorescence parameters of slow fluorescence kinetics were measured after 15 min–dark period using the *PAM* (pulse amplitude modulation) *Chlorophyll Fluorimeter* (*Walz*, Germany) on attached leaves. Measuring irradiance was 0.35 μmol m^{-2} s^{-1}, actinic irradiation 200 μmol m^{-2} s^{-1}, 700 ms saturated flashes of 2 500 μmol m^{-2} s^{-1} after 10, 250 and 265 s. The *DA 100 Data Acquisition System* (*Walz*, Germany) was used for sampling, control and calculation. The minimum Chl fluorescence yield in the dark-adapted state (F_0), maximum photochemical efficiency (F_v/F_m; F_v - variable Chl fluorescence yield in the dark-adapted state; F_m - maximum Chl fluorescence yield in the dark-adapted state), maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2 (F_v/F_0), NPQ = $(F_m-F_m')/F_m'$ (F_m'-maximum Chl fluorescence yield in the light-adapted state), effective quantum yield of photochemical energy conversion in PS2 (Φ_2), were calculated according to Roháček (2002).

6.3 Analysis of pigment content

The contents of photosynthetic pigments i.e. Chl *a,b* and carotenoids (V, A, Z, L, N and β-carotene) were determined from 3-6 lyophilised leaf discs from the same leaf (0.5 cm^2 per one disc) extracted in 200 µl of acetone with small amount of $MgCO_3$ in grinding mortar and than transferred with 800 µl of aceton to a centrifuge tube. The diluted extract was centrifuged 1 – 2 min at low speed. Sediment was re-extracted in 200 µl of acetone and added to original extract. The pigment extract was concentrated by nitrogen evaporation of all acetone and re-dissolved in 50 µl of acetone just before analysis. Samples were measured by HPLC (*SpectraPhysics*, USA) using a reverse-phase column *Sepharon* SGX C18, 5 µm particle size, 150 mm x 3 mm (*Tessek*, Czech Republic). The solvent system was acetonitrile:methanol: water (80:12:6, v:v:v) for 10 min followed by 100 % methanol for 25 min, at flow rate 1 ml min^{-1}, the detection wavelength 445 nm (Tichá *et al*., 1998, Mýtinová *et al*., 2006) DEPS was calculated as ratio (Z + 0.5A)/(V + A + Z).

6.4 Protein extraction for measuring of AOE activities

Soluble proteins were extracted from leaves by 10 s homogenisation (*Turrax, IKA*, Germany) in 0.1 M Tris-HCl (Tris(hydroxymethyl)aminomethane; pH 7.8) extraction buffer contented 1 mM dithiotreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 % (w/v) Triton X-100, 5 mM Asc. The ratio of buffer to weight of leaf was 5 ml g^{-1} fresh weight (FW).

The extracts immersed into ice bath were incubated for 1 min in ultrasound (*Tesla UC 006 DM1*, ČR), then left for 30 min in dark and ice, and centrifuged (20 000 g, 10 min, 4°C; *Biofuge 28 RS, HERAEUS*, Germany). Supernatant was filtrated through four layers of gauze. One quarter of each filtered supernatant was passed by centrifugation (500 g, 2 min, 4°C; *Universal 16 R, Hettich*, Germany) through mini columns with 200 µl of Sephadex G-25 for desalting and later used for SOD assay. Samples were frozen in liquid nitrogen and stored at – 70°C up to analysis.

6.5 Activities of antioxidant enzymes

Activities of AOE (APX, GR and SOD) were measured spectrophotometrically (*Hitachi U 3300*, Japan) in stirred cuvettes at 25°C.

6.5.1 Ascorbate peroxidase

Activity of APX was determined in 0.1 ml of protein extract as a decrease of reduced Asc concentration at 290 nm (Nakano *et al*., 1981). The reaction proceeded in 0.1 M HEPES-EDTA (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.0; 1 mM EDTA), 0.5 mM Asc and 0.88 mM H_2O_2. The samples were measured against reference cell with the same content but without extract.

6.5.2 Glutathione reductase

The GR activity was assayed according to Goldberg and Spooner (1983) in 0.2 ml of protein extract as a decrease of NADPH concentration at 340 nm. The reaction ran in 0.1 M Tris-HCl-EDTA (pH 7.8; 1 mM EDTA), 0.13 mM GSSG and 0.1 mM NADPH. The samples were measured against referential cell with the same content but without GSSG.

6.5.3 Superoxide dismutase

The method for determination of SOD activity according Ukeda *et al*. (1997) is based on inhibition of reduction by superoxide of XTT (sodium 3,3´-{-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) as a detection molecule. Production of superoxide was provided by conversion of xanthine catalysed by XO. One unit of the SOD activity was defined as amount of the enzyme required for 50 % inhibition of reaction rate of XTT reduction by superoxide. Activity in 0.01 ml of protein extract was measured at 470 nm in 50 mM Na_2CO_3 – EDTA (pH 10.2; 1 mM EDTA), 0.1 mM xanthine, 0.025 mM XTT and 56 mU/ml XO. Blank sample with maximal reduction rate of XTT did not contain protein extract.

6.5.4 Catalase

Activity of CAT was measured polarographically using the oxygen electrode (*Hansatech Instruments*, Great Britain) (Thomas *et al*., 1998). Rate of oxygen evolution was registered as change of electric current, which was transferred to the voltage signal and

registered by a printer. For calibration of oxygen electrode the line of water saturated by oxygen was registered (0.253 µl O_2/1 ml H_2O at 25°C) than the sodium dithionite for oxygen elimination was added, that gives zero signal:

$Na_2S_2O_4 + O_2 + H_2O \rightarrow NaHSO_4 + NaHSO_3$

Real concentration of oxygen is given by the difference between saturated solution and solution without oxygen. Reaction cell contained 0.1 M sodium phosphate buffer (pH 7.0), 2 mM H_2O_2 and 0.01 - 0.05 ml of protein extract.

6.6 Protein content

Naformátováno: Odrážky a číslování

Protein content was determined spectrophotometrically according to Bradford (1976). Method is based on shift of absorbing maximum of Coomassie Brilliant Blue G-250 after it is bound on protein. 2 to 10 µl of protein extract were mixed with 0.79 - 0.798 ml of water and 0.2 ml of Coomassie Bio-Rad and the absorption was measured at 595 nm. The bovine serum albumin was used as a standard at a concentration range 0 – 10 µg ml^{-1} for calibration.

6.7 Native polyacrylamide gel electrophoresis

We used native polyacrylamide gel electrophoresis (Laemmli, 1970) for isoenzyme separation (*Hoefer Scientific Instrument*, USA). For determination of APX and SOD isoenzyme activity we used 12.5 % running gel with 10 % glycerol, and 6 % stacking gel. CAT isoenzymes were detected using 7.5 % running gel with 10 % glycerol, and 6 % stacking gel (running gel: 1.13 M Tris-HCl (pH 8.9); stacking gel: 0.08 M Tris-HCl (pH 6.7)). For the gel preparation we used the mixture of acrylamide and bisacrylamide (30 % acrylamide + 0.8 % bisacrylamide). We applied 40 µl of extracts. Electrophoresis (electrode buffer: 0.05 M Tris–HCl and 0.38 M glycine, pH 8.3; 300 V, 20 mA per gel, 4°C) ran 2 h for SOD and APX, and 4 h for CAT detection.

6.7.1 Determination of APX isoenzymes

For APX isoforms determination the gel was pre-run (300 V, 15 mA, 30 min) using cathodic Tris-glycine buffer containing 2 mM Asc. After electrophoresis the gel was equilibrated for 30 min in 50 mM sodium phosphate buffer (pH 7) with 2 mM Asc. The solution was changed every 10 min and than the gel was incubated for 20 min in 50 mM

sodium phosphate buffer (pH 7) with 4 mM Asc and 2 mM H_2O_2. Gel was stained in sodium phosphate buffer (pH 7.8) containing 28 mM N,N,N',N'-tetramethylethylendiamine (TEMED) and 2.45 mM nitro blue tetrazolium chloride (NBT) (Mittler and Zilinskas, 1993).

6.7.2 Determination of SOD isoenzymes

To determine SOD isoenzymes the gel was incubated 20 min at 25°C in dark in 50 ml of solution containing 0.25 mM NBT, 1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), 23 mM TEMED and 0.2 mM riboflavin. After incubation the gel was illuminated with UV transilluminator (*Kaiser*, Germany) ca. 15 min. Visualisation of isoenzymes insensitive to KCN (inhibition of Cu,Zn-SOD) and H_2O_2 (inhibition of Fe-SOD and Cu,Zn-SOD) was achieved before staining by incubation in 50 mM potassium phosphate buffer (pH 7.8) with 2 mM KCN or 5 mM H_2O_2 respectively for 30 min at 25°C in dark (Beauchamp and Fridovich, 1971).

6.7.3 Determination of CAT isoenzymes

Gel for determination of CAT was incubated 10 min in 0.01 % H_2O_2 and than stained in 10 % $FeCl_3$ and 10 % $K_3Fe(CN)_6$ for 10 min (Woodbury *et al*., 1971).

6.8 Cytokinin oxidase/dehydrogenase activity

The method described by Motyka *et al*. (1996, 2003) was used for extraction and partial purification of CKX activity. Tobacco leaves (2 to 4.5 g FW) were homogenised in cold extraction buffer (0.1 M Tris-HCl, pH 7.5). After filtration through an acid-activated polyvinylpolypyrrolidone column, centrifugation and removal of nucleic acids by polyethyleneimine (Polymin P, 1 % v/v, pH 7.5), proteins were precipitated by 80 % saturation of ammonium sulphate.

The CKX activity was determined by *in vitro* assays based on conversion of [2-^{3}H]N^6-(Δ^2-isopentenyl)adenine ([2-^{3}H]iP; prepared by Dr. Jan Hanuš, Isotope Laboratory, Institute of Experimental Botany AS CR v.v.i., Prague, Czech Republic) to [^{3}H]adenine. The assay mixture (50 µL final volume) included 100 mM 3-{[tris(hydroxy-methyl)methyl]amino}propanesulfonic acid buffer (TAPS-NaOH) containing 75 µM 2,6-dichloroindophenol (pH 8.5), 2 µM substrate ([2-^{3}H]iP, 7,4 TBq mol^{-1}) and enzyme preparation equivalent to 250-500 mg FW (control) or 0.25-12.5 mg FW (AtCKX2). After

incubation at 37°C the reaction was terminated by addition of 10 µl EDTA (200 mM) and 120 µl 95 % (v/v) ethanol. Separation of the substrate from the product of the enzyme reaction was achieved by a *Series 200 HPLC Quaternary Pump* (*Perkin Elmer*, USA) coupled to *235C Diode Array Detector* (*Perkin* Elmer) and *RAMONA 2000* flow-through radioactivity detector (*Raytest*, Germany) on the column Luna C_{18} (2) (50 mm/4.6 mm/3 µm) connected with two C_{18} guard cartridges as described in Blagoeva *et al*. (2004).

6.9 Cytokinin analysis

CKs were extracted and purified according to Dobrev and Kamínek (2002). Frozen leaf samples (ca. 1 g FW) were ground in liquid nitrogen and extracted overnight with 10 ml methanol/water/formic acid (15/4/1 v/v/v, pH ca. 2.5, - 20°C). Deuterium-labelled internal CK standards (*Apex Organics*, *Honigton*, UK; Kamínek *et al*., 2000), each at 50 pmol per sample, were added at the beginning of extraction. The extracts were purified using Si-C_{18} columns (*SepPak Plus*, *Waters*, *Milford*, MC, USA) and *Oasis MCX* mixed mode (cation exchange and reverse-phase) columns (150 mg, *Waters*, USA) and fractions containing CK bases, ribosides and riboside monophosphates were evaporated. LC-MS analysis was performed as described in Lexa et al. (2003) using a *Rheos 2000 HPLC* gradient pump *(Flux Instruments*, Switzerland) and *HTS PAL autosampler* (*CTC Analytics*, Switzerland) coupled to an *Ion Trap Mass Spectrometer Finnigan MAT LCQ-MS^n* equipped with an electrospray interface. Detection and quantification were carried out using a Finnigan LCQ operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with deuterated CKs as internal standards. The levels of 27 different CK derivatives were measured. The detection limit was calculated for each compound as 3.3 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve.

6.10 Ascorbate content

Asc was extracted from leaves homogenised in grinding mortar with 2 ml of metaphosphoric acid [1.5 % (w/v) metaphosphoric acid, 3.5 % (w/v) polyvinylpyrrolidon] per 0.6 g FW and centrifuged at 7 000 g for 10 min at 4°C. The supernatant was separated and centrifuged at 14 000 g, 5 min at 4°C. Part of extract was used for determination of reduced Asc and second part for total Asc. For reduction all oxidised ascorbate 450 µl of 0.2 M Tris-

HCl and 50 µl of 0.26 M DTT were added to 600 µl of the extract. The mixture was incubated for 25 min at room temperature and dark. The reaction was stopped by addition of 10 µl of 85 % orthophophoric acid. For calibration were used concentrations of Asc in the range of 0 - 1 mM. Samples were measured by HPLC (*ECOM*, Czech Republic) using a reverse-phase column *Watrex Nucleosil* 120-5-C18, 5 µm particle size, 125×4 mm (*ECOM*, Czech Republic). The solvent system was followed by acetic acid (pH 3) for 7 min, at flow rate 1 ml min^{-1}, the detection wavelength 244 nm (Tausz *et al*., 1996). Data were captured by PC - software *Clarity* (*DataApex*, Czech Republic).

6.11 Determination of protein oxidation as content of carbonyl groups

This method (Levine *et al.*, 1990) is based on condensation of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyl groups and determination (365 nm) of the originating product dinitrophenylhydrazon by spectrophotometry. The quantity of carbonyl groups, generated after reaction of proteins and ROS, reflects the level of oxidative damage to proteins.

Proteins were extracted from frozen leaves by homogenisation with phosphate buffer (0.2 M Na-phosphate buffer, pH 7; 2 mM EDTA; 0.2 mg phenylmethylsulphonylfluoride; 0.2 mg Leupeptine; 0.2 mg Pepstatine A) in ratio 5 ml g^{-1} FW. The homogenate was filtred through eight layers of gauze and than centrifuged (15 000 g, 25 min, 4°C; *Biofuge 28 RS*, *HERAEUS*, Germany). For elimination of nucleic acids 300 µl of 10 % streptomycine sulfate dissolved in 50 mM HEPES (pH 7.2) was added to 2.7 ml of supernatant since streptomycine precipitates and bounds nucleic acids. The solution was incubated for 15 min and than centrifuged (15 000 g, 10 min, 4°C *Biofuge 28 RS*, *HERAEUS*, Germany).

The supernatant was portioned out in 0.5 ml to four microtubes. To each microtube 0.5 ml of 20 % of trichloroacetic acid was added for protein precipitation. After centrifugation (22130 g, 15 min, 4°C; *Universal 16 R, Hettich*, Germany) 500 µl of 10 mM DNPH in 2 M HCl was added to three microtubes and to that one used as a blank only 2 M HCl. The sample was well shaken and incubated for 1 hour at room temperature with shaking every 15 min. After 1 hour 500 µl of 20 % TCA was added and sample was centrifuged (22 130 g, 5 min, room temperature; *Universal 16 R, Hettich*, Germany). For elimination of free DNPH 1 ml of ethanol:ethylacetate (1:1) was added to sediment. After 10 min sample was centrifuged

(22 130 g, 5 min, room temperature; *Universal 16 R, Hettich*, Germany). Wash with ethanol:ethylacetate was repeated three times.

Proteins in sediment were dissolved in 0.6 ml of 6 M guanidine (dissolved in 20 mM KH_2PO_4; pH 2.3 was adjusted by trifluoroacetic acid) for 15 min at 37 °C. After centrifugation absorbation of samples was measured at 365 nm. The blank with only HCl instead of DNPH was used as a reference. Concentration of carbonyl groups was calculated according to formula:

c (nmol ml^{-1}) = A_{365} (sample – blanc) 10^6/ ε, where ε = 22 000 $M^{-1}cm^{-1}$.

Results are expressed as nmol carbonyl groups mg $proteins^{-1}$. Proteins were measured in blank at 280 nm against 6 M guanidine.

6.12 Statistic evaluation

All results were analysed by analysis of variance using the NCSS 6.0 Jr programme (*NCSS*, USA), statistical significance of differences was evaluated by post-hoc Scheffé's test at $P<0.05$.

The Chl fluorescence parameters and pigment concentration were measured in four different series, the activities of AOE and protein concentration and oxidative damage in three series, activity of CKX and Asc content in two series, and CK concentration in one. The zymograms were prepared from the one, which was chosen as representative of activities of AOE.

The repetition of measurement differs in relation to estimated parameter. Activities of AOE were measured four times, protein content three times, pigment and Asc concentration two times, and activity of CKX, CK content, oxidative damage to proteins and Chl fluorescence parameters only one time. Resultant values were calculated from three plants for one serie.

7 Results

7.1 Markers of senescence and antioxidant system in leaves of tobacco with cytokinin oxidase/dehydrogenase gene overexpression

7.1.1 Characterization of tested plants

The results were derived from 6 different series. In all repeated series we observed very different phenotype of transgenic plants compared to controls as a consequence of enhanced expression of *AtCKX2*. Transgenic plants were lower with shorter internodes and fewer leaves. Leaves were smaller and thicker. AtCKX2 had also more extended root system compared to the controls (Fig. 3,4). Transgenic plants developed 12 - 7 leaves while control plants 20 - 10 leaves depending on series. In age of about 12 weeks (the time of sampling) the bottom oldest leaves of control plants were fully yellow in contrast to AtCKX2, which had all leaves still green or the oldest started only little to pale (Fig. 3).

Figure 3: Tobacco plants before collecting samples.

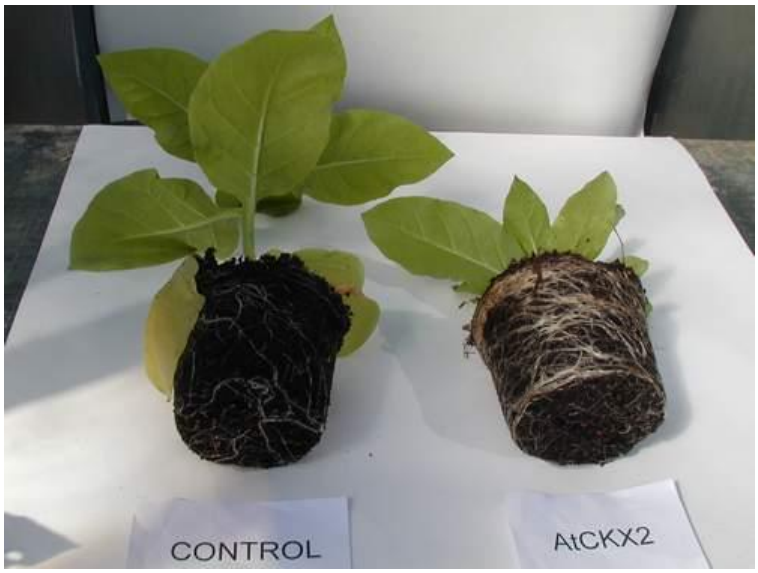

Figure 4: Root system of tobacco plants

For the experiments, leaves were divided according to their position and thus according age into three groups:

Characterization of the leaf groups:

1. Young leaves - the highest ones
2. Mature leaves - leaves from the middle of the plant shoot
3. Old leaves - leaves from the bottom of the plant.

7.1.2 Fresh weight

The FW of leaves was related to a lafe area increased with age in both WT and transgenic plants, but it was significantly higher in the AtCKX2 then in the controls (Fig. 5)

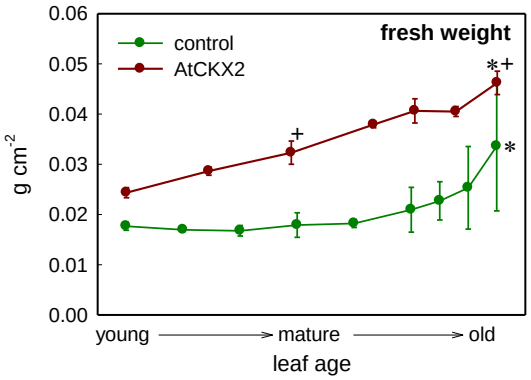

Figure 5: The FW of leaves of control and AtCKX2 plants. Asterisks indicate significance of differences from the respective young leaves. Crosses indicate significant differences between transgenic and control plant.

7.1.3 Activity of cytokinin oxidase/dehydrogenase and cytokinin content

The activities of CKX were measured by Ing. Václav Motyka, CSc. (Laboratory of Hormonal Regulations in Plants, IEB AS CR, v.v.i.). The overexpression of *AtCKX2* caused substantially pronounced enhancing of CKX activity in all three stages compared to WT (190-980-fold) (Fig. 6). The activity of CKX in control plants was several orders of magnitude lower when compared with transgenic plants. The changes with leaf age were statistically insignificant but it was the highest in the youngest leaves of WT tobacco. In transgenic plants the activity decreased with leaf age.

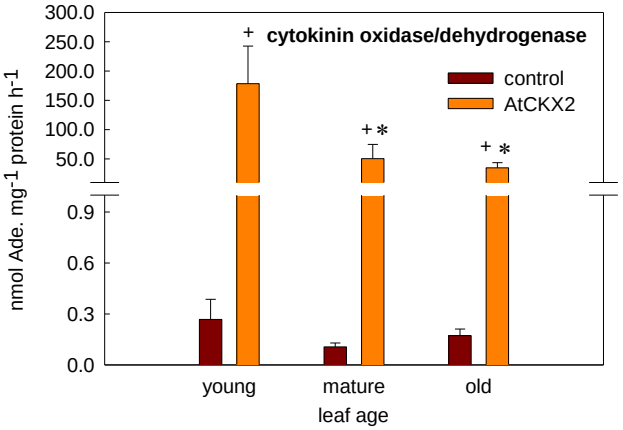

Figure 6: Activity of CKX in young, mature and old leaves of control and transgenic AtCKX2 tobacco. Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significant differences between transgenic and control plants.

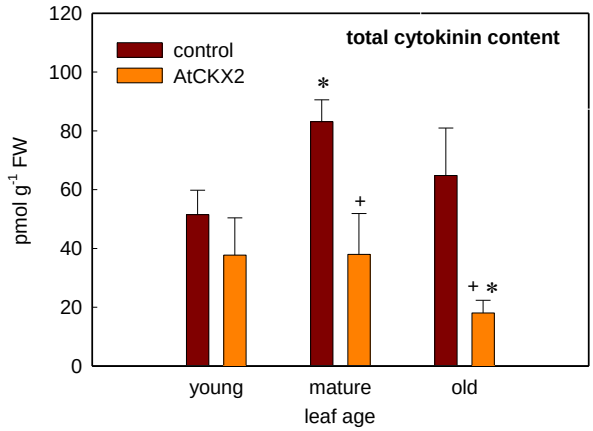

Figure 7: Total CK content in young, mature and old leaves of control and transgenic AtCKX2 tobacco. Asterisks indicate significance of differences from the respective young leaves. Crosses indicate significance of differences of transgenic plants from control plant.

The CK content was determined by Ing. Petre Ivanov Dobrev, CSc. (Laboratory of Hormonal Regulations in Plants, IEB AS CR, v.v.i.). As a result of overexpression of *AtCKX2* gene and thus the increase of CKX activity the endogenous CK content was significantly reduced in

transgenic plants in comparison with WT (Fig. 7). The highest concentration of CKs was in the mature leaves of the WT as well as AtCKX2. In WT the lowest content was found out in the young leaves but in AtCKX2 plants in the old leaves. The highest difference in CK content between both plant types was found in old leaves

7.1.4 Markers of senescence

For characterization of the physiological status of the leaves of both tobacco types investigated we measured the slow Chl fluorescence kinetics (Fig. 8), Chl *a* and *b* content (Fig. 9), protein content (Fig. 10) and oxidative damage to proteins (Fig. 11).

7.1.4.1 Slow chlorophyll fluorescence kinetics

We calculated parameters from slow Chl fluorescence kinetics that have been used to describe photochemical and non-photochemical processes occurring in thylakoid membranes of chloroplasts in examined plants (Fig. 8).

The ratio F_v/F_m was almost constant in AtCKX2 leaves of different age in contrast to WT where the decline of F_v/F_m from the mature to the old leaves was observed. The same course was in case of the parameter $\Phi 2$ and F_v/F_0 but F_v/F_0 parameter started to decrease already from the young leaves. Parameter F_0 had opposite course in controls. F_0 was again unchanged in transgenic plants but was enhanced from the young leaves of controls but in the oldest ones the significant decrease was observed. The NPQ was similar in both transgenic and control plants and decreased with age. It was slightly higher in almost all stages of transgenic leaves development.

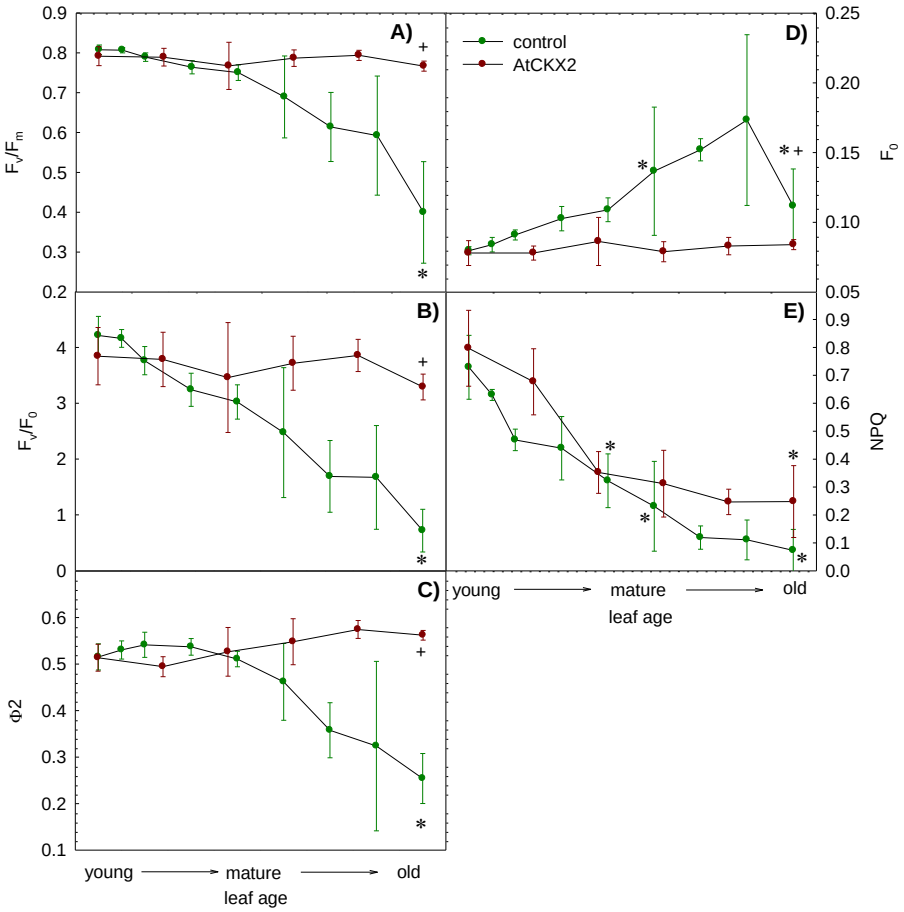

Figure 8: Maximum photochemical efficiency, *i.e.* F_v/F_m (A), maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2, *i.e.* F_v/F_0 (B), effective quantum yield of photochemical energy conversion in PS2, *i.e.* Φ2 (C□□□), minimum Chl fluorescence yield recorded at very low light intensity (at dark-adapted state), *i.e.* F_0 (D), and non-photochemical quenching NPQ (E) in young, mature and old leaves of WT and transgenic tobacco AtCKX2. Asterisks indicate significance of the differences from the respective young leaf. Crosses indicate significance of differences of transgenic plants from WT plant.

7.1.4.2 Chlorophyll content

The content of photosynthetic pigments as well as carotenoids were measured by Mgr. Daniel Haisel (Laboratory of Stress Physiology, IEB AS CR, v.v.i.). The content of Chl *a* and *b* was enhanced in transgenic plants in all stages of leaf age but the age dependence was comparable in both plants (Fig. 9).

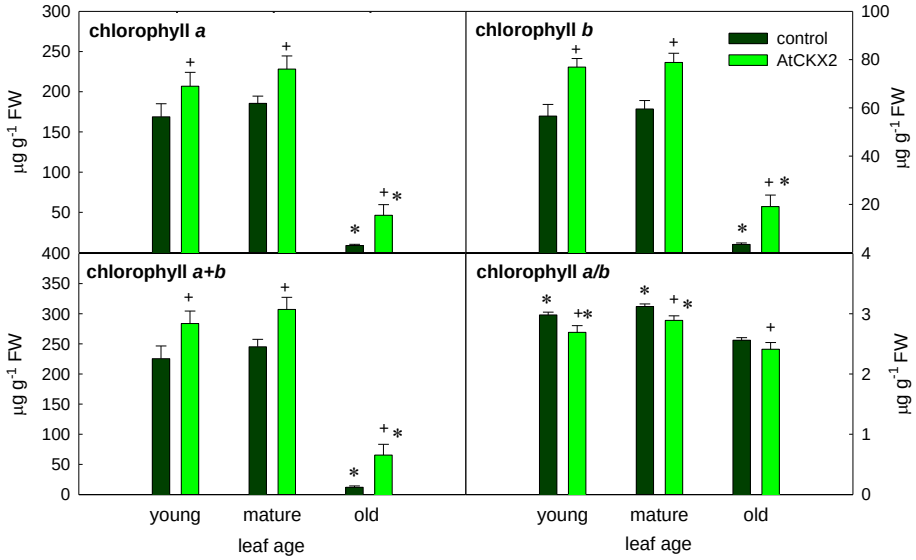

Figure 9: A photosynthetic pigment concentration in young, mature and old leaves of control and transgenic tobacco AtCKX2 and Chl *a/b* ratio. Asterisks indicate significance of the differences from the respective young leaf. Crosses indicate significant differences of transgenic plants from control plants.

The highest concentrations of Chl *a+b* were found out in the mature leaves and the lowest content in the oldest ones. The Chl *a/b* ratio declined in the AtCKX2 plants and was also the highest in the mature leaves of both genotypes.

7.1.4.3 Protein content and oxidative damage to proteins

The soluble protein content (Fig. 10), another marker of senescence, decreased with leaf age in both genotypes. While there was no difference between the young leaves of WT and transgenic plants, the concentration was significantly higher in old transgenic leaves

compared to the WT leaves of the same age. The oxidative damage was determined by quantity of carbonyl groups on protein molecules (Fig. 11). This quantity decreased from the youngest leaves in AtCKX2 and control plants and then there was little enhancement followed by a rapid drop in both plants. Less carbonyl protein groups in young leaves of AtCKX2 compared to WT were formed.

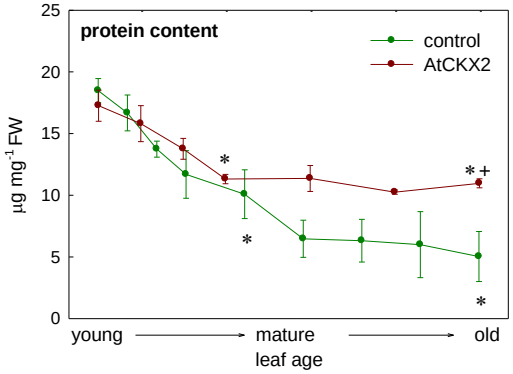

Figure 10: Protein concentration changes during ageing in leaves of control and transgenic tobacco AtCKX2. Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significant differences of transgenic plants from control plant.

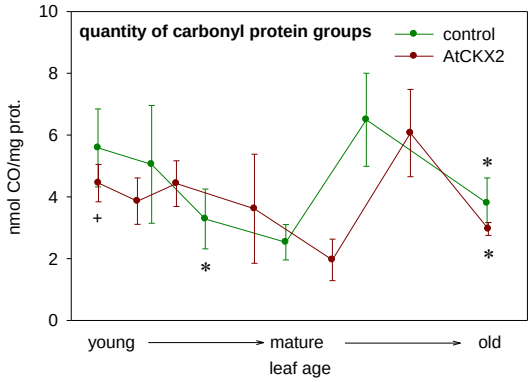

Figure 11: Quantity of carbonyl protein groups in leaves during ageing of control and transgenic tobacco AtCKX2. Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significance of the differences of transgenic plants from control plant.

7.1.5 Low-molecular weight antioxidants

We investigated the effect of lowered endogenous CK content on antioxidant capacity during leaf ageing in this study. As the non-enzymatic antioxidants, level of β-carotene (Fig. 12), pigments of xanthophyll cycle (Fig. 13), DEPS (Fig. 14), L (Fig. 15), N (Fig. 15) and Asc (Fig. 16) contents were measured.

7.1.5.1 β-carotene content

The concentration of β-carotene was enhanced in all leaf stages of AtCKX2 compared to the control plants (Fig. 12). The control plants exhibited the highest β-carotene content in mature leaves however the AtCKX2 in the youngest ones. The most extreme increase compared to WT was in the young leaves of AtCKX2 plants. Rather slight differences in β-carotene content between WT and AtCKX2 plants were observed in the old leaves.

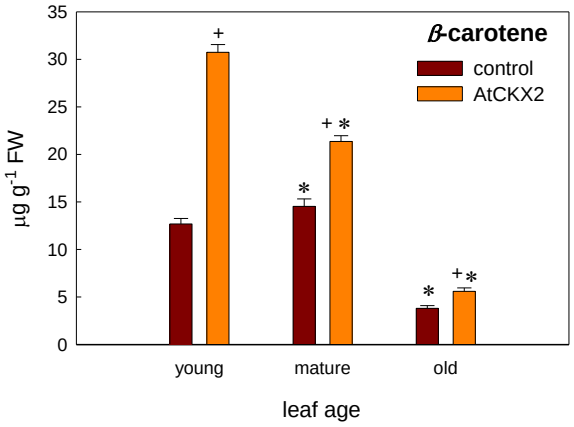

Figure 12: Concentration of β-carotene in leaves during ageing of control and transgenic tobacco AtCKX2. Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significant difference of transgenic plants from control plant.

7.1.5.2 The content of xanthophyll pigments

The content of all xanthophyll cycle pigments (Fig. 13) was higher in all three stages of AtCKX2 especially in mature leaves. The V content was the highest in the mature leaves of both genotypes and represented the majority of total amount of xanthophyll cycle pigments, thus it had the same course during ageing as the sum V+A+Z. Contrariwise the A and Z contents had maxima in the youngest leaves both in the control and in AtCKX2. The content was higher in the young and mature leaves of AtCKX2 plants, in old it was similar to the controls, while Z content was higher in the mature and old leaves of AtCKX2.

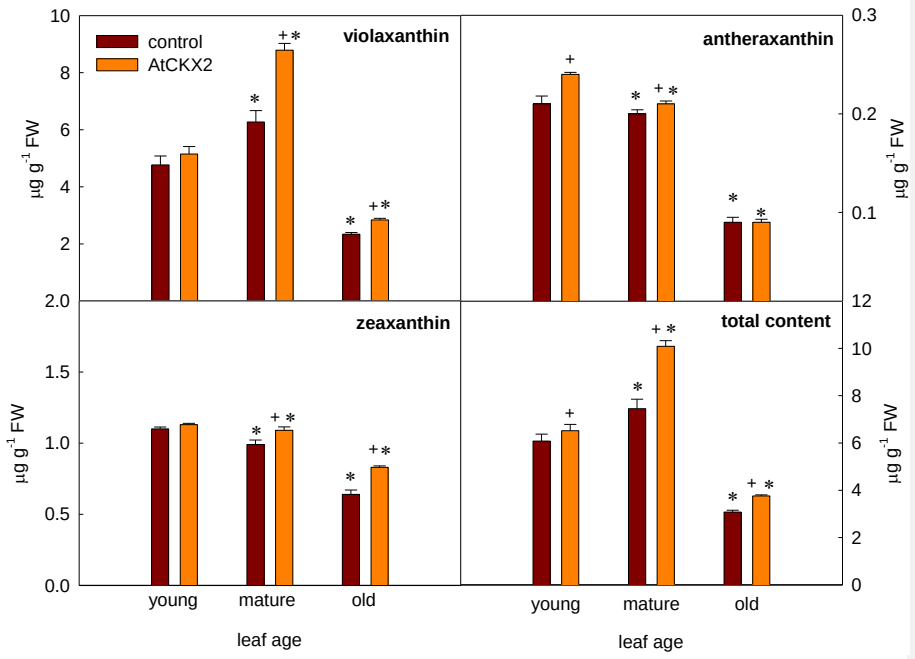

Figure 13: Xanthophyll cycle pigment content (violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), total content V+A+Z) in young, mature and old leaves of control and transgenic tobacco AtCKX2. Asterisks indicate significance of the differences from the respective young leaf. Crosses indicate significance of the differences of transgenic plants from control plant.

The DEPS (Fig. 14) was the lowest in the mature leaves and was higher in the old leaves compared to young ones in both plants. In the young and mature leaves of AtCKX2 it was slightly lower then in controls.

L and especially N concentration (Fig. 15) was remarkably higher in transgenic plants compared to the WT. N as well as L was higher in mature leaves in both kinds of tobacco. All xanthophyll pigments showed a decline in concentration in the oldest leaves of both analysed plants.

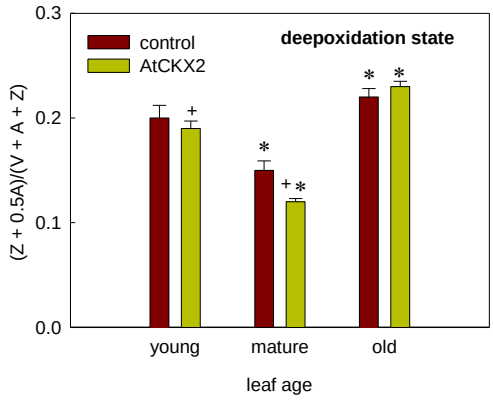

Figure 14: DEPS of the xanthophyll cycle in young, mature and old leaves of control and transgenic tobacco AtCKX2. DEPS is calculated as (zeaxanthin + 0,5*antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin). Asterisks indicate significance of the differences from the respective young leaf. Croses indicate significance of the differences of transgenic plants from control plant.

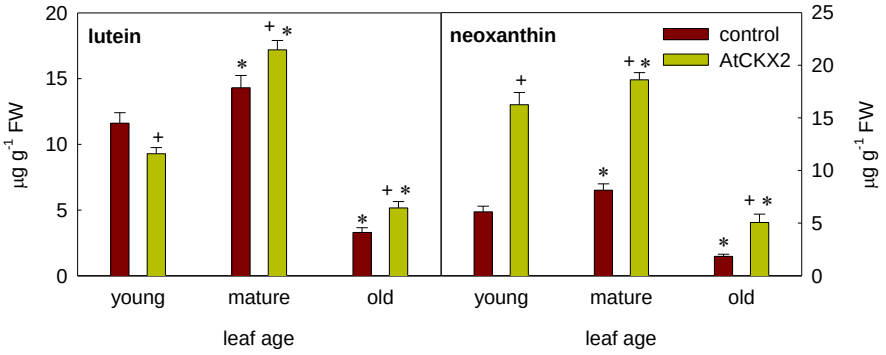

Figure 15: L content and N content in young, mature and old leaves of control and transgenic tobacco AtCKX2. Asterisks indicate significance of the differences from the respective young leaf. Crosses indicate significance of the differences of transgenic plants from control plants.

7.1.5.3 Ascorbate content

The total as well as dehydroascorbate content (DHA) (Fig. 16) decreased during ageing in both WT and AtCKX2 plants. Even DHA concentrations showed comparable values in both plants.

The content of reduced Asc (Fig. 16) decreased with leaf age in AtCKX2. On the contrary in control plant it increased at first and then from it declined from the mature leaves to the oldest ones. These results mean that the ratio of Asc/DHA (Fig. 16) was almost constant during leaf ageing of AtCKX2, contrary to the control plants where this ratio reached the marked maximum in the mature leaves and then it declined. Differences between both tobacco plants were also in the time course of ratio Asc/(Asc+DHA) (Fig. 16). This ratio also increased from the youngest leaves to mature ones and then it decreased with leaf age in WT. The slight decline from young leaves was observed in AtCKX2 plants.

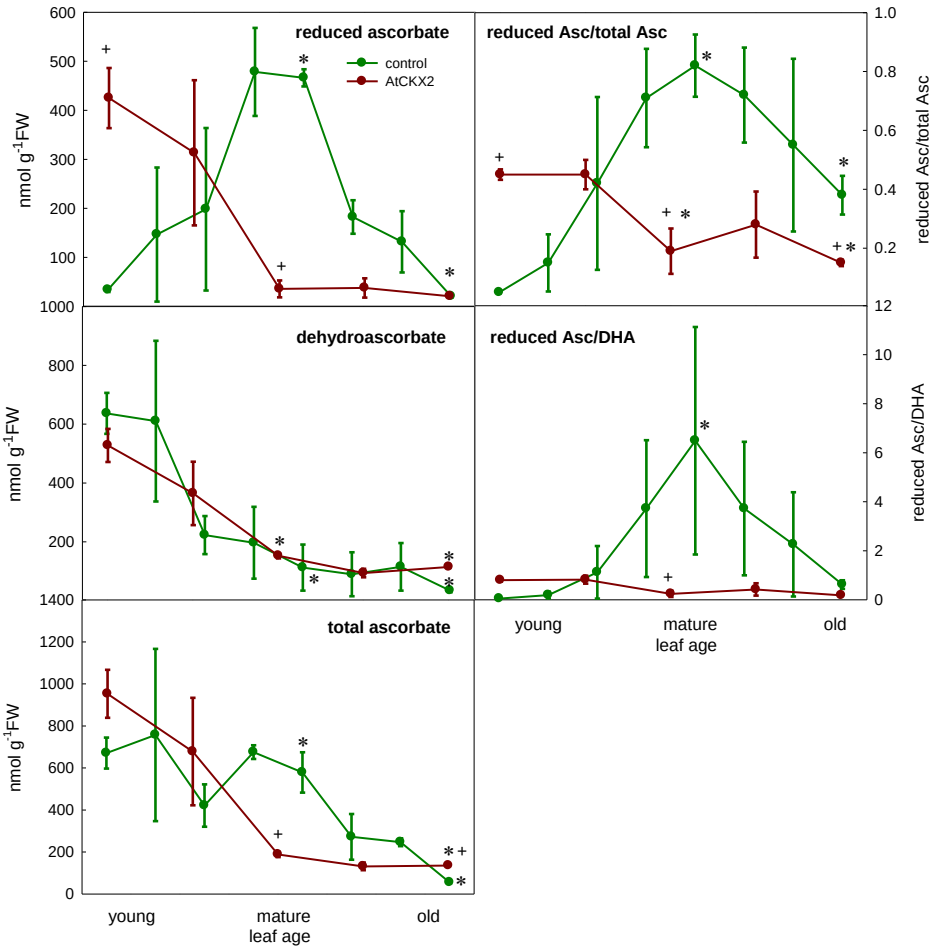

Figure 16: Reduced Asc, DHA and total Asc content and ratio reduced Asc/total Asc and reduced Asc/DHA in young, mature and old leaves of control and transgenic tobacco AtCKX2. Asterisks indicate significance of the differences from the respective young leaf. Crosses indicate significance of the differences of transgenic plants from control plant.

7.1.6 Antioxidative enzymes

7.1.6.1 Total activities of antioxidative enzymes

The activities of AOE were related to the leaf protein content or to the FW. Interestingly they displayed dissimilar results. In contrast to activities related to proteins the activities of AOE related to FW were more significantly age-dependent. They were the highest in the young leaves on the top of the plant and decreased to the old senescent leaves, this held true for both the WT and transgenic plants. There were only two exceptions concerning namely enzyme activities of APX and GR.

The APX activity (Fig. 17) was very similar in both WT and transformed plants except an enhancement in the oldest AtCKX2 leaves when related to FW (Fig. 17A). The similarity in activities between both tobacco types could be seen also when the activities are related to proteins (Fig. 17B), but the courses of both these relations are different.

The age-dependence of the GR activity related to FW (Fig. 18A) was not as significant as observed in case of other AOE but there was the most significant enhancement in AtCKX2 compared to WT. Contrariwise the significant increase of GR activity related to proteins (Fig. 18B) with age in WT was detected. The GR activity related to FW was ca. twice higher in transgenic plants than in the WT at all stages of the leaf development (but it is true for activities related to protein).

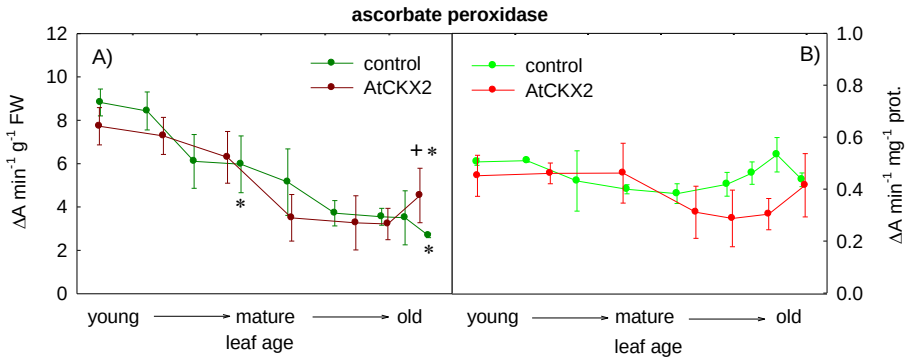

Figure 17: Activities of APX in leaves of different ages in control and transgenic tobacco AtCKX2 related to FW (A) and protein (B). Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significant differences of transgenic plants from control plant.

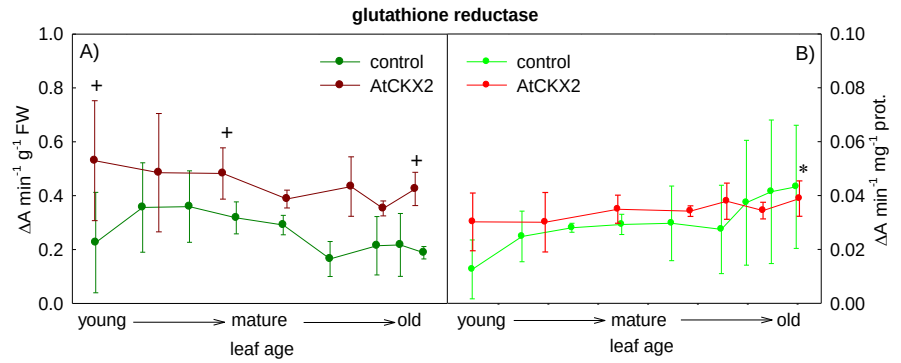

Figure 18: Activities of GR in leaves of different ages in control and transgenic tobacco AtCKX2 related to FW (A) and protein (B). Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significant differences of transgenic plants from control plant.

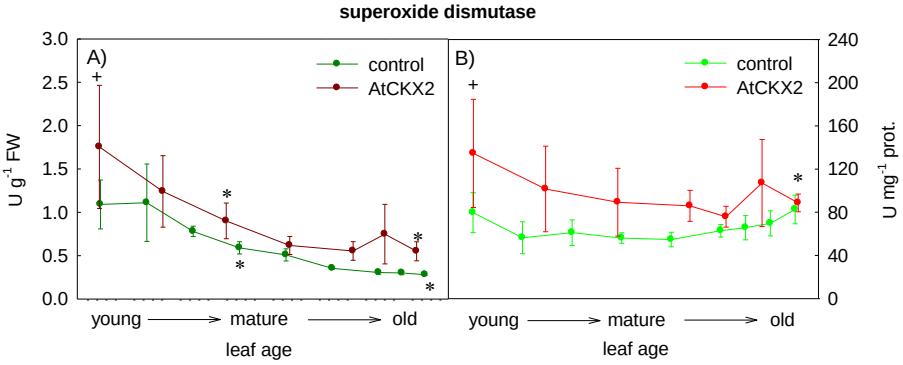

Figure 19: Activities of SOD in leaves of different ages in control and transgenic tobacco AtCKX2 related to FW (A) and protein (B). Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significant differences of transgenic plants from control plant.

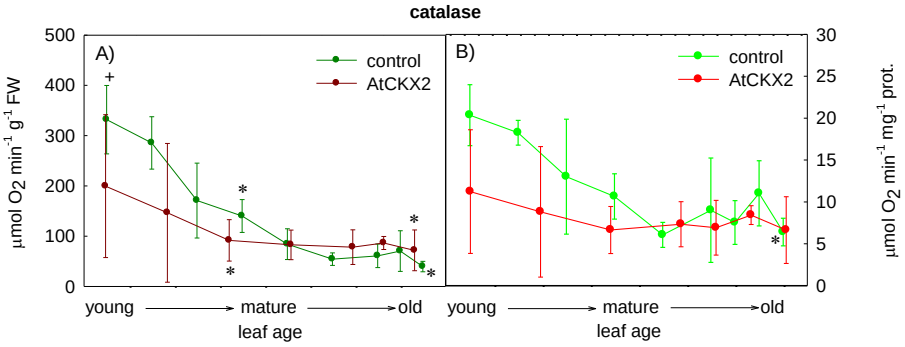

Figure 20: Activities of CAT in leaves of different ages in control and transgenic tobacco AtCKX2 related to FW (A) and protein (B). Asterisks indicate significance of the differences from the respective young leaves. Crosses indicate significant differences of transgenic plants from control plant.

Analogous to GR, the activity of SOD related to FW (Fig. 19A) and also to proteins (Fig. 19B) was enhanced in AtCKX2 leaves in comparison to the WT at all leaf stages but not as significant as in case of in GR activity. The age-dependence of SOD activities was detected in case of both relative unites.

On the contrary, lower CAT activity (Fig. 20A,B) was found in the young leaves of transgenic plants compared to the WT, from the mature leaves to the old ones it become similar in both plants. The course of activities related to both FW and proteins were similar.

7.1.6.2 Isoenzyme composition

The effect of age on distribution of individual antioxidant isoenzymes in leaves of control and AtCKX2 tobacco was investigated by native electrophoresis (Fig. 21). Three SOD isoenzymes (Mn-SOD, Fe-SOD and Cu,Zn-SOD) were detected on the basis of an inhibition with 2 mM KCN (inhibition of Cu,Zn-SOD) or 5 mM H_2O_2 (inhibition of Cu,Zn-SOD and Fe-SOD). The Cu,Zn-SOD isoenzymes were distiguished in three bands differing in their mobility on a polyacrylamide gel. Activities of all isoenzymes decreased with the leaf age in

both control and transgenic plants. The Fe-SOD exhibited the highest activity of all SOD isoenzymes in the whole course of the leaf development. The most marked decline was apparent for Mn- and Cu,Zn-SOD2, while the slowest decrease was apparent for Fe-SOD isoenzyme. Herewith, the intensity of the latter isoenzyme in old leaves of the transgenic plants changed to the lowest extent.

The identical APX isoenzyme composition and behaviour during leaf ageing was observed in both WT as well as in transgenic plants (Fig. 22). Three APX isoforms (APX1, APX2 and APX3) differing in their responses to the leaf developmental stage were detected. While the APX1 and APX2 activities decreased with the leaf age, the activity of APX3, which was the lowest of the three isoenzymes and exhibited the highest mobility, showed a reverse course and increased during the leaf development.

Interestingly, two isoforms of CAT were distinguishable in control leaves (CAT1 and CAT2) but only one in leaves of transgenic plants (CAT1 or CAT2) (Fig. 23).

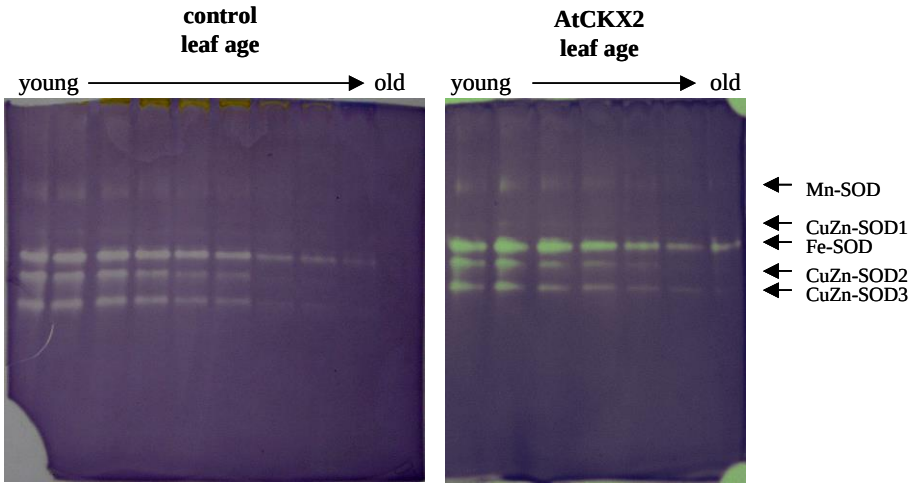

Figure 21: Zymograms of SODisoenzymes in leaves of different age in control (left) and transgenic (right) tobacco AtCKX2. Arrows show isoenzymes described in the text.

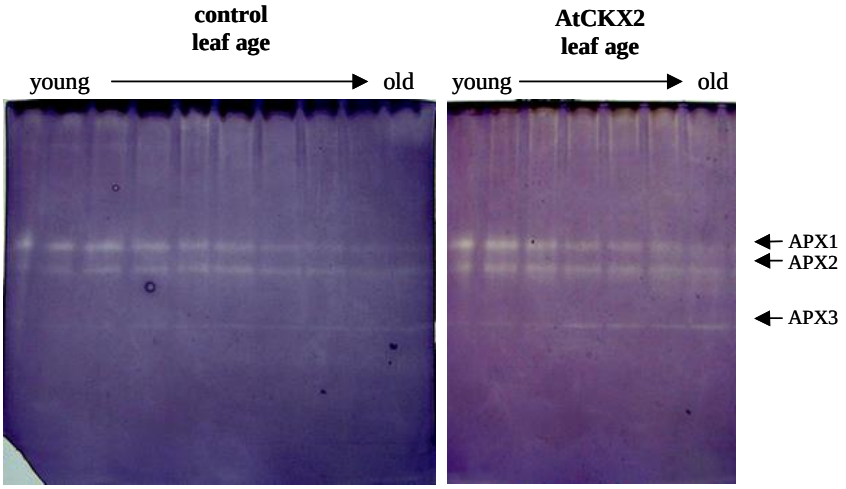

Figure 22: Zymograms of APX isoenzymes in leaves of different age in control (left) and transgenic (right) tobacco AtCKX2. Arrows show isoenzymes described in the text.

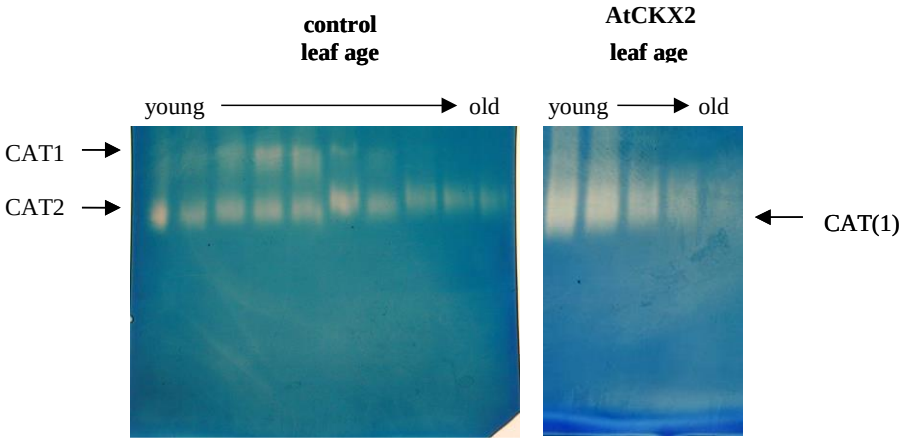

Figure 23: Zymograms of CAT isoenzymes in leaves of different age in control (left) and transgenic (right) tobacco AtCKX. Arrows show isoenzymes described in the text.

7.2 Effect of abiotic stresses on tobacco leaves with cytokinin oxidase/dehydrogenase gene overexpression

7.2.1 Differences of appearance of plants under the stress or non-stress condition

The impact of individual stresses on plant appearance differed between WT and AtCKX2 tobacco plants. The WT plants responded the most sensitively to drought compared to other stresses, they were lower in comparison with non-stressed plants and displayed pronounced signs of wilting with yellowish bottom leaves. The effect of drought on phenotype of AtCKX2 tobacco was less marked and visible. The appearance of drought stressed transgenic plants was similar to corresponding non-stressed ones but the leaves on the bottom were more pale than in unstressed plants. On the other hand, the most harmful stress for AtCKX2 was treatment with zinc. These plants were as high as their non-stressed tobacco, but their bottom leaves were substantially yellow. Resembling but less pronounced effect was found in the WT. AtCKX2 and WT plants showed similar responses to salt stress with their bottom leaves only a slightly lighter compared to unstressed plants (Fig. 24).

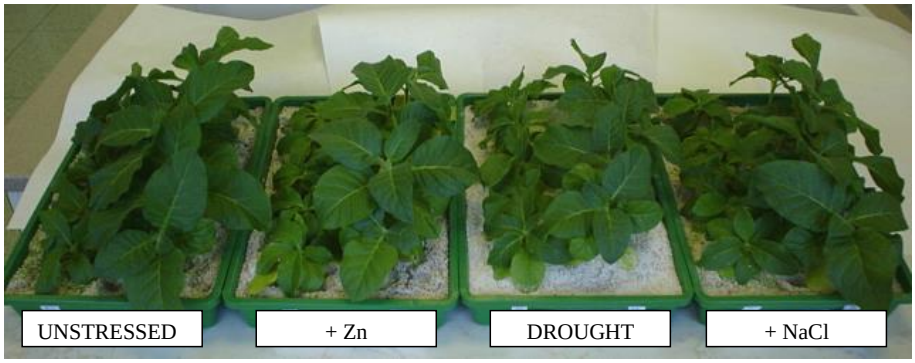

Figure 24: Tobacco AtCKX2 (left side of each tray) and WT plants (right side of each tray) during different growing condition (from the left - normal non-stressed conditions, watered with enhanced $ZnSO_4$ concentration, drought stress and enhance salinity)

7.2.2 Pigment content

Changes in total Chl content were used as a marker of stress impact on tobacco leaves. Content of Chl in AtCKX2 leaves was higher compared to WT even in non-stressed conditions (Fig. 25A). Upon all stresses, the Chl content decreased in both control and AtCKX2 plants. The lowest Chl content was observed in salt stressed WT leaves. The only case when Chl amount in AtCKX2 leaves was lower than in WT tobacco was observed after zinc treatment. The Chl content was equal in both tobacco types following drought stress.

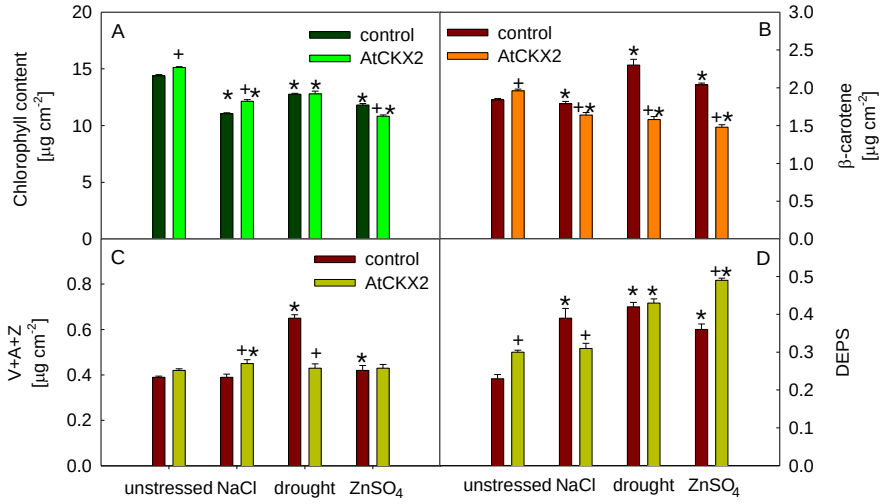

Figure 25: Chl content (*a*+*b*) (A), β-carotene (B), total xanthophyll cycle pigments (V+A+Z) (C), and DEPS of xanthophyll cycle pigments calculated as (Z + 0.5A)/(V + A + Z) (D) in leaves of WT and AtCKX2 transgenic plants grown in unstressed conditions, and under salt and drought stress and high $ZnSO_4$ concentration. Z, V and A represent zeaxanthin, violaxanthin and antheraxanthin contents, respectively. Asterisks indicate significant differences of particular stress from unstressed conditions. Crosses indicate significant differences of transgenic plants from control.

The content of β-carotene was also higher in AtCKX2 leaves in non-stressed conditions and stress responses in both tobacco groups differed (Fig. 25B). In WT, the β-carotene content increased in response to stresses except for salt stress when it was not changed. In AtCKX2, its concentration decreases in case of all three types of stress. β-carotene content declined in transgenic plants with the highest difference between WT and AtCKX2 observed after drought stress.

In WT the xanthophyll cycle pigments significantly increased in drought stress compare to non-stress conditions, lower enhancement was also detected after supply of $ZnSO_4$ (Fig. 25C). In the contrast the only difference between non-stress and stress condition in AtCKX2 plants was during salt stress.

The content of xanthophyll cycle pigments was also higher in AtCKX2 in non-stress conditions compared to the WT (Fig. 25D). In response to stresses, DEPS increased in all cases, but the extent differed in both plant types. Following salt stress, the DEPS was higher in WT while after zinc treatment it was higher in AtCKX2.

7.2.3 Activity of antioxidant enzymes in leaves and roots

The activities of APX, GR, SOD and CAT were estimated separately in leaves and roots of both WT and AtCKX2 tobacco during non-stress and stress conditions.

Under all conditions, including non-stress, the activities of APX were significantly higher and activities of CAT markedly lower in both WT and AtCKX2 roots (Fig.26B and 29B) compared to their respective leaves (Fig. 26A and 29A).

In non-stressed plants, the activities of APX and SOD in roots (Fig. 26B and 28B) and GR in leaves (Fig. 27A) of transgenic plants were significantly higher compared to WT plants. On the other hand, the activity of CAT in non-stressed leaves (Fig. 29A) was lower in AtCKX2 than in WT.

During the salt stress, the activities of only SOD increased in WT leaves (Fig. 28A) and the APX activity increased in WT roots (Fig. 26B). On the other hand, the activity of CAT decreased in leaves of WT (Fig. 29A). Concerning transgenic AtCKX2 tobacco, the activities of SOD decreased and CAT increased in leaves of salt stressed plants. In roots of these transgenic plants also the activity of APX (Fig. 26B) and SOD (Fig. 28B) were reduced in response to salinity.

The activities of all studied AOE were unchanged during drought stress in leaves of WT except CAT declined (Fig. 29A). In WT roots the activities of APX (Fig. 26B), and GR

(Fig. 27B) increased due to the lack of water. In AtCKX2 drought stressed leaves, only the activity of CAT (Fig. 29B) was different from WT, it was elevated, while in AtCKX2 roots the activities of APX (Fig. 26B) and SOD (Fig. 28B) were depressed upon drought stress.

The effect of zinc application to WT led to a decrease of APX and CAT activities in leaves (Fig. 26A and 29A). The SOD activity increased in both leaves (Fig. 28A and roots (Fig. 28B) of WT in these conditions. In AtCKX2 plants zinc caused a high increase in the activity of APX (Fig 26A) in leaves (Fig. 26B). On the other hand, the decrease was observed in activity of SOD in leaves (Fig. 28A) and in activities of APX (Fig. 26B) and GR (Fig. 27B) in roots.

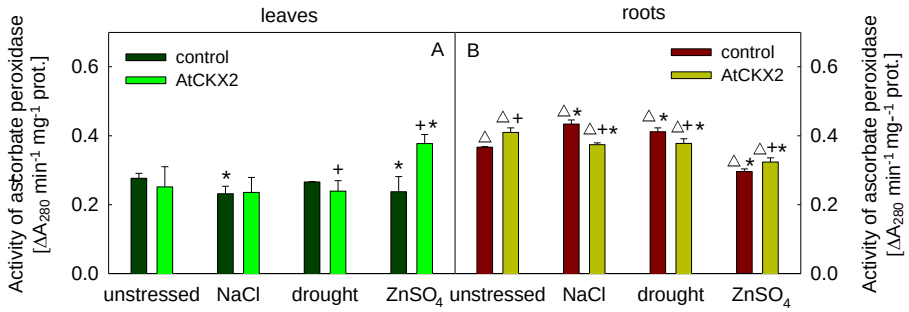

Figure 26: The activity of APX in WT and AtCKX2 transgenic plants leaves (A) and roots (B) grown in unstressed conditions, under salt and drought stress, and after application of $ZnSO_4$. Asterisks indicate significant differences of particular stress from unstressed conditions. Crosses indicate significant differences of transgenic plants from WT. Triangles indicate significant differences of roots from leaves within the same plant and condition.

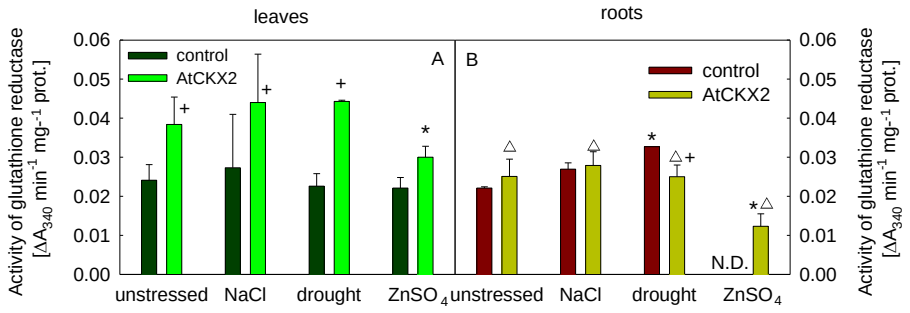

Figure 27: The activity of GR in WT and AtCKX2 transgenic plants leaves (A) and roots (B) grown in unstressed conditions, under salt and drought stress, and after application of $ZnSO_4$. Asterisks indicate significant differences of particular stress from unstressed conditions. Crosses indicate significant differences of transgenic plants from WT. Triangles indicate significant differences of roots from leaves within the same plant and condition. N.D. - not detected.

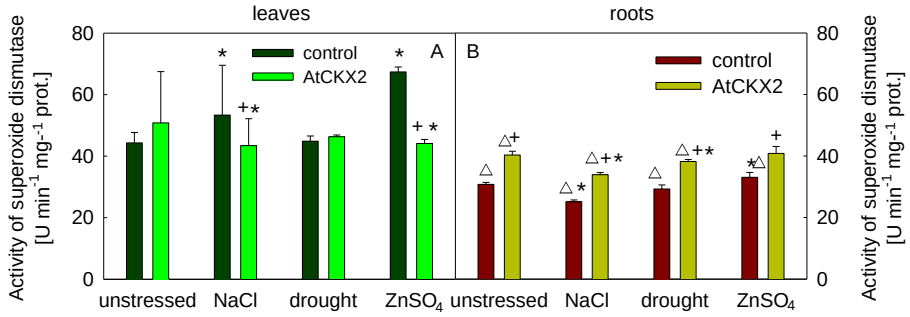

Figure 28: The activity of SOD in WT and AtCKX2 transgenic plants leaves (A) and roots (B) grown in unstressed conditions, and under salt and drought stress and high $ZnSO_4$ concentration. Asterisks indicate significant differences of particular stress from unstressed conditions. Crosses indicate significant differences of transgenic plants from WT. Triangles indicate significant differences of roots from leaves within the same plant and condition.

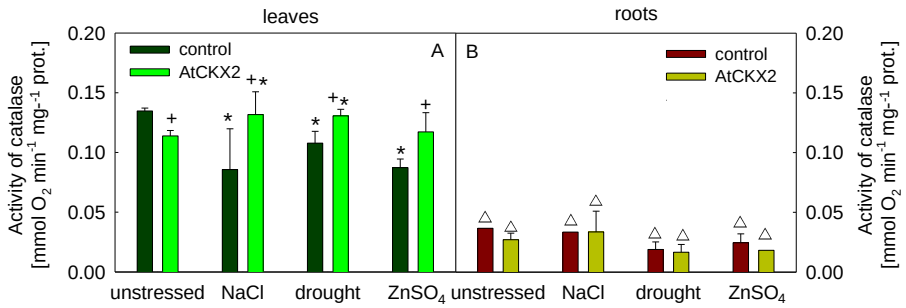

Figure 29: The activity of CAT in WT and AtCKX2 transgenic plants leaves (A) and roots (B) grown in unstressed conditions, under salt and drought stress, and after application of $ZnSO_4$. Asterisks indicate significant differences of particular stress from unstressed conditions. Crosses indicate significant differences of transgenic plants from WT. Triangles indicate significant differences of roots from leaves within the same plant and condition.

8 Discussion

8.1 Influence of cytokinin deficiency in tobacco leaves on antioxidative system during the leaf senescence

Werner *et al.* (2001) presented transgenic tobacco plants with enhanced overexpressing *Arabidopsis* gene *AtCKX2* with reduced endogenous CK content. These plants do not have only different phenotype but their leaves turned yellow later than the leaves of controls, their development was retarded, and started flowering up to 3-months later (Werner *et al*., 2001). Such transgenic plants represent unique model for investigation of the effects of lowered CK content on the physiological processes such as ageing and stress responses.

For confirmation of increased overexpression of *AtCKX2* in transgenic tobacco we analysed leaves at different developmental stages detached from 12 weeks old transgenic and WT tobacco plants for the CKX activity and CK content. At all three developmental stages of the leaf, the over-expression of *AtCKX2* gene was found to cause a considerable elevation of the enzyme activity thereby the substantial reduction of total CK levels. This fact unambiguously demonstrates that transgenic plants could be used as a tool to investigate the relevance of CK deficiency in antioxidative enzymatic protection during ageing.

Chl content, PS2 photochemistry, protein content and the level of protein oxidation in leaves of different age in WT and transgenic tobacco were further estimated as markers of physiological status of leaves. A decline in Chl content has been widely used as a senescence marker (Jordi *et al.*, 2000; Stessman *et al.*, 2002; Jiao *et al.*, 2003; Weng *et al.*, 2005). Despite the fact that the oldest leaves of transgenic plants were still green contrary to the WT, that had been already yellow, we observed a decline of Chl content in both plants with leaf age. Chl content was higher in all leaf developmental stages (old, mature, and young) in transgenic tobacco. We supposed that the level of Chl in senescence stage would still decrease in these plants. We can deduce from a decline in Chl *a/b* ratio that during senescence the Chl *a* is preferentially degraded. The same results were also found out in ageing of bean cotyledons (Wilhelmová *et al.*, 1997).

Together with Chl content also protein content is commonly used as index of senescence (Gan, 2004). Various proteolytic processes are known to be activated during leaf senescence. Increased expression of polyubiquitin genes and increased ubiquitin-conjugation activity have been reported (Belknap and Garberino, 1996) and the activity of the 20S

proteasome is retained during senescence (Roberts *et al*., 2002). Proteins can be subjected to oxidation by ROS under ageing or stress conditions and protein oxidation is often used as a marker of oxidative stress. Carbonylation is the most commonly occurring oxidative protein modification and there is no evidence that carbonylation is reversible (Møller *et al*., 2007).

We found out that soluble protein content is lowered in older leaves in controls than in AtCKX2 plants but interestingly the oxidative damage to proteins, described by level of carbonylation, is comparable in both plant types. There is evident increase of oxidatively modilated soluble proteins followed by sudden decline with age progression. Johannson *et al*. (2004) described that in *Arabidopsis*, protein carbonylation in total protein extract increased during the vegetative phase, but decreased sharply and staying relatively low till and during senescence. It seems that protein oxidation promotes their low stability or breakdown. Their rapid degradation could be caused by a change in conformation exposing more hydrophobic residues, which are recognized by protease (Møller *et al*., 2007).

As the level of Chl may not always precisely reflect decline in leaf performance, the photosynthetic activity has thus been used as another criterion for leaf senescence (Gan, 2004). Fluorescence parameter F_v/F_m has been frequently used as an indicator of the photoinhibition or other kind of injury occurred in the PS2 complex (Roháček, 2002). A lot of scientists reported that the decrease of this parameter during senescence is a result of decline of PS2 function (*e.g*. Lu *et al.*, 2001, Procházková and Wilhelmová, 2004, Weng *et al.*, 2005, Wilhelmová *et al.*, 2005). It is evident from our observations that the photochemical efficiency in transgenic plants was almost unchanged during all leaf developmental stages contrary to controls where a considerable decline was evident. Based on the determination of photochemical efficiency the oldest leaves of transgenic plants were not apparently senescent yet. Similar conclusions were obvious from a parameter F_v/F_0 which can be used as a sensitive indicator of the maximum efficiency of photochemical processes in PS2 and/or the potential photosynthetic activity of healthy as well as stressed plants (Roháček, 2002). Later onset of senescence in old transgenic leaves was also confirmed by fluorescence parameter Φ2 reflecting the actual performance of PS2. Φ2 also decreased during ageing only in WT plants.

The magnitude of the F_0 is influenced by two factors (Schnettger *et al.*, 1994). One is the loss of the photosynthetic pigments, resulting in a decline of F_0, and the other is damage to the PS2 reaction centres, which can contribute to an increase in F_0. Therefore we propose that the increase of F_0 observed in WT tobacco leaves with their age could reflect progressive damage to PS2 reaction centres, whereas the final decrease of F_0 could be consequence of a

detachment Chl from complexes and its rapid degradation that we observed as well *via* Chl content determination. Transgenic plants did not show such time course and evidently their old leaves did not undergo senescence.

NPQ has been widely used as an indicator of excess-radiant energy dissipation to heat in the PS2 antenna complex and its increase is associated with enhancement of A and Z contents (Gilmore, 1997). In our results NPQ decreased with age as well as contents of xanthophyll pigments A and Z. Guiamét *et al.* (2002) reported that the decline in NPQ in senescent leaves probably depended on growth condition. Evidently, the efficiency of a plant to dispose excess energy decreased with age. This could result in increased ROS production.

We can conclude from the presented data that the transgenic plants aged more slowly, the lowest leaves were not in senescence stage yet, contrary to the same ones in controls. They maintained higher Chl content, level of soluble proteins and photosynthetic performance in the oldest leaves.

It is generally known that ageing is an oxidative process due to the constantly increasing concentration of ROS such as H_2O_2 superoxide and hydroxyl radicals and singlet oxygen (Scebba et al., 2001; Peñarrubia and Moreno, 2002). We reported previously that their scavenging enzymes entitled as antioxidative decreased during leaf ageing (Procházková *et al*., 2001; Procházková and Wilhelmová, 2007A). It was shown also that CKs play an important role in regulation of ageing, being recognised as a class of phytohormones postponing senescence onset (Van Staden *et al*., 1988; Mok, 1994; Gan and Amasino, 1996). The effect of CKs on antioxidative defence has been studied up to now only in plants exhibiting enhanced levels of CKs (Synková *et al*., 2006). Several research groups investigated effects of exogenous CK application on antioxidant protection (Gidrol *et al*., 1994; Clarke *et al*., 2002). In this respect, CK-deficient plants seem to be highly informative and complement to this type of studies as the lack of CKs should address processes that are indeed limited and regulated by CKs.

Pigments of xanthophyll cycle provide protection against damage to photosynthetic apparatus from excess radiation. We observed that total content of xanthophyll cycle pigments attained maximum value in the mature leaves and decreased in the oldest ones in both AtCKX2 and control plants. The time courses for contents of total xanthophylls were similar for both tobacco types. The majority of their total amount was represented by V that indeed had the same courses.

On the contrary A and Z contents have declined already in mature leaves in both plants, nevertheless DEPS was significantly enhanced in old leaves. Decreasing V, A and Z

contents and enhanced DEPS during ageing were also observed by other authors (Yang *et al.*, 2001, Munné-Bosch and Peñuelas, 2003). The same courses as V had also contents of N and L. Both at first increased and than in old leaves declined. It is in agreement with other authors (Lu *et al.*, 2001, Munné-Bosch and Peñuelas, 2003). All measured xanthophylls were enhanced almost in all leaf stages of transgenic plants compared to controls. The higher xanthophyll concentrations in the transgenic plants contribute to better photoprotection in chloroplasts. We deduce that the higher content of xanthophyll pigments that mediate better photoprotection could contribute to the later onset of senescence in transgenic plants.

The most significant differences between control and transgenic plants were in case of N. It is interesting, because N as well as V is precursors of ABA, which stimulates senescence. As ABA was reduced in AtCKX2 (Václav Motyka, personal communication), a regulation independent on precursor levels had to participate in ABA synthesis.

Further, we studied content of a non-enzymatic antioxidant β-carotene, an important lipophillic protective compound in chloroplasts: its age-dependent alterations as well as effect of diminished CK level on its concentration. The primary function of β-carotene consists probably in the quenching of high energy-excited triplet Chl and $^1O_2$ produced *via* the energy transfer from this triplet state of Chl (Telfer, 2002). As reported earlier, the age-dependent increase in oxidative stress is accompanied by a decline in chloroplastic antioxidant defence (Munné-Bosch and Alegre, 2002). In our experiments, β-carotene content decreased with age in WT as well as in transgenic plants, however, its concentration was higher in AtCKX2 at all leaf stages. The most significant difference in β-carotene level was found in the youngest leaves with respect to the discrepancy between the occurrence of its concentration maxima. The maximum of β-carotene content in AtCKX2 was found in the young leaves in contrast to the WT, where the highest β-carotene concentration occurred in mature leaves. As carotenoids play an important role in maintaining the integrity of photosynthetic membranes under oxidative stress (Havaux, 1998; Munné-Bosch and Alegre, 2002), the significantly higher content of β-carotene indicated an enhanced ability of protection against photodamage.

The non-enzymatic antioxidant Asc plays an important role in antioxidant defence as a reductant. Asc seems to regulate the activity of APX. When the level of Asc increased or decreased, APX activity was modulated in the same direction (De Gara and Tommasi, 1999). The level of total Asc in leaves of rice exhibited a consistent increase with the leaf age (Yang *et al*., 2001) as well as at the final stage of bean cotyledons development (Procházková and Wilhelmová, 2007B). We detected that the level of total Asc declined with leaf age in both WT and transgenic tobacco plants similarly as in castor bean leaf (Jongeboled *et al*., 2004).

The course of reduced Asc concentration differed in both our plants. In AtCKX2 with delayed senescence the reduced Asc declined continuously whereas in controls it reached the maximum in the mature leaves and than started to decrease. This is in the contrast to results with cucumber plants with delayed senescence by triadimefon. Here a decrease of reduced Asc in senescent leaves was prevented (Feng *et al*., 2003). The DHA in both tobacco plants decreased with leaf age, it might be due to the transformation of DHA into oxalic acid (Yang and Loewus, 1975).

It is a generally known phenomenon that activities of AOE decrease with plant age. We got two age-dependency patterns according to reference units *i.e.* FW or protein content. It is difficult to establish which one is more correct, because neither of these parameters was stable during ageing. The protein content decreased with leaf age contrariwise the FW per leaf area increased reflecting the leaf thickness was proportional to age. Regarding the opposite courses of the protein content and FW during leaf ageing, it is evident that activities of AOE differed in their trends during ageing according to the particular reference unit. The FW of leaves changed during ageing in both plants very closely (FW of old leaves was ca. 190 % of young ones in both plant types) and to a lesser extent. On the other hand, the slope of protein decline markedly differed (protein content in old leaves was 27 % and 63 % of those in young ones, for controls or AtCKX2, respectively). In our opinion for comparison of AtCKX2 and controls, it is more convenient to choose the FW as a relative unit. Accordingly, we prefer the FW to describe the activities of AOE during ageing. We found out the downward trend in activities of APX, GR, SOD and CAT during ontogeny of both tobacco genotypes. Following a native electrophoretic separation we detected three SOD isoenzymes, namely Mn-SOD, Fe-SOD and three bands of Cu,Zn-SOD. Activities of all SOD isoenzymes decreased with leaf age. The Fe-SOD, localised in chloroplasts, represented a major portion of total SOD activity. This held true especially in old leaves of AtCKX2 plants, where this isoenzyme contributed to high SOD activity. The APX activity was found to be represented by three isoforms. Two of them decreased with plant age while the other associated with very low activity increased. This could explain the enhancement of APX activity observed at the end of the leaf development in AtCKX2 plants.

The effect of CKs on antioxidative defence has been studied up to now only in plants exhibiting enhanced levels of CKs. Several research groups investigated effects of exogenous CKs application on antioxidant protection. It was observed that DZ (Clarke *et al*., 2002) or zeatin riboside (Gidrol *et al*., 1994) treatment enhanced activities of CAT and SOD, zeatin riboside having also increased the GR activity (Clarke *et al*., 2002). Based on these data it has

been suggested that enhanced CKs correlate with high activities of AOE. This assumption was confirmed in transgenic tobacco Pssu-*ipt* plants where overproduction of CKs resulted in stimulation of activities of AOE throughout the plant ontogeny (Synková *et al*., 2004; 2006). In contrast to these facts, we found out rather surprisingly that activities of GR and SOD were enhanced in tobacco leaves with decreased concentration of CKs throughout the whole life span. The activity of CAT in the oldest leaves of AtCKX2 transgenic plants was comparable to controls, but lower than in controls at the very beginning of the leaf development. This lowering of CAT activity might be a consequence of CK depletion as de Boer and Feierabend (1974) reported stimulation of the activity of CAT by CKs in developing rye leaves. On the other hand, Toyama *et al*. (1995) showed that an application of 50 μM 6-benzylaminopurine inhibited CAT in etiolated cucumber cotyledons. Petit-Paly *et al*. (1999) demonstrated the effect of 6-benzylaminopurine on CAT activity in tobacco suspension-cultured cells and observed that the enzyme activity slightly decreased during the first hour after the treatment but increased thereafter.

It seems possible that the decrease in the level of total CKs, assumed as potential non-enzymatic antioxidants (Gidrol *et al*., 1994), resulting from the AtCKX2 gene overexpression, could promote the enhancement of certain antioxidant defence as a compensatory effect. It is apparent from changes in activities of some AOE. The increase of several antioxidant defence in young leaves of AtCKX2 plants in relation to a decline of inactive and/or weakly active CKs might be of a special interest. In our experiments, we observed elevated antioxidant protection functional already in the youngest leaves. On the basis of these results we hypothesize that the lack of CKs in young developing leaves could induce oxidative stress that is balanced by elevated another antioxidant defence. The induced antioxidant defence could be indirect effect of lowered CK concentration, but obviously this means a higher tolerance to oxidative stress occurring particularly in aged leaves. There is enough evidence that high activities of AOE correlate with delayed senescence (Lacan and Baccou, 1998). It is plausible that the higher antioxidant defence might contribute to the retardation of ageing as a consequence of postponed onset of senescence in leaves with a deficiency of CKs.

8.2 Effect of abiotic stresses on antioxidants in tobacco with retardation of ageing

Salinity, drought and treatment with Zn^{2+} salts have been considered to induce oxidative stress and thus reduce growth parameters of plants. AOE prevent harmful accumulation of ROS generated during oxidative stress. Plants differ in their antioxidant defence that could be the basis of their different tolerance to stress (Sumithra *et al*., 2006). In this part of study, we tested the tolerance to abiotic stresses of transgenic tobacco overexpressing the *AtCKX2* gene. The higher antioxidant capacity of a plant is assumed to be associated with an improved tolerance to environmental stresses.

Instead, the WT and AtCKX2 tobacco plants differed in their responses to particular stresses. According to their appearance after stress treatments, the WT plants were quite sensitive to drought stress (the plants are lower compared to non-stressed plants) and in lesser extent also to other stresses. On the other hand, the appearance of AtCKX2 plants was changed only after zinc-induced stress, but just rather slightly by mild yellowing of the oldest leaves. Only during this type of stress the Chl content was lower in leaves of AtCKX2 than in leaves of WT plants. A special attention was given to the study of stress effect on the β-carotene and pigments of xanthophyll cycle contents and activities of AOE.

The level of xanthophylls and β-carotene were very different in stressed and unstressed WT and also in AtCKX2 plants. Differing changes in carotenoids during various stresses were also reported previously. Also within only one type of stress the reaction is not similar but differs in various plants and experiments. Zhang and Kirkham (1996) reported that in sorghum and sunflower drought did not affected carotenoid content in either crop, whereas Ünyayar *et al*. (2005) described significant increase in the content of β-carotene and pigments of xanthophyll cycle in two tomato species, in pea plants the drought stress led to reduction of the total level of carotenoids (Moran *et al*., 1994).

APX and SOD activities were found higher in roots of 8 week-old AtCKX2 plants compared to WT plants under non-stressed conditions. On the other hand, only activity of GR was markedly higher in leaves of AtCKX2 plants under non-stressed conditions in comparison with WT while activity of CAT was even lower. It is apparent, that mainly in roots, but almost not at all in leaves, the AOE defence of non-stressed plants is better in AtCKX2 than in WT plants.

The salt stress had marked impact on WT plants as apparent from pronounced decline of Chl content in comparison with non-stressed conditions. Also activities of APX and CAT decreased in leaves of WT tobacco. On the other hand, CAT activity was elevated and SOD activity decreased in salt stressed AtCKX2 plants. Enhancement in the AOE activities after salt treatment has been reported previously in a number of other plant species such as pea (Hernandéz *et al*., 1999), beet (Bor *et al*., 2003), cucumber (Zhu *et al*., 2004) and tomato (Ünyayar *et al*., 2005) and was associated with salinity resistance.

It is evident from literature data that different plant species differ considerably in their AOE activities in response to water deficit (Moran *et al*., 1994, Zhang and Kirkham 1996, Ünyayar *et al*., 2005). In this work we observed that activities of AOE in WT leaves were almost not affected during water deficit in contrast to roots where APX and GR activities increased after the stress treatment. However, the activity of CAT increased in leaves and the SOD and APX activities decreased in roots of AtCKX2. In spite of the fact that the WT plants as well as their leaves were highly reduced in size upon drought stress compared to AtCKX2, the content of Chl was equal in leaves of both tobacco types. The explanation for better endurance of AtCKX2 tobacco in water deficit may consists in their thicker and smaller leaves in normal growth conditions.

Zinc plays a dual role in a regulation of plant growth and development. At low concentrations it is indispensable for normal plant growth as a cofactor of more than 300 enzymes and proteins involved in cell division, nucleic acid metabolism, and protein synthesis (Hacisalihoglu *et al*., 2003). Zinc might also participate in oxidative stress-induced expression of genes encoding antioxidative defence enzymes such as APX and GR (Cakmak, 2000). On the other hand, when present at higher concentrations zinc plays a negative toxic role in plants by inactivating the cellular antioxidant pool, disrupting the metabolic balance, eventually enhancing the amount of ROS (Triphati and Gaur, 2004). However, the intensity as well as the kind of Zn^{2+} effect does not depend only on zinc concentration but also on tolerance of particular plant species. In this work, based on our previous experiments (Mýtinová *et al*., 2005) where 5 mM concentration of Zn^{2+} caused oxidative stress with visible symptoms of yellowing of the bottom leaves of both WT and transgenic plants. Based on the decline in Chl content it is evident that AtCKX2 plants were heavily impaired compared to WT. The AOE activities were affected by zinc in different way in WT and AtCKX2. While a decrease in activity of APX and CAT appeared after zinc treatment in WT leaves, a decrease in GR and SOD activities in AtCKX2 leaves was found. In transgenic AtCKX2 roots the decline in APX and GR activities was observed. This finding is in contrast to increased activities of AOE

reported in other plant species (Bonnet *et al*., 2000, Tripathi and Gaur, 2004). In our work, the SOD activity in leaves and roots of WT plants increased. It might be connected with higher zinc supply, which is essential in Cu,Zn-SOD.

In this aspect the opposite effect was observed in leaves of AtCKX2 tobacco. Here the same zinc concentration seemed to be more harmful. Zinc application was the most devastating for AtCKX2 plants among all stresses and simultaneously also SOD activity declined in AtCKX2 leaves.

According to several authors, the differences in responses between roots and leaves of a particular plant as well as between WT plants and AtCKX2 to zinc treatment could be attributed to different time course of stress response (Clijsters *et al*., 1999, Cuypers *et al*., 2001). We actually observed that the AtCKX2 were in different developmental stage than the WT. Moreover, the root system of AtCKX2 transgenic plants was highly dispersed and spreading within a soil compared to WT, which could hence promote higher intake of the metal by AtCKX2 plants.

There are only few reports comparing differences in the antioxidant protection between leaves and roots. It is obvious that the roots are directly and primarily exposed to stresses originating from the soil as drought, salinity as well as metal application. Cuypers *et al*. (2002) interestingly found only limited impact of zinc on roots of *Phaseolus* compared to its primary leaves, which was substantiated by the fact that zinc was readily transported to the plant shoot. Dixit *et al*. (2001) reported different antioxidant responses to cadmium treatment in roots and leaves of pea and also Liu and Huang (2000) reported distinct activities of AOE during heat stress in creeping bentgrass. In our experiments, the stress-induced responses of AOE activities often differed between leaves and roots, both in WT and transgenic plants. In general, the APX activity was higher and CAT activity lower in roots compared to leaves in both plant types in non-stress and even in stress conditions. The SOD activity was lower in WT roots under non-stress conditions and upon salt stress, however, in all other stresses the activities of SOD in roots and leaves were comparable. Interestingly, activity of GR was enhanced in roots of WT plants compared to leaves in response to salt and drought stress.

To summarize, the responses of AOE activities to individual stresses differed in leaves and roots of both WT and AtCKX2 tobacco plants. Interestingly, responses of CAT activity in leaves were opposite: it always decreased in WT plants but increased (except for zinc treatment) in AtCKX2 tobacco. On the other hand, the SOD activity increased in WT but decreased after all stresses except for drought in AtCKX2 plants. Similarly, the APX activity

prevailingly increased in roots of WT tobacco while mostly decreased in roots of AtCKX2 plants.

The higher tolerance of AtCKX2 plants could be attributed to noticeably higher activity of GR in leaves and APX, SOD and CAT in roots displayed already under control conditions. The higher activity of GR in leaves of AtCKX2 observed under non-stressed conditions increased even more in response to two of three stresses. It is known that GR provides satisfactory level of reduced GSH, leading to favourable redox state of this compound. The importance of GR was already reported in resistance to e.g. drought stress (Contour-Ansel *et al*., 2006). Although transgenic tobacco plants possess, at least partially, better antioxidant protection in non-stressed conditions, they were not confirmed to be more tolerant to abiotic stresses. Their sensitivity and response to stresses was probably modified compared to WT, which need not to be directly associated with transformation. Analogously with this supposition, Vaillant *et al*. (2005) reported different sensitivity in four different members of *Datura* species to zinc stress. The difference between transgenic and WT tobacco in their stress responses could be associated with divergent sensitivity to imposed stress in consequence of protracted development of AtCKX2. The WT and AtCKX2 tobacco may thus differ in their general stress response.

9 Conclusions

Leaves of transgenic tobacco with reduced level of CK aged more slowly, contrary to the control leaves. AtCKX2 plants maintained higher Chl content and protein content, and also higher photosynthetic performance in the oldest leaves.

We observed elevated antioxidant protection (both enzymatic and low-molecular) functional already in the youngest leaves of AtCKX2.

Enhancement of certain antioxidant defence could be caused compensatory effect of the decrease in the level of total CKs, assumed as potential non-enzymatic antioxidants or as a result of oxidative stress induced by the lack of CKs in young developing leaves.

It is plausible that the higher antioxidant defence might contribute to retardation of ageing with a consequence of postponed onset of senescence in leaves with a deficiency of CKs.

The responses of AOE activities to individual stresses differed between leaves and roots of both WT and AtCKX2 tobacco plants.

The higher tolerance of AtCKX2 plants could be attributed to noticeably higher activity of GR in leaves and APX, SOD and CAT in roots displayed already under control conditions. Although transgenic tobacco plants possess, at least partially, better antioxidant protection in non-stressed conditions, they were not confirmed to be more tolerant to abiotic stresses.

The difference between transgenic and WT plants in their stress responses could be associated with divergent sensitivity to imposed stress in consequence of protracted development of AtCKX2.

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11 Attachemants

List of Publications

Publications in impact journals:

Mýtinová, Z., Haisel, D., Wilhelmová, N.: Photosynthesis and protective mechanisms in transgenic tobacco leaves with overexpressed cytokinin oxidase/dehydrogenase and thus lowered cytokinin content during ageing. *Photosynthetica* 44: 599-605, 2006.

Wilhelmová, N., Wilhelm, J., Fuksová, H., Mýtinová, Z., Procházková, D., Schwippelová, Z., Vytášek, R.: The effect of plant cytokinin hormones on the production of ethylene, nitric oxide, and protein nitrotyrosine in ageing tobacco leaves. *BioFactors* 27: 203-211, 2006.

Mýtinová, Z., Motyka, V., Haisel, D., Malbeck, J., Dobrev, P., Wilhelmová, N. Effect of cytokinin deficiency on antioxidative enzymatic defence during ageing of transgenic AtCKX2 tobacco leaves during ageing. *Plant Science*, submitted.

Mýtinová, Z., Haisel, D., Motyka, V., Gaudinová, A., Wilhelmová, N.: Effect of various abiotic stresses on the activity of antioxidative enzymes and phytohormone levels in wild type and transgenic tobacco plants overexpressing Arabidopsis thaliana cytokinin oxidase/dehydrogenase gene. *Biologia Plantarum*, submitted.

Mýtinová, Z., Wilhelmová, N., Haisel, D., Motyka,V.: Influence of decreased level of endogenous cytokinins on antioxidative mechanisms in tobacco leaves during ageing. *Biologia Plantarum* 49, Abstr. p. 4-9, S24, 2005.

Publication in other journals and proceedings:

Mýtinová, Z., Wilhelmová, N., Motyka, V., Haisel, D., Malbeck, J., Dobrev, P.: Oxidační poškození a antioxidační ochrana v listech tabáku se zvýšenou expresí cytokininoxidasy/dehydrogenasy během stárnutí. *Chemické listy* 5, Abstr. p. 397, 2006.

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Prohlašuji, že výsledky jiných autorů (Ing. Václav Motyka, CSc.; Ing. Petre Ivanov Dobrev, CSc.; Ing. Petre Ivanov Dobrev, CSc.) byly použity ze společných publikací a se souhlasem spoluautorů a školitele.

Ing. Zuzana Mýtinová RNDr. Naďa Wilhelmová, CSc.