Charles University in Prague
Faculty of Science

Plant physiology and anatomy

Ing. Zuzana Lubovská

Odpověď antioxidačních enzymů na abiotický stress. Vliv snížené hladiny cytokininů.

Antioxidant enzymes response to abiotic stress. Impact of decreased cytokinin level.

Ph.D. Thesis

Supervisor: RNDr. Naďa Wilhelmová, CSc.

Praha, 2015

Antioxidant enzymes response to abiotic stress. Impact of decreased cytokinin level.

Prohlašuji, že jsem tuto disertační práci vypracovala samostatně, na základě vlastních pokusů a s použitím citované literatury. Tuto práci, ani její část, jsem nepředložila k získání jiného akademického titulu.

.....……..

Zuzana Lubovská

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor **RNDr. Naďa Wilhelmová, CSc.** for her advice, friendly atmosphere in laboratory, her continuous support during my Ph.D. study and for giving room to work my own way.

I am obligated to **RNDr. Radomíra Vaňková, CSc.**, whose thought gave birth to this work. I am honoured that I worked on her project and I would like to thank her for rewarding experience and her patience.

I also wish to thank consultant **RNDr. Helena Štorchová, CSc.** for teaching me primer design and PCR. I truly appreciate her conscientious leadership.

I thank **Lenka Kolčabová** for her help and her smile and humanity she brings for everybody.

Finally, I would like to thank **Prof. Xia Li, Ph.D.** for kind welcome in her laboratory, knowledge she gave me and for working space she offered me for gene sequencing. I am honoured I could work in her laboratory, time I spent there brought me priceless experience for professional as well as personal life.

This work was supported by the Grant Agency of the Czech Republic, grant no. 206/06/1306.

1. Content

2. Abstract

The response of the antioxidant enzyme system to drought, heat and a combination of these stresses was followed in tobacco plants overexpressing *cytokinin oxidase/dehydrogenase1* (*AtCKX1*) under the root-specific *WRKY6* promoter (*W6:CKX1* plants*)* and under the constitutive *35S* promoter (*35S:CKX1* plants) and in the corresponding wild type (WT). *CKX1* over-producing lines developed a bigger root system, which contributed to their stress tolerance. The high stress tolerance of *35S:CKX1* plants was also associated with a dwarf shoot phenotype and changed leaf morphology.

The genes for chloroplastic antioxidant enzymes, stroma ascorbate peroxidase (sAPX), thylakoid ascorbate peroxidase (tAPX) and chloroplastic superoxide dismutase (FeSOD), which are responsible for scavenging of ROS produced via electron transfer during photosynthesis, were all strongly transcribed in control conditions. All the tested stresses down-regulated expression of these genes in WT and *W6:CKX1* plants, whereas expression was maintained in the stress-tolerant *35S:CKX1* plants during drought and heat stresses.

Enzymes supposed to play a general role in stress protection, namely cytosolic ascorbate peroxidase (cAPX), catalase 3 (CAT3) and cytosolic superoxide dismutase (CuZnSOD), were activated under stress conditions. Heat shock, alone or combined with drought, induced the expression of *CAT3*, *CuZnSOD* and especially that of *cAPX* in all genotypes, whereas prolonged drought alone was not associated with their increased expression. Transcription of genes for peroxisomal enzymes *CAT1* and *CAT2*, unlike *CAT3*, was extensive under control conditions. Heat shock alone, unlike a combination of drought and stress, induced *CAT1* but reduced *CAT2* expression in WT and *W6:CKX1*. In the more stress tolerant *35S:CKX1* genotype, *CAT1* expression was enhanced under all stress conditions. The activity of mitochondrial superoxide dismutase (MnSOD), which removes superoxide generated by electron leakage in respiratory electron transport chain, positively correlated with cytokinin content, being much lower in *35S:CKX1* plants than in WT. Defence mediated by the antioxidant enzyme system possesses high plasticity, being independently regulated at the level of gene expression and activity. The effect of down-regulation of cytokinin levels on the antioxidant enzyme system seems to be indirect, mediated by stress tolerance under unfavourable conditions or by modulation of photosynthesis under control conditions.

3. Abbreviations

35S:CKX1 - *N. tabacum* overexpressing *CKX1* from *A. thaliana* under *35S* promoter

A. thaliana - *Arabidopsis thaliana*

APX - ascorbate peroxidase(s)

ARR - *Arabidopsis* response regulators

ATP - adenosine triphosphate

ADP - adenosine diphosphate

C - control conditions

CAT - catalase(s)

CK - cytokinin(s)

CKX - cytokinin oxidase/dehydrogenase(s)

CuZnSOD - coper zinc superoxide dismutase

D - drought (8 days water withdrawal)

D+H - drought followed by heat

ERD1 - early response to dehydration 1

FeSOD - iron superoxide dismutase(s)

H - heat (2 hours 40 °C)

iP - N^6-isopentenyladenine

IPT - isopentenyl transferase

MnSOD - manganese superoxide dismutase(s)

N. - *Nicotiana*

PAGE - polyacrylamide gel electrophoresis

PCA - principal component analysis

Pssu - *Pisum sativum* ribulose-1,5-biphosphate carboxylase small subunit promoter

ROS - reactive oxygen species

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

RWC - relative water content

sAPX - stromatal ascorbate peroxidase

SOD - superoxide dismutase(s)

tAPX - thylakoidal ascorbate peroxidase

W6:CKX1 - *N. tabacum* overexpressing *CKX1* under *WRKY6* promoter

WT - wild type

4. Preface

Due to sessile life-style, maintaining metabolic equilibrium in their organisms is crucial for plant surviving. Inner defence mechanisms, among others, comprise of cytokinins (CK) and antioxidant enzyme system.

Hormones CK are indispensable for the growth regulation and plant development and also play an important role in stress responses. CK are the latest identified hormones among auxins, abscisic acid and gibberellins, and their role in stress response is the fewest explored, especially in comparison to their antagonist, the abscisic acid.

Exogenous CK act like stress protectants and also some transgenic plants with increased level of endogenous CK showed improved stress tolerance. However, plants with diminished level of CK had also improved tolerance to abiotic stress. Mechanism of improved stress tolerance in plants with reduced level of endogenous CK was not clarified yet.

To activate antioxidant system reaction, we treated plants with abiotic stressors which are agriculturally important, drought and heat. Drought is a frequent stress that reduces plant biomass production and crop yield. In nature, drought is often accompanied by heat stress.

Reaction to drought and heat stresses combination is remarkable but insufficiently explored field. Plant response to drought and heat combination is not a sum of defence reactions against single stresses, in addition some defence mechanisms are contradictory, e.g. water management by stomata.

Both, CK as well as antioxidant enzymes play role in stress defence. We have evaluated antioxidant enzymes interplay with CK, more precisely, impact of reduced CK level on antioxidant enzymes in response to drought or/and heat. We examined if CK stress protective function was substituted by improved antioxidant system in plants with decreased CK level. We also studied antioxidant enzymes reaction to drought and heat combination. Antioxidant enzymes were observed on both levels, transcripts as well as active proteins what brings another importance to this work.

5. Introduction

Background for this thesis includes three main topics: CK, plants under stress conditions and antioxidant system. This overview of present state of knowledge deals in more detail with special topics as cytokinin oxidase/dehydrogenase (CKX), transgenic plants with modified CK metabolism, formation of reactive oxygen species (ROS), abiotic stresses, especially drought and heat stress, and antioxidant enzymes. Particular attention is dedicated to interconnections between mentioned topics, in chapters antioxidant enzymes under abiotic stress, and antioxidant enzymes and CK. A special part is focused on plants with changed level of endogenous CK.

4.1. Cytokinins

In comparison with other hormones as auxin and abscisic acid, CK are the last discovered. CK have irreplaceable function in many processes. They are known to play a role in cell division, cell growth, cell differentiation, apical dominance (Sachs and Thimann 1967, Tanaka *et al*. 2006), axillary bud growth (Napoli *et al.* 1999), leaf senescence (Kim *et al*. 2006), phloem and xylem formation, primary root development, root proliferation (Werner *et al.* 2001), stomata opening, chloroplast structure, starch accumulation, chlorophyll preservation, phyllotaxis (Giulini *et al.* 2004), reproductive competence (Ashikari *et al.* 2005), nutritional signalling (Takei *et al.* 2002, Samuelson and Larsson 1993), sink strength increase (Roitsch and Ehneß 2000), as well as in both biotic and abiotic stress defence (Choi *et al.* 2011, Nishiyama *et al.* 2011).

4.1.1. Types of cytokinins

Hormones CK the mostly often naturally occur as N^6-substituted adenine derivatives with unsaturated isoprenoid side chain (Mok and Mok 2001). Presence of adenine with purine ring substituted in N^6 position is a requirement for CK activity. Plants synthesize several different CK: *trans*-zeatin, N^6-isopentenyladenine (iP), *cis*-zeatin, dihydrozeatin, iP sugar

derivatives, kinetin (N^6-furfuryladenine), and 6-benzylaminopurine. Phenylurea-type CK as diphenylurea or thidiazuron display also CK activity, but they have not been found in plants.

Free CK bases are regarded as the most active forms. They are converted in plants to less active or inactive forms, which are more suitable for transport and storage. There are four reactions how CK are transformed: 1) reversible conversion of bases to ribosides or ribotides, 2) reversible O-glycosylation or acetylation of side chain, 3) purine N-glycosylation and conjugation with alanine in position N-9, and 4) reversible reduction of side chain.

Ribosides and ribotides probably serve for quick and reversible modulation of amount of highly active CK and are probably used as transport form.

CK can be conjugated at O- or N-positions with sugar moiety in reaction catalysed by urine-glycosyltransferase, where uridine diphosphate serves as a donor of sugar molecule. O-glucosides are regarded as storage or transport forms, but they can act without previous hydrolysis, thus are quite active. Their CK activity could be restored by deglycosylation in plastids by β-glucosidase. CK O-glycosides are stored mainly in vacuole (Sakakibara 2006).

N-7,9-glucosides and conjugates with alanine show low activity and probably serve to regulate CK activity. N-3-glucosides have quite high activity and their role is not fully clear. By contrast, N-glucosides with glucose at 7- and 9-position of purine ring are irreversibly inactivated, probably are employed for inactivation of CK forms that cannot be inactivated by CKX. Localization of N-glucosides in a cell is not clearly defined, however, accumulation of O- or N-glucosides could be species specific (Vaňková *et al.* 1999). Formation of dihydrozeatin could be a protecting strategy because dihydrozeatin is not degraded by CKX.

4.1.2. Biosynthesis

Hormones CK are present in all parts of a plant. Formerly, tissues with the highest CK concentrations, i.e. root tips, shoot apices and immature seeds, were considered the main place of CK biosynthesis (Letham 1994, Emery *et al.* 2000). Nowadays it is accepted that CK are synthesized at various sites of a plant and the synthesis is precisely regulated to exact concentrations. Cellular concentration of endogenous CK is in order of nM. At the level of a green cell, main subcellular compartment of CK *de novo* synthesis are plastids.

The majority of natural CK include isoprenoid side chain. The first reaction in CK biosynthesis is catalysed by isopentenyl transferase (IPT). The IPT is a key enzyme in CK

biosynthesis which was successfully employed to produce transgenic plants with elevated level of endogenous CK, which contributed to exploration of CK role. Enzyme IPT uses dimethylallyl diphosphate as prenyl donor and transfers its isopentenyl group to an adenine nucleotide (preferentially adenosine triphosphate - ATP, adenosine diphosphate - ADP, but also adenosine monophosphate - AMP). Biosynthesis of the prenyl donor dimethylallyl diphosphate follows the mevalonate pathway and to a lesser extent the methylerythritol phosphate pathway with hydroxymethylbutenyl diphosphate as an intermediate in plastids. Thus, in higher plants, the major initial product is an iP nucleotide, such as iP riboside 5´-triphosphate or iP riboside 5´-diphosphate. In *Arabidopsis*, iP nucleotides are converted into *trans*-zeatin nucleotides that are the prominent active CK by cytochrome P450 monooxygenases that hydroxylate isopentenyl chain, called CYP735A1 and CYP735A2 (Takei *et al.* 2004). In the last step biologically inactive CK are converted into active forms. It is likely that there are at least two CK activation pathways. To become biologically active, iP- and *trans*-zeatin nucleotides are converted into nucleobase forms by dephosphorylation and deribosylation (Chen and Kristopeit 1981a, 1981b). The activation can be further mediated directly by enzyme CK nucleoside 5´-monophosphate phosphoribohydrolase, called Lonely guy, a component of the last step of CK biosynthesis. Enzyme Lonely guy catalyses a removing of phosphoribose moiety (Kurakawa *et al.* 2007).

In lower amount CK are also produced by recycled tRNA molecules. Isopentenyl chain is bound at 3´-end of anticodon of polynucleotide and subsequently is released by hydroxylation. CK produced this way are in *cis*-conformation. The prenylation of the adenines is carried out by tRNA-IPT (Procházka *et al.* 2003).

Biosynthesis of CK is regulated by auxin, inorganic nitrogen sources, such as nitrate, by 24-epibrassinolide (Yuldashev *et al.* 2012), by light, and another factors. Also Lonely guy represents a regulation step in CK concentration, because it is specially expressed in the shoot meristem tip (Kurakawa *et al.* 2007).

4.1.3. Inactivation

The major pathway of CK degradation is irreversible oxidative cleavage by CKX. Until identification of CKX double activity, this enzyme was named cytokinin oxidase. CKX is a flavin adenine dinucleotide-containing oxidoreductase and it catalyses selective cleavage

of unsaturated N^6 side chains from adenine (Armstrong 1994, Jones and Sreiber 1997). Due to CKX ability to degrade CK, its overexpression was successfully utilized for examination of changes caused by CK lack in plants.

Substrates for this enzyme are free CK, as zeatin and iP, and their N-glucosides, and conjugates with alanine. O-glucosides and CK with saturated side chain are not cleaved by CKX (Armstrong 1994).

There are several CKX proteins, which differ in cellular localization, pH optimum, electron acceptors, and substrates. The best established situation is in *Arabidopsis* which contains seven genes coding for CKX, i.e. *AtCKX1- AtCKX7*.

AtCKX1 and AtCKX7 significantly prefer CK glycosides, especially N^6-(2-isopentenyl)adenine 9-glucoside, under weakly acidic conditions (Kowalska *et al.* 2010). Isoforms AtCKX2 and AtCKX4 showed high turnover rates of isoprenoid CK and their ribosides, utilizing 2,6-dichlorophenolindophenol or 2,3-dimethoxy-5-methyl-1,4,-benzoquinone as electron acceptors *in vitro*. AtCKX1, 3, 5 and 7 were weakly active in reaction with isoprenoid CK (Galuszka *et al.* 2007). Furthermore, AtCKX1 showed higher rate of degradation of N^6-(2-isopentenyl)adenine di- and triphosphates than monophosphates (Kowalska *et al.* 2010). AtCKX6 activity was almost undetectable (Galuszka *et al.* 2007). Moreover, AtCKX3 showed preference for degradation of N^6-(2-isopentenyl)adenine 9-ribosides-5´-monophosphate (Kowalska *et al.* 2010). All CKX isoforms preferentially catalysed degradation of isoprenoid CK, but in lower reaction rates also aromatic CK were decomposed (Galuszka *et al.* 2007). CK which are not degraded by CKX are deactivated by conversion to N-7 and N-9-glucosides.

In *Arabidopsis*, CKX localization was examined. *CKX1* promoter:*GUS* fusion gene showed that the promoter activity is targeted to shoot apex, lateral root meristem, and growing parts of young flowers, in roots it was localized in vicinity of vascular cylinder at site of growing lateral roots and its expression increased with their growth. The same research also showed that AtCKX1 and AtCKX3 are localized to vacuole, AtCKX2 is an extracellular protein, AtCKX4 is targeted to endomembrane system, AtCKX5 to extracellular region (Werner *et al.* 2003).

4.1.4. Transport and signalling

CK are mobile phytohormones and act as signal molecules at long as well as short distance. Due to selective transport, CK accumulate in xylem and phloem thus they are able to be transported through plant and bring long distance signal typically from roots, especially their tips, to shoot. Extracellular CK free bases and ribosides can diffuse into cells or CK can be transferred by selective transport, e.g. by purine permeases.

Within a cell, CK are mainly synthesized *de novo* in plastids. CK glycosides accumulate in vacuole and are deglucosylated CK in plastids.

CK signal transduction is mediated by two-component system composed from histidine kinase and response regulator. Histidine kinase is usually a transmembrane receptor with signal sensing domain and signal transducing domain. Response regulator includes receiver domain. Histidine kinase perceives signal through signal sensing domain. Then conserved His residue in transmitted domain is phosphorylated, using ATP as phosphate donor. Then the phosphoryl group is transferred to conserved Asp of receiver domain of response regulator.

Three *Arabidopsis* histidine kinases (AHK2, AHK3, AHK4/CRE1/WOL) were found to act as transmembrane CK receptors. Concomitantly 22 genes were found to be coding for response regulator. There are two primary classes of *Arabidopsis* response regulators (ARR, named type-A and type-B). Type-B ARR act as transcription factors and induce expression of CK primary responsive genes, including type-A ARR, they act as negative regulator of CK signal. Into this regulation, Lonely guy is also directly involved. With decreasing Lonely guy level amount of CK increases and CK inducible ARR decreases (Kurakawa *et al.* 2007). CK response factors are a part of CK response pathway, belong to AP2 *Arabidopsis* family, are up-regulated by CK two-component signalling pathway and together with type-B ARR mediate expression of CK regulated genes (Rashotte *et al*. 2006). Twenty-three genes were identified as ARR1 direct-target genes in *Arabidopsis* (Taniguchi *et al.* 2007).

A model how CK in *Arabidopsis* control development of root and shoot meristem was created. CK are identifiable in root meristem quiescent centre, stem cell niche and transition zone. Such structures are under control of genes *Plethora*, *Scarecrow,* and *Shortroot*. These genes are regulated by CK via AHK3/ARR1 and ARR12 (Scheres 2007, Aida *et al.* 2004, Sabatini *et al.* 2003). Also gene *Retinoblastoma-related* plays a role in root development (Wildwater *et al.* 2005, Wyrzykowka *et al.* 2006). In shoot, *Shoot meristemless* gene maintains cell division, prevents cell differentiation, and activates *ATP/ADP-IPT 7,* a key CK

biosynthesis gene. Furthermore, *Wuschel* (Williams *et al.* 2005) is necessary for maintenance of stem cells, and directly represses expression of some *type-A ARR* (Shani *et al.* 2006, Leibfried *et al.* 2005).

4.1.5. Transgenic plants with modulated cytokinin level

Transgenic plants with either increased or decreased level of endogenous CK are a useful tool for studying CK role. The most often approach to increase endogenous CK is overexpression of *IPT*, producing the key enzyme in CK biosynthesis. On the contrary, depletion of endogenous CK was achieved by insertion of *CKX* gene coding for the enzyme which catalyses irreversible CK degradation.

In *Arabidopsis*, *IPT* belongs to gene family consisting of seven genes (Kakimoto 2001, Takei *et al.* 2001). Particular *IPT* differ in subcellular localization and five of them are targeted into plastids, one into cytosol, and one into mitochondria (Kasahara *et al.* 2004). Except intracellular localization, *IPT* are also diverse in expression patterns, *Arabidopsis IPT1* is mostly expressed in xylem precursor cell files of root tip, leaf axils, ovules, *IPT3* in phloem tissues, *IPT5* in root primordia, columella root caps, young inflorescences, fruit abscission zones, *IPT7* in endodermis of the root elongation zone, trichomes, young leaves, pollen, and in immature seeds are expressed *IPT1*, *IPT4*, and *IPT8* (Miyawaki *et al.* 2004).

Plants with enhanced endogenous CK level overexpressing gene coding for IPT under control of *Pisum sativum* ribulose-1,5-biphosphate carboxylase small subunit promoter (*Pssu*), tetracycline-dependent promoter, dexamethasone inducible promoter, or cauliflower mosaic virus *35S* promoter were created. For example, *N. tabacum* L. cv. Petit Havana SR1 overexpressing *IPT* gene under control of *Pssu* promoter (*Pssu:ipt*) contained higher level of zeatin, dihydrozeatin, zeatin riboside, and iP than WT (Cortleven *et al.* 2009). These transgenic plants showed higher level of FeSOD transcripts, higher protein concentration of chloroplastic FeSOD, and ferredoxin-NADP reductase than WT (Cortleven *et al.* 2011). *Pssu:ipt* plants also contained higher amount of chlorophyll, beta-carotene, lutein, and neoxantin than WT (Cortleven *et al.* 2012). In these plants higher degree of grana stacking was observed (Synková *et al.* 2006a). *Pssu:ipt* plants showed higher activity of peroxidases, SOD, CAT and glutathione-S-transferase (Synková *et al.* 2006b).

Several tobacco plants with diminished level of endogenous CK were created. These plants overexpress various *CKX* from *Arabidopsis* under various promoters. Werner *et al.* (2001) created tobacco plants overexpressing *CKX1-4* under constitutive promoter (*35S:CKX1*, *35S:CKX2*, *35S:CKX3*, *35S:CKX4*) and overexpressing *CKX1* under *WRKY6*, a senescence inducible promoter which is mainly active in roots (*W6:CKX1*).

Transgenic tobacco plants overexpressing *CKX* under *35S* promoter, have smaller apical meristem, only 5- 6 % of final leaf cell number of that in non-transgenic plants, developed stunted shoots but enlarged root system, their root system was developing more rapidly and is more branched, but development of whole plants is retarded (Werner *et al.* 2001).

Transgenic tobacco plants *35S:CKX1* showed lower CK concentration, especially of zeatin riboside and iP (Cortleven *et al.* 2009) what is in accordance with substrate preference of CKX1 (Galuszka *et al.* 2007). However, other CK metabolites were elevated (Cortleven *et al.* 2009). These plants contained decreased level of chlorophylls and carotenoids in comparison to WT (Cortleven *et al.* 2012). Tobacco *35S:CKX1* had less developed vasculature with larger spacing between veins and more flat veins (Werner *et al.* 2001). *35S:CKX1* also differed from WT in protein level of fructose bisphosphate aldolase, it was higher in *35S:CKX1* (Cortleven *et al.* 2011).

Tobacco overexpressing *CKX1* under *WRKY6* promoter did not show such different habit from WT as *35S:CKX1*. Plants *W6:CKX1* had a larger root system than WT, however, shoot part had similar habit to WT (Werner *et al.* 2010). Promoter *WRKY6* was the most active in roots and senescent leaves (Robatzek and Somssich 2001). *WRKY6* from *Arabidopsis* supresses its own promoter activity and its expression is the most dominant during leaf senescence (Robatzek and Somssich 2007).

Tobacco overexpressing *CKX2* from *Arabidopsis*, a gene which is targeted to extracellular secretion pathway (Galuszka *et al.* 2007), under *35S* promoter (*35S:CKX2*) also showed decreased level of CK (Mýtinová *et al.* 2010, Polanská *et al.* 2007) alongside it had decreased level of abscisic acid (Mýtinová *et al.* 2010). The highest CKX2 activity was observed in young leaves. There was a discrepancy between rise in CKX2 activity and decline of CK concentration, which was not as significant as CKX activity. This difference could be probably caused by the fact that only some CK were accessible to CKX (Mýtinová *et al.* 2011). These plants showed postponed senescence, delayed flower formation, larger root system, and decreased level of CK and abscisic acid (Mýtinová *et al.* 2010). Plants *35S:CKX2* showed higher level of chlorophylls in leaves of different age and showed lower ratio of

chlorophyll a/b, beta-carotene, and DEPS than WT (Mýtinová *et al.* 2011). On the other hand, Werner *et al.* (2008) found reduced content of chlorophyll in *35S:CKX2* but it could be affected by different relative units. These transgenic plants had higher activity of glutathione reductase and superoxide dismutase (SOD) in leaves, higher activity of APX in roots, and lower activity of CAT in young leaves (Mýtinová *et al.* 2011). Transgenic plants were more tolerant to drought however they were more sensitive to zinc treatment than WT. After stress treatments, APX activity increased in WT roots and in *35S:CKX2* roots decreased. CAT activity after stresses decreased in WT, but increased in transgenic tobacco. Contrariwise, SOD activity was enhanced by stress in WT and was reduced by stress in *35S:CKX2* (Mýtinová *et al.* 2010). Increased level of protective xanthophyll pigments could participate on their postponed senescence (Mýtinová *et al.* 2006).

Further, plants constitutively overexpressing *CKX3* had lower level of abscisic acid (Polanská *et al.* 2007).

Also *A. thaliana* plants overexpressing individual genes from *CKX* family were created. Genes *AtCKX1- AtCKX6* were overexpressed under promoter *35S* (Werner *et al.* 2003). These plants showed similar traits as the tobacco plants with diminished level of CK.

Despite the exogenous application of CK postpone plant senescence, onset of senescence in tobacco plants with decreased level of CK interestingly did not occur sooner than in WT (Mýtinová *et al.* 2006), similar effect was observed in leaves of *Arabidopsis* with lower level of endogenous CK (Werner *et al.* 2003). Not surprisingly, in transgenic tobacco plants overexpressing *IPT*, senescence was supressed (Rivero *et al.* 2007). Moreover, *35S:CKX1*, *35S:CKX2* and *Pssu:ipt* were more tolerant to drought (Werner *et al.* 2010, Mýtinová *et al.* 2010, Rivero *et al.* 2007).

4.2. Abiotic stress

Plants are sessile organisms which developed many ways how to cope with adverse environmental conditions. Their strategies were grouped according to main principals as escape from stress, avoidance of stress, and stress tolerance. For example, to prevent getting damaged by drought, plants shorten their life cycle or complete the most sensitive developmental stage before period when usually drought appears. This strategy is classified as escape from stress (Blanvillain *et al.* 2011). To avoid stress reaction caused by drought, plants enhance root system and/or reduce evapotranspiration. In case of another strategy to postpone stress, plants enhance osmoprotectant concentration, reduce cell size and/or improve elasticity. If these strategies fail in adverse conditions, stress reaction bursts out. However, in case when environmental conditions change gradually, plants can adjust themselves, i.e. they acclimatize.

Stress reaction of plant is a dynamic process. In the first phase, the alarm phase, acute damage appears. After first stress reaction, restitution and hardening follow. Than second phase, the phase of resistance, arrives. Depending on stress length and intensity, adjustment happens or chronic damage appears and a phase of exhaustion comes (Fig. 1).

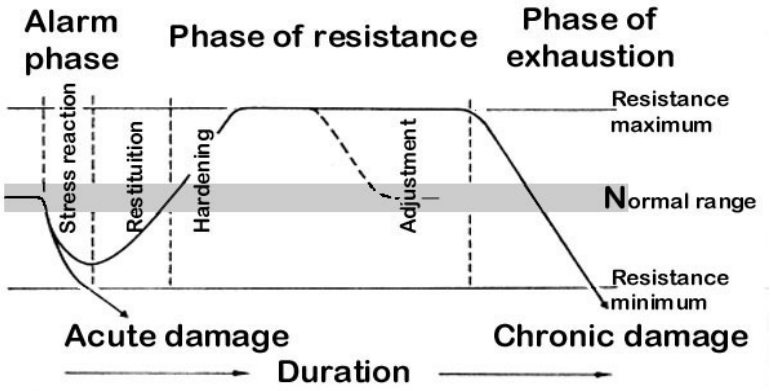

Figure 1: Stress phases according to Larcher (1987).

Except dynamics coming from stress duration, response to stress is also influenced by plant species and its developmental stage. Stress defence could be mediated differently also in different tissue types or even in the single cells.

In next section, drought and heat, the two stressors which often appear in natural conditions and cause serious agricultural losses, are characterized in detail.

4.2.1. Drought

Under drought, primary damages are caused by lack of water. Water deficit is one of the most important and influential conditions occurring in natural environment and causing the highest percentage of agricultural losses.

Within acclimatization to drought, plants enhance root system, reduce leaf area, develop thicker cuticula impregnated by wax, build rigid cell wall, synthesise osmoprotectants, and create smaller cells (Wilson *et al.* 1980). Also cell membrane stability is important in drought tolerance, partitioning of stem and reserve mobilisation. Thus, drought tolerance is a complex quantitative trait.

During drought, water loss is minimized by closing stomata, rolling leaves, and maximizing of water uptake. To reduce osmotic stress, plants accumulate osmoprotectants which maintain cell turgor and protect membranes and proteins from damage (Madden *et al.* 1985, Kaplan and Guy 2004).

Energy balance changes under drought. Starch is under optimal conditions stored as osmotically inert granules, which serve as main carbohydrate source and is mobilized to provide soluble sugars during stress (Todaka *et al.* 2000, Kaplan and Guy 2004, Basu *et al.* 2007, Kempa *et al.* 2008). Furthermore, photosynthesis is inhibited. Under mild drought stomata have been closed before decreasing of photosynthesis rate, thus decline in photosynthesis is probably caused by restriction of CO_2 diffusion into leaves (Chaves 1991, Cornic 2000), not by photosystem damage. In addition, elevated concentration of soluble hexokinases suppresses expression of photosynthesis-associated genes (Smeekens 1998), as proved in plants overexpressing hexokinases (Quirino *et al.* 2000). During drought, old leaves shed, unlike younger leaves where often its photosynthesis rate has been even increased (Ludlow and Ng 1974).

4.2.2. Heat

Temperature optimum differs among plant species, generally transient elevation of temperature about 10-15 °C above optimum is considered as heat stress (Wahid *et al.* 2007).

Heat acclimatization involves changes in leaf orientation, cell size reduction, increased densities of stomata and trichomes, and greater xylem vessels. On subcellular level membrane lipid composition changes, grana in chloroplasts are swelling and/or they are less stacked, content of vacuoles forms clumps (Zhang *et al.* 2005).

Under heat, ascorbate content decreases, compatible solutes, especially glycinebetaine, heat shock proteins, carotenes, lutein, total chlorophyll, and alfa-tocopherols are accumulated (Pinto-Marijuan *et al.* 2013). Photosynthetic tissues are protected by xanthophylls (Demmig-Adams & Adams 1996a, b) and lutein epoxides cycles (García-Plazaola *et al.* 2007) which limit absorption of light energy by chlorophylls by increasing the rate of thermal energy dissipation by carotenoids (Marijuan *et al.* 2012).

Serious heat stress disrupts mitochondrial membranes and mitochondria content leaks (Zhang *et al.* 2005). Photosynthesis is inhibited by heat, thus CO_2 assimilation rate decreases, and contrariwise transpiration increases (Marijuan *et al.* 2012). Oxygen evolving complex, cofactors in photosystem II, ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), and ATP generating system are the most sensitive constituents of photosynthetic apparatus to heat stress. In fact, oxygen evolving complex loses two of four Mn from Mn-containing cluster (Enami *et al.* 1998). Defect of photosystems is caused by their inhibited repair caused by elevated temperatures (Nishiyama *et al.* 2006, Murata *et al.* 2007). Rubisco is heat resistant, however loss in its activity is caused by heat sensitivity of Rubisco activase (Sharkey 2005). In longer heat duration, D1 protein is damaged by ROS, and *de novo* synthesis of D1 protein is inhibited and proteins of reaction centres are cleaved off. Membrane fluidity is changed and thylakoid membranes lose their integrity.

In sensing of heat, probably changes in membrane fluidity are the most important signal. Heat stress signalization involves mitogen activated kinases cascades which are connected to heat shock proteins, Ca^{2+}, H_2O_2, salicylic acid, and abscisic acid concentration (Wahid *et al.* 2007). Also, dehydration responsive element binding protein 2A was identified to be connected to heat defence (Sakuma *et al.* 2006b).

4.2.3. Combined stress

Plants have often to sustain unfavourable conditions in natural environment where more stress factors act simultaneously. Because drought and heat often occur together and both negatively affect plant growth and yield, it is important to study this combination of stresses (Mittler 2006). Besides the response to combined stresses is not just a sum or average of partial reactions for each stress thus it is necessary to study stress combinations, not only separate stressors.

A few obvious examples demonstrate that reaction to combined stress has to be different from reaction to separate drought or heat. During drought, plants close stomata to save water and avoid or postpone water deficit. Contrariwise during heat, plants open stomata to evaporate water to cool down the organism. When both stresses act simultaneously, closed stomata were observed (Rizhsky *et al*. 2004). Moreover, in tobacco plants during drought the respiration decreased. Contrariwise during heat, it increased. Under combination of these stresses respiration was higher than during heat alone (Rizhsky *at al.* 2002, Rhizsky *et al.* 2004). Further, cell proline concentration differs under drought and heat. Proline is known to accumulate during drought and to improve drought tolerance (Dobrá *et al.* 2010), however proline synthesis is not enhanced after heat shock and also not after combination of drought and heat stress (Rizhsky *et al.* 2004). Raised level of proline makes heat tolerance even worse (Rizhsky *et al.* 2004, Dobra *et al.* 2010, Lv *et al.* 2011). Besides, from total amount of 1833 genes which transcripts change during drought, heat or their combination, expression of only 77 the same genes changes under all three kinds of particular conditions (Rizhsky *et al.* 2004).

Combination of drought and heat caused changes in plant metabolism. *Arabidopsis* plants exposed to combination of drought and heat accumulated sucrose, maltose, glucose, melibiose, and mannitol (Rizhsky *et al*. 2004). In tobacco, expression of genes connected to photosynthesis and pentose phosphate pathway changed. Expression of photosynthesis-associated genes was suppressed, in contrast to transcripts coding for proteins from pentose phosphate pathway and glycolysis that were more abundant than under control conditions. This points at modified flow of sugars through these pathways under stress combination (Rizhsky *et al*. 2002b).

4.2.4. Oxidative stress

Oxidative stress appears as secondary stress under adverse conditions when balance between prooxidant and antioxidant reactions is shifted in favour of the former. All kinds of stress conditions, including drought, heat or ageing, cause accumulation of reactive oxygen species (ROS) which disrupts cellular redox homeostasis. ROS are produced constitutively in organisms, however under adverse conditions, production of ROS can usually increase. Equilibrium of ROS concentration is maintained by antioxidant system.

4.3. Reactive oxygen species

Relatively stable oxygen is potentially able to create its active forms, the ROS, which are in higher amount toxic for cells. Despite this risk, plants use aerobic metabolism because it is more efficient than anaerobic, as yield from one glucose molecule is 38 ATP by aerobic metabolism or 8 ATP by anaerobic. Presence of ROS in cells still appears inconsistent.

On the one hand these molecules have capability to damage cell compounds. Due to their high reactivity, ROS are able to disrupt cell homeostasis, they oxidize proteins, lipids, carbohydrates and nucleic acids, ROS can disrupt all cell compartments. If ROS induce plasma membrane lipid peroxidation, cell content including H_2O_2, leaks out and cell death occurs. Damage to intracellular membranes leads to disruption of electron transport in mitochondria and/or chloroplasts and induces pigment degradation, thus chloroplasts lose their ability to assimilate carbon (Scandalios *et al.* 1993). On the other hand ROS are involved in signalling, development (Gapper and Dolan 2006), organogenesis (Shin 2005), program cell death, cell wall formation (Fry *et al.* 2001), cell wall lignification, and stress responses.

4.3.1. ROS types, production and localization

Free radicals as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), perhydroxy radical ($HO_2^{\bullet}$) and alkoxy radicals, and non-radical molecules as hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are overall called ROS. The major site for the production of $^1O_2$, H_2O_2 and O_2^- are photosynthetic and respiration electron transport chain, reaction centres of

photosystem I and II, excited chlorophyll, NADPH oxidase, fatty acid β-oxidation, glycolate oxidase, oxalate oxidase, xanthine oxidase, peroxidases, amine oxidase (Mittler *et al.* 2002).

More precisely, in chloroplasts, photosystem I and II are the main source of singlet oxygen and $O_2^{\bullet-}$. When energy is utilized insufficiently during photosynthesis, the singlet oxygen is generated from excited chlorophyll triplet state which reacts with triplet oxygen (Gill and Tuteja 2010). From total electron flow through photosystem I, up to 10% of electrons reduce O_2 to $O_2^{\bullet-}$ (Foyer and Noctor 2003). In mitochondria, complex I, ubiquinone and complex III of electron transport chain generate $O_2^{\bullet-}$. H_2O_2 is also produced during respiration in electron transport chain in mitochondria. Roughly 2 % of oxygen utilized in mitochondria is converted to H_2O_2 (Chance *et al.* 1973).

Hydroxyl radical can be produced in presence of reduced metals (Fe^{2+} or Cu^{+}) in so called

Fenton reaction:

$$M^{(n)+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\bullet}OH + OH^- \quad \text{(where M is Fe or Cu)}$$

Oxidized metal is re-reduced by superoxide:

$$M^{(n+1)+} + O_2^{\bullet-} \rightarrow M^{(n)+} + O_2$$

Taking together both equations, the net reaction represents Haber Weiss reaction:

$$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + OH^- + {}^{\bullet}OH$$

(Wilhelm *et al.* 2011).

4.3.2. Role of ROS in signalling

Although ROS are potentially dangerous, they play important role in signalling. The most important signalling molecule among ROS is H_2O_2 for which membranes are permeable and it can diffuse within or outside a cell (Feierabend 2005). There are further special membrane pores, called aquaporins that can transport it (Gerd *et al.* 2007). In comparison with other ROS, H_2O_2 is not highly reactive. Despite H_2O_2 relative stability, some metalloproteins, e.g. CuZnSOD (Beyer *et al.* 1987), and some enzymes of Calvin cycle, e.g. fructose bisphosphatase, sedoheptulose bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase, are sensitive to it (Scandalios *et al.* 1997).

Production of H_2O_2 in a cell can reach 4000 $nmol.m^{-2}.s^{-1}$ H_2O_2 in chloroplast (Foyer and Noctor 2003) and already $6x10^{-4}$ M H_2O_2 can inhibit 90% of CO_2 assimilation in isolated chloroplasts (Kaiser *et al.* 1979).

In contrast to H_2O_2, superoxide cannot move across membrane and it stays in place of its origin. The life time of $^1O_2$ in a cell has been measured to be approximately 3 ms (Gill and Tuteja 2010). Despite its quick turnover, also singlet oxygen was found to play role in signalling (Kim *et al.* 2008, 2009).

Several components involved in ROS signal transduction pathway, e.g. mitogen-activated protein kinase kinase kinases AtANP1 and NtNPK1, mitogen-activated protein kinases AtMPK3/6 and Ntp-46MAPK (Kovtun *et al.* 2000, Samuel *et al.* 2000), calmodulin (Harding *et al.* 1997, Desikan *et al.* 2001), and protein tyrosine phosphatases were identified. Singlet-oxygen-mediated signalling also includes plastid nucleus-encoded protein Executer1 and -2 (Kim *et al*. 2008).

4.4. Antioxidant enzymatic system

Scavenging of ROS is essential to keep ROS at harmless levels. Plants evolved antioxidant system which consists of nonenzymatic and enzymatic components. Nonenzymatic part is composed of low weight molecules, e.g. reduced glutathione, cysteine, mannitol, ascorbate, tocopherol, flavonoids, some alkaloids, carotenoids, polyamines. Enzymatic part consists of enzymes of at least five different gene families i.e. ascorbate peroxidases (APX), catalases (CAT), glutathione peroxidases, peroxiredoxins, and peroxidases, catalysing H_2O_2 decomposition, and superoxide dismutases (SOD) which catalyse dismutation of superoxide into oxygen and H_2O_2. Scavenging of H_2O_2 and $O_2^{\bullet-}$ prevents OH^- production in Haber-Weis and Fenton reaction (Mittler 2005).

In all plants, antioxidant enzymes occur in several isoforms, generally these isoforms are quite conserved among plant species (Teixeira *et al.* 2005). Individual isoforms differ in subcellular localization (Fig. 2) and in promoter sequences, which means in function. Some isoforms originate from alternative splicing or polymorphism (Nadif *et al.* 2005, Mak *et al.* 2007), or from formation of heteromeric instead of homomeric molecules (Zimmermann *et al.* 2006, Lleídas *et al.* 1998, Myouga *et al.* 2008).

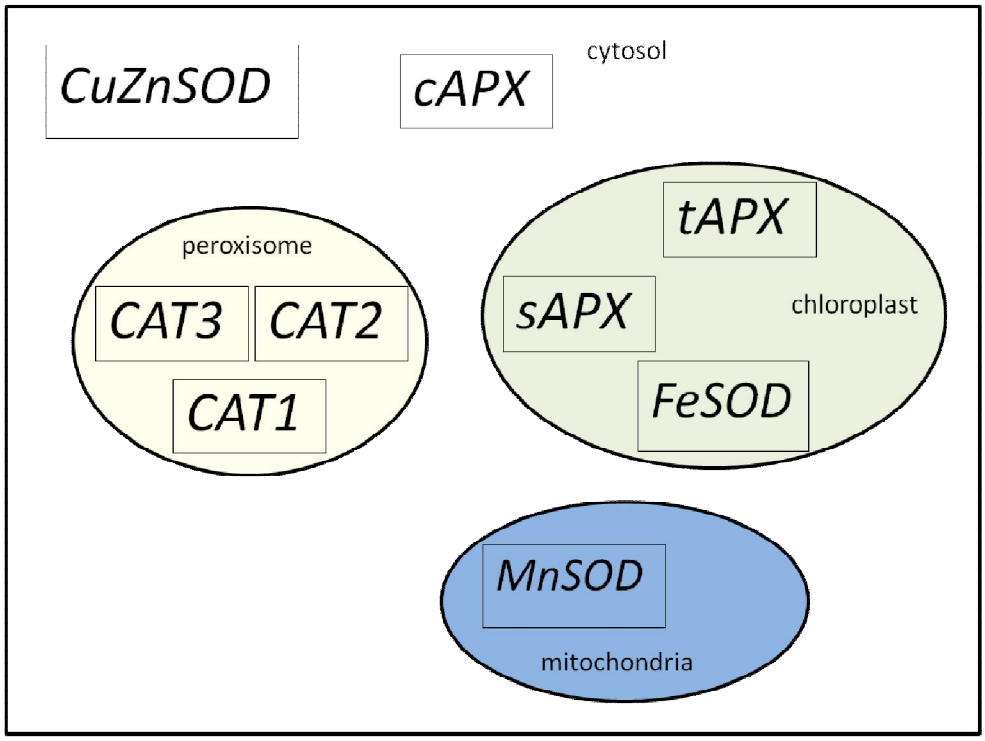

Figure 2: Cell compartments localization of antioxidant enzymes studied within this work.

4.4.1. Ascorbate peroxidase

In contrast to other enzymes scavenging H_2O_2, APX is unique for algae and plants (Asada and Takahashi 1987). Enzyme APX is a part of ascorbate-glutathione cycle which is comprised of another three enzymes, monodehydroascorbate reductase, glutathione-dependent dehydroascorbate reductase and glutathione reductase. This system acts in chloroplasts (Chew *et al*. 2003), however its enzymes are also present in another cell compartments, as mitochondria, peroxisome, cytosol and apoplast (Asada and Takahashi 1987).

Enzyme APX catalyses reaction

$$2 \text{ ascorbate} + H_2O_2 \rightarrow 2 \text{ monodehydroascorbate} + 2\ H_2O.$$

As reducing substrate, APX prefers ascorbic acid which is present in almost all cell compartments. Function of APX is dependent on reduced ascorbic acid (Asada and Takahashi

1987) or in some cases on reduced glutathione depending on monodehydroascorbate reductase activity and dehydroascorbate concentration (Polle *et al*. 2001). APX have high affinity to H_2O_2 as a substrate (micromolar as well as submicromolar concentration) unlike CAT (milimolar concentration) (Mittler *et al*. 2005).

Enzyme APX is coded by multigene family. The best defined situation is in *Arabidopsis*, successively from seven to nine APX isoforms were identified (Zilinskas and Mittler 1993, Jespersen *et al.* 1997, Chew *et al.* 2003). *Arabidopsis* genome contains three genes coding for cytosolic APX (Shigeoka *et al.* 2002), two chloroplastic APX, three microsomal APX isoforms (Panchuk *et al.* 2005), and one mitochondrial (Mittler *et al.* 2005). As mentioned above, *Arabidopsis* chloroplastic APX are coded by two different genes (Shigeoka *et al.* 2002). In addition, *Arabidopsis* stromatal APX (sAPX) has two targets of action, i.e. chloroplast stroma and intermembrane mitochondrial space (Chew *et al.* 2003).

In contrast to *Arabidopsis*, other plant species vary in number of genes coding for chloroplastic APX. For example in pumpkin, spinach, and tobacco, both chloroplastic APX are coded by one gene. In these species, stromatal and thylakoidal APX (tAPX) arise from alternative splicing (Mittler *et al.* 2005).

Isoforms of APX differ in localization as well as in action. In *Arabidopsis*, cytosolic *APX1* and microsomal *APX3* were the most abundant transcripts, contrariwise cytosolic *APX2* and microsomal *APX5* expression was low (Panchuk *et al*. 2005). *Arabidopsis* chloroplastic APX prevent photosynthetic apparatus from oxidation, whereas cytosolic APX fulfil general stress defence under stress caused by ozone, light, heat, heavy metals (Panchuk *et al*. 2005). Furthermore, leaf and plant age influenced APX expression. *Arabidopsis tAPX* expression was strongly age-dependent and decreased from young to old leaves as well as it decreased with age of a plant. While, *sAPX* expression was reduced in old leaves of old plants, on the contrary five *APX* genes were not age-dependent (Panchuk *et al*. 2005).

Plants overexpressing antioxidant enzymes were used to study role of antioxidant defence. Transgenic plants overexpressing FeSOD showed increased cytosolic APX activity (Van Camp *et al.* 1996a). Overexpression of CuZnSOD increased transcripts and activity of cytosolic APX in tobacco. The same reaction was induced by H_2O_2 application on leaf discs (Sen *et al.* 1993).

4.4.2. Catalase

Enzymes CAT are present in all aerobic organisms. CAT belong to three phylogenetically unrelated CAT groups: 1. typical monofunctional CAT, 2. bifunctional CAT-peroxidases, and 3. non-heme Mn-containing CAT. Monofunctional CAT are the most abundant in organisms, likewise in plants. CAT-peroxidases are found in bacteria and some fungi. Finally, non-heme CAT occur in *Lactobacillus plantarum* and two thermophilic bacteria. Monofunctional CAT have been further divided according to phylogenetic relationships into clades I-III when plant CAT belong to clade I which includes small CAT subunits (55-60 kDa) (Smirnoff 2008).

Plant CAT subunits are coded by small gene family. Willekens *et al.* (1995) suggested CAT classification on the basis of their expression profiles. Class I CAT are expressed in photosynthetically active tissues, they are connected to photorespiration and are positively regulated by light. Class II CAT are expressed in seeds and young seedlings. They scavenge H_2O_2 produced during fatty acid degradation in glyoxysomes. Class III CAT are expressed in vascular tissues and in maize mitochondria (Scandalios *et al.* 1997). Awkwardly, the names of CAT genes differ among plant species and researchers (Tab. 1).

Table 1: Matching of CAT genes naming in different plants. Terminology within this whole text is unified according to Willekens (*et al.* 1994), i.e. for example *CAT2* from *Arabidopsis* is here presented as *CAT1*.

Class	**tobacco**	***Arabidopsis***	**maize**
Class I	*CAT1*	*CAT2*	*CAT2*
Class II	*CAT2*	*CAT3*	*CAT3*
Class III	*CAT3*	*CAT1*	*CAT1*
author	Willekens *et al.* 1994	Du *et al.*2008	Scandalios *et al.* 1997

Within a cell, CAT are located in peroxisomes and glyoxysomes where they scavenge H_2O_2 formed in oxidation reactions by oxidases (Scandalios *et al.* 1997). Despite high H_2O_2 production in chloroplast, chloroplasts contain no CAT, and H_2O_2 is scavenged by ascorbate-glutathione cycle. Hydrogen peroxide is also present in cytosol and endoplasmic reticulum (Chance *et al.* 1979), thus it is presumed that CAT is also present in cytosol.

CAT provide rapid H_2O_2 scavenging. Rate of its reaction is 10^7 min^{-1}. CAT catalyse two different reactions depending on H_2O_2 concentration. Overall equation catalysed by CAT is

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

This reaction runs in two steps. In the first step, CAT interact with H_2O_2.

$$\text{CAT-Fe(III)} + H_2O_2 \rightarrow \text{CAT Fe(IV)=O} + H_2O$$
$$\text{CAT Fe(IV)=O} + H_2O_2 \rightarrow H_2O + \text{CAT-Fe(III)} + O_2$$

H_2O_2 in active site reacts not only with Fe but interacts also with amino acids Asn 147 and His74. A proton transfer from one oxygen atom of H_2O_2 to the other is mediated via His 74 causing elongation and breaking of O-O bond. The result is CAT Fe(IV)=O, water, and heme radical. In the second step this form of CAT interacts with second H_2O_2 producing water, O2 and CAT-Fe(III)

Second step is dependent on substrate concentration. The unique characteristic of CAT is no need of reductant under high H_2O_2 concentrations (Asada and Takahashi 1987), where CAT decompose substrate in catalatic reaction. Thus in the second step, under high H_2O_2 concentration, product of first step, reacts with second H_2O_2 molecule. Water and oxygen are evolved.

Under low H_2O_2 concentration ($<10^{-6}$ M) CAT has peroxidation effect and hydrogen donor is e.g. ethanol or ascorbic acid. In the second step, under low H_2O_2 concentration, product from first step is reduced by hydrogen donors. Overall reaction could be expressed as

$$H_2O_2 + H_2R \rightarrow 2H_2O + R$$

The exact mechanism is not recognized yet.

As apparent from CAT classification, *CAT1* from *N. plumbaginifolia* is expressed mainly in leaves. Alongside, *CAT1* is also expressed in stamen, sepals and senescing petals. *CAT2* transcripts are the most abundant in stem and also in whole flower. Finally, *CAT3* is expressed in flower and in small amount in other parts of plant (Willekens *et al.* 1994).

Expression of CAT is also influenced by circadian rhythm, when CAT1 and CAT2 transcripts change during day cycle. CAT1 expression is high in the morning and CAT2 expression is high in the evening, as proved for tobacco (Willekens *et al.* 1994), *Arabidopsis* (Du *et al.* 2008), and wheat (Luna *et al.* 2004).

CAT proteins consist of four subunits which together contain four Fe protoporfyrin IX prosthetic groups (Smirnoff 2008). CAT enzymes are homo- as well as heterotetramers, thus they appear in many isoforms (Lledias *et al.* 1998). Due to such variability, number of CAT isoforms on level of active protein is hard to detect. In *N. plumbaginifolia* four CAT were identified which are present in all plant organs (Willekens *et al.* 1994). In *N.tabacum* var. Havana six bands of active CAT protein were proved (Zeltich *et al.* 1991), in *N. tabacum* cv. Xanthi at least 12 CAT isoforms were detected on native IEF gel (Siminis *et al.* 1994).

4.4.3. Superoxide dismutase

Several isoforms of SOD according to metal cofactor present in active centre were identified. Plants contain three different cofactors in SOD, Fe (FeSOD), Mn (MnSOD) a Cu with Zn (Cu/ZnSOD) (Bannister *et al.* 1987).

Tobacco plants contain SOD with all three cofactors. In *Nicotiana* (*N.*) *plumbaginifolia* one MnSOD, one FeSOD, and three CuZnSOD were identified (Ragusa *et al.* 2001). In the same plant, other authors isolated two MnSOD isoforms (Van Camp *et al.* 1996) and found three FeSOD (Van Camp *et al.* 1994). In *N. sylvestris* only three active SOD isoforms were characterized (Priault *et al.* 2007). In *N. tabacum* five SOD isoforms were found, one MnSOD, one FeSOD, and three CuZnSOD (Sheng *et al.* 2004). Differences between observations could be explained by plant variety, age of analysed material, or tissue type (Van Camp *et al.* 1996 and 1997), or growing conditions. In addition, presence of SOD was also proved in peroxisomes and extracellular space in other plants (Bowler *et al.* 1994). In *Arabidopsis* seven SOD isoforms were identified. According to phylogenetic tree, FeSOD is the oldest isoform and CuZnSOD was evolved independently on FeSOD and MnSOD (Panchuk *et al.* 2002).

Enzymes SOD are present in all cell compartments and catalyse conversion of superoxide radical to H_2O_2.

$$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

MnSOD and FeSOD have similar primary, secondary and tertiary structure. MnSOD probably developed from FeSOD when Fe^{2+} ions were less abundant in environment, thus organisms utilized more available Mn^{3+} ions (Fridovich *et al*. 1986).

Enzyme MnSOD has homodimer or homotetramer structure with one Mn atom. MnSOD is inhibited by neither by KCN nor by H_2O_2 (Bowler *et al.* 1994) in contrast to other isoenzymes.

MnSOD is present in both prokaryotes and eukaryotes. Plant and bacterial MnSOD have 65 % similarity. Human genome codes for a single MnSOD gene, however human body contains more kinds of *MnSOD* transcripts arising by alternative polyadenylation or alternative splicing (Alscher *et al.* 2002), this differs from plants.

Within plant cell, MnSOD is present in mitochondria and peroxisomes. Previously, its nonspecific activity was also observed in thylakoid membrane of green algae, cyanobacteria and spinach (Okada *et al.* 1979, Hayakawa *et al.* 1985, Palma *et al.* 1986). Within mitochondria, MnSOD is present in matrix (Bowler *et al.* 1989) where it scavenges superoxide produced during respiration.

According to some authors, in tobacco one MnSOD is present (Ragusa *et al.* 2001, Sheng *et al.* 2004), however two MnSOD isoforms were isolated from *N. plumbaginifolia* (Van Camp *at al.* 1996a). These MnSOD have 88 % identity of amino acid sequence and they differ in promoter region (Van Camp *et al.* 1996b). One promoter was highly active in pollen, anthers and slightly active in vegetative parts. Within leaf this activity was strongest in phloem and stomata. Activity of the second promoter was observed in leaves, stems, roots, flowers and pollen. Promoter activities correlated with MnSOD protein localization (Van Camp *et al.* 1997).

MnSOD activity differed not only among tissues, also leaf age influenced presence of active enzyme. Tobacco upper leaves were mostly protected by MnSOD (Priault *et al.* 2007). Reduced MnSOD1 activity in lower leaves is presumably connected to decreased respiration (Van Camp *et al.* 1996b). Despite MnSOD is dominant SOD in mitochondria, tobacco plants with knockout MnSOD did not suffer by its absence (Van Camp *et al.* 1997).

Despite FeSOD probably was the first evolved SOD isoform (Bannister *et al.* 1991), animals and yeast do not contain it. Similarity of plant with cyanobacteria sequence and FeSOD absence in non-photosynthetic bacteria (Bowler *et al.* 1994) points at plastidic origin

of plant FeSOD and in the course of time its gene was transferred into nuclear genome (Van Camp *et al.* 1990).

Organisms contain two different FeSOD groups. First group are homodimeric FeSOD consisting of two 20 kDa protein subunits with one or two Fe atoms in the reaction centre. These FeSOD are in e.g. *Escherichia coli, Gingko biloba, Brassica campestris*. FeSOD in the second group are tetramers with molar weight 80- 90 kDa containing 2- 4 Fe atoms in active centre (Alscher *et al.* 2002). The latter FeSOD are in higher plants.

FeSOD are present in chloroplast and in cytosol. Tobacco chloroplastic stromatal FeSOD (Van Camp *et al.* 1990) scavenge superoxide produced in photosystem I (Asada 1999) and was reported as FeSOD protecting plasma membrane and photosystems (Van Camp *et al.* 1996a).

In *Arabidopsis*, three FeSOD isoforms were identified (Kliebenstein *et al.* 1998), also *N. plumbaginifolia* contains three FeSOD isoforms (Van Camp *et al.* 1994). FeSOD active enzymes are abundant in tobacco leaves and its activity is relatively stable among younger and older leaves (Slooten *et al.* 1995). However in *N. sylvestris* its activity was increasing from upper to lower leaves (Priault *et al.* 2007). In chloroplasts of majority of plants, FeSOD activity is dominant. In some plants, e.g. *Arabidopsis*, FeSOD is even the only isoform which is able alone to scavenge all superoxide formed during photosynthesis (Van Camp *et al.* 1997). However in some plants, e.g. in maize or rice, FeSOD were under level of detection, role of FeSOD is covered by CuZnSOD in these plants (Bowler *et al.* 1994).

Once when oxygen concentration in atmosphere increased, thus Fe^{2+} was inaccessible but insoluble Cu^{1+} was oxidised to soluble Cu^{2+} and started to be used as metal cofactor in SOD active centre. Isoforms CuZnSOD are primarily present in eukaryotes but also in some bacteria (Alscher *et al.* 2002).

According to a structure, two groups of CuZnSOD are distinguished. One group are cytoplasmic and periplasmic isoforms with homodimer structure. Second group are chloroplastic and extracellular isoforms with homotetramer structure (Bordo *et al.* 1994). All subunits act independently and functional interaction among them is not obligatory for the enzyme activity (Fridovich *et al.* 1986).

In plants several CuZnSOD isoforms are known, i.e. chloroplastic, cytoplasmic, peroxisomal and CuZnSOD bound to cell wall. Chloroplastic CuZnSOD is a soluble enzyme present is stroma which is localized on stromatal side of thylakoid membrane where photosystem I is exposed (Ogawa *et al.* 1995). Assumed role of CuZnSOD in apoplast is

connected to lignification and nuclear CuZnSOD accounts for a defence against mutations caused by O^{2-} (Ogawa *et al.* 1996).

Proportional content of CuZnSOD isoforms differs among plants. Spinach contains two cytoplasmic (Kanematsu *et al.* 1990) and one chloroplastic CuZnSOD isoform (Ogawa *et al.* 1996). Another study showed that 40 % of spinach CuZnSOD are present in apoplast and 25 % in nucleus (del Río *et al.* 1987). In water melon, peroxisomal CuZnSOD showed 18 % of total cellular CuZnSOD activity (del Río *et al.* 1987). In majority of plants the most dominant SOD isoform is FeSOD, contrariwise in pea CuZnSOD is more active than FeSOD (Barón *et al.* 1988).

N. tabacum and *N. plumbaginifolia* contain three CuZnSOD isoforms (Van Camp *et al.* 1994, Ragusa *et al.* 2001) and CuZnSOD in *N. plumbaginifolia* are encoded by a single gene (Hérouart *et al.* 1994). Tobacco CuZnSOD are present in cytosol (Tsang *et al.* 1991), chloroplast or mitochondria (Van Camp *et al.* 1994). Tobacco chloroplastic CuZnSOD is probably not connected to photosynthetic activity. CuZnSOD in tobacco were detectable only in immature leaves (Slooten *et al.* 1995, Van Camp *et al.* 1996, 1997, Kurepa *et al.* 1997), as well as in another plants, i.e. in tomato (Kardish *et al.* 1994). However in younger tobacco leaves CuZnSOD activity was higher than FeSOD (Slooten *et al.* 1995).

4.5. Cytokinins under stress conditions

Hormones CK play important role under stress, their concentration change under stress conditions and influences plant response to stress.

Further we focus our interest on CK role in response to drought, heat and combination of those stresses which often appear in natural environment (Mittler *et al.* 2006).

4.5.1. Cytokinin level under drought

Generally, from point of view of total CK content on level of whole plant, CK tend to decrease under drought. However, a closer look shows that situation is more complicated. Trends in CK regulation differ according to drought intensity, among plant species, in plants

of different age, as well as within plant parts on level of tissue and a cell kind. Ratio of CK types and forms is not less important. Thus examples of research on detailed CK levels during stress will be precisely specified.

Xylem sap from 12 days drought treated maize showed decrease in zeatin and zeatin riboside concentration (Alvarez *et al.* 2008). Pospíšilová (*et al.* 2005) measured changes in biologically active CK (*trans*-zeatin, dihydrozeatin, *trans*-zeatin-9-riboside, dihydrozeatin-9-riboside, isopentenyladenine, isopentenyladenine-9-riboside), *cis*-derivatives of zeatin (*cis*-zeatin, *cis*-zeatin-9-riboside), storage CK (*trans*-zeatin-O-glucoside, *trans*-zeatin-9-riboside-O-glucoside, dihydrozeatin-9-riboside-O-glucoside), inactive CK (*trans*-zeatin-7-glucoside, dihydrozeatin-7-glucoside, *trans*-zeatin-9-glucoside, dihydrozeatin-9-glucoside, isopentenyladenine-7-glucoside, isopentenyladenine-9-glucoside), and CK phosphates in maize, sugar beet, French bean and tobacco after water withdrawal causing mild or severe drought stress. In maize leaves under severe stress, concentration of all CK kinds increased (Pospíšilová *et al.* 2005). Sugar beet leaves, in the same study, contained increased level of *cis*-derivatives of Z, inactive CK, and CK phosphates after severe drought. In leaves of French bean under mild stress, CK phosphates decreased, while the other CK kinds stayed unchanged, under severe drought, all measured CK types increased (Pospíšilová *et al.* 2005).

In rice having intact roots dried in air for 24 hours, CK zeatin and zeatin riboside, isopentenyladenine and isopentenyladenosine in xylem sap decreased (Bano *et al.* 1993). In xylem sap of *Helianthus annuus* L. under mild drought, when soil water potential was -0.3 MPA, *trans*-zeatin riboside concentration did not change, while it decreased after severe stress, when soil water potential reached -1.2 MPa (Shashidhar *et al.* 1996). Zeatin-type CK were studied in tomato leaves and xylem sap after 5-day drought. Concentration of zeatin-type CK in bulk leaves decreased, contrarily their concentration in xylem sap increased (Kudoyarova *et al.* 2007).

Tobacco plants contained high level of inactive CK in comparison to other tested plants under optimal as well as under drought. In tobacco cultivated in perlite, amount of storage CK and CK phosphates increased after mild and severe drought (Pospíšilová *et al.* 2005). When upper, middle and lower leaves and roots of 42 days old *N. tabacum* plants cultivated in soil were analysed separately, content of CK among those parts differed after drought treatment. Within the same study, three different drought intensities were applied, mild 1-day drought, 6-day drought considered as moderate drought and severe drought lasting for 11 days. Mild drought did not seriously influence CK content. After moderate stress, active CK concentration decreased in all leaves and increased in roots, thus also total CK

content changed. Under severe drought, total CK increase in upper leaves and roots, whereas it decreased in middle and older leaves. Concentration of active CK decreased in all leaves and increased in roots after severe drought (Havlová *et al.* 2008). In another studies, the same tobacco plants were treated with 10-day drought. Drop of all active CK was observed (Macková *at al.* 2013, Dobrá *et al.* 2010).

The 40-days tobacco plants were subjected to 2-day up to 15-days water cessation. Content of CK-nucleobases, CK–nucleosides and nucleotides, CK–N-glucosides, and CK–O-glucosides was measured in apical, middle and lower leaves separately. Content of CK-nucleobases decreased on second day of drought and stayed on the same level until last day, CK-nucleobases were not influence by drought in middle and lower leaves. Content of CK-nucleosides and –nucleotides were not seriously influenced. Concentration of CK-N-glucosides rose in all leaves from beginning of drought treatment. Level of CK-O-glucosides gradually increased with enhancing stress intensity in all leaves (Rivero *et al.* 2007).

CK showed high dynamics in response to drought in dependence on stress intensity and leaf age, what is particularly evident from work of Havlová (*et al.* 2008) and Rivero (*et al.* 2007), however it is also apparent from comparison of those experiments that, apart from the tested treatments and tissues, also other factors strongly influence CK concentration, because several results differed or were even opposite.

4.5.2. Cytokinin level under heat

Heat is less studied stress than drought, however role of CK under this stress was already partly investigated.

In bean plant, high temperature reduced content of zeatin riboside and isopentenyl adenosine in roots but did not influence them in leaves (Udompraset *et al.* 1995). Reduction of CK was also observed in bentgrass leaves in high temperature (Wang *et al.* 2004). Also in wheat kernels CK decreased after heat (Banowetz *et al.* 1999a, b). In young maize leaves heat caused a decrease in CK level as well (Caers *et al.* 1984). Furthermore, 3 days after pollination maize kernels were subjected to 4-day or 8-day 35 °C treatment. CK analysis revealed that zeatin disappeared after both tested heat treatments and zeatin riboside content decreased and peaked later after shorter heat, after longer heat kernels contained no zeatin riboside (Cheikh and Jones 1994).

In tobacco plants concentration of zeatin decreased after 2 hours of heat at 45 ^{o}C (Medford *et al.* 1989). The latest study of CK also proved a decrease in level of active CK in tobacco treated with heat shock, 40 °C for 2 hours (Macková *et al.* 2013). Contrarily to active CK, *cis*-zeatin metabolites were slightly increased after heat shock (Macková *et al.* 2013). Smart and colleagues (1991) concluded that heat increased CK concentration in tobacco, however in this study the CK elevation could be considered as recovery reaction because measurement was done up to 4 hours after heat treatment.

4.5.3. Cytokinins and stress combination

Issue of stress combination is generally less studied than impact of drought or heat imposed separately, thus studies about role of CK under combined stress are not numerous.

The total CK content was measured in upper and lower leaves and roots of 42 days old *N. tabacum* plants under stress combination being comprised from 10–day water cessation followed by 2 hours of 40 °C heat shock. Under a single stress, drought or heat, decrease of CK content was observed. After stresses combination, content of active CK further decreased in all leaves and roots (Dobrá *et al.* 2010, Macková *et al.* 2013).

4.5.4. Exogenous cytokinins and stress

Application of CK represents one of principal ways how to investigate CK role in plant stress defence. Exogenous CK positively influenced photosynthesis, initiated stomatal apertures opening and transpiration.

Application of 6-benzylaminopurine and N-2-chloro-4-pyridyl-N´-phenylurea on *Phaseolus vulgaris* treated with drought improved recovery of photochemical activity in those plants (Metwally *et al.* 1997). Application of 6-benzylaminopurine on drought treated bean plants also delayed leaf senescence, improved photosynthetic rate, increased chlorophyll content and stomatal conductance (Rulcová *et al.* 2001).

Positive as well as negative impact of exogenous CK was observed in plants under heat stress. Interestingly, application of kinetin on tobacco plants prior to heat increased damage by this stress, while kinetin application after heat treatment improved recovery of

plants (Itai *et al.* 1978). Also bean plants pre-treated with zeatin riboside, did not show improved heat tolerance (Udompraset *et al.* 1995).

Creeping bentgrass (*Agrostis palustris* L.) was protected from 35°C heat injury by zeatin riboside injection into root zone. Those plants showed alleviated membrane injury and higher chlorophyll content (Liu and Huang 2002).

4.5.5. Plants with changed levels of endogenous cytokinins in stress

Besides exogenous CK application, another approach to study CK role is using plants with changed level of endogenous CK. Plants overexpressing *IPT*, the enzyme catalysing the rate-limiting step in CK biosynthesis, were used to study impact of increased concentration of endogenous CK. On the other hand, due to genetically manipulated plants, it is also possible to explore plants with depleted CK level. Reduction of endogenous CK can be achieved by overexpression of *CKX*, the enzyme irreversibly degrading CK or by down- regulation of CK biosynthesis (Miyawaki *et al*. 2006).

As CK have protective function but were observed to decrease under drought it is possible to expect that increased level of endogenous CK improve stress tolerance by promoting higher CK level also under stress conditions. This statement is valid for plants overexpressing *IPT* under senescence- or stress inducible promoter (Rivero *et al.* 2007 and 2009, Synková *et al.* 2001, Merewitz *et al.* 2011a, 2011b, and 2012, Zhang *et al.* 2010). Senescence- and stress inducible promoters induced increased level of CK when they decreased in wild plants and also without undesirable habit modification as observed in other types of promoters. By such transformation of cassava (Zhang *et al.* 2010), bentgrass (Merevitz *et al.* 2011), rice (Peleg *et al.* 2011), tomato (Ghanem *et al.* 2011), and tobacco (Rivero *et al.* 2007) plants with improved drought tolerance were produced. Expression of *IPT* under constitutive promoter resulted in diminished root growth thus worsened drought tolerance due to hampered water accessibility (Alvarez *et al.* 2008, Smigocki and Owens 1989).

Transgenic plants overexpressing *IPT* showed, except improved drought tolerance, also better tolerance to heat (Xu *et al.* 2009). Crosstalk between CK and proteome connected to heat stress tolerance was studied in bentgrass *Agrostis stolonifera* overexpressing *IPT*

under constitutive or heat shock protein gene promoter. Those plants showed changes in proteins connected to energy metabolism, storage and stress defence (Xu *et al.* 2010).

To study impact of diminished endogenous CK level, transgenic plants overexpressing *CKX* are valuable tool. Enzyme CKX irreversibly degrades CK by side chain oxidative cleavage (Galuszka *et al.* 2001). *Arabidopsis CKX* gene family is comprised of seven members, *AtCKX1 – AtCKX7* (Galuszka *et al.* 2007), which differ in their subcellular localization and substrate specificity. *AtCKX1* and *-3* are directed into vacuole, *AtCKX2, -4, -5, -6* are present in endoplasmic reticulum, and *AtCKX7* shows cytosolic localization (Werner *et al.* 2003). Despite CK role in stress is important and despite CK act as antioxidants and protect plants from stress, transgenic plants with decreased level of endogenous CK were, quite surprisingly, also more tolerant to drought (Werner *et al.* 2010, Mýtinová *et al.* 2010). Plants overexpressing *CKX* had also improved heat tolerance (Macková *et al.* 2013).

On the assumption that CK under drought decrease, it is possible to expect that decreased level of CK improve stress tolerance by promoting acclimatization. On the contrary, plants having dysfunction in cytokinin receptor histidine kinase 1 were drought sensitive (Tran *et al.* 2007). Besides, *A. thaliana* overexpressing *CKX4* were more sensitive to high light stress (Cortleven *et al.* 2014).

4.5.6. Effect of cytokinins on stress responses and their protective mechanism

Hormones CK have stress protective function. However, simultaneously in some aspects CK mediated signalization goes against survival strategy under drought. For example, CK stimulate metabolism which is inhibited during drought. CK have antagonistic function towards abscisic acid, which initiates stomata closing to avoid water loss. Further, shoot growth is stimulated by CK alongside they suppress root growth. By contrast, plants suffering from dehydration inhibit shoot growth and under long term drought they enhance root growth to achieve water source. Such facts may seem to be contradictory.

Protective role of exogenous CK under drought was confirmed (Metwally *et al.* 1997). For example, appropriate concentration of exogenous CK improved net photosynthesis after drought (Rulcová *et al.* 2001). In creeping bentgrass exogenous CK slowed down senescence and hindered lipid peroxidation (Liu and Huang 2002). Application of 6-benzylaminopurine inhibited leaf senescence and stimulated proline accumulation (Alvarez *et al.* 2008, Zalabák *et*

al. 2013). Exogenously applied 6-benzylaminopurine further protected cell membranes, light-harvesting a/b binding protein, large and small subunit of Rubisco in wheat leaves senescing under dark (Zavaleta-Mancera *et al.* 2007). Moreover, endogenous CK gradient caused by drought treatment with the highest CK level in the upper leaves points at improved protection of young leaves (Macková *et al.* 2013). Differences among tobacco leaves in CK content were also observed in study of Smart *et al.* (1991).

CK also improved heat tolerance. Exogenous zeatin riboside application to *Agrostis palustris* L. under heat inhibited lipid peroxidation, delayed senescence, preserved chlorophyll content and caused retaining of SOD and CAT activities (Liu and Huang 2002).

Because plants with both increased and decreased level of endogenous CK, showed improved stress tolerance, more ways how CK protect plants against stress exist. CK tend to decrease under stress conditions (Hare *et al.* 1997, Zalabák *et al.* 2013). Thus, improved drought tolerance of plants overexpressing *IPT* under senescence- or stress inducible promoter could be associated with enhanced supply of CK which under drought in WT decrease. In view of the fact that CK concentration decrease under drought, decreased level of CK, in plants overexpressing *CKX*, could act as acclimatization.

4.6. Antioxidant enzymes and abiotic stress

Antioxidant enzyme system plays key role in stress defence, thus also in stress signalling through ROS control. If ROS production increases during stress within certain limits, antioxidant system keeps ROS in appropriate concentration. The response of antioxidant enzymes to stress was often studied, but the framework of this defence is still not well understood. Information about antioxidant system defence comes from studies how stress stimulates or suppresses expression or activity of antioxidant enzymes. Another information about antioxidant system arises from transgenic plants overexpressing or lacking some antioxidant enzyme(s) (Gill and Tuteja 2010). Unfortunately, expression and activity are often studied separately regarding antioxidant enzyme as well as particular stress.

Serious difficulty originate from high sensitivity and thus also wide variability in response which is individual for each stress and often differs among plant species, even differs within a single plant according to its organ or a tissue.

The further text will be focused on the main antioxidant enzymes, APX, CAT, and SOD, and only on three abiotic stresses often appearing in natural conditions, drought, heat and their combination.

4.6.1. Antioxidant enzymes under drought

Despite production of ROS increases during stress, expression of chloroplastic *APX*, *tAPX*, *sAPX* and *FeSOD* decrease in tobacco after drought (Rizhsky *et al.* 2002). Also in another study, both expression and activity of sAPX and tAPX decreased in tobacco plants after one week drought (Rivero *et al*. 2007).

Cytosolic APX are supposed to have a more general stress protective function and expression of its gene increased in tobacco leaves under drought conditions (Rizhsky *et al.* 2002). Isoform APX1 was relatively active under optimal conditions and its activity was even higher under stress conditions, including drought (Shigeoka *et al.* 2002). Also pea cytosolic APX gene expression and activity increased under drought (Mittler *et al.* 1994). Furthermore, APX1 is postranscriptionally regulated under recovery from drought (Mittler *et al.* 2005).

In wheat, CAT activity after drought increased, expression of *CAT1* and *CAT2* decreased after drought treatment in comparison to optimal conditions (Luna *et al*. 2004). Also in tobacco, expression and activity of CAT decreased after drought (Rivero *et al.* 2007). In another study on tobacco, *CAT3* expression increased after drought treatment (Rizhsky *et al.* 2002). Tomato plants overexpressing CAT showed improved drought stress tolerance (Mohamed *et al*. 2003).

Activity of CuZnSOD in tomato leaves stayed unchanged under long term drought (Kardish *et al*. 1991). In another work, activation of cytosolic CuZnSOD was observed, while chloroplastic CuZnSOD stayed unchanged in drought treated plant (Bowler *et al.* 1992). Pea chloroplastic CuZnSOD expression increased after drought, as well as its activity (Mittler and Zilinskas 1994). Tobacco cytosolic CuZnSOD expression and activity was not significantly changed after one week drought (Rivero *et al*. 2007).

4.6.2. Antioxidant enzymes under heat

Heat stress is less frequently studied stress than drought, but there are several data about response of antioxidant system to high temperatures.

A dynamics in *APX* expression under heat was described in *Arabidopsis*. Transcription of *APX1* coding for cytosolic *APX* was up-regulated by heat. Cytosolic isoform *APX2* is heat inducible, it is not expressed under optimal conditions and its expression appears after heat shock. Microsomal *APX3* and *APX4* transcripts increased after short term heat period, but another microsomal isoform *APX5* showed a weak expression under different temperature regimes. Cytosolic *APX6*, and chloroplastic *sAPX*, and *tAPX* did not show clear response to heat treatments (Panchuk *et al.* 2002).

In tobacco plants, expression of cytosolic *APX* also increased, while stromatal *APX* decreased after heat, and *tAPX* transcripts were not changed significantly (Rizhsky *et al.* 2002).

4.6.3. Antioxidant enzymes under combined stresses

There are only several studies of antioxidant enzymes under combination of drought and heat, although it is quite common in natural conditions.

N. tabacum plants were treated with 6-7 days long drought period or/and heat shock 37 °C for 1 hour followed by 44 °C for 6 hours. Combination of heat and drought increased transcription of *CuZnSOD*, cytosolic *APX*, and *CAT3*. Contrarily, *sAPX* expression decreased after stresses combination (Rizhsky *et al*. 2002).

Poa pratensis L. showed decrease in CAT and SOD activity during up to 21-days water withdrawal and temperature 26-37 °C (Liu *et al.* 2008).

The 23-day old plants *Jatropha curcas* were subjected to 5 days water withdrawal or gradually increased temperature up to 43°C or their combination. Plants showed drop of CAT activity after drought, increase in its activity after heat, after stresses combination CAT activity was lower than under optimal conditions and higher than after drought. Activity of APX increased after a single treatment with drought and heat, and increased even more after their combination. Activity of SOD was not changed by drought, increased after heat and contrarily decreased after stresses combination (Silva *et al.* 2010).

The 21-day old cotton plants were treated with 10-day drought or/and 6 hours temperature high 30-45 °C. Activity of SOD increased under drought, however stayed unchanged after heat and stresses combination. Also CAT activity increased under drought, its reaction to heat and combined treatment differed between tested cultivars. Activity of CAT decreased in less drought tolerant cultivar, but increased in drought tolerant cultivar. Activity of APX was enhanced under drought and response to other stresses again differed between cultivars, increased in less tolerant and stayed unchanged in more tolerant drought cotton cultivar (Sekmen *et al.* 2014).

4.7. Crosstalk between antioxidant system and cytokinins

Hormones CK are also perceived as antioxidants and their cross-talk with the antioxidant system has been observed. For example, they are able to directly scavenge superoxide anions (Gidrol *et al.* 1994), to inhibit lipoxygenase activity (Swamy and Suguna 1974) and to directly interact with NO (Liu *et al.* 2013).

The fact that CK directly or indirectly influence antioxidant defence was previously observed. Exogenously applied 6-benzylaminopurine induced increase of CAT and APX activities in wheat (Zavaleta-Mancera *et al.* 2007). Tobacco plants with decreased level of endogenous CK evinced changes in activities of antioxidant enzyme system. Activity of GR was elevated while that of CAT was declined in transgenic plants. SOD and APX activities were not significantly affected by CK depletion (Mýtinová *et al.* 2010). Tobacco overexpressing *IPT* under light-inducible promoter of the Rubisco enzyme of *Pisum sativum*, showed higher abundance of chloroplastic FeSOD protein (Cortleven *et al.* 2011).

4.8. Crosstalk between antioxidant enzyme system, cytokinins and stress

Both, CK and antioxidant enzymes play role in plant stress defence, however their interaction under stress is not fully clarified.

Application of zeatin riboside into root zone changed antioxidant response of *Agnostis stolonifera* to heat stress. In this CK treated grass, SOD and CAT activities, which were

decreasing during 56 days heat treatment, were higher than in untreated plants (Liu and Huang 2002). Also ageing is a stress causing oxidative damage. Tobacco plants overexpressing *CKX2* under constitutive promoter showed in chloroplasts of older leaves higher SOD activity and lower APX activity than WT (Procházková and Wilhelmová 2009). Increase in total SOD activity was probably caused particularly by CuZnSOD which stayed active in old leaves of old tobacco plants overexpressing *CKX2*, as observed by Mýtinová *et al.* (2011) who also detected increased SOD and lowered APX activity in those plants in comparison to WT during ageing.

Plants *A. thaliana* overexpressing *CKX4*, which showed increased sensitivity to high light stress, had reduced ROS-scavenging capacity. In those plants under high light, total SOD activity was lower than in stressed WT (Cortleven *et al.* 2014). These plants contained reduced level of carotenoids, more precisely xanthophylls, and also reduced APX activity (Cortleven *et al.* 2014).

In plants overexpressing *IPT* under senescence associated receptor protein kinase promoter treated with drought cytosolic SOD activity was much higher than in WT under drought. This transgenic tobacco also differed in *sAPX*, *tAPX* and *CAT* expression which in WT decreased however in transgenic tobacco increased. Cytosolic *SOD* expression stayed unchanged in WT, but in *IPT* overexpressors it was also up-regulated (Rivero *et al.* 2007).

Exploring interaction among antioxidant system, CK, and stress response is aim of this study.

6. Aims

- Since both, CK and antioxidant enzymes, participate on stress tolerance, we examined if decreased level of endogenous CK due to overexpression of *A. tahaliana CKX1* influence antioxidant enzyme system stress response.

Hypothesis:

"Stress response of antioxidant enzymes in plants overexpressing *CKX1* differs from that in WT."

- CK as well as antioxidant enzymes are involved in stress defence and both contribute to stress tolerance. We tested if decreased CK level could be substituted by enhanced antioxidant enzymatic system.

Hypothesis:

"Plants overexpressing *CKX1* have improved antioxidant enzymatic system."

- We investigated antioxidant enzyme system response to stress. Ageing is also a kind of stressor causing oxidative damage. In addition, CK play important role in leaf senescence. We tested if antioxidant defence differs within leaf gradient.

Hypothesis:

"Antioxidant enzyme response differs in leaves of different age."

7. Material and methods

7.1. Plant material

Non transformed tobacco *N. tabacum* L. cv. Samsun NN (WT) and two transgenic lines overexpressing a gene coding for CKX1 from *Arabidopsis thaliana* were analysed in this study. Line *W6:CKX1-29* designated as *W6::CKX1* had *CKX1* gene inserted under *WRKY6* promoter which is the most active in roots and senescent leaves (Werner *at al.* 2010). Line *35S:CKX1-50* named *35S::CKX1* overexpressed *CKX1* constitutively under promoter *35S* (Werner *et al.* 2001). The seeds were kind gift of Prof. Thomas Schmülling from Freie Universität Berlin, Germany.

Tobacco plants were sown in sterilized soil (Garden Substrate B, Rašelina Soběslav, Czech Republic) without added fertilizer into 350 ml pots. Plants were cultivated in a growth chamber (Sanyo MLR 350H, Japan) at temperature 25/23°C, 16 h/ 8 h light/dark photoperiod at 130 µmol m^{-2} s^{-1} and air humidity 80 %.

Fifty days after germination separately upper, middle and lower leaves (Fig. 3), and roots were collected for analyses. Samplings were executed always at the same day time in the morning.

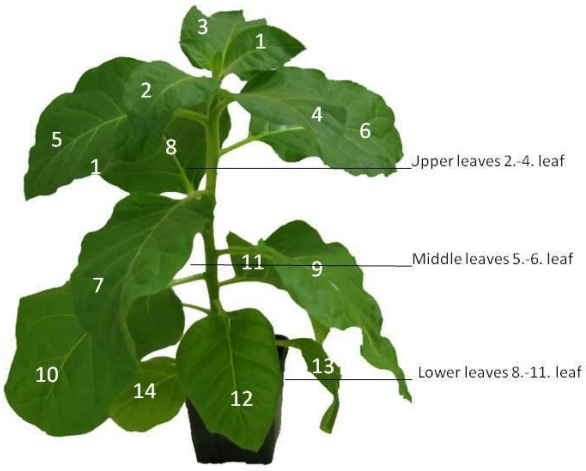

Figure 3: Leaf position in WT with marking of upper, middle and lower leaves as collected for experiments.

7.2. Experimental design

Within this study crosstalk among CK, abiotic stress and antioxidative enzymatic system was investigated. As regards abiotic stress, drought and heat stress and their combination were studied. Response of antioxidative enzymatic system to CK depletion in stress conditions was investigated on level of transcripts and activities of corresponding proteins.

To bring about drought, plants at age 42 days after germination were not watered for 8 days and were kept at air humidity 35 %. To cause heat shock plants had been moved to a chamber set at 40 °C for 2 hours. When combined stress was applied, described drought treatment was followed by the heat shock (Fig. 4). Ion leakage was measured after 24 hours recovery after drought. Spectrophotmetrically and polarografically detected activities were also observed after 24 hours recovery from drought and stresses combination.

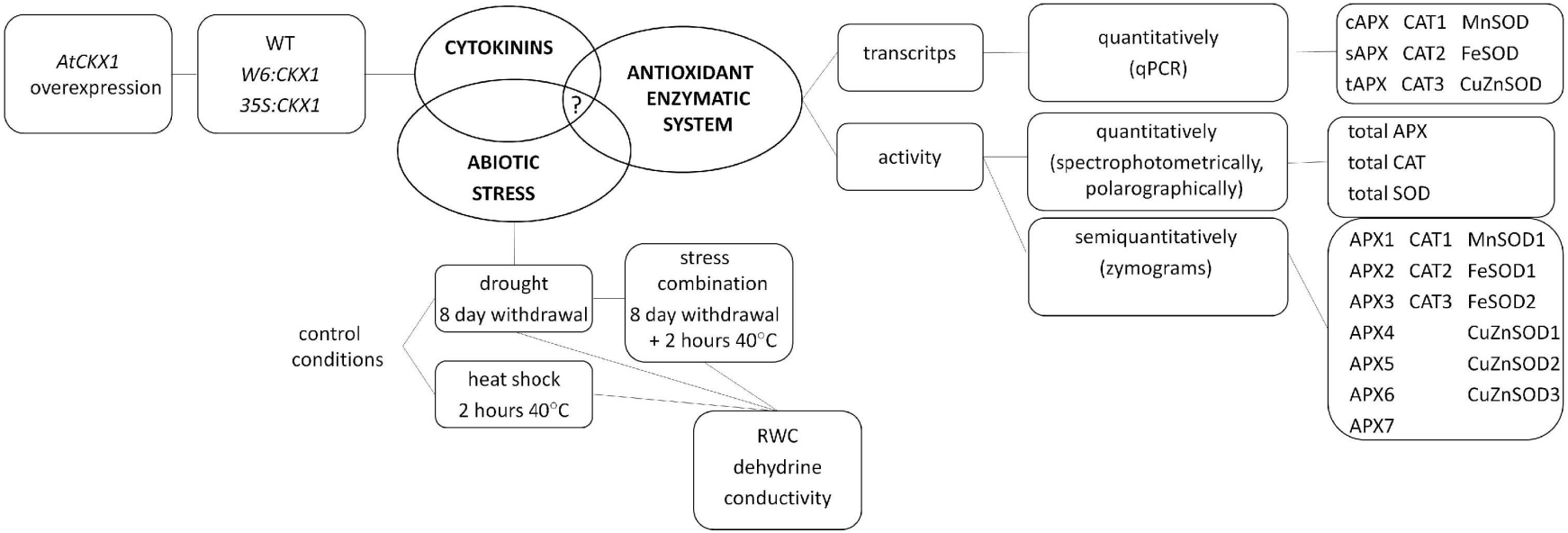

Figure 4: Diagram shows experimental design, time sequence of treatments and observed variables.

7.3. Relative water content

Fresh middle leaves were harvested, weighted (fresh mass, FW) (Kern ABJ 80-4M, Kern & Sohn GmbH, Balingen, Germany), then hydrated overnight with tap water in the beakers to full turgidity and weighted (water saturated mass, SM). Subsequently leaves were dried at 88°C for 24 h (dry mass, DM) and weighted. Relative water content was calculated as Weatherley (1950):

RWC (%) = (FM- DM)/(SM – DM) x 100.

7.4. Ion leakage

Plasma membrane integrity was established as electrolyte leakage on the basis of sample conductivity. For conductivity measurement 10 leaf discs (10 x 3.14 cm^2) per sample were collected, rinsed by distilled water and let soak in 10 ml distilled water for 24 h at 4°C, their conductivity was Ci. Subsequently the samples were autoclaved (120°C, 20 min, Chirana PS20A, Czechoslovakia), their conductivity was Cf. Relative damage to plasma membrane integrity was calculated as Ci/Cf * 100.

Ion leakage measurements consist of three independent biological repetitions and each sample had three technical replicates. Membrane damage was measured in upper, middle and lower leaves. Simultaneously, under control and drought conditions, we also measured ion leakage separately for each leaf from the youngest to the oldest leaf.

7.5. Sequence analysis

Gene sequences of known antioxidative enzymes were studied. Sequences of genes of antioxidative enzymes from different plants were chosen from NCBI database (National Centre for Biotechnology Information, Bethesda, USA). Acquired sequences were aligned and compared by means of software VectorNTI (Invitrogen, Carlsbad USA). Phylogenetic tree was created by software FigTree (Andrew Rambaut, UK). Similarity of sequences among different plants was evaluated.

To create comprehensive picture of antioxidative enzymes genes in tobacco, several unknown genes were sequenced. Tobacco RNA was isolated from 50 mg fresh leaves by 1 ml TRIzol reagent in homogenizer followed by chloroform extraction and isopropanol precipitation. The homogenized sample was incubated 5 min. in room temperature, then 200 µl of chloroform was added, vortexed and incubated 15 min. in room temperature. Then the sample was centrifuged at 12.000 g for 4 min. To supernatant 400 µl of isopropyl alcohol was added, incubated for 30 min. at -20°C, centrifuged at 12.000 g for 10 min. Gained pellet was twice rinsed by 70 % ethanol, then let dried and dissolved in water with diethyl pyrocarbonate. Five'- and 3'-rapid amplification of cDNA ends (RACE) with SMARTer™ RACE cDNA Amplification Kit (Clontech, Palo Alto, USA). Primers for 5'- and 3'-RACE PCR were designed according to EST sequences listed in Tab. 2. Products from PCR were cloned into complement cells DH52 via pMD19-T Simple Vector (TaKaRa, Tokyo, Japan) and sequenced.

7.6. Expression analysis

Expression of nine genes coding for antioxidative enzymes were analysed, namely *cAPX*, *sAPX*, *tAPX*, *CAT1*, *CAT2*, *CAT3*, *MnSOD*, *FeSOD*, and *CuZnSOD*.

For expression analysis tips of leaves and roots were harvested, immediately frozen in liquid nitrogen, and stored in -80 °C. Roughly 0.02 g of fresh weight was collected.

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Isolated RNA was stored in -80 °C for short term. Thus obtained RNA was treated by DNase I (DNA-free™ DNase Treatment and Removal Reagents, Ambion, Huntingdon, UK). Concentration of RNA was determined spectrophotometrically by nano cell spectrophotometer (BioMate3, Thermo electron corporation, Madison, USA).

To synthetize cDNA, DNase-treated RNA was mixed with 35 µM oligo dT primers ($dT_{23}dV$) and heated 5 min at 70 °C, subsequently chilled on ice. Then premix containing 5x M-MLV buffer, 10 mM dNTP, Protector RNase inhibitor (Roche Applied Science, Mannheim, Germany), and M-MLV Reverse Transcriptase RNase H Minus, Point Mutant (Promega, Madison, USA) according to manufacturer instruction was added. First strand cDNA was synthesized at 42 °C for 70 min.

Known tobacco sequences of antioxidant enzymes acquired from NCBI were used for blast in TAIR database (The Arabidopsis Information Resource, Stanford, USA). According to *Arabidopsis* sequences introns of matching tobacco genes were identified. Then PCR primers for genes of interest were designed according to tobacco sequences by means of software Primer3 (Rosen and Skaletsky, Cambridge, USA). Characteristics of designed primers were tested in VectorNTI (Invitrogen) before optimization. Sequences of designed primers are listed in Tab. 3.

Quantitative PCR reaction ran in Light Cycler 1.2 (Roche Applied Science) using SYBR mix FastStart DNA^{PLUS} SYBR GreenI (Roche Applied Science) with 20x diluted cDNA at following program: initial denaturation 5 min at 95 °C, 45 cycles of denaturation for 10 s at 95°C, appropriate annealing temperature for 10 s and 10 s at 72°C for extension. Used primers were chromatography purified (Metabion, Martinsried, Germany). Efficiencies of reaction were calculated according to calibration curves from serial dilution of cDNA. Actin was used as a housekeeping gene. Primers for actin, *dehydrin* and *CKX1* were previously designed by Jana Dobrá from Institute of Experimental Botany AS CR in Prague. Run-to-run differences were corrected by calibrator. Relative amount of transcripts was calculated as follows: $E_T^{\,CpT\,(C)\,-\,CpT\,(S)} \times E_R^{\,CpR\,(S)\,-\,CpR\,(C)}$. Where E_T/E_R is efficiency of target/reference amplification, CpT/CpR is cycle number at target/reference detection threshold, the crossing point (Cp). Letter S labels sample, letter C means calibrator.

7.7. Enzyme activity measurement

Total activities of antioxidative enzymes were detected. Activity of APX and SOD were measured spectrophotometrically. Activity of CAT was measured polarographically using oxygen electrode.

7.7.1. Protein extraction

Soluble proteins were extracted from 2 g of frozen leaves or roots by 10 s homogenization (Ultra-turrax TP 18/10, Ika-Werk, Staufen, Germany) in 10 ml Tris-HCl extraction buffer containing 0.1 M Tris-HCl pH 7.8, 10^{-3} M dithiothreitol (DTT), 10^{-3} M

ethylenediaminetetraacetic acid (EDTA Na_2), 1 % (v/v) Triton X-100, 5 x 10^{-3} M ascorbic acid (Asc). For release of membrane associated proteins, samples were treated with ultrasound (Tesla UC 006 DM1, Orava, Czechoslovakia) for 2 min, subsequently incubated on ice in dark for 30 min, centrifuged 10 min 20 000 g at 4°C (Biofuge 28RS, Heraeus Sepatech, Hanau, Germany), and then filtered through 8 layers of gauze. Samples for spectrofotometric determination of SOD activity were desalted by passing (centrifugation 2 min 18 g at 4°C, 3K30, Sigma, Osterode, Germany) through Sephadex G-25. Samples were frozen in liquid nitrogen and stored at -70 °C.

7.7.2. Protein determination

Protein content was determined according to Bradford (1976) using Bradford Reagent (Sigma-Aldrich, Saint Louis, USA) and measured by spectrophotometer (U-3300, Hitachi, Tokyo, Japan) at A_{595}. Calibration curve and measurements were done in range where it shows linear function, i.e. 0-9 μg protein/ml. Bovine serum albumin (BSA, Sigma-Aldrich) was used as a standard protein for calibration. Samples for calibration curve construction contained extraction buffer instead of sample. Standard with zero protein concentration was use as reference.

7.7.3. Ascorbate peroxidase activity

Total activity of APX was assayed spectrophotometrically (U-3300, Hitachi) with magnetically-stirred cells thermostated at 25°C. It was determined by monitoring the decrease of reduced Asc concentration at wavelength 290 nm (Nakano and Asada 1981). Reaction mixture contained Hepes-EDTA (4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.0) containing 0.1 M Hepes, 0.5 mM Asc, 0.88 mM H_2O_2 with 0.1 ml of protein extract in total volume 3 ml.

In reference cell, protein extract was replaced by extraction buffer used for protein isolation. Activity of APX was expressed as absorbance change per minute and related to one mg of protein ($\Delta A\ min^{-1}\ mg^{-1}$ prot.).

7.7.4. Catalase activity

Total CAT activity was determined polarographically by means of oxygen electrode (Hansatech Instruments, King´s Lynn, UK) thermostated at 25 °C (VEB MLW, Prüfgeräte-Werk, Medingen, Germany). Reaction catalysed by CAT ran in phosphate buffer (pH 7.0) containing 0.1 mM sodium phosphate, 10 mM H_2O_2 and 0.05 ml protein extract (Thomas *et al.* 1998). Activity was detected as oxygen evolution during reaction catalysed by CAT. The oxygen production rate was registered as change of electric current, which was transferred to voltage signal during 40 s.

For calibration of the electrode, line of water saturated with oxygen was registered (0.253 µmol O_2/ml H_2O), than sodium dithionite, which eliminates oxygen, was added and zero signal was measured. Catalase activity was determined as oxygen evolution rate per minute related to protein content (µmol O_2 min^{-1} mg^{-1} prot.).

7.7.5. Superoxide dismutase activity

Total SOD activity was measured spectrophotometrically (U-3300, Hitachi) with magnetically-stirred cells thermostated at 25°C at A_{470}. Reaction catalysed by SOD ran in mixture containing 50 mM Na_2CO_3, 0.1 mM EDTA, (pH adjusted to 10.2), 3 mM xanthine, 0.75 mM sodium 3,3´-{-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT), and xanthine oxidase (11.2 mU/ 3 ml, XO, Sigma-Aldrich) with addition 0.01 ml of protein extract (Ukeda *et al.* 1997). Blank giving the maximum colour reaction of superoxide generated in a mixture with XTT was measured without a sample.

One unit of SOD activity was defined as amount of the enzyme required for 50 % inhibition of reaction rate of XTT as a detection molecule reduced by superoxide. Units of activity were related to protein content (U mg^{-1} prot.).

7.8. Detection of isoenzymes of antioxidant enzymes on native gel

Presence of particular APX, CAT, and SOD active isoforms was determined by using specific dye after separation on native polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970).

Protein extracts used for native PAGE for APX and SOD were prepared by the identical method as for protein extraction for activity measurement. Protein extraction for determination of CAT isoforms is described below.

Used acrylamide gels contained acrylamide and bisacrylamide in ration 75:2. All native PAGE for isoenzyme separation ran in vertical electrophoretic chamber (Hoefer Scientific Instrument, San Francisco, USA; with Power Pac 300, Bio-Rad, Hercules, USA). Gels size was 10 x 10 cm, electrophoretic chamber was cooled to 4°C in a fridge during the procedure.

The activity of isoforms was semiquantitatively evaluated by Multi Gauge software (Fuji film, Science Lab 2002, Japan). We choose a marker sample and measured activity of its antioxidant enzymes. This sample was added in each gel. Magnitude and band intensity of all samples was related to bands of marker sample. Each sample was simultaneously run on three gels and each gel was evaluated three times.

Relative mobility of bands was characterized as migration distance of the top of gel divided by total length of gel.

7.8.1. Ascorbate peroxidase isoforms

Gel for APX isoform separation was comprised from 7.5 % (w/v) polyacrylamide stacking gel containing Tris-HCl stacking buffer (0.06 M Tris-HCl, pH 6.7) and 12.5 % (w/v) resolving gel with 10 % (w/v) glycerol and Tris-HCl running buffer (0.35 M Tris-HCl, pH 8.9) were prepared. Gel was pre-run for 30 min at 20 mA and maximum voltage 300 V with 2 mM Asc in Tris-HCl electrophoretic buffer (25 mM Tris-HCl with 0.19 M glycine, pH 8.3). Samples containing 20 μg protein were run on a gel at 20 mA and maximum voltage 300 V for 90 min.

After electrophoresis the gel was equilibrated for 30 min in 0.1 M phosphate buffer (pH 7) with 2 mM Asc and then the gel was incubated for 20 min in 0.1 M phosphate buffer

(pH 7) with 4 mM Asc and 2 mM H_2O_2. Gel was stained in phosphate buffer (pH 7.8) containing 28 mM N,N,N´,N´-tetramethylenediamine and 2.45 mM nitro blue tetrazolium chloride (NBT). Achromatic bands developed where H_2O_2 was removed by APX. Reaction was stopped by 10 % (v/v) acetic acid (Mittler and Zilinskas 1993).

7.8.2. Catalase isoforms

For native electrophoresis 1 g of frozen leaves was homogenized (Ultra-turrax TP 18/10, Ika) in 10 ml buffer (0,1 M Tris-HCl, 20 % (w/v) glycerol, 3. 10^{-2} M DTT). Membrane associated proteins were released by ultrasound (UC 006 DM1, Tesla) for 2 min, subsequently centrifuged 10 min 20 000 g at 4°C (Biofuge 28RS, Heraeus Sepatech), then filtered through 8 layers of gauze. Samples were frozen in liquid nitrogen and stored at -70°C (Zimmermann *et al.* 2006).

Catalase isoenzymes were separated on 6 % (v/v) stacking gel in stacking buffer (0,125 M Tris-HCl, pH 6.8), and on 7.5 % (v/v) resolving gel in resolving buffer (0.375 M Tris-HCl, pH 6.8) (Frugoli *et al.* 1996). Samples containing 4 µg of proteins were applied on gel. Separation lasted for 16 hours at voltage 70-80 V in Tris-HCl electrophoretic buffer (25 mM Tris-HCl with 0.25 M glycine, pH 8.3).

Gel for CAT determination was incubated 10 min in 0.01 % (v/v) H_2O_2 and then stained in 10 % (w/v) $FeCl_3$ and 10 % (w/v) $K_3Fe(CN)_6$ for 10 min.

7.8.3. Superoxide dismutase isoforms

Resolving and stacking buffers had the same composition as for APX gel, chapter 6.8.1. Separation of SOD isoenzymes was carried out on 6 % (w/v) stacking and 12 % (w/v) resolving gel with 10 % (w/v) glycerol, and at 20 mA for 2 h in the same electrophoretic buffer as for other antioxidant enzymes. Twenty µg of proteins was used for analysis.

After electrophoresis, gel was incubated on a shaker in a phosphate buffer (pH 7.8) containing from 0.5 M KH_2PO_4, 0.5 M EDTA, 23 mM N,N,N´,N´-tetramethylenediamine, 0.2 mM riboflavin, and 0.25 mM NBT at room temperature in dark for 20 min. Subsequently the gel was rinsed with distilled water and placed under two 16 W lamp (Prolite basic, Kaiser

Fototechnik, Buchen, Germany) until achromatic bands developed. Reaction was stopped by 10 % (v/v) acetic acid (Beauchamp and Fridovich 1971). Determination of isoenzymes insensitive to KCN, i.e. MnSOD and FeSOD, and insensitive to H_2O_2, i.e. MnSOD, was achieved 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 before staining (Beauchamp and Fridovich 1971).

7.8.4. Quantification of isozymes activity on zymograms

Images of gels were acquired by means of scanner (Epson Perfection V700 Photo, Nagano, Japan, with software SilverFast Launcher, LaserSoft Imaging, Kiel, Germany) without any modification of basic setting and saved in raw format. Obtained pictures were analysed by MultiGauge software (FujiFilm, Tokyo, Japan). Intensity of bands was evaluated as amount of pixels and corrected by subtraction of background signal.

Gel to gel variability was eliminated by using calibrator, a sample present in all gels. Total enzymatic activity of proteins of interest in calibrator sample was measured as described above. Intensity of all bands in calibrator was related to measured activity of calibrator. Activity of isoforms was calculated on the base of ration pixel amount to measured activity in calibrator. Calculated activity was related to mg of protein.

7.9. Statistical evaluation

Three independent biological repetitions were performed. All variables were measured at least three times within each biological experiment. Except for PCR, which was determined once due to its high reproducibility.

All data were normalized by square root transformation. Data obtained for variables were analysed by multivariate analysis of variance using programme Statistica (StatSoft, Tulsa, USA). Statistically important differences were proved by post-hoc Tukey test at level of significance $P < 0.05$. Data for leaves and roots were analysed separately.

Correlations among measured variables were analysed by correlation matrix and analysis principal component analysis (PCA). Significant correlations among variables were identified on the basis of Spearman correlation coefficient ($p < 0.001$).

Table 2: Nucleotide accession numbers for antioxidant enzymes genes in *A. thaliana* and EST accession numbers for antioxidant enzymes in tobacco.

gene	*Arabidopsis thaliana*	*Nicotiana tabacum*
APX2	NM_111798	E4355481, EB683997, EB429932
APX3	NM_119666	DV999892
APX4	NM_116970	FS415726, FS387894
CuZnSOD2	NM_128379	DW003050
CuZnSOD3	NM_121815	EB436784, EB679844
FSOD3	NM_122237	BP530823

Table 3: Accession numbers for antioxidant enzymes genes in tobacco and designed primers used in our experiments. Antioxidant enzymes names are given as commonly accepted according to previous authors.

Gene	Accession number	Primer sequence
cAPX	NTU15933	F: CCTCTCTGATCCTGCTTTCC R: CAACTCTTCCTCCTATCGCA
sAPX	AB022274	F: GGAAGCCCATGCTAAACTGA R:CAAACGGCAGAACTAAAGACG
tAPX	AB022273	F: AGCAACCTTGGAGCCAAAT R: GCTCTCTCTTTCCGGTTGAG
CAT1	NTU93244	F: CACTTATGAGATCCGCACCA R: TCACCTCCTCCGAACCATTA
CAT2	NTU07627	F: GTCAGAAGGTCGCTTCTCGT R: CTGCAACTTGAGACGTTTTCC
CAT3	Z36977	F: TCCAAAGTCCCACATTCAGG R: CACCGAGATCATCAAACAGG
MnSOD	AB093097	F: CATGACGCCATTTCCAAAG R: GGGGCAAGATTCTTCCAGA
FeSOD	A09032	F: TTCCAATGATGGATTGTGGA R: AACTGACTGCTTCCCACGAC
CuZnSOD	EU123521	F: AGCATGGTGCTCCTGAAGAT R: TCCAATGATGGATTGTGGA

8. Results

8.1. Plant characterization

Wild-type (WT) tobacco plants and two transgenic lines overexpressing *Arabidopsis thaliana CKX1* under control of the *35S* or *WRKY6* promoters were used to investigate the impact of modulation of endogenous CK levels on the antioxidant enzyme system in response to abiotic stress caused by drought, heat and their combination.

Plants WT and *W6:CKX1* had similar shoot habits, however, *W6:CKX1* had enhanced root system and were two internodes shorter at eight weeks when the experiments were performed. The *35S:CKX1* plants developed smaller and thicker leaves, shorter internodes (Fig. 5), and considerably enhanced root system in comparison to WT (Fig. 6). Wild type and *35S:CKX1* plants produced 12 - 14 leaves, while *W6:CKX1* 10 - 13 leaves when harvested at the age of 50 days after germination.

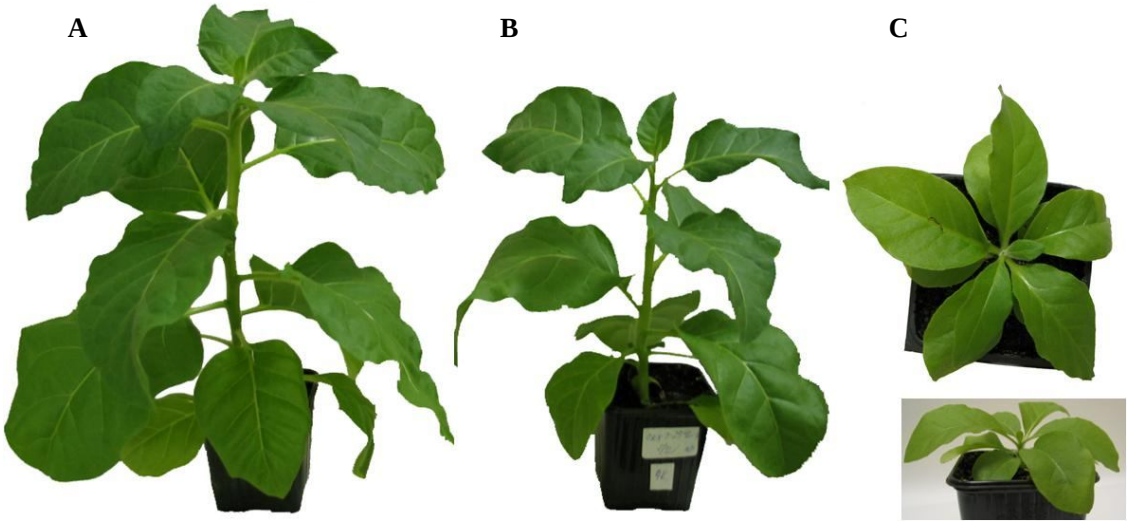

Figure 5: Tobacco plants at the age of 50 days after germination cultivated under optimal conditions. *N. tabacum* Samsun NN (A) WT, and transgenic lines (B) *W6:CKX1* and (C) *35S:CKX1*.

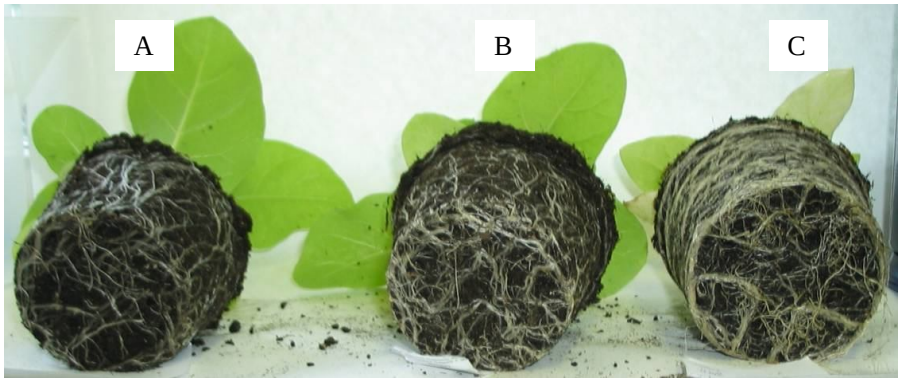

Figure 6: Root system of *N. tabacum* Samsun NN (A) WT, (B) *W6:CKX1*, (C) *35S:CKX1* at the age of 50 days after germination cultivated in optimal conditions.

In *W6:CKX1* plants, *CKX1* gene was expressed under promoter *WRKY6*, activity of which was high in roots and moderate in senescent leaves (Fig. 7). Expression of *CKX1* in *W6:CKX1* showed a gradient within a shoot, when the highest concentration of transcripts was in lower leaves. Further, under control conditions *CKX1* expression was the highest in roots within these plants. In *W6:CKX1* treated with drought and drought in combination with heat, amount of *CKX1* transcripts decreased. After heat shock *CKX1* expression decreased only in *W6:CKX1* roots.

Plants *35S:CKX1* expressed *CKX1* constitutively under control of *35S* promoter. In this genotype, *CKX1* expression was the lowest in roots within entire plant in all cultivation conditions and decreased only in leaves of plants treated with drought and heat combination.

Expression of inserted gene was significantly higher in *35S:CKX1* than in *W6:CKX1* plants under all conditions and in all tested plant parts (Fig. 7). It was higher 20- 200 times in *35S:CKX1* than in *W6:CKX1*. Thus employed transgenic genotypes considerably differed in *CKX1* expression level.

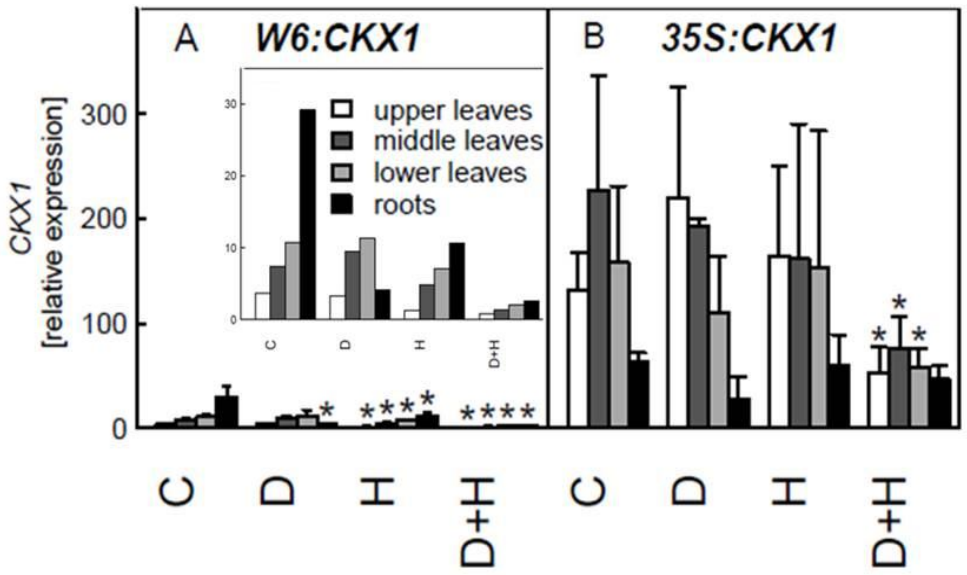

Figure 7: Transcript level of inserted *A. thaliana CKX1* in leaves and roots of two transgenic tobacco plants (A) *W6:CKX1* and (B) *35S:CKX1*. C -control hydrated conditions, D - drought stress (8-d dehydration), H - heat (40 °C for 2 h), D + H - combined stress (8-d dehydration + 40 °C for 2 h). Data represent mean values + SD from three biological repetitions. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype.

8.2. Plant responses to stresses

To induce drought, heat stress, and a combination of drought and heat, tobacco plants were not watered for eight days or/and exposed to heat shock at 42°C for 2 hours. The impact of stresses on the physiological state of plants was evaluated by determination of RWC and by expression level of the dehydration marker gene (*early response to dehydration 1*, *ERD1*).

After eight days of water withdrawal, whole WT plants were wilted (Fig. 8A). Plants *W6:CKX1* were also wilted, however, they were able to maintain turgor in the upper and partly in the middle leaves (Fig. 8B). The lowest leaves of *W6:CKX1* had slightly reduced chlorophyll content. Also young and middle leaves of *35S:CKX1* did not lose turgor, lower leaves were wilted and had lowered chlorophyll (Fig. 8C). After heat shock, all plants appeared as under control conditions and after stress combination all genotypes were similar to those after drought (picture not shown).

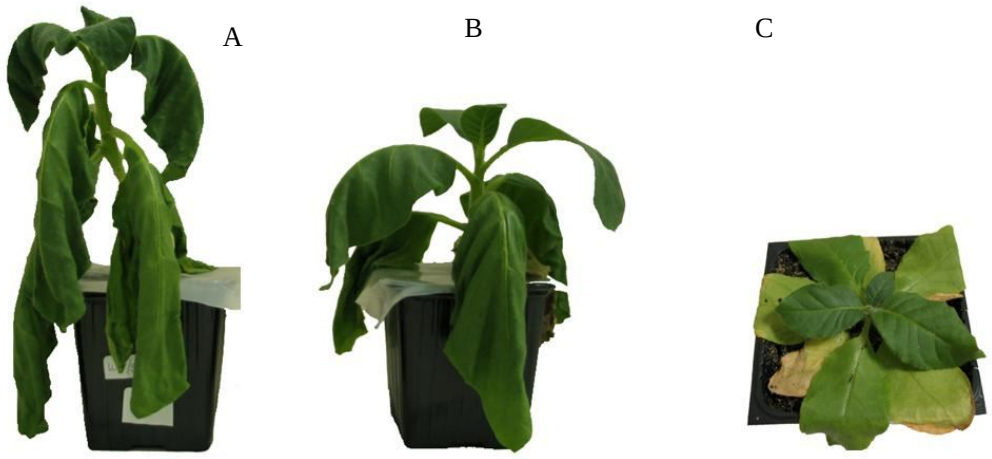

Figure 8: Tobacco plants at the age of 50 days after germination subjected to 8-day water withdrawal. *N. tabacum* Samsun NN (A) WT, and transgenic lines (B) *W6:CKX1*, (C) *35S:CKX1*.

Relative water content reflected the observed plant wilting. After drought and combined stress, middle leaves of WT and *W6:CKX1* had decreased comparably their RWC. Under control conditions their RWC was on average 80 % of full saturation. RWC dropped to 45 %, and 50 %, after drought and combined stress, respectively. Under control conditions RWC in plants *35S:CKX1* was similar as in WT. After drought and combined stress it decreased only to 70 %. Thus *35S:CKX1* lost less water than other two genotypes after drought and combined stress (Fig. 9). After heat shock no plants showed RWC difference in comparison to control conditions.

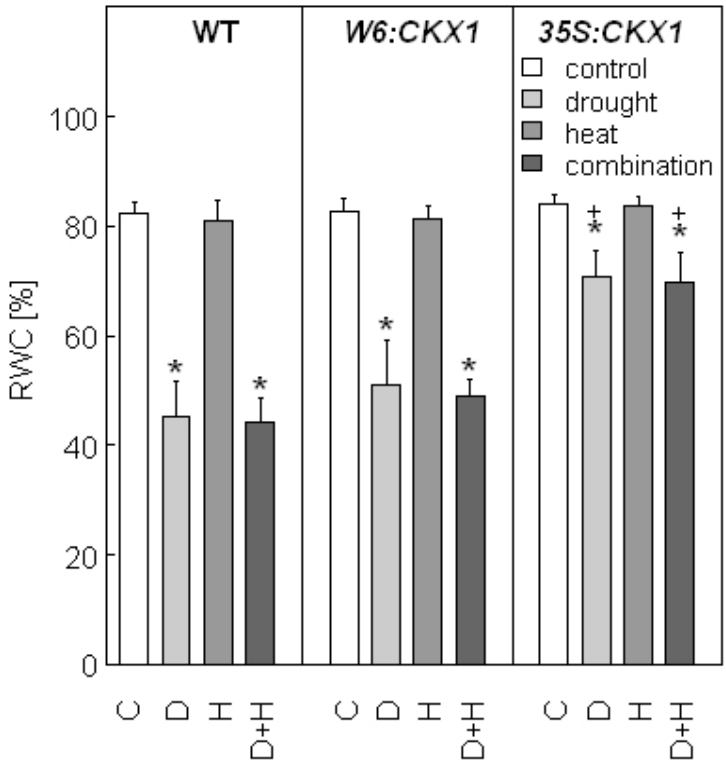

Figure 9: Relative water content (RWC) of middle leaves of *N. tabacum* Samsun NN WT, *W6:CKX1*, and *35S:CKX1* plants under C - control conditions, D - drought, heat, and D+H - stress combination. Data represent mean values + SD from three biological repetitions. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype. Symbol + indicates significant ($p \leq 0.05$) difference between transgenic plants and WT in particular plant part under given conditions.

Also expression of dehydrin is an indicator of drought intensity. Under control conditions, *ERD1* expression was very low in all tested plants. After drought and combined stress, *ERD1* expression increased in all tested plants and was higher than in control conditions. Heat shock alone did not significantly change *ERD1* expression. However, drought, both alone or in combination with heat significantly increased *ERD1* expression in all tested plants, but to a lower extent in *35S:CKX1* than in *WT* and *W6:CKX1* (Fig. 10).

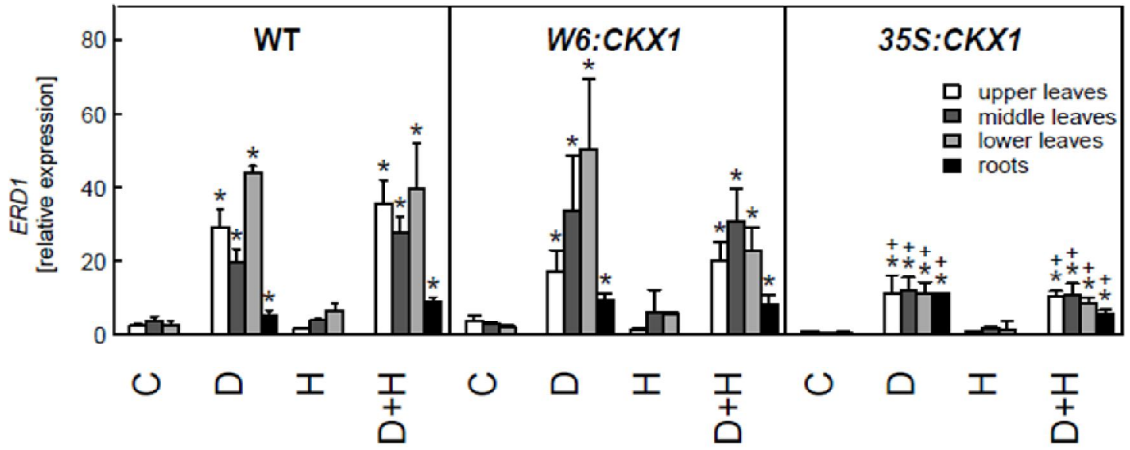

Figure 10: Dehydrin *ERD1* expression levels in upper, middle and lower leaves or roots of *N. tabacum* Samsun NN WT, *W6:CKX1*, and *35S:CKX1* plants under C - control, D - drought, heat, and D+H - stress combination. . Data represent mean values + SD from three biological repetitions. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype. Symbol + indicates significant ($p \leq 0.05$) difference between transgenic plants and WT in particular plant part under given conditions.

Membrane electrolyte leakage of leaf discs was determined as conductivity of incubation medium due to membrane damage of leaves. Conductivity in all tested plants under control conditions was comparable (Fig. 11, Fig. 12) and it showed slight dependence on leaf age in WT and *W6:CKX1* (Fig. 11). Under drought, ion leakage grew steeply with leaf age. After 24 hour after re-watering, all plants recovered and reached similar level as under control conditions (Fig. 11). Ion leakage assay showed lower membrane injury in *35S:CKX1* than in other two genotypes, what is in agreement with other measured stress markers.

Heat treatment did not cause significant elevation of membrane damage in any tested plants (Fig. 12).

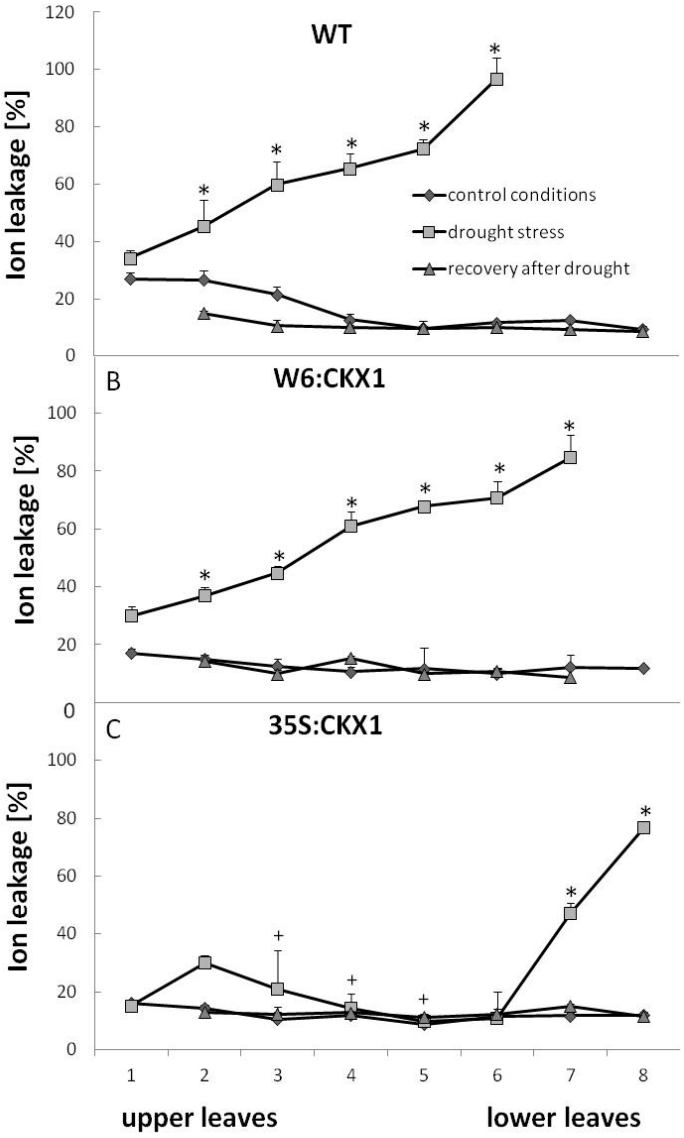

Figure 11: Membrane damage in all leaves from first upper to eight lower leaf within *N. tabacum* Samsun NN (A) WT, (B) *W6:CKX1*, and (C) *35S:CKX1* under control conditions, after 8-day water withdrawal, and after 24 hours recovery from drought. Measurements from one representative serie. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype. Symbol + indicates significant ($p \leq 0.05$) difference between transgenic plants and WT in particular plant part under given conditions.

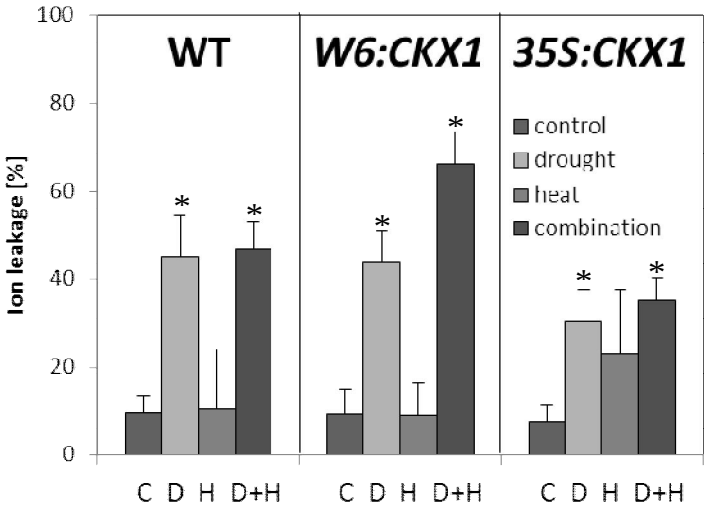

Figure 12: Damage of membranes measured as ion leakage in middle leaves of *N. tabacum* Samsun NN WT, *W6:CKX1*, and *35S:CKX1* under C - control conditions, D - drought, heat, and D+H - stress combination. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype. Symbol + indicates significant ($p \leq 0.05$) difference between transgenic plants and WT in particular plant part under given conditions.

8.3. Ascorbate peroxidase

8.3.1. Sequence analysis of *APX* genes

Sequences of three *APX* isoforms from tobacco are known (Tab. 3). In *A. thaliana*, sequences of eight *APX* isoforms are known (Tab. 2). We used these sequences to identify further tobacco *APX* sequences according to expressed sequence tags. On the basis of expressed sequence tags we designed primers for RACE.

Sequence analysis, comparing tobacco and *A. thaliana APX* genes, confirmed these genes code for cytosolic, stromatal and thylakoidal APX. Additionally, three *APX*, which we sequenced, are involved in sequence analysis. Analysis of conserved domains proved these sequences to belong to APX gene family. Phylogenetic analysis shows that new sequences are putative cytosolic *APX2*, microsomal *APX3* with transmembrane segment, and microsomal *APX4* (Fig. 13).

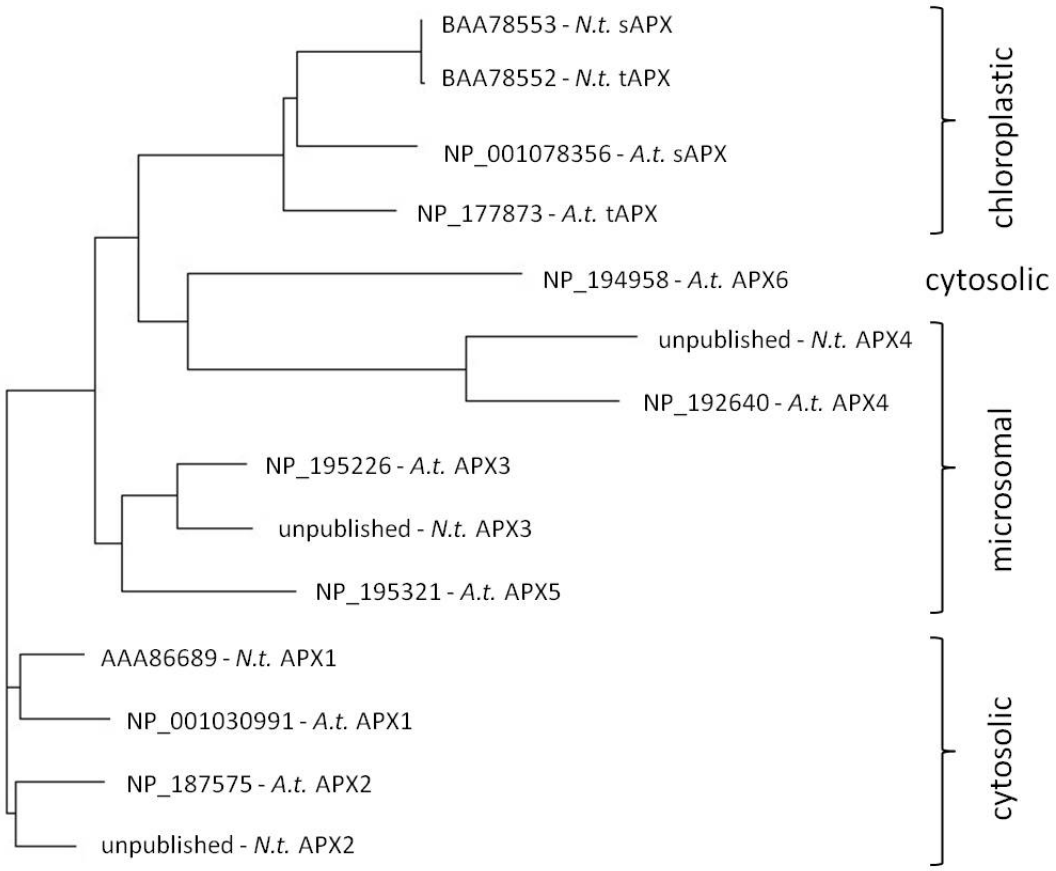

Figure 13: Phylogenetic tree of *N. tabacum* (*N. t.*) and *A. thaliana* (*A. t.*)APX enzymes and their localization within a cell. Tobacco isoforms are marked according to their homology with *A. thaliana* isoforms. New sequenced isoforms are marked as unpublished.

8.3.2. Expression of *APX*

Expression of three *APX* genes, one cytosolic (*cAPX*) and two chloroplastic (*sAPX*, *tAPX*) in WT, *W6:CKX1*, and *35S:CKX1* in response to particular stresses was examined. Primers for chloroplastic *APX* were designed to distinguish between the stromatal and thylakoidal *APX* arising from a common pre-mRNA by alternative splicing.

In all tested genotypes, expression of *cAPX* was markedly enhanced by heat shock, but not by drought, in both shoots and roots (Fig. 14A). The elevation of expression was lowest in *35S:CKX1*, the most heat stress-tolerant genotype. After heat shock, an expression gradient increasing from upper to lower leaves appeared in all genotypes. The application of heat at the end of the drought period in WT and *W6:CKX1* was associated with lower expression levels than that caused by heat alone (Fig. 14A).

Transcripts of *sAPX* were abundant in all leaves of all tested plants under control conditions. These transcripts were also abundant in roots, especially in *W6:CKX1* genotype. After stress treatments, changes of *sAPX* expression in *W6:CKX1* was similar to WT. Under all tested stress conditions, the expression of *sAPX* in those genotypes was down-regulated. In contrast, *35S:CKX1* plants maintained the same transcript levels under drought or heat. A combination of both stresses was required to decrease *sAPX* expression in mature and old leaves of this *CKX1* overexpressor (Fig. 14B).

Transcripts of *tAPX*, as well as *sAPX*, were abundant under control conditions in both leaves and roots of all tested plants. Expression of *tAPX* decreased after all applied stress conditions in both leaves and roots of WT and *W6:CKX1* plants compared to control conditions. It diminished after heat in *35S:CKX1*. The *tAPX* transcription exhibited a gradient decreasing from upper to lower leaves in all tested genotypes under all tested conditions (Fig. 14C).

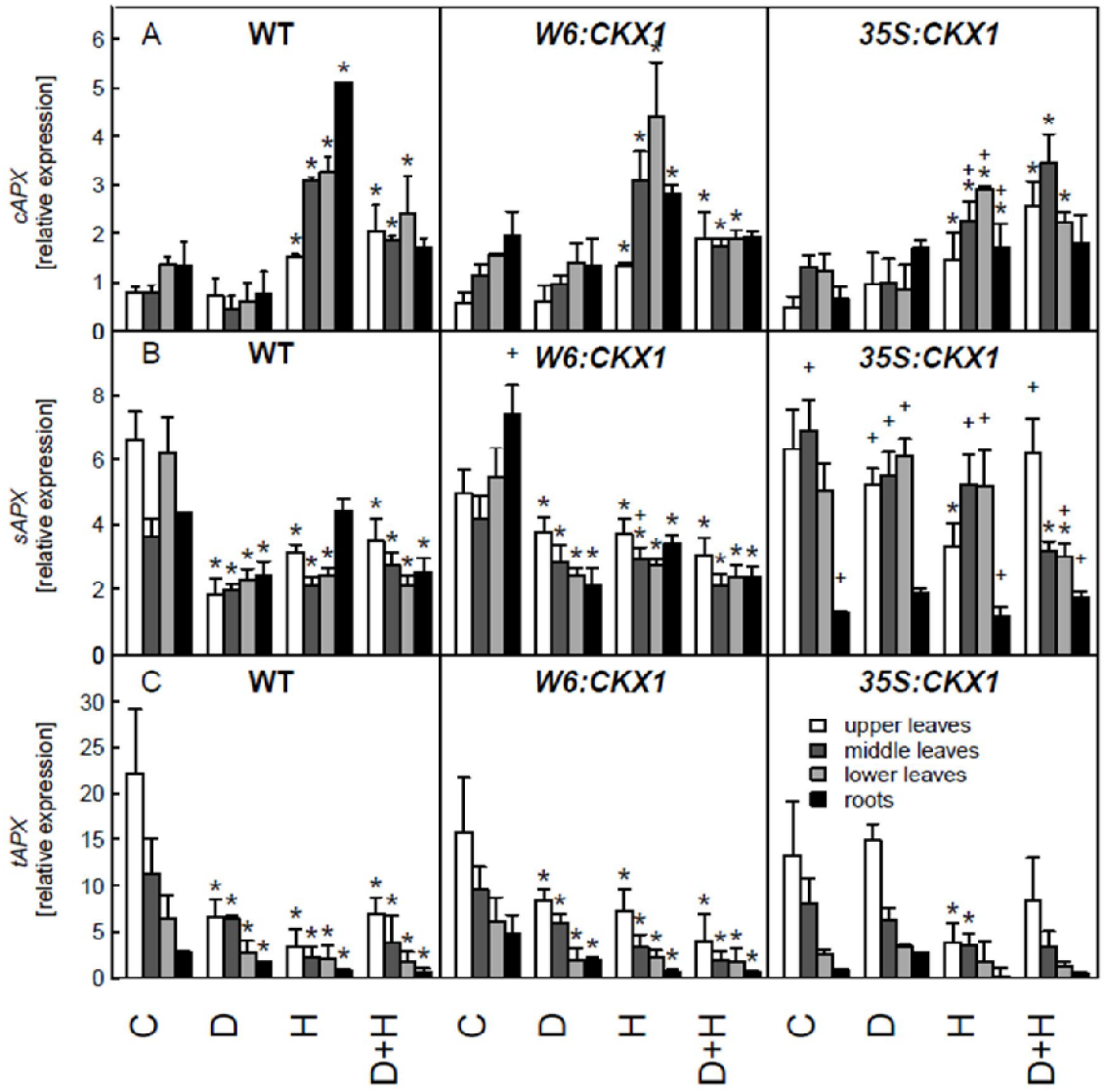

Figure 14: Effect of drought or/and heat on *APX* expression in upper, middle, and lower leaves or roots of *N. tabacum* Samsun NN WT, *W6:CKX1*, and *35S:CKX1*. (A) cytosolic (*cAPX*), (B) stromatal (*sAPX*), (C) thylakoidal (*tAPX*). C - control, D - drought, heat, and D+H - stress combination. Data represent mean values + SD from three biological repetitions. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype. Symbol + indicates significant ($p \leq 0.05$) difference between transgenic plants and WT in particular plant part under given conditions.

8.3.3. Activity of APX

The activity of APX isoforms was detected on native PAGE gels after appropriate staining. According to zymograms, all studied lines of tobacco produced nine active APX isoforms. Isoforms were named according to their order in ascending mobility from the slowest (APX1) to that with the highest mobility (APX9) (Fig. 15).

Image analysis of zymograms was used to measure activity of four most dominant APX isoforms, namely APX1, APX5, APX7, and APX9.

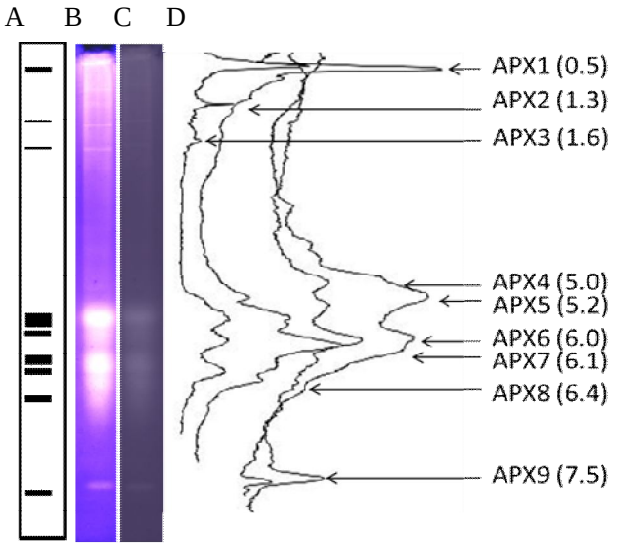

Figure 15: Representative pattern of APX isoforms visualised on (A) graphic diagram, (B) zymogram with edited colours for a highlighting the bands, (C) original zymogram, (D) densitogram. Zymogram shows APX isoforms in mixture of six samples. Densitogram shows four representative samples. Relative mobility is marked in brackets.

APX1 in WT decreased after all applied stress conditions, as well as in *W6:CKX1*, except in upper leaves after drought. APX1 activity in whole *35S:CKX1* plants under all tested conditions was very low in comparison to other two lines, and its activity decreased only after combination of drought and heat, not after a single stress. APX1 activity was higher in WT than in transgenic plants under control conditions and after combined stress. APX1 isoform was not detectable in roots of any plant (Fig. 16A).

In all genotypes under all conditions, the most abundant isoform was APX5. APX5 activity increased from upper to lower leaves in WT and *W6:CKX1* under control conditions,

after drought and after heat, after stress combination did not showed the same gradient. Gradient of APX5 activity was opposite, decreasing from upper to lower leaves in *35S:CKX1* plants under control conditions, after a single stress. After heat shock, activity of APX5 in WT decreased, contrary in both transgenic lines increased. APX5 activity was not detectable in roots of all tested plants (Fig. 16B).

Activity of isoform APX7 also showed an increasing gradient from upper to lower leaves in WT under all tested conditions. Drought and heat shock increased APX7 activity in *W6:CKX1*, thus APX7 activity was higher in *W6:CKX1* than in other two lines under those conditions. APX7 did not show any detectable activity in roots of all tested plants (Fig. 16C).

APX9 was in all tested plants the only APX activity detectable in roots by native PAGE gel. APX9 decreased after single drought and single heat shock in shoots of WT (Fig. 16D).

Spectrophotometrically determined total APX activity in leaf extracts increased in WT after drought and combined stress, and in *W6:CKX1* in all leaves after all applied stress treatments. APX activity of WT and *W6:CKX1* was also increased after recovery from drought and stresses combination. The APX activity was not significantly elevated in *35S:CKX1* under stress conditions and it was lower in *35S:CKX1* than in WT after recovery from stresses combination (Fig. 17).

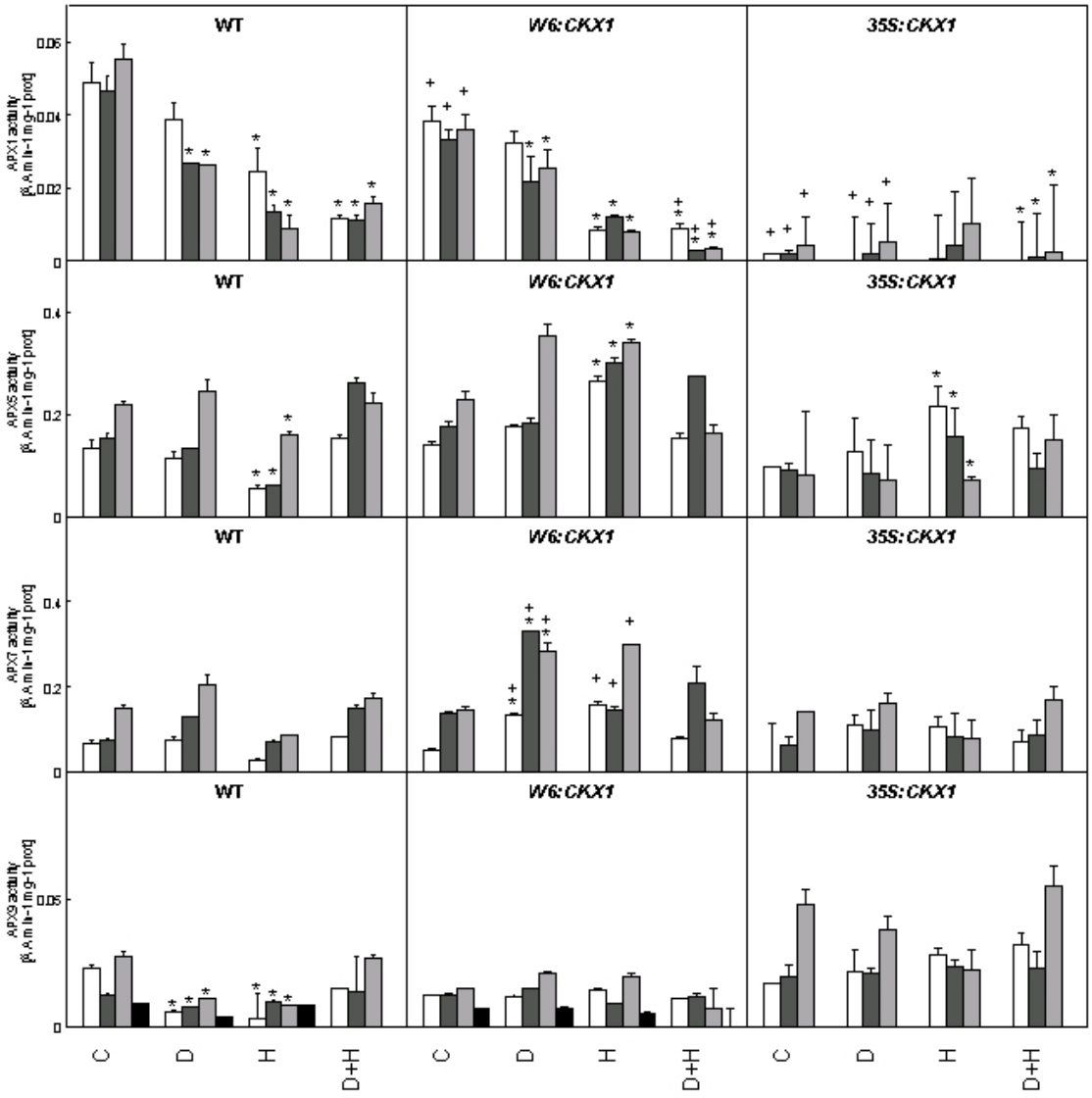

Figure 16: Activity of APX1, APX5, APX7 and APX9 in upper, middle and lower leaves and roots of WT, *W6:CKX1* and *35S:CKX1*. Activity was quantified according to native electrophoresis zymograms. C - control, D - drought, heat, and D+H - stress combination. Data represent mean values + SD from one representative series. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype. Symbol + indicates significant ($p \leq 0.05$) difference between transgenic plants and WT in particular plant part under given conditions.

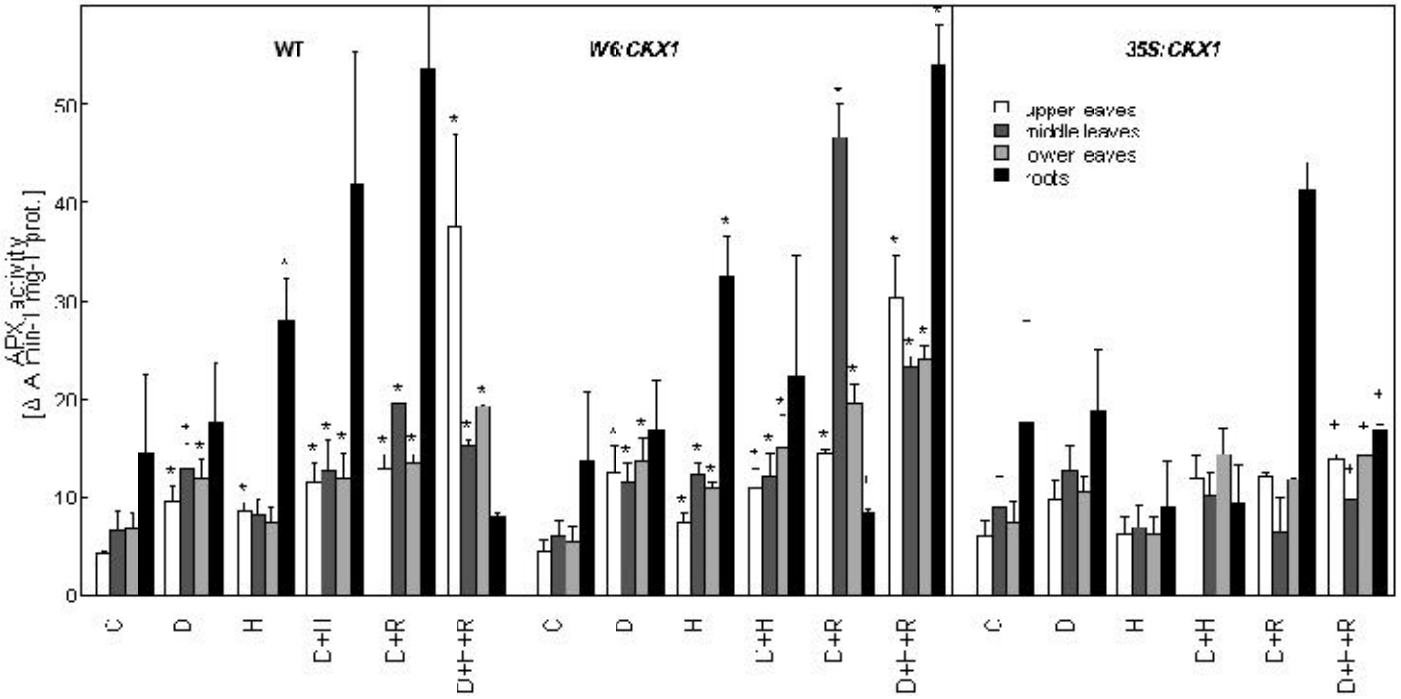

Figure 17: Spectrophotometrically detected total APX activity in upper, middle, and lower leaf or root extracts from *N. tabacum* Samsun NN WT, *W6:CKX1*, and *35S:CKX1*. C - control, D - drought, heat, and D+H - stress combination, D+H - recovery after drought, D+H+R - recovery after stresses combination. Data represent mean values + SD from three biological repetitions. Symbol * indicates significant difference ($p \leq 0.05$) between particular stress treatment and control conditions in the corresponding part and genotype. Symbol + indicates significant ($p \leq 0.05$) difference between transgenic plants and WT in particular plant part under given conditions.

8.4. Catalase

Three genes are known to code for subunits of tetrameric CAT complexes. Herein, *CAT*s are named in accordance with genes in *N. plumbaginifolia* (Willekens *et al.* 1994), i.e. *CAT1*, *CAT2* and *CAT3*.

8.4.1. Expression of *CAT*

Transcripts of *CAT1* were abundant in all genotypes under control conditions. Its expression decreased after drought and combined stresses in WT and *W6:CKX1* leaves. In contrast, it increased after drought in *35S:CKX1*. Heat shock activated *CAT1* transcription in all genotypes. A *CAT1* expression gradient in favour of upper leaves was observed in *35S:CKX1* plants after all stress treatments and in all plants after heat stress. The expression of *CAT1* was not detectable in roots of any genotype (Fig. 18A).

Also transcripts of *CAT2* were abundant under control conditions in all tested plants. *CAT2* expression under drought was lower in *35S:CKX1* than in WT. Expression of *CAT2* decreased in shoots of all tested genotypes after heat shock and combined stresses. After drought and combined stress, there was a gradient in *CAT2* expression, which increased from upper to lower leaves (Fig. 18B).

Expression of *CAT3* was very low in all explored genotypes under control conditions but was up-regulated slightly by drought and strongly by heat alone or in combination with drought. The observed *CAT3* elevation occurred in all genotypes, except in *35S:CKX1* after drought. Low expression levels of *CAT3* were detected in the roots under all treatments (Fig. 18C).

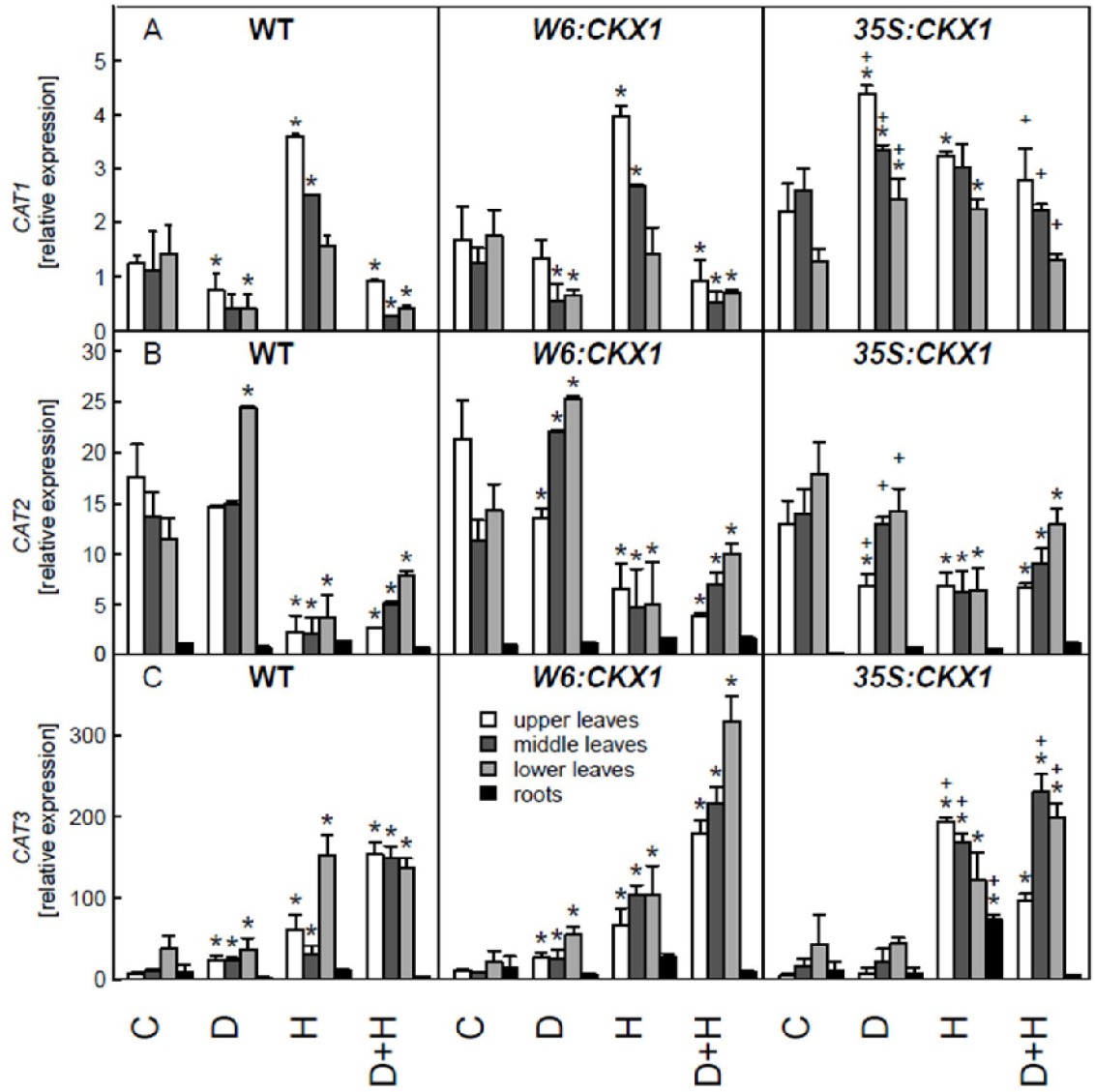

Figure 18: Expression levels of *CAT* genes in upper, middle and lower leaves and roots of *N. tabacum* Samsum NN WT, *W6:CKX1* and *35S:CKX1*. (A) *CAT1*, (B) *CAT2*, (C) *CAT3*. Other designation as described in Fig. 14.

8.4.2. Activity of CAT

Bands displaying CAT activity identified on zymograms were named according to their increasing mobility in the gel, from the isoform with the lowest mobility, CAT1, to CAT9 with the highest mobility (Fig. 19). It should be kept in mind that numbering of *CAT* genes does not match that of the proteins.

No significant differences in the activity of particular isoforms were observed among individual experimental conditions. Activity of measured CAT isoforms when comparable among genotypes did not show significant differences (Fig. 20).

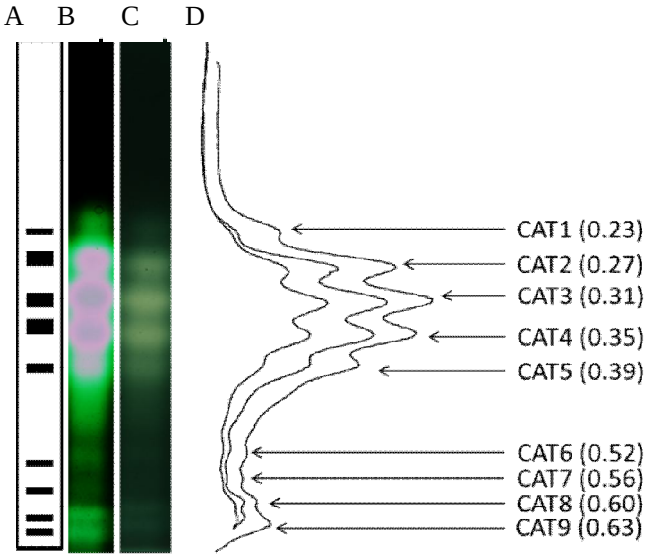

Figure 19: Representative pattern of CAT isoforms visualised on (A) graphic diagram, (B) zymogram with edited colours, (C) original zymogram, (D) densitogram. Zymogram is mixture of six samples. Densitogram shows three representative samples. Relative mobility is marked in brackets.**Figure 20:** Activity of CAT1 - CAT5 in upper, middle and lower leaves and roots of WT, *W6:CKX1* and *35S:CKX1*. Activity was quantified according to native electrophoresis zymograms. Other designation as described in Fig. 16.

Also polarographic measurement of total CAT activity in leaf extracts did not show any significant difference among tested variants. In *35S:CKX1* plants, gradient of CAT activity descending from upper to lower leaves was observed after heat and stresses combination (Fig. 21). Only a weak CAT activity was detectable in all tested roots.

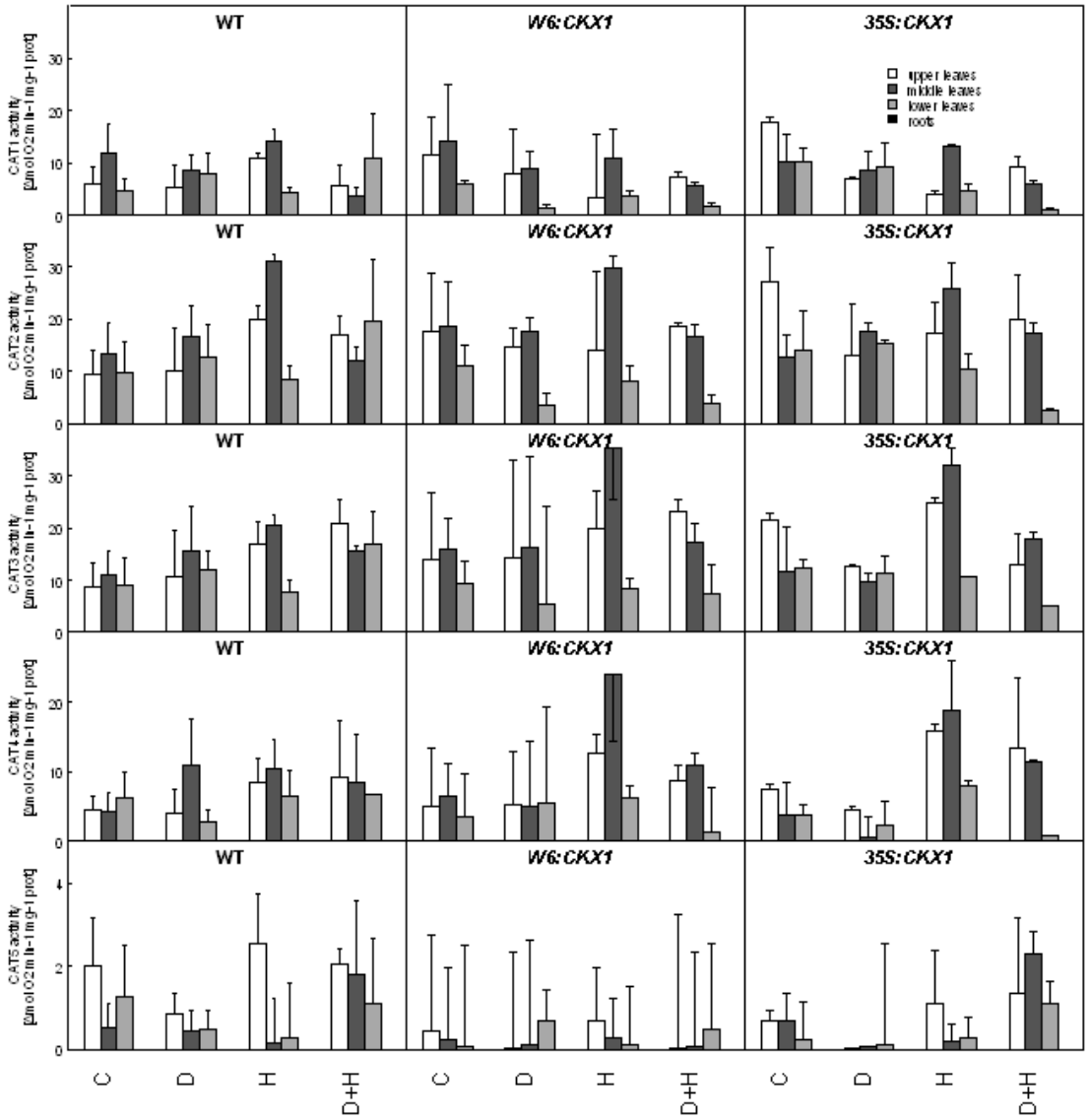

Figure 20: Activity of CAT four most active tetramers in upper, middle and lower leaves and roots of WT, *W6:CKX1* and *35S:CKX1*. Activity was quantified according to native electrophoresis zymograms. Other designation as described in Fig. 16.

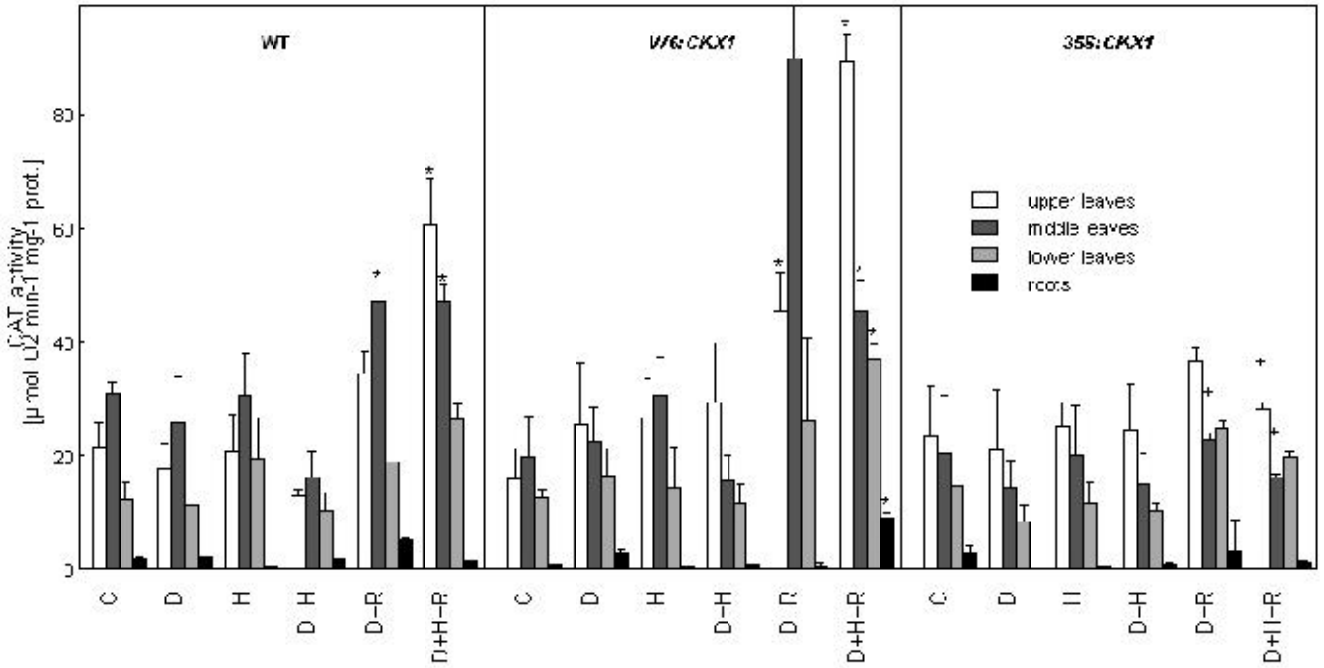

Figure 21: Total CAT activity in leaf extracts of upper, middle and lower leaves and roots from WT, *W6:CKX1* and *35S:CKX1* detected polarographically. Other designation as described in Fig. 17.

8.5. Superoxide dismutase

8.5.1. Sequence analysis of *SOD* genes

Three different *SOD* isoforms from tobacco are published in gene databases (Tab. 3), in *A. thaliana* are known sequences of seven *SOD* isoforms. Analogous to method for APX RACE sequencing, we attempted to identify sequences of SOD of *N. tabacum*. We sequenced another three *SOD* isoforms in *N. tabacum.*

Previously known sequences are *MnSOD*, *FeSOD*, and *CuZnSOD*, newly sequenced SOD isoforms correspond with chloroplastic *FeSOD3*, chloroplastic *CuZnSOD2*, and peroxisomal *CuZnSOD3* (Fig. 22).

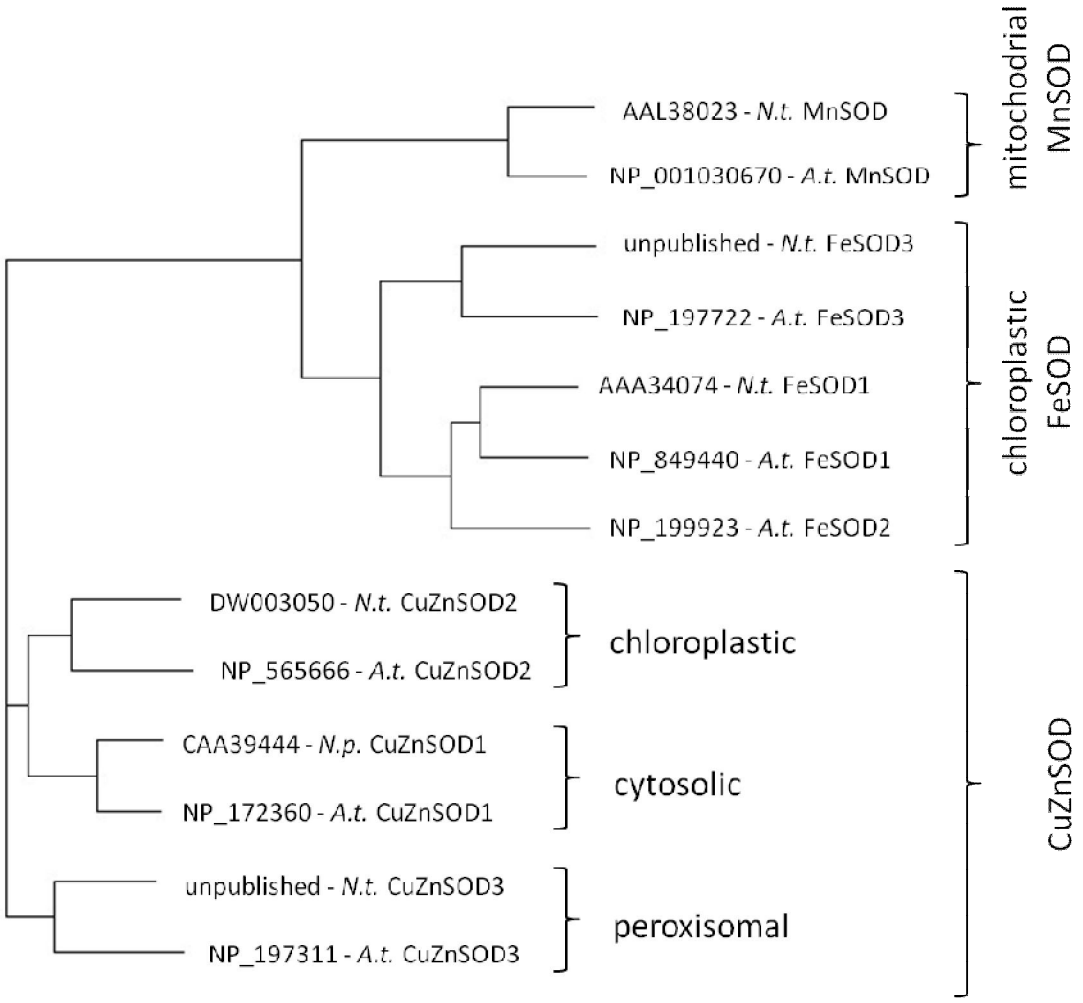

Figure 22: Phylogenetic tree of *N. tabacum* (*N. t.*), *N. plumbaginifolia* (*N. p.*) and *A. thaliana* (*A.t.*) SOD isoforms and their localization within a cell. Tobacco isoforms are marked according to their homology with *A. thaliana* isoforms. Newly sequenced isoforms are marked as unpublished.

8.5.2. Expression of *SOD*

Concerning expression analysis, we focused on three *SOD* genes, mitochondrial *MnSOD,* chloroplastic *FeSOD* and cytosolic *CuZnSOD*.

The expression of the *MnSOD* isoform declined after all stress treatments in WT leaves. A significant decrease was observed in *W6:CKX1* and *35S:CKX1* leaves after combined stresses. Transcripts of *MnSOD* were more abundant under all stress conditions in *35S:CKX1* in comparison to other genotypes. A gene expression gradient decreasing from upper to lower leaves was observed in WT plants under control conditions. Drought reversed this gradient, *MnSOD* expression was the lowest in upper leaves. High levels of *MnSOD* mRNA, not very much affected by stress, were detected in roots in all tested genotypes under all experimental conditions (Fig. 23A).

Expression of *FeSOD* showed a descending gradient of transcripts from upper towards lower leaves in WT and *W6:CKX1* under control conditions and after heat treatment. In WT leaves, transcription of *FeSOD* decreased markedly after drought and combined stresses in comparison to control conditions, especially in the upper leaves. In *W6:CKX1* and *35S:CKX1* leaves *FeSOD* also decreased after combined stresses. In control and single stress treated *35S:CKX1* middle leaves, *FeSOD* expression was higher than in WT. After heat shock, *FeSOD* expression was higher in middle and lower leaves of both transgenic plants in comparison to WT. The *FeSOD* was also expressed in roots of all genotypes (Fig. 23B).

Expression of *CuZnSOD* was low in control conditions and after drought in leaves of all genotypes. Drought caused substantial decrease in *CuZnSOD* expression in upper leaves of all tested plants (Fig. 23C). In contrast, strong promotion of *CuZnSOD* expression was observed following heat stress alone but after combined stress only in transgenic plants. This stress induction was the most prominent in *35S:CKX1* plants and in the roots of WT after heat treatment.

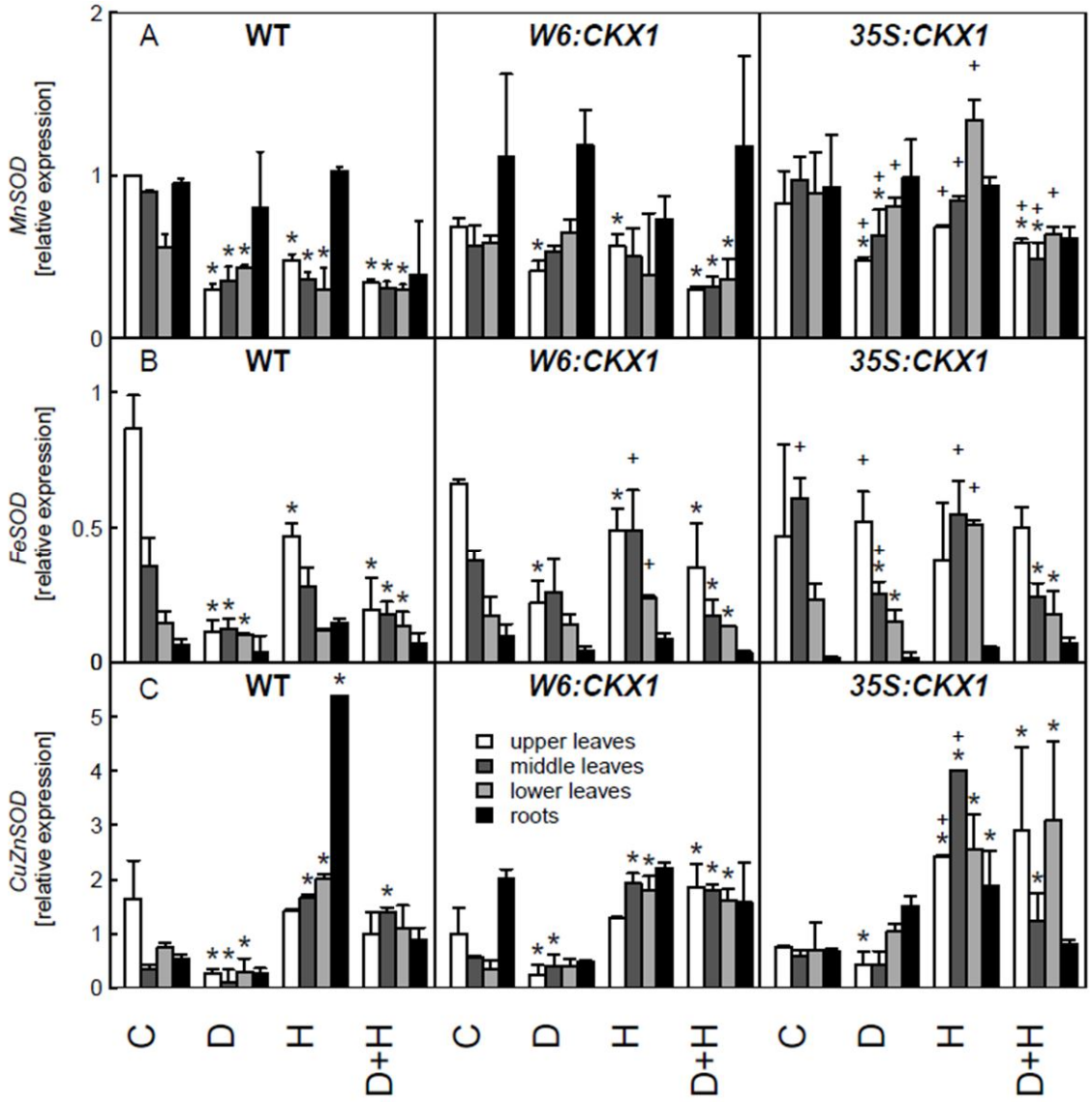

Figure 23: Expression levels of *SOD* genes in upper, middle and lower leaves and roots of *N. tabacum* WT, *W6:CKX1* and *35S:CKX1*. (A) mitochondrial *MnSOD*, (B) chloroplastic *FeSOD*, (C) cytosolic *CuZnSOD*. Other designation described in Fig. 14.

8.5.3. Activity of SOD

Distribution and activity of individual SOD isoforms were analysed by native PAGE. For tobacco, six SOD isoforms were identified on zymograms (Fig. 24). By selective H_2O_2 and/or KCN inactivation MnSOD, FeSOD, and CuZnSOD isoforms were distinguished, there were one MnSOD, two FeSOD, and three CuZnSOD isoforms in *N. tabacum* cv. Samsun NN.

Isoforms were named according to cofactor and their increasing mobility in PAGE gel from the slowest to the most rapid: MnSOD1, FeSOD1, FeSOD2, CuZnSOD1, CuZnSOD2, CuZnSOD3 (Fig. 24).

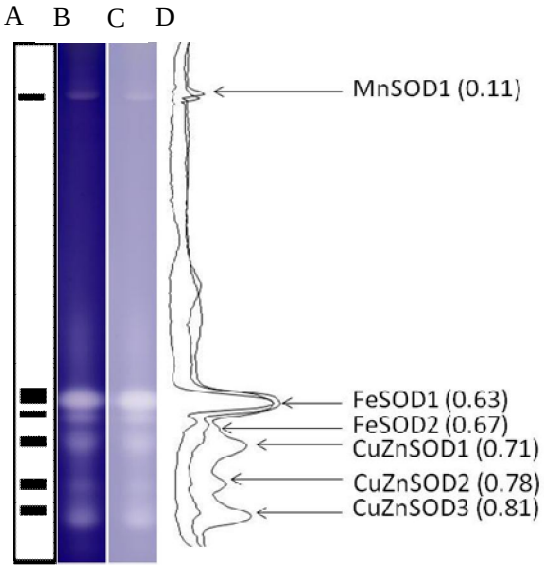

Figure 24: Representative pattern of SOD isoforms visualised on (A) graphic diagram, (B) zymogram with edited colours, (C) original zymogram, (D) densitogram. Zymogram is from mixed sample from six different samples. Densitogram shows four representative samples. Relative mobility is marked in brackets.

According to image analysis of zymograms, MnSOD1 activity was lower in *35S:CKX1* leaves than in WT under all tested conditions. The activity of MnSOD1 exhibited decreasing gradient from upper to lower leaves in WT in all conditions. Activity of MnSOD1was detected in roots, but only in negligible amount (Fig. 25A). Protein MnSOD1 was synthesized on the basis of *MnSOD* mRNA.

FeSOD1 was the most abundant isoform within whole plants of all three tested lines. Furthermore, there was a FeSOD1 activity gradient highest in upper leaves and decreasing to lower leaves in all tested lines under all growth conditions, but this activity gradient was not statistically provable. FeSOD1 activity increased after drought and combined stress in *W6:CKX1* plants. FeSOD1 was active also in roots of all tested plants (Fig. 25B).

FeSOD2 was also active in roots in all plants, however FeSOD2 activity was approximately 2 - 4x lower than activity of FeSOD1 (Fig. 25C). FeSOD2 increased in several cases after stress treatment and never decreased after stress.

CuZnSODs were the most abundant in upper leaves and there was a decreasing gradient from upper to lower leaves of all three CuZnSOD isoforms in all tested lines under control conditions as well as after all stress treatments (Fig. 26).

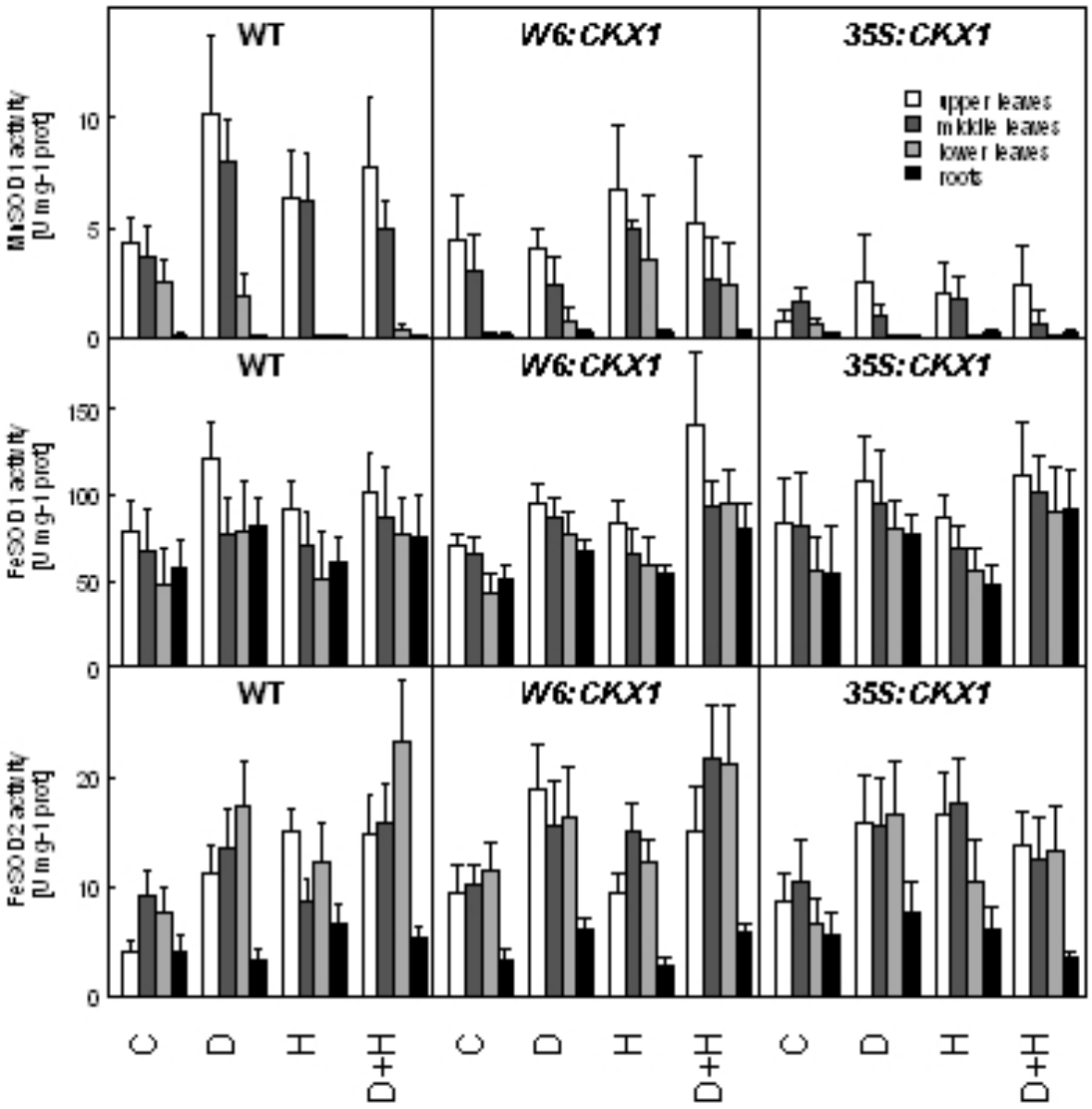

Figure 25: Activity of MnSOD1, FeSOD1, and FeSOD2 in upper, middle and lower leaves and roots of WT, *W6:CKX1* and *35S:CKX1*. Activity was quantified according to native electrophoresis zymograms. Other designation as described in Fig. 16.

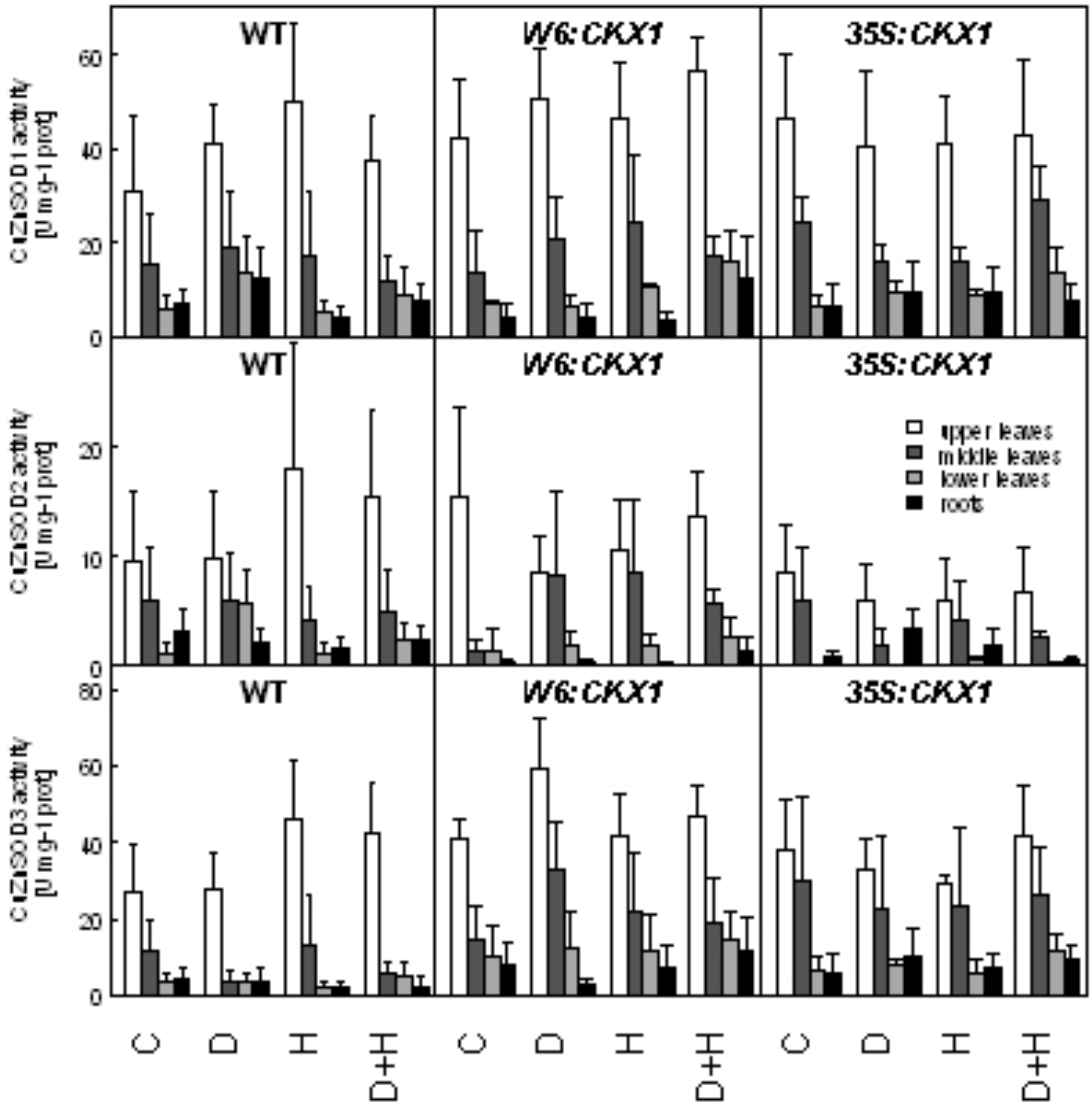

Figure 26: Activity of CuZnSOD1 – 3 in upper, middle and lower leaves and roots of WT, *W6:CKX1* and *35S:CKX1*calculated according to native electrophoresis zymograms. Other designation as described in Fig. 16.

Total SOD activity was measured spectrophotometrically in leaf extracts (Fig. 27), however any significant difference among tested variants were proved.

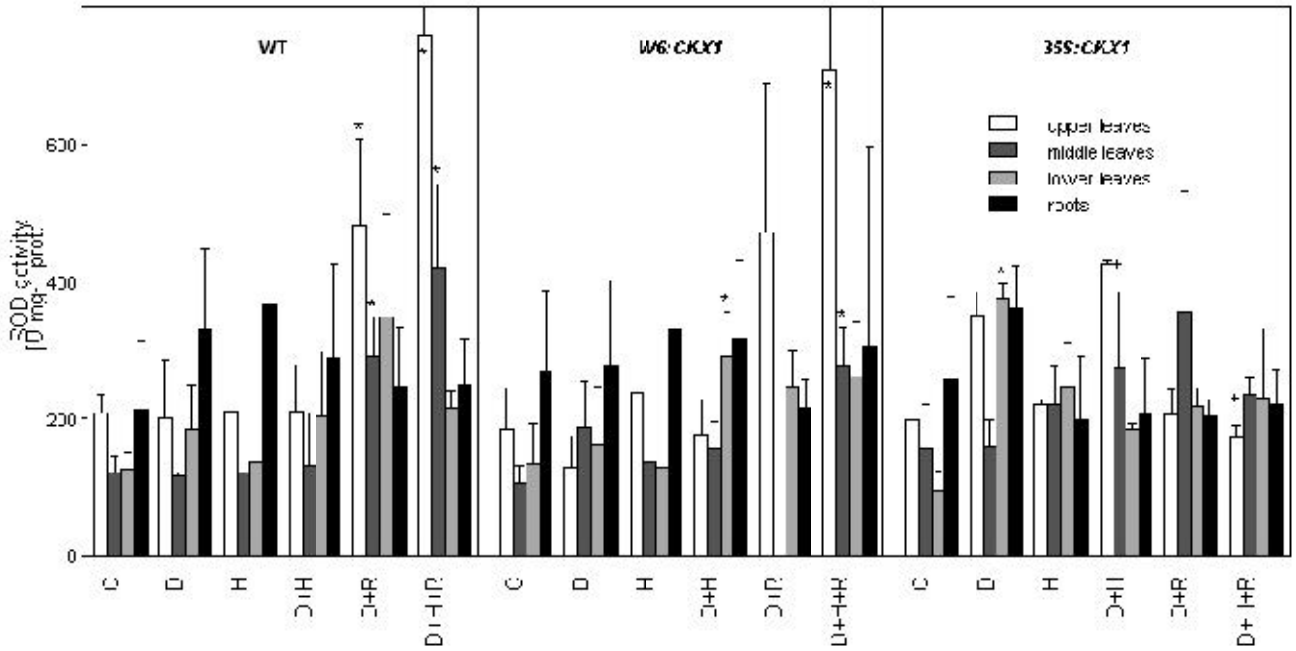

Figure 27: Total SOD activity of leaf extracts from upper, middle and lower leaves and roots from WT, *W6:CKX1* and *35S:CKX1* detected detected spectrophotometrically. Other designation as described in Fig. 17.

8.6. Correlation among enzymes

Relationships between expressions of individual antioxidant enzymes were studied by correlation analysis and principal component analysis (PCA). Pearson coefficient on normalized data (not shown) displayed significant correlations between the same pairs of transcripts as Spearman coefficient on not normalized data with only two exceptions. *AtCKX1* and *CAT3* did not correlate, due to excessively low values in some cases, when analysed parametrically. Below only Spearman coefficient is used.

Statistical analysis showed a weak positive correlation between the expression of the *CKX1* transgene and that of *CAT1* (0.48), and between *CKX1* and *MnSOD* (0.52). Expression of *FeSOD* and *CAT1* negatively correlated with *ERD1* (-0.52, -0.51, respectively). The *FeSOD* and *CAT1* transcript levels correlated positively (0.57) (Tab. 4).

A positive correlation among expression levels of genes for the chloroplastic enzymes *sAPX*, *tAPX*, and *FeSOD* was found. Stromatal and thylakoidal *APX* correlated strongly with each other (0.87). The correlation of expression of these two genes with *FeSOD* was lower (0.57 and 0.56, respectively). Expression of both chloroplastic *APXs* correlated with that of *CAT2* (0.64 and 0.69, respectively) (Tab. 4).

Expression of genes encoding enzymes with general stress protective function correlated positively, *cAPX* with *CuZnSOD* (0.62), and *CAT3* correlated weakly with *CuZnSOD* (0.38). Transcripts of *cAPX* negatively correlated with *CAT2* (-0.54) (Tab. 4).

Cytosolic *APX* with *CuZnSOD* (0.64), *sAPX* correlated positively in root tips with *FeSOD* (0.61) and *CAT3* (0.65) (Tab. 5).

In the principal component analysis all chloroplastic isoforms (*sAPX*, *tAPX*, *FeSOD*) were in vicinity and also *cAPX*, *CAT3* and *CuZnSOD* were grouped (Fig. 28).

Correlation relationships and expression trends are summarized in a diagram (Fig. 29).

Table 4: Correlation matrix for antioxidant enzymes transcripts in leaves. Statistically significant differences are indicated in bold ($p \leq 0.001$).

ERD1	*cAPX*	*sAPX*	*tAPX*	*CAT1*	*CAT2*	*CAT3*	*MnSOD*	*FeSOD*	*CuZnSOD*	variable
-0,27	0,05	0,25	0,09	**0,48**	0,09	-0,06	**0,52**	0,28	0,02	*AtCKX1*
	0,03	-0,35	-0,35	**-0,57**	-0,03	0,10	**-0,48**	**-0,52**	-0,16	*ERD1*
		-0,05	-0,30	0,22	**-0,54**	0,31	-0,10	-0,01	**0,62**	*cAPX*
			0,87	**0,41**	**0,64**	**0,66**	0,25	**0,57**	0,17	*sAPX*
				0,29	**0,69**	**0,47**	0,13	**0,56**	-0,05	*tAPX*
					-0,09	0,10	**0,38**	**0,57**	0,16	*CAT1*
						0,33	0,18	0,14	-0,34	*CAT2*
							-0,28	0,14	**0,38**	*CAT3*
								0,43	0,07	*MnSOD*
									0,29	*FeSOD*

Table 5: Correlation matrix for antioxidant enzymes transcripts in roots. Statistically significant differences are indicated in bold ($p \leq 0.001$).

ERD1	*cAPX*	*sAPX*	*tAPX*	*CAT1*	*CAT2*	*CAT3*	*MnSOD*	*FeSOD*	*CuZnSOD*	variable
-0,35	-0,09	-0,32	-0,16	0,09	-0,14	0,05	0,05	-0,21	0,22	*AtCKX1*
	0,05	-0,3	-0,05	0,14	0,01	-0,27	0,16	-0,24	-0,23	*ERD1*
		0,19	-0,1	-0,25	-0,26	0,08	0,14	0,58	**0,64**	*cAPX*
			0,75	0	0,34	**0,65**	-0,17	0,61	0,13	*sAPX*
				0,24	0,42	0,39	0,09	0,22	-0,13	*tAPX*
					-0,18	0,02	0,06	-0,2	-0,2	*CAT1*
						0,37	0,12	0,1	-0,17	*CAT2*
							-0,04	0,35	0,31	*CAT3*
								-0,03	-0,19	*MnSOD*
									0,28	*FeSOD*

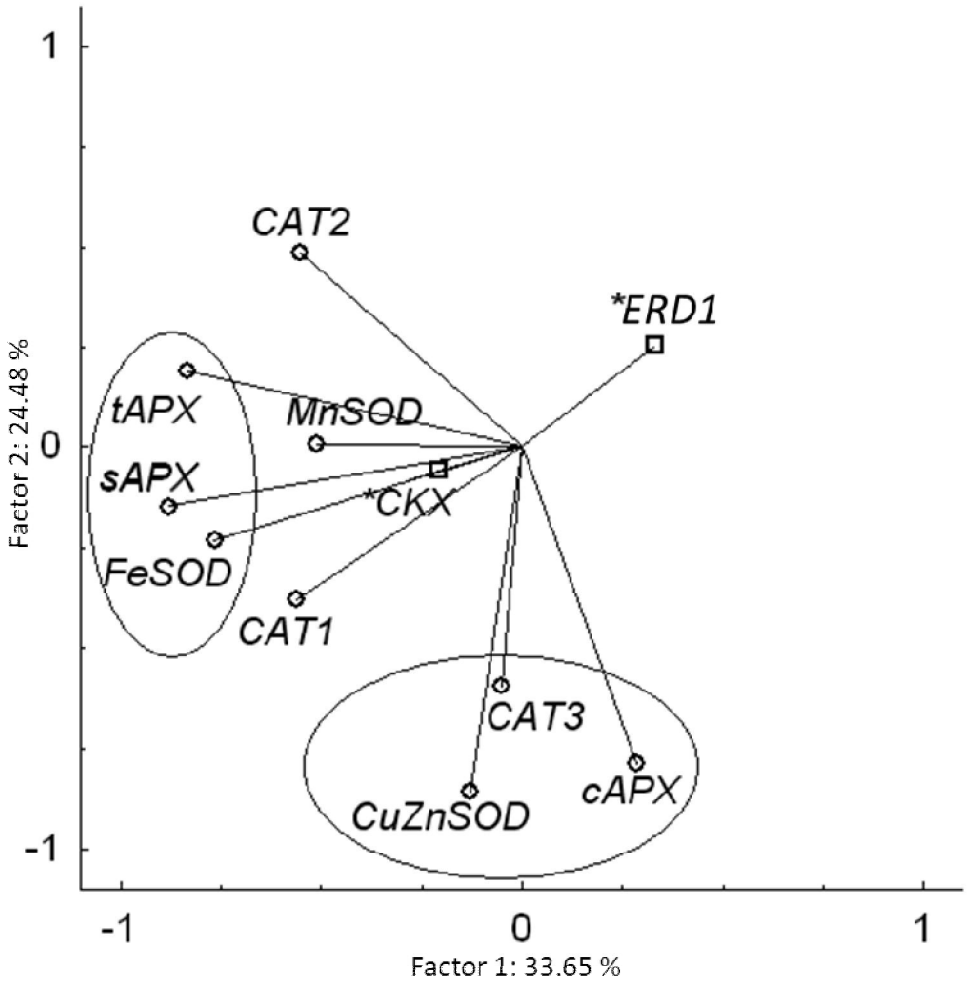

Figure 28: Principal component analysis showing grouping according to functionally specialized antioxidant enzymes.

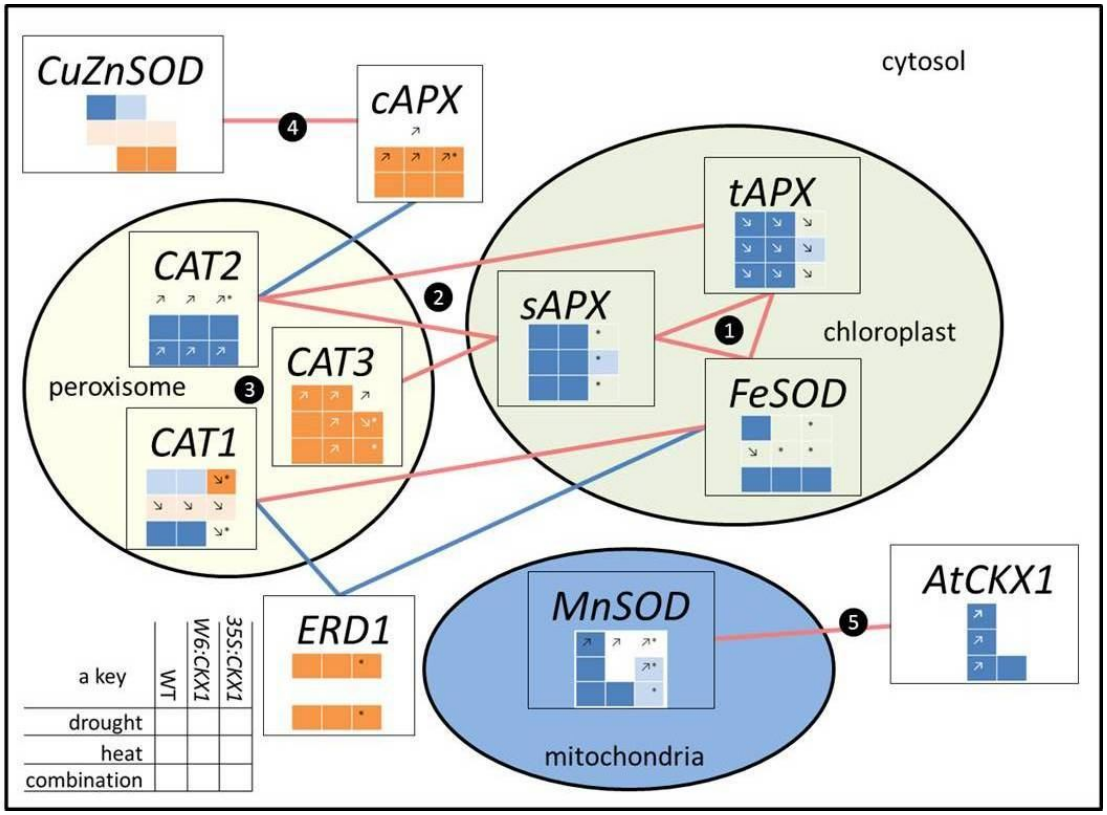

Figure 29: Diagram of dynamics and relationships between transcripts of antioxidant enzymes in tobacco leaves. Red and blue lines represent positive and negative correlations, respectively, with Spearman correlation coefficient > |0.5| (according to Tab. 4). Significant differences among transcripts under different conditions and genotypes (according to Tab. 4) are showed by matrix next to each isoform. Orange and blue colour mark increase or decrease, respectively, in all leaves in comparison to control condition. The cases where difference was significant in most of the leaves are labelled in light colours. ↗ and ↘ mark gradients increasing and decreasing from upper to lower leaves, respectively. * points to significant difference between WT and transgenic plant. Key for matrix see in left bottom corner. Notes: 1) Chloroplastic transcripts, *sAPX*, *tAPX* and *FeSOD*, were closely interconnected. 2) Situation in chloroplast is closely linked to those in peroxisomes, especially *CAT2* with both chloroplastic *APX*. 3) No correlation was among *CAT*, they are not redundant and have independent regulation. 4) Cytosolic *CuZnSOD* correlates with another generally stress protective enzyme, cAPX. 5) *MnSOD* is indirectly influenced by CK through their role in source/sink distribution of sugars. Thus *MnSOD* was influenced by *AtCKX1* expression.

9. Discussion

Both, CK as well as antioxidant enzyme system play key roles in stress response in plants. However, their interactions are not clarified fully yet and were studied quite rarely. To elucidate interaction between CK and antioxidant enzyme system, we investigated expression and activity of antioxidant enzymes in tobacco with depleted CK content. The plants with increased CK level is more frequently employed system for such studies. We chose complementary model with depleted CK content. This was achieved by the overexpression of the enzyme responsible for CK degradation, the *cytokinin oxidase/dehydrogenase*, under the control of root specific promoter in *W6:CKX1* plants or constitutive promoter in *35S:CKX1*. Such plants were subjected to drought, heat or combination of both stresses.

CK act as antioxidants and increase abiotic stress tolerance, however, tobacco with decreased level of CK display quite surprisingly better tolerance to drought and heat. We examined how is the stress response of antioxidant enzyme system affected by CK depletion in transgenic tobacco in comparison with the wild type. We hypothesized that modified antioxidant enzyme system stress defence contributes to an improved stress tolerance to transgenic tobacco.

Quite recently, several plants overexpressing *CKX* were found to be more tolerant to drought, heat or salinity (Werner *et al.* 2010, Mýtinová *et al.* 2010, Macková *et al.* 2013). We also observed improved tolerance of both transgenic lines to drought, heat and stress combination. This is apparent from several parameters reflecting stress impact. Transgenic tobacco maintained higher RWC and *DEH* transcripts, the marker of drought stress, was on lower level than in WT, these plants were also obviously less wilted than WT. In dependence on abiotic stress defence strategy, both down-regulation (Werner *et al.* 2010, Macková *et al.* 2013) as well as up-regulation of endogenous CK level (Metwally *et al.* 1997, Rulcová *et al.* 2001, Rivero *et al.* 2007 a 2009, Merewitz *et al.* 2011a, 2011b, 2012, Zhang *et al.* 2010), have a positive effects on stress tolerance what indicates that plants may adopt different strategies to cope with abiotic stresses. The mechanism of CK action in stress responses has not yet been fully elucidated. Drought tolerance of plants with lowered CK content seems to be associated with their enhanced root system, slower growth rates and altered leaf morphology (Werner *et al.* 2010, Nishiyama *et al.* 2011). On the other hand, stress- or senescence-induced CK up-regulation in transgenic plants seems to diminish negative stress effects by stabilizing photosynthesis. The overexpression of *CKX1* gene fused with root-specific promoter *WRKY6*,

enables plants to utilize both approaches. High *CKX1* expression under control conditions positively affects root growth, whilst the promoter switches-off *CKX1* expression under stress conditions. This mechanism was found to lead to CK elevation during the early stages of the stress response (Macková *et al.* 2013).

We found that modulation of CK content affected antioxidant enzyme system under both control and stress conditions. The function of antioxidant enzyme system is to maintain ROS levels at non-harmful concentrations. ROS are formed constitutively under normal conditions, but their production is elevated under stress, which consequently leads to the stimulation of antioxidant enzyme system. The response of antioxidant enzyme system to the stresses that are very common in nature, i.e. drought and heat, was addressed. As a combination of these stresses frequently occurs and as it is not possible to predict plant response to combined stress based on their reaction to individual stresses (Mittler 2005, Rizhsky *et al.* 2004), a combination of drought and heat stresses was applied as a treatment in this experiment.

Our expression study of nine genes encoding components of the antioxidant enzyme system in tobacco plants with modulated CK content under drought and heat, alone and in combination, revealed a complicated picture. Our results indicate a complex regulation of antioxidant enzyme system at the level of gene expression, which is influenced by CK. The assayed antioxidant enzymes may be grouped according to their functions: (1) chloroplastic enzymes protecting the photosynthetic apparatus - sAPX, tAPX, FeSOD; (2) enzymes with general stress defence function - cAPX, CAT3, cytosolic CuZnSOD; (3) peroxisomal protecting enzymes - CAT1, CAT2 and CAT3 removing H_2O_2 produced by photorespiration in stress; and (4) mitochondrial MnSOD enzyme removing ROS produced during respiration.

Chloroplastic enzymes

Under optimal conditions, electron transport in the chloroplasts is assumed to be the principal ROS source in leaf cells during daylight. Genes of chloroplastic antioxidant enzymes, *sAPX*, *tAPX*, and *FeSOD*, were all strongly transcribed under control conditions. The chloroplastic enzymes protect individual components of the photosynthetic apparatus. The enzyme sAPX scavenges H_2O_2 in stroma where it serves as second defence after primary protection by tAPX and SOD, which are bound in the vicinity of photosystem I (Asada 2006). The tAPX is functionally associated with photosystem I (Yabuta *et al.* 2002, Asada 2006).

Chloroplastic FeSOD is responsible for the elimination of superoxide produced in photosystem I and protects both photosystems (Van Camp *et al.* 1997, Zhang *et al.* 2011). Despite functional and spatial specialization of chloroplastic antioxidant enzymes, their expression patterns were highly coordinated, as indicated by correlation coefficients and PCA.

Transcription of *sAPX* and *FeSOD* in leaves of *W6:CKX1* plants in control conditions did not differ significantly from WT, what corresponded well to the similar CK content in leaves of both genotypes (Macková *et al.* 2013). The *35S:CKX1* plants, which have reduced CK levels, exhibited expression of *sAPX* and *FeSOD* in upper leaves under control conditions comparable with WT. The transformant showed, however, a much higher expression of both genes in middle leaves in comparison with WT. This correlated well with their relatively high photosynthetic activity, namely of photosystem II, reported in this genotype by Cortleven and Valcke (2012). Surprisingly, expression of *sAPX* was also relatively high in roots, particularly in roots of *W6:CKX1* plants. Expression of chloroplastic *APX* genes was previously observed in roots of spinach (Yoshimura *et al.* 2002), but their role in roots has not yet been elucidated. The expression of *tAPX*, which is functionally associated with photosystem I (Yabuta *et al.* 2002, Asada 2006), did not significantly differ between any of the genotypes under investigation.

Stress conditions bring about changes in the metabolic activity of plants. The photosynthetic activity is depressed during both drought (Ramachandra *et al.* 2004, Rivero *et al.* 2009) as well as elevated temperature (Allakhverdief and Kreslavski 2008). Photosystem II is one of the most sensitive protein complexes in the entire cell (Adir *et al.* 2003). Its key component, D1 protein, is easily damaged by ROS, which contributes to down-regulation of photosynthesis by so called photoinhibition. It could be one of the protective mechanisms against overreduction of photosynthetic apparatus (Somersalo and Krause 1990).

We observed a decrease in expression of chloroplastic *sAPX* in all tested stress conditions in WT and *W6:CKX1* plants. This could reflect a lower ROS production in their chloroplasts as a result of reduced photosynthesis under stress. However, in *Arabidopsis* after drought, *sAPX* expression slightly increased (Koussevitzky *et al.* 2008). In case of heat treatment, our data are in accordance with Rizhsky *et al.* (2002) who found diminished *sAPX* expression in tobacco after heat stress. Opposite reaction was observed in *Arabidopsis*, where *sAPX* expression increased under elevated temperature (Panchuk *et al.* 2002). Plants *35S:CKX1* maintained higher *sAPX* expression than WT under stress. This might be related to a greater tolerance of *35S:CKX1*genotype to stress conditions and/or to structural changes in their photosystem II that were found by Cortleven and Valcke (2012).

Expression of *tAPX* was also lowered in stress in both WT and *W6:CKX1*, but it was generally maintained in *35S:CKX1* plants. Drop in *tAPX* expression after drought and stress combination was observed in *Arabidopsis* (Koussevitzky *et al.* 2008). On the contrary, tAPX expression increased after heat stress in *Arabidopsis* (Panchuk *et al.* 2002). In tobacco, changes of *tAPX* expression after stresses were not significant (Rizhsky *et al.* 2002). Maintenance of high tAPX expression in *35S:CKX1* could be plausibly connected to lower stress sensitivity of this genotype (Werner *et al.* 2008).

In the case of *FeSOD*, we observed its decrease in upper leaves of WT and *W6:CKX1* after heat stress, what was observed earlier in tobacco after 2 hours of heat by Tsang *et al.* (1991). These authors also reported that *FeSO*D transcripts increased whenever oxidative stress appeared in chloroplasts. Thus we can deduce that probably after two hours of heat treatment, the photosynthesis was supressed and production of ROS in chloroplasts did not increased in our plants.

Compared to WT, *FeSOD* transcription in leaves was higher under stress conditions in both transgenic lines, particularly in *35S:CKX1*. Thus, *FeSOD* expression seems to correlate positively with stress tolerance, as was formerly suggested by Van Camp *et al.* (1996a).

We noticed a decrease in *tAPX* and *FeSOD* expression with leaf age in all tested genotypes. A similar trend was found in *Arabidopsis* (Panchuk *et al.* 2005) and partially in tobacco (Priault *et al.* 2007). This could be attributed to the lowered photosynthetic activity in older leaves (Panchuk *et al.* 2005, Mýtinová *et al.* 2006, Šesták *et al.* 1978). On the contrary, expression of *sAPX* did not display dependence on leaf age in tobacco, similarly as in *Arabidopsis*, where it decreased only in the latest stages of senescence (Panchuk *et al.* 2005).

Our data indicate a relationship between CK level and the antioxidant defence response in chloroplasts mediated indirectly by improved stress tolerance.

Enzymes with general stress defence function

N. tabacum and *N. plumbaginifolia* contain three CuZnSOD isoforms (Van Camp *et al.* 1997, Ragusa *et al.* 2001) and CuZnSOD in *N. plumbaginifolia* are encoded by a single gene (Hérouart *et al.* 1994).

Certainly, enzymes believed to play a general role in stress protection, i.e. cAPX, CAT3 (Panchuk *et al.* 2005) and cytosolic CuZnSOD were expressed to a lower extent in optimal conditions than under stress, which accords with previously published data from

Arabidopsis (Du *et al.* 2008). The transcript levels were comparable in WT and both transgenic lines under control conditions, indicating that overexpression of inserted *AtCKX1* did not induce any imbalance with result in oxidative stress. However this statement cannot be overgeneralized. Contrarily *A. thaliana* overexpressing *CKX4* showed reduced antioxidant capacity (Cortleven *et al.* 2014).

The expression profiles of genes of this group showed a similar response to applied stresses in all tested genotypes, as indicated both by strong correlation between *cAPX* and *CuZnSOD* and a weaker one between *CAT3* and *CuZnSOD*. Principal component analysis also indicates similar expression profile, all these three genes are grouped.

Heat shock and stress combination induced a high level of expression of *CAT3*, *CuZnSOD* and especially of *cAPX*, whereas drought did not elevate their expression. These results are in agreement with the reported stimulation of *cAPX* expression in heat stressed tobacco (Rizhsky *et al.* 2002) and *Arabidopsis* (Panchuk *et al.* 2002). Besides this, protection by cAPX was found to be crucial in response to drought combined with heat, when cAPX-deficient mutant was more sensitive to this stress combination (Koussevitzsky *et al.* 2008). No effect of stress combination on *CAT3* expression was previously observed in tobacco (Willekens *et al.* 1995).

Despite the general similarity of the stress responses of these enzymes, reactions of WT differed from those of the *35S:CKX1* transformants. The expression of *cAPX* was similar in WT and *W6:CKX1* plants under heat stress, but was lower in *35S:CKX1* plants. On the other hand, expression of *CAT3* and *CuZnSOD* was higher in *35S:CKX1* than in WT. It is apparent that the expression of these protective antioxidant enzymes under heat is modified in plants with lower CK content, which are more tolerant to this stress (Macková *et al.* 2013). Hypothetically, plants overexpressing *CKX1* could be more tolerant against stress due to their higher CuZnSOD activities. It was previously found out that tobacco or potato overexpressing cytosolic CuZnSOD were more tolerant of ozone (Pitcher *et al.* 1996) or of paraquat (Perl *et al.* 1993).

Peroxisomal protecting enzymes

The peroxisomal enzymes CATs, are homo- or heterotetramers, consisting of three different subunits coded by genes *CAT1*, *CAT2*, and *CAT3* (Willekens *et al.* 1994). We found extensive transcription of *CAT1* and *CAT2*, but not *CAT3*, in leaves of all genotypes under control conditions. Our data accord with the report of Luna *et al.* (2005).

Stress conditions generally reduce photosynthesis in favour of photorespiration. Thus, drought and heat promote photorespiration, which is associated with production of H_2O_2 in peroxisomes. Subsequently, the demand for antioxidant defence in peroxisomes increases. However, increased temperature causes not only photoinhibition of photosynthesis but also photoinactivation of CAT (Willekens *et al.* 1995, Herwing *et al.* 1992).

The *CAT3* expression was strongly stimulated by all stresses, predominantly by their combination. CAT3 seems to provide majority of H_2O_2 scavenging in peroxisomes under stress conditions.

Different expression profiles as well as the lack of correlation among them indicate different function and regulation of *CAT* isoforms. Generally, the opposite trends in *CAT1* and *CAT2* expression, both in reactions to stress and also expression profiles within leaf gradient, indicate an absence of *CAT1* and *CAT2* functional redundancy. *CAT1* expression in combined stress was similar to the expression in drought. Such behaviour differed from that of a group of other general stress protective enzymes and indicates that not all antioxidant enzymes react to the last applied stress.

Mitochondrial MnSOD expression and activity

Mitochondrial MnSOD removes superoxide generated by electron leakage to molecular oxygen in a respiratory electron transport chain (Bowler *et al.* 1989, Van Camp *et al.* 1996b). The gradient in MnSOD activity in favour of upper leaves found in WT agrees with the negative correlation between the expression as well as the activity of MnSOD and leaf age in tobacco, reported by Priault *et al.* (2007).

It is further known that both expression and activity of MnSOD in tobacco can be stimulated by exogenous application of sucrose (Bowler *et al.* 1989). Taking into account that CK control sink/source distribution of sugars, it is possible to anticipate that CK regulate MnSOD through modulation of carbohydrate metabolism via sink/source dynamics (Cowan *et al.* 2005, Roitsch and Ehneß 2000). Indeed changes in MnSOD expression and activity among leaves of different ages correspond to this assumption. Young leaves that are a sink for sugars

(Turgeon 1989) contained high level of *MnSOD* transcripts as well as high activity of MnSOD1, a protein coded by this gene, in WT under control conditions. The opposite situation was observed in old leaves that are carbohydrate sources and their respiration was lower (Ducet and Rosenberg 1962). Old leaves produced lower amount of *MnSOD* transcript and had also lower MnSOD1 activity.

Moreover, CK probably play role in signalling for MnSOD expression, which is indicated by the fact that *35S:CKX1* plants that contain reduced concentration of soluble sugars as well as of CK (Werner *et al.* 2008) exhibited lower MnSOD1 activity.

In contrast to low MnSOD1 activity, expression of *MnSOD* was very abundant in *35S:CKX1* plants. The discrepancy between the activity and transcript levels was reported also for other antioxidant enzymes, e.g. APX (de Campos *et al.* 2011). The lack of correspondence may be given by transcript stability, post-transcriptional or post-translational regulations (de Carvalho *et al.* 2013). Our results indicate that the level of *MnSOD* transcript does not directly determine MnSOD1 activity.

Antioxidant enzymes on the level of active protein

APX are coded by multigene family and a number of APX isoforms differs among employed plants and also differs according to authors, e.g. in *Arabidopsis* were identified eight (Zilinskas and Mittler 1993) or seven (Jespersen *et al.* 2010) APX isoforms. We identified nine APX enzymes in zymograms in tobacco, what corresponds to a number of APX genes. The most active isoforms were APX1, APX5, APX7 and APX9 in our tobacco ecotypes and growing conditions.

Number of CAT genes, coding for CAT subunits, is probably definitive and equals three, however number of CAT proteins varies. For example, in *N.tabacum* cv. Petit Havana six bands of CAT were found (Zeltich *et al.* 1991), in *N. tabacum* cv. Xanthi 12 CAT isoforms were identified on native IEF gel (Siminis *et al.* 1994). We discovered nine CAT isoenzymes. However this acquired number might not be definitive, because one dimensional native electrophoresis used in these studies has separation limits, and with two dimensional system better resolution could yet be achieved.

We did not observe significant changes in activity of CAT isoforms as well as on level of total CAT activity in different stress conditions. Even not after heat shock, which inhibits

CAT protein synthesis, for example in *Secale cereale* (Hertwing *et al.* 1992) and also CAT activity was found to be photoinhibited under high temperature (Willekens *et al.* 1995). Differences, which were manifested among *CAT* transcripts, could be connected to changing need of variable combination of CAT subunits in CAT heterotetramers on the level of active protein. Unfortunately, system of organizing and combining of CAT subunits in tetramers is not clear yet. Moreover, CAT proteins are not clearly distinguishable on native electrophoresis because CAT isoforms evince heterogeneity originating also from polymorphism, epigenetic variations, and/or oxidation of proteins (Kunce and Trelease 1986).

Although *CAT* transcripts are also present in roots according to our and Du *et al.* (2008) results, CAT activity was not detectable in roots either on PAGE gels or in extracts.

We identified six SOD isoforms in *N. tabacum* cv. Samsun NN, namely one MnSOD, two FeSOD and three CuZnSOD. Individual authors differ in opinion of SOD isoform number, for instance in *N. tabacum* five SOD isoforms were found, namely one MnSOD, one FeSOD, three CuZnSOD (Sheng *et al.* 2004), as well as in *N. plumbaginifolia* (Ragusa *et al.* 2001). Furthermore, VanCamp *et al.* (1996b) isolated two MnSOD isoforms from *N. plumbaginifolia* and found three FeSOD (Van Camp *et al.* 1994), in *N. sylvestris* only three active SOD isoforms were characterized (Priault *et al.* 2007). Differences between observations could be explained by plant species, age of analysed material, analysed tissue (Van Camp *et al.* 1996a, b and 1997).

FeSOD was identified as tobacco chloroplastic stromatal isoform (Van Camp *et al.* 1990) scavenging superoxide produced by photosystem I (Asada 1999) and was reported to protect plasma membrane and photosystem II in tobacco (Van Camp *et al.* 1996a). FeSOD1 was the most active SOD isoform within whole plants, except upper leaves where CuZnSOD activity was also high, under all tested conditions in all tobacco lines.

Isoenzyme FeSOD1 displayed an activity gradient decreasing from upper to lower leaves. In *N. sylvestris*, FeSOD activity gradient was increasing from upper to lower leaves (Priault *et al.* 2007), we suppose that the *N. sylvestris* FeSOD corresponds to our FeSOD2, which tend to show activity gradient increasing from upper to lower leaves.

Investigations of *FeSOD* expression often reveal gradient sloping forward lower leaves what indicates that these *FeSOD* transcripts could match up mRNA for FeSOD1 protein with the same trend.

FeSODs were the main isoforms active in roots despite they are localized to chloroplasts where it protects photosystems.

CuZnSOD in tobacco is present in cytosol (Tsang *et al.* 1991), chloroplast or mitochondria (Van Camp *et al.* 1994). We identified three CuZnSOD isoforms in *N. tabacum* cv. Samsun NN, similarly as in *N. plumbaginifolia* (Van Camp *et al.* 1994, Ragusa *et al.* 2001). CuZnSOD in *N. plumbaginifolia* is encoded by a single gene (Hérouart *et al.* 1994), however, our results showed there are more genes coding for CuZnSOD in *N. tabacum.*

Tobacco CuZnSODs are usually detectable only in immature leaves (Van Camp *et al.* 1996a, 1997, Kurepa *et al.* 1997). In agreement with previous results, we detected the high CuZnSOD activity in upper leaves, however we also detected CuZnSOD activity in older leaves. A low CuZnSOD1 and -3 activity was in middle leaves, and a very low activity of CuZnSOD3 in lower leaves in WT. CuZnSOD activity is dependent on developmental stage and leaf age with high activity in young leaves (Van Camp *et al.* 1996a). As transgenic plants showed slowed down development and postponed onset of senescence (Werner *et al.* 2001, Schmülling, 2002, Mýtinová *et al.* 2011), transgenic plants could be expected to contain significantly increased CuZnSOD activity in comparison to WT. However this was not found out in our experiments.

Stimulation of CuZnSOD activity by stress treatment was observed in tobacco earlier (Kurepa *et al.* 1997, Van Camp *et al.* 1994, Van Camp *et al.* 1996a, Bowler *et al.* 1991, Hérouart *et al.* 1994). Our experiment did not bring any significant increase after stress treatment.

Our data contributed to creation of a picture depicting antioxidant system defence in stress. We brought a new point of view and put in context CK impact with both expression and activity of antioxidant enzyme system, which were previously studied only separately. Connection of modulated CK levels and stress combination provoked reactions of antioxidant enzyme system that revealed interrelationships among antioxidant enzyme system components and also between antioxidant enzyme system and CK.

In conclusion, tobacco plants with down-regulated CK content differed in their stress tolerance as well as in antioxidant enzyme system responses compared to WT. Enhanced stress tolerance was reflected by better preservation of photosynthetic activity under stress conditions, as indicated by higher expression of photosynthesis related antioxidant genes. The impact of down-regulation of CK levels on antioxidant enzyme system seems to be indirect, mediated by the positive effect on plant stress tolerance or on photosynthesis.

The defence mediated by antioxidant enzyme system exerts a high plasticity. It is specifically regulated under various stress conditions, often exhibiting difference between the

response to a single stress and to combined stresses. The distinct contrast is apparent for example after application of heat at the end of drought either increase expression of cytosolic genes (*cAPX*, *CuZnSOD*) and *CAT3*, while chloroplastic and mitochondrial related gene expression decrease (*sAPX*, *tAPX*, *MnSOD*, *FeSOD*).

10. Conclusions

- We accept hypothesis that stress response of antioxidant enzymes in plants overexpressing *CKX1* differs from that in WT. Transcripts of *sAPX*, *CAT1*, *CAT3*, *MnSOD*, *FeSOD* and *CuZnSOD* plus APX and MnSOD activity differed between WT and *35S:CKX1*. *MnSOD* and *FeSOD* expression also differed in *W6:CKX1*. These antioxidant enzymes were indirectly influenced by diminished CK level. Chloroplastic enzymes were probably influenced through modified photosystems, what in turn impact peroxisomal CAT because there were strong correlations between chloroplastic and peroxisomal enzymes expressions. MnSOD was probably affected through sugar metabolism in which source/sink management CK plays role.

- Transgenic plants *35S:CKX1* showed increased expression of *sAPX*, *CAT1*, *CAT3*, *MnSOD* and *FeSOD* namely in stress conditions, however, we reject null hypothesis that improved stress tolerance of plants overexpressing *CKX1* is caused by enhanced antioxidant enzyme system. We conclude that increased antioxidant enzyme expression was not a cause of improved stress tolerance, but probably a consequence. In addition, activity of APX and MnSOD, which were enhanced in WT under stress, did not increase in *35S:CKX1*. Improved stress tolerance of transgenic plants was probably connected to changes of habit, as enhanced root system, or modified photosystem, which provided advantage for these plants to endure stress conditions.

- Leaf age influenced antioxidant enzyme expression and activity. Transcripts of *tAPX* and *CAT1* showed gradient decreasing from upper to lower leaves. Contrariwise, *CAT2*, *CAT3*, *MnSOD* transcripts and MnSOD, FeSOD1, as well as CuZnSOD1 - 3 activity were lowest in upper and highest in lower leaves.

The defence mediated by antioxidant enzymatic system exerts a high plasticity. It is specifically tuned under various stress conditions, exhibiting difference between the response to a single stress and to combined stresses. The application of additional stress may either increase (*cAPX*, *CAT3*, *CuZnSOD*) or decrease (*sAPX*, *tAPX*, *FeSOD, MnSOD*) the expression

of genes coding for antioxidant enzymes after sole stress. The impact of down-regulation of CK levels on antioxidant enzyme system seems to be indirect, mediated by the positive effect on plant stress tolerance or on photosynthesis.

11. References

Alscher, R. G., Erturk, N., Heath, L. S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. Journal of Experimental Botany **53**: 1331-1341.

Bano, A., Dorffling, K., Bettin D., Hahn, H. (1993). Abscisic acid and cytokinins as possible root-to-shoot signals in xylem sap of rice plants in drying soil. Functional Plant Biology **20**: 109–115.

Adir, N., Zer, H., Shochat, S., Ohad, I. (2003). Photoinhibition-a historical perspective. Photosynthesis Research **76**: 343–370.

Aida, M., Beis, D., Heidstra, R., Willemsen, V., Blilou, I., Galinha, C., Nussaume L., Noh, Y.-S., Amasino R., Scheres, B. (2004). The *PLETHORA* genes mediate patterning of the *Arabidopsis* root stem cell niche. Cell **119**: 109-120.

Allakhverdiev, S.I., Kreslavski, V.D., Klimov, V.V., Los, D.A., Carpentier, R., Mohanty, P. (2008). Heat stress: an overview of molecular responses in photosynthesis. Photosynthesis Research **98**: 541–550.

Alvarez, S., Marsh, E. L., Schroeder, S. G., Schachtman, D. P. (2008). Metabolomic and proteomic changes in the xylem sap of maize under drought. Plant, Cell & Environment **31**: 325-340.

Armstrong, D.J. (1994). Cytokinin oxidase and the regulation of cytokinin degradation. In Mok D.W., Mok M.C. (Eds.) Cytokinins: Chemistry, Activity, and Function, pp. 139–154. CRC Press, Boca Raton, FL.

Asada, K., Takahashi, M. (1987). Production and scavenging of active oxygen in chloroplasts. In Kyle D.J., Osmond C.B., Arntzen C.J. (Eds) Photoinhibition, pp. 227–287. Elsevier, Amsterdam.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiology **141**: 391–396.

Asada, K. (1999). The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Biology **50**: 601-639.

Bannister, J. V., Bannister, W. H., Rotilio, G. (1987). Aspects of the structure, function, and applications of superoxide dismutas. Critical Reviews in Biochemistry and Molecular Biology **22**: 111-180.

Bannister, W. H., Bannister, J. V., Barra, D., Bond, J., Bossa, F. (1991). Evolutionary aspects of superoxide dismutase: the copper/zinc enzyme. Free Radical Research **12**: 349-361.

Bano, A., Hansen, H., Dörffling, K., Hahn, H. (1994). Changes in the contents of free and conjugated abscisic acid, phaseic acid and cytokinins in xylem sap of drought stressed

sunflower plants. Phytochemistry **37**: 345–347.

Banowetz, G. M., Ammar, K., Chen, D. D. (1999a). Postanthesis temperatures influence cytokinin accumulation and wheat kernel weight. Plant, Cell & Environment **22**: 309-316.

Banowetz, G. M., Ammar, K., Chen, D. D. (1999b). Temperature effects on cytokinin accumulation and kernel mass in a dwarf wheat. Annals of Botany **83**: 303-307.

Barón, M., Lachica, M., Chueca, A., Sandmann, G. (1990). The role of copper in the structural organization of Photosystem II in chloroplast membranes. In Baltscheffsky M. (Ed.) Current Research in Photosynthesis, pp. 303-306. Springer Netherlands.

Basu, P., Ali, M., Chaturvedi, S. (2007). Osmotic adjustment increases water uptake, remobilization of assimilates and maintains photosynthesis in chickpea under drought. Indian Journal of Experimental Biology **45**: 261.

Beauchamp, C., Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry **44**: 276–287.

Beyer Jr, W. F., Fridovich, I. (1987). Effect of hydrogen peroxide on the iron-containing superoxide dismutase of *Escherichia coli*. Biochemistry **26**: 1251-1257.

Bienert, G. P., Møller, A. L., Kristiansen, K. A., Schulz, A., Møller, I. M., Schjoerring, J. K., Jahn, T. P. (2007). Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. Journal of Biological Chemistry **282**: 1183-1192.

Blanvillain, R., Wei, S., Wei, P., Kim, J. H., Ow, D. W. (2011). Stress tolerance to stress escape in plants: role of the OXS2 zinc-finger transcription factor family. The EMBO Journal **30**: 3812-3822.

Boon, E. M., Downs, A., Marcey, D. (2007). Catalase: H_2O_2: H_2O_2 Oxidoreductase. http://www.callutheran.edu/BioDev/omm/catalase/cat1.htm.

Bordo, D., Djinovic, K., Bolognesi, M. (1994). Conserved patterns in the Cu, Zn superoxide dismutase family. Journal of Molecular Biology **238**: 366-386.

Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M., Inzé, D. (1991). Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. The EMBO Journal **10**: 1723.

Bowler, C., Montagu, M. V., Inze, D. (1992). Superoxide dismutase and stress tolerance. Annual Review of Plant Biology **43**: 83-116.

Bowler, C., Alliotte, T., De Loose, M., Van Montagu, M., Inzé, D. (1989). The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. The EMBO Journal **8**: 31- 38.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of

microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**: 248–254.

Caers, M., Vendrig, J. C. (1984). Effects of high temperature on roots of etiolated maize seedlings: photosynthetic activity and chloroplast ultra-structure. In Sybesma C. (Ed.) Advances in Photosynthesis Research, pp. 271-274. Springer Netherlands.

Cornic, G. (2000). Drought stress inhibits photosynthesis by decreasing stomatal aperture–not by affecting ATP synthesis. Trends in Plant Science **5**(5): 187-188.

Cortleven, A., Valcke, R. (2012). Evaluation of the photosynthetic activity in transgenic tobacco plants with altered endogenous cytokinin content: lessons from cytokinin. Physiologia Plantarum **144**: 394–408.

Cortleven, A., Noben, J.-P., Valcke, R. (2011). Analysis of the photosynthetic apparatus in transgenic tobacco plants with altered endogenous cytokinin content: a proteomic study. Proteome Science **9**: 1–14.

Cortleven, A., Remans, T., Brenner, W.G., Valcke, R. (2009). Selection of plastid- and nuclear-encoded reference genes to study the effect of altered endogenous cytokinin content on photosynthesis genes in *Nicotiana tabacum*. Photosynthesis Research **102**: 21–29.

Cortleven, A., Nitschke, S., Klaumünzer, M., Abdelgawad, H., Asard, H., Grimm, B., Riefler, M., Schmülling, T. (2014). A novel protective function for cytokinin in the light stress response is mediated by the Arabidopsis histidine kinase2 and Arabidopsis histidine kinase3 receptors. Plant Physiology **164**: 1470-1483.

Cowan, A.K., Freeman, M., Björkman, P.-O., Nicander, B., Sitbon, F., Tillberg, E. (2005). Effects of senescence-induced alteration in cytokinin metabolism on source-sink relationships and ontogenic and stress-induced transitions in tobacco. Planta **221**: 801–814.

Demmig-Adams, B., Adams, W.W. (1996a). The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends in Plant Science **1**: 21-26.

Demmig-Adams, B., Adams, W.W. (1996b). Xanthophyll cycle and light stress in nature: uniform response to excess direct sunlight among higher plant species. Planta **198**: 460-470.

Desikan, R., Soheila, A. H., Hancock, J. T., Neill, S. J. (2001). Regulation of the Arabidopsis transcriptome by oxidative stress. Plant Physiology **127**: 159-172.

Dobrá, J., Motyka, V., Dobrev, P., Malbeck, J., Prášil, I.T., Haisel, D., Gaudinová, A., Havlová, M., Gubiš, J., Vaňková, R. (2010). Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. Journal of Plant Physiology **167**: 1360–1370.

Du, Y.-Y., Wang, P.-C., Chen, J., Song, C.-P. (2008). Comprehensive functional analysis of the catalase gene family in *Arabidopsis thaliana*. Journal of Integrative Plant Biology **50**: 1318–1326.

Ducet, G. Rosenberg, A. (1962). Leaf respiration. Annual Review of Plant Physiology **13**: 171–200.

Emery, R.J., Ma, Q. Atkins, C.A. (2000). The forms and sources of cytokinins in developing white lupine seeds and fruits. Plant Physiology **123**: 1593–1604.

Enami, I., Kamo, M., Ohta, H., Takahashi, S., Miura, T., Kusayanagi, M., Tanabe, S., Kamei, A., Motoki, A., Hirano, M., Tomo, T., Satoh, K. (1998). Intramolecular cross-linking of the extrinsic 33-kDa protein leads to loss of oxygen evolution but not its ability of binding to photosystem II and stabilization of the manganese cluster. Journal of Biological Chemistry **273**: 4629-4634.

Feierabend J. (2005). Catalases in plants: molecular and functional properties and role in stress defence. In Smirnoff N. (Ed.) Antioxidants and Reactive Oxygen Species in Plants, pp. 101- 140. Blackwell Publishing.

Fita, I., Rossmann, M. G. (1985). The active center of catalase. Journal of Molecular Biology **185**: 21-37.

Foyer, C. H., Noctor, G. (2003). Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiologia Plantarum **119**(3): 355-364.

Fridovich I. (1986). Superoxide dismutases. Advances in Enzymology and Related Areas of Molecular Biology **58**: 61- 97.

Frugoli, J.A., Zhong, H.H., Nuccio, M.L., McCourt, P., McPeek, M.A., Thomas, T.L., McClung, C.R. (1996). Catalase is encoded by a multigene family in *Arabidopsis thaliana* (L.) Heynh. Plant Physiology **112**: 327–336.

Fry, S.C., Dumville, J.C., Miller, J.G. (2001). Fingerprinting of polysaccharides attacked by hydroxyl radicals in vitro and in the cell walls of ripening pear fruit. Biochemical Journal **357**: 729-737.

Galuszka, P., Frébort, I., Šebela, M., Sauer, P., Jacobsen, S., Peč, P. (2001). Cytokinin oxidase or dehydrogenase? European Journal of Biochemistry **268**: 450–461.

Galuszka, P., Popelková, H., Werner, T., Frébortová, J., Pospíšilová, H., Mik, V., Köllmer, I., Schmülling, T., Frébort, I. (2007). Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. Journal of Plant Growth Regulation **26**: 255–267.

Gapper, C. Dolan, L. (2006). Control of plant development by reactive oxygen species. Plant Physiology **141**: 341–345.

García-Plazaola, J. I., Matsubara, S., Osmond, C. B. (2007). The lutein epoxide cycle in higher plants: its relationships to other xanthophyll cycles and possible functions. Functional Plant Biology **34**: 759-773.

Ghanem, M. E., Albacete, A., Smigocki, A. C., Frébort, I., Pospíšilová, H.,

Martínez-Andújar, C., Acosta, M., Sánchez-Bravo, J., Lutts, S., Dodd, I.C., Pérez-Alfocea, F. (2011). Root-synthesized cytokinins improve shoot growth and fruit yield in salinized tomato (*Solanum lycopersicum* L.) plants. Journal of Experimental botany **62**: 125-140.

Gidrol X, Lin WS, Dégousée N, Yip SF, Kush A. (1994). Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. European Journal of Biochemistry **224**: 21–28.

Gill, S.S. Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry **48**: 909–930.

Giulini, A., Jing W., Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. Nature **430**: 1031-1034.

Harding, S. A., Oh, S. H., Roberts, D. M. (1997). Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species. The EMBO Journal **16**: 1137-1144.

Hare, P., Cress, W., Van Staden, J. (1997). The involvement of cytokinins in plant responses to environmental stress. Plant Growth Regulation **23**: 79–103.

Havlová, M., Dobrev, P.I., Motyka, V., Štorchová, H., Libus J., Dobrá, J., Malbeck, J., Gaudinová, A., Vaňková, R. (2008). The role of cytokinins in responses to water deficit in tobacco plants over-expressing trans-zeatin O-glucosyltransferase gene under *35S* or *SAG12* promoters. Plant, Cell & Environment **31**: 341–353.

Hayakawa, T., Kanematsu, S., Asada, K. (1985). Purification and characterization of thylakoid-bound Mn-superoxide dismutase in spinach chloroplasts. Planta **166**: 111-116.

Herouart D, Van Montagu M, Inzé D. (1994). Developmental and environmental regulation of the *Nicotiana plumbaginifolia* cytosolic Cu/Zn-superoxide dismutase promoter in transgenic tobacco. Plant Physiology **104**: 873–880.

Hertwig B, Streb P, Feierabend J. (1992). Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. Plant Physiology **100**: 1547–1553.

Chance, B., Boveris, A., Oshino, N., Loschen, G. (1973). The nature of the catalase intermediate in its biological function. In King, T.E.; Mason, H.S. In Morrison, M. (Ed.) Oxidases and Related Redox Systems, pp. 350-353. University Park Press.

Chance, B., Sies, H., Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. Physiological Reviews **59**: 527-605.

Chaves, M. (1991). Effects of water deficits on carbon assimilation. Journal of Experimental Botany **42**: 1–16.

Cheikh, N., Jones, R.J. (1994). Disruption of maize kernel growth and development by heat stress (role of cytokinin/abscisic acid balance). Plant Physiology **106**: 45–51.

Chen, C.-M., Kristopeit, S.M. (1981a). Metabolism of cytokinin dephosphorylation of cytokinin ribonucleotide by 5′-nucleotidases from wheat germ cytosol. Plant Physiology **67**: 494-498.

Chen, C.-M., Kristopeit, S.M. (1981b). Metabolism of cytokinin: deribosylation of cytokinin ribonucleoside by adenosine nucleosidase from wheat germ cells. Plant Physiology **68**: 1020-1023.

Chew, O., Whelan, J., Millar, A.H. (2003). Molecular definition of the ascorbate-glutathione cycle in Arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants. Journal of Biological Chemistry **278**: 46869–46877.

Choi, J., Choi, D., Lee, S., Ryu, C.-M., Hwang, I. (2011). Cytokinins and plant immunity: old foes or new friends? Trends in Plant Science **16**: 388–394.

Itai, C., Benzioni, A., and Munz, S. (1978). Heat stress: Effects of abscisic acid and kinetin on response and recovery of tobacco leaves. Plant and Cell Physiology **19**: 453–459.

Jaspers, P., Kangasjärvi, J. (2010). Reactive oxygen species in abiotic stress signaling. Physiologia Plantarum **138**: 405–413.

Jespersen, H., Kjrd, I., Stergaard, L., Welinder K. (1997). From sequence analysis of three novel ascorbate peroxidases from *Arabidopsis thaliana* to structure, function and evolution of seven types of ascorbate peroxidase. Biochemical Journal **326**: 305–310.

Jones, R.J. Schreiber, B.M. (1997). Role and function of cytokinin oxidase in plants. Plant Growth Regulation **23**: 123–134.

Kaiser, W. M. (1979). Reversible inhibition of the Calvin cycle and activation of oxidative pentose phosphate cycle in isolated intact chloroplasts by hydrogen peroxide. Planta **145**: 377-382.

Kanematsu, S., Asada, K. (1989). CuZn-superoxide dismutases in rice: occurrence of an active, monomeric enzyme and two types of isozyme in leaf and non-photosynthetic tissues. Plant and cell physiology **30**: 381-391.

Kaplan, F., Guy, C.L. (2004). β-Amylase induction and the protective role of maltose during temperature shock. Plant Physiology **135**: 1674–1684.

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., Yamaguchi, S., Sakakibara, H. (2004). Distinct isoprenoid origins of cis-and trans-zeatin biosyntheses in *Arabidopsis*. Journal of Biological Chemistry **279**: 14049-14054.

Kempa, S., Krasensky, J., Dal Santo, S., Kopka, J., Jonak, C. (2008). A central role of abscisic acid in stress-regulated carbohydrate metabolism. PLoS One **3**: e3935.

Kim, C., Meskauskiene, R., Apel, K., Laloi, C. (2008). No single way to understand singlet oxygen signalling in plants. EMBO Reports **9**: 435–439.

Kim, H.-S., Kim, Y.-S., Hahn, K.-W., Joung, H., Jeon, J.-H. (2009). Reactive oxygen species: regulation of plant growth and development. Advances in Botanical Research **52**: 25–46.

Kim, H. J., Ryu, H., Hong, S. H., Woo, H. R., Lim, P. O., Lee, I. C., Sheen, J., Nam, H.G., Hwang, I. (2006). Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proceedings of the National Academy of Sciences of the U S A **103**: 814-819.

Kliebenstein, D.J., Monde, R.-A., Last, R.L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiology **118**: 637–650.

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R. (2008). Ascorbate peroxidase 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. Journal of Biological Chemistry.

Kowalska, M., Galuszka, P., Frébortová, J., Šebela, M., Béres, T., Hluska, T., Šmehilová, M., Bilyeu, K.D., Frébort, I. (2010). Vacuolar and cytosolic cytokinin dehydrogenases of *Arabidopsis thaliana*: Heterologous expression, purification and properties. Phytochemistry **71**: 1970–1978.

Kudoyarova, G.R., Vysotskaya, L.B., Cherkozyanova, A., Dodd, I.C. (2007). Effect of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (*Solanum lycopersicum* L.) xylem sap and leaves. Journal of Experimental Botany **58**: 161–168.

Kunce, C.M., Trelease, R.N. (1986). Heterogeneity of catalase in maturing and germinated cotton seeds. Plant Physiology **81**: 1134–1139.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakubara, H., Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445**: 652-655.

Kurepa, J., Hérouart, D., Van Montagu, M., Inzé, D. (1997). Differential expression of CuZn-and Fe-superoxide dismutase genes of tobacco during development, oxidative stress, and hormonal treatments. Plant and Cell Physiology **38**: 463-470.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680–685.

Larcher, W. (1987). Stress bei pflanzen. *Naturwissenschaften* **74**: 158-167.

Leibfried, A., To, J. P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., Lohmann, J. U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438**: 1172-1175.

Letham, D.S. (1994). Cytokinins as phytohormones: sites of biosynthesis, translocation, and function of translocated cytokinin. In Mok D.W., Mok M.C. (Eds.) Cytokinins: Chemistry, Activity, and Function, pp. 57-80. CRC Press, Boca Raton, FL.

Liu, J., Xie, X., Du, J., Sun, J., Bai, X. (2008). Effects of simultaneous drought and heat stress on Kentucky bluegrass. Scientia Horticulturae **115**: 190-195.

Liu, X., Huang, B. (2002). Cytokinin effects on creeping bentgrass response to heat stress. Crop Science **42**: 466–472.

Lledías, F., Rangel, P., Hansberg, W. (1998). Oxidation of catalase by singlet oxygen. Journal of Biological Chemistry **273**: 10630-10637.

Ludlow, M. M., Ng T.T. (1974). Water stress suspends leaf ageing. Plant Science Letters **3**: 235-240.

Luna, C.M., Pastori, G.M., Driscoll, S., Groten, K., Bernard, S., Foyer, C.H. (2005). Drought controls on H2O2 accumulation, catalase (CAT) activity and CAT gene expression in wheat. Journal of Experimental Botany **56**: 417–423.

Lv, W.-T., Lin, B., Zhang, M., Hua, X.-J. (2011). Proline accumulation is inhibitory to *Arabidopsis* seedlings during heat stress. Plant Physiology **156**: 1921–1933.

Macková H., Hronková M., Dobrá J., Turečková V., Novák O., Lubovská Z., Motyka V., Haisel D., Hájek T. Prášil I.T., Gaudinová A., Štorchová H., Ge E., Werner T., Schmülling T., Vaňková R. (2013). Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. Journal of Experimental Botany **64**: 2805-2815.

Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P., Janoff, A.S. (1985). Protection of large unilamellar vesicles by trehalose during dehydration: retention of vesicle contents. Biochimica et Biophysica Acta-Biomembranes **817**: 67–74.

Mak, J., Ho, S., Yu, W., Choo, K., Chu, C., Yew, W., Lam, W., Chan-Yeung, M., and others (2007). Polymorphisms and functional activity in superoxide dismutase and catalase genes in smokers with COPD. European Respiratory Journal **30**: 684–690.

Medford, J. I., Horgan, R., El-Sawi, Z., Klee, H. J. (1989). Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. The Plant Cell Online **1**: 403-413.

Merewitz, E.B., Du, H., Yu, W., Liu, Y., Gianfagna, T., Huang, B. (2012). Elevated cytokinin content in ipt transgenic creeping bentgrass promotes drought tolerance through regulating metabolite accumulation. Journal of Experimental Botany **63**: 1315–1328.

Merewitz, E.B., Gianfagna, T., Huang, B. (2011a). Photosynthesis, water use, and root viability under water stress as affected by expression of SAG12-ipt controlling cytokinin synthesis in *Agrostis stolonifera*. Journal of Experimental Botany **62**: 383–395.

Merewitz, E.B., Gianfagna, T., Huang, B. (2011b). Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an ipt gene for cytokinin synthesis. Journal of Experimental Botany **62**: 5311–5333.

Metwally, A., Tsonev, T., Zeinalov, Y. (1998). Effect of cytokinins on the photosynthetic apparatus in water-stressed and rehydrated bean plants. Photosynthetica **34**: 563–567.

Mittler, R., Zilinskas, B.A. (1994). Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought. The Plant Journal **5**: 397–405.

Mittler, R. (2005). Ascorbate peroxidase. In Smirnoff N. (Ed.) Antioxidants and Reactive Oxygen Species in Plants, pp. 88- 100. Blackwell Publishing.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science **7**: 405–410.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. Trends in Plant Science **11**: 15–19.

Mittler, R. Zilinskas, B.A. (1993). Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. Analytical Biochemistry **212**: 540–546.

Mittler, R., Zilinskas, B. A. (1993). Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. Analytical Biochemistry **212**: 540-546.

Miyawaki, K., Matsumoto-Kitano, M., Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. The Plant Journal **37**: 128–138.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G., Kakimoto, T. (2006). Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proceedings of the National Academy of Sciences USA **103**: 16598-16603.

Mohamed, E. A., Iwaki, T., Munir, I., Tamoi, M., Shigeoka, S., Wadano, A. (2003). Overexpression of bacterial catalase in tomato leaf chloroplasts enhances photo-oxidative stress tolerance. Plant, Cell & Environment **26**: 2037-2046.

Mok, D.W.S., Mok, M.C. (2001). Cytokinin metabolism and action. Annual Review of Plant Biology **52**: 89-118.

Murata, N., Takahashi, S., Nishiyama, Y., Allakhverdiev, S. I. (2007). Photoinhibition of photosystem II under environmental stress. Biochimica et Biophysica Acta - Bioenergetics **1767**: 414-421.

Myouga, F., Hosoda, C., Umezawa, T., Iizumi, H., Kuromori, T., Motohashi, R., Shono, Y., Nagata, N., Ikeuchi, M., Shinozaki, K. (2008). A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in *Arabidopsis.* The Plant Cell Online **20**: 3148–3162.

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

Mýtinová, Z., Motyka, V., Haisel, D., Gaudinová, A., Lubovská, Z., Wilhelmová, N. (2010). Effect of abiotic stresses on the activity of antioxidative enzymes and contents of phytohormones in wild type and AtCKX2 transgenic tobacco plants. Biologia Plantarum **54**: 461–470.

Mýtinová, Z., Motyka, V., Haisel, D., Lubovská, Z., Trávníčková, A., Dobrev, P., Holík, J., Wilhelmová, N. (2011). Antioxidant enzymatic protection during tobacco leaf ageing is affected by cytokinin depletion. Plant Growth Regulation **65**: 23–34.

Nadif, R., Mintz, M., Jedlicka, A., Bertrand, J.-P., Kleeberger, S.R., Kauffmann, F. (2005). Association of CAT polymorphisms with catalase activity and exposure to environmental oxidative stimuli. Free Radical Research **39**: 1345–1350.

Nakano, Y. Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and Cell Physiology **22**: 867–880.

Napoli, C. A., Beveridge, C. A., Snowden, K. C. (1999). Reevaluating concepts of apical dominance and the control of axillary bud outgrowth. Current Topics in Developmental Biology **44**: 128-170.

Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D.T., Kojima, M., Werner, T., Vaňková, R., Yamaguchi-Shinozaki, K., Shinozaki, K., Kakimoto, T., Sakakibara, H., Schmülling, T., Tran, L. S. P. (2011). Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. The Plant Cell Online **23**: 2169–2183.

Nishiyama, Y., Takechi, K., Nanjo, Y., Murata, N., Hayashi, H. (2006). Acclimation of photosystem II to high temperature in a suspension culture of soybean (*Glycine max*) cells requires proteins that are associated with the thylakoid membrane. Photosynthesis Research **90**: 223-232.

Ogawa, K. I., Kanematsu, S., Takabe, K., Asada, K. (1995). Attachment of CuZn-superoxide dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immuno-gold labeling after rapid freezing and substitution method. Plant and Cell Physiology **36**: 565-573.

Ogawa, K. I., Kanematsu, S., Asada, K. (1996). Intra-and extra-cellular localization of "cytosolic" CuZn-superoxide dismutase in spinach leaf and hypocotyl. Plant and Cell Physiology **37**: 790-799.

Okada, S., Kanematsu, S., Asada, K. (1979). Intercellular distribution of manganese and ferri superoxide dismutases in blue-green algae. FEBS Letters **103**:106- 110.

Palma, J. M., Sandauo, L. M., Del Río, L. A. (1986). Manganese superoxide dismutase and higher plant chloroplasts: a reappraisal of a controverted cellular localization. Journal of Plant Physiology **125**: 427-439.

Panchuk, I.I., Volkov, R.A., Schöffl, F. (2002). Heat stress-and heat shock transcription factor-dependent expression and activity of ascorbate peroxidase in *Arabidopsis*. Plant Physiology **129**: 838–853.

Panchuk, I.I., Zentgraf, U., and Volkov, R.A. (2005). Expression of the Apx gene family during leaf senescence of *Arabidopsis thaliana*. Planta **222**: 926–932.

Peleg, Z., Reguera, M., Tumimbang, E., Walia, H., Blumwald, E. (2011). Cytokinin-mediated source/sink modifications improve drought tolerance and increase grain yield in rice under water-stress. Plant Biotechnology Journal **9**: 747–758.

Perl, A., Perl-Treves, R., Galili, S., Aviv, D., Shalgi, E., Malkin, S., Galun, E. (1993). Enhanced oxidative-stress defense in transgenic potato expressing tomato Cu, Zn superoxide dismutases. Theoretical and Applied Genetics **85**: 568-576.

Kardish, N., Magal, N., Aviv, D., Galun, E. (1994). The tomato gene for the chloroplastic Cu, Zn superoxide dismutase: regulation of expression imposed in transgenic tobacco plants by a short promoter. Plant Molecular Biology **25**: 887-897.

Pintó-Marijuan, M., Joffre, R., Casals, I., De Agazio, M., Zacchini, M., García-Plazaola, J.-I., Esteban, R., Aranda, X., Guàrdia, M., Fleck, I. (2013). Antioxidant and photoprotective responses to elevated CO2 and heat stress during holm oak regeneration by resprouting, evaluated with NIRS (near-infrared reflectance spectroscopy). Plant Biology **15**: 5–17.

Pitcher, L. H., Zilinskas, B. A. (1996). Overexpression of copper/zinc superoxide dismutase in the cytosol of transgenic tobacco confers partial resistance to ozone-induced foliar necrosis. Plant Physiology **110**: 583-588.

Polanská, L., Vičánková, A., Nováková, M., Malbeck, J., Dobrev, P. I., Brzobohatý, B., Vaňková, R., Macháčková, I. (2007). Altered cytokinin metabolism affects cytokinin, auxin, and abscisic acid contents in leaves and chloroplasts, and chloroplast ultrastructure in transgenic tobacco. Journal of Experimental Botany **58**: 637-649.

Polle, A. (2001). Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant Physiology **126**: 445–462.

Pospíšilová, J., Vágner, M., Malbeck, J., Travníčková, A., Baťková, P. (2005). Interactions between abscisic acid and cytokinins during water stress and subsequent rehydration. Biologia Plantarum **49**: 533–540.

Priault, P., Vidal, G., De Paepe, R., Ribas-Carbo, M. (2007). Leaf age-related changes in respiratory pathways are dependent on complex I activity in *Nicotiana sylvestris*. Physiologia Plantarum **129**: 152–162.

Procházková, D., Wilhelmová, N. (2009). Antioxidant protection during ageing and senescence in transgenic tobacco with enhanced activity of cytokinin oxidase/dehydrogenase. Biologia Plantarum **53**: 691–696.

Quirino, B. F., Noh, Y. S., Himelblau, E., Amasino, R. M. (2000). Molecular aspects of leaf senescence. Trends in Plant Science **5**: 278-282.

Ragusa, S., Cambria, M. T., Scarpa, M., Di Paolo, M. L., Falconi, M., Rigo, A., Cambria, A. (2001). Properties of Purified Cytosolic Isoenzyme I of Cu, Zn-Superoxide Dismutase from *Nicotiana plumbaginifolia* Leaves. Protein Expression and Purification **23**: 261-269.

Rashotte, A. M., Mason, M. G., Hutchison, C. E., Ferreira, F. J., Schaller, G. E., Kieber, J. J. (2006). A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway.Proceedings of the National Academy of Sciences USA **103**: 11081-11085.

Rivero, R.M., Kojima, M., Gepstein, A., Sakakibara, H., Mittler, R., Gepstein, S., Blumwald, E. (2007). Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proceedings of the National Academy of Sciences USA **104**: 19631–19636.

Rivero, R.M., Shulaev, V., Blumwald, E. (2009). Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Physiology **150**: 1530–1540.

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R. (2004). When defense pathways collide. The response of *Arabidopsis* to a combination of drought and heat stress. Plant Physiology **134**: 1683–1696.

Rizhsky, L., Hallak-Herr, E., Van Breusegem, F., Rachmilevitch, S., Barr, J. E., Rodermel, S., Dirk, I., Mittler, R. (2002). Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. The Plant Journal **32**: 329-342.

Rizhsky, L., Liang, H., Mittler, R. (2002). The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiology **130**: 1143–1151.

Robatzek, S., Somssich, I. (2002) Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes & Development **16**: 1139–1149.

Robatzek, S., Somssich, I.E. (2001). A new member of the *Arabidopsis* WRKY transcription factor family, AtWRKY6, is associated with both senescence-and defence-related processes. The Plant Journal **28**: 123–133.

Roitsch T, Ehneß R. (2000). Regulation of source/sink relations by cytokinins. Plant Growth Regulation **32**: 359–367.

Rulcová, J., Pospíšilová, J. (2001). Effect of benzylaminopurine on rehydration of bean plants after water stress. Biologia Plantarum **44**: 75–81.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., Scheres, B. (1999). An Auxin-Dependent Distal Organizer of Pattern and Polarity in the *Arabidopsis* Root. Cell **99**: 463-472.

Sabatini, S., Heidstra, R., Wildwater, M., Scheres, B. (2003). SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. Genes & Development **17**: 354-358.

Sachs, T., Kenneth V.T (1967). The role of auxins and cytokinins in the release of buds from dominance. American Journal of Botany **54**: 136-144.

Sakuma, Y., Maruyama, K., Qin, F., Osakabe, Y., Shinozaki, K., Yamaguchi-Shinozaki, K. (2006). Dual function of an *Arabidopsis* transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. Proceedings of the National Academy of Sciences USA **103**: 18822–18827.

Samuelson, M. E., Larsson, C. M. (1993). Nitrate regulation of zeation riboside levels in barley roots: effects of inhibitors of N assimilation and comparison with ammonium. Plant Science **93**: 77-84.

Scandalios JG, Guan L, Polidoros AN. (**1997)**. Catalases in plants: gene structure, properties, regulation, and expression. Cold Spring Harbor Monograph Series **34**: 343–406.

Scandalios, J.G. (1993). Oxygen stress and superoxide dismutases. Plant Physiology **101**: 7-12.

Sekmen, A. H., Ozgur, R., Uzilday, B., Turkan, I. (2014). Reactive oxygen species scavenging capacities of cotton (*Gossypium hirsutum*) cultivars under combined drought and heat induced oxidative stress. Environmental and Experimental Botany **99**: 141-149.

Shani, E., Yanai, O., Ori, N. (2006). The role of hormones in shoot apical meristem function. Current Opinion in Plant Biology **9**: 484-489.

Sharkey, T. D. (2005). Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. Plant, Cell & Environment **28**: 269-277.

Shashidhar, V., Prasad, T., Sudharshan, L. (1996). Hormone signals from roots to shoots of sunflower (Helianthus annuus L.). Moderate soil drying increases delivery of abscisic acid and depresses delivery of cytokinins in xylem sap. Annals of Botany **78**: 151–155.

Sheng, L., Zheng, X., Tong, H., Liu, S., Du, J., Liu, Q. (2004). Purification and characterization of cytosolic isoenzyme III of Cu, Zn-superoxide dismutase from tobacco leaves. Plant Science **167**: 1235–1241.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., Yoshimura, K. (2002). Regulation and function of ascorbate peroxidase isoenzymes. Journal of Experimental Botany **53**: 1305–1319.

Shin, R., Berg, R.H., Schachtman, D.P. (2005). Reactive oxygen species and root hairs in Arabidopsis root response to nitrogen, phosphorus and potassium deficiency. Plant and Cell Physiology **46**: 1350–1357.

Scheres, B. (2007). Stem-cell niches: nursery rhymes across kingdoms. Nature Reviews Molecular Cell Biology **8**: 345-354.

Schmülling, T. (2002). New insights into the functions of cytokinins in plant development. Journal of Plant Growth Regulation **21**: 40–49.

Silva, E.N., Ferreira-Silva, S.L., Fontenele, A. de V., Ribeiro, R.V., Viégas, R.A., Silveira, J.A.G. (2010). Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in *Jatropha curcas* plants. Journal of Plant Physiology **167**: 1157–1164.

Siminis, C.I., Kanellis, A.K., Roubelakis-Angelakis, K.A. (1994). Catalase is differentially expressed in dividing and nondividing protoplasts. Plant Physiology **105**: 1375–1383.

Slooten, L., Capiau, K., Van Camp, W., Van Montagu, M., Sybesma, C., Inzé, D. (1995). Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. Plant Physiology **107**: 737–750.

Smart, C.M., Scofield, S.R., Bevan, M.W., Dyer, T.A. (1991). Delayed leaf senescence in tobacco plants transformed with tmr, a gene for cytokinin production in Agrobacterium. The Plant Cell Online **3**: 647–656.

Smeekens, S. (1998). Sugar regulation of gene expression in plants. Current Opinion in Plant Biology **1**: 230-234.

Smigocki, A. C., Owens, L. D. (1989). Cytokinin-to-auxin ratios and morphology of shoots and tissues transformed by a chimeric isopentenyl transferase gene. Plant Physiology **91**: 808-811.

Somersalo, S. Krause, G.H. (1990). Photoinhibition at chilling temperatures and effects of freezing stress on cold acclimated spinach leaves in the field. A fluorescence study. Physiologia Plantarum **79**: 617–622.

Swamy, P., Suguna, P. (1992). Influence of calcium chloride and benzyladenine on lipoxygenase of Vigna unguiculata leaf discs during senescence. Physiologia Plantarum **84**: 467–471.

Synková, H. Valcke, R. (2001). Response to mild water stress in transgenic Pssu-ipt tobacco. Physiologia Plantarum **112**: 513–523.

Synková, H., Schnablová, R., Polanská, L., Hušák, M., Šiffel, P., Vácha, F., Malbeck, J., Macháčková, I., Nebesářová, J. (2006a). Three-dimensional reconstruction of anomalous chloroplasts in transgenic ipt tobacco. Planta **223**: 659-671.

Synková, H., Semorádová, S., Schnablová, R., Witters, E., Hušák, M., Valcke, R. (2006b). Cytokinin-induced activity of antioxidant enzymes in transgenic Pssu-ipt tobacco during plant ontogeny. Biologia Plantarum **50**: 31-41.

Šesták, Z., Solárová, J., Zima, J., Václavík, J. (1978). Effect of Growth Irradiance on Photosynthesis and Transpiration in *Phaseolus vulgaris* L. Biologia Plantarum **20**: 234–238.

Takei, K., Sakakibara, H., Sugiyama, T. (2001). Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. Journal of Biological Chemistry **276**: 26405-26410.

Takei, K., Takahashi, T., Sugiyama, T., Yamaya, T., Sakakibara, H. (2002). Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinin. Journal of Experimental Botany **53**: 971-977.

Takei, K., Yamaya, T., Sakakibara, H. (2004). *Arabidopsis* CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-zeatin. Journal of Biological Chemistry **279**: 41866-41872.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. The Plant Journal **45**: 1028-1036.

Taniguchi, M., Sasaki, N., Tsuge, T., Aoyama, T., Oka, A. (2007). ARR1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions. Plant and cell physiology **48**: 263-277.

Teixeira, F. K., Menezes-Benavente, L., Galvão, V. C., Margis-Pinheiro, M. (2005). Multigene families encode the major enzymes of antioxidant metabolism in *Eucalyptus grandis* L. Genetics and Molecular Biology **28**: 529-538.

Thomas, D.J., Avenson, T.J., Thomas, J.B., and Herbert, S.K. (1998). A cyanobacterium lacking iron superoxide dismutase is sensitized to oxidative stress induced with methyl viologen but is not sensitized to oxidative stress induced with norflurazon. Plant Physiology **116**: 1593–1602.

Todaka, D., Matsushima, H., Morohashi, Y. (2000). Water stress enhances β-amylase activity in cucumber cotyledons. Journal of Experimental Botany **51**: 739–745.

Tran, L.-S.P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., Yamaguchi-Shinozaki, K. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. Proceedings of the National Academy of Sciences USA **104**: 20623–20628.

Tsang, E., Bowler, C., Hérouart, D., Van Camp, W., Villarroel, R., Genetello, C., Van Montagu, M., Inzé, D. (1991). Differential regulation of superoxide dismutases in plants exposed to environmental stress. The Plant Cell Online **3**: 783–792.

Turgeon, R. (1989). The sink-source transition in leaves. Annual Review of Plant Biology **40**: 119–138.

Udompraset, N., Li, P. H., Davis, D. W., Markhart, A. H. (1995). Root cytokinin level in relation to heat tolerance of *Phaseolus acutifolius* and *Phaseolus vulgaris*. Crop Science **35**: 486-490.

Van Camp, W., Willekens, H., Bowler, C., Van Montagu, M., Inzé, D., Reupold-Popp, P., Sandermann, J., Langebartels, C. (1994). Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. Nature Biotechnology **12**: 165-168.

Van Camp, W., Bowler, C., Villarroel, R., Tsang, E., Van Montagu, M., Inze, D. (1990). Characterization of iron superoxide dismutase cDNAs from plants obtained by genetic complementation in *Escherichia coli*. Proceedings of the National Academy of Sciences USA **87**: 9903–9907.

Van Camp, W., Capiau, K., Van Montagu, M., Inze, D., Slooten, L. (1996a). Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. Plant Physiology **112**: 1703-1714.

Van Camp, W., Hérouart, D., Willekens, H., Takahashi, H., Saito, K., Van Montagu, M., Inzé, D. (1996b). Tissue-specific activity of two manganese superoxide dismutase promoters in transgenic tobacco. Plant Physiology **112:** 525-535.

Van Camp, W., Inzé, D., and Van Montagu, M. (1997). The regulation and function of tobacco superoxide dismutases. Free Radical Biology and Medicine **23**: 515–520.

Vaňková, R. (1999). Cytokinin glycoconjugates-distribution, metabolism and function. In Strnad, M., Peč, P., Beck, E. (Eds.) Advances in Regulation of Plant Growth and Development: pp. 67-78.

Wahid, A., Gelani, S., Ashraf, M., Foolad, M. (2007). Heat tolerance in plants: an overview. Environmental and Experimental Botany **61**: 199–223.

Wang, J., Letham, D. S., Cornish, E., Stevenson, K. R. (1997). Studies of cytokinin action and metabolism using tobacco plants expressing either the ipt or the GUS gene controlled by a chalcone synthase promoter. I. Developmental features of the transgenic plants. Functional Plant Biology **24**: 661-672.

Wang, Y. Y., Zhou, R., Zhou, X. (1994). Endogenous Levels of ABA and Cytokinins and their Relation to Stomatal Behavior in Dayflower (*Commelina communis* L.). Journal of Plant Physiology **144**: 45-48.

Werner, T., Motyka, V., Strnad, M., Schmülling, T. (2001). Regulation of plant growth by cytokinin. Proceedings of the National Academy of Sciences **98**: 10487-10492.

Werner, T., Holst, K., Pörs, Y., Guivarc'h, A., Mustroph, A., Chriqui, D., Grimm, B., Schmülling, T. (2008). Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. Journal of Experimental Botany **59**: 2659–2672.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., Schmülling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. The Plant Cell Online **15**: 2532–2550.

Werner, T., Nehnevajova, E., Köllmer, I., Novák, O., Strnad, M., Krämer, U.,

Schmülling, T. (2010). Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. The Plant Cell Online **22**: 3905–3920.

Wildwater, M., Camplio, A., Perez-Perez, J.M., Heidstra, R., Blilou, I. Korthout, H., Chatterjee, J., Mariconti, L., Gruissen, W., Scheres, B. (2005) The *RETINOBLASTOMA-RELATED* Gene Regulates Stem Cell Maintenance in *Arabidopsis* Roots. Cell **123**: 1337-1349.

Willekens H., Langebartels C., Tire C., Van Montagu M., Inzé D., Van Camp W. (1994). Differential expression of catalase genes in *Nicotiana plumbaginifolia* (L.). Proceedings of the National Academy of Sciences, **91**: 10450-10454.

Willekens, H., Inzé, D., Van Montagu, M., and Van Camp, W. (1995). Catalases in plants. Molecular Breeding **1**: 207–228.

Williams, L., Grigg, S. P., Xie, M., Christensen, S., Fletcher, J. C. (2005). Regulation of *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes. Development **132**: 3657-3668.

Wilson, J. R., Ludlow, M. M., Fisher, M. J., Schulze, E. (1980). Adaptation to water stress of the leaf water relations of four tropical forage species. Functional Plant Biology **7**: 207-220.

Wyrzykowska, J., Schorderet, M., Pien, S., Gruissem, W., Fleming, A. J. (2006). Induction of differentiation in the shoot apical meristem by transient overexpression of a retinoblastoma-related protein. Plant Physiology **141**: 1338-1348.

Xu, Y., Gianfagna, T., Huang, B. (2010). Proteomic changes associated with expression of a gene (ipt) controlling cytokinin synthesis for improving heat tolerance in a perennial grass species. Journal of Experimental Botany **61**: 3273–3289.

Xu, Y., Tian, J., Gianfagna, T., Huang, B. (2009). Effects of SAG12-ipt expression on cytokinin production, growth and senescence of creeping bentgrass (*Agrostis stolonifera* L.) under heat stress. Plant Growth Regulation **57**: 281–291.

Yabuta, Y., Motoki, T., Yoshimura, K., Takeda, T., Ishikawa, T., and Shigeoka, S. (2002). Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. The Plant Journal **32**: 915–925.

Yoshimura, K., Yabuta, Y., Ishikawa, T., Shigeoka, S. (2002). Identification of a cis element for tissue-specific alternative splicing of chloroplast ascorbate peroxidase pre-mRNA in higher plants. Journal of Biological Chemistry **277**: 40623–40632.

Yuldashev, R., Avalbaev, A., Bezrukova, M., Vysotskaya, L., Khripach, V., Shakirova, F. (2012). Cytokinin oxidase is involved in the regulation of cytokinin content by 24-epibrassinolide in wheat seedlings. Plant Physiology and Biochemistry **55**: 1–6.

Zalabák, D., Pospíšilová, H., Šmehilová, M., Mrízová, K., Frébort, I., Galuszka, P.

(2013). Genetic engineering of cytokinin metabolism: prospective way to improve agricultural traits of crop plants. Biotechnology Advances **31**: 97-117.

Zavaleta-Mancera, H.A., López-Delgado, H., Loza-Tavera, H., Mora-Herrera, M., Trevilla-García, C., Vargas-Suárez, M., Ougham, H. (2007). Cytokinin promotes catalase and ascorbate peroxidase activities and preserves the chloroplast integrity during dark-senescence. Journal of Plant Physiology **164**: 1572–1582.

Zelitch, I., Havir, E.A., McGonigle, B., McHale, N.A., Nelson, T. (1991). Leaf catalase mRNA and catalase-protein levels in a high-catalase tobacco mutant with O_2 -resistant photosynthesis. Plant Physiology **97**: 1592–1595.

Zhang, J. H., Huang, W. D., Liu, Y. P., Pan, Q. H. (2005). Effects of Temperature Acclimation Pretreatment on the Ultrastructure of Mesophyll Cells in Young Grape Plants (*Vitis vinifera* L. cv. Jingxiu) Under Cross-Temperature Stresses. Journal of Integrative Plant Biology **47**: 959-970.

Zhang, P., Wang, W.-Q., Zhang, G.-L., Kaminek, M., Dobrev, P., Xu, J., Gruissem, W. (2010). Senescence-Inducible Expression of Isopentenyl Transferase Extends Leaf Life, Increases Drought Stress Resistance and Alters Cytokinin Metabolism in Cassava. Journal of Integrative Plant Biology **52**: 653–669.

Zhang, Y., Ding, S., Lu, Q., Yang, Z., Wen, X., Zhang, L., Lu, C. (2011). Characterization of photosystem II in transgenic tobacco plants with decreased iron superoxide dismutase. Biochimica et Biophysica Acta -Bioenergetics **1807**: 391–403.

Zimmermann, P., Heinlein, C., Orendi, G., Zentgraf, U. (2006). Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. Plant, Cell & Environment **29**: 1049–1060.

12. List of publications

Publications in impact journals:

Lubovská Z., Dobrá J., Štorchová H., Wilhelmová N., Vaňková R. (2014). Cytokinin oxidase/dehydrogenase overexpression modifies antoxidant defence against drought, heat and their combination in tobacco. Journal of Plant Physiology **171**:1625-1633 (IF=2.6). I acknowledge that my contribution to the above paper is 75 percent.

Macková H., Hronková M., Dobrá J., Turečková V., Novák O., Lubovská Z., Motyka V., Haisel D., Hájek T. Prášil I.T., Gaudinová A., Štorchová H., Ge E., Werner T., Schmülling T., Vanková R. (2013). Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression, Journal of Experimental Botany **64**: 2805-2815. (IF=5.24). I acknowledge that my contribution to the above paper is 10 percent.

Mýtinová Z., Motyka V., Haisel D., Lubovská Z., Trávníčková A., Dobrev P., Holík J., Wilhelmová N. (2011). Antioxidant enzymatic protection during tobacco leaf ageing is affected by cytokinin depletion, Plant Growth Regulation **65**: 23-34. (IF=1.63). I acknowledge that my contribution to the above paper is 15 percent.

Mýtinová Z., Motyka V., Haisel D., Gaudinová A., Lubovská Z. and Wilhelmová N. (2010). Effect of abiotic stresses on the activity of antioxidative enzymes and contents of phytohormones in wild type and AtCKX2 transgenic tobacco plants. Biologia Plantarum **54**: 461-470. (IF=1.58). I acknowledge that my contribution to the above paper is 15 percent.

Publications in other journals and proceedings:

Lubovská Z., Wilhelmová N., Štorchová H., Vanková R. (2011). Expression and activity of superoxide dismutase isoforms is connected to cytokinin level and environmental conditions. XI. mezioborové setkání mladých biologů, biochemiků a chemiků. Chemické listy 105: 407-408.

Lubovská Z., Xia Li, Štorchová H., Vaňková R., Wilhelmová N. (2010). Characterization of new ascorbate peroxidase and superoxide dismutase genes in *Nicotiana tabacum*. Bulletin ČSEBR 36.

Lubovská Z., Wilhelmová N., Havlová M., Štorchová H., Vaňková R. (2010). Presence of superoxide dismutase isoforms under drought and heat in tobacco with decreased level of cytokinins. Bulletin ČSEBR 145.

Lubovská Z., Wilhelmová N., Havlová M., Vaňková R., Štorchová H. (2009). Impact of cytokinins on antioxidant enzymes isoforms under drought and heat. Vliv abiotických a biotických stresorů na vlastnosti rostlin 2009. Sborník příspěvků 387- 382.

Lubovská Z., Wilhelmová N., Havlová M., Vaňková R. (2008). Impact of drought and heat on antioxidant enzymes in tobacco overexpressing cytokininoxidase dehydrogenase. Vliv abiotických a biotických stresorů na vlastnosti rostlin 2008. Sborník příspěvků 254.

Lubovská Z., Wilhelmová N., Havlová M., Vaňková R. (2008). The impact of drought and heat stress on antioxidant enzyme activities in tobacco plants over-expressing gene for cytokinin oxidase/dehydrogenase. Fresh Insights in Plant Affairs Nové Hrady 2008. Book of Abstracts 25.

Lubovská (Frýdlová) Z., Wilhelmová N., Havlová M., Vaňková R. (2008). Antioxidant enzymes activities in tobacco with diminished level of cytokinins under drought. Bulletin ČSEBR 108.

Frýdlová Z., Wilhelmová N., Havlová M.,Vaňková R. (2007). Activities of antioxidant enzymes in tobacco with dimininshed level of cytokinins under drought. 11.dny fyziologie rostlin aneb Konference experimentální biologie rostlin. Sborník příspěvků 108.

Frýdlová Z., Wilhelmová N., Havlová M., Vaňková R. (2007). Antioxidant enzyme activities in tobacco with increased level of proline treated by drought and heat. Chem. Listy 101: 435.

Frýdlová Z., Wilhelmová N., Gubiš J., Havlová M.,Vaňková R. (2007). Impact of drought and heat on antioxidant enzymes in tobacco with elevated level of proline. Vliv abiotických a biotických stresorů na vlastnosti rostlin 2007, VÚRV. Sborník příspěvků 184 - 188.