Charles University in Prague, Faculty of Science
Department of Experimetnal Plant Biology

Ph.D. study program: Plant physiology and anatomy

Summary of the Ph.D. Thesis

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

Zuzana Lubovská

Supervisor: RNDr. Naďa Wilhelmová, CSc.
Supervisor-consultant: RNDr. Helena Štorchová, CSc.

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Contents

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* (*CKX1*) 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 reactive oxygen species 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 or by changes in source/sink of sugars metabolism.

Introduction

Drought is a frequent stress that reduces plant biomass production and crop yield. Drought promotes stomata closure and inhibition of photosynthesis (Chaves 1991, Cornic 2000), dampens respiration (Rizhsky *et al.* 2002), initiates leaf rolling, and accumulation of osmoprotectants (Madden *et al.* 1985, Kaplan and Guy 2004), including soluble sugars (Todaka *et al.* 2000, Kaplan and Guy 2004, Basu *et al.* 2007, Kempa *et al.* 2008). In nature, drought is often accompanied by heat stress. In contrast, heat stress induces stomata opening, increases transpiration and respiration, and affects the accumulation of solutes such as glycine betaine and sugars (Marijuan *et al.* 2012, Wahid *et al.* 2007, Allakhverdiev *et al.* 2008). Thus, drought and heat often stimulate contradictory responses in plants, e.g. in stomata movement, respiration or proline accumulation (Rizhsky *et al.* 2004, Dobrá *et al.* 2010, Lv *et al.* 2011). When plants are subjected to drought and heat simultaneously, defence against the combined stresses differs from the response to a single stress. From the total number of 1833 transcripts known to be influenced by drought, heat or their combination, only 77 transcripts were affected in a common way (Rizhsky *et al.* 2004). Each stress, including drought and heat, is associated with an oxidative stress due to the enhanced production of reactive oxygen species (ROS). On one hand, ROS serve as a signal in the regulation of development (Gapper and Dolan 2006, Shin 2005), stimulation of programmed

cell death, cell wall formation (Fry *et al.* 2001), and activation of stress responses (Gechev *et al.* 2006). On the other hand, those molecules are highly reactive and have the capability to disrupt cell redox homeostasis with consequent damage to cell components (Scandalios *et al.* 1993). ROS are continuously produced in organisms, but, their production increases under stress (Gill and Tuteja 2010). The main source of hydrogen peroxide and superoxide radicals are photosynthetic and respiratory electron transport chains, photosystem I and II, overexcited chlorophyll, NADPH oxidase, fatty acid β-oxidation, glycolate oxidase, oxalate oxidase, xanthine oxidase, peroxidase and amine oxidase (Mittler 2002). Hydrogen peroxide and superoxide radical can react with transition metals in the Haber-Weiss reaction, producing OH^{-}, a strong oxidizing agent. In order to maintain the delicate equilibrium of ROS concentrations, plants evolved both enzymatic and non-enzymatic antioxidant systems.

The main enzymatic ROS scavengers are ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD). Antioxidant enzymes exist in several isoforms which differ in their subcellular localization and expression control, which enables their specific regulation and thus modulates their unique role. Some isoforms originate from the alternative splicing or polymorphism (Nadif *et al.* 2005, Mak *et al.* 2007), or from the formation of both heteromeric and homomeric complexes (Zimmermann *et al.* 2006, Lleídas *et al.* 1998, Myouga *et al.* 2008). The enzyme APX is a component of the ascorbate-glutathione cycle in chloroplasts, mitochondria, microsomes and cytosol, where it maintains an appropriate level of H_2O_2 (Shigeoka *et al.* 2002). The function of APX is dependent on the presence of reduced ascorbic acid (Asada *et al.* 1987), which is then recovered by monodehydroascorbate reductase and dehydroascorbate reductase in dependence on NADPH and glutathione, respectively (Polle *et al.* 2001). Ascorbate peroxidase has high affinity to H_2O_2 as a substrate in micromolar and submicromolar concentrations. In *Arabidopsis*, nine genes coding for APX are known. Enzyme CAT scavenges H_2O_2, especially in peroxisomes where H_2O_2 is produced during photorespiration. Catalase decomposes H_2O_2 in milimolar

concentrations without requirement of any donor molecule (Mittler *et al.* 2005). In most of plant species, three genes coding for CAT subunits are present. These CAT subunits form various homo- and heterotetramers giving functional enzymes (Lledías *et al.* 1998, Zimmermann *et al.* 2006). Superoxide dismutase (SOD) catalyses dismutation of superoxide into oxygen and H_2O_2 in chloroplasts, mitochondria, peroxisomes, and cytosol. There are three types of SOD isoenzymes according to different metal cofactors - Mn (MnSOD), Fe (FeSOD), or Cu and Zn (CuZnSOD).

Cytokinins (CK) are plant hormones indispensable for the regulation of growth and development (Mok *et al.* 2001, Spíchal 2012), which also play an important role in stress responses (Ha *et al.* 2012). Exogenously applied CK were found to act like stress protectants (Metwally *et al.* 1998, Rulcová *et al.* 2001). Accordingly, the elevation of endogenous CK levels, achieved by overexpression of isopentenyl transferase, coding for the rate-limiting enzyme in CK biosynthesis, under stress- or senescence-inducible promoter, resulted in improved drought tolerance (Rivero *et al.* 2007 and 2009, Merewitz *et al.* 2011a, 2011b, and 2012, Zhang *et al.* 2010). A positive impact of exogenously applied CK (Itai *et al.* 1978, Liu *et al.* 2002) or elevated endogenous CK (Xu *et al.* 2009) was also observed in plants exposed to heat. However, plants with diminished levels of endogenous CK, caused by overexpression of *cytokinin oxidase/dehydrogenase* (*CKX*), the gene encoding the main enzyme for CK degradation, are also highly tolerant to drought or heat (Werner *et al.* 2010, Mýtinová *et al.* 2010, Macková *et al.* 2013). Plants overexpressing *CKX* are unique tool for studying decreased level of CK. Tobacco plants overexpressing *AtCKX1* under the constitutive promoter *35S* (*35S:CKX1*) exhibited a stunted shoot phenotype and enlarged root system (Werner *et al.* 2001). They exhibit delayed development and higher FeSOD activity (Cortleven *et al.* 2011). Diminished CK content was also associated with decreased chlorophyll and carotenoid levels (Cortleven *et al.* 2012). The modulated development and habit of *AtCKX1* overexpressors may alter *per se* stress responses. In order to avoid negative effects of *CKX* overexpression on shoot growth, the root-specific promoter *WRKY6* was used in the present study (Werner *et al.* 2010). The

transgenic line *W6:CKX1* displayed a shoot habit quite similar to WT and maintained an enhanced root system. The *W6:CKX1* plants were found to be more tolerant to drought (Werner *et al.* 2010).

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). Exogenously applied CK increased CAT and APX activity (Zavaleta-Manceraa *et al.* 2007), and also enhanced the activity of CAT and SOD after heat (Liu *et al.* 2002). Modulation of endogenous CK levels was reported to affect the antioxidant system under both control and stress conditions (Cortleven *et al.* 2012, Mýtinová *et al.* 2010 and 2011).

Aims

Antioxidant enzymes as well as CK contribute significantly to stress tolerance. In this study we have evaluated their interplay of antioxidant enzymes with CK under drought and/or heat stress conditions. Antioxidant transcript levels and enzyme activities were estimated in tobacco plants overexpressing *CKX1* under the control of either constitutive *35S* or root-specific *WRKY6* promoters.

- We examined if decreased level of endogenous CK due to overexpression of *A. thaliana CKX1* influence antioxidant enzyme system response.
  Hypothesis: Stress response of antioxidant enzymes in plants overexpressing *CKX1* differs from that in WT.

- We tested if decreased CK level was substituted by enhanced antioxidant enzymatic system.
  Hypothesis: Plants overexpressing *CKX1* have improved antioxidant enzyme system.

- Ageing is also a kind of stressor causing oxidative damage, alongside, CK play important role in leaf senescence. We tested if antioxidant defence differs within leaf gradient.
  Hypothesis: Antioxidant enzyme response differs in upper and lower leaves.

Materials and methods

Seeds of two transgenic lines of *Nicotiana tabacum* L. cv. Samsun NN overexpressing a gene for cytokinin oxidase/dehydrogenase from *Arabidopsis thaliana* (*CKX1*) either under the root-specific promoter *WRKY6* (*W6:CKX1*) or the constitutive promoter *35S* (*35S:CKX1*) and the corresponding wild-type (WT) were sown in sterilized soil and cultivated in a growth chamber at 25/23 °C, 16 h photoperiod at 130 μmol m^{-2} s^{-1} and air humidity 80 %. Six-week old plants were subjected to 8-day water withdrawal (cessation of watering at air humidity 35 %). Heat was applied for 2 hours at 40 °C. Stress combination was achieved by application of the heat at the end of drought period. Upper, middle and lower leaves, and roots were harvested.

Soluble proteins were extracted from 2 g of frozen leaves or roots homogenized in 10 ml buffer (0.1 M Tris-HCl, 1 mM dithiotreitol, 1 mM Na_2EDTA, 1 % Triton X-100, 5 mM ascorbic acid, pH 7.8). For isolation of membrane associated proteins, samples were after 2-min ultrasonic treatment incubated on ice in the dark for 30 min, centrifuged (20,000 g, 10 min, 4 °C), and filtered. Samples for spectrophotometrical determination of SOD activity were desalted by passing through Sephadex G-25 (18 g, 2 min, 4 °C). Samples were frozen in liquid nitrogen and stored at -70 °C. Extract for CAT native electrophoresis was isolated from 1 g of frozen leaves homogenized in 10 ml buffer (0.1 M Tris-HCl, 20 % glycerol, 30 mM DTT, pH 8) (Frugoli *et al.* 1996). The homogenate

was processed as described above. Protein content was determined according to Bradford (1976).

APX and SOD activities were assayed spectrophotometrically. Total APX activity was determined by monitoring the decrease of absorbance at 290 nm (A_{290}) according to Nakano and Asada (1981). Total SOD activity was measured at 470 nm according to Ukeda *et al.* (1997). CAT activity was determined polarographically according to Thomas *et al.* (1998).

The isoenzymes were separated by native polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). The APX activity was visualized using 20-µg protein samples according to Mittler and Zilinskas (1993). The CAT isoenzymes were separated by PAGE using 4-µg protein samples (Zimmermann *et al.* 2006). To determine SOD isoenzymes, 20-µg protein samples were run on PAGE, isoforms with different cofactors were identified with an aid of specific inhibitors (Beauchamp and Fridovich 1971). Activities in gels were analysed densitometrically using Multi Gauge software (Fuji film, Science Lab 2002, Japan).

Total RNA from leaf and root tips was extracted using RNeasy Plant Mini Kit (Qiagen, Germany). DNaseI-treated RNA was transcribed with M-MLV Reverse Transcriptase RNase H Minus, Point Mutant, according to the manufacturer's protocol. Primers were designed by software Primer3 (Koressaar and Remm 2007). Quantitative PCR was run in Light Cycler 1.2 (Roche, Switzerland) using FastStart DNA^{PLUS} SYBR GreenI (Roche, Switzerland). The transcript levels were normalised using the *Act9* gene as a reference.

Results from three independent biological experiments were evaluated by analysis of variance using the Statistica 10 programme (StatSoft, USA). Statistically important differences were proved by post-hoc Tukey test ($p < 0.05$). Significant correlations among variables were identified on the basis of Spearman correlation coefficient ($p < 0.001$). Data were also tested by principal component analysis.

Results and Discussion

As antioxidant enzymes contribute significantly to stress tolerance and CK exhibit cross-talk with the antioxidant enzymes, we have evaluated interplay of antioxidant enzymes with CK under drought and/or heat. Antioxidant transcript levels, as well as enzyme activities, were estimated in tobacco overexpressing *CKX1* under the control of either constitutive *35S* (transgenic line *35S:CKX1*) or root-specific *WRKY6* (transgenic line *W6:CKX1*) promoters. For detection of transcripts we used qPCR, further we measured total antioxidant activity spectrophotometrically and polarographically and activity of individual enzyme isoforms was detected semiquantitatively from native zymograms.

The *35S:CKX1* plants developed smaller and thicker leaves, shorter internodes and considerably enhanced root system in comparison to WT. In order to avoid negative effects of *CKX* overexpression on shoot growth, the root-specific promoter *WRKY6* was used (Werner *et al.* 2010). The WT and *W6:CKX1* plants had similar shoot habits. According to relative water content, dehydration marker gene, as well as, ion leakage measurement, both transgenic plants showed lower stress impact than WT.

We detected antioxidant enzymes on the level of expression as well as active enzyme. The assayed antioxidant enzymes may be grouped according to their functions: (1) chloroplastic enzymes - sAPX, tAPX, FeSOD; (2) enzymes with general stress defence function - cAPX, CAT3, cytosolic CuZnSOD; (3) peroxisome protecting enzymes - CAT1, CAT2 and CAT3; and (4) mitochondrial MnSOD, enzyme removing ROS produced during respiration.

The chloroplastic enzymes protect individual components of the photosynthetic apparatus. 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). The enzyme sAPX scavenges H_2O_2 in

stroma, where it serves as the second defence after primary protection by tAPX and FeSOD (Asada 2006). The expression patterns of chloroplastic antioxidant enzymes were highly coordinated, as indicated by correlation coefficients (Fig. 6).

Under optimal conditions, the principal ROS source in plants during daylight is electron transport in the chloroplasts. All chloroplastic antioxidant enzymes were strongly transcribed in leaves of all genotypes under control conditions. Surprisingly, expression of *sAPX* was also relatively high in roots, particularly in *W6:CKX1* (Fig. 1B). 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.

Within this study we subjected tobacco plants to 8-day water withdrawal. Heat was applied for 2 hours at 40 °C. Stress combination was achieved by application of the heat at the end of drought period. Stress conditions bring about changes in the metabolic activity of plant cells. The photosynthetic activity is depressed during both drought (Rivero *et al.* 2009) as well as elevated temperature (Allakhverdief *et al.* 2008). A decrease in *sAPX* and *tAPX* expression in all stress conditions was observed in WT and *W6:CKX1* (Fig. 1B, C). This down-regulation could reflect a diminished ROS production in their chloroplasts as a result of reduced photosynthesis. Our data are in accordance with Rizhsky *et al.* (2002) who found diminished *sAPX* expression in tobacco after heat and de Carvalho *et al.* (2013) who reported decrease in chloroplastic *APX* transcripts after drought. In spite of the fact that exogenous CK as well as enhanced endogenous CK levels (Rivero *et al.* 2009) were reported to increase the activity of antioxidant enzymes, we detected under stress conditions higher *sAPX* expression also in *35S:CKX1* plants which have diminished CK content (Fig. 1B). Potential explanation may be that *sAPX* expression reflects predominantly plant stress tolerance. This assumption is in accordance with strong positive effect of elevated proline content on both stress tolerance and chloroplastic *APX* expression in *Swingle citrumelo* (de Carvalho *et al.* 2013). Positive correlation of *FeSOD* expression with stress tolerance was suggested already by Van Camp *et al.* (1996a). Our results showing higher *FeSOD* expression in both

CKX1 overexpressors, especially in more tolerant *35S:CKX1*, (Fig. 3B) are in accordance with this conclusion.

Enzymes participating in stress protection, i.e. cAPX, CAT3 and cytosolic CuZnSOD, were expressed to a relatively low extent under control conditions (Fig. 1A, 2C, 3C). Under optimal conditions, their transcription was comparable in WT and both transgenic lines which indicated that overexpression of *AtCKX1* did not induce any oxidative stress. The expression profiles of these genes showed a similar response to applied stresses in all genotypes, as indicated both by strong correlation between *cAPX* and *CuZnSOD* and a weaker one between *CAT3* and *CuZnSOD* (Fig. 6). Heat and stress combination activated *CAT3*, *CuZnSOD* and especially *cAPX* (Fig. 1– 3). These results are in line with the reported stimulation of *cAPX* expression in heat stressed tobacco (Rizhsky *et al.* 2002) and *Arabidopsis* (Panchuk *et al.* 2002).

The peroxisomal enzymes CAT, are present in plants in several isoforms. In tobacco three genes were detected - *CAT1*, *CAT2*, and *CAT3* (Willekens *et al.* 1994). We found extensive transcription of *CAT1* and *CAT2*, unlike *CAT3*, in leaves under control conditions. Our data accord with the report of Luna *et al.* (2005) on wheat and Du *et al.* (2008) on *Arabidopsis.*

Drought and heat promote photorespiration, which is associated with production of H_2O_2. Subsequently, the demand for antioxidant defence in peroxisomes increases. *CAT1* expression was stimulated by heat stress, being repressed by drought and combined stresses in WT and *W6:CKX1* plants. Mild up-regulation of *CAT2* expression was observed in drought, the other stresses had adverse effects. 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. Our results are in accordance with Du *et al.* (2008) who found different regulation of individual *CAT* isoforms by drought, cold, oxidative stress and abscisic acid application. Thus, different expression profiles as well as the lack of correlation among them indicate different function and regulation of *CAT* isoforms.

Mitochondrial MnSOD removes superoxide generated by electron leakage 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 reported by Priault *et al.* (2007). It is known that both expression and activity of MnSOD in tobacco are 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 can regulate MnSOD by modulation of carbohydrate metabolism via sink/source dynamics (Cowan *et al.* 2005, Roitsch and Ehneß 2000). The potential CK function is also indicated by the coincidence of lower CK content as well as reduced concentration of soluble sugars in *35S:CKX1* plants (Werner *et al.* 2008) and their lower MnSOD1 activity (Fig. 4). Idea of cross-talk between CK and MnSOD is also supported by correlation analysis (Fig. 6).

In contrast to low MnSOD1 activity, *MnSOD* transcription was high in *35S:CKX1* (Fig. 3A, 4). The discrepancy between the activity and transcript levels were 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). It may be, however, also caused by inhibition of MnSOD enzymatic activity in *35S:CKX1* plants.

We detected nine APX isoforms on native electrophoresis gels (Fig. 5A) what corresponds to number of genes coding for APX in *Arabidopsis*. CAT has only three genes which code for three CAT subunits from which many CAT isoforms arise, we distinguished nine isoforms (Fig. 5B). Formerly, from six (Zeltich *et al.* 1991) to twelve (Siminis *et al.* 1994) CAT isoforms were found in tobacco. Further, we identified six SOD isoforms (Fig. 5C), previous publications differs in amount of different isoforms (VanCamp *et al.* 1996b, Priault *et al.* 2007).

In conclusion, tobacco with down-regulated CK content differed in their stress tolerance as well as in antioxidant enzyme

system responses compared to WT. Enhanced stress tolerance of plants with decreased level of CK was reflected by better preservation of photosynthetic activity under stress conditions, as indicated by higher expression of photosynthesis-related antioxidant genes. The defence mediated by antioxidant enzyme system exerts a high plasticity. It is specifically regulated under various stress conditions, exhibiting differences between the response to a single stress and to combined stresses. The application of heat at the end of drought, which strongly increased the stress severity, further decreased the expression of chloroplast and mitochondria related genes, while the expression of cytosolic genes and *CAT3* was enhanced. The impact of down-regulation of CK levels on antioxidant enzyme system seems to be indirect, mediated by chloroplast modification, changes in carbohydrate metabolism, or the positive effect on plant stress tolerance.

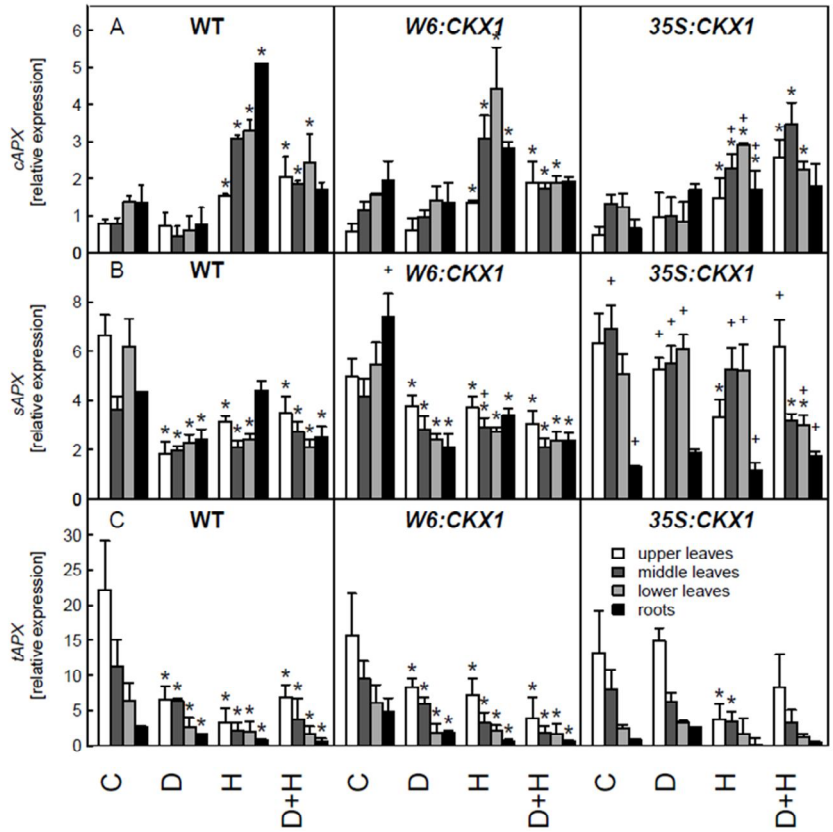

Fig.1: Effect of drought or/and heat on *APX* expression in upper, middle, and lower leaves or roots of *N. tabacum* Samsun NN WT, *W6:CKX1*, and *35S:CKX1*. (A) cytosolic (*cAPX*), (B) stromatal (*sAPX*), (C) thylakoidal (*tAPX*). C - control, D - drought, H - heat, and D+H - stress combination. Data 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.

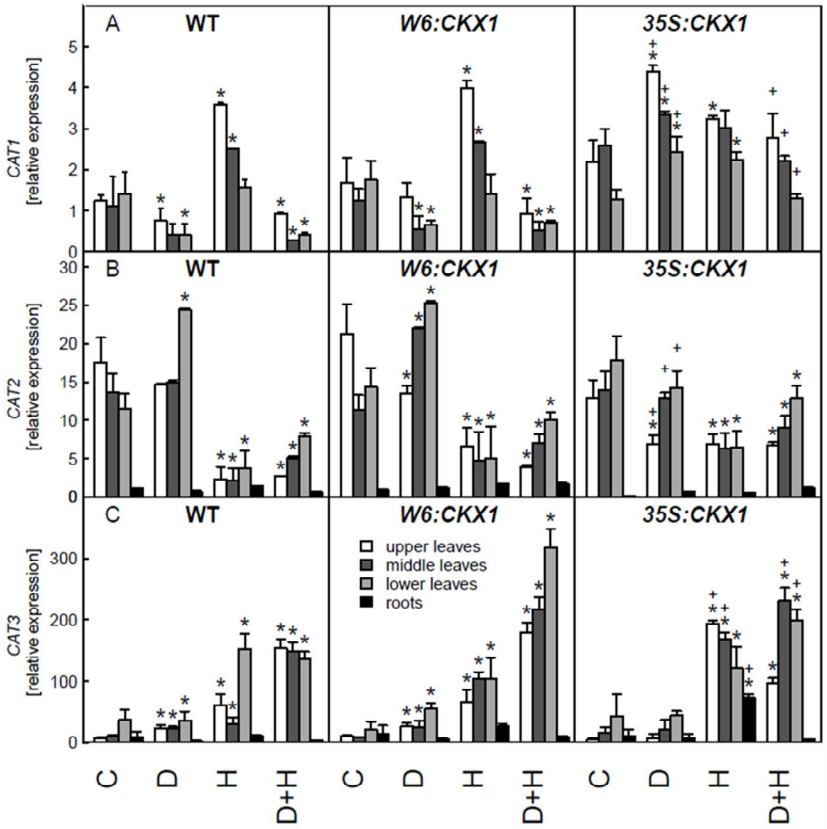

Fig. 2: Expression levels of *CAT* genes in upper, middle and lower leaves and roots of *N. tabacum* Samsum NN WT, *W6:CKX1* and *35S:CKX1*. (A) *CAT1*, (B) *CAT2*, (C) *CAT3*. Other designation as described in Fig. 1.

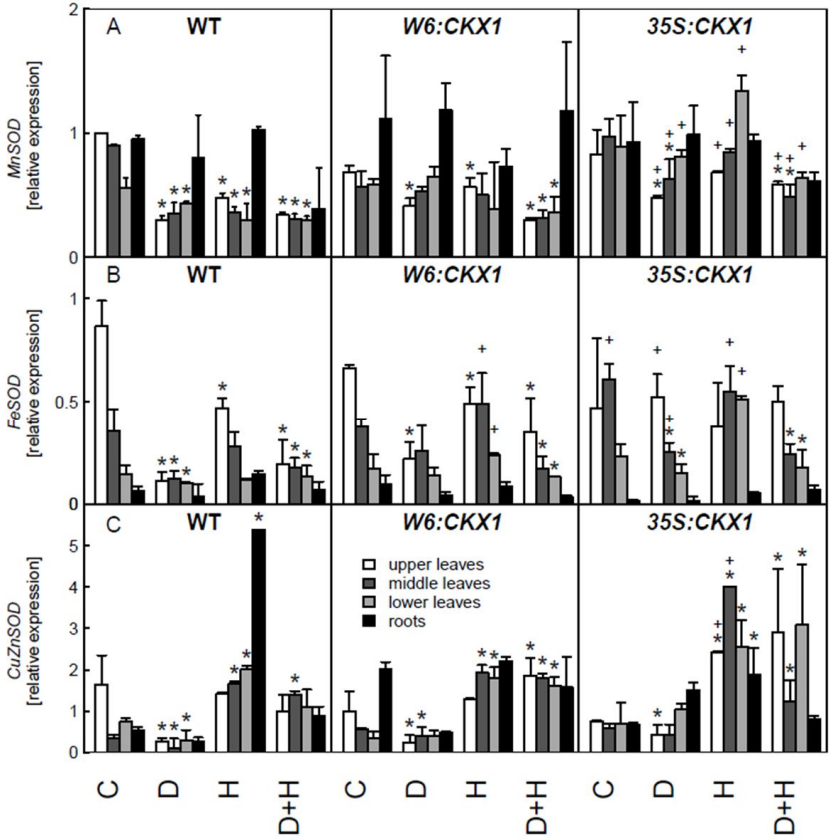

Fig. 3: Expression levels of *SOD* genes in upper, middle and lower leaves and roots of *N. tabacum* WT, *W6:CKX1* and *35S:CKX1*. (A) mitochondrial *MnSOD*, (B) chloroplastic *FeSOD*, (C) cytosolic *CuZnSOD*. Other designation described in Fig. 1.

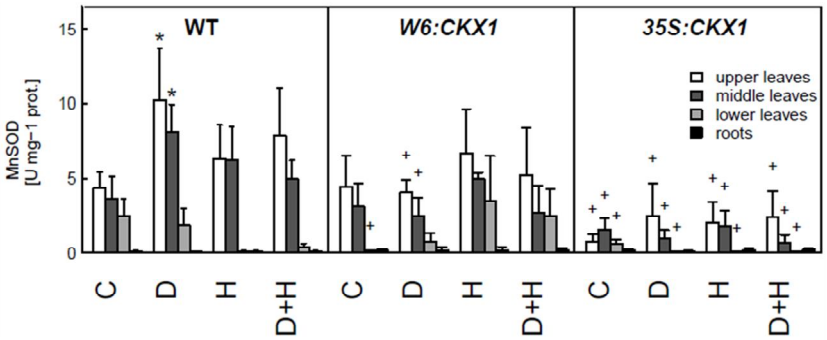

Fig. 4: Activity of MnSOD1 in upper, middle and lower leaves and roots of WT, *W6:CKX1* and *35S:CKX1*. Activity was quantified according to native electrophoresis zymograms. Other designation as described in Fig. 1.

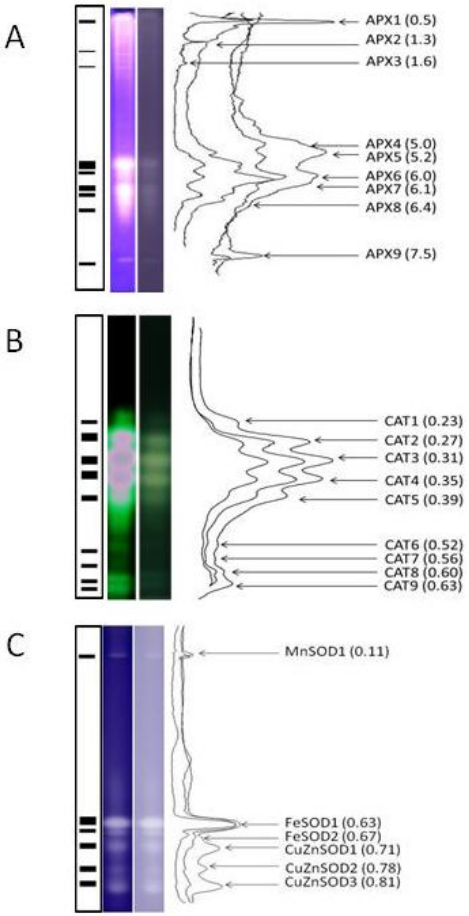

Fig. 5: Diagrams, zymograms, zymograms with edited colours and densitograms (from right) of (A) ascorbate peroxidase (APX), (B) catalase (CAT), and (C) superoxide dismutase (SOD). The activity of all individual isoforms was detected in representative mixed samples of WT tobacco leaves and roots. Relative mobility is marked in brackets

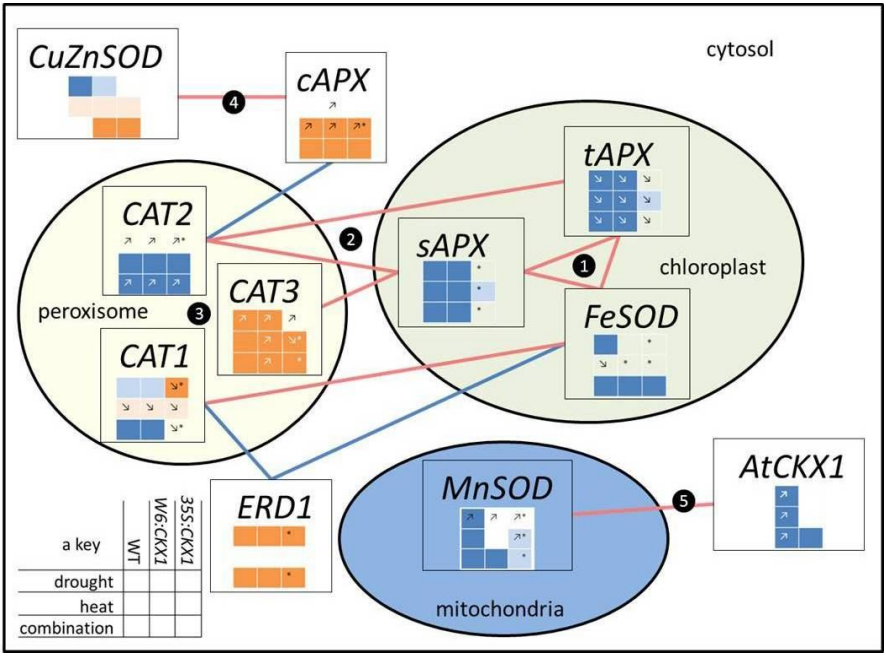

Fig. 6: Diagram of dynamics and relationships between transcripts of antioxidant enzymes in tobacco leaves. Red and blue lines represent positive and negative correlations, respectively, with Spearman correlation coefficient > |0.5|. Significant differences among transcripts under different conditions and genotypes are showed by matrix next to each isoform. Orange and blue colour mark increase or decrease, respectively, in all leaves in comparison to control conditions. 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 (marked as numbered black dots): 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.

Conclusions

- Hormones CK as well as antioxidant enzyme system play role in stress reaction, thus we suppose CK level influence antioxidant enzymes response. 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 photosystem, what also impact peroxisomal CAT because there were strong correlations between chloroplastic and peroxisomal enzymes. MnSOD was probably affected through sugar metabolism in which source/sink management CK plays role.

- Despite increased level of CK improves stress tolerance, plants with decreased CK concentration showed higher stress tolerance than WT. We tested if lack of CK was substituted by improved antioxidant enzyme system. Transgenic plants *35S:CKX1* showed increased expression of *sAPX*, *CAT1*, *CAT3*, *MnSOD* and *FeSOD*, However, we conclude that increased antioxidant enzyme expression was not a cause of improved stress tolerance, but 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 under 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.

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Curriculum vitae

Address: Laboratory of Biology of the Cell Nucleus,
Institute of Molecular Genetics ASCR, v.v.i.
Vídeňská 1083, 142 20, Prague 4, Czech Republic

E-mail: zuzanalubovska@gmail.com

Research experience

2014 – present

Technician, Laboratory of Biology of the Cell Nucleus,
Institute of Molecular Genetics,
The Academy of Sciences of the Czech Republic
Position Summary: Sample preparation for electron microscopy (high preasure freezing, cryosubstitution, preembeding, sectioning, immunolabeling on sections), sample observing on electron microscope.

2006 – 2014

PhD Student, Laboratory of Stress Physiology,
Institute of Experimental Botany,
The Academy of Sciences of the Czech Republic
Position Summary: Experiment design, experiment execute, data evaluation, statistical analysis and data interpretation, presentations of results, manuscript writing.
Key Achievements: Four publications. Applicant is first author of one of them and co-author of three.

2009 (3 months)

Intern, Centre for Agricultural Resources Research,
Institute of Genetics and developmental Biology,
Chinese Academy of Sciences

Position Summary: Creation genome library, detection of genes by Rapid Amplification of cDNA Ends method.
Key Achievements: Six newly sequenced tobacco genes coding for antioxidant enzymes.

2005 - 2006

Laboratory Technician, Department of Virology,
Division of Plant Health,
Crop Research Institute.
Position Summary: RNA isolation, PCR, ELISA, virus infection transduction by insect.

Education

2006 - present

Doctoral study in field of **Plant Anatomy and Physiology**,
Department of Experimental Plant Biology,
Faculty of Science, Charles University in Prague.

Supervisor: RNDr. Naďa Wilhelmová, CSc.
Thesis Title: Antioxidant enzymes response to abiotic stress. Impact of decreased cytokinin level.

2001 - 2006

Master´s study program in field of **Horticulture**,
Faculty of Horticulture, Mendel University in Brno.

Supervisor: Doc. RNDr. Miroslav Pidra, CSc.
Thesis Title: Analysis of *Capsicum annum* mutants with increased nitrate uptake

2005 (1 semester)

Helsingin University, Department of **molecular biology**

Publications

Publications, peer reviewed:

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=2,77)

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 (IF=5,24)

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Supervisor: RNDr. Naďa Wilhelmová, CSc.

Consultant: RNDr. Helena Štorchová, CSc.

Principal grant investigator: RNDr. Radomíra Vaňková, CSc.

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