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The effect of autoregulated *ipt* gene expression, exogenous cytokinins and nitrate on cytokinin metabolism and leaf senescence

Ph.D. THESIS

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Declaration

I hereby declare that the work presented in this manuscript is my own and was carried out entirely with help of literature and aid specified in the enclosed manuscripts.

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Abbreviations

Isoprenoid cytokinins	see Fig. 5
ABA	abscisic acid
ACC	1-amino-cyclopropane-1-carboxylic acid
ADP	adenosine 5' -diphosphate
AMP	adenosine 5' -monophosphate
Arabidopsis	*Arabidopsis thaliana* (L.) Heynh.
ARR	arabidopsis response regulator
AtCKX	Arabidopsis cytokinin oxidase/dehydrogenase
AtIPT	Arabidopsis isopentenyltransferase
ATP	adenosine 5' -triphosphate
AtPUP	Arabidopsis purine transporter
AtRNT	nitrate transporter gene
BAP	N^6-benzyladenine, *meta*-topolin
BFN	bifunctional nuclease associated with senescence from Arabidopsis
CAB	Chl a/b binding protein
CKX	cytokinin oxidase/dehydrogenase
CRE/AHK	cytokinin receptor
c-Z	*cis*-zeatin
DHZ	dihydrozeatin
DHZR	dihydrozeatin riboside
DMAPP	dimethylallyl diphosphate
DR protein	defence-related protein
ELISA	enzyme-linked immuno-sorbent assay
FPC	farnesyl diphosphate synthase
Fru	fructose
GA	gibberelic acid
GC	gas chromatography
GLN	glutamin synthetase gene
Glu	glucose
GUS	beta-glucyronidase
HN	high nitrogen (corresponds to near to optimum nitrogen)
HPLC	high-performance liquid chromatography
HR	hypersensitive response
Chl a/b	chlorophyll a/b ratio
IAA	indole-3-acetic acid
iP	isopentenyl adenine
iPG	isopentenyl adenine glycoside
iPMP	isopentenyl 5'-monophosphate
iPP	isopentenyl disphosphate
iPR	isopentenyl adenine riboside
iPRDP	isopentenylriboside 5'-diphosphate
iPRMP	isopentenylriboside 5'-monophosphate
iPRTP	isopentenylriboside 5'-triphosphate
IPT	isopentenyltransferase
MEP	methylerithritol phosphate
MS	mass spectrometry
MVA	mevalonic acid
MYB	Myb family transcription factor
N	nitrogen
NR	nitrate reductase

PAP	Myb transcription factor genes; putative/production of anthocyanin pigment protein
PR protein	pathogen-related protein
RIA	radio-immuno assay
SAG	senescence associated gene
SAG12	*Arabidopsis* senescence associated gene
SARK	senescence-associated receptor-like kinase
Suc	sucrose
t-Z	*trans*-zeatin
t-ZR	*trans*-zeatin riboside
WT	wild type
Z	zeatin (*cis* and *trans* isomers are not distinguished)
ZMP	zeatin 5'-monophosphate
ZmRR	maize response regulator
ZR	zeatin riboside (*cis* and *trans* isomers are not distinguished)
ZRDP	zeatin riboside 5'-diphosphate
ZRED	zeatin reductase
ZRMP	zeatinriboside 5'-monophosphate
ZRTP	zeatinriboside 5'-triphosphate

1 Leaf senescence as a programmed process

Aging and senescence are recognized as distinguished processes in plant biology. The simplest differentiation can be that aging is a passive, non programmed process while the senescence represents an active, internally programmed and regulated sequence of events leading to sustaining of development of some organs, namely seeds, on the expenses of others (namely leaves). Both are degenerative and both can lead to death, either directly by cellular collapse or indirectly by increased vulnerability to external forces as e.g. non-friendly environment, diseases and predators (Noodén and Guiamét 1996). Loss of viability (ability to germinate) of seeds over time is a classical illustration of aging in plants (Noodén and Guiamét 1996).

1.1 Senescence syndrome

The term “senescence” is generally used to describe the senescence syndrome, which includes both causal and secondary reactions. The senescence processes are described mainly on leaves, the first place with visible symptoms of senescence.

Leaves are specialized photosynthetic organs and the plant invests much energy and nutrients in leaf formation. After a photosynthetic period, the leaves enter their last stage of development: senescence (Quirino et al. 2000, Bleecker and Patterson 1997). The senescence consists of coordinated degradation of macromolecules and of mobilization of metabolites. Nutrients such as nitrogen, phosphorus and metals that were invested in the leaf development are reallocated to younger leaves and to growing seeds, or are stored for the next growing season (Buchanan-Wollaston 1997). Indeed, leaf senescence can be viewed as a recycling program at the organism level (Quirino et al. 2000) leading to loss of homeostasis at the cell or whole plant level (Noodén 1988).

The earlier phases of senescence are characterized by chlorophyll loss, which is related to membrane degradation mainly of thylacoids in chloroplasts (Smart 1994). The apparent chlorophyll loss is the most widely used measure of senescence in green tissues (Noodén and Guiamét 1996). On the other hand, the mitochondria, nuclei and vacuolar membranes persist with little visible change until very late stage of senescence. Mitochondrion is the main organelle of energetic metabolism and senescence appears to require energy, which is probably the reason why the integrity of the mitochondria is maintained to the late phases of senescence (Noodén 1988). The plasma membrane seems to be the last membrane which becomes altered and its decomposition probably marks the collapse of homeostasis, i.e. cell death (Noodén and Guiamét 1996).

On metabolic level the senescence is accompanied by overall enhancement of RNA, protein and lipid degradation (Smart 1994). There is an increase in the activity of proteases, nucleases and enzymes involved in nitrogen, lipid and carbohydrate metabolism that has a great impact on respiration and photosynthesis. Consistently with protein degradation, the number of ribosomes is declining while the mobile amino acids are beeing accumulated. Although protein and RNA synthesis also decline during senescence, some synthesis continues and certain enzymes and mRNAs may increase (Noodén and Guiamét 1996). Indeed, an activation or increase of key dismantling enzymes such as special proteases or lipases rather than a decrease in “house-keeping” or self-maintenance proteins may be caused by senescence. DNA and its coding capacity remain stabile to the late stages of senescence. However, total DNA decreases slightly probably due to chloroplast DNA degradation (Noodén and Guiamét 1996).

Taking together, it implies that leaf senescence requires dismantling of the photosynthetic apparatus to mobilize the nitrogen (N) allocated in photosynthetic proteins that represent the main deposit of organic nitrogen in plants (Takei et al. 2002). This process can be regulated by sugars, cytokinins, light and availability of mineral nitrogen. These compounds/factors act in an interactive manner (Wingler et al. 1998; Takei et al. 2002).

1.2 Developmental and stress induced senescence

Leaf senescence is an integral part of plant development. The onset of senescence can be induced by both internal and external factors inducing and/or controlling age-dependent and premature senescence, respectively (Bleecker and Patterson 1997). If the plant proceeds through the reproductive phase of life and reach a certain stage of ontogeny, senescence will be initiated in the leaf even if the plant is growing in favourable conditions (Smart 1994). In monocarpic plants, pollination and beginning of seed development usually generate a signal for initiation of leaf senescence (Noodén 1988).

Leaf senescence can be also induced by external factors such as phytohormone application (Smart 1994) and/or by environmental stress, such as temperature, drought or desiccation, water logging, low light intensity or nutrient deficiency, pathogen attack and detachment from the plant (Weaver et al. 1998, Smart 1994, Bleecker and Patterson 1997). At the molecular level, age-mediated leaf senescence is the most similar, but not identical process, to senescence induced by darkness in detached leaves and to ethylene treatment (Quirino et al. 2000).

1.2.1 Dark and light induced senescence

Dark-induction is frequently used to induce rapid and uniform senescence (Noodén 1988) of whole plant or detached plant organs namely leaves (Weaver et al. 1998, Ananieva et al. 2004). The senescence of detached leaves that starts after leaf shading may be due to deprivation of leaf of its source of cytokinin, which could result in induction of senescence associated genes (SAGs, see in 1.3). It seems that darkness might be a separate inducer of senescence, which acts cumulatively with the drop in cytokinin levels caused by detachment alone (Weaver et al. 1998). Appositively, the incubation of detached leaves of *Arabidopsis thaliana* in light did not induce obvious visible yellowing, although chlorophyll levels declined in older leaves or during incubation (Weaver et al.1998). On the contrary, incubation of detached leaves under high continuous light promoted senescence, most probably indirectly by blocking the export of over-accumulated assimilates (Noodén et al. 1996). The senescence was induced also in illuminated leaves, but it started after a longer period of incubation than in dark. Interestingly, placing of intact *Arabidopsis* plants to darkness did not induce the senescence characterized by chlorophyll loss and *SAG12* expression but delayed it. However, when individual leaves of intact plants were darkened, senescence was induced in covered leaves indicating that the induction of senescence is highly localized (Weaver and Amasino 2001). Accordingly, the application of a cytokinin (*meta*-topolin) to detached wheat leaves incubated under continuous light in contrast to incubation in darkness accelerated senescence characterized by pronounced increase in excitation pressure on photosystem II and deepoxidation state of xantophyll cycle pigments, accumulation of starch grains in chloroplasts and stimulation of lipid peroxidation (Vlčková et al. 2006). Generally, we can say that light at low or moderate intensities delays senescence while darkness promotes senescence (Noodén and Guiamét 1996).

1.2.2 Lipid metabolism and sugars in relation to leaf senescence

Sugars are the main product of photosynthesis and a decline in photosynthetic activity can play an important role in the initiation of senescence. Accumulation of carbohydrates, namely sucrose and starch in photosynthetically active leaf may be linked to the initiation of senescence (Noh and Amasino 1999a). It is well known that elevated sugar levels repressed the expression of photosynthetic-associated genes, presumably via product-negative-feedback system (Quirino et al. 2000) and the decline of photosynthesis of aging leaves may act as a senescence–inducing signal (Hensel et al. 1993). Pourtau et al. (2006) reported that external supply of sugars induces leaf yellowing, the first visible symptom of senescence. However, the signalling function of sugars during senescence was not definitely proved.

Growth on glucose in combination with low nitrogen supply induces a strong accumulation of sugars during senescence and changes in gene expression that is characteristic for developmental senescence (Pourtau et al. 2006).

A rapid and continuous decline of *SAG12* mRNA levels in response to sucrose, glucose and fructose treatments indicates that the SAG12 regulatory pathway can be effectively down-regulated by the products of photosynthesis. However, *SAG13* expression was not repressed by the same treatment, indicating that the rapid decline of senescence activities by sugar may be specific for developmentally controlled senescence (Noh and Amasino 1999a).

The metabolism of fatty acids is closely associated with sugar metabolism and exploitation of phloem-mobile sucrose in signalling. In *Arabidopsis,* similarly to other plants, the nutrients and metabolites are translocated from senescing leaves to developing seeds (Himelblau and Amasino 2001). If the onset of senescence is characterized by chlorophyll decline reflecting dismantling of thylacoids, the breakdown of thylacoid membrane is initiated early in the leaf senescence cascade according to prediction. As chloroplasts senesce, galactolipid fatty acids from thylacoids are de-esterified and converted to phloem-mobile sucrose to be translocated out of the senescing leaf (Kaup et al. 2002).

1.2.3 Defence response in relation to senescence process

The pathogen attack represents one of the konds of stress which might induce events similar to developmental senescence. Actually, there is an overlap of leaf senescence and pathogen-defence programs (Quirino et al. 2000). Some authors assume the existence of a common factor, such as a stress or post-physiological response (e.g. increased enhancement of ethylene production), that induces the expression of certain genes associated with both senescence and defence responses. For example, a decline in photosynthesis might be an inducer of senescence. In cells undergoing some of defence response, a slowing-down of photosynthesis might initiate the senescence program at a late defence response. In addition, the senescence program might include features of the pathogen-defence response to protect the senescing leaf against opportunistic pathogens. Common regulators of both programs can exist and mutants affected in pathogen response might also be affected in senescence and vice versa (Quirino et al. 2000).

Some genes induced during leaf senescence encode products that are similar to the pathogenesis–related proteins (PR proteins) or more generally defence related proteins (DR proteins). PR proteins are associated with the hypersensitive response (HR, disease resistant response) that results from incompatible pathogen interaction (van Loon et al. 1994). One explanation for the induction of DR genes in senescing leaves is that infections commonly accompany senescence and DR-gene expression is a normal pathogenesis response that usually attends senescence. However, the DR genes are still induced during leaf senescence in *Arabidopsis* plants grown in sterile conditions (Quirino et al. 2000). It seems that the expression of DR genes might be an integral part of the senescence program rather than a direct response to pathogen infection (Quirino et al 2000).

1.3 Genetic control of senescence

Senescence, as a genetically programmed process, is under control of endogenous regulatory factors (Noodén and Guiamét 1996). This implies that senescence is an active process (Buchanan and Wollaston 1997).

As already mentioned in chapter 1.1 and 1.2, the total RNA and protein content is decreasing during senescence and the expression of many genes is switched off (Lohman et al. 1994, Noodén et al. 1997). However, the earlier studies with inhibitors of protein synthesis suggested that senescence is not induced primarily by overall shutting genes off but also by up-regulation of some genes. It has been postulated some time ago that the senescence process may depend on *de novo* transcription of nuclear genes, and molecular studies have proved that this is just the case (Weaver et al. 1998, He and Gan 2002).

The genes which are significantly up-regulated during leaf senescence are generally called SAGs and thay encode some proteases, nucleases and proteins involved in nitrogen and lipid metabolism. They were identified as cDNAs, showing senescence-specific or senescence-preferred expression in many plant species. Their spatial expression was described by Grbic (2002). Different SAGs responded differently to various senescence-inducing factors and their expression patterns in dependence on plant development stage were also quite diverse (Weaver et al. 1998). Some SAGs are expressed in pre-senescence tissue and their expression is increased during senescence, while other SAGs were more specific for senescence, being expressed only during this process. These results indicate the possibility of the presence of multiple pathways in the regulation of senescence (Gan and Amasino 1995, Weaver et al. 1998). In young leaves (prior to full expansion) *SAG12* was not significantly induced by induction pathways that can enhance senescence (Noh and Amasino 1999a). This suggests that there are regulatory pathways that are exclusively activated by developmental signals rather than by environmental or hormonal stimuli. It was shown that *SAG12* can be induced in "senescence-primed" leaves by their detachment and by application of abscisic acid (ABA) and ethylene (Weaver et al. 1998). Therefore it is thought that once leaves started to senesce, the environmental or hormonal factors activating senescence may enhance the progression of developmental senescence which involves the activation of *SAG12*.

Smart (1994) proposed six classes of genes, according to their expression and function of their products, which can be involved in leaf senescence. Class 1 (not specific to senescence) contains genes that control essential metabolic activities of the cell and are expressed at a constant level during the whole life of the leaf. Class 2 and 3 genes are expressed in green leaves, but their activity can influence senescence at later stages. These genes are active before senescence starts and are switched off before any senescence signs occur. The genes which code proteins activated during senescence belong to class 2 and the genes which code proteins causing the initiation of senescence by their absence are included into class 3. Class 4 is represented by regulatory genes, which are expressed immediately prior to senescence or at its beginning. These genes are expressed for a relatively short time. Class 5 includes the genes involved in the mobilization processes occurring specifically during senescence and class 6 consists of genes associated with the mobilization of storage products that may also play a role during other developmental stages. The "mobilizing" genes can be expressed from the onset of senescence to the leaf death.

Buchanan and Wollaston (1997) proposed four new classes of genes expressed during leaf senescence of *Brasica napus* according to their expression patterns. Class 7 includes genes whose mRNA level is gradually increased over the period of leaf development from the youngest leaves and through the senescence stages. Class 8 genes are expressed also during the whole life of the leaf, but the corresponding mRNA levels are low in early stages of leaf development and increase dramatically at a specific stage of senescence. Expression of genes from class 9 is induced specifically in some stages of senescence, but their expression does not continue to the last stages of senescence. Their period of expression is short. Class 10 consists of the genes expressed similarly as genes from class 6 (they are expressed strongly in young leaves and than again during senescence). However, they differ from genes of class 6, being expressed significantly also in mature leaves. The range of different expression patterns implies that the transcription of these senescence related genes is not regulated by the same mechanism. There probably exist a variety of regulatory pathways involved in controlling the individual enzymatic steps involved in coordination of degradative process. The regulatory proteins that control the expression of these genes should be present at various times during senescence (Fig. 1.; Buchanan-Wollaston 1997, Noh and Amasino 1999a).

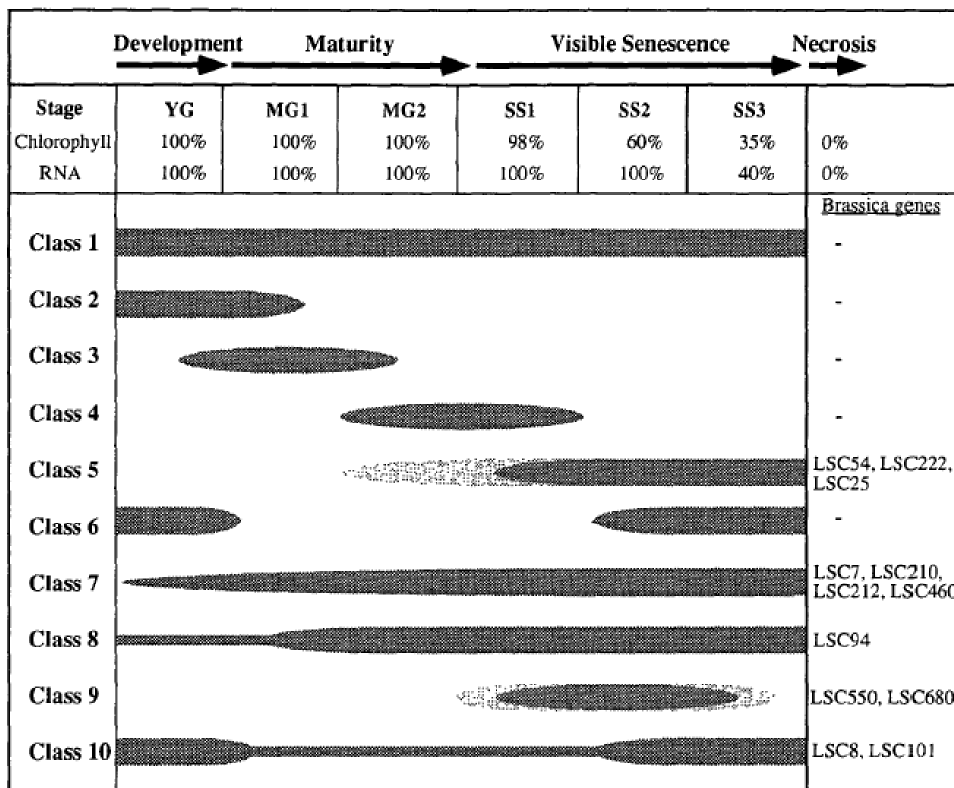


Fig. 1. Expression of genes during leaf development. Patterns of expression of senescence-related genes during leaf development can be used to divide the genes into classes. Classes 1-6 are as proposed by Smart (1994). Classes 7 – 10 are proposed following analysis of different expression patterns of a range of genes that show induced expression during leaf senescence in *Bracisa napus.* The stages of development are the following: YG – fully expand green leaves of young plants; MGI – mature green leaves starting flowering; MG2 – mature green leaves starting siliqued development, SS1, SS2 and SS3 – senescing leaves showing 98%, 60% and 35% of green leaf chlorophyll levels, resp. Levels of chlorophyll and RNA in each sample are as in the percentage of the level in mature green leaves. The *Brasica* genes that represent each class are indicated. According to Buchanan-Wollaston (1997).

1.3.1 Senescence-specific gene expression

Most of the SAGs examined in Arabidopsis thaliana by Weaver et al. (1998) were expressed at a basal level in young leaves, suggesting that SAGs products can play a role in non-senescent tissue. Many of the SAGs were gradually induced with leaf aging and expression predicted visible yellowing. In pea leaves, the mRNA of cystein proteinase starts to accumulate rapidly at a time when the degradation processes (especially protein degradation) have not begun yet (Pourtau et al. 2006).

Hajouj et al. (2000) cloned a SAG gene from bean leaves which was exclusively expressed during senescence. Based on DNA and amino acids sequences they predicted that the gene encodes senescence-associated receptor-like kinase (SARK). SARK, similarly to many SAGs from *Arabidopsis* (Weaver et al. 1998), was expressed in later stages of leaf maturation, but appears shortly prior to the symptoms of senescence characterized by decreases in chlorophyll level and the level of the mRNA corresponding to photosynthetic gene encoding Chla/b binding protein (CAB). The SARK mRNA was detectable before senescence was inducted by exogenous factors such as leaf detachment, darkness incubation and application of ethylene precursor, 1-amino-cyclopropane-1-carboxylic acid

(ACC), suggesting that SARK can be involved in regulation of developmental senescence (Hajouj et al. 2000).

Degradation of nucleic acids is an integral part of processes involved in plant development, including senescence. Perez-Amador et al. (2000) identified and characterized *Arabidopsis* gene *BFN1*, whose transcript corresponds to a nuclease associated with senescence. It encodes nuclease type I enzymes, which are responsible for the degradation of RNA and single-stranded DNA. *BFN1* mRNA level was expressed in the whole plant before senescence, but its level was significantly increased in senescing tissues. Generally, a number of genes involved in induction of senescence and senescence signalling have been characterized and cloned, allowing experimental intervention in senescence-associated process. This knowledge gives us an efficient tool for control of progress of senescence and potentially for improvements of crop plants.

1.3.2 External factors influencing SAGs expression and its relation to developmental process

The incubation of detached leaves in darkness and also in light induces both visible yellowing and expression of all of 11 SAGs examined by Weaver et al. (1998). The light-induced as well as ethylene-induced expression of SAGs was weaker than that induced by the darkness. Most SAGs that responded positively to darkness were detected in both young and old leaves. It seems that for SAGs, the dark response and the age-mediated response are additive (Weaver et al. 1998). In prolonged darkness, SAGs expression declined similarly as declined the corresponding mRNA levels. The *SAG12*, according to its expression patterns is considered as the gene which comes closest to specificity for natural senescence and appears to be the best so far known molecular marker of age-dependent senescence (Weaver et al. 1998). *SAG12* expression was induced over 900-fold by glucose in *Arabidopsis* during late stages of senescence (Pourtau et al. 2006). Sugar-inducible late senescence is consistent with hypothesis claiming operation of a product-negative-feedback system participating in control of senescence (Quirino et al. 2000). Two MYB transcription factor genes, *PAP1* and *PAP2*, were identified as senescence associated genes that are induced by glucose (Pourtau et al. 2006). The growth of *Arabidopsis thaliana* plants on glucose containing medium induced genes for nitrogen remobilization that are typically enhanced during the developmental senescence, including glutamine synthetase gene *GLN1;4* and the nitrate transporter gene *AtRNT2.5*. Taken together, these results indicate that *Arabidopsis* leaf senescence is induced by hexose accumulation (mainly glucose and fructose) rather than by starvation and is not repressed by sugars (Pourtau et al. 2006). Hexoses accumulate in senescing *Arabidopsis* leaves. External glucose treatment mainly induced genes that are characteristic for developmental senescence, such as *SAG12*, and genes of pathways for nitrogen mobilisation (Pourtau et al. 2006).

The induction of *SARK* gene, encoding one of potentional senescence-associated kinase (Hajouj et al. 2000), was also increased and accelerated by detaching leaves and their incubation in the dark or following ACC application. On the contrary, cytokinin application delayed senescence and transcription of *SARK* mRNA. The putative function of the *SARK* gene and the fact that its expression was detected before any senescence symptoms occurred suggests that *SARK* expression may regulate some pathways of the age-dependent senescence program.

He and Gan (2002) cloned a leaf senescence-associated gene *SAG101* from *Arabidopsis. SAG101* transcript displayed acyl hydrolase activity and was significantly expressed after the onset of senescence. The acyl hydrolase disturbs cell membranes by attacking the phospholipids bilayer, and the initial attack on membranes marked the onset of leaf senescence. Released membrane fatty acids, including linolic acid, can serve as precursors for jasmonic acid (He and Gan 2002).

Some plant hormones, such as ethylene, jasmonic acid and ABA are known as enhancers of senescence. In *Arabidopsis* leaves treated with ABA or ethylene the age-

related SAGs were induced, similarly as in the whole plant replaced into dark (Weaver et al. 1998). Although there are common biochemical pathways in the senescence systems of detached and attached leaves, different genes may participate in the two systems (Weaver et al. 1998, Becker and Apel 1993). This may be due to fact that various stress factors, such as wounding or darkness, causes stress responses that visually mimic the senescence syndrome but the resultant expression of the stress-induced genes may not participate in the natural senescence syndrome (Weaver et al. 1998). Indeed, the functions of some of the identified SAGs are related primarily to protecting cells from stress (Weaver et al. 1998). *SARK* is expressed in intact leaves prior to senescence-related biochemical changes; it is possible that this gene is more directly associated with the senescence program than with the stress response that may be caused by senescence (Hajouj et al. 2000).

1.3.3 Transgenic plants with delayed senescence

Transgenic plants with altered cytokinin biosynthesis and metabolism have been used to overcome the problems associated with the uptake, translocation and metabolism of exogenously applied cytokinins (Chang et al. 2003). An elegant example is the autoregulatory system controlling cytokinin levels in response to senescence in tobacco plants developed by Gan and Amasino (1995). They transformed tobacco plants with isopentenyltransferase (*IPT*; EC 2.5.1.27) gene from *Agrobacterium tumefaciens* under the control of the promoter for *SAG12,* a senescence specific *Arabidopsis* gene encoding a Cys proteinase (Lohman et al. 1994, Fig. 2). Expression of this chimeric gene *SAG::IPT* is induced at the beginning of senescence, and cytokinins are synthesized till they reach the level which prevents the further progress of senescence. If the senescence is stopped, the SAG12 promoter is switched off and the cytokinin content falls down till the senescence is re-induced to start a new round of *SAG12::IPT* expression. This autoregulatory system prevents cytokinin overproduction that could lead to an abnormal plant development (Gan and Amasino 1995). Autoregulated cytokinin production results in prolonged photosynthetic life span due to delayed decline of chlorophyll and enzymes involved in photosynthetic metabolism (Wingler et al. 1998). Transgenic tobacco plants showed prolonged flowering, more flowers and increased seeds production as well as the yield. The senescence-related yellowing of older leaves started only in wild type plants in the early stages of flowering. The differences in leaf yellowing well correlated with chlorophyll and protein content which decreased strongly as the leaves aged. Photosynthesis rate remains higher in transgenic plants (Wingler et al. 1998). Similarly, *SAG::IPT* transgenic *Lactuca sativa* plants exhibited significantly delayed developmental and postharvest leaf senescence in mature heads and in this way prolonged life of lettuce heads (McCabe et al. 2001).

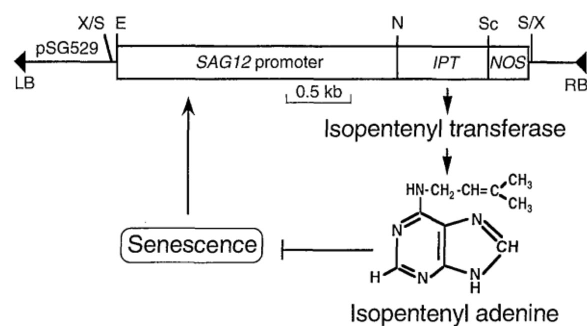


Fig. 2. Rationale of the autoregulatory senescence-inhibition system. The senescence specific SAG12 promoter was fused to *IPT*. At the onset of senescence P-SAG 12 directs the expression of *IPT* which results in an increased level of cytokinin that in turn inhibits senescence. The inhibition of senescence attenuates expression from $P_{SAG\ 12}$ to prevent

The SAG12 promoter can drive the senescence-specific expression of either *ipt* or □□glucuronidase (*GUS*) in tobacco (Gan and Amasino 1995) suggesting the conservation of a regulation sequence between *Arabidopsis* and tobacco (Noh and Amasino 1999b). However, this promoter region does not contain consensus sequence for known DNA-binding proteins. It seems that the regulation of developmental senescence involves a new or divergent class of transcription factors (Quirino et al. 2000).

The timing of *SAG12* expression was examined by many authors. The spatial expression pattern of this senescence-specific gene was described by Grbic (2002) who used *GUS* under P-SAG12 regulation. SAG12 promoter was active, after the beginning of senescence process, in all the cells of senescing leaves. The *GUS* gene product accumulated also in the senescing corolla limb, corolla abscission zone and in anthers and pistils of pollinated flowers. The expression pattern is consistent with the proposed role of SAG12 in mobilizing nutrients from senescing organs; SAG12 was expressed also in the phloem cells used to transport nutrients out of senescing leaves (Grbic 2002). Accumulation of GUS in specific domains of senescing flowers suggests that age-dependent factors are not sufficient to activate SAG12 in these tissues. Instead, a more complex regulatory network seems to control the SAG12 expression in tobacco reproductive organs.

Similarly to *SAG::IPT* in tobacco, the *SAG12::IPT* in *Petunia* also exhibited increased IPT levels after pollination (Chang et al. 2003). Flowers from transformed plants were less sensitive to exogenous ethylene and required longer treatment to induce endogenous ethylene production. Accumulation of ABA, the hormone which regulates flower senescence, was significantly greater in wild type (WT) plants confirming that floral senescence was delayed in IPT plants.

1.4 The role of leaf senescence in monocarpic plants

Monocarpic senescence starts after the plant begins to produce seeds (Noodén 1988). In annual plants, monocarpic senescence is characterized by a syndrome of physiological changes (Noodén 1988) during the reproductive phase (Noodén and Guiamét 1996). The onset of reproductive phase of plant development as well as of senescence of monocarpic plants is characterized by stop of vegetative growth followed by massive mobilization of nutrients, such as nitrogen and organics from the mature leaves to other plant parts. The processes are highly regulated and coordinated. In annual plants, which include most agriculture crops, the mobilizable nutrients and metabolites from the entire plant are used for biosynthesis of store compounds that ultimately deposited in the developing seeds (Mauk et al. 1990). Towards the end of seed filling, the leaves become visibly yellow and drop off, leading to the death of the plant (Noodén and Letham 1993). Disassembly of the photosynthetic apparatus and leaf abscission are parallel but separately regulated components of monocarpic senescence (Noodén 1988). In barley, three phases of senescence could be distinguished after the flag leaves reached their final lengths: (1) early decrease in photosynthetic capacity and D1 protein level, (2) decline in chlorophyll content and (3) decrease in cytochrome level and small unit of rubisco. All these events result into a decline of photosynthetic activity earlier than the chlorophyll content declined (Humbeck et al. 1996). With the onset of reproductive phase the flux of cytokinins and minerals via the xylem sap and their flux into the shoot decline (van Staden et al. 1988). This sequence of events suggests that pollination represents a principal signal for initiation processes subordinated to sacrificing of plant body for efficient seed production.

2 The role of plant hormones in control of leaf senescence

All the major plant hormones are directly and/or indirectly implicated as being involved in the senescence process. However cytokinins and ethylene definitely have a role in the regulation of senescence (Smart 1994). Very often their effect is associated with control of plants growth and development in general and is not senescence-specific.

2.1 Plant hormones as messengers of physiological signals

The correlative controls of plant development are mediated by hormones. The classical example of correlative control of senescence is the ability of roots to retard leaf senescence

by producing an antisenescence hormone cytokinin (van Staden et al. 1988) or the ethylene mediated senescence of flowers after pollination (Smart 1994).

There are at least four signalling pathways mediated by plant hormones that can play a role in the regulation of genes involved in leaf senescence (Buchanan-Wollaston 1997). Cytokinins represent the first group of potential (negative) signals needed for the onset of senescence. Senescence process can only be initiated when the cytokinins in the leaf fall below their threshold level. Preservation of cytokinin levels inhibits transcriptional regulation of senescence enhanced genes and prevents the onset of senescence. The second group includes signals arising from developing sinks. Removal of developing sinks delayed senescence. However, this process can also be reversed (Crafts-Brandner 1991). The signal from blossom and developing seeds seems to be weaker than the cytokinin threshold signal since the senescence started in the transgenic tobacco plants after repression of IPT expression in leaves (Gan and Amasino 1995). However, in wild type plants cytokinin levels seem to be influenced by the signal from developing generative sinks. Another hormone, which can be involved in plant development as a senescence signal, is ethylene. It is known from mutant studies showing delayed leaf senescence, that ethylene changes the timing of senescence. An increased ethylene level enhances senescence process, but only in leaves that are already programmed to start senescence. Ethylene does not appear to activate senescence-related genes directly, but probably modulates the activation of the corresponding genes by other signals. Ethylene may have a role in repressing the expression of genes involved in photosynthesis (Grbic and Bleecker 1995). Levels of photosynthates and other metabolites can also be considered as plant signal influencing senescence progress. The earliest sign of senescence initiation is the decline in carbon fixation rates which, in *Arabidopsis* and some other plants, appear after the full leaf expansion is reached (Hensel et al 1993). The decline in photosynthesis, which is manifested by reduced sugars availability, can be a signal for the induction of expression of senescence-related genes (Buchanan-Wollaston 1996).

2.2 The role of exogenous cytokinins in the progress of leaf senescence

The ability of cytokinin to delay leaf senescence of was discovered just fifty years ago (Richmond and Lang 1957). Although they do not necessarily block the expression of all SAGs (Becker and Apel 1993), Gan and Amasino (1995) believe that cytokinins are strong antagonists of senescence. A delay in SAG expression by cytokinins was also detected in detached leaves (Weaver et al. 1998). The control of senescence by cytokinins proceeds at the transcriptional level because the presence of cytokinins inhibits an expression of senescence-related genes either directly or indirectly via a signalling pathway (Buchanan and Wollaston 1996). Weaver et al. (1998) proposed that an external supply of cytokinin may substitute the decline of internal cytokinin levels caused by leaf detachment.

Exogenous application of cytokinins inhibits the degradation of chlorophyll and photosynthetic proteins (Badenoch-Jones et al. 1996). Weaver et al. (1998) incubated detached leaves of *Arabidopsis thaliana* either in water or in cytokinin solution in the dark. In addition to the delay of visible yellowing by applied cytokinins the expression of most of the SAGs was repressed. The cytokinin effect was stronger in younger leaves, which were presumably not yet developmentally ready to senesce, compared to the older ones which would have begun to senesce even in the absence of detachment. A similar effect in response to applied benzyl adenine (BAP) to detached bean leaves was described by Hajouj et al. (2000). Exogenously applied BAP repressed the initiation of *SARK* expression in darkness. Similarly to natural leaf senescence, the appearance of SARK following this treatment was also visualized prior to the decrease in chlorophyll level (Hajouj et al. 2000).

Not only leaf but also blossom senescence is repressed by exogenously applied cytokinins (Cook and van Staden 1988; Upfold and van Staden 1990). The effect of cytokinin treatment depends on cytokinin form, concentration, pulse versus continuous application or the stage of development at which the blossom was treated. Zeatin (Z) and its combination with some mineral nutrients reversed senescence-inducing influence of the developing pods

in soybean. While this combination may not stop the decline in foliar chlorophyll, it does prevent total loss and death (Mauk et al. 1990).

2.3 The role of ethylene

The gaseous hormone ethylene functions as a senescence-promoting signal in many organs, particularly blossomes and fruits (Noodén and Guiamét 1996). ACC, the immediate precursor of ethylene (Yang and Hoffman 1984), enhanced senescence processes accelerating chlorophyll breakdown and the appearance of *SARK* mRNA in detached bean leaves (Hajouj et al. 2000). The fact that ACC induce *SARK* expression is consistent with the notion that once the leaf reaches its mature stages, ethylene accelerates the same parameters of leaf senescence as those which characterize the developmental senescence (Grbic and Bleecker 1995, Weaver et al. 1998). The treatment of young *Arabidopsis* leaves by ethylene did not result in increased expression of senescence-related genes (Grbic and Bleecker 1995), but it accelerated the senescence process in already senescing leaves (Smart 1994). Decrease respiration, which coincided with rapid chlorophyll degradation. Thus, transcription of senescence-related genes is not achieved by ethylene unless the leaf is "ready" to senesce. Therefore, the ethylene signalling pathway is probably not directly involved in regulation of transcription of senescence-specific genes during leaf senescence but its presence/activation probably increases the sensitivity of signalling pathway to the "age-related factors". Correspondingly, in the absence of ethylene, transmission of the signals leading to the senescence occurs later, possibly due to the requirement for a higher concentration of "age-related factors" (Buchanan-Wollaston 1997).

It seems that although ethylene has an effect on senescence, it is not an essential regulator of the process. Moreover, its effect is species-specific. In many plant species ethylene appears to have no role in ripening or senescence; for example in the ripening of fruits of non-climacteric plants such as strawberry, in the senescence of some flowers such as day lilies and in leaf senescence of some plants, in particular the monocots, there is no requirement for ethylene signalling (Smart 1994).

2.4 The role of other hormones

The ABA is commonly known to induce senescence. Weaver et al. (1998) sprayed whole *Arabidopsis thaliana* plants with ABA. The treatment induced relatively quickly visible yellowing in older leaves and rapid increase of mRNA levels of *Cor15*, an ABA-responsive gene. About half of the SAGs responded to ABA. The response was stronger in the older than younger leaves and was consistent with the induction of yellowing. Similarly to other hormones influencing senescence process, the ABA affect leaves that are ready to senesce, accelerating the senescence progress (Weaver et al. 1998, Smart 1994). Increased endogenous ABA level following pollination in Petunia is probably a primary response to senescence, rather than an initiator of the process as is indicated by the delay of senescence in *SAG12::IPT* petunias (Chang et al. 2003). In plants growing on glucose medium with low content of nitrogen, senescence was accelerated and sugars were accumulated. However, the ABA treatment did not influence sugar induced leaf senescence (Pourtau et al. 2004).

Some senescence associated genes are affected not only by cytokinins but also by ABA and auxin. ABA activates directly *SAG13*, which seemes as a good molecular marker of early senescence, although basal level of *SAG13* was detected in both younger and older leaves (Weaver et al. 1998). As mentioned above, ABA and ethylene accelerate senescence process.

The rapid repression of *SAG12* mRNA levels by auxin treatment indicates a relatively direct regulation of *SAG12* expression by auxin (Noh and Amasino 1999a), although a longer effect of auxins did not avoid later increase of *SAG12* expression. Its promoter was repressed on molecular level by sugars (Suc, Glu, Fru), auxin and cytokinins, such as kinetin and BAP, but not by adenine (Noh and Amasino 1999a). Polyamines, especially spermidin

(Sood and Nagar 2003), and gibberellic acid (GA) (Jordi et al. 1994) retarded leaf senescence characterized by chlorophyll loss. Jasmonic acid and some of its derivatives promote senescence in both intact plants (Ananieva et al. 2004) and detached leaves (Parthier 1990).

3 The role of cytokinins in root to shoot signalling

It was believed that cytokinins are predominantly synthesized in the root tips and transmitted to the shoot as a root-synthesized signal (Reviewed in Beck 1996, Aloni et al. 2005). However, the functioning of cytokinins in long-distance and/or paracrine signaling has accompanied the cytokinin research from its early beginning. Engelbrecht (1972) in her classical study could detect cytokinin activity in separated bean leaves only after the rooting of petioles. The rooting was associated with occurrence of cytokinin activity in petiole sap and with delay of leaf blade senescence known to be suppressed by cytokinins. Following the rooting of petioles the bioassayed cytokinin activity spread upwards to leaf blade, indicating that roots are the main site of cytokinin biosyntheis and that synthesized cytokinins are transported from roots acropetally to the leaf. This contrasts with her and others' earlier finding that the retention of chlorophyll in tobacco leaves is restricted to the area of the blade to which kinetin is supplied and that labeled amino acids migrate to and accumulate in the treated parts of leaf (Mothes et al. 1961). However, cytokinin biosynthesis is not limited to roots. Tissues of some organs including shoots, immature seeds, young leaves and shoot meristems are capable to produce cytokinins (Chen and Petschow 1978, Chen et al. 1985, Chen and Chang 2001) (for reviews see Letham 1994).

In spite of the efforts of number of researches, the biosynthetic pathway(s) of cytokinin in roots, and in higher plants in general, had not been identified for a long time. A breakthrough was achieved by Kakimoto (2001) and Myiawaki et al. (2004), who identified and cloned a number of *Arabidopsis IPTs* and characterized their spatial expression patterns. This allowed detection of several sites of cytokinin biosynthesis in addition to roots including those in young leaves and in vasculature (Miyawaki et al. 2004) (see chapter 4.1 below). This contradicts the hypothesis of exploitation of cytokinins as long-distance mediators in root-shoot communication and supports the study of Faiss et al. (1997) who suggested that effect of cytokinins is much localized corresponding to a short-distance paracrine signalling.

Biological functioning of both, the long-distance and short-distance transport of cytokinins, assumes localization of a cytokinin perception and transduction system(s) at the site(s) of hormone action. Kakimoto (1996) identified two component system mediating perception and transduction of cytokinin signal. It is similar to the bacterial two-component phosphorelay paradigm (reviewed in Kakimoto 2003). Cytokinin perception on cell surface and subsequent changes in gene expression in the nucleus determine the signalling events in plant cell (Maxwel and Kieber 2004). One output appears to be the interaction of type-A ARRs with phytochrome and another is the regulation of gene expression by the type-B ARRs but other outputs surely remain to be uncovered. Two important additional potential outputs are the regulation of protein stability and calcium signalling (Maxwel and Kieber 2004).

3.1 Cytokinins in xylem exudates

It was reported many times that cytokinins are mainly synthesized in roots. However, the place of their action is in the "opposite" part of plant, in the shoots, namely in shoot meristems. Thus the cytokinins need to be transported by xylem to the aerial part of the plant. The xylem transpiration stream was proved to be the main route of cytokinin transport in higher plants (see Aloni et al. 2005 and references therein). Transport of cytokinins to leaves via the vascular tissue to sites with high water loss results in the establishment of basal-to-apical gradient of free cytokinins.

As the dominant cytokinins of xylem sap were determined in soybean Z and zeatinriboside (ZR), which are also very active in preventing leave senescence (Noodén and

Letham 1993). In tobacco leaves, the cytokinin bases (*trans*-zeatin (t-Z) and dihydrozeatin (DHZ)) and their ribosides were identified as major xylem cytokinins, while ribotides (t-Z- and isopentenyl (iP)- nucleotides) were present at low levels and *O*-glucosides were either absent or present under detection limits (Singh et al. 1992). Similarly, in rice, the main cytokinins in xylem sap were t-ZR and t-Z and isopentenylglycoside (iPG). The amounts of bioactive cytokinins (iP, t-Z, dihydrozeatin riboside (DHZR)) were increased after panicle (i.e. sink) removal (Shinano et al. 2006). Badenoch-Jones et al. (1996) reviewed occurrence of number of cytokinins in xylem sap of different plant species. The t-ZR, DHZR, Z, iP and isopentenylriboside (iPR) were reported to occur in xylem sap of dicotyledonous plants while in monocots t-ZR, t-Z, c-Z and iP-type cytokinins were detected in measurable amounts.

The positive effect of xylem sap ZR on delay of leave senescence corresponds to the declining of cytokinin level in xylem sap following the initiation of senescence. Based on this finding Noodén et al (1990) suggested that reduced level of cytokinins in the leaf can cause the onset of senescence. Similarly, the onset of reproductive phase is followed by the decline of cytokinins and minerals flux through the xylem and initiation of leaf senescence (van Staden et al. 1988). During the development of soybean pods, just before seeds are filled, the cytokinin and nutrient levels in xylem declined markedly (Mauk et al. 1990), and this fall was reflected in decreased foliar photosynthetic activity. As soon as the seeds stop to accumulate their dry weight, the leaves still growing on the plant can restore their own dry mass. This late increase probably reflects the Z-mediated preservation effect on foliar assimilation process (Mauk et al. 1990).

Decrease in xylem flux seems to be a necessary but not unique reason to start leaf senescence (Noodén and Letham 1993). The [^{3}H]-labelled cytokinins, taken-up by plant to the xylem, flowed primarily to the leaves, where they were rapidly metabolized preferentially to adenine and its riboside. Therefore Noodén and Letham (1993) concluded that continuous supply of cytokinin is required and that the changes in cytokinin flux appear to be a sensitive signalling mechanism controlling the senescence.

In rice, the flux of cytokinins from roots to shoots decreased continuously after rice panicle initiation, whereas it increased when the panicle was removed (Shinano et al. 2006).

3.2 Cytokinins as a signal of N availability

Nitrate is the major source of inorganic nitrogen for most of higher plants. Nitrate itself is the primary signal molecule which induces the transcription of nitrate assimilation and related genes. Because the nitrate can induce the expression of genes involved in its utilization by itself, it seems that nitrate can function as both an assimilation substrate and a signalling molecule.

In his inspiring study Beck (1996) proposed that cytokinin trafficking from roots via the xylem to shoots serves not only as a mechanism for cytokinin delivery but also as a pathway transferring physiological signals. In this context, Takei et al. (2002) suggested that cytokinin metabolism and translocation could be commonly modulated by nitrogen availability in higher plants (Fig. 3). Thus, in addition to nitrate, cytokinin could be another root-to-shoot signal communicating nitrogen availability.

3.2.1 Cytokinin accumulation in roots in response nitrate

The accumulation of cytokinins in roots and shoots in response to re-supply of plant with nitrate was repeatedly demonstrated for different species of plants (reviewed in Kaminek et al. 2003). Maize plants responded to the re-supply of nitrate to roots in a space and time dependent manner. Levels of endogenous cytokinins were sequentially increased in roots, xylem sap and shoots. The iP ribotide, the primary product of cytokinin biosynthesis (Takei et al. 2001a), was the first accumulated cytokinin in maize roots, after the nitrogen re-supply (Takei et al. 2001b, 2002). The early production of iP ribotide and the decrease of t-Z-*O*-glucoside explain the accumulation of iP- and Z-type cytokinins. In conclusion, not only increased cytokinin biosynthesis, but also the release of cytokinins from temporary inactive

storage conjugates is responsible for the cytokinin accumulation induced by nitrate (Takei et al. 2001a). Accordingly, Dodd and Beveridge (2006) observed a declinine of cytokinin level in xylem sap, if the pea plants were deprivated of nitrogen.

Takei et al. (2001a) and Golovko et al. (2002) characterized 9 *IPT* genes in *Arabidopsis. AtIPT5* transcript correlated with the concentrations of NO_3^- and NH_4^+ in the grown medium, but its expression was only slightly affected, while *AtIPT3* expression was rapidly induced by nitrate followed by the accumulation of cytokinins (Takei et al. 2004a). It seems that *AtIPT3* is a key determinant of cytokinin biosynthesis in response to rapid changes in the availability of nitrate, whereas *AtIPT5* reacts to long term treatment of both ammonium and nitrate (Takei et al. 2004a). In summary, cytokinin biosynthesis in roots can be regulated by nitrate that enhances NO_3^- responsive *IPT3*. Cytokinin accumulation in roots is followed by increase of cytokinin flux via xylem to shoots. Cytokinins delivered to leaves are capable to activate expression of nitrate-specific response regulators.

Ammonium ions, other source of nitrogen for plant, can also influence plant development. Application of ammonium ions affected both cell division and elongation in the leaves of tobacco. Growth inhibition of leaves was associated with reduction of Z and ZR levels in xylem sap. No inhibition effect was detectable, if NH_4^+ was applied together with NO_3^-. It seems that the presence of nitrate is required for cytokinin biosynthesis and/or root to shoot transfer of cytokinins at a level that is sufficient to mediate normal leaf morphogenesis (Walch-Liu et al. 2000). In detached tobacco leaves ammonium ion- and/or nitrate treatment induced accumulation of Z and indicated that cytokinin could be synthesized in leaves in response to inorganic nitrogen sources (Singh et al. 1992). Ammonium ions also delayed tobacco leaf senescence, probably due to elevated endogenous cytokinin level (Sign et al. 1992).

3.2.2 *Effects of cytokinins on uptake and assimilation of nitrate*

Cytokinins may not only signal nitrogen availability to the shoots but also enhance nitrate uptake and translocation by activation of meristematic and reproductive sinks. Cytokinin enhanced strength of sink seems to be a driving force for N uptake, assimilation and allocation within the plant (Kamínek et al. 2003). Indeed, net nitrate uptake increased significantly in wheat plants incubated in nutrient solution and treated by cytokinins on leaves. Cytokinin, delivered to N starving plants, was more efficient in improvement of N economy and grain yield than when applied to wheat plants sufficiently supplied with mineral nitrogen (Trčková and Kamínek 2000). The effect of cytokinins on nitrate uptake is accompanied by stimulation of chloroplast development and delay of chloroplast ageing that maintain supply of photoassimilates and energy required for inorganic N assimilation. The supply of leaves with nitrate is promoted by cytokinin-enhanced transpiration and probably also by their involvement in the control of NO_3^- uptake in response to cytokinin-induced nitrate reductase (NR) (Gaudinová 1990), NO_3^- assimilation, and translocation (Trčková and Kamínek 2000). The ability of cytokinins to induce NR and to provide carbohydrates improves conditions for N assimilation.

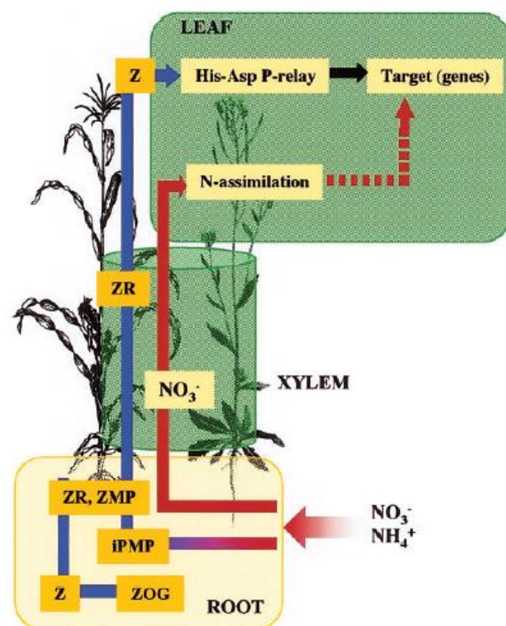


Fig 3. The scheme of multiple routes communicating nitrogen availability of roots to the shoots. Z – zeatin; ZR – zeatin riboside; ZOG – zeatin glucoside; IPMP – isopentenyladenine monophosphate; NO_3^- - nitrate; NH_4^+ - ammonium ion; His-Asp-P-relay – histidine – asparagine – phosphorelay. According to Takei et al. (2002).

Lowering of nitrate concentration in xylem sap and apoplast following its assimilation may stimulate nitrate uptake.

Trčková and Kamínek (2000) observed differences in nitrate uptake rates between plants grown in high and low nitrate nutrient solutions. Cytokinin was far more effective in stimulation of NO_3^- uptake when applied to low NO_3^- nutrient solution while the response of plats grown under optimum NO_3^- supply was very low. The NO_3^- uptake capacity was influenced more than uptake rate following cytokinin application, probably due to a more developed root system in cytokinin treated plants (Trčková and Kamínek 2000). It seems that cytokinins in addition to nitrate represent a root-to-shoot signal communicating nitrogen availability (Takei et al. 2001b).

Nitrate and cytokinin supply significantly increased NR activity, but not in flag leaf of plants grown on high nitrate medium (HN). It is possible that high NR activity in flag leaves of HN plants is induced not only by nitrate, as its substrate, but also by endogenous cytokinins (Gaudinová 1990). Accordingly, the addition of nitrogen to nitrogen-starved maize increased the level of isopentenyladenineriboside 5'-monophosphate (iPRMP), preceding elevation of t-ZRMP, t-ZR and t-Z in roots (Takei et al. 2001b). Banowetz (1992) documented light dependent effect of exogenous BAP on NR induction in wheat seedlings. The inducible effect depended on the light conditions under which the seedlings were grown prior to BAP treatment. Light influenced the endogenous concentration of cytokinin, which was 20-fold higher compared to etiolated plants. Similarly, Gaudinová (1990) reported that kinetin induced NR in the absence of nitrate in light grown seedlings. Appositively, the level of zeatin-*O*-glucoside (ZOG), the storage form of cytokinin, decreases gradually after replenishment of nitrate in the maize roots (Takei et al. 2002).

3.2.3 Sequence of processes involved in signalling of nitrogen availability

Takei et al. (2001b) described spatial and temporal accumulation pattern of cytokinin species in roots, xylem sap and leaves during re-supply of nitrate (NO_3^-) to maize grown under nitrogen-starving conditions. The t-ZRMP and t-Z, followed by t-ZR, were the first cytokinins accumulated after nitrogen addition to the roots. The dominant cytokinin in xylem sap was t-ZR, whereas t-Z predominated in leaves. The rate of xylem flow increased somewhat later, similarly as the cytokinin concentration in the exudate, and was followed by the increase of t-Z in leaves and by enhanced expression of transcripts of ZmRR1 and ZmRR2 genes that function as nitrogen-responsive regulators (Takei et al. 2001b; 2002). The time dependent accumulation pattern of cytokinins suggests that cytokinins are transported across the roots to shoots in response to nitrogen availability and function as signalling molecules transducing nitrogen status of roots to the shoots and that the Z-type cytokinins induce the ZmRR1 gene expression (Takei et al. 2001b). Interestingly, nitrate did not induce the nitrogen-responsive ZmRR1 in detached maize leaves, suggesting the existence of another long-distance signal of nitrogen availability (Takei et al. 2001a). Genes which are induced directly by nitrate can be called as nitrate-specific responsive genes (for example NR). Other group includes genes broadly responsive to nitrogen, which respond slowly and the inducible effect of nitrogen can be replaced by other compounds (Takei et al. 2002).

3.2.4 Control of nitrate uptake and leaf senescence in transgenic plants

Transgenic tobacco plants expressing *IPT* gene under SAG12 promoter and grown under N-limiting conditions showed increased cytokinin content (mainly Z and ZR) in both senescing and younger leaves (Jordi et al. 2000). The senescence-associated decline in N levels was delayed in transgenic plants. Nitrogen accumulation in roots was not affected, but the N translocation to younger leaves was progressively reduced. It seems that older leaves compete against the young ones for N. The Z-type cytokinin level increased strongly in old leaves, but only slightly in younger ones. Jordi et al. (2000) suggested three explanations for

the event described above: (1) Cytokinins are transported from older to younger leaves, (2) the SAG12 promoter is not strictly senescence-specific or (3) young leaves increase their cytokinin production in response to the increased sink activity of the older leaves. Because the SAG12 promoter was repeatedly described as a specifically senescence induced promoter, N deficiency induced senescence was used as an activating factor for senescence in his experiments. Senescing old leaves in the *SAG::IPT* plants remained strong sinks for N (Jordi et al. 2000).

4 Modulation of cytokinin levels

Cytokinins in cooperation with auxin play a key role in the regulation of cell division and differentiation. Their effect on physiological processes does not depend only on their endogenous contents but also on the hormone perception and signalling. However, numerous data indicate that regulations by hormone concentration are more effective and operational than those realized via hormone signalling.

Hormones as the key compounds regulating plant development must quickly react to changed environmental and developmental stimuli. The re-establishment of their homeostasis should follow changes in hormone levels. It implies that metabolic regulation of cytokinin levels in plant cell must cause rapid and transient changes of hormone concentration. While the transient change in cytokinin concentrations in relation to that of auxin is essential for induction of such specific morphogenetic processes, as e.g. adventitious bud formation and tillering, the re-establishment of cytokinin homeostasis is essential for the continuation of initiated events. Based on this paradigma Kaminek et al. (1997) proposed a complex network of mechanisms regulating cytokinin levels (Fig. 4).

Once the cytokinin accumulation achieves a threshold level it can be further enhanced in competent cells by a positive feedback mechanism (habituation). Cytokinin accumulation can be caused not only by stimulation of cytokinin biosynthesis but also by (1) inhibition of cytokinin degradation by cytokinin oxidase/dehydrogenase (CKX), (2) suppression of cytokinin inactivation by *N*- and *O*-glycosylation, and by (3) uptake from cell exterior. Alternatively, the accumulated cytokinin can be down-regulated by induction of CKX, inactivation by *N*- and *O*-glucosylation, and in seeds of cereals also by binding to specific cytokinin-binding proteins. The ability of cytokinins to induce their own accumulation was demonstrated by application of BAP or the synthetic urea-type cytokinin thidiazuron, which resulted in an increased of endogenous cytokinin level in cytokinin-dependent cells or tissue cultures of tobacco (Motyka et al. 2003) and sugar beet (Vaňková et al. 1991).

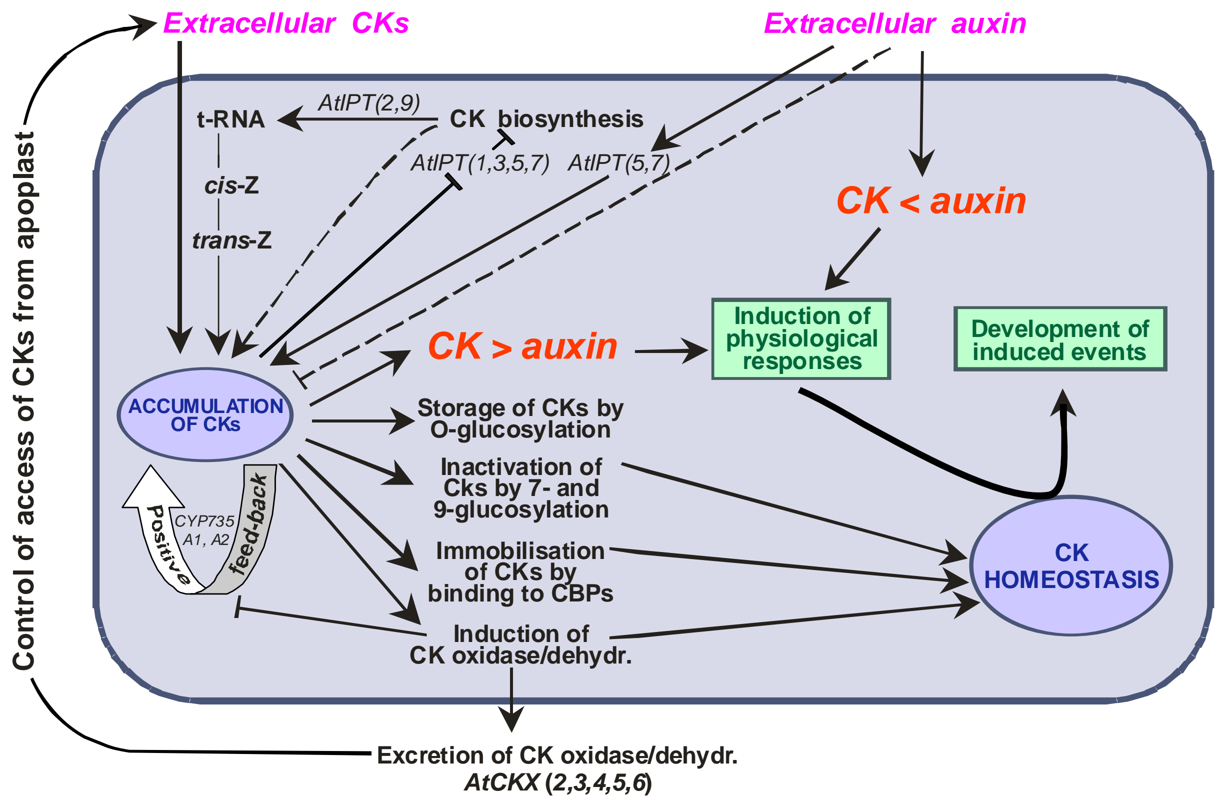
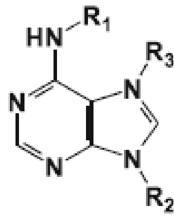


Fig. 4. A model of regulation of cytokinin content in relation to that of auxin in plant cells. Dashed line corresponds to the iPRMP-independent biosynthetic pathway (Kamínek et al. 2006).

4.1 Biosynthesis of cytokinins

The natural plant cytokinins are adenines which are substituted at the N^6 terminal by either an isoprene-derived side chain (isoprenoid cytokinins) or an aromatic derivative side chain (aromatic cytokinins). There operates a metabolic network of interconversion of cytokinins and its conjugates In plants (Fig. 5; Sakakibara 2004).



R_1	R_2	R_3	R_4	Common name	Abbreviation
CH_3	H	-	-	6-*N*-(2-isopentenyl)adenine	iP
	R	-	-	6-*N*-(2-isopentenyl)adenosine	iPR
	RP	-	-	6-*N*-(2-isopentenyl)adenosine 5´-monophosphate	iPRMP
CH_3	-	G	-	6-*N*-(2-isopentenyl)adenine 7-glucoside	iP7G
CH_2	G	-	-	6-*N*-(2-isopentenyl)adenine 9-glucoside	iP9G
	H	-	H	*trans*-zeatin	Z
	R	-	H	*trans*-zeatin riboside	ZR
CH_2OR_4	RP	-	H	*trans*-zeatin riboside 5´-monophosphate	ZRMP
	-	G	H	*trans*-zeatin 7-glucoside	Z7G
CH_3	G	-	H	*trans*-zeatin 9-glucoside	Z9G
CH_2	H	-	G	*trans*-zeatin *O*-glucoside	ZOG
	R	-	G	*trans*-zeatin riboside *O*-glucoside	ZROG
	H	-	H	*cis*-zeatin	*cis*Z
CH_3	R	-	H	*cis*-zeatin riboside	*cis*ZR
	-	G	H	*cis*-zeatin 7-glucoside	*cis*Z7G
	G	-	H	*cis*-zeatin 9-glucoside	*cis*Z9G
CH_2OR_4, CH_2	H	-	G	*cis*-zeatin *O*-glucoside	*cis*ZOG
	R	-	G	*cis*-zeatin riboside *O*-glucoside	*cis*ZROG
	H	-	-	dihydrozeatin	DHZ
CH_2OH	R	-	-	dihydrozeatin riboside	DHZR
	-	G	H	dihydrozeatin 7-glucoside	DHZ7G
CH_3	G	-	H	dihydrozeatin 9-glucoside	DHZ9G
CH_2	H	-	G	dihydrozeatin *O*-glucoside	DHZOG
	R	-	G	dihydrozeatin riboside *O*-glucoside	DHZROG

H, hydrogen; R, ß-D-ribofuranosyl; RP, ß-D-ribofuranosyl-5´-monophosphate; G,ß-D-glucopyranosyl

Fig. 5. Structures and abbreviations of isoprenoid cytokinins (Kaminek et al. 2000).

Although biosynthesis of cytokinins was primarily detected in roots and roots are supposed to be the main site of cytokinin biosynthesis the expression of *IPT*, the main cytokinin biosynthetic gene was also detected in aerial plant organs (Myiawaki et al. 2004). This corresponds to earlier suggestions that the meristematic tissues, such as immature kernel (Blackwell and Horgan 1994), shoot apical meristem (Koda and Okazawa 1980) and young leaves with still dividing cells have a capacity to synthesize cytokinins *de novo* (Faiss et al. 1997). There was shown an ability of young leaves, in which the cells still divide, to synthesize the cytokinins *de novo* in tobacco plants (Golovko et al. 2002). Lateral root meristems were the main sites of iPRMP-dependent biosynthesis of cytokinins (Nordström et al. 2004). In contrast, Miyawaki et al. (2004) did not find any expression of *IPT* gene encoding the isopentenyltrasferase in the apical meristem of *Arabidopsis* plants.

Two different pathways, direct and indirect, have been proposed for the biosynthesis of isoprenoid cytokinins (Fig. 6). Cytokinins synthesized via the indirect pathway are derived from degradation of tRNA species containing cytokinin as an integral part of their molecule. The tRNA cytokinin biosynthesis has been shown to occur at the polymer level during tRNA posttranscriptional processing (Hall 1973). The isopentenyl side chain of cytokinins in tRNA is derived from mevalonic acid (MVA) and attached to adenosine residue next to the 3’ end

of the tRNA anticodon to give the iP moiety. Piaggesi et al. (1997) proved the incorporation of MVA into ZR and iP in a higher plant tissue but the mechanism was at that time unclear. According to present knowledge, tRNA is isopentenylated by specific tRNA:isopentenyltransferase (*AtIPT2* and *AtIPT9*) using dimethylallyl diphosphate (DMAPP) as the site chain donor (Miyawaki et al. 2006). Some tRNA species of almost all organisms contain modified cytokinin moieties next to the 3'end of anticodon. Golovko et al. (2002) cloned and characterized a tRNA-IPT from *Arabidopsis thaliana,* which were divided into three subclasses, depending on degree of adenine modification. The tRNAs of higher plants contain c-Z, which exhibits very low cytokinin activity as compared to tRNAs of other organisms which contain iP or its 2-methyltio derivatives. This feature of tRNA-dependent pathway suggests that cytokinins derived from tRNA do not substantially interfere with hormonal regulations in higher plants (Kamínek 1974, Miyawaki et al. 2006).

Two different direct cytokinin biosynthetic pathways were detected in plants. The iPRMP-dependent pathway involves isopentenylation of free adenine nucleotides (AMP, ADP or ATP) catalyzed by IPT (IPT1, IPT3-IPT8) using DMAPP as N^6-side chain precursor to form iPR mono-di-or triphoshate (Kakimoto 2001, Takei et al. 2001a), which is hydroxylated to corresponding Z-derivatives (ZRMP, ZRDP, ZRTP) by cytokinin-specific hydroxylases (Takei et al. 2004b). The hydroxylation step must be extremely rapid since iPMP, iP are rarely found in most plants (Piagessi et al. 1997).

Plant IPT genes were firstly identified in *Arabidopsis* (Takei et al. 2001b, Kakimoto 2001; Golovko et al. 2002). The *Arabidopsis* genome encodes 9 genes (*AtIPT1* to *AtIPT9*), which show similarity to *Agrobacterium* gene. Plant *IPTs* encode either tRNA modifying enzyme tRNA- IPT (tRNA-IPT; no *2* and *9*) or the iPRphosphate-forming enzyme (IPT; no. *1, 3 - 8*) (Takei et al. 2001b). AtIPTs are expressed in various non-photosynthetic cells and tissues including the root stele, lateral root primordium (*AtIPT5*) and phloem companion cells (*AtIPT3* and *7*; Takei et al. 2001b). The expression of *AtIPT3* in phloem companion cells (Takei et al. 2004a) implies a contribution to long distance signalling via the vascular systems.

The alternative iP-independent pathway catalyzes attachment of hydroxylated side chain donor of terpenoid character to AMP producing directly t-Z ribotide (Åstot et al. 2000). The rate of cytokinin biosynthesis is dependent on the availability of N^6-side chain precursors (Manzano et al. 2006). Precursors of cytokinin N^6-isoprenoid side chain are synthesized through two different pathways: (1) the methylerythritol phosphate (MEP) pathway which produces isopentenyl disphosphate (iPP) and dimethylallyldiphosphate (DMPP) from glyceraldehyde-3 phosphate and pyruvate in the plastids (Esenreich et al. 2001) and (2) the MVA pathway synthesizing these intermediates from acetylCoA in the cytosol (Esenreich et al. 2001).

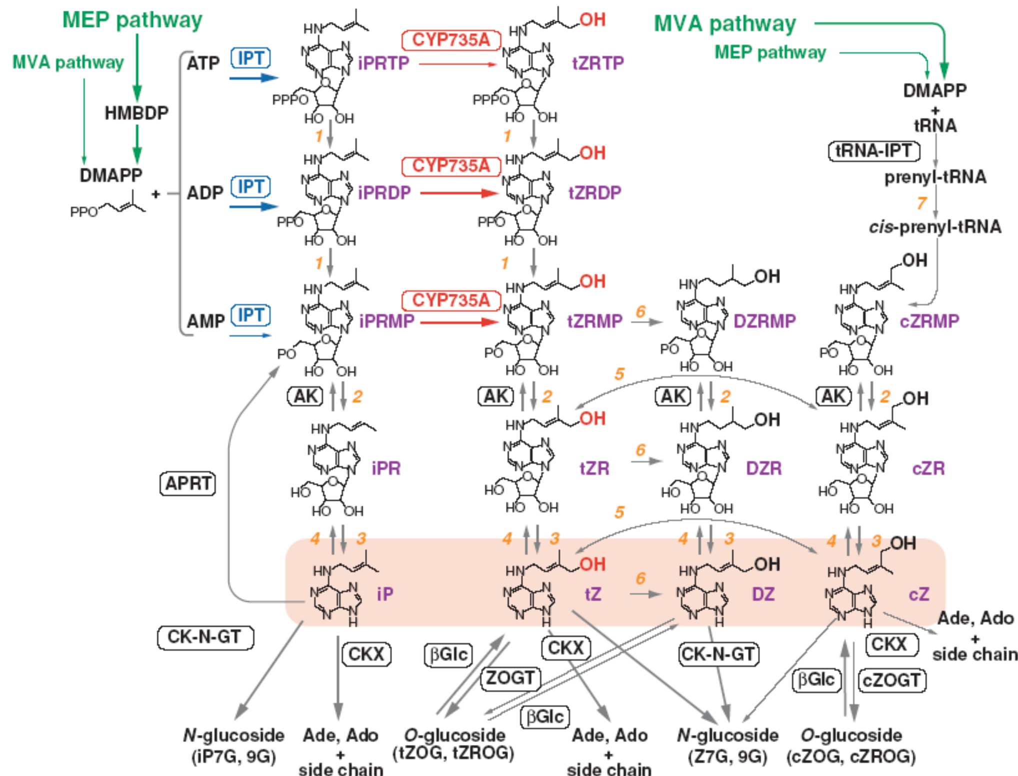


Fig. 6. Current model of isoprenoid cytokinin biosynthesis pathway in *Arabidopsis.* Current model of isoprenoid cytokinin (CK) biosynthesis pathways in *Arabidopsis*. Isoprenoid side chains of *N*6-(_2-isopentenyl)adenine (iP) and *trans*-zeatin (tZ) predominantly originate from the methylerythritol phosphate (MEP) pathway, whereas a large fraction of the *cis*-zeatin (cZ) side chain is derived from the mevalonate (MVA) pathway (*green arrows*). Plant adenosine phosphate-isopentenyltransferases (IPTs) preferably utilize ATP or ADP as isoprenoid acceptors to form iPRTP and iPRDP, respectively (*blue arrows*). Dephosphorylation of iPRTP and iPRDP by phosphatase (*1*), phosphorylation of iPR by adenosine kinase (AK), and conjugation of phosphoribosyl moieties to iP by adenine phosphoribosyltransferase (APRT) create the metabolic pool of iPRMP and iPRDP. APRT utilizes not only iP but also other CK nucleobases. The CK nucleotides are converted into the corresponding tZ-nucleotides by CYP735A (*red arrows*). iP, tZ, and the nucleosides can be catabolized by CKX to adenine (Ade) or adenosine (Ado). cZ and tZ can be enzymatically interconverted by zeatin *cis-trans* isomerase (5). tZ can be reversibly converted to the *O*-glucoside by zeatin *O*-glucosyltransferase (ZOGT) and β-glucosidase (βGlc). CK nucleobases also can be converted to the *N*-glucoside by CK *N*-glucosyltransferase (CK-N-GT). The width of the arrowheads and lines in the green, blue, and red arrows indicates the strength of metabolic flow. Flows indicated by black arrows are not well characterized to date. tZRDP, tZR 5_-diphosphate; tZRTP, tZR 5_-triphosphate; *2*, 5_-ribonucleotide phosphohydrolase; *3*, adenosine nucleosidase; *4*, purine nucleoside phosphorylase; *6*, zeatin reductase; *7*, CK *cis*-hydroxylase. (Modified by Sakakibara et al. 2006).

4.1.1 Control of cytokinin biosynthesis by availability of N^6- side chain donors

The N^6-side chain of isoprenoid cytokinins is derived from DMAPP and its isomer isopentenyldiphosphate (iPP). The former is predominantly synthesized via the methylerythriol phosphate pathway (MEP) from glyceraldehyde 3-phosphate and pyruvate in the plastids (Eisenreich et al. 2001), while biosynthesis of iPP proceeds via the mevalonic acid pathway (MVA) from acetyl-CoA in the cytosol (Newman and Chappell 1997; Esenreich et al. 2001). With respect to this central position of iPP and DMAPP in biosynthesis of a wide spectrum of isoprenoids the two intermediates may become limiting for biosynthesis of isoprenoids including cytokinin side chain. Constitutive overexpression of two different farnesyl diphosphate synthases (FPC) that catalyzes condensation of two IPP units with

DMAPP to produce geranyl diphosphate led to a reduction of cytokinin levels and initiation of senescence symptoms in detached leaves of Arabidopsis. The original level of cytokinins was restored and senescence symptoms were eliminated by concomitant overexpression of 3-hydroxy- 3-methylglutaryl-Coenzyme A reductase that catalyzes biosynthesis of MVA (Manzano et al. 2004; 2006). With respect to the central position of DMAPP and MVA in biosynthesis of isoprenoids their levels in plant cells need to be tightly regulated. Evidently, availability of DMAPP and MVA as cytokinin side-chain precursors may regulate cytokinin biosynthesis.

4.2 Metabolism of cytokinins

The cytokinins occur as free bases, ribosides, glycoconjugates and nucleotides in plants. The variation and distribution of cytokinin species are ubiquitous and depend on the plant species, tissue and developmental stage. According to their function, cytokinins can be classified into four groups: bioactive forms (e.g. t-Z and iP), translocation forms (ZR) (Takei et al. 2001a) and nucleotides which occur in abundance in xylem and floem sap (Sakakibara 2004), storage and inactivated forms. Since the identification of cytokinin receptors in *Arabidopsis* plants only cytokinins recognized by the receptors are considered as active forms. Using two different receptors, CRE1/AHK4 and AHK3, Spíchal et al. (2004), found that the former is activated exclusively by cytokinin bases but the AHK3 recognizes also cytokinin ribosides and c-ZR which confirm these cytokinin forms as active compounds (Romanov et al. 2006). This corresponds to the earlier testing of activity of different cytokinins in various bioassays where cytokinin bases and ribosides exhibited the highest activities (Skoog and Ghani 1981).

4.2.1 Irreversible degradation by cytokinin oxidase/dehydrogenase

The enzyme CKX was isolated from maize kernels and expressed in heterologous hosts moss protoplasts (Houba-Hérin et al. 1999) or recombined yeast (Morris et al. 1999). Seven distinct CKX genes (*AtCKX1 - AtCKX7*) were identified in *Arabidopsis* genome (Bilyeu et al. 2001) and subsequently number of CKX homologs were detected in some other plant species including cereals (Galuzska et al. 2004). Overexpression of four distinct *AtCKX* genes in *Arabidopsis* plants led to a decrease in cytokinin levels that was accompanied with corresponding changes in plant phenotype (Werner et al. 2003).

Isoprenoid cytokinin bases and ribosides bearing unsaturated and non-glycosylated side chain are irreversibly degraded by removal of N^6 isoprenoid side chain by CKX, which actually behaves as dehydrogenase (Galuszka et al. 2001). The products of this reaction are adenine or adenosine and aldehyde of the corresponding side chain (Fig 7). Because the iP and Z and their ribosides are very good substrates of the enzyme, CKX plays a crucial role in down-regulation of broad spectrum of cytokinins. Accordingly, the transgenic tobacco plants overexpressing CKX showed significantly decreased levels of all cytokinins (Werner et al. 2003). The activity of CKX enhanced considerably in response to application of exogenous cytokinins (Chatfield and Armstrong 1986, Motyka and Kamínek 1992) or heterologous overexpression of IPT gene that was consequently followed with increase of endogenous cytokinin levels (Motyka et al. 2003). Both CKX substrate and non-substrate cytokinins were capable of increasing the CKX activity *in vivo* shortly after their application (Motyka and Kaminek 1992). This indicates that cytokinins enhance CKX activity either by reaction with CKX repressor or by affecting the conformation of CKX as an allosteric enzyme (Kaminek et al. 2003).

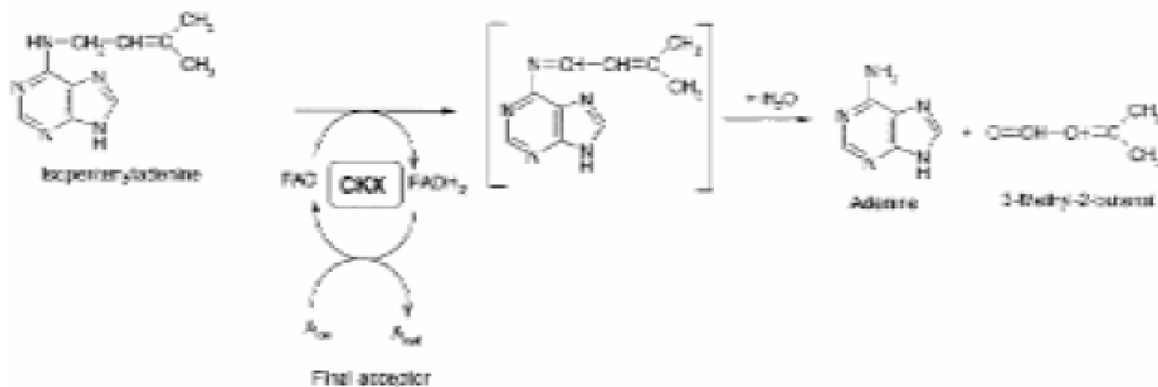


Fig. 7. Reaction schema of cytokinin oxidase/dehydrogenase.

4.2.2 O- and N-glycosylation

The physiological activity of cytokinins can be decreased or eliminated by their conjugation with sugar or alanine moieties on the purine ring at positions 7 and 9. This so called *N*-glycosylation or *N*-alanylation, respectively, catalyzed by cytokinin-specific glycosyltransferase (Hou et al. 2004) or cytokinin alanine synthetase (Entsch et al. 1983), results in irreversible inactivation of cytokinins. So far, this is the only known way how to inactivate cytokinins which are not the substrates or only weak substrates for CKX (DHZ- and BAP-type cytokinins).

The reaction of glucose or xylose with the terminal methyl group of the N^6-isopentenyl side chain of Z leads to formation of *O*-glycosides. The *O*-glycosylation is a reversible inactivation of cytokinins providing a storage pool of cytokinins. *O*-glucosides can be converted back by hydrolysis catalyzed by more or less cytokinin-specific β-glucosidase (Brzobohatý et al. 1993, Mok and Mok 2001, Vaňková 1999). Some authors presume physiological activity of *O*-glycosides. Conversion of Z to ZOG seems to be important for its transport via xylem sap and its role in stimulation of chlorophyll formation in cucumber leaves (Kato et al. 2002). Higher physiological activity of *O*-glycosides compared to *N*-glycosides was demonstrated, in legumes (Mok et al. 1990). However, it is not possible to exclude that cytokinin *O*-glucosides are hydrolyzed to corresponding bases or ribosides during the duration of the assays for cytokinin activity or during the testing of responses of cytokinin receptors.

4.2.3 Saturation of cytokinin N^6-side chain

Some physiological processes in plants (e.g. germination, seedling growth) are controlled predominantly by cytokinins with saturated N^6-side chain (DHZ-type cytokinins). The involvement of transport of DHZ type cytokinins was reported in a number of plant species (Letham 1994). The enzyme responsible for reduction of Z double bond on N^6-side chain, zeatin reductase (ZRED; EC 1.3.1.69) was detected in *Phaseolus* embryos (Martin et al. 1989). DHZ-type cytokinins are resistant to CKX attack (Armstrong 1994). In this respect, the conversion of Z- to DHZ-type cytokinins may preserve cytokinin biological activity, especially in tissues with high CKX activity. The reaction catalyzed by ZRED is NADPH dependent (Gaudinová et al. 2005; Martin et al. 1989) and requires metal cofactor.

4.2.4 Cis-trans isomerisation of zeatins

t-Z is a major and ubiquitous cytokinin in higher plants. As compared to t-Z the *cis*-zeatin (c-Z) was traditionally described as an adjunct with low activity and rare occurrence.

However, there is accumulating evidence that c-Z and its derivatives are present in high amounts namely in roots of some plant species. Martin et al. (2001) demonstrated the existence of maize gene *cis*-ZOG1 encoding an *O*-glucosyltransferase specific to c-Z. The c-Z and c-ZR and their *O*-glucosides were detected in all tested maize tissues, including immature kernels containing very high levels of the *O*-glucoside of c-ZR (Veach et al. 2003). A distinct *cis*-ZOG2 gene was detected by Veach et al. (2003) in *Arabidopsis.* The clear preference of the two glucosyltransferases for c-Z and the presence of *O*-glucosides of c-Z and its riboside in maize lead to obvious conclusion that c-Z *O*-glucosylation is a natural metabolic process in maize. An inspiring view on the function of c-Zs was brought by partial purification of zeatin *cis-trans* isomerase activity that catalyzes *in vitro* stereospecific conversion of c-Zs to the biologically active *trans*-isomers (Bassil et al. 1993; Mok and Mok 2001). This finding has indicated that c-ZR released from tRNA during its degradation could be converted to the highly active t-ZR. However, functioning of *cis-trans* isomerase *in vivo* has not be confirmed so far and very recent results of Miyawaki et al. (2006) strongly supported the earlier suggestion that regulation of t-Z cytokinins is strictly separated from the tRNA turnover.

4.3 Other factors influencing cytokinin levels

Cytokinins affect important developmental evens and reactions to environmental factors. The regulation of their level must be very quick and precise to maintain their homeostasis. Besides, the intracellular biosynthesis and metabolism of cytokinins and the excretion of cytokinins from plant cells represents a mechanism controlling intracellular concentration of this hormone (Petrášek et al. 2002). Alternatively, cytokinins can be imported from the apoplast into the cell. Recently identified high-affinity purine transporters (AtPUP1 and AtPUP2) recognize also Z and could mediate the transport of this and maybe also some other cytokinins into the cell (Bürkle et al. 2003; Hirose et al. 2005). Both cytokinin excretion and uptake may regulate cytokinin levels in the symplast and apoplast.

Cytokinins slow down the process of senescence; however, sugars accumulation can block the effect of cytokinins especially in low light, which induces the onset of senescence. Light also interacts with sugars by partially reducing the effect of sugar repression (Wingler et al. 1998).

4.3.1 Cytokinin / auxin interaction

There are two different types of auxin/cytokinin interactions. While the two plant hormones act synergistically in promotion of cell division, regulation of other processes associated with organelle, cell and tissue differentiation proceeds in an antagonistic manner (Skoog and Miller 1957, Miyazawa et al. 1999). Down- as well as up-regulation of cytokinin biosynthesis and accumulation by auxin corresponds to the dual functions of cytokinin/auxin ratios in control of plant development. Different regulation of various biosynthetic pathways by auxin corresponds to requirement of synergistic and antagonistic interaction of two phytohormones (Kamínek et al. 2003; 2006). Once a certain physiological and/or morphogenetic process is induced by the enhanced cytokinin/auxin ratio, the excretion of plant cytokinins to cell exterior can result in a decrease of cytokinin/auxin ratio to the level corresponding to the hormone homeostasis that is required for further progression of induced physiological and morphological events as indicated in Fig. 4. According to this scheme, the accumulation of auxin either due to the increase of its influx/efflux ratio and intracellular biosynthesis inhibits cytokinin accumulation and decrease cytokinin content with respect to that of auxin. This may be favourable for induction of “auxin specific” physiological and developmental events, such as for example cell enlargement, root formation and apical dominance (Kaminek et al. 2003).

Recent investigations of regulation of expression of different *IPTs* by auxin have been very helpful for understanding of molecular basis of regulation of cytokinin levels by auxin. The cytokinin content and its biosynthesis via IPRMP-independent pathway were rapidly

suppressed by direct auxin effect (Nordström et al. 2004). Elevated cytokinin levels resulted in long-term (slower) reduction of auxin content and synthesis rate of IAA. Both these reductions were observed with delay, indicating the existence of indirect mechanism of regulation, probably mediated by change in development (Nordström et al. 2004). On the other hand, auxin up-regulation of *AtIPT5* and *AtIPT7* in Arabidopsis plants directed tissue specific enhancement of cytokinin biosynthesis via the iPRMP (iPRDP, iPRTP)- dependent pathway (Miyawaki et al. 2004, Sakakibara 2005).

In addition to the control of cell cycle, cell division and differentiation operational control of cytokinin/auxin ratios is required for regulation of many other processes. Auxin and cytokinin show opposite effects on amyloplast development by regulation of the expression of genes required for starch biosynthesis. Many of differentiating amyloplasts grown in auxin-depleted medium were observed to redifferentiate into proplastids after auxin addition. Auxin repressed amyloplast development whereas cytokinin accelerated starch accumulation (Miyazawa et al. 1999).

5 Exploitation of mechanisms regulating cytokinin levels to improve plant productivity

Cytokinins, in cooperation with auxins, control developmental processes, which are decisive for productivity of crop plants. These include organ regeneration, control of apical dominance which has an impact on tillering and stem branching, formation and maintenance of photosynthetic apparatus, grain setting and filling as well as acquisition of essential resources, including carbon skeleton and mineral nutrients, and their efficient assimilation and allocation (Fig. 8)

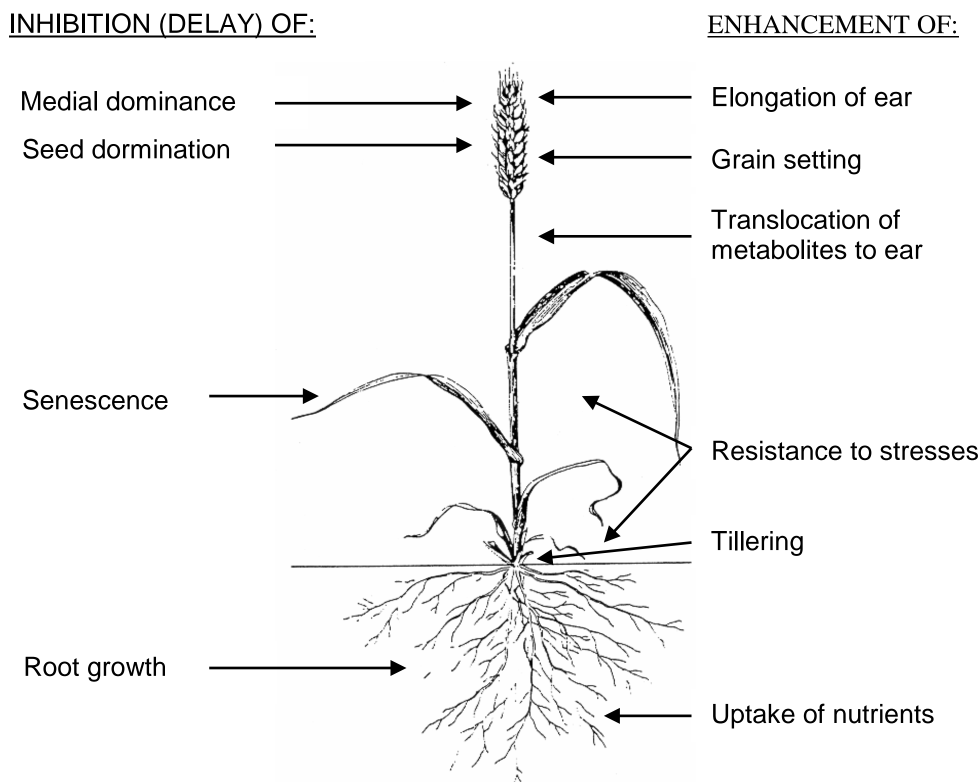


Fig. 8. Cytokinin control of physiological processes affecting proctivity of cereal plants (Kamínek et al. 2003).

5.1 The control of apical dominance

It has been believed that the long-distance transport of auxin and cytokinins affects their ratios at the site of action and thus controls major developmental events in plants, including apical dominance.

Cytokinins release lateral buds from apical dominance and stimulate tillering. This was demonstrated by application of cytokinins to wheat (Shanahan et al. 1985), barley (Ryshke and Leike 1989) and rice (Buu and Chu 1983) and it correlates well with the changes of exogenous cytokinins (Harisson and Kaufman 1983).

Cytokinins suppress the dominance of early developed grains in the middle part of the ear (so called “medial” actually “semibasal” dominance) and enhance the development of grains in distal parts of ear in wheat (Trčková et al. 1992). Cytokinin also suppressed the dominance of peripheral grains within the spike by supporting the development of the retarded middle grain. Moreover, application of cytokinins to maize and wheat at pollination and anthesis, respectively, increased the number of fully developed grains per ear by reducing the number of aborting ones in wheat and maize. Cytokinins reduce the detrimental effect of heat and limited nitrogen supply respectively on grain abortion (Trčková et al. 1992, Dietrich et al. 1995, Jones et al. 1996).

However, there are situations (systems) where the auxin/cytokinin ratio dogma concerning the control of apical dominance is not applicable. Apical dominance, which generally decreases when cytokinin content is increased, was not changed significantly in the cytokinin-hypersensitive mutants of *Arabidopsis* (Kubo and Kakimoto 2000). Moreover, Beveridge et al. (1997) analyzing branching mutants of pea suggested that hormone-like signals other than auxin and cytokinin are also involved in the control of apical dominance.

5.2 The control of sink strength

Source-sink relationships and relationships between the reproductive and vegetative development may play a crucial role in the onset of senescence (Pic et al. 2002). Stimulation of grain setting is closely related to sink formation. Reproductive sink strength in cereals can be defined as their potential capacity for starch accumulation (Jones et al. 1996). At the whole plant level it may be increased by enhancement of the number of amyloplasts per endosperm cell, the number of endosperm cells per seed and the number of seeds per plant. Cytokinin increased the number of seeds per plant by stimulation of formation and survival of secondary fertile tillers and/or grain setting within the ear of wheat (Trčková et al. 1992, Dietrich et al. 1995).

The accumulation of cytokinins after pollination in developing seeds (Banowetz et al. 1999) corresponds with the onset of endosperm cellularisation (Kamínek et al. 1999) and maximal mitotic activity in maize grains (Dietrich et al. 1995). This corresponds to the schema that cytokinins are involved in stimulation of cell division also at an early stage of endosperm development.

Fertilized ovaries destined to set pods in *Lupinus albus* accumulate cytokinins. The proportion of *cis* and *trans* isomers of zeatin in ovaries was initially 10:1 and declined to less than 1:1. In ovaries destined to abort the proportion remains high. During early pod set, accumulation of cytokinin was accounted for by xylem and floem translocation, both containing more than 90% of c-Z. During embryogenesis and seed development, the cytokinin content increased in endosperm, indicating synthesis of the hormone *in situ* (Emery et al. 2000). The spectrum of cytokinin forms in transport pathways is not constant and it changes during plant development and can also reflect the differences in dynamics of water movement and nitrogen status of the plant (Sakakibara et al. 1998).

As mentioned under 3.2, the cytokinin content reflects nitrogen availability. Shinano et al. (2006) suggest the autonomy of leaf, which is regulated by an endogenous program of nitrogen translocation from the leaf regardless of cytokinin level in xylem sap. In favour of leaf autonomy in terms of nitrogen translocation during the ripening stage of the rice plant evince the following evidence: (1) the nitrogen and chlorophyll levels in the leaves were not

affected by panicle manipulation, (2) following panicle removal nitrogen decreased in the leaves and then either accumulated in the stems or was lost from the plants entirely. In legume crops leaf senescence and nitrogen translocation from the leaves were repressed by sink manipulation (Noodén and Letham 1993). It seems that there exists some indirect mechanism of senescence regulation, at least in rice (Shinano et al. 2006). However, the nitrogen uptake was stopped by the panicle removal treatment, thus the uptake of nitrogen by the root appears to be regulated by the sink requirements regardless of carbohydrate status (Shinano et al. 2006).

5.3 Delay of senescence induced by stress stimuli

The increase of endogenous cytokinin content impacts source-sink relations to delay ontogenic changes wherein senescence is a necessary process. The *SAG12::IPT* transgenic tobacco plants show delayed biomass allocation in response to water deficit stress compared to WT plants. Transformants exhibited a delayed remobilisation of leaf reserves to sink. Their seeds germinated slowly and the leaf senescence was delayed. The age-dependent decline in photosynthetic metabolism was delayed (Wingler et al. 1998) and nitrogen translocation to non-senescing leaves was reduced (Jordi et al. 2000). These changes were interpreted as cytokinin-induced modification of source-sink relations. It was hypothesized, that sugar accumulation impacts cytokinin activity, causing a nutrient deficiency in young leaves (Cowan et al. 2005). Transgenic tobacco plants display not only delayed senescence and altered source-sink relations but also delayed germination and retarded response to water deficit stress (Cowan et al. 2005). The leaves of tobacco showing slight yellowing (early senescent leaves) differed from nonsenescent leaves only in decreased Z level, suggesting that Z may play a key role in control of senescence. More senescent leaves showed a decrease of cytokinin bases, ribosides and glucosides, but not of nucleotide levels. (Singh et al. 1992)

It is known that cytokinins increase the stress tolerance induced by heat, salinity and nutrient deficiency (reviewed in Kamínek et al. 2003). Smigocki (1995) generated transgenic plants of *Nicotiana plumbaginifolia* and *Nicotiana tabacum* with *ipt* gene fused to the wound-inducible proteinase-inhibitor-IIK gene promoter. This chimeric gene shown wound inducible expression of *ipt* gene in leaves and correlated with enhanced insect resistance, elevated content of Z and ZR, pronounced apical dominance prior to flowering, and increased chlorophyll content (Smigocki 1995).

6 Methods of determination of cytokinin content and cytokinin degradation

6.1 Analysis of cytokinins

Over 40 different cytokinins differing in chemical and physical properties have been identified in plants. Cytokinins are present in plant cells and tissues in extremely low concentrations ($<10^{-7}$ M). As derivatives of adenine, the cytokinins occur in plants in presence of overwhelming amounts of structurally closely related compounds. This constellation raises very high demands on selectivity and sensitivity of methods of cytokinin analysis.

Cytokinins were discovered on the basis of their activity in very specific and precise tobacco callus bioassay (Naylor et al. 1954) and were based on laborious estimation of their biological activities with respect to synthetic standards in more or less purified preparations (Miller et al. 1956). Early methods of cytokinin analysis included simple fractionation of plant extracts by partition in different solvents (see e.g. Hembberg and Westlin 1973, Powell 1964) and trapping of cytokinin bases and ribosides on cation-exchange polystyrene and cellulose sorbents as Dowex 50, DEAE cellulose and DEAE Sephadex (Horgan and Scott 1987), followed by their fractionation using paper and/or thin-layer chromatography (Pačes et al.

1971, Friedrich et al. 1972). For separation of cytokinins from enriched samples, like hydrolyzates of tRNAs, liquid chromatography (LC) on LH20 Sephadex gave very reproducible results (Burrows et al.1971).

Considerable improvement in separation of different cytokinins was achieved by introduction of high performance liquid chromatography (HPLC) in cytokinin analysis. It permits a rapid high-resolution purification of cytokinins prior their determination. The HPLC using new sorbents allowing reversed-phase LC provided a very useful and revolutionary tool for analysis of cytokinins (Cannes et al. 1975, Challice 1975) as well as of other plant hormones from a single plant extract (Prinsen et al. 1995).

The commercial availability of dual-mode sorbents bearing both reversed-phase and ion-exchange characters has allowed purification and separation of auxins, ABA, cytokinin ribotides and other cytokinins (bases, ribosides and glucosides) on one column from a single plant extract (Dobrev and Kamínek 2002). This very fast and efficient purification method has been widely used in number of laboratories (e.g. Takei et al. 2004, reviewed in Ge et al. 2006).

Successful preparation of antibodies against cytokinins provided an efficient tool for cytokinin purification and determination. The advantages of immunochemical techniques are their sensitivity, high specificity and very short analysis time. Both poly- and monoclonal antibodies have been used for this purpose. Immunoaffinity LC using antibodies recognizing a broad spectrum of cytokinins appeared especially useful for cytokinin purification (McDonald and Morris 1985). Unfortunately, the antibodies do not bind *N*-7 and *O*-cytokinin glucosides which need to be purified separately (Banowetz 1994; Jordi et al. 2000).

Various methods were used for quantitation of cytokinins, beginning with the comparison of physiological activities of purified plant extracts with respect to synthetic cytokinin standards (Friedrich et al. 1972), followed by measurement of UV absorbencies of highly purified preparations (Nicander et al. 1993) and immunochemical RIA and ELISA methods (Weiler 1980; Jordi et al. 2000).

The physico-chemical methods based on mass spectrometric (MS) detection have been increasingly used for plant hormone analysis. Combination of MS with HPLC or GC and exploitation of labeled (mostly deuterated) internal cytokinin standards allowed very fast simultaneous quantitation of large number of cytokinins and their unequivocal identification in a single run. The GS/MS, which was used for cytokinin analysis already in 1987 (Palni et al.), requires obstructive cytokinin derivatization. For this and some other reasons, the HPLC/MS is preferred wherever the expensive instrumentation is accessible. This method, which is becoming a standard in analysis of cytokinins in plant materials (Redig et al. 1996; Sun et al. 2003), has been increasingly used since 1993 when the first LC/MS system was developed for determination of cytokinins present in biologically relevant concentrations in plant samples (Yang et al. 1993). The sensitivity of MS method was increased by 200 to 1000 times by adaptation of micocapillary LC/MS for cytokinin analysis (Prinsen et al. 1998). In spite of high selectivity and sensitivity of HPLC/MS methods, an effective purification of cytokinins from plant extracts is required for removal impurities which may decrease the MS responses of analyzed cytokinins and corresponding internal reference standards and protect the HPLC columns and mass spectrometer from spoiling.

6.2 Determination of cytokinin oxidase/dehydrogenase activity

Irreversible degradation of cytokinins is catalyzed by CKX, enzyme that selectively cleave unsaturated cytokinin isoprenoid side chain, resulting in the formation of adenine/edenosine and the corresponding side chain aldehyde (Armstrong 1994). The rate of this reaction can be determined *in vitro* using either radioisotope or spectrometric assays. The radioisotope methods are based on the measurement of conversion of purine ring-radiolabelled substrate cytokinins, most frequently the iP, t-Z or their ribosides in presence of enzyme preparation. The labeled products of cytokinin degradation (adenine or adenosine) are separated from unreacted cytokinin substrates by appropriate chromatographic procedures. The radioisotope method is very specific and sensitive allowing measurement of

cytokinin degradation in concentrations corresponding to those of endogenous cytokinins in plant cells and tissues (2μM- 10μM) (Chatfield and Armstrong 1986, Laloue and Fox 1989, Motyka and Kamínek 1994). A disadvantage of low substrate cytokinin concentration in the assay mixture is gradual decrease of the substrate cytokinin during incubation and a correspondingly time-dependent decrease of substrate conversion to the product. In order to get reasonable results, the concentration of the substrate should not decrease under 85% of the original level. To meet such a requirement, the suitable enzyme concentration in the incubation solution and time period of incubation should be determined in a pilot experiment.

The colorimetric assay is based on determination of formation of Schiff base between the enzymatic reaction product (aldehyde of the side chain) and an electron acceptor like p-aminophenol (Liberto-Minotta and Tipton 1995, Galuszka et al. 2001, Laskey et al. 2003). The first indication of the fact that the enzyme behaves like dehydrogenase provided high stimulation of CKX activity when assayed in of copper-immidazole buffer. The replacement of copper-imidazole with a suitable artificial electron acceptor almost removed the requirements of the CKX-catalyzed reaction for oxygen (Frébortová et al. 2004). An advantage of this assay is in possibility to measure continuously colour development, which allows easy monitoring of kinetics of enzyme reaction. However, serious disadvantages are the requirements of more extensively purified enzyme preparations and the use of substrate cytokinin in concentration far above those occurring in plant tissues (≥40 μM, usually 150 - 250 μM) (Liberto-Minotta and Tipton 1995, Frébortová et al. 2004).

7 References

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8 Aims and conclusions

8.1 Aims of the study

I. To evaluate the effects of expression of cytokinin biosynthetic *ipt* gene under the control of senescence associated promoter (*SAG12::ipt*) in wheat plants on delay of senescence, cytokinin content, uptake and allocation of nitrate and grain formation.

II. To analyze the xylem sap of oat and/or wheat plants for potential CKX activity and if present to characterize some of its biochemical properties and eventual responses to an environmental signal that is known to affect cytokinin levels in roots.

III. To detect activities and characterise selected biochemical properties of cytokinin oxidase/dehydrogenase (CKX) and zeatin reductase (ZRED) and their involvement in establishment of cytokinin homeostasis in wheat or someother plant species exhibiting significant enzyme activity.

IV. To compare the effects of isoprenoid and aromatic cytokinins and the ways of their applications on progress of senescence, endogenous cytokinin levels, cytokinin uptake and translocation and chloroplast ultrastracture of detached oat and wheat leaves.

V. To summarize the improvements of methods of cytokinin analysis and determination of activities of enzyme(s) catalyzing cytokinin metabolism elaborated for achievement of the above specified objectives.

8.2 Conclusions of the study

I. The *ipt* expression under the senescence-specific *SAG12* promoter resulted in delay of leaf senescence, increase of content of bioactive cytokinins and nitrate reductase activity in leaves as well as to enhancement of nitrate influx in plants grown under limited N supply. However, the *SAG12::ipt* plants did not differ from WT plants in grain yield components including the number of grains and grain weight. Results suggest that delay of leaf senesce of wheat plants also delays translocation of nutrients and metabolites from leaves to developing grains after anthesis and in this way interferes with the reproductive strategy wheat as strictly monocarpic plant which is based on a programmed fast translocation of metabolites and nutrients from senescing leaves to the reproductive sinks shortly after anthesis.

II. Responding to the mostly vague statements that the physiological effects of cytokinins are dependent on plant genotype and affected by various internal factors we tested the efficiency of and aromatic (3OHBAR) and an isoprenoid (t-ZR) cytokinin on retention of chlorophyll in detached oat and wheat leaf apices. The efficiency of the two cytokinins in preservation of chlorophyll was different in the oat and wheat depending on local or whole adaxial leaf surface cytokinin application. The oat and wheat leaf pieces differed in the rates of uptake and the dynamics of [^{3}H]t-ZR and [^{3}H]3OHBAR translocation which was very fast in wheat and slower but steadily increasing with time in oat. The oat and wheat leaf apices also differed in the metabolism of applied t-ZR namely in its conversion to the storage dihydrozeatin-*O*-glucoside prevailing in oat and to the inactive t-Z *N9*-glucoside prevailing in wheat. The t-Z was more efficient in stimulation of cytokinin oxidase/dehydrogenase (CKX)

than 3OHBAR and the activity of the enzyme was more increased by the two cytokinins in oat than in wheat leaf apices. The difference in activities of both cytokinins in retention of chlorophyll in wheat and oat can be minimized by their pulse application followed by incubation of leaf apices floating on water.

III. Root pressure xylem sap from de-topped oat plants contains CKX activity that is associated with a glycosylated protein. The pH optimum of the enzyme (8.5) is much higher than pH of root xylem sap (6.1) indicating suppression of cytokinin degradation by the enzyme during its transport via the xylem flow. Reported alkalization of the xylem sap in leaf apoplast and its enhancement in response to NO_3^- and water availability may create favorable conditions for metabolic degradation of co-transported cytokinins and thus decrease of cytokinin/ABA ratio at sites of high transpiration.

IV. Simultaneous determination of CKX and ZRED activities in pea leaves allowed comparisons of the actual roles of the two enzymes in control of cytokinin levels in plants, which represents a novel approach toward the investigation of the mechanisms maintaining hormonal homeostasis. Characterization of CKX activity revealed the existence of a non-glycosylated CKX isoform with relatively high pH optimum (pH 8.5) in pea leaves. The presence of non-glycosylated CKX and/or CKX with a very low degree of glycosylation is generally rather uncommon in plants, and the finding in pea suggests a more abundant occurrence as well as possible relevance and function in some legume genotypes. Also, the detection of ZRED activity in leaves (that is, vegetative organs) of pea is novel, because so far this enzyme activity had been isolated only from Phaseolus embryos (that is, generative organs; Martin et al. 1989; Mok et al. 1990). Although the proportion of ZRED to CKX activities was found to vary in a relatively broad range in pea leaves, a close relationship between conversions of Z-type cytokinins catalyzed by ZRED and their degradation by CKX is obvious. The fact that ZRED activity converts cytokinins to forms protected from breakdown by CKX underlines a potential role of ZRED in cytokinin homeostasis.

V. The modified Bieleski’s, as compared to two other tested extraction solvents, sufficiently suppressed dephosphorylation of cytokinin riboside monophosphates and reduced the extraction of compounds decreasing the RISRs of tested deuterated cytokinins. This solvent, lacking $CHCl_3$, is easier and safer to handle and appeared to be the most suitable for extraction of cytokinins. Purification of cytokinins using mixed-mode-SPE, as compared to DEAE Sephadex RP-C18 method, was powerful in the removal UV absorbing contaminants providing preparations exhibiting high RISRs of deuterated counterparts of natural cytokinins. This method was found simpler, faster and more operational. It also allows more complex plant hormone analysis by providing a partially purified fraction containing auxin and abscisic acid.

I. The Effect of Senescence-induced *ipt* Expression on Cytokinin Levels, Nitrate Uptake and Allocation and Grain Yield in Wheat

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(submitted)

II. Factors Affecting Delay of Chlorophyll Degradation by Cytokinins in Senescing Oat and Wheat Leaves

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(submitted)

III. Cytokinin Oxidase/dehydrogenase Activity in Oat Xylem Sap and Its Regulation by Exogenous Nitrate

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(submitted)

IV. The Involvement of CytokininOxidase/Dehydrogenase and Zeatin Reductase in Regulation of Cytokinin Levels in Pea (Pisum sativum L.) Leaves

Alena Gaudinová, Petre I. Dobrev, Blanka Šolcová, Ondřej Novák, Miroslav Strnad, David Friedecký and Václav Motyka

(Journal of Plant Regulations 24: 188 – 200)

V. Improvement of Methods of Cytokinin and CKX Analysis

V. - 1. Efficiency of Different Methods of Extraction and Purification of Cytokinins

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V. - 2. Increase in Sensitivity of Cytokinin Oxidase/dehydrogenase Radioisotope Assay by Introducing Electron Acceptor(s) to the Reaction Solution Containing Crude Enzyme Preparations from Pea (*Pisum sativum* L. cv. Gotik) Leaves

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(submitted)