Palacký University in Olomouc

Faculty of Science

Department of Biochemistry

Transgenic barley with altered cytokinin content

Ph.D. THESIS

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I hereby declare that this thesis has been written solely by me and that all the sources used in this thesis are cited and included in the reference part.

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Abstrakt

Ječmen (*Hordeum vulgare* L.) je celosvětově pěstovaná plodina, patřící mezi 4 zemědělsky nejvýznamnější obilniny (kukuřice, pšenice, rýže, ječmen). Pro transformaci ječmene byly použity geny pro cytokinin dehydrogenasu. Cytokinin dehydrogenasa (CKX) je enzym zodpovědný za ireverzibilní degradaci růstových regulátorů - cytokininů. Hlavní funkcí cytokininů je kontrola buněčného dělení a orgánové diferenciace, déle pak kontrola růstu adventivních pupenů, prodlužování listů a oddálení senescence. Již dříve bylo prokázáno, že transgenní cytokinin-deficientní rostliny vykazují pozměněný fenotyp se zakrslou nadzemní částí a zvětšeným kořenovým systémem. Zvětšený kořenový systém je výhodnou vlastností, která by mohla být využita v zemědělství, například pro lepší zakotvení rostlin v zemi, vyšší toleranci vůči stresu v období sucha a zvýšení příjmu živin z půdy.

Pro transformaci ječmene byly vybrány geny *gHvCKX2* a *ZmCKX1* řízené konstitutivním ubikvitinovým promotorem. Přibližně 500 embryí bylo transformováno genomovou formou ječmenného genu *HvCKX2*. Zregenerovala pouze jedna transgenní rostlina. Nadzemní část transgenní rostliny byla zmenšená a vytvářela výrazně méně stvolů ve srovnání s kontrolními rostlinami. Zvětšený kořenový systém nebyl pozorován. Po transformaci 993 embryí kukuřičným genem *ZmCKX1* bylo získáno 13 transgenních rostlin. Kořenový systém těchto rostlin byl značně zvětšen, zatímco nadzemní část zůstávala zakrslá. Rostliny začaly hynout po 7 až 11 týdnech od převedení do hlíny, nejspíše jako důsledek cytokininové deficience vyvolané zvýšenou aktivitou CKX. Následně byly vytvořeny transgenní rostliny s kořenově-specifickou expresí genu *ZmCKX1*, které měly zvětšený kořenový systém beze změny fenotypu nadzemní části.

Druhá část práce sleduje expresní profily genů zapojených do metabolismu a percepce cytokininů během vývoje kukuřice a odpovědi na salinitní a

osmotický stres. Exprese genů je diskutována v souvislosti s obsahem všech cytokininových metabolitů. Salinitní a osmotický stres indukuje expresi některých biosyntetických genů, což vede k mírnému zvýšení aktivních forem cytokininů trvající několik dnů po vyvolání stresových podmínek. Nicméně, nelze předpokládat přímý efekt cytokininů na zprostředkování aktivace odpovědi na stres, a to kvůli pomalým změnám hladin cytokininových metabolitů. Rostliny se nejspíše během stresu vyrovnávají se sníženou rychlostí růstu zvýšenou produkcí cytokininů.

Klíčová slova	*Agrobacterium*, cytokininy, cytokinin dehydrogenasa, *Hordeum vulgare*, ječmen, osmotický stres, promotor, transformace
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Abstract

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops grown worldwide. Genes for cytokinin dehydrogenase (CKX, EC1.5.99.12) were used for barley transformations. Cytokinin dehydrogenase is an enzyme responsible for the irreversible degradation of plant growth regulators called cytokinins. Main function of cytokinins is control of cell division and organ differentiation. Cytokinin deficiency in transgenic plants showed interesting changes in plant morphology - retarded shoot growth and enhanced root growth. Enhanced root system can be exploited for a better anchorage of crop plants in soil, increased tolerance to drought stress or for improving their nutrient uptake.

The maize *CKX1* gene (*ZmCKX1*) and the genomic form of barley *CKX2* gene (*gHvCKX2*) controlled by ubiquitin promoter were used for barley transformation. Approximately 500 immature barley embryos were transformed with the *gHvCKX2* gene and only one transgenic plant was obtained. The transgenic plant created notably fewer culms compared to control plant. A significant change in root phenotype was not confirmed. Thirteen transgenic barley plants were formed after transformation of 993 embryos with the *ZmCKX1* gene. Root systems of all transgenic plants were significantly enhanced, whereas aerial parts were reduced. Transgenic plants started to die after 7 to 11 weeks probably as a result of cytokinin deficiency. Therefore, transgenic plants with root-specific expression of the *ZmCKX1* gene were prepared.

The last chapter is focused on expression profiling of genes involved in cytokinin biosynthesis, degradation and perception in maize during development and stress responses. Expression of several cytokinin biosynthetic genes was induced in seedlings exposed to salt and osmotic stresses. Nevertheless, a direct effect of cytokinins on mediation or activation of stress responses was not proved due to slow changes in metabolite levels. Plants

perhaps undergo a reduction of growth rates, maintained by abscisic acid accumulation in stressed tissues, by enhanced cytokinin levels.

Keywords	*Agrobacterium*, barley, cytokinins, cytokinin dehydrogenase, *Hordeum vulgare*, osmotic stress, promoters, transformation
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Number of appendices	-
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CONTENT

SOUHRN

Tématem disertační práce je příprava transgenního ječmene s pozměněným obsahem cytokininů. Ječmen se využívá převážně pro přípravu sladu v pivovarnictví a lihovarnictví, ale také jako krmivo pro zvířata nebo jako součást lidské výživy. Proto je ječmen rostlinou vhodnou pro genetické transformace, které mohou pozměnit jeho vlastnosti pro dané použití. První kapitola disertační práce je literární rešerší zabývající se metodami transformace a shrnuje nejčastější problémy související s genetickou transformací ječmene.

Pro transformaci ječmene byly použity geny pro cytokinin dehydrogenasu. Cytokinin dehydrogenasa (CKX, EC 1.5.99.12) je enzym zodpovědný za ireverzibilní degradaci růstových regulátorů - cytokininů. CKX štěpí cytokininy a jejich glykosidy na adenin či jeho cukerný konjugát a aldehyd odpovídající N^6-substituentu. Hlavní funkcí cytokininů je kontrola buněčného dělení a orgánové diferenciace, déle pak kontrola růstu adventivních pupenů, apikální dominance, tvorby chloroplastů nebo oddálení senescence. CKX geny byly vybrány pro transformaci ječmene jelikož studie s transgenními rostlinami tabáku a *Arabidopsis thaliana* s vloženými CKX geny z *A. thaliana* prokázaly vliv snížené hladiny cytokininů na fenotyp rostlin. Rostliny měly zakrslou nadzemní část a zvětšený kořenový systém. Zvětšený kořenový systém u transgenních rostlin se zvýšenou aktivitou CKX je výhodnou vlastností, která by mohla být využita v zemědělství, například pro lepší zakotvení rostlin v zemi a pro zvýšení příjmu živin z půdy. Rovněž by se takové rostliny mohly lépe vyrovnávat s abiotickým stresem, konkrétně v období sucha.

Pro transformaci ječmene byly vybrány geny *gHvCKX2* a *ZmCKX1.* Přibližně 500 embryí bylo transformováno genomovou formou ječmenného genu *HvCKX2* pod kontrolou ubikvitinového promotoru. Zregenerovala pouze jedna transgenní rostlina. Přítomnost vloženého transgenu v genomu rostliny byla potvrzena pomocí PCR z genomové DNA a to amplifikací fragmentu

obsahujícího sekvenci genu a terminátoru, a fragmentu obsahujícího sekvenci promotoru a genu. Semikvantitativní RT-PCR odhalila, že exprese genu *gHvCKX2* byla přibližně 20x vyšší v transgenní rostlině v porovnání s kontrolní rostlinou. Specifická aktivita enzymu CKX v transgenní rostlině, stanovovaná se substrátem iP9G specifickým pro enzym HvCKX2, byla 4,3x vyšší v kořenech a 16,8x vyšší v listech v porovnání s kontrolou. Také Western blotem byla potvrzena přítomnost vyššího množství HvCKX2 proteinu. Transgenní rostlina vykazovala fenotypové změny – nadzemní část byla menší a vytvářela výrazně méně stvolů. Rostlina nevykvetla a předčasně uhynula. Zvětšený kořenový systém nebyl pozorován. Po transformaci asi 1000 embryí kukuřičným genem *ZmCKX1* řízeným ubikvitinovým promotorem bylo získáno 13 transgenních rostlin. Real-time PCR potvrdil expresi genu *ZmCKX1*. Aktivita CKX měřená s IP jako substrátem byla zvýšena v listech 48-1271x a v kořenech 3-301x. Kořenový systém těchto rostlin byl značně zvětšen, zatímco nadzemní část zůstávala zakrslá. Rostliny začaly hynout po 7 až 11 týdnech od převedení do hlíny, nejspíše jako důsledek zvýšené aktivity CKX.

Z genomové DNA byly amplifikovány 2 ječmenné kořenově-specifické promotory, které byly otestovány pomoci reporterového genu *gus* v *Arabidopsis thaliana*. Promotor HvRAF (root abundant factor) prokázal specifickou expresi v kořenové špičce a cévních svazcích kořene. Promotor HvPHT1-1 (phosphate transporter gene) řídil *gus* expresi v kořenovém vlášení. Pletivově specifické promotory byly použity společně s genem *ZmCKX1* pro transformaci ječmene. Po transformaci 1200 embryí s kořenově-specifickým promotorem PHT zregenerovalo 226 rostlin. Pro transformaci s ječmenným kořenově-specifickým promotorem RAF bylo použito 1400 embryí a zregenerovalo 170 rostlin. Rostliny byly ověřovány pomocí real-time PCR s použitím genomové DNA. Specifická aktivita enzymu CKX (iP jako substrát, pH 6,5) byla u transgenních rostlin s PHT promotorem v první generaci zvýšena v kořenech 2,0x-6,1x a kořenový systém byl zvětšen v porovnání s kontrolními rostlinami. Tři transgenní rostliny byly použity pro imunohistochemickou lokalizaci kukuřičného proteinu. ZmCKX1 protein exprimovaný pod PHT promotorem byl lokalizován pomocí specifické protilátky proti ZmCKX1 převážně v rhizodermis a kořenových vláscích. Transgenní rostliny s RAF promotorem měly specifickou

aktivitu CKX v kořenech (iP jako substrát, pH 6,5) zvýšenou nejvýše 1,7x. Zvětšený kořenový systém nebyl pozorován.

Poslední kapitola pojednává o změnách exprese genů zapojených v biosyntéze, degradaci a percepci cytokininů během odpovědi na abiotický stres. Expresní profily byly stanovovány v kukuřici staré 1 týden, která byla vystavena salinitnímu nebo osmotickému stresu. Množství transkriptu jednotlivých genů bylo porovnáváno s hladinou jednotlivých cytokininových metabolitů. Salinitní a osmotický stres indukoval expresi některých biosyntetických genů, což mělo za následek mírné zvýšení aktivních forem cytokininů. Role cytokininů jako zprostředkovatelů aktivace odpovědi na stres nebyla potvrzena, protože hladiny metabolitů se měnily jen pomalu. Hladina *cis*-zeatinu, nejhojnějšího cytokininu v mladé kukuřici, byla zvýšena během prvotní fáze odpovědi na stres. Byla vyslovena hypotéza, že tento cytokinin může pocházet z RNA, která degraduje v důsledku vystavenému stresu. Zvýšenou hladinou cytokininů se rostliny nejspíše vyrovnávají se snížením rychlosti růstu, které je řízeno akumulací kyseliny abscisové ve stresovaných pletivech.

SUMMARY

This Ph.D. thesis is focused on the preparation of transgenic barley with altered cytokinin content. The first chapter deals with barley transformation and its main difficulties.

Genes for cytokinin dehydrogenase (CKX, EC1.5.99.12) were used for barley transformations. CKX is an enzyme responsible for the irreversible degradation of plant growth regulators cytokinins. The enzyme cleaves preferentially isoprenoid cytokinins and their N9-glycosides to adenine or its sugar conjugate and an aldehyde corresponding to the N^6-side chain. Accurately balanced levels of cytokinins in plants are responsible for local promotions of cell division and correct organ differentiation.

The maize *CKX1* gene (*ZmCKX1*) and the genomic form of barley *CKX2* gene (*gHvCKX2*) driven by the ubiquitin promoter and the maize *CKX1* gene driven by the root-specific promoter were used for barley transformation. The constitutive expression of *ZmCKX1* gene and over-expression of *gHvCKX2* gene were lethal for plants. We observed retarded growth of shoots and root systems of plants expressing the *ZmCKX1* gene was significantly enhanced. Approximately 500 immature barley embryos were transformed with the *gHvCKX2* gene controlled by the constitutive ubiquitin promoter and only one transgenic plant was obtained. Presence of the inserted *gHvCKX2* gene construct in the barley genome was determined by PCR amplification of the fragment flanking transgene and terminator sequence, and the fragment including part of promoter and transgene sequence. Semi-quantitative determination of the *HvCKX2* expression level showed significant increase in transgenic leaves compared to control plant. Western blot analysis proved an elevated amount of HvCKX2 protein in transgenic leaves and roots. The specific activity determined with iPR as a substrate was increased 18.2-fold in transgenic leaves and 2.3-fold in transgenic roots compared to the control

plants. The leaves and roots of transgenic barley showed a 16.8-fold and 4.3-fold increase in specific activity with iP9G as a substrate, respectively. Thirteen transgenic barley plants were formed after transformation of 993 embryos with the *ZmCKX1* gene. The expression of *ZmCKX1* gene was confirmed by real-time PCR. The leaves of transgenic barley lines showed a 48-fold to 1271-fold increase in specific CKX activity. The specific activity in transgenic roots showed a 3-fold to 301-fold increase. The root system of all transgenic plants was significantly enhanced, whereas aerial parts were reduced. Transgenic plants started to die 7 to 11 weeks after transfer to soil.

Two barley root-specific promoters were amplified from genomic DNA. The activity of promoters was confirmed by expression of the reporter gene (*gus*) in *Arabidopsis thaliana*. Expression of *gus* driven by the HvPHt1-1 promoter (barley phosphate transporter gene) was localized mainly in the epidermal layer, including the trichoblast (root-hair) cells and also in stele. *Gus* expression driven by the HvRAF promoter (barley root abundant factor) was localized in vascular bundles and root tips. 1200 and 1400 immature embryos were used for barley transformation with the construct *HvPHT1-1::ZmCKX1* and *HvRAF::ZmCKX1*, respectively. Presence of transgenes integrated into the barley genome was tested by real-time PCR. Transformation with a construct *HvPHT1-1::ZmCKX1* resulted in 200 regenerated plants. CKX activity in roots of four transgenic plants showed a 2.0-fold to 6.1-fold increase. Expression of the *ZmCKX1* gene was also detected by immunolocalization using a primary antibody raised against recombinant ZmCKX1 protein. Maize CKX protein was localized mainly in the rhizodermis and root-hairs and was also evident in stele. Transgenic plants produced larger root biomass compared to wild-type plants. 141 plants were obtained after transformation of barley immature embryos with the *ZmCKX1* gene driven by HvRAF promoter. Unfortunately, specific activity of CKX enzyme was increased 1.3-fold to 1.7-fold only in 5 plants and no change in root phenotype was distinguished.

The last chapter is focused on expression profiling of genes involved in cytokinin biosynthesis, degradation and perception in maize during development and stress responses. Changes in gene expression and total transcript levels were associated to the levels of different cytokinin metabolites.

Expression of several cytokinin biosynthetic genes was induced in seedlings exposed to salt and osmotic stresses. This induction probably led to a moderate increase of active forms of cytokinins, which lasted several days during acclimatization to stress. Nevertheless, a direct effect of cytokinins on mediation or activation of stress responses was not proved due to slow changes in metabolite levels. *Cis*-zeatin and its derivatives were found as the most abundant cytokinin in maize seedlings. Levels of this zeatin isomer were significantly increased during early stress response and its origination from degraded RNA as a consequence of immediate stress response is hypothesized. Plants perhaps undergo a reduction of growth rates, maintained by abscisic acid accumulation in stressed tissues, by enhanced cytokinin levels.

AIMS OF THE WORK

- Compilation of a literature review dealing with barley transformation and its difficulties

- Establishment of barley transformation procedure

- Amplification and cloning of barley tissue-specific promoter sequences and proving their specificity by *gus* expression in *Arabidopsis thaliana*

- Preparation of binary vectors suitable for plant transformations

- Constitutive over-expression of cytokinin dehydrogenase genes in barley

- Root-specific expression of cytokinin dehydrogenase gene in barley

- Phenotype evaluation of barley transgenic lines

- Characterization of maize genes involved in cytokinin metabolism and their expression during osmotic stress

INTRODUCTION

Genetic engineering is a useful approach for creating crop species with improved qualities. Since cereals belong to an agriculturally important crop species, many innovations in transformation techniques were implemented during the last few years. The first successes in cereal transformation were achieved using particle bombardment. Progression in transformation technologies revealed that monocotyledonous plants are also susceptible to transformation by *Agrobacterium tumefaciens.* Barley, the fourth major cereal crop in the world, represents an important crop for feed and food production.

This study describes barley transformation with cytokinin dehydrogenase genes (CKX). Cytokinin dehydrogenase is an enzyme responsible for irreversible degradation of plant growth regulators, called cytokinins that play a crucial role in plant growth and developmental processes. Natural cytokinins are N^6-substituted purine derivatives. CKX enzyme inactivates cytokinins by cleavage of unsaturated isoprenoid side chains. Cytokinin-deficient plants have shown interesting developmental alterations in the shoot and root systems (Werner et al. 2001 and 2003). Shoot development of cytokinin-deficient plants was retarded which lead to dwarf habit. In contrast, root growth was increased with enhanced root branching. Over-expression of CKX in roots, causing enlarged root systems, could be used in transformation of crop species. Enhanced root systems can positively influence plant growth, for example by increased nutrient uptake or during a drought period. Therefore, one of the aims of this study was to create transgenic barley plants over-expressing CKX gene.

Abiotic stresses, such as salinity or drought, are limiting factors for productivity in agriculture. These environmental stresses have unfavorable effects on plant growth and yield. Dehydration and osmotic stress are caused by reduced availability of water. For a long time, cytokinins have been considered to be involved in plant responses to stress. In this thesis, expression profiles of the entire gene families of CK biosynthetic and degradation genes in maize during development and stress responses are described, and discussed in relation to the levels of different cytokinin metabolites.

CHAPTER 1:

GENETIC TRANSFORMATION OF BARLEY (*Hordeum vulgare* L.) - LIMITING FACTORS

Genetic transformation of barley (*Hordeum vulgare* L.): limiting factors

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Abstract

This review summarizes main difficulties involved in barley (*Hordeum vulgare* L.) transformation. The most commonly used procedures for genetic transformation in barley are *Agrobacterium tumefaciens* and particle bombardment mediated methods. While different barley cultivars are used for genetic engineering with varying sensitivity, recent improvements in regeneration and transformation techniques are described and summarized. Furthermore, some of the transformation complicating factors, in particular somaclonal variation and transgene insertion sites, are discussed in more detail.

Key words: *Agrobacterium*, albinism, barley, genetic transformation, particle bombardment, somaclonal variation, transgene insertion site

Introduction

Genetic transformation of crop species is a tool for improving agronomic traits. Monocotyledonous species include a great number of agriculturally important crops such as the cereals: maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.). Barley is a cereal crop utilized for its malting quality in brewing and distilling, and for its nutrition as animal feed or human aliment.

Microprojectile bombardment was the first successful method for barley transformation (Wan and Lemaux 1994, Ritala *et al.* 1994). Different tissues were used for microprojectile DNA transfer - immature embryos, callus derived from immature embryos, microspores and shoot meristematic cultures (Wan and Lemaux 1994, Ritala *et al.* 1994, Hagio *et al.* 1995, Jähne *et al*. 1994, Zhang *et al.* 1999). Holm *et al.* (2000) transformed barley by microinjection of DNA into zygote protoplasts, but transgene expression was rarely achieved. Fertile transgenic barley was also obtained by direct gene transfer to protoplasts – via polyethylenglycol-mediated uptake or electroporation (Kihara *et al.* 1998, Funatsuki *et al.* 1995, Salmenkallio-Marttila *et al*. 1995). However, a disadvantage of this method is the time-consuming initiation of embryogenic cell suspension cultures. The statement that *Agrobacterium* is not possible to use for transformation of monocotyledons (Potrykus 1990) has been refuted after the achievements with cereals (Chan *et al.* 1992, Hiei *et al.* 1994, Cheng *et al.* 1997, Ishida *et al.* 1996). Successful transformation of barley by *Agrobacterium tumefaciens* was first reported by Tingay *et al.* (1997). The efficiency of T-DNA delivery to cereal cells was improved by using the super-virulent *Agrobacterium* strain AGL1. The modified transformation procedure used by Tingay *et al.* (1997) consisted of wounding immature embryos without embryonic axis by shooting the scutellum surface with 1 μm gold particles and subsequent inoculation with the AGL1 strain. The bombardment prior to co-cultivation induced a wound response. Acetosyringone is now used as an agent causing chemically induced wound response. However, Bartlett *et al.* (2008) did not confirm the effectiveness of acetosyringone use during co-cultivation to achieve high transformation efficiencies.

A substantial advantage of *Agrobacterium*–mediated transformation (Karami *et al.* 2009) compared to microprojectile bombardment is the possibility to transfer large segments of DNA with only minimal rearrangement (Hiei *et al*. 1997, Shibata and Liu 2000). Other advantages include low copy number integration, higher transformation efficiency and a higher percentage of stable T-DNA inherited as a simple Mendelian trait. On the contrary, transgene silencing and rearrangements have been frequently observed in transformants produced by direct DNA delivery (Travella *et al.* 2005). In Travella´s study, all barley lines produced by *Agrobacterium* transformation integrated between one and three copies of the transgenes, with minimal rearrangements, whereas 60% of the barley lines transformed by particle bombardment integrated more than eight copies of the transgenes, with many rearrangements. Multi-copy insertion of a transgene has often been associated with gene silencing in transgenic plants produced by direct DNA delivery (Pawlowski and Somers 1996). However, single-copy and single-locus transformants also undergo silencing (Kohli *et al.* 1999, Stoger *et al.* 1998).

The main goal of barley transformation is improvement of its quality traits by expression of new genes. The transformation procedure was already utilized in research focusing on improving characteristics such as malting quality (Wang *et al.* 2000 and 2001, Manoharan *et al.* 2006), disease resistance (Nuutila *et al.* 1999, Kihara *et al.* 2000, Tull *et al*. 2003), amino acid composition (Lange *et al.* 2007, Hansen *et al.* 2007), and quality of grains used as feed (Xue *et al.* 2003). Transgenic barley tolerating high levels of some toxic chemicals were also examined in a polluted environment (Kim *et al.* 2003, Delhaize *et al.* 2004). Interestingly, barley seeds were chosen as a bioreactor for molecular farming (Schünmann *et al.* 2002, Joensuu *et al.* 2006). Companies like ORF Genetics (Iceland) and Maltagen Forschung GmbH (Germany) have started to produce pharmaceutical proteins (growth factors, cytokines, oral vaccines, food additives) in transgenic barley lines with robust endosperm-specific expression.

In recent years, a considerable progress in barley transformation has been achieved. This review summarizes particular improvements during transformation procedures which have led to increasing transformation efficiency and successful regeneration or transformation of different barley

cultivars. Somaclonal variation and transgene insertion sites, which are in connection with transgene stability, are discussed in more detail.

Transformation efficiency

Most published barley transformation experiments showed low transformation efficiency (Table 1). An innovation in transformation procedures was the use of an intermediate step when calli are exposed to low light levels on callus induction media (approximately 10-30 µmol m^{-2} s^{-1}, Cho *et al.* 1998) or regeneration media (75 µmol m^{-2} s^{-1}, Bartlett *et al.* 2008) resulting in a reduction in the frequency of regenerated albino plants (Figure 1). Copper is thought to be an important microelement. Media containing increased concentrations of copper (5 µM) ameliorated callus quality and regenerability (Dahleen 1995). Other modifications in microelement concentrations for improved plant regeneration included increased H_3BO_3 (0.75 mM) and decreased $FeSO_4$ (0.05 mM; Dahleen and Bregitzer 2002). The regeneration frequency was also increased by adjustment of NH_4^+ concentration in the regeneration medium. Wan and Lemaux (1994) used regeneration medium where the concentration of NH_4NO_3 was ten fold lower than that of MS medium (Murashige and Skoog 1962) and furthermore, glutamine was added. Nuutila *et al.* (2000) demonstrated that the higher concentrations of organic nitrogen are necessary during the early embryogenesis, whereas inorganic nitrogen in the form of nitrates is required for shoot development. Amino acids (glutamine, proline or casein hydrolysate – mixed source of amino acids) provide a source of reduced nitrogen. Synthetic 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methoxy-3,6-dichlorobenzoic acid (dicamba) are the most commonly used auxins in barley callus-induction media. Also 4-amino-2,5,6-trichloropicolinic acid (picloram) was successfully used in the media during cereal transformation (Przetakiewicz *et al.* 2004). In Trifonova´s *Agrobacterium*-mediated transformation study, dicamba and 2,4-D promoted similar regeneration frequencies; however, dicamba was superior in supporting long-term regeneration ability in barley. In addition, dicamba (in the callus induction and maintenance media) was generally superior to 2,4-D in promoting transient expression and subsequent stable transformation (Trifonova *et al.* 2001). According to Castillo *et al.* (1998),

dicamba was superior to 2,4-D and picloram in the induction and maintenance of embryogenesis and regeneration capacity. Another study showed that optimal ratios between 2,4-D and the cytokinin benzylaminopurine (BAP) helped to maintain the regeneration of green plantlets better than dicamba and BAP (Jiang *et al.* 1998). Addition of BAP increased the quality of calli and its regenerability and reduced the incidence of albinism (Cho *et al.* 1998). However, low cytokinin levels should be maintained for initial callus induction because of the fact that high cytokinin levels decrease the initial growth rate of the callus. Thus, callus initiation on medium with auxin alone and inclusion of an intermediate sub-culture medium containing auxin, cytokinin and copper before regeneration increased green shoot production (Cho *et al.* 1998). Our research is focused on transformation of barley with cytokinin-depletion genes (cytokinin dehydrogenase gene, CKX). For callus formation, after inoculation with *Agrobacterium,* we use medium containing 2,4-D and BAP. Since plant regeneration on hormone-free medium failed, probably due to transgene action, we regenerated plants on medium with 1 mg dm^{-3} of BAP. We also successfully used the specific inhibitor of CKX, together with 2,4-D, for callus induction and maintenance on sub-culture medium. Inhibitor induced regeneration with efficiency comparable to BAP (unpublished data). Rooting of regenerated shoots was performed on medium without hormones.

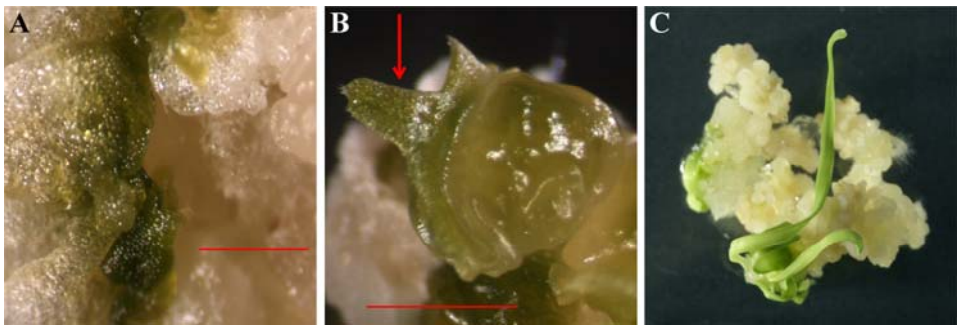

Figure 1: Plant regeneration from embryogenic callus of barley cultivar Golden Promise. A: callus exposed to low light levels (80 µmol m^{-2} s^{-1}), bar 1 mm; B: green regenerative structure (arrow shows developing shoot), bar 1 mm; C: regeneration of shoot in full light (160 µmol m^{-2} s^{-1}).

Bartlett *et al.* (2008) developed a protocol with average transformation efficiencies of 25%. Moreover, they reported that their recent experiments yielded transformation efficiencies of over 50%. Immature embryos were transformed with *Agrobacterium tumefaciens* (strain AGL1) harboring pBract vectors enabling selection on hygromycin. Copper at 5 μM was used in callus induction and transition sub-culture media. Regeneration medium did not contain additional copper (copper concentration was the same as in Murashige and Skoog medium). Dicamba (2.5 mg dm^{-3}) was used in the callus induction medium, 2,4-D (2.5 mg dm^{-3}) and benzylaminopurine (0.1 mg dm^{-3}) were added to the transition medium. The regeneration medium contained no hormones. All medium components, with exception of phytagel, were filter sterilized to prevent the adverse effects of autoclaving. It is thought that filter-sterilized media gives improved results, since autoclaving could induce unwanted interactions between medium components and the production of toxic products from sugar breakdown. It was also reported that high and stable transgene expression can be kept by incorporating of the intron at a specific position within the coding sequence (Bartlett *et al.* 2009). Hensel *et al.* (2008, 2009) established a transformation protocol that gave efficiency up to 86.7 stable transgenics per 100 immature embryos of cultivar Golden Promise. The co-cultivation medium was improved by adding 800 mg dm^{-3} of L-cysteine and 500 μM acetosyringone. Infected embryos showed no sign of necrosis or abortion, probably due to the anti-oxidative effect of L-cysteine.

The most important factor influencing high-throughput transformation is the availability of immature embryos from good quality plants grown under strictly controlled conditions. Plants should not be sprayed with fungicides or insecticides. Optimal growth conditions, especially appropriate watering, must be kept through the whole lifespan of embryo-donor plants. Transformation efficiency increased from 5% to 70% (Table 1) when we substituted our greenhouse-grown donor plants (cv. Golden Promise) with plants grown under above mentioned conditions (Bartlett *et al.* 2008). Another essential factor for obtaining a high-number of transformants is use of an appropriate selection agent. In our hands, hygromycin selection works much more effectively then selection on the herbicide bialaphos.

Method	Efficiency*	Selection gene	Promoter/Gene of interest	Goal	Reference
PB, IEs/ microspore-derived Es	7.9%/ 0.3%	Ubi1/*bar*, 35S Adh1 intron/*bar*	Reporter gene: Ubi1, Adh1/*uidA* (35S/BYDV-PAV)	First transformation by PB	Wan and Lemaux 1994
AG, IEs	1.7-7%	Ubi1/*bar*	Reporter gene: Act1/*gus*	First *Agrobacterium*-mediated transformation	Tingay *et al.* 1997
PB, IEs	15.2%	Ubi1/*bar*	Hybrid high-pI α-amylase/ thermostable endo-(1,4)-β-glucanase	Improved malt quality	Nuutila *et al.* 1999
AG, IEs	13%	35S/*hpt*	hairpin RNA construct: Ubi1/BYDV-PAV polymerase	Resistence to BYDV (PAV strain)	Wang *et al.* 2000
AG, IEs	2-12%	35S/*hph*	Barley high-pI α-amylase/ α-amylase, α-glucosidase	Marker gene elimination	Matthews *et al.* 2001
PB, IEs	1.4%	Ubi1/*bar*	Barley high-pI α-amylase/α-amylase (*alkBA*)	Increased activity Improved malt quality	Tull *et al.* 2003
AG, IEs	17.9%	35S/*hpt*	Reporter genes: Act1/*gus*, Ubi1/*gfp*	Marker-free transgenic plants	Coronado *et al.* 2005
AG, IEs	4.4%, 9.2%	35S/*hph*	Reporter genes: Ubi1/*gfp*, *gus*	Comparison of reporter gene expression	Murray *et al.* 2004
PB, IEs	1%	Ubi1/*bar*	Barley trypsin inhibitor promoter/faeG	Grains containing vaccine	Joensuu *et al.* 2006
AG, ovules	3.1%	35S/*hpt*	Reporter gene: Ubi1/*gfp*	Ovule transformation	Holme *et al.* 2008
AG, IEs	5.4%	35S/*hpt*	Ubi/antisense C-hordein encoding gene	More balanced AA composition	Lange *et al.* 2007
AG, IEs	25%	35S/*hpt*	Reporter gene: Ubi1/*luc*	Improvement of transformation	Bartlett *et al.* 2008
AG, IEs	4-86.7%	35S/*hpt*	Reporter genes: Ubi1/*gfp*, Act1/*gus*	Improvement of transformation	Hensel *et al.* 2008
AG, IEs	2.8%	Ubi/*bar*	Ubi/*gus*	Improvement of transformation	Our unpublished data
AG, IEs	5-74%	35S/*hpt*	Ubi/*dapA*	Increased content of free-lysine	Our unpublished data

Table 1. Efficiency of selected barley transformations (cultivar Golden Promise).
*These data may not represent the true efficiency since genetically identical clones could be included. Efficiency is expressed as the number of plants per 100 transformed embryos/ovules. AA - amino acid, AG - *Agrobacterium*-mediated transformation, BYDV - barley yellow dwarf virus, IEs - immature embryos, PB - particle bombardment.

Barley cultivars

Most transformation protocols were developed for the model cultivar, Golden Promise, which is not agriculturally important. However, these protocols are not suitable for many commercially important barley cultivars, which have low plant regeneration frequencies (Bregitzer *et al*. 1998a) or callus-induction response rates (Jiang *et al.* 1998). Many studies have compared regeneration ability of different barley cultivars in relation to medium composition. The increase in regenerability caused by changes in the transformation protocol described by Cho *et al.* (1998) resulted in successful transformation of the North American barley cultivar – Galena and Harrington.

Separate autoclaving of certain culture media components and reducing the amount of callus per Petri dish improved regeneration of the cultivars Harrington, Morex and Hector. Regeneration improvement in response to various concentrations of copper and 2,4-D were more genotype specific (Bregitzer *et al.* 1998a). Castillo *et al*. (1998) studied the regeneration of different barley cultivars by comparing three different auxins used for callus induction. Dicamba was superior 2,4-D and Picloram for the induction and maintenance of callus. Dahleen and Bregitzer (2002) reported significant improved plant regeneration of cultivars Morex and Harrington by modifying iron and boric acid concentrations, and by adding BAP (0.1 mg dm^{-3}) to maintenance medium. Under the same conditions, improvement was also observed in plant regeneration of other North American cultivars – Foster, Drummond, Conlon, Colter, 90Ab321, Baronesse and Crystal. Chang *et al*. (2003) developed a plant regeneration system for the cultivar Morex. The optimal size of embryos for callus formation and following production of green plants with fewer albinos was 0.5-1.5 mm. Callus-induction medium contained 3 mg dm^{-3} 2,4-D or dicamba. Shoot regeneration was performed on medium with 0.5-1.0 mg dm^{-3} BAP and shoots were rooted with 0.2 mg dm^{-3} indole butyric acid in the medium. Twelve spring barley cultivars registered in the Czech Republic were tested for callus induction and regeneration capacity. Most cultivars created more regenerated plants after callus induction with 2,4-D compared to picloram or dicamba. Cultivars Atribut, Forum and Scarlett were selected as the most suitable cultivars for further study (Šerhantová *et al.* 2004).

Manipulation with ethylene production and action during tissue culture cultivation increased plant regeneration of cultivars Golden Promise and Morex (Jha *et al.* 2007). Increased ethylene production, caused by addition of 1-aminocyclopropane 1-carboxylic acid, within weeks 8 to 10 of *in vitro* culturing enhanced the regeneration of Morex. On the contrary, blocking of ethylene activity by silver nitrate during weeks 5 to 10 increased regeneration of Morex (almost 2-fold) and Golden Promise (1.5-fold). He and Jia (2008) developed an efficient plant regeneration system from mature embryos with endosperm of highland barley (var. nudum Hk. f.), which can be used for genetic transformation. Successful formation of embryogenic calli from leaf base segments of seven semi-winter, commercial cultivars and following regeneration was recently reported (Li *et al.* 2009).

Transgenic barley (cultivar Harrington) was obtained by bombardment of shoot meristematic cultures (SMCs) derived from germinated seedlings (Zhang *et al.* 1999). Advantages of SMCs are high plant regeneration and enhanced genomic stability in comparison to embryogenic callus. Shoot meristematic cells do not go through a callus or de-differentiation phase; vegetative shoots can be directly induced from shoot meristematic cultures. Australian cultivars Schooner, Sloop and Chebec were also successfully transformed by *Agrobacterium* (Wang *et al.* 2001, Murray *et al.* 2004). Roussy *et al.* (2001) tested five Nordic cultivars (Baronesse, Cecilia, Filippa, Mentor, Pongo) for transformation and regeneration capacity. Pongo, Baronesse and Filippa showed the best results. Manoharan and Dahleen (2002) successfully transformed the barley cultivar Conlon by bombardment of embryo-derived callus. Hensel *et al.* (2008) improved transformation conditions (see above) for the spring cultivars Helium, Optic, PF17048-51, PF18147-52, W122/37.1 and the winter cultivar Tafeno.

Holme *et al*. (2008) reported that plant regeneration from young barley embryos derived from *in vitro*-cultured ovules is genotype independent. Cultivars Femina, Salome, Corniche and Alexis, which are known to have poor response in other types of tissue cultures, were successfully transformed using the protocol for *Agrobacterium* infection of ovules.

Selection of transgenic homozygous plants

Barley is a self-pollinated plant. Foreign genes (transgenes) integrated into the barley genome are transmitted to progenies in a Mendelian manner. In practical breeding, homozygous plants with a stable transgene are required. The process used for identification of transgenic homozygous plants requires screening of a large number of plants in the T1 and T2 generations; therefore, it is a labour–intensive process (Massiah *et al*. 2001). Homozygous transgenic plants of barley can be rapidly produced by androgenic segregation through anther culture (Müllerová *et al*. 2000), embryogenic pollen culture (Coronado *et al*. 2005) and microspore culture (Ritala *et al*. 2005). The anther culture technique in T1 and T2 progeny of transgenic barley cv. Golden Promise was successfully applied. The transgenic line HB1A (T2) was transformed with plasmid pAHC25 (Christensen and Quail 1996) containing both the *gus* and *bar* genes. Transgenic T1 lines HH3E and HH1A were co-transformed with plasmids pAL51 (Lonsdale *et al*. 1995) and pAMFIT (provided by prof. C. Fogher). The following parameters were studied: androgenic response, green and albino plant frequency, ploidy level of green and albino regenerated plants, and segregation of transgenes in the androgenic progeny (Müllerová *et al*. 2000). The androgenic material from transformed donor plants is important for evaluation of the transgene distribution and for study of selection against transgenic cells in haploid and diploid plants. Production of doubled haploid transgenic barley through anther or microspore culture can be used for the fast creation of homozygous transgenic plants.

Kumlehn *et al.* (2006) published that *Agrobacterium* infection of androgenetic pollen cultures can be used for barley transformation. After infection with *Agrobacterium tumefaciens* strains LBA4404 and GV3101, about 31% and 69%, respectively, of the primary transgenic plants carried a single copy of the transgene. Four out of 20 T1 lines did not segregate for the reporter gene. Thus, identification of homozygous plants is possible one generation earlier in comparison to conventional transformation procedures. This method has the promise of becoming a tool for transformation of diverse barley cultivars since most genotypes are susceptible to doubled haploid formation via pollen culture. Shim *et al.* (2009) reported improvements in transformation of isolated

barley microspores. The transgenic frequency was improved 8 to 10-fold over previous reports on bombardment of microspores. Their results indicated that the best procedure included a 4 h prebombardment cultivation on high-osmotic medium (0.5 M mannitol plus sorbitol) at either 4 or 25 °C. Additionally, arabinogalactan protein was added to the microspore culture medium and the actin promoter was used.

Albinism

Albinism is a common problem during barley transformation (Kasha *et al.* 1990). Albinism can be influenced by genetic background (Foroughi-Wehr *et al.* 1982), physiological state of the donor plants (Goldstein and Kronstadt 1986), exposure to bialaphos (Wan and Lemaux 1994), time in culture (Bregitzer *et al.* 1995a) and culture conditions (Ziauddin and Kasha 1990, Kao *et al.* 1991). Wan and Lemaux (1994) also mentioned that ammonia released by non-transformed cells during selection (Tachibana *et al.* 1986) can contribute to the occurrence of albinism.

The exposure of calli to light early in the selection process reduced the incidence of albinism (Cho *et al.* 1998). A possible explanation is that chlorophyll biosynthesis is induced by light-dependent enzymes (Holtorf *et al.* 1995). The creation of green sectors on calli caused by exposure to light ensures that green plants will be regenerated. Albinism is also linked to changes in plastid DNA that occur during re-differentiation (Mouritzen *et al.* 1994).

The study of Bregitzer and Campbell (2001) identified one quantitative trait locus (QTL) for green plant regeneration and at least one for albino plants. They also confirmed previously reported associations of three QTLs with green plant regeneration (Komatsuda *et al*. 1995, Mano *et al*. 1996).

Somaclonal variation

Genetic changes arising from *in vitro* culture was termed somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variation is a serious problem during barley transformation. Structural rearrangements and variation in chromosome number can be found in plants from *in vitro* culture. For agronomic application, it is essential to improve a particular trait without changing the basic genetic background. Some factors are known to affect chromosomal instability in regenerated, nontransgenic plants. These include: plant species, genotype, initial ploidy level, explant source, medium composition, growth regulators, and time in culture (Constantin 1981). For example, polyploidy and aneuploidy in barley are positively correlated with increasing time in culture (Ziauddin and Kasha 1990, Wang *et al.* 1992).

Barley plants (cultivar Golden Promise) derived from non-transgenic and transgenic callus obtained via microprojectile bombardment were compared regarding ploidy (Choi *et al.* 2000a). Variation in ploidy was greater in transgenic compared to nontransgenic plants. Fifty four percent of the transgenic lines were diploid (2n = 2x = 14) and 46% of the transgenic lines were tetraploid (2n = 4x = 28) or aneuploid around the tetraploid level. Only 0-4.3% of the nontransgenic plants were tetraploid. Ploidy changes in transgenic plants were more frequent, probably due to additional stresses during transformation. The DNA introduction process involved exposure of cells to vacuum, cellular damage due to microprojectiles, and potential loss of cell turgor following particle impact. Moreover, transformed tissues grow in the presence of dead tissue during selection and this may cause cellular stress. Choi *et al.* (2000a) also tested two different culturing procedures. These varied in: media used for culture initiation, *in vitro* culture regimes during selection process and inclusion of osmotic media (bombardment of osmotically treated embryos). Differences in the frequency of chromosomal aberrations were not observed. However, ploidy changes occur most probably early during the selection process (selection on bialaphos). Similar results were described by Bregitzer *et al.* (1998b). Particle bombardment of immature embryos of the cultivar Golden Promise induced greater somaclonal variation during culture than in those without bombardment. One of the many potential mutagenic

factors taken into account was the use of phosphinothricin, which results in a locally altered pH and increased ammonia levels (Bregitzer *et al*. 1998b). Choi *et al.* (2001) published that selection on bialaphos or hygromycin B and osmotic treatment (cultivation of embryos on medium with 0.2 M mannitol and 0.2 M sorbitol for 4 h before bombardment) cause extensive cytological aberrations in transgenic barley. Their investigation did not prove a significant effect of bombardment itself on frequency of aberrations in cells of calli (cultivar Golden Promise); however, damage caused by the particles to immature embryos was found to be significantly less than that of Galena (Koprek *et al.* 1999). Based on our unpublished data, we confirm such observations since the transformants produced from particle bombardment of Golden Promise showed tetraploidy in high frequency when selected on bialaphos.

Choi *et al.* (2000a) found that cells of callus tissue were aneuploid around the diploid chromosome number, but no non-transgenic and transgenic plants were aneuploid around the diploid number. However, high numbers of transgenic plants were aneuploid around the tetraploid level. The authors suggested that polyploid cells were probably more buffered to the effects of gene losses in aneuploids due to gene dosage. Other chromosomal changes, such as point mutations, small deletions or insertions or methylation polymorphism, were also observed (Choi *et al.* 2001).

Choi *et al.* (2000a) observed phenotypic variation related to ploidy. Tetraploid or near-tetraploid plants had delayed growth rates, broader leaves, thicker roots, spikes that did not emerge completely from leaf sheaths and seeds that were longer in comparison to those of diploid plants. Aneuploid plants (mostly with chromosome numbers of 26 or 27) had an abnormal grass-like phenotype with sterility. The sterility was probably related to the instability of chromosome number during an abnormal meiosis. Morphological variation was also observed in 18 cultivars of barley lines derived from tissue culture (Ullrich *et al*. 1991). Height, heading date, spike length, spikelet density, grain yield, and malting quality were negatively affected in tissue-culture-derived plants of several barley cultivars (Bregitzer and Poulson 1995, Bregitzer *et al.* 1995b). Prostrate and spreading growth habit, excessive dwarfism, extremely slow development, several types of chlorophyll mutations, and premature death were

observed in many transgenic barley lines (genes *bar*, *uidA*, BYDVcp.) of cultivar Golden Promise (Bregitzer *et al*. 1998b).

Cho *et al*. 1999 published that T0 plants from 6 of 12 independent transgenic lines were tetraploid and out of these 6 lines, plants from only one gave a ratio consistent with 35:1 segregation of GUS expression. This means that cells were diploid and became tetraploid after DNA integration. Plants from the rest of the lines gave a ratio consistent with a 3:1 segregation of expression. Thus, DNA integrations had already occurred in tetraploid cells (Choi *et al.* 2000a).

Bregitzer *et al*. (2002) tested three different tissue culture systems – standard embryogenic callus tissues (SEC; Wan and Lemaux 1994), shoot meristematic cultures (SMC; Zhang *et al.* 1999), and modified embryogenic callus tissues (MEC; Cho *et al*. 1998). Agronomic traits (heading date, plant height, grain yield and quality) were measured for plants regenerated from cultures of two barley genotypes and compared with those from uncultured controls. SEC and MEC tissues were derived from scutellar tissues of immature embryos under different cultivating conditions and SMC tissues were derived from axillary meristems of *in vitro*-germinated immature embryos. Long term culture capable of high levels of plant regeneration is an advantage of MEC and SMC. A disadvantage of SMC is slow initial development. The greatest reductions in agronomic performance were from plants derived from standard embryogenic callus tissues. On the contrary, plants derived from highly differentiated, meristematic tissues had the least reduction in agronomic performance. SEC-derived plants were generally shorter than MEC- and SMC-derived plants. SMC-derived plants had the highest yield and grain weight whereas SEC-derived plants had the lowest values. Moreover, MEC and SMC tissues showed minimal losses of plant regeneration (even in cultures older than one year). This increase in plant regeneration may reflect greater genomic stability. Nevertheless, Bregitzer *et al*. (2002) did not report that SMC tissues are superior to the MEC tissues with respect to somaclonal variation.

Bregitzer *et al*. (2008) recently published a new approach to reduce negative changes in transgenic barley. Single backcrosses of transgenic lines with wild-

type were used for eliminating agronomic and quality alterations caused by somaclonal variation.

Transgene insertion sites – transgene stability

It is desirable to integrate a transgene into a position in the genome where transcription is active. The transgene insertion site influences transgene stability. However, it is still not possible to target a transgene into a particular site of a host genome since homologuous recombination is not routinely implemented in higher plant cells.

Transgene insertion into a host plant genome is thought to be a fully random process. A study focused on distribution of transgene insertion sites in barley revealed the presence of transgenes only on five (2H, 3H, 4H, 5H, and 6H) of the seven barley chromosomes (Salvo-Garrido *et al*. 2004). Fluorescent *in situ* hybridization was used for determination of the physical position of transgenes. Subsequent confirmation of the precise location of the transgenes was determined by genetic mapping. Most of the integration sites were found on chromosomes 5H – 30%, 4H – 30%, and 6H – 22%. Salvo-Garrido *et al.* (2004) detected the most transgene insertions in the telomeric and subtelomeric regions of both the long and the short arms (39%). Seventeen percent of the insertions were in the centromeric regions. Other regions of the long arms contained 22% of the insertions, and the short arms also contained 22%. Moreover, specific regions were found on chromosomes 4H and 5H with clusters of transgene insertions (on the short arm of chromosome 4H, on both the long arm and the short arm of chromosome 5H) suggesting that these areas are probably more susceptible for transgene insertion than other regions. Genomic regions flanking the transgene were gene-rich areas. It is estimated that only 12% of the barley genome contains coding sequences (Barakat *et al*. 1997). Therefore, it is evident that integration of transgenes is not a fully random process. These conclusions support studies in *Arabidopsis thaliana* and rice. Qin *et al.* (2003) observed “hot regions” in the *Arabidopsis* genome that contained more insertions than others and most of the insertion sites were located in, or close to, genes. Also, Barakat *et al.* (2000) confirmed the

presence of transgenes in gene-rich regions of *Arabidopsis* and rice by localizing T-DNA in fractions of DNA separated according to their GC levels. Sha *et al.* (2004) examined T-DNA flanking regions in rice and they detected preferential insertion into the coding areas of the genome.

Choi *et al.* (2002) used fluorescence *in situ* hybridization for raw mapping of transgenes and screening for homozygous transgenic barley prepared by microprojectile bombardment. No preferential integration sites among the chromosomes were found; however, within a chromosome a distal preference for transgene integration was observed. Transgenes in distal and telomeric regions of the chromosomes were observed in 58% of the transgenic lines. Other integration sites were in centromeric and subtelomeric regions, and in satellite regions of the chromosomes.

Not only the transgene locus but also stress can affect transgene stability. Plant transposable elements can be activated by stress, mainly during *in vitro* culture (Grandbastien 1998). These elements can be inserted into novel sites, and therefore can cause somaclonal variation. Meng *et al*. (2006) observed no effect of environmental stress on transgene expression stability or methylation status in multi- or single-copy transgenic lines; however, environmental stresses (water, nutrient deprivation and elevated temperature) caused numerous morphological changes. On the contrary, complete or partial heritable transgene silencing was observed after six generations of stable expression (*uidA* and *bar* gene driven by the maize *ubiquitin*-1 promoter) in one multi-copy subline following passages *in vitro*. Transcriptional gene silencing correlated with methylation in the 5´UTR (5´nontranslated exon) and intron of the *ubi1* promoter complex and condensation of chromatin around the transgenes. The subline contained stable, transcription-competent, inverted repeats of the transgene and the 3´LTR (Long Terminal Repeat) from a gypsy-like barley retrotransposon Sabrina-1. Meng *et al*. (2006) concluded that the transgene locus itself may affect its tendency to silence after *in vitro* culture and transgene silencing might result from host defense mechanisms activated by changes in plant development programming and/or stress imposed during *in vitro* growth.

Koprek *et al*. (2001) reported that transposon-mediated single-copy gene delivery increases transgene expression stability. Barley plants expressing the maize *Ac* (*Activator*) transposase were crossed with plants containing one or more copies of the selection gene inserted between the inverted-repeat *Ds* (*Dissociation*) ends. Transgene expression in F2 progeny with the transposed *Ds*-selection gene in different locations was 100% stable, while stable expression in plants without activated transposition of the selection gene was only 23%. Analysis of the integration site in single-copy plants showed that the transposed transgene was inserted into transcriptionally active regions of the genome, whereas the original location of the transgene was in redundant or highly repetitive genomic regions.

Perspectives

Plant genetic engineering is a powerful tool for the study of gene function, and for the realization of increased yields through plant breeding. Barley is the fourth most important cereal of the world and the second most important cereal grown in the Czech Republic. In 2009 it was 450 tonnes per hectare, and 30% was used for malt production. Natural selection and plant breeding has resulted in more than 100 varieties being developed in the Czech Republic, and they now occupy approximately 57% of the total barley cultivation. Application of new technologies is therefore of considerable interest to the scientific community. Transformation technologies have been successfully utilized in barley improvement for the production of elite cultivars with desired characteristics such as high and stable yields, feed and malting quality, resistance to pathogens, stress tolerance and high-level expression of valuable recombinant proteins in barley grains (Dahleen and Manoharan 2007, Goedeke *et al.* 2007, Godwin *et al.* 2009).

Development of transformation protocols which would be effective with minimal negative aspects, such as albinism, somaclonal variation or transgene inactivation is a main goal of laboratories working on transformation of barley and other cereals. Many innovations have resulted in resolution of these problems and also increased the efficiency of transformation using agriculturally

important cultivars. The recent work of Bartlett *et al.* (2008) showed a big increase in transformation efficiency of the model cultivar Golden Promise. If their procedure is applicable for cultivars of agricultural importance, it will be beneficial for future biotechnology procedures. In the future, transgenic barley can play an important role in high-quality food and feed production and in various industrial applications.

A novel strategy for plant genetic manipulation was recently developed (Shukla *et al*. 2009, Townsend *et al*. 2009). Designed zinc-finger nucleases (ZFNs) induced a double-strand break at the target locus. ZFNs were used to modify endogenous loci in plants of maize and tobacco protoplasts. Shukla *et al.* (2009) reported that simultaneous expression of ZFNs and delivery of a simple heterologous donor molecule leads to precise targeted addition of a herbicide-tolerance gene at the intended locus. Modified maize plants transmit genetic changes to the next generation. Shukla *et al.* (2009) chose as a target the *IPK1* gene encoding inositol-phosphate kinase that catalyzes the final step in phytate biosynthesis in seeds. Insertional disruption of the target locus, *IPK1*, results in both herbicide tolerance and alteration of inositol phosphate profile in developing seeds. This new promising strategy can have relevance for precise genetic manipulation of other cereal plant species such as barley in future (e.g. targeted gene knock-out).

Various techniques are under development for the removal of selectable marker genes in barley transgenic lines (Matthews *et al*. 2001, Xue *et al.* 2003); as production of marker-free plants is necessary to public concerns over the safety of genetically engineered crops. Chloroplast genetic engineering offers several advantages over nuclear genetic engineering, including gene containment in non-transgenic pollen and enhanced gene expression. Chloroplast transformation technology could be developed for barley and used for production of valuable recombinant proteins, such as those which need to be produced in large quantities and those which have prokaryotic origin.

Our effort is focused on preparation of transgenic barley with altered cytokinin content and increased lysine content. Transgenic barley with increased activity of cytokinin dehydrogenase (CKX, enzyme responsible for

cytokinin degradation) showed retarded growth of the aerial part and enhanced proliferation of the root system. Hence, root-driven expression of CKX in barley is a promising genetic manipulation to improve agricultural important traits such as higher tolerance to drought and better nutrient uptake. Furthermore, silencing of CKX expression in the aleurone layer of the barley grain could result in the accumulation of active cytokinins leading to the activation of cell-wall invertases, key enzymes in regulation of nutrition flow from source to sink tissues (Roitsch and González 2004). Thus, the spatial increase of cytokinin level can have a direct effect on yield by strengthening sink activity of the grain. Genetic engineering is also useful for improving *nutritional* quality, such as vitamins, essential amino acids and mineral content of crops. Cereal grains such as wheat, barley and maize contain insufficient levels of lysine. We prepared several lines of barley plants overexpressing dihydrodipicolinate synthase, the key enzyme in the biosynthesis of lysine and other essential amino acids. These plants are currently being tested for increased free-lysine content.

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Abbreviations:

2,4-D - 2,4-dichlorophenoxyacetic acid, Act1 - rice actin 1, Adh1 - alcohol dehydrogenase 1, BAP - benzylaminopurine, BYDV - barley yellow dwarf virus, CKX - cytokinin dehydrogenase, DON - deoxynivalenol, *gfp* - green fluorescent protein, *gus* - β-glucuronidase, IPK - inositol-phosphate kinase, *luc* - luciferase, LTR - long terminal repeat, MEC - modified embryogenic callus, QTL - quantitative trait loci, SEC - standard embryogenic callus, SMC - shoot meristematic culture, Ubi1 - maize ubiquitin 1, UTR - untranslated region, ZFN - zinc-finger nuclease.

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CHAPTER 3:

CHARACTERIZATION OF NEW MAIZE GENES PUTATIVELY INVOLVED IN CYTOKININ METABOLISM AND THEIR EXPRESSION DURING OSMOTIC STRESS IN RELATION TO CYTOKININ LEVELS

Characterization of New Maize Genes Putatively Involved in Cytokinin Metabolism and Their Expression during Osmotic Stress in Relation to Cytokinin Levels[1][W]

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Plant hormones, cytokinins (CKs), have been for a long time considered to be involved in plant responses to stress. However, their exact roles in processes linked to stress signalization and acclimatization to adverse environmental conditions are unknown. In this study, expression profiles of the entire gene families of CK biosynthetic and degradation genes in maize (*Zea mays*) during development and stress responses are described. Transcript abundance of particular genes is discussed in relation to the levels of different CK metabolites. Salt and osmotic stresses induce expression of some CK biosynthetic genes in seedlings of maize, leading to a moderate increase of active forms of CKs lasting several days during acclimatization to stress. A direct effect of CKs to mediate activation of stress responses does not seem to be possible due to the slow changes in metabolite levels. However, expression of genes involved in cytokinin signal transduction is uniformly down-regulated within 0.5 h of stress induction by an unknown mechanism. cis-Zeatin and its derivatives were found to be the most abundant CKs in young maize seedlings. We demonstrate that levels of this zeatin isomer are significantly enhanced during early stress response and that it originates independently from de novo biosynthesis in stressed tissues, possibly by elevated specific RNA degradation. By enhancing their CK levels, plants could perhaps undergo a reduction of growth rates maintained by abscisic acid accumulation in stressed tissues. A second role for cytokinin receptors in sensing turgor response is hypothesized besides their documented function in CK signaling.

Abiotic stresses, especially drought and soil salinity, are among the major limiting factors of plant growth and production in the environment. Activation of signaling pathways as a consequence of acquiring tolerance toward stress leads to rapid responses such as stomata closure or long-term changes in metabolism, growth, and development. Modification of gene expression profiles induced by stress response signal transduction can be mediated by phytohormones. Rapid elevation of abscisic acid (ABA) concentration in plant tissues exposed to water deficit is well documented (for review, see Schachtman and Goodger, 2008). ABA in particular reduces transpiration rates and activates at least two downstream signal transduction pathways involving MYB-like and MYC-like transcription factors (Abe et al., 2003). A recent microarray analysis of Arabidopsis cytokinin (CK) receptor mutants clearly showed that CK-mediated signaling can also be involved in stress responses. Knockout lines of two out of three CK receptors were strongly tolerant of drought and salt stress due to up-regulation of many stress-inducible genes (Tran et al., 2007). Alteration in the CK content in plants exposed to various stresses has been frequently reported. For instance, trans-zeatin (*t*Z) and trans-zeatin riboside (*t*ZR) contents decreased rapidly in the elongation zone of barley (*Hordeum vulgare*) leaves within several minutes after salinity stress induction. However, concentrations of both CK types increased in the nonelongated part of the leaf blade, indicating a possible reduction of cell division and CK translocation (Fricke et al., 2006). A significant long-term decrease of active isoprenoid CK content was observed in barley roots and shoots after exposure to a higher concentration of NaCl (Kuiper et al., 1990). It was assumed that the water-deficit-induced CK deprivation in leaves is mainly attributed to decelerated transport of root-borne CK via xylem (Davies and Zhang, 1991). Comprehensive CK analysis in wild-type tobacco (*Nicotiana tabacum*) leaves exposed to drought showed a gradual decrease in bioactive CK levels during stress progress

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accompanied by elevated activity of CK degradation enzymes. Severe stress, however, led to the accumulation of all CK forms in the roots (Havlová et al., 2008).

Maize (*Zea mays*) is one of the most important crop species used especially for direct human consumption and animal feed, besides today being a significant plant source for chemical feedstock. Together with model plants Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), maize has been extensively studied on the molecular level, and although the full genome sequence has not been officially published yet, there is enough information accessible in public databases (e.g. The Institute for Genomic Research Maize Database; Chan et al., 2006) to describe coherent gene families. In the Arabidopsis genome, there are more than 50 genes functionally studied that are directly involved in CK signal transduction and metabolism. In 2004, three genes coding for CK-responsive His kinases (CK receptors; HKs) were functionally described in maize (Yonekura-Sakakibara et al., 2004). Surprisingly, in contrast to known CK receptors from Arabidopsis, all three receptors responded to cis-zeatin (*cZ*), which had for a long time been considered to be inactive. Genetic information about other signal transduction pathway components, His-phosphotransfer proteins and CK primary response regulators (RRs), is accessible from previous works (Sakakibara et al., 1998, 1999; Asakura et al., 2003). One out of 10 recently described rice *RR* genes was shown to be up-regulated in seedlings exposed to a high concentration of salt (Jain et al., 2006).

CKs are generated in plant tissues by two biosynthetic pathways, de novo synthesis and isoprenylated tRNA degradation. The bulk of isoprenoic CKs are synthesized due to the activity of ATP/ADP isopentenyltransferases (IPTs), whereas the production of *cZ* in plants seems to be attributed mostly to tRNA degradation (Kasahara et al., 2004; Miyawaki et al., 2006). Several putative *ATP/ADP IPTs* and one *tRNA-IPT* gene were mined from a maize bacterial artificial chromosome (BAC) clone library. One of them, *ZmIPT2*, was recently proved functional and shown to contribute to CK de novo biosynthesis during kernel development (Brugière et al., 2008). CK homeostasis in plants is primarily maintained by different compartmentation of biosynthesis and degradation. While de novo biosynthesis is mostly bound to plastids (Kasahara et al., 2004), CK catabolic processes take place in vacuoles and apoplast (Werner et al., 2003). The irreversible degradation of free CK bases and their derivatives is catalyzed by CK oxidase/dehydrogenases (CKXs), encoded as well by a small gene family. Nevertheless, there is evidence for several maize CKXs (Massonneau et al., 2004); only one member of maize CKX enzymes, ZmCKX1, was studied in detail in connection with plant stress responses (Brugière et al., 2003). Elevated levels of *ZmCKX1* transcript were observed in developing kernels during various abiotic stresses. A positive effect on CKX expression in detached leaves was also induced by ABA, which suggests a possible role of this stress hormone in triggering CK degradation machinery.

The goal of this work was to clarify the role of CKs in the responses of maize seedlings to water deficit and salinity stress. The results provide (1) a comprehensive characterization of gene families involved in CK biosynthesis and degradation in maize, (2) a detailed characterization of the transcript abundance in different tissues and organs and their changes following stress treatment, and (3) together with the signaling and response network characterization, support for the concept of succession of gene expression changes that fit the changes in enzyme activities and endogenous contents of phytohormones.

RESULTS

Sequence Analysis of Genes Involved in CK Metabolism and Perception

Homology searches were done by BLASTn software on the maize genome sequencing project database (http://www.maizesequence.org/index.html) with currently known sequences as templates (Massonneau et al., 2004; Brugière et al., 2008) and rice orthologous sequences (Sakamoto et al., 2006; Hirose et al., 2007). BAC clones in the database cover almost 95% of information of the rough draft of the maize genome, as was announced on December 2008. Matched sequences with E values $\leq 2e^{-7}$ were checked for IPT and CKX conserved motifs. Contig sequences showing E values significantly below the threshold that contained insertion or deletion in open reading frames (ORFs) were excluded from the study. Eleven putative ORFs showing high similarity to 10 rice *IPTs* (Sakamoto et al., 2006) were identified. Seven of them are identical to maize *IPTs* recently annotated (*IPT1*, *IPT2*, and *IPT4–IPT8*; Brugière et al., 2008); hence, the numbering is kept due to the reference. The numbering, contig accession numbers to BAC libraries, locations of exons, as well as chromosome positions are summarized in Supplemental Table S1. Two out of four nonannotated sequences share high mutual homology (90.3%) and therefore were numbered as *IPT3* and *IPT3b*. The third one shows the highest homology to *IPT6* (82.4%) and was numbered as *IPT9*. A putative ortholog to a prokaryotic-origin *tRNA-IPT* gene (*IPT10*) highly homologous to rice *OsIPT10* and Arabidopsis *IPT9* was found (Sakamoto et al., 2006) on chromosome 6.

Two maize *CKX* genes were proved to encode functional enzymes (*CKX1* and *CKX3*; Houba-Hérin et al., 1999; Morris et al., 1999; Massonneau et al., 2004), and expression profiles of another three genes were analyzed (*CKX2*, *CKX4*, and *CKX5*; Massonneau et al., 2004). Data mining from the maize genome database revealed another eight sequences showing ORFs homologous to annotated CKX proteins. They were numbered upwardly from *CKX6* to *CKX12* (Sup-

plemental Table S1). The ORF found on chromosome 8 shares 93.4% homology to annotated *CKX4* on chromosome 3 and has been annotated as *CKX4b*. Positions of additional close paralogs *CKX2* and *CKX3* (93.5% homology) were found on the same chromosome segments as *CKX4* and *CKX4b*, respectively, indicating a common ancestral origin of these genes and the possibility that they segregated during the same duplication event. Patterns of recent chromosome duplication between maize chromosomes 3 and 8 have been reported (Gaut, 2001). Similar duplicates have been identified for gene pairs *CKX7*/*CKX8* (94.2%) and *CKX11*/*CKX12* (91.2%). Recently, the subcellular localization to cytosol and enzymatic activity of CKX10 have been demonstrated (Šmehilová et al., 2009). It seems to be the sole maize CKX isoform missing any signal peptide that contributes to CK depletion in the cytosol. Amino acid alignments of all maize IPTs and CKXs are presented in Supplemental Figures S1 and S2, and phylogenetic trees with rice and Arabidopsis orthologs are shown in Supplemental Figures S3 and S4.

Concerning genes involved in CK perception and primary response, the maize genome database screening confirmed loci for all annotated receptor and type A response regulator genes (Sakakibara et al., 1999; Asakura et al., 2003; Yonekura-Sakakibara et al., 2004). Several single nucleotide polymorphisms were detected among the annotated sequences and sequences generated from genome sequencing projects, which can be associated with different cultivars either used by or caused by the inaccuracies in the sequencing data. In the case of the *RR3* gene, another locus on chromosome 5 carrying an ORF for a closely homologous gene (83.9%) was found and marked as *RR3b*. Primers for this response regulator were designed to amplify targets from both sequences. Primers amplifying *HK1* were designed according to the annotated sequence NM_001111389 (BAC clone AC195458.3; chromosome 5). However, hybridization with the annotated sequence for receptor HK1a2 (NM_001112387; AC185637.3), which has not been functionally tested, is probable due to shared 92.4% homology. Only one locus was found for the receptor HK3, the corresponding transcript of which was previously shown to be alternatively spliced (Yonekura-Sakakibara et al., 2004). Two alternative versions of HK3 transcripts were described as full-length and functional HK3a and a nonfunctional version missing the third exon. Alignment of the genomic locus (BAC clone AC185417.3) revealed that the third exon (449 bp) is flanked by at least one large intron (third intron, 7,371 bp; sequence of the second intron is interrupted by two not yet arranged subcontigs). The size of the introns probably led to a splicing mistake and exon skipping, resulting in a folding of a nonfunctional receptor without the input domain.

One primer pair was designed to sense the signal of two genes encoding two very homologous cis-zeatin *O*-glucosyltransferases (*c*ZOGT; Veach et al., 2003). A 9-cis-epoxycarotenoid dioxygenase seems to be the key regulatory enzyme in ABA release from carotenoid structures (Schwartz et al., 2003). The structure of 9-cis-epoxycarotenoid dioxygenase gene as well as its responsiveness to abiotic stress were first described in maize (Tan et al., 1997). The sequence of this gene (*VP14*, viviparous phenotype; U95953) was used to design a primer pair; however, the amplification was very weak and unspecific. A BLAST search from the Maize Genome Project revealed two homologous ORFs to the VP14 sequence (homology 93.5% and 99.1%). Thus, new sets of primers were designed to avoid all polymorphism, and the most efficient one listed in Supplemental Table S1 was used in this study. A similar approach was employed to design primers for closely related galactinol synthase genes (GALS; Zhao et al., 2004) and betaine aldehyde dehydrogenase (BADH).

Expression Patterns of *IPT* and *CKX* Genes in Maize

To determine which of the biosynthetic and degradation enzymes can be involved in stress responses, the *IPT* and *CKX* transcript accumulation was analyzed by real-time PCR using cDNA prepared from 7-d-old roots and leaves as well as from various tissues of mature plants along with developing and germinating kernels. The analysis was complemented by genes involved in the CK signal perception and primary CK response described previously (Asakura et al., 2003; Yonekura-Sakakibara et al., 2004).

The absolute transcript levels of all studied genes are summarized in Table I. Values for genes whose transcript level was less than one copy per nanogram of isolated total RNA are not listed, due to the possibility of being unreliable. Concerning *IPT* genes, two genes for maize tRNA-IPT (*IPT1* and *IPT10*) were highly abundant and constitutively expressed in all tested organs. Other *IPT* transcripts showed spatial and temporal patterns of expression. Expression of *IPT2* is solely bound to developing kernels, as recently published by Brugière et al. (2008). *IPT5* is relatively highly present in all vegetative tissues, especially in roots and tassels. Transcripts of *IPT4*, *IPT6*, *IPT7*, and *IPT9* were also detected together with *IPT5* in roots, and these five IPT enzymes probably mainly contribute to the CK pool rising in the root during maize growth. In leaves, the total adenylate *IPT* transcript level is almost 1 order of magnitude lower than in roots, and in addition to *IPT5*, only *IPT8* and *IPT9* transcripts were determined above the defined threshold.

The transcript levels of *CKX* genes seem to be no higher than of those encoding IPTs. The only cytosolic CK degradation enzyme, CKX10, is relatively highly expressed in all tissues with the exception of endosperm. Other CKXs probably have a redundant role or can be expressed in specialized types of cells in different tissues; hence, it is visible from their low presence in all materials tested (e.g. CKX7 or CKX9). There are paralogs predominantly bound to reproductive

Table I. *Transcript abundance in different maize organs*

Abundance is expressed as gene copy number in 1 ng of total RNA amplified by qPCR, normalized to 18S RNA, and recalculated as primer pair efficiency. Total RNA from each sample was transcribed in two independent reactions, and PCR was performed in duplicate. Mean values with SD are shown.

Gene	Embryo	Endosperm	Pedicel	Silk	Tassel	Immature Ear	Coleoptile + Radicle	7-d-Old Root	Mature Root	7-d-Old Leaf	Mature Leaf
IPT genes											
IPT1	101.9 ± 33.6	110.8 ± 6.6	83.4 ± 5.4	112.3 ± 12.9	111.9 ± 4.8	114.2 ± 17.8	63.0 ± 8.9	71.6 ± 12.0	111.2 ± 14.9	125.0 ± 17.8	38.8 ± 10.6
IPT2	10.9 ± 2.2	89.4 ± 13.8	303.3 ± 17.2	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
IPT3	<1.0	<1.0	1.1 ± 0.3	<1.0	2.9 ± 0.6	1.2 ± 0.2	<1.0	<1.0	3.5 ± 0.2	<1.0	1.1 ± 0.7
IPT3b	<1.0	<1.0	<1.0	<1.0	1.1 ± 0.2	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
IPT4	1.9 ± 0.5	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	1.0 ± 0.4	8.3 ± 0.3	<1.0	<1.0
IPT5	5.1 ± 0.7	<1.0	34.5 ± 6.2	32.3 ± 4.2	353.7 ± 29.0	66.4 ± 13.7	3.7 ± 1.0	49.7 ± 12.5	169.3 ± 7.9	14.0 ± 9.3	33.2 ± 4.9
IPT6	<1.0	1.7 ± 0.6	<1.0	2.1 ± 0.4	8.5 ± 2.1	1.6 ± 0.3	1.1 ± 0.2	8.3 ± 2.7	19.2 ± 4.5	<1.0	<1.0
IPT7	<1.0	<1.0	2.1 ± 0.2	<1.0	11.2 ± 2.3	<1.0	<1.0	1.6 ± 0.4	<1.0	<1.0	<1.0
IPT8	8.5 ± 1.1	35.8 ± 3.8	4.2 ± 1.1	3.0 ± 0.5	7.3 ± 4.2	<1.0	1.4 ± 0.9	<1.0	<1.0	6.4 ± 1.4	8.8 ± 0.9
IPT9	2.2 ± 1.2	<1.0	3.2 ± 0.7	<1.0	7.9 ± 1.2	<1.0	<1.0	2.7 ± 0.6	23.2 ± 3.6	2.7 ± 0.3	<1.0
IPT10	51.5 ± 2.3	25.1 ± 1.6	16.7 ± 1.3	8.9 ± 0.6	40.9 ± 3.1	19.7 ± 2.8	48.0 ± 3.4	35.9 ± 2.7	11.2 ± 3.6	47.7 ± 1.0	46.9 ± 2.4
CK oxidase/dehydrogenase genes											
CKX1	422.9 ± 13.5	37.2 ± 7.4	95.2 ± 12.0	73.6 ± 14.0	190.0 ± 31.7	87.1 ± 15.5	2.1 ± 0.2	54.3 ± 4.5	116.7 ± 5.4	1.6 ± 0.1	1.5 ± 0.1
CKX2	23.2 ± 2.7	35.8 ± 2.8	78.8 ± 8.4	19.4 ± 2.5	224.6 ± 12.6	20.9 ± 1.8	6.2 ± 0.7	18.7 ± 2.1	37.1 ± 1.6	27.5 ± 3.7	609.4 ± 24.2
CKX3	15.3 ± 1.9	53.7 ± 0.7	38.7 ± 4.2	65.2 ± 6.8	12.4 ± 1.0	36.6 ± 2.2	2.9 ± 0.3	25.6 ± 0.9	5.8 ± 0.2	160.4 ± 5.3	10.2 ± 0.6
CKX4	<1.0	1.6 ± 0.7	57.1 ± 5.1	13.9 ± 0.4	2.5 ± 0.4	2.6 ± 0.1	<1.0	2.6 ± 0.1	11.1 ± 0.2	1.1 ± 0.1	3.8 ± 0.2
CKX4b	7.9 ± 0.4	<1.0	37.7 ± 6.5	21.7 ± 2.0	17.4 ± 3.8	7.4 ± 1.2	2.0 ± 0.2	26.3 ± 2.8	763.9 ± 96	20.3 ± 4.6	21.0 ± 1.8
CKX5	<1.0	1.8 ± 0.3	7.4 ± 2.0	54.6 ± 2.4	23.5 ± 1.2	2.5 ± 0.6	<1.0	<1.0	6.1 ± 0.4	<1.0	<1.0
CKX6	16.4 ± 3.6	<1.0	32.2 ± 3.3	4.2 ± 0.3	74.0 ± 3.4	16.6 ± 1.2	38.0 ± 1.7	221.2 ± 12.0	99.1 ± 11.0	93.4 ± 7.0	20.4 ± 1.7
CKX7	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
CKX8	6.6 ± 0.5	<1.0	<1.0	<1.0	<1.0	<1.0	2.1 ± 0.3	38.0 ± 19.6	2.6 ± 0.1	1.1 ± 0.2	4.6 ± 0.7
CKX9	<1.0	<1.0	<1.0	<1.0	<1.0	1.3 ± 0.1	<1.0	<1.0	<1.0	1.8 ± 0.7	<1.0
CKX10	141.6 ± 1.1	13.3 ± 0.7	229.3 ± 30.2	647.2 ± 56.1	1,120.1 ± 48.9	351.2 ± 28.7	219.2 ± 18.4	61.2 ± 18.4	126.2 ± 10.4	310.9 ± 25.1	76.6 ± 18.0
CKX11	2.0 ± 0.1	<1.0	<1.0	1.0 ± 0.2	3.5 ± 0.3	1.3 ± 0.3	1.8 ± 0.1	2.5 ± 0.3	4.6 ± 0.3	71.7 ± 4.6	2.9 ± 0.7
CKX12	9.5 ± 2.7	13.4 ± 0.8	<1.0	<1.0	3.8 ± 1.6	<1.0	1.5 ± 0.5	2.4 ± 0.2	2.7 ± 0.4	10.3 ± 0.6	3.5 ± 1.9
CK receptor genes											
HK1	1,712.9 ± 40.9	821.7 ± 38.7	1,117.1 ± 54.8	1,243.1 ± 42.0	1,439.8 ± 45.8	2,237.4 ± 75.9	838.2 ± 45.4	2,078.9 ± 45.6	763.4 ± 27.5	596.3 ± 30.5	322.6 ± 17.5
HK2	668.2 ± 47.0	818.8 ± 61.5	573.1 ± 29.5	1,849.3 ± 149.2	2,344.8 ± 88,3	2,233.0 ± 155.0	1,166.6 ± 147.0	4,812.8 ± 202.2	1,095.5 ± 85.7	2,394.4 ± 102.5	1,322.8 ± 147.8
HK3	141.0 ± 9.8	340.4 ± 41.5	158.0 ± 25.4	362.5 ± 36.6	273.1 ± 30.0	225.1 ± 17.7	178.2 ± 18.2	641.5 ± 54.4	195.6 ± 9.8	150.8 ± 7.9	240.6 ± 13.2
CK response regulator type A genes											
RR1	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	55.5 ± 4.7	189.4 ± 32.4	<1.0	449.1 ± 19.7	<1.0
RR2	5.6 ± 1.4	<1.0	29.7 ± 3.8	<1.0	11.2 ± 0.8	4.3 ± 0.3	38.7 ± 7.2	116.4 ± 11.7	19.1 ± 3.7	66.6 ± 5.8	92.9 ± 24.0
RR3	16.8 ± 2.7	<1.0	<1.0	<1.0	<1.0	3.3 ± 0.2	<1.0	<1.0	<1.0	0.3 ± 0.1	<1.0
RR4	118.2 ± 24.7	20.3 ± 3.0	201.7 ± 41.0	39.2 ± 4.9	435.3 ± 33.7	112.6 ± 6.7	615.6 ± 47.5	979.5 ± 54.5	190.8 ± 5.6	303.5 ± 24.2	356.0 ± 26.8
RR5	36.8 ± 9.1	44.8 ± 4.8	67.6 ± 7.5	69.2 ± 9.7	108.6 ± 9.0	28.7 ± 0.9	88.0 ± 3.9	53.8 ± 7.4	76.7 ± 5.4	36.2 ± 2.1	189.0 ± 27.8
RR6	41.2 ± 2.7	16.0 ± 1.5	1,007.0 ± 71.5	102.9 ± 21.5	1,693.5 ± 105.6	217.6 ± 16.4	835.1 ± 121.0	4,691.6 ± 350.4	920.2 ± 65.5	441.4 ± 25.9	2,362.1 ± 95.5
RR7	35.2 ± 10.5	4.7 ± 0.7	139.6 ± 18.0	37.1 ± 4.3	250.0 ± 39.7	62.0 ± 4.5	1,705.3 ± 254.7	4,993.6 ± 387.2	846.8 ± 25.7	1,693.5 ± 74.0	460.1 ± 28.6
Other genes											
cZOGT	7.5 ± 1.5	29.3 ± 3.8	111.1 ± 18.0	341.5 ± 23.9	806.7 ± 54.2	153.9 ± 28.0	2,188.7 ± 63.4	817.9 ± 29.2	279.3 ± 21.4	258.8 ± 19.6	29.0 ± 2.4
bGLU	38.3 ± 4.3	3.1 ± 1.1	11.2 ± 1.5	523.1 ± 38.6	67.7 ± 4.8	2,531.6 ± 89.4	150,129 ± 14,771	32,448 ± 2,110	8,512 ± 1,324	11,081 ± 865	10.8 ± 2.5
GALS	54.8 ± 9.7	<1.0	213.1 ± 14.5	5,134.0 ± 196.7	6,543.6 ± 266.6	126.7 ± 9.4	28.5 ± 5.8	134.0 ± 11.8	2,250.2 ± 92.7	3,605.2 ± 178.6	1,3087.2 ± 489.2
VP14	143.6 ± 14.7	12.0 ± 2.7	605.0 ± 47.8	218.4 ± 38.0	926.6 ± 72.4	568.4 ± 28.4	35.3 ± 2.7	31.5 ± 5.1	607.1 ± 57.1	41.1 ± 4.4	664.4 ± 29.7

organs (i.e. CKX4 and CKX5). Among the *CKX* genes that most likely contribute to the pool of CK degradation activity in tissues exposed to stress conditions are *CKX1*, *CKX6*, and *CKX8* expressed in young roots and *CKX3*, *CKX6*, and *CKX11* expressed in young leaves. In the control plants, broad changes were observed in transcript levels of several *CKX* genes between the first and second weeks of plant development when stress was applied. For example, approximately a 6-fold increase in the copy number of *CKX1* between 7 and 10 d of root development or a 3-fold decrease of *CKX6* expression in the same tissue was seen. These kinds of fluctuations were also observed in leaves (data not shown). A dramatic increase of *CKX2* and *CKX4b* expression was detected in mature leaves and roots, respectively. However, the level of all *CKX* transcripts with the exception of cytosolic *CKX10* was negligible in coleoptile and radicle (2-d-old plantlet), probably due to the demand for a high concentration of active CKs in these rapidly developing tissues. The above summarized data are in good accord with previously reported expression profiles of CKX1 to CKX5 (Massonneau et al., 2004) obtained by semi-quantitative reverse transcription-PCR.

Changes in Expression Profiles during Stress Responses

Relative to expression of all *CKXs*, *IPT* was quantified by real-time reverse transcription-PCR in tissues exposed to the two stresses or to an exogenous supply of CK for 0.5 h (Fig. 1, A and C) or 3 d (Fig. 1, B and D). Expression of *HK* and *RR type-A* genes was followed at six time points from the initiation of the stress experiment (Fig. 2). Since the expression of *IPT* and *CKX* genes is generally low in plant tissues, transcripts of most of them are hard to detect by any convenient hybridization technique. Nevertheless, the changes in expression of several *RR type-A* genes and the gene responsible for glucosylation of *cZ* were followed by northern-blot analysis. These data correspond well to those obtained by quantitative PCR (qPCR) analysis (Supplemental Fig. S5; Supplemental Methods S1). Isoforms of the β-actin gene (accession no. U60508) that was selected from two other actin genes and a glyceraldehyde phosphate dehydrogenase gene as the most constantly expressed ones in young leaves and roots were used as references to normalize expression levels among the tested samples.

Generally, CK treatment caused persistent down-regulation of de novo adenylate *IPT* genes detectable in whole plants 0.5 h after the treatment, whereas the transcript levels of two genes contributing to tRNA-linked CK biosynthesis did not seem to have altered. On the other hand, the levels of *CKX* genes were up-regulated; however, the extent of up-regulation that was observed 3 d after the treatment differed in intensity. For instance, *CKX1* and *CKX4b* showed extensive up-regulation, while other genes were regulated more moderately. Interestingly, *CKX3*, which is strongly expressed in leaves, showed a significant down-regulation after the CK treatment. As expected, expression of all type A response regulators were induced in the roots immediately after CK addition to the nutrient solution and then was slightly reduced after 24 h of treatment. However, expression was still higher in comparison with untreated maize until the end of the experiment. Similar up-regulation of *RR* genes was observed in the leaves, with an approximately 0.5-h delay likely caused by transportation of the CK signal to aerial part of the plant. The levels of receptor transcripts were not significantly altered after exogenous CK application.

Regulation of CK metabolic gene expression in stressed tissues is not straightforward (Fig. 1). There were only two *IPT* genes induced 0.5 h after osmotic stress application in roots, while the expression of all other genes tested was not immediately influenced in leaves or in whole plant by salinity stress application. The most abundant *CKX* gene in root tissue, *CKX6*, and the *CKX3* gene were down-regulated 0.5 h after either stress induction. However, other less abundant *CKX* genes were up-regulated. The situation in leaves appeared likewise: *CKX3*, *CKX6*, and *CKX11* were slightly down-regulated, whereas the expression of the other genes was not significantly changed. As well, the transcript for the sole cytosolic CKX enzyme does not seem to be regulated in fast response to stress.

Long-term actions of NaCl and water deficit in plant tissues affect transcript levels of most *CKX* and *IPT* genes. All abundant *IPT* genes are up-regulated to some extent. While in leaves salinity causes significantly increased expression levels of both de novo and tRNA-linked synthesis genes, in roots a significant enhancement of two major de novo synthesis gene expressions, *IPT5* and *IPT6*, was observed in osmotically stressed plants. Increase of *CKX* transcript level was more evident in leaf tissues than in roots. Whereas most of the genes were up-regulated in roots of salt-stressed plants including *CKX6*, *CKX8*, and *CKX10*, long-term osmotic stress preserved elevated expression of *CKX6* and two less abundant genes, *CKX3* and *CKX2*. Interestingly, the transcript level of another relatively abundant root gene, *CKX1*, was found in stressed roots below the levels of control plants. Although the gene is not highly expressed in leaves, its significant up-regulation under stress conditions was observed, indicating a possible tissue specificity of *CKX1* promoter activity to the stress response.

Regarding the genes encoding CK receptors, the onset of stress application had no noticeable effect on their regulation. During acclimatization, elevated levels of receptor transcripts appeared only in the leaves of maize seedlings grown under stress conditions. The levels of all type A genes were jointly down-regulated immediately after the stress induction in whole plants. A further stress response slowly increased RR type A levels back to normal or even above the control levels. However, the profile of the up-regulation was not uniform. One hour after the stress treatment, levels of *RR5* and *RR6* transcripts rose

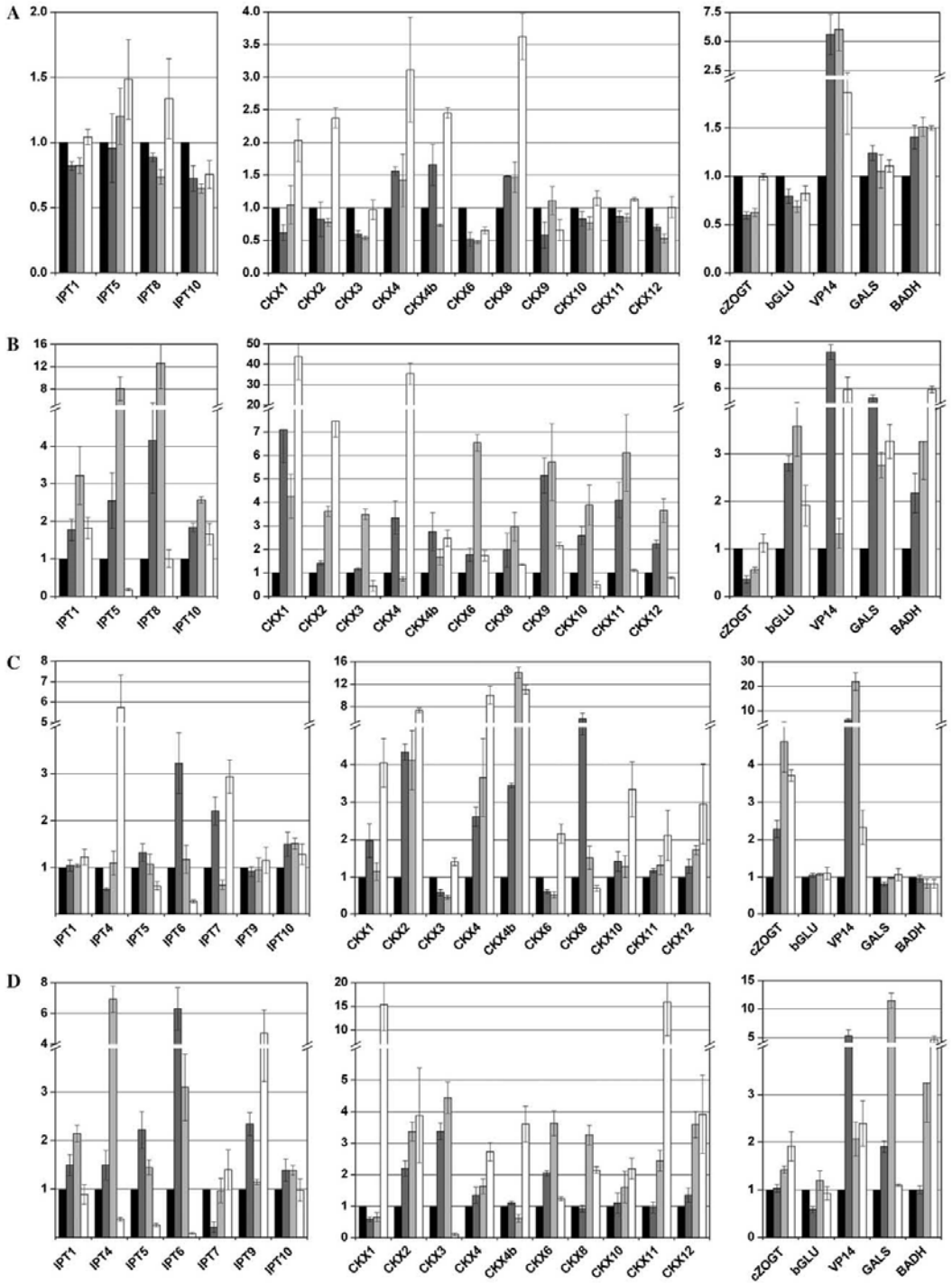

Figure 1. (*Legend appears on following page.*)

above the control levels in roots exposed to high salinity. On the other hand, expression of the most abundant response regulator gene in maize seedlings, *RR7*, was significantly down-regulated until the third day of the plant acclimatization.

From a huge family of maize glucosyl transferases and glucosidases, there are only three functionally described enzymes participating in specific glycosylation and deglycosylation of CK molecules (Brzobohatý et al., 1993; Veach et al., 2003). Two genes whose products are responsible for *O*-glucosylation of *cZ* derivatives seem to be regulated alternatively in different tissues. While *cZOGT* transcripts in roots increase in response to stress, their levels in leaves are diminished. Thus, the gene for β-glucosidase *Zm-p60.1* (*bGLU1*) encoding the enzyme that catalyzes the reverse reaction shows an opposite expression profile. While water deprivation enhanced its level in leaves, in roots it was slightly down-regulated. As expected, the level of pooled transcripts coding for VP14, the key enzyme in ABA release, was exceedingly up-regulated immediately after the stress application, and this level of transcription was maintained for 3 d, particularly in osmotically stressed tissues. The expression levels of other genes (*GALS* and *BADH*) whose products are probably involved in the production of the osmoprotective compounds galactinol and betaine were not found to increase before the late stage of stress.

Changes in CKX Activity during Stress Responses

Efficiency of degradation of N^6-isopentenyladenosine (iPR) at physiological pH 6.0 and in the presence of quinone electron acceptor was followed for a period of 10 d after stress induction. CKX activity was detected separately in root tissue and upper parts of plants. Total activity was considerably higher in the roots (5.45 pkat mg^{-1} extracted proteins in 7-d-old control plants) than in leaves (0.43 pkat mg^{-1}). Although the total activity during the stress experiment fluctuated slightly, probably due to diurnal changes (data not shown), generally a slight decrease was observed each day of maize growth (3.75 and 0.28 pkat mg^{-1} in roots and leaves, respectively, in 17-d-old maize). Activation of CKX enzymes after exogenous CK application was previously well described (Brugière et al., 2003). The increase was detectable in roots 0.5 h after CK addition to the nutrient solution and within 1 h in the leaves (Fig. 3). Stress did not have any immediate effect on CKX activity (within 1 h); however, a significant decrease in the activity was observed in the root tissue 3 h after the application of osmotic or salinity stress. During plant acclimatization to the stress conditions, degradation efficiency gradually grew in the whole plant. A 3- to 4-fold increase was detected in roots exposed to both stresses. In the upper part of the plant, a significant enhancement of CKX activity was detected only in the plants exposed to high salinity, reaching an almost 6-fold increase on the 10th d of acclimatization to stress (Fig. 3).

CK Content and Changes during Stress Responses

CK content was determined in leaves and roots separately (Supplemental Figs. S6 and S7). All aromatic CKs as well as *N7*- and *N3*-glucosides present in all tested tissue samples were under the limit of detection (less then 0.05 pmol g^{-1} dry weight), with the exception of plants treated by benzyladenine (BAP; Supplemental Fig. S8). Generally, *cZ* and its derivatives were the most abundant types of CKs in the maize seedlings, followed by *tZ* and N^6-isopentenyladenine (iP) derivatives. Levels of dihydrozeatin derivatives were markedly lower, with only *N9*-glucoside, riboside-5′-monophosphate, *O*-glucoside, and riboside *O*-glucoside detectable in roots. Free bases and ribosides, which are considered to be the active CKs (A-CKs), were less abundant than glucosidic forms. *cZ* accumulated mainly as its *O*-glucoside, whereas *tZ* and iP accumulated as *N9*-glucosides in roots. Riboside-5′-monophosphates of all three CK types, considered to be the nonactive biosynthetic intermediates, were more abundant than active forms, with the exception of *tZR5′MP* in leaves.

One-half hour after the induction of abiotic stress, few changes in the content of active CKs were observed (Fig. 4). Slight fluctuations among various types of A-CKs appeared, but the total levels of A-CKs in stressed leaves copied those in the control plants still at 3 h after the stress induction. In stressed roots, levels of A-CKs approximately doubled, mainly due to accumulations of *cZ* and its riboside (*cZR*), whereas concentrations of other less abundant A-CKs were reduced within the same time interval. After 1 d under the stress, the total level of A-CKs dropped back to normal; however, a significant increase in the levels of iP and *tZ* types was observed in roots. When plants acclimatized to the stress conditions, the pool of A-CKs remained elevated compared with control plants, which was detectable in roots after the third day of stress treatment. In leaves, there was a noticeable decrease of *cZ* and its riboside 1 d after the stress induction; however, as in roots, accumulation of *tZ*

Figure 1. Expression profiles of genes involved in CK metabolism and perception in maize leaves (A and B) and roots (C and D) exposed to osmotic stress (dark gray bars), salinity stress (light gray bars), and exogenous BAP application (white bars). Total RNA was isolated from tissues exposed to stress and CK stimuli for 0.5 h (A and C) or 72 h (B and D) from at least two independent biological replicates. cDNA from each sample was obtained at least in two independent reactions and run in two separate PCRs. The actin gene was used as an endogenous control, and untreated tissue samples were used as calibrators (black bars) in which expression level was set to 1. Only genes with abundance above one copy per 1 ng of total isolated RNA are included.

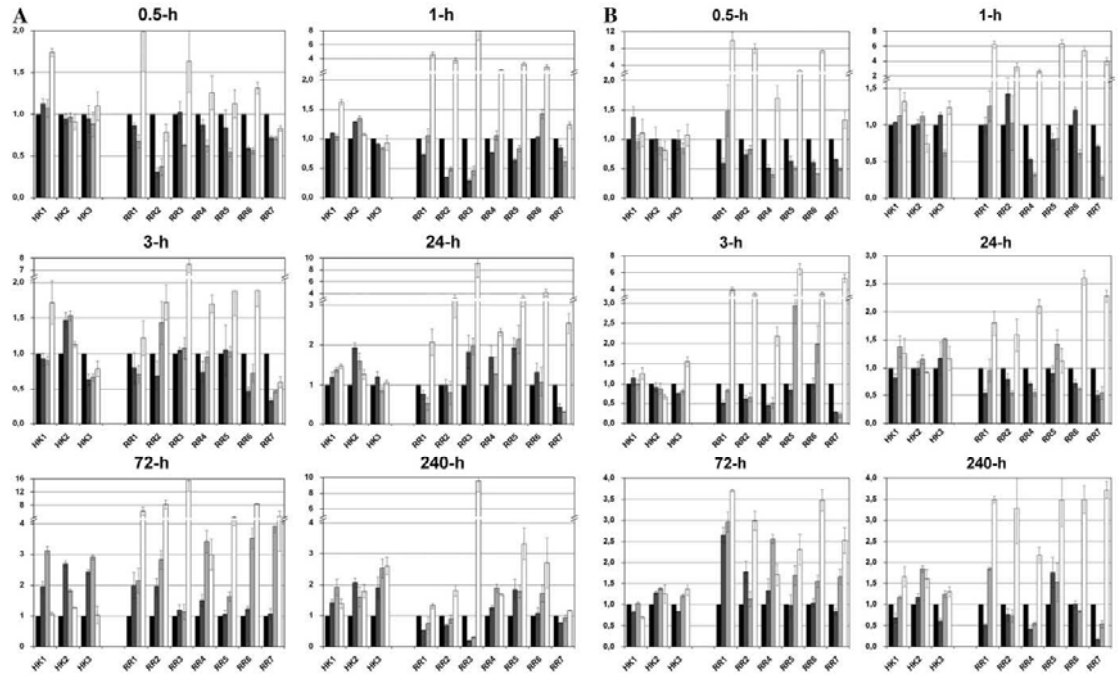

Figure 2. Expression profiles of genes involved in CK perception in maize leaves (A) and roots (B) exposed to osmotic stress (dark gray bars), salinity stress (light gray bars), and exogenous BAP application (white bars). Total RNA was isolated from tissues exposed to stress and CK stimuli at six time points from at least two independent biological replicates. cDNA from each sample was obtained at least in two independent reactions and run in two separate PCRs. The actin gene was used as an endogenous control, and untreated tissue samples were used as calibrators (black bars) in which expression level was set to 1. Only genes with abundance above one copy per 1 ng of total isolated RNA are included.

and iP types, followed later by *c*Z and *c*ZR increase, was observed in the course of acclimatization. In the roots, growth of A-CK content was similar or slightly higher than in leaves after 3 d of salinity stress. A higher pool of A-CKs was observed in leaves of osmotically stressed plants.

A rapid elevation of *c*Z and its riboside in roots occurred within 3 h of stress and was accompanied by a 2- to 3-fold increase of its riboside-5′-monophosphate (*c*ZR5′MP), which was observed in roots that were salt stressed for 0.5 h. Furthermore, levels of N^6-isopentenyladenosine-5′-monophosphate (iPR5′MP) and *t*ZR5′MP in the stressed samples diminished after a 3-h interval but started to elevate quickly within the first day of stress response. Thus, *t*ZR5′MP level in roots was 4- to 5-fold higher than the control after 72 h. In leaves, iPR5′MP was similarly elevated in the same time interval and *t*ZR5′MP was below the detection limit. Regarding the glucosides of isoprenoid CKs, the levels in stressed tissues compared with control plants started to distinctly rise after an extended period of stress, with noticeable change on the third day in both roots and leaves. Only *c*Z did not preferably accumulate in stressed roots as *N*- or *O*-glucoside (*c*ZOG, *c*ZROG, *c*Z9G). In the leaves of osmotically and salt-stressed plants, however, increasing accumulation of *c*ZOG and *c*ZROG was observed on the third day of plant acclimatization or later.

ABA and Its Metabolites in Stressed Tissues

As expected, levels of free ABA were elevated in whole plants 30 min after stress application (Fig. 5). In leaves, levels increased approximately 2-fold, while a more robust 4-fold ABA accumulation was detected in salt-stressed roots. Interestingly, free ABA accumulation returned to control levels in salt-stressed plants 72 h after the stress treatment while being kept significantly elevated in osmotically stressed plants. However, high levels of ABA catabolic products such as phaseic acid and dihydrophaseic acid in salt stress samples, which are biologically inactive, indicate a massive degradation of released ABA within the first 3 d. Free ABA level reduction back to the control level is likely related to long-term acclimatization to salt stress. On the other hand, levels of ABA glucosyl ester (ABAGE) were constantly elevated in leaves under both stress treatments. However, ABAGE con-

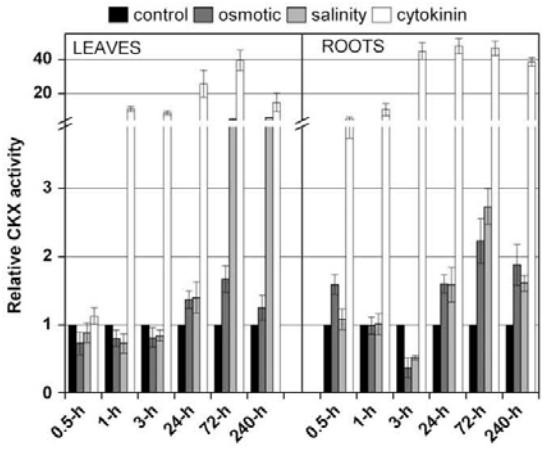

Figure 3. Changes in relative CKX activities during stress and CK treatment. Total activity in control leaf and root tissues (0.5 h) was determined as 0.43 and 5.45 pkat mg $^{-1}$, respectively, with iPR as substrate under the conditions described in "Materials and Methods." Mean values from free replicate measurements are shown with SD.

centration was insignificant in roots. Neophaseic acid, 9′-OH-ABA, ABA alcohol, and ABA aldehyde were below detection limits in all analyzed samples.

Exogenously applied 10 μM BAP to unstressed maize plants dramatically increased the concentration of ABA and related compounds (Fig. 5). A massive accumulation of free ABA and its degradation products was detected in leaves after 3 d of growing in a nutrient solution with CK. The concentrations of ABA and phaseic acid were even 2- and 10-fold higher, respectively, compared with osmotically stressed tissues. Increased levels of dihydrophaseic acid indicate increased metabolism of ABA in roots, despite the fact that the levels of ABA and its glucosyl ester were comparable with the control plants. Hence, the accumulation of ABA metabolites matches the observed reduction in leaf size of plants exposed to an oversupply of CK. The influence of exogenously applied CK on ABA levels in plants was formerly tested only in the halophyte *Mesembryanthemum crystallinum* without any positive correlation (Thomas et al., 1992). Nevertheless, those authors did not specify which parts of the plant were used for analysis.

DISCUSSION

Molecular mechanisms regulating responses to abiotic stresses have been extensively studied during the last decade. For instance, the essential role of mitogen-activated protein and salt overly sensitive kinase cascades and the function of transcription factors containing ABA-responsive elements are undoubtedly well established. CKs are an important signal traveling from roots to the shoots in response to nutrient insufficiency (Takei et al., 2004); however, conclusions concerning their role in drought, osmotic, and salinity responses or signalization are derived from inferences with no cause/effect data (Kuiper et al., 1990; Davies and Zhang, 1991; Kudoyarova et al., 2007; Tran et al., 2007). In this article, we, to our knowledge for the first time, describe complete gene families of CK biosynthetic and degradation genes in maize and regulation of their expression in a short-term response to osmotic and salinity stress as well as during the plant acclimatization to these adverse environmental stimuli. We also present the expression of CK signaling components with the aim to pursue all of the expression profiles in the context of endogenous CK and ABA levels and in particular the levels of the active forms.

Seedlings of 7-d-old maize were studied due to the fact that CK metabolism and CK responsiveness in young tissues begin extensively on the seventh day of development. This is supported by relatively high expression levels of many genes encoding biosynthetic and degradation enzymes as well as CK signaling components (Table I). During germination and the early phase of seedling development, CKs are probably still supplied in the form of glucosides from the kernel, as becomes evident from the massive expression of the β-glucosidase gene and the marginal expression of all *IPT* and *CKX* genes detected in 2-d-old radicles and coleoptiles. On the other hand, in developing kernels, where the CK role in response to water

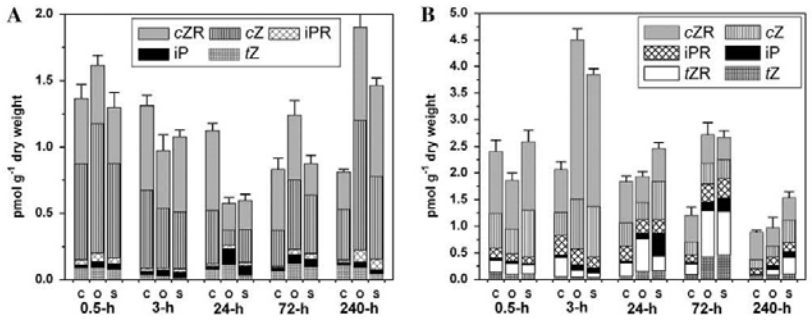

Figure 4. Changes in active CK content in maize leaves (A) and roots (B) during responses to osmotic (O) and salinity (S) stress compared with untreated plants (C). All values are derived from at least two biological replicates that were determined in at least two technical replicates. Error bars show the sums of SD values of all represented CKs. Levels of A-CKs not shown were under the detection limit of the method.

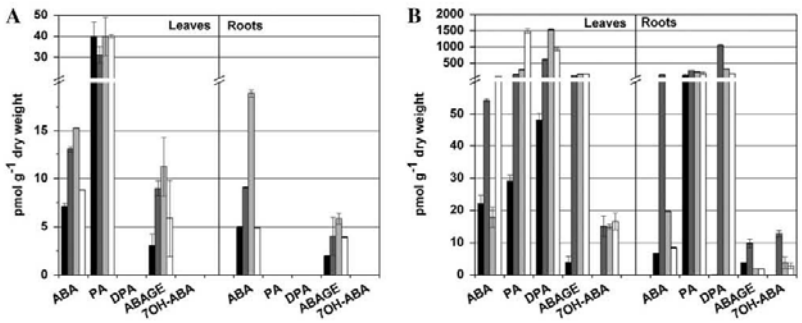

Figure 5. Endogenous levels of ABA and its derivatives in untreated maize roots and leaves (black bars) and tissues exposed to osmotic stress (dark gray bars), salinity stress (light gray bars), and exogenous BAP application (white bars) for 0.5 h (A) and 72 h (B). All bars show mean values of two replicates with indicated SD. DPA, Dihydrophaseic acid; PA, phaseic acid.

stress was studied previously (Yang et al., 2001; Brugière et al., 2003), only specific genes for de novo biosynthesis (*IPT2*), degradation (*CKX1* and *CKX4*), and signal response (*RR3*) are active. Total content of active CKs reaches a maximum in 1-week-old seedlings (Fig. 4) and later slowly declines as CKs are converted to nonactive glycosylated forms. The observed peak of active CKs in 7-d-old seedlings is probably due to meristematic activity and rapid elongation promoted by induction of de novo biosynthesis and depletion of kernel-stored CK forms. Recently, similar accumulations of active CK forms were observed in 6-d-old pea (*Pisum sativum*) seedlings, while in 9-d-old seedlings the levels started to decline (Stirk et al., 2008).

Immediately after the stress induction, we did not observe any significant decline or increase in CK content, with the exception of *c*ZR5′MP, as was previously demonstrated in several studies (Mustafina et al., 1998; Fricke et al., 2006). A constant level of active CKs 30 min after stress induction is in accordance with unchanged activities of degradation enzymes. Nevertheless, expression of CK metabolic genes is already influenced, especially in osmotically stressed roots. Genes for two de novo *IPTs* (*IPT6* and *IPT7*) were up-regulated, while the main root-expressed degradation gene, *CKX6*, was down-regulated, resulting in an approximately 2-fold decrease in CKX activity and accumulation of active CKs in stressed roots during first 24 h of stress application. A more pronounced positive effect of the stress on CK biosynthesis was observed after 3 d, when also the level of the most abundant transcript, *IPT5*, was significantly elevated in whole seedlings. Regulation mechanisms of the expression of *CKX* gene family members are not uniform, as is evident from the expression profiles under stress conditions and BAP treatment. For instance, most *CKX* genes are significantly up-regulated after exogenous application of BAP (*CKX1*, *CKX2*, *CKX4b*); however, the expression of *CKX3* is approximately 10-fold less than the levels in untreated tissue. Accordingly, two out of 11 rice *CKX* genes were down-regulated 2 h after *t*Z application on rice seedlings, whereas the others were up-regulated (Hirose et al., 2007). Tissue-dependent variations in *CKX* gene regulation are apparent as well; whereas the stress status induces *CKX1* expression in leaves, it has an opposite effect in roots. The direct effect of ABA on the amount of *CKX1* transcripts in maize leaves, as was demonstrated by Brugière et al. (2003), is not likely in roots, where higher ABA levels stimulated by stress do not induce *CKX1* expression. On the other hand, significant elevation of *CKX1* transcripts was observed later, 42 d after pollination, and could be attributed to the accumulation of ABA in the desiccating kernels, as the total level of active CKs is distinctively decreased in this stage of development (Brugière et al., 2003). No correlation is evident in the leaves where ABA as well as *CKX1* transcript levels are kept high under long-term osmotic stress, although in salt-exposed plants at the same moment active ABA concentration returns to normal level but *CKX1* is still up-regulated. This suggests that no short-term feedback mechanism exists to return *CKX1* transcript level back to normal after induction by ABA. Alternatively, degradation products of ABA, which are still highly accumulated in salt-stressed tissues, might to some extent keep ABA responsiveness, as was demonstrated for 7′-OH-ABA in barley (Hill et al., 1995). Hence, levels of free ABA correlate with growth rates of acclimatized plants, while in NaCl-treated seedlings, elongation growth is to some extent restored and polyethylene glycol (PEG)-treated seedlings display significantly less elongation of leaves within a 7-d period of stress action, probably due to the permanent increase in free ABA level.

Levels of *c*ZR5′MP were significantly increased in roots exposed to a high NaCl concentration 0.5 h after the application (Supplemental Fig. S7). A similar increase was also observed later in osmotically stressed roots. Dephosphorylation and deglycosylation of *c*ZR5′MP resulted afterward in an increased pool of *c*Z and its riboside in stressed plants detectable 3 h after application. Interestingly, the elevated level of *c*ZR5′MP is accompanied by a significant up-regulation of a gene encoding *c*Z *O*-glucosyltransferase 0.5 h after the stress induction (Fig. 1C), resulting in *c*ZOG and *c*ZROG accumulation detected after 3 h in roots of both stressed samples. This indicates that the expression of the enzyme is activated rapidly by

its substrate or its precursor. Thus, *O*-glucosylation is a mechanism by which the plant cell could react very fast to the disturbed hormone homeostasis induced by physiological stimuli. Since expression of *IPT* genes was not initially up-regulated in salt-stressed samples, the 3-fold elevated level of *c*ZR5′MP well supports previous studies where the origin of *c*Z-type CKs is attributed to prenyl-tRNA degradation (for review, see Sakakibara, 2006). Recently, a massive and rapid endonucleolytic cleavage of different types of mature tRNAs as well as rRNA has been observed in yeast and Arabidopsis exposed to oxidative and some other stresses (Thompson et al., 2008). Interestingly, enhanced tRNA cleavage does not significantly reduce the pool of mature tRNAs in yeast exposed to hydrogen peroxide. Although the authors focused only on specific fragmentation of tRNA within the anticodon loop, prenylation of tRNA right at this tRNA structure indicates that the described stress-induced fragmentation may lead to a release of free modified nucleotides from destabilized small RNA structures or they may be released by postulated cytotoxic tRNAses (Tomita et al., 2000). Processes connected with the release of prenylated nucleotides from RNA molecules could be linked to the specific cell compartments called stress granules or RNA granules, which rapidly form in all kinds of eukaryotic cells within 15 to 30 min after various stress stimuli. Although the primary function of these granules is thought to be the degradation of specific mRNAs, the granules also contain other enzymes linked to various forms of RNA decay (for review, see Anderson and Kedersha, 2006). The contribution of tRNA-released CKs to their pool in stressed tissues is well demonstrated in earlier work on NaCl-stressed maize (Atanassova et al., 1997). Hydrolyzed RNA from long-term stressed samples contained more CK bases than the control. In accordance, the expression of both tRNA-IPTs was elevated 72 h after stress induction; however, these genes seem to be more or less constitutively expressed during maize development. Nevertheless, further research needs to be done (e.g. stress responses of tRNA-IPT mutants) to confirm such a rapid release of modified nucleotides from degraded RNA. The dynamics of stress-induced CK changes needs to be viewed also from the point of cellular compartmentation. As the only known CK receptors are situated on the cytoplasmic membrane (Kim et al., 2006), the efflux rates of CKs out of the cell that were not characterized so far might be the limiting factor of CK-mediated transduction.

From our study, it is evident that stress response followed by acclimatization caused CK imbalance by increased rates of metabolism. Up-regulation of the majority of *CKX* genes leads to higher CK degradation activities in plants acclimatized to osmotic and salinity stress. Nevertheless, the enhanced catabolism is accompanied by similarly accelerated de novo biosynthesis and, most likely together with CK release from RNA and other CK-linked metabolism, such as β-glucosidase activity in leaves, contributes to moderate accumulation of active CKs in stressed plants. Apparent CK accumulation in 72-h stressed roots is subsequently transposed to the upper part of plants, being promoted by increased root levels of either *t*ZR or *O*-glucosides, which are thought to be a transport form of CKs (Bano et al., 1993; Hansen and Dörffling, 2003). Root-to-shoot trafficking of CKs could also explain certain discrepancies in this study regarding a later increase in *c*ZOG in stressed leaves that contrast with down-regulation of *cZOGT* and up-regulation of *bGLU* genes. Thus, the supply of this form from roots is most likely. Hence, it is premature to discuss all changes in expression profiles, since almost nothing is known about substrate specificities and turnover rates of determined gene products. For instance, our recent work clearly shows that diverse Arabidopsis isoforms of CKX significantly differ in their preference for *c*Z and *t*Z isoforms (Pertry et al., 2009) and that the degradation of differently glycosylated CKs is pH dependent (Galuszka et al., 2007). Alkalinization of xylem sap and apoplast observed under water-deficient conditions (for review, see Schachtman and Goodger, 2008) might therefore induce significant changes in CKX substrate preferences. It will be a question in subsequent studies to describe substrate specificities and cellular compartmentation of maize CKX and IPT enzymes. As visible from the phylograms (Supplemental Figs. S3 and S4), maize proteins cluster independently of Arabidopsis orthologs. Thus, CK homeostasis in cereal plants can be functionally diverse based on details emerging from the study of the dicot model Arabidopsis (Galuszka et al., 2007).

In the early phase of stress treatment, when plants were apparently wilted, expression of all CK response regulator genes was markedly down-regulated (Fig. 2), although the levels of active CKs were not altered. Complementation studies with Arabidopsis CK receptors previously showed that variations in osmotic pressure can involve changes in the activity of these receptors and subsequent regulation of downstream phosphorylation in yeast in the presence of CKs (Reiser et al., 2003). However, it is not clear whether the reduction in CK sensitivity is directly caused by double antagonistic responsiveness of CK receptors to CK molecules and turgor changes or the CK signaling transduction is affected by other relative turgor receptors and/or signaling pathways. Recently, the antagonistic function of CK receptors and another related His kinase (AHK1) in abiotic stress signaling has been demonstrated by studying gain- and loss-of function mutants in Arabidopsis (Tran et al., 2007).

The dynamics of *RR* gene expression observed within the first day of stress action could contribute not only to increased turgor sensing but also to fluctuation of specific CK forms. Active *c*Z forms reached 3-fold higher levels in 3-h stressed roots than in the control. The primary increase in *c*ZR5′MP detected in salt-stressed roots is accompanied by *RR1* transcript elevation followed by *RR5* and *RR6* expression increase detected at 1 and 3 h after the stress application,

respectively, while the other *RR* genes, especially *RR4* and *RR7*, are still expressed below the control level. Transcript levels of these response regulators decreased as the levels of *cZ* types declined 24 h after the stress application. At the same time point, however, expression of most *RR* genes in the leaves returned to control levels or started to be slightly upregulated, despite the fact that the levels of *cZ* types dropped 2-fold. Such an enhancement can be attributed to the increase of iP and the lower amount of *tZ* in the stressed leaves. Thus, the responsiveness of the CK transduction pathway in maize seems to be uniformly influenced by the changes in turgor as well as specifically (at least to some extent) by different types of CKs.

Generating data from full-genome transcriptional profile screening exploiting Affymetrix chips is easy via Genevestigator software. Although there are no accessible results from experiments done on Affymetrix maize genome array so far, data from 166 Affymetrix Rice Genome arrays, including several providing expression information from abiotic stress treatments, were recently released (Zimmermann et al., 2008). Since there is a high degree of genetic synteny between maize and rice genomes, we were able to unambiguously locate nearly identical rice homologs to almost all studied genes from maize (Supplemental Figs. S3 and S4). Beyond sequence similarity, transcripts of these orthologs demonstrate comparable levels of abundance. Expression profiles of all CK-related genes from the experiment where 7-d-old rice seedlings were exposed for 3 h to drought and salinity stress induced by 200 mM NaCl (Jain et al., 2007) are summarized in Supplemental Table S2. Interestingly, changes in the expression of CK metabolic and perception genes in stressed rice are closely comparable to those we described in maize 0.5 h after stress induction. Thus, all genes for type A response regulators were downregulated 3 h after salinity stress application and almost all when seedlings were dried. Transcripts of rice *CKX* genes, showing highest homology to ZmCKX3 and ZmCKX6, generally the most abundant maize seedling orthologs, were as well significantly below control levels in both stress samples. These CKXs probably contribute to the decrease in CKX activity observed 3 h after stress application in maize. Concerning the genes for de novo IPTs, two of them showing highest signals on the chip were slightly upregulated. This documented agreement in expression profiling between our results obtained from maize and published rice microarray data demonstrates that initial fluctuation and later accumulation of CKs induced by stress stimuli found here in maize seedlings can be a general response to abiotic stress at least in crop plants from the Poaceae family.

In conclusion, we demonstrated that CKs do not have a direct function in stress signaling similar to ABA due to a slow response of metabolic genes to stress induction. Thus, CK levels start to significantly alter in comparison with unstressed tissues as late as several hours after stress action, with significant accumulation after plant acclimatization to the adverse conditions. Therefore, restriction of growth rate under abiotic stress does not seem to be caused by the limited amount of active CKs due to accelerated degradation, as was assumed previously. On the other hand, at least in stressed maize seedlings, reduction of growth is linked only to the level of active ABA, which directly regulates stress responses and can have an impact on ethylene production that was shown to have a direct negative effect on shoot growth as well (for review, see Sharp and LeNoble, 2002). Hereafter, accumulation of active CKs among other processes is just the way plants try to overcome stress status and release themselves from growth inhibition. Hence, crop plants with enhanced endogenous levels of active CKs, engineered for instance by knockout of particular *CKX* genes, can be tested in the future as new transgenic cultivars with increased drought and salinity tolerance.

MATERIALS AND METHODS

Plant Material

Maize seeds (*Zea mays* 'Cellux'; Morseva) were imbibed in tap water and germinated in the dark on wetted filter paper. After 2 d, the germinated seedlings were transferred to aerated hydroponic tanks filled with Hoagland nutrient solution. The plants were grown in a growth chamber with a 16-h light period (250 μE m^{-2} s^{-1}) at 27°C and an 8-h dark period at 20°C. During 1 week of growth, the nutrient solution was exchanged two times and finally supplied with 175 mM NaCl and 25 mM $CaCl_2$, 20% PEG 6000, or 10 μM BAP. Concentrations of salt and PEG were set up to be osmotically equivalent to cause a similar decline in leaf water potential (Ueda et al., 2003). PEG lowers the osmotic potential of the external medium and reduces water availability for root tissues, unlike NaCl, which crosses plant membranes and has a direct toxic effect on plant cells. Calcium chloride was added to the NaCl solution to eliminate severe sodium-induced calcium deficiency, which leads to extensive membrane destabilization and appeared to be more limiting to shoot growth than Na toxicity per se (Grieve and Fujiyama, 1987). Stress treatment was set up 3 h after turning on the light. Root and leaf tissues of 10 plants were harvested at 0.5, 1, 3, 8, 24, 72, and 240 h after the stress induction, immediately frozen in liquid nitrogen, and lyophilized for hormone analysis. Control plants were grown in parallel and harvested at the same time points to exclude hormonal variation due to circadian rhythms. The experiment was carried out in three period-independent replicates, and the plant material from each replicate collected at each time point was processed separately with the exception of 0.5-h time point collection, which was run in four replicates. CK content at each time point was statistically evaluated from three biological replicates, where each replicate sample was measured twice. Changes in expression levels were determined from two biological replicates each done at least in four technical replicates with the exception of 0.5-h time point, which was done in four biological replicates.

Tissues for screening *IPT* and *CKX* expression profiles were collected from maize plants grown in a greenhouse during summer.

CKX Activity Assay

Plant material was powdered with liquid nitrogen using a hand mortar and pestle and extracted with a 2-fold excess (v/w) of 0.2 M Tris/HCl buffer, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride and 0.3% Triton X-100. Cell debris was removed by centrifugation at 12,000*g* for 10 min.

The assay was performed according to the method described previously (Frébort et al., 2002). Samples were incubated in a reaction mixture composed of 100 mM McIlvaine buffer, pH 6.0, 0.5 mM electron acceptor 2,3-dimethoxy-5-methyl-1,4-benzoquinone, and 0.25 mM substrate iPR for 2 to 10 h at 37°C. For determination of specific activities, protein contents in the samples were assayed according to Bradford (1976) with bovine serum albumin as a standard.

qPCR Analysis

Total RNA for reverse transcription was isolated using the RNAqueous kit and Plant RNA Isolation Aid solutions (Ambion). To minimize bias in qPCR data, isolated RNA was treated twice by Ambion's TURBO DNase-free kit to remove all traces of genomic DNA contamination. First-strand cDNA was synthesized by RevertAid H Minus Moloney murine leukemia virus reverse transcriptase and oligo(dT) or random hexamer primers (Fermentas). Diluted cDNA samples were used as templates in real-time PCRs containing POWER SYBR Green PCR Master Mix or TaqMan Gene Expression Master Mix (Applied Biosystems), 300 to 900 nM of each primer, and 250 nM specific 5′ 6-carboxyfluorescein- and 3′ nonfluorescent quencher-labeled minor groove binder probe, respectively. Primers for all genes were mostly designed to cover the 3′ end of the ORF to minimize inaccuracy caused by possible partial degradation of mRNA usually starting from the 5′ end. However, some gene family members share high mutual homology, which does not allow this kind of design. Moreover, some primer combinations caused strong unspecific amplification, disturbing correct fluorescence reading. In these cases, amplification with specific TaqMan probes was exploited (i.e. *IPT5*, *IPT6*, *CKX1*, *CKX4*, *CKX5*, and *CKX8*) or primers were designed to the nonhomologous 5′ end sequences (i.e. *CKX2*, *CKX3*, and *RR2*) to avoid cross-reactivity of primer pairs to highly homologous paralogs. The primers for PCR exploiting SYBR Green chemistry were designed using Primer Express 3.0 software (Applied Biosystems). TaqMan probes together with corresponding primers were designed by Applied Biosystems customer service. The primer and probe sequences are listed in Supplemental Table S1. RNA from every biological replicate was at least transcribed in two independent reactions, and each cDNA sample was run in at least two technical replications on the StepOnePlus Real-Time PCR System in a default program (Applied Biosystems). To ensure that primers amplified the desired gene sequence, amplicons for every used primer pair were produced by standard Taq polymerase, cloned into pDRIVE vector, and sequenced by a commercial sequencing service. For each pair of primers, plasmid DNA was used afterward as template to generate a calibration curve for determining the efficiency of PCR. Cycle threshold values were normalized with respect to the 18S small RNA subunit gene (expression during development and in different organs) or the β-actin gene (expression in stressed samples) and the efficiency of amplification.

CK Analysis

The procedure used for CK purification was a modification of the method described by Faiss et al. (1997). Three fractions were obtained by this procedure: the first one contained free bases, ribosides, and *N*9-glucosides; the second fraction was enriched by nucleotides; and the third fraction contained *O*-glucosides. Deuterium-labeled CK internal standards (OlChemIm) were added, each at 1 pmol per sample, to check the recovery during the purification and to validate the determination (Novák et al., 2008). The samples were purified using immunoaffinity chromatography based on wide-range specific monoclonal antibodies against CKs (Novák et al., 2003). The methanolic eluates from immunoaffinity columns were evaporated to dryness, and the obtained solids were dissolved in 20 μL of the mobile phase used for quantitative analysis. The samples were analyzed with an ultraperformance liquid chromatograph (Acquity UPLC; Waters) coupled to a Quatro micro API (Waters) triple quadrupole mass spectrometer equipped with an electrospray interface. The purified samples were injected onto a C18 reverse-phase column (BEH C18, 1.7 μm, 2.1 × 50 mm; Waters). The column was eluted with a linear gradient (0 min, 10% B; 0–8 min, 50% B; flow rate of 0.25 mL min^{-1}; column temperature of 40°C) of 15 mM ammonium formate (pH 4.0; A) and methanol (B). Quantification was achieved by multiple reaction monitoring of $[M+H]^+$ and the appropriate product ion. The quantification was performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous CK to the appropriate labeled standard was determined and further used to quantify the level of endogenous compounds in the original extract, according to the known quantity of the added internal standard.

Analysis of ABA and Its Metabolites

Approximately 50-mg aliquots of plant tissue were homogenized and extracted for 1 h in 750 μL of ice-cold methanol:water:acetic acid (10:89:1, v/v) containing sodium diethyldithiocarbamate (400 μg g^{-1} dry weight) as an antioxidant. Internal standard mixtures, containing 50 pmol each of [$^2H_2$]ABA alcohol, [$^2H_2$]ABA aldehyde, (−)-7′,7′,7′[$^2H_3$]phaseic acid, (−)-7′,7′,7′[$^2H_3$]dihydrophaseic acid, (−)-8′,8′,8′[$^2H_3$]neophaseic acid, (+)-4,5,8′,8′,8′[$^2H_5$]ABAGE, (−)-5,8′,8′,8′[$^2H_4$]7′-OH-ABA, and (+)-3′,5′,5′,7′,7′,7′[$^2H_6$]ABA, were added to the samples. The homogenates were centrifuged (21,000*g*, 10 min, 4°C) after 1 h of extraction, and the pellets were then reextracted in the same way for 30 min. The combined extracts were purified by solid-phase extraction on Oasis HLB cartridges (60 mg, 3 mL; Waters), evaporated to dryness, and finally analyzed by ultraperformance liquid chromatograph-electrospray ionization (−/+)-tandem mass spectrometry.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of all maize putative IPT proteins.

Supplemental Figure S2. Alignment of all maize putative CKX proteins.

Supplemental Figure S3. Phylogenetic tree of all ZmIPT, OsIPT, and AtIPT proteins.

Supplemental Figure S4. Phylogenetic tree of all ZmCKX, OsCKX, and AtCKX proteins.

Supplemental Figure S5. Northern blots of selected genes in tissues exposed to stress and CK.

Supplemental Figure S6. Fluctuation of CK content in maize leaves during exposure to osmotic and salinity stress.

Supplemental Figure S7. Fluctuation of CK content in maize roots during exposure to osmotic and salinity stress.

Supplemental Figure S8. CK content in maize exposed to BAP compared with untreated plants.

Supplemental Table S1. Sequences of primers and TaqMan probes used for qPCR.

Supplemental Table S2. Expression profiles of genes involved in CK metabolism and perception in rice seedlings exposed to drought and salinity stress.

Supplemental Methods S1. Northern-blot analysis.

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CONCLUSION

Genetic transformation of crop plants is an important method, useful not only for analysis of gene function, but also for creating plants with new agronomically important traits. Progress in barley transformation technologies during the last several years led to development of high-throughput transformation methods (e.g. Bartlett *et al*. 2008). Nevertheless, further technical improvements would be necessary for future applications, especially for transformation of commercial cultivars.

This study demonstrates that constitutive expression of maize cytokinin dehydrogenase 1 gene in a monocotyledonous plant (barley) has the same effect on plant phenotype as was described for transgenic dicotyledonous plants with CKX-overexpression (*Arabidopsis thaliana*, tobacco, Werner *et al*. 2001 and 2003). Transgenic plants showed retarded growth of shoots, whereas the root system of these plants was significantly enhanced. This indicates a potential use of transgenic plants with site-specific altered cytokinin content in the agricultural industry in the future. Moreover, silencing of the *HvCKX1* gene in barley resulted in a higher plant yield and root weight (Zalewski *et al*. 2010) which supports the supposition of possible implementation of transgenic plants with manipulated cytokinin content into agriculture in several years.

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Basic course in laboratory work, KBC/LABT (2006/7, 2007/8, 2008/9, 2009/10)

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	Towards functional characterization of a clade of ABC transporters in barley

Posters on conferences

1. Galuszka, P., Šromová, L., **Vyroubalová, Š.,** Jablonská. H., Frébort, I.: Changes of cytokinin oxidase/dehydrogenase levels during senescence in cereals. Second international symposium, Auxins and cytokinins in plant development, Praha (June 7-12, 2005). Poster P1-5, Biologia plantarum, 2005, vol. 49S, p. S5.

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3. Vyroubalová, Š., Nadolska-Orczyk, A., Galuszka, P.: Transformation of plants with genes mediating cytokinin degradation. Future Trends in Phytochemistry: A Young Scientists Symposium, Olomouc, Czech Republic (June 28 - July 1, 2006). Poster by Š. Vyroubalová, Book of abstracts p.93.

4. Galuszka, P., Šmehilová, M., **Vyroubalová, Š.,** Frébort, I.: Role of cytokinin breakdown in plant development and senescence. 11th IAPTC&B Congress, Beijing, China (August 13-18, 2006). Book of abstracts p.89.

5. Vyroubalová, Š., Ohnoutková, L., Galuszka, P.: Transformation of barley (*Hordeum vulgare* L.) with cytokinin dehydrogenase gene. World Congress on In Vitro Biology, Tucson, Arizona, USA (June 14-18, 2008). Poster by Š. Vyroubalová. In vitro Cellular and Developmental Biology, volume 44, p. S68.

6. Vyroubalová, Š., Ohnoutková, L., Galuszka, P.: Transgenic barley (*Hordeum vulgare* L.) with enhanced expression of cytokinin dehydrogenase gene. XXI. Biochemický sjezd, České Budějovice, Czech Republic (September 14-17, 2008). Poster by Š. Vyroubalová. Book of abstract p. 82.

7. Vyroubalová, Š., Galuszka, P.: Barley (*Hordeum vulgare* L.) transformation with constitutive and root-specific promoters controlling cytokinin dehydrogenace expression. ACPD 2009, Praha (June 10-14, 2009). Poster by Š. Vyroubalová. Book of abstracts, p. 62.

8. Vyroubalová, Š., Galuszka, P., Frébort, I.: Genetic transformation of barley (*Hordeum vulgare* L.) with cytokinin dehydrogenase gene under constitutive and root-specific promoters. 21st IUBMB abd 12th FAOBMB international congress of biochemistry and molecular biology, Shanghai, China (August 2-7, 2009). Poster by Š. Vyroubalová. Abstracts, p. 197.

Talk on conference

1. Vyroubalová, Š., Ohnoutková, L., Galuszka, P. Transformation of plants with genes mediating cytokinin degradation. XI. Setkání biochemiků a molekulárních biologů, Brno, January 31 – February 1, 2007. Book of abstracts, p. 42-43. Lecture by Š. Vyroubalová.

2. Galuszka, P., **Vyroubalová, Š**., Václavíková, K., Turečková, V., Novák, O.: Metabolism and possible function of cytokinin during abiotic stress in maize. ACPD 2009, Praha, June 10-14, 2009. Book of abstracts, p. 95. Lecture by P. Galuszka.

3. Vyroubalová, Š., Galuszka, P., Frébort, I.: Transgenic barley with altered cytokinin content may have higher tolerance to abiotic stress. 3rd International SMBBM congress of biochemistry and molecular biology, Marrakesh, Marocco, April 20-24, 2009. Abstracts, p. 107. Lecture by I. Frébort.

Publications

Vyroubalová, Š., Ohnoutková, L., Galuszka, P.: Transformation of barley (*Hordeum vulgare* L.) with cytokinin dehydrogenase gene. In vitro Cellular and Developmental Biology-Animal 44, Supplement 1: S68, 2008. Abstract - World Congress on In Vitro Biology, IF – 0.576

Vyroubalová, Š., Václavíková, K., Turečková, V., Novák, O., Šmehilová, M., Hluska, T., Ohnoutková, L., Frébort, I., Galuszka, P. Characterization of new maize genes putatively involved in cytokinin metabolism and their expression during osmotic stress in relation to cytokinin levels. Plant Physiology 151: 433-447, 2009. IF - 6.11

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Vyroubalová, Š., Galuszka P.: Transgenic barley (*Hordeum vulgare* L.) with altered cytokinin content (manuscript)