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Cytokinin oxidase/dehydrogenase in *Pisum sativum* plants during vegetative development. Influence of UV-B irradiation and high temperature on enzymatic activity

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Key words: Cytokinin oxidase/dehydrogenase, Development, High temperature, Senescence, Stress, UV-B irradiation

Abstract

Cytokinin oxidase/dehydrogenase (EC 1.5.99.12) specific activity was determined in leaves and roots of two *P. sativum* cultivars (cv. Scinado and cv. Manuela) during vegetative development and the effect of UV-B irradiation or elevated temperature was assessed. The measurement of CKX activity during development showed localisation of this enzyme to roots. The reduction in CKX activity in leaves after UV-B irradiation and the increased levels of the enzyme in high temperature-treated plants suggests that the enzymes from the CKX gene family have a different expression during stress responses provoked by different factors and probably are tissue specific. Differences regarding cytokinin oxidase/dehydrogenase activity stress response were observed between the two pea cultivars.

Abbreviations: CKX – cytokinin oxidase/dehydrogenase

Introduction

Plant tissues exhibit high but transient levels of cytokinins during specific periods of development (Jones and Schreiber 1997). In previous studies it was shown that cytokinin levels and cytokinin oxidase/dehydrogenase activity are closely related (Jäger et al. 1997; Auer et al. 1999).

Rapid changes in the hormonal levels usually correspond to initiation of major developmental processes in plants like organ formation, while hormonal homeostasis is required for further development of the initiated events (organ growth) (Kaminek et al. 1997).

Treatment with cytokinins can delay leaf senescence and it has been suggested that a reduction in the cytokinin concentration below a threshold level could serve as a signal to trigger leaf senescence (Gan and Amasino 1995). In recently performed experiments with transgenic tobacco plants overexpressing *Arabidopsis thaliana ckx* genes, visual leaf senescence did not occur early. Older leaves remained green (with the exception of intercostal regions) leading to a prolonged lifespan. This phenotype suggested that cytokinins were not an efficient physiological signal that triggered the onset of senescence (Schmülling 2002). It has been suggested that cytokinins are important regulatory factors of plant meristem activity and morphogenesis with opposing roles in shoots and roots (Werner et al. 2001).

In our study we investigated the changes in CKX activity after UV-B irradiation and high

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Effect of temperature stress on the endogenous cytokinin content in *Arabidopsis thaliana* (L.) Heynh plants

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Key words: *Arabidopsis thaliana* (L.) Heynh, cytokinins, ELISA, ethylene-insensitive mutant *(eti5),* temperature stress

Abstract

The levels of three endogenous cytokinin equivalents: zeatin (Z), *iso*-pentenyladenine (iP) and dihydrozeatin (dZ) in two *Arabidopsis thaliana* (L.) Heynh genotypes – wild type (wt) and ethylene-insensitive mutant (*eti5*), were compared using enzyme immunoassay (ELISA). Cytokinin content was measured after exposure to low (4 °C for 24 h in darkness) or high temperature (38 °C for 24 h in darkness). Measurements were performed immediately and 24, 48 and 120 h after treatments. It was found that at normal growth conditions *eti5* plants contained more endogenous cytokinins compared to the wild type. At both temperature treatments mutant plants had decreased total cytokinin levels. Wild-type plants treated with high temperature (HT) exhibited reduced total cytokinins (with the exception of rates at 48 h), while low temperature (LT) treatment resulted in elevated total amount of the studied equivalents (except at 24 h). The obtained results suggested that HT had greater effect on cytokinin levels than LT since it caused more profound changes in the total content. We assume that this was due to the natural chilling tolerance of *Arabidopsis* plants.

List of abbreviations: dZ – dihydrozeatn, dZR – dihydrozeatin riboside, ELISA – enzyme linked immunosorbent assay, HT – high temperatute, iP – *iso*-pentenyladenine, iPA – *iso*-pentenyladenosine, LT – low temperature, SE – standard error, wt – wild type, Z – zeatin, ZR – zeatin riboside

Introduction

Senescence as programmed ageing process leads to plant death (Dangl *et al.* 2000). According to Nooden *et al.* (1997) leaf senescence resembles processes occurring at oxidative stress. Levels of reactive oxygen species increased during senescence likewise after environmental stress (Merzlyak and Hendry 1994). The plant responses to different environmental stresses are specifically mediated by plant hormones. Cytokinins play an important role in several aspects of plant growth, metabolism and development at normal growth conditions. The mechanisms by which environmental changes affect cytokinins are still not clear but their adaptive function is undoubted. It is suggested that cytokinins

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\Cytokinin oxidase/dehydrogenase (CKX) activity in wild and ethylene-insensitive mutant *eti5* type of *Arabidopsis thaliana* (L.) Heynh plants and the effect of cytokinin N^1-(2-chloro-4-pyridyl)-N^2-phenylurea on enzymatic activity and leaf morphology

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Key words: *Arabidopsis thaliana* (L.) Heynh, cytokinin oxidase/dehydrogenase, cytokinin 4PU-30, ethylene-insensitive (*eti5*) mutant.

Abstract

The specific activity of cytokinin oxidase/dehydrogenase (EC 1.5.99.12) (CKX) was determined in leaves of wild type (*wt*) and ethylene-insensitive mutant (*eti5*) of *Arabidopsis thaliana* (L.) Heynh plants. Comparative studies showed that this mutation has lower basal CKX activity than *wt*. Application of 4PU-30 (N^1-(2-chloro-4-pyridyl)-N^2-phenylurea) resulted in decreased CKX activity in both *wt* and mutant plants. The treatment increased leaf blade thickness and the volume of chlorophyll-containing cells per unit leaf area in *wt* but these changes were not observed in the *eti5* mutant. The reduction in chlorophyll "a" and "b", as well as in carotenoids content in the treated *wt* tissues resulting from altered leaf morphology was not detected in *eti5* plants.

List of abbreviations: 4PU-30 - N^1-(2-chloro-4-pyridyl)-N^2-phenylurea, CKX - cytokinin oxidase/dehydrogenase; iP – *iso*-pentenyladenine; SE - Standard error; FW - fresh weight; dZR - dihydrozeatin riboside, iPA – *iso*-pentenyladenosine, *wt*- wild type, ZR - zeatin riboside; dZ – dihydrozeatin; Z – zeatin.

Introduction

Transgenic and hormone mutant plants are convenient model systems for the investigation of various physiological processes. Earlier, it has been established that the ethylene-insensitive mutant *eti5* of *Arabidopsis* has characteristics of delayed senescence accompanied by enhanced ethylene biosynthesis (Harpham *et al.* 1991), a higher amount of leaf pigments and soluble proteins (Sergiev *et al.* 2003), as well as elevated endogenous polyamines (Todorova *et al.* 2002) and cytokinins (Kudryakova *et al.* 2001, Todorova *et al.* 2005) than the wild type. *Eti5* mutation is a very curious case of co-existence of two factors, which counteract each other in relation to senescence – firstly higher ethylene levels promoting it and secondly higher cytokinin content that delays the process. The abnormally elevated cytokinin levels in *eti5*

TWO PEA VARIETIES DIFFER IN CYTOKININ OXIDASE/ DEHYDROGENASE RESPONSE TO UV-B IRRADIATION

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Summary. Cytokinin oxidase/ dehydrogenase (CKX EC: 1.5.99.12) and cytokinin changes after UV-B irradiation (302 nm) in two pea varieties with different vegetation periods have been studied. Treatment caused total inhibition of CKX and reduced endogenous cytokinins in the slower-growing "Manuela", while it induced the enzymatic activity and positively influenced hormonal content in leaves of the faster-growing cultivar – "Scinado". Results suggest presence of diverse *ckx* alleles in the genomes of both varieties, which are characterized with different basal endogenous cytokinin concentration.

Key words: cytokinins, cytokinin oxidase/ dehydrogenase, *Pisum sativum*, UV-B.

Abbreviations: BSA – bovine serum albumin, *cis*Z – *cis* zeatin, *cis*ZR – *cis* zeatin riboside, *cis*ZRP – *cis* zeatin riboside monophosphate, CK – cytokinins, CKX – cytokinin oxidase/ dehydrogenase, HPLC – high performance liquid chromatography, iP – isopentenyl adenine, iPR – isopentenyl adenine riboside, iPRP – isopentenyl adenine riboside monophosphate, PMSF – phenylmethylsulfonyl fluoride.

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ORIGINAL PAPER

Response of cytokinin pool and cytokinin oxidase/dehydrogenase activity to abscisic acid exhibits organ specificity in peas

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Jiří Malbeck · Alena Trávníčková ·
Ivana Macháčková**

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Abstract Changes in cytokinin pool and cytokinin oxidase/dehydrogenase activity (CKX EC: 1.5.99.12) in response to increasing abscisic acid (ABA) concentrations (0.5–10 μM) were assessed in the last fully expanded leaves and secondary roots of two pea (*Pisum sativum*) varieties with different vegetation periods. Certain organ diversity in CKX response to exogenous ABA was observed. Treatment provoked altered cytokinin pool in the aboveground parts of both studied cultivars. Specific CKX activity was influenced significantly basically in roots of the treated plants. Results suggest that ABA-mediated cytokinin pool changes are leaf-specific and involve certain root signals in which CKX activity presents an important link. This enzymatic activity most probably regulates vascular transport of active cytokinins from roots to shoots.

Keywords Abscisic acid · Cytokinins · Cytokinin oxidase/dehydrogenase · Organ-specific response · *Pisum sativum*

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Introduction

Abscisic acid (ABA) mediates environmental stress signals resulting from drought, high salinity, and low temperature, which is usually manifested at the physiological level by controlling germination, stomatal movements, and growth (Hoth et al. 2002). Balance between abscisic acid and cytokinins, was found to be one of the basic factors which control the stomatal aperture effectively (Das et al. 1976).

Significant cross talk occurs between various plant hormone signal transduction cascades initiating also the process of ageing. Ethylene and ABA normally induce the early onset of senescence. Generally endogenous levels of cytokinins (together with those of auxin and gibberellins) decline before and with the appearance of senescence (Van Staden et al. 1988).

Antagonistic relationship between cytokinin and ABA was demonstrated in an earlier study where application of abscisic acid inhibited the cytokinin-stimulated formation of buds in the moss *Funaria hygrometrica* (Valadon and Mummery 1971). The induction of shoot buds in the moss *Funaria hygrometrica* is a classic quantitative bioassay for cytokinins. Later Christianson (2000) localized the inhibitory action of ABA to a particular time in the process of bud formation. Experiments transferring protonema between cytokinin and cytokinin plus ABA show that ABA does not interfere with the initial perception of cytokinin. ABA acted later by blocking the cytokinin-mediated stable commitment of nascent buds—the so-called second cytokinin-mediated event in the process of bud formation. This event was also responsible for the concentration-dependent regulation of the overall number of buds that will be produced from individual protonemata (Christianson 1998). Obtained results revealed that initial perception of cytokinin and the second cytokinin-mediated

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Photosynthetic Responses of Nitrate-Fed and Nitrogen-Fixing Soybeans to Progressive Water Stress

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ABSTRACT

The effect of nitrogen (N) source (nitrogen fixation or nitrate assimilation) and progressive water stress on pigment content, carbon assimilation and changes in the activity of certain photosynthetic (Rubisco and phosphoenol pyruvate carboxilase) and photorespiratory enzymes (glutamate synthetase and glycolate oxidase) during vegetative development of soybean plants was studied. Glycolate oxidase declined by 13% in nitrogen-fixing plants under water deficit, and increased in nitrate-fed ones. Nodulated plants were less sensitive to drought than nitrate-fed individuals; although as general growth was inhibited under drought stress in both experimental models. Results support the importance of nitrogen source in soybean responses to water stress. Difference in sensitivity of nitrate-fed and nitrogen-fixing plants towards water stress seems to be related not to nitrogen assimilation process itself, but to complex interactions with photorespiratory flux and stomatal conductance.

Keywords: carbon assimilation, different nitrogen sources, *Glycine max* L., nitrate assimilation, nitrogen fixation, nitrogen nutrition, water stress

INTRODUCTION

Factors which determine activity of photosynthesis are diversely influenced under water stress (Lawlor and Cornic, 2002). Hsiao (1973) described the different effects that water deficit produced on plants at physiological, biochemical and molecular levels, with photosynthetic rate reduction being one of the most

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DROUGHT-INDUCED LEAF PROTEIN ALTERATIONS IN SENSITIVE AND TOLERANT WHEAT VARIETIES

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Summary. Wheat plants with a fully developed first leaf were subjected to severe but recoverable water stress. Leaves from drought tolerant (Katya and Zlatitza) and drought sensitive (Sadovo and Miziya) varieties in control, drought and recovery conditions were used for the experiments. The physiological response of drought tolerant varieties did not differ from the one of drought sensitive varieties at early seedling stage under these conditions. A comparative study of the ultrathin sections by transmission electron microscopy from control and drought stressed plants revealed prominent changes in mitochondrion fine structures. The relative cell area occupied by mitochondria was reduced in the drought sensitive varieties. An increased quantity of abscisic acid (ABA) was detected in drought stressed wheat plants. The drought sensitive varieties (Miziya and Sadovo) possessed higher azocaseinolytic activity. An immunoblotting analysis was performed for some specificity detection of protein response under drought conditions of Rubisco, Rubisco activase (RA), Rubisco binding protein (RBP), dehydrins (DHN), some heat shock proteins (HSP) and ATP dependent calpain protease (Clp) proteins. The obtained results

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MILD TEMPERATURE STRESS MODULATES CYTOKININ CONTENT AND CYTOKININ OXIDASE/DEHYDROGENASE ACTIVITY IN YOUNG PEA PLANTS

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Cytokinin oxidase/dehydrogenase (CKX: EC 1.5.99.12) is able to provide a means for the rapid turnover of its substrate and it has been considered responsible for changes in the cytokinin pool in an adverse environment. Mild temperature stresses (10°C and 33°C average) were applied to young pea plants of two varieties (cvs. Manuela and Scinado) in order to assess the response of the cytokinin pool and CKX activity to altered growth conditions. Both temperature treatments increased the isopentenyl adenine (iP) and isopentenyl adenine riboside (iPR) contents in stressed plants. This trend was far more pronounced in the leaves. Low temperature additionally resulted in elevated *cis* zeatin riboside (*cis*ZR) and CKX activity. Heat did not influence the enzymatic activity in the leaves, while opposing trends were observed in the root-derived CKX activity of the two tested varieties. The data suggest that variance in the temperature provokes adaptive reactions in the cytokinin pool, which is maintained by CKX activity.

Key words: cytokinins, cytokinin oxidase/dehydrogenase, pea, temperature stress

Abbreviations: *cis*Z – *cis* zeatin, *cis*ZR – *cis* zeatin riboside, *cis*ZRP – *cis* zeatin riboside monophosphate, CK – cytokinins, CKX – cytokinin oxidase/dehydrogenase, iP – isopentenyl adenine, iPR – isopentenyl adenine riboside, iPRP – isopentenyl adenine riboside monophosphate.

Introduction

Hormonal changes are controlled by numerous signals and enzymatic pathways which govern plant acclimation to environmental stress (Mok and Mok, 2001). CK titres are usually modified by unfavourable growth conditions such as drought, water deprivation, excess salinity, changes in nutrient solutions, pathogen infection and wounding (Hare et al., 1997), high metal concentration (Atanasova et al., 2004) and herbicide treatment (Atanasova et al., 2005). Cytokinin changes, together with alterations in other endogenous plant growth regulators, may initiate physiological mechanisms involved in various protective plant stress responses.

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RESEARCH PAPER

Abscisic acid and late embryogenesis abundant protein profile changes in winter wheat under progressive drought stress

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Keywords
Dehydrins; early vegetative growth; immunoblotting; one-step RT-PCR; SDS-PAGE; TaLEA; WZY2.

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ABSTRACT

Three varieties (cv. Pobeda, Katya and Sadovo) of winter wheat (*Triticum aestivum*), differing in their agronomic characteristics, were analysed during progressive soil water stress and recovery at early vegetation stages. Changes in abscisic acid content, SDS–PAGE and immunoblot profiles of proteins that remained soluble upon heating were monitored. Initially higher ABA content in control Pobeda and Katya corresponded to earlier expression of the studied late embryogenesis abundant (LEA) proteins. A combination of higher ABA content, early immunodetection of dehydrins, and a significant increase of *WZY2* transcript levels were observed in drought-stressed leaves of the tolerant variety Katya. One-step RT-PCR analyses of some acidic dehydrin genes (*WCOR410b*, *TADHN*) documented their relatively constant high expression levels in leaves under drought stress during early vegetative development. Neutral *WZY2* dehydrin, *TaLEA2* and *TaLEA3* transcripts accumulated gradually with increasing water deficit. Delayed expression of *TaLEA2* and *TaLEA3* genes was found in the least drought-tolerant wheat, Sadovo. The expression profile of *WZY2* revealed two distinct and separate bands, suggesting alternative splicing, which altered as water stress increased.

INTRODUCTION

Rainfall deficit during the growing season of spring crops (from sowing to full maturity) is a common phenomenon in Southeast Europe (Tran *et al.* 2002; Koleva & Alexandrov 2008). Water limiting conditions during both vegetative and reproductive stages are particularly damaging to wheat crops, and identification of markers for precision breeding of drought-tolerant wheat varieties is very important. Being a complex multigene process, drought tolerance has some common, universal elements.

Abscisic acid plays an important regulatory role in plant development during water deprivation. It is considered a potentially useful trait in selecting for drought tolerance in crops (Mugo *et al.* 2000). Plant physiological responses to ABA are, in large part, brought about by changes in gene expression and post-translational modifications (Leung & Giraudat 1998; Schroeder *et al.* 2001). This phytohormone stimulates osmotic adjustment and induces synthesis of protective proteins, including some late embryogenesis abundant (LEA) proteins. LEA proteins generally participate in processes related to desiccation tolerance of plant seeds during their late stages of development, but also accumulate in vegetative tissues during dehydration stress (Bray 1993; Close 1997). They are characterised by a high glycine content, high hydrophilicity and low secondary structure in purified form (Garay-Arroyo *et al.* 2000), and have been divided into five groups: LEA D19 (group I), LEA D11 (group II, also termed dehydrins), LEA D7 (group III), LEA D113 (group IV) and LEA D95 (group V) (Ingram & Bartels 1996).

Accumulation of dehydrins (DHNs) in plants is a common response to drought. They are unified by the presence of one or more copies of a highly conserved amphipathic α-helix-forming domain (the K-segment) of a 15-residue consensus sequence, EKKGIMDKIKEKLPG, near the C or N terminal of the molecule (Close 1997). Many dehydrins contain a serine-rich S-segment, which might be phosphorylated, producing important post-translational modifications, responsible for correct protein location under stress. According to the arrangement of their different domains and clustering analysis, DHNs can be divided into five subtypes: Y_nSK_2, K_n, K_nS, SK_n and Y_2K_n. Immunolocalisation and sub-cellular fractionation studies have established that DHNs are present in the nucleus or cytoplasm (Close 1997; Egerton-Warburton *et al.* 1997; Danyluk *et al.* 1998). *DHN* loci are multigenic and present in clusters on different chromosomes (Campbell & Close 1997). There are 13 *DHN* genes in barley (Rodriguez *et al.* 2005), which implies that in hexaploid wheat there should be more than three times this number (Tommasini et al. 2008). The dispersed nature of the *DHN* family and the inherent cross-hybridisation of genes both require the use of gene-specific tools, such as RT-PCR for expression analysis (Tommasini *et al.* 2008). Close *et al.* (2000) found that dehydrin expression in Triticeae and maize varies. These authors discovered that some genes coding for mainly low-molecular weight and some alkaline Y_nSK_2-type DHNs are induced only

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Short communication

Proteolytic activity and cysteine protease expression in wheat leaves under severe soil drought and recovery

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ABSTRACT

The involvement of acidic proteases in soil drought response of winter wheat (*Triticum aestivum* L.) at seedling stage in three cultivars differing in water stress tolerance was studied. Withholding irrigation for seven days resulted in severe drought stress corresponding to 60% leaf water deficit. Stressed plants were recovered by providing optimal water supply for 3 days. Reversible changes in leaf pigment and protein content were registered, being least expressed in the drought-resistant cultivar Katya. Protein loss was inversely related to the increase in total proteolytic activity at pH 5 and in aminopeptidase activity at pH 7. Quantitative differences among the cultivars were established only for azocaseinolytic activity (pH 5). The drought-resistant cultivar (Katya) showed relatively little increase in acid protease activity whereas the highest values of this activity were detected in cultivar Pobeda. In-gel staining for cysteine-activated proteases revealed four to five separate activity bands. The upper band, specifically inhibited by E-64, was raised at severe drought. Transcript abundance of two wheat cysteine proteases – *Ta.61026* putative thiol protease, and *WCP2* peptidase of papain type was analyzed by RT-PCR. Gene expression of the cysteine proteases under study was suppressed in the drought-tolerant cultivar, while in the less resistant ones it remained unchanged or augmented. The results suggest that lower proteolytic activity and decreased expression of certain cysteine protease genes under water deficit during early developmental stage could be regarded as an indicator for drought resistance of winter wheat cultivars.

1. Introduction

Drought stress is one of the most serious environmental factors that can severely limit plant growth, performance and productivity of agricultural crops and the predicted long-term effects of global warming include more frequent drought episodes in the future [6]. Development of adequate agricultural strategies to counteract this effect and minimize drought-related yield loss could be orientated to marker-assisted selection of crop cultivars with improved drought resistance [11]. In this respect, identification of suitable molecular markers for drought tolerance seems to be of particular importance.

Plants respond to water stress generally by synthesis of ABA, inhibition of photosynthesis and respiration, accumulation of osmotically active compounds, synthesis of protective proteins such as dehydrins and chaperones, adjusting sink/source allocation and senescence acceleration [6]. Suppression of drought-induced leaf senescence in transgenic tobacco plants, which accumulate cytokinins under stress, has been linked to enhanced expression of dehydrins and heat shock proteins and to increased drought tolerance [24]. Enhanced expression of genes coding for proteases is a common event both in senescence and under various environmental stresses [19] which is necessary for reorganization of plant metabolism, remodeling of cell protein components, degradation of damaged or unnecessary proteins and nutrient remobilization [9,12]. However, some experimental evidence suggests that proteases involved in drought response differ from those expressed specifically in senescence [3,16].

Genome studies revealed a striking diversity of protease genes which reflects the diversity in their specific functions of regulating the fate of many different proteins [17]. Proteases in the MEROPS database (http://merops.sanger.ac.uk) have been subdivided into families and clans on the basis of structural and evolutionary relationships [22]. For example, the *Arabidopsis* genome contains over 800 proteases, which are distributed in 60 families belonging to 30 different clans [33]. The pattern of expression of these

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Abbreviations: DCI, 3,4-dichloroisocoumarin; E-64, L-transepoxysuccinyl-leucylamido-[4-guanidino]butane; FW, fresh weight; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; RT-PCR, reverse transcription polymerase chain reaction; TW, turgid weight; WD, water deficit.

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Combined drought and heat stress in wheat: changes in some heat shock proteins

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Abstract

The influence of combined and individually applied drought and heat stress was studied in two wheat (*Triticum aestivum* L.) cultivars: resistant cv. Katya and susceptible cv. Sadovo. Relative water content decreased and electrolyte leakage increased due to individual and combined application of both stresses. Initial heat shock protein profile has been outlined *via* SDS electrophoresis of leaf extracts. The results obtained were confirmed by immunoblotting with anti-HSP70 monoclonal antibodies, anti-HSP110 polyclonal antibodies and anti-αβ-crystalline polyclonal antibodies. The effect of simultaneously applied water stress and heat shock resembled the alterations in protein expression provoked only by water stress and differed significantly from the changes occurring after the individual application of heat stress.

Additional key words: electrolyte leakage, immunoblotting, relative water content, *Triticum aestivum*.

Introduction

Plants in the field are frequently subjected to abiotic stresses that affect adversely their growth, development and productivity (Chaves *et al.* 2004, Kotak *et al.* 2007). They developed different mechanisms to respond to external conditions (Vierling 1991, Waters *et al.* 1996, Schoffi *et al.* 1998, Lee *et al.* 2000, Smykal *et al.* 2000, Ferguson 2004, Wang *et al.* 2004). Drought and heat stress are among the factors causing the most severe damage (Wang *et al.* 2004, Sumesh *et al.* 2008, Santos *et al.* 2009). Each of these stresses has been extensively studied but little is known about their combined impact on wheat plants (Kregel 2002, Rizhsky *et al.* 2002, 2004, Mittler 2006). The combination of high temperature and water deficit is quite common in dry and semi-dry regions across the world and claims extensive agricultural losses (Mittler *et al.* 2001, Rizhsky *et al.* 2002, Moffat 2002, Kotak *et al.* 2007, Sumesh *et al.* 2008).

Similar responses to combined drought and heat stress have been described in *Arabidopsis* and tobacco (Rizhsky *et al.* 2002, 2004). It was found out that a combination of drought and heat stress provokes cessation of conventional protein synthesis, accompanied by increased translation of heat shock proteins (HSPs) and other stress related proteins (Vierling 1991, Schoffi *et al.* 1998, Mittler 2006, Caeiro *et al.* 2008, Lin *et al.* 2008).

HSPs were first discovered in 1962 and described as a set of proteins with expression induced by heat shock and a variety of other stresses (Ritossa 1962, Wang *et al.* 2004). They have been described as highly conserved polypeptides which play an important role for survival under both normal and extreme conditions (Vierling 1991, Schoffi *et al.* 1998, Kregel *et al.* 2002, Kotak *et al.* 2007). HSP production is an essential component of thermotolerance (Vierling 1991, Schoffi *et al.* 1998,

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Abbreviations: D - drought stress; DH - combined drought and heat stress; EDTA - ethylendiaminetetracetic acid; H - heat stress; HSP - heat shock protein; PMSF - phenylmethanesulfonyl fluoride; RLS and RSS - Rubisco large and small subunits, respectively; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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SHORT COMMUNICATION

Expression of selected heat shock proteins after individually applied and combined drought and heat stress

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Klimentina Demirevska · Urs Feller

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Abstract Drought and heat stress are among the abiotic factors causing the most severe damage on plant crops. Their combination is quite common in dry and semi-dry regions worldwide and little is known about its effect on heat shock protein (HSP) profile in wheat plants. The expression of four HSP genes (*Hsp* 17.8, *Hsp* 26.3, *Hsp* 70 and *Hsp* 101b) in *Triticum aestivum* L. plants subjected to individually applied water deprivation or high temperature and their combination was monitored via one-step RT-PCR analysis. Changes in the expression levels of small HSPs (smHSPs), HSP70 and HSP100 were established also by SDS-PAGE. The combination of drought and heat induced HSP expression more effectively than the individually applied stresses. The induction of HSPs displayed greater rate in the drought-tolerant wheat variety Katya than in the drought-sensitive cv. Sadovo. The results obtained in wheat plants suggested that the effect of separately applied drought and heat shock cannot be extrapolated to their combination.

Keywords Heat shock proteins · Drought · Heat stress · Combined drought and heat stress · Wheat · One step RT-PCR analysis

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Abbreviations

D	Drought
DH	Combined drought and heat stress
EDTA	Ethylendiaminetetracetic acid
H	Heat stress
HSP	Heat shock protein
PMSF	Phenylmethanesulfonyl fluorid
RLS and RSS	Respectively Rubisco large and small subunits
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Abiotic stress is the primary reason for farm production loss worldwide, occasioning average yield reductions of more than 50% for major crops (Larkindale and Knight 2002; Valliyodan and Nguyen 2006; Tayyar 2010), among which is bread wheat (*Triticum aestivum* L.). In nature, individual stresses, such as drought and high temperatures, usually do not occur separately (Howarth and Ougham 1993). Combined effect of water deficit and heat shock on cereals has not been studied in details and published data show that it differs from the sum of the separately conducted ones, and cannot be extrapolated and integrated between them (Mittler 2006; Sreenivasulu et al. 2007). Previous studies report that plant response to combined drought and heat (DH) stress differed from the reaction to other stresses such as pathogen attack, cold or salt (Hasegawa et al. 2000; Rizhsky et al. 2002; Mittler 2006; Jäger et al. 2008).

Plants react to stress (such as low and high temperatures, drought, salinity, flooding and cold) in a similar manner by production of HSPs—part of the cell-specific protection

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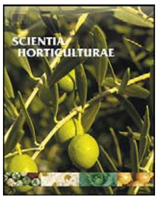

Drought stress tolerance of red and white clover–comparative analysis of some chaperonins and dehydrins

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ABSTRACT

The ecophysiological function linked to the contribution of the legume to the nitrogen supply of the sward, as well as its high content in pastures, make red (*Trifolim pratense*) and white clover (*Trifolium repens*) forage plants of economic importance in temperate regions. The study aimed to characterize the expression profiles of some important stress tolerance related proteins in red clover (cv. "Start"), and two white clover cultivars – cv. "Haifa" (medium-leafed) and cv. "Debut" (small-leafed). An attempt has been made to explain the differences in red and white clover potential to sustain unfavorable environmental conditions regarding the obtained results from protein and gene profiling. Rubisco binding protein (RBP), low molecular weight heat shock proteins (HSP), calpains (ClpA and ClpP) and dehydrins (DHN) responded significantly to drought. White clover cv. "Debut" developed the lowest leaf water deficit (WD) compared to the one detected in red clover and the medium leafed white clover cultivar "Haifa". Immunoblot and RT-PCR showed that the small leafed white clover cv. "Debut" copes with drought stress most efficiently. Detailed gene expression analysis revealed that Y_2SK type dehydrins were subjected to alternative splicing under drought and the transcripts were strongly induced in the more drought tolerant small-leafed white clover cultivar "Debut". A homolog sequence of Y_2K dehydrin in *T. pratense* (GenBank ID: JF748409) and *T. repens* (GenBank ID: JF748410) have been amplified and an increased transcipt levels of JF748409 were detected in *T. pratense* drought stressed leaves. The expression pattern of SK_2 transcripts showed that this dehydrin type has been down-regulated in both white clover cultivars under water deprivation.

1. Introduction

Red and white clovers are forage legumes with excellent feed quality. Red clover (*Trifolium pratense*), a tall growing and short lived perennial, is used for hay, pasture, silage, green chop, soil improvement, and wildlife habitats. On the other hand white clover (*Trifolium repens*), a low growing perennial, is best suited for grazing, it can also be used for soil improvement and reclaiming disturbed land. White clover is easy to establish and is more drought-tolerant than red clover though it has lower yield. There are different types of white clover: small-leafed, medium-leafed (intermediate), and large-leafed, such as ladino. The small-leafed types are lower yielding, but more persistent than the intermediate and ladino types (Carlson et al., 1985). These forage legumes work well as a cover crop in crop rotations and are a popular choice in organic rotations because clovers fix nitrogen, improve forage quality and yield, extend the grazing season and improve animal performance. When renovating, clover is sown in the spring into already established pasture grasses that have either been mowed or grazed very short. Clovers may also be sown in late summer; however, problems with drought can occur in these later plantings since water deficit affects grass growth and development. Better understanding of physiological mechanisms of stress tolerance would benefit clover breeding and management programs. The interspecific differences in drought responses between species have been studied relatively little (Yin et al., 2005).

Plants have developed various strategies to survive water-deficit and to improve their drought tolerance (Xoconostle-Cázares et al., 2011). Defense mechanisms in genotypes with tolerance to drought are due to several physiological and biochemical pro-

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Abbreviations: Clp, calpain; cv., cultivar; DHN, dehydrin; EDTA, ethylene-diamine-tetra-acetic acid disodium salt; FW, fresh weight; HSP, heat shock protein; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; RA, rubisco activase; RBP, rubisco binding protein; RLS, rubisco large subunit; RLS-C, C terminus of rubisco large subunit; RLS-N, N terminus of rubisco large subunit; RSS, rubisco small subunit; RT-PCR, reverse transcription polymerase chain reaction; TW, turgid weight; WD, water deficit.

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Chapter 1

THE RESPONSE OF PLANTS TO DROUGHT STRESS: THE ROLE OF DEHYDRINS, CHAPERONES, PROTEASES AND PROTEASE INHIBITORS IN MAINTAINING CELLULAR PROTEIN FUNCTION

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ABSTRACT

Abiotic stresses with a dehydration component (drought, salt, and freezing) involve, as a common feature, increased numbers of inactive proteins – denatured, aggregated or oxidatively damaged. Maintaining proteins in their functional conformation, preventing aggregation of non-native proteins, refolding of denatured proteins to their native conformation and removal of non-functional and potentially harmful polypeptides are all vital for cell survival under dehydration stress. To achieve this, plants respond to drought by synthesis of protective proteins such as dehydrins and chaperones and by degradation of irreversibly damaged proteins by proteases. Here we review the important cellular functions of dehydrins, chaperones, proteases and protease inhibitors, together with their role in the response to drought, that make them potential biochemical markers for assessing drought tolerance.

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New Phytologist

Review

Tansley review

Ethylene in vegetative development: a tale with a riddle

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Contents

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Key words: ACC, Arabidopsis, auxin, cell-type specificity, cell wall remodeling, development, ethylene.

Summary

The vegetative development of plants is strongly dependent on the action of phytohormones. For over a century, the effects of ethylene on plants have been studied, illustrating the profound impact of this gaseous hormone on plant growth, development and stress responses. Ethylene signaling is under tight self-control at various levels. Feedback regulation occurs on both biosynthesis and signaling. For its role in developmental processes, ethylene has a close and reciprocal relation with auxin, another major determinant of plant architecture. Here, we discuss, in view of novel findings mainly in the reference plant Arabidopsis, how ethylene is distributed and perceived throughout the plant at the organ, tissue and cellular levels, and reflect on how plants benefit from the complex interaction of ethylene and auxin, determining their shape. Furthermore, we elaborate on the implications of recent discoveries on the control of ethylene signaling.

I. Introduction

Ethylene is a gaseous plant hormone that affects multiple aspects of plant development, although it is best known as the ripening hormone (Abeles *et al.*, 1992). Physiological effects are detectable at ambient levels as low as $0.1\ \mu l\ l^{-1}$. The first discovery of ethylene effects dates back over a century (Neljubow, 1901). Neljubow demonstrated that ethylene causes horizontal growth of pea seedlings, inhibition of elongation and radial swelling (‘triple response’). Conclusive evidence that ethylene is produced by plants was presented by Gane in 1934. Although playing a primary role in ripening, abscission and senescence, many aspects of vegetative growth are also influenced by ethylene (Smalle & Van Der Straeten, 1997). First, it has a positive effect on the germination of several plant species. Furthermore, it influences growth in both darkness and light. In ethylene-treated etiolated Arabidopsis seedlings, the ‘triple response’ is seen as an inhibition of elongation and radial swelling of the hypocotyl, and an exaggerated apical hook. In general, primary and lateral root elongation is inhibited, whereas root hair development is

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RESEARCH ARTICLE

Drought, high temperature, and their combination affect ultrastructure of chloroplasts and mitochondria in wheat (*Triticum aestivum* L.) leaves

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Plants experience a number of limiting factors, as drought and heat, which are often coinciding stress factors in natural environment. This study evaluated the changes in mesophyll cell ultrastructure in the leaves of two varieties of winter wheat (*Triticum aestivum* L.), differing in their drought tolerance, under individual or combined drought and heat treatment. Although the individual stress factors affected leaf ultrastructure, the damaging effect of the combined drought and heat was more pronounced and manifested certain differences between genotypes. Chloroplasts and mitochondria were affected in a variety-specific manner under all adverse treatments. The organelles of the drought-tolerant Katya were better preserved than those in the sensitive variety Sadovo. Leaf ultrastructure can be considered as one of the important characteristics in the evaluation of the drought susceptibility of different wheat varieties.

Keywords: combined drought and heat stress; wheat; ultrastructure; mitochondria; chloroplasts; plastoglobules

Introduction

In nature, plants are affected by changes in environmental conditions, which may negatively impact their growth, development, and productivity (Saini and Westgate 1999; Mahajan and Tuteja 2005). Drought and heat shock are common stress factors that often reduce crop yield by more than 50% (Larkindale and Knight 2002; Macar and Ekmekçi 2009; Tayyar 2010). Basically, this reduction depends on the duration and severity of the stress (Utrillas and Alegre 1997). Although drought and heat shock have been extensively studied, little is known about plant responses to their combination (Feller 2006). A series of recent reports demonstrated that plant reactions to this combined stress are unique and cannot be extrapolated from individually applied drought and heat (Rizhsky et al. 2002; Wang et al. 2004; Mittler 2006; Fábián et al. 2008; Jäger et al. 2008). So far, most of the studies related to combined drought and heat effects focus on physiological and biochemical parameters (Baniwal et al. 2004; Grigorova et al. 2011a, 2011b), but very few papers address changes at the ultrastructural level. Generally, cell organelles react quickly to environmental stress with a series of adaptation and avoidance mechanisms (Miller et al. 1971; Crèvecoeur et al.1976; Velikova et al. 2009). Leaf cell organelles from different plant species show different changes that imply for a variety of stress survival strategies (Mittler et al. 2001; Kosakivska et al. 2008). Plants subjected to drought and heat face changes in osmotic potential, resulting in unavoidable consequences for the structure and functioning of chloroplasts and mitochondria. In sorghum, the main drought-induced damages of the chloroplasts include excessive swelling of the outer chloroplast membrane, distortion of the stroma and intergranal lamellar system, accumulation of lipid droplets and reduction of starch amounts in the chloroplasts (Giles et al. 1976; Olmos et al. 2007; Vassileva et al. 2011). Conversely, in the mesophyll chloroplasts of *Cynodon dactylon* L., an increase in starch granule content and undulating dilated thylakoids are observed (Utrillas and Alegre 1997). The damaging effect of heat stress on the chloroplasts is mainly based on the decrease in total membrane length and inhibition of grana stacking (Hugly et al. 1989). The reduced light absorbance under moderate high temperatures stress can be explained by the swollen chloroplasts and increased number of plastoglobules in Arabidopsis leaves (Zhang et al. 2010). It has been suggested that plastoglobules participate in plant stress response, because their number increases in the leaves of different species under biotic and abiotic stresses (Eymery and Rey 1999). Proteomic and ultrastructural studies of plastoglobules confirm their essential role in protecting thylakoid membranes from oxidative damages by intensive synthesis and storage of lipids (such as carotenoids,

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ORIGINAL PAPER

Antioxidant response to drought in red and white clover

Irina Vaseva · Yasar Akiscan · Lyudmila Simova-Stoilova · Anelia Kostadinova · Rosa Nenkova · Iwona Anders · Urs Feller · Klimentina Demirevska

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Abstract Antioxidant response to drought in red (*Trifolium pratense* L., cv. "Start") and white clover (*Trifolium repens* L, cv. "Haifa" and cv. "Debut") grown as soil cultures was evaluated in water-deprived and recovered plants. Drought provoked oxidative stress in leaves confirmed by the considerable changes in electrolyte leakage, malondialdehyde, hydrogen peroxides and proline contents. Immunoblot of Δ-1-pyrroline-5-carboxylate synthetase (P5CS), which catalyzes the first two steps in proline biosynthesis, revealed strong induction of the enzyme in red clover plants submitted to drought. Water-deprived white clover plants exhibited distinct P5CS profiles. This was related to different drought tolerance of the studied *T. repens* cultivars. Isoenzyme analyses of superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) demonstrated certain differences in antioxidant defence among the tested varieties. It was confirmed that MnSOD (in both *T. repens* and *T pratense*) and FeSOD (in *T. repens*) isoforms were the most affected by drought. The red clover cultivar "Start" exhibited the lowest FeSOD and POX activities which could contribute to its poor performance under water deprivation.

Keywords Drought stress · Isoenzyme analysis · ROS detoxifying enzymes · Δ1-pyrroline-5-carboxylate synthetase · Red clover (*Trifolium pratense* L.) · White (*Trifolium repens* L.) clover

Abbreviations

CAT	Catalase
EL	Electrolyte leakage
FW	Fresh weight
GS	Glutamine synthetase
MDA	Malondialdehyde
PAGE	Polyacrylamide gel electrophoresis
P5CS	Δ1-pyrroline-5-carboxylate synthetase
PGP	Phosphoglycolate phosphatase
POX	Peroxidase
Pro	Proline
R	Recovery
ROS	Reactive oxygen species
SOD	Superoxide dismutase
WD	Water deficit

Introduction

Water scarcity resulting from global climate change is accompanied by more frequent and more severe summer droughts in many regions (Seneviratne et al. 2006; Koleva and Alexandrov 2008; Jaeger and Seneviratne 2011). This causes drought stress in plants and limits crop yield worldwide (Hamdy et al. 2003). The agricultural practice

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ORIGINAL PAPER

Protein changes and proteolytic degradation in red and white clover plants subjected to waterlogging

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Rosa Nenkova · Urs Feller · K. Demirevska

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Abstract Red (*Trifolium pratense* L., cv. "Start") and white clover varieties (*Trifolium repens* L., cv. "Debut" and cv. "Haifa") were waterlogged for 14 days and subsequently recovered for the period of 21 days. Physiological and biochemical responses of the clover varieties were distinctive, which suggested different sensitivity toward flooding. The comparative study of morphological and biochemical parameters such as stem length, leaflet area, dry weight, protein content, protein pattern and proteolytic degradation revealed prominent changes under waterlogging conditions. Protease activity in the stressed plants increased significantly, especially in red clover cv. "Start", which exhibited eightfold higher azocaseinolytic activity compared to the control. Changes in the protein profiles were detected by SDS-PAGE electrophoresis. The specific response of some proteins (Rubisco, Rubisco-binding protein, Rubisco activase, ClpA and ClpP protease subunits) toward the applied stress was assessed by immunoblotting. The results characterized the red clover cultivar "Start" as the most sensitive toward waterlogging, expressing reduced levels of Rubisco large and small subunits, high content of ClpP protease subunits and increased activity of protease isoforms.

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Keywords ClpA and ClpP protease subunits · Proteolysis · Rubisco · Red clover (*Trifolium pratense* L.) · White clover (*Trifolium repens* L.) · Waterlogging

Abbreviations

Clp	ATP-dependent protease belonging to serine protease
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
M	Protein marker
MW	Molecular weight
P	Band with proteolytic activity
PHMB	Para-chloro-mercuribenzoate
PMSF	Phenylmethylsulfonyl fluoride
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RA	Rubisco activase
RBP	Rubisco-binding protein
RLS	Rubisco large subunit
RSS	Rubisco small subunit
RT	Room temperature
RuBP	Ribulose-1,5-bisphosphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

Introduction

Waterlogging may occur as a result of natural seasonal changes in climate, but often it is a consequence of misbalanced redistribution of water resources caused by anthropogenic activities (Directive 2007/60/EC 2007). Globally, flooding of soils leads to significant reduction of soil quality, which negatively influences the quality of agricultural production. Subsequently this reflects on the

SHORT COMMUNICATION

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Natural antisense transcripts of *Trifolium repens* dehydrins

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Keywords: Dehydrins, natural antisense transcripts, sense-antisense pairs, splice variants, Trifolium repens

The recently described complex nature of some dehydrin-coding sequences in *Trifolium repens* could explain the considerable variability among transcripts originating from a single gene.[1] For some of the sequences the existence of natural antisense transcripts (NATs), which could form sense-antisense (SAS) pairs, was predicted. The present study demonstrates that *cis*-natural antisense transcripts of 2 dehydrin types (Y_nK_n and Y_nSK_n) accumulate in white clover plants subjected to treatments with polyethylene glycol (PEG), abscisic acid (ABA), and high salt concentration. The isolated Y_nK_n *cis*-NATs mapped to sequence site enriched in alternative start codons. Some of the sense-antisense pairs exhibited inverse expression with differing profiles which depended on the applied stress. A natural antisense transcript coding for an ABC F family protein (a *trans*-NAT) which shares short sequence homology with Y_nSK_n dehydrin was identified in plants subjected to salt stress. Forthcoming experiments will evaluate the impact of NATs on transcript abundances, elucidating the role of transcriptional and post-transcriptional interferences in the regulation of dehydrin levels under various abiotic stresses.

Dehydrins (group 2 LEA proteins) tend to accumulate late in embryogenesis and in response to stress leading to cell dehydration (e.g., drought, low temperature, and salinity).[2] The expression pattern of group 2 LEA genes is frequently associated with higher tolerance of crop plants to abiotic stresses such as cold[3] and drought.[4-6] All dehydrins have at least one conserved, lysine-rich 15-amino acid domain, EKKGIMDKIKEKLPG, named the K-segment near the C-terminus, and some of them may have a track of Ser residues (the S-segment), and/or a consensus motif (in one or more copies), T/VDEYGNP (Y-segment) located near the N-terminus.[2] The number and order of the Y-, S-, and K-segments define different dehydrin sub-classes: Y_nSK_n, Y_nK_n, SK_n, K_n and K_nS.[2] Detailed analyses of promoter regions of some DHN genes provided evidence for a close relationship between dehydrin expression patterns and the various upstream and downstream *cis*-regulatory elements present in the sequences.[7-11] Published data outlined that the regulation of expression of some dehydrin genes is elaborate and could be a result of several interacting factors.[8,10]

Results from a recent study[1] aimed at the identification of different dehydrin types in white clover (*Trifolium repens*) demonstrated the complex nature of dehydrin-coding sequences, which may lead to a high variability among the transcripts originating from a single gene. The aim of the present study was to confirm experimentally the existence of previously predicted dehydrin NAT transcripts and to compare their abundance in white clover grown under different abiotic stress treatments.

Natural antisense RNAs could potentially regulate the expression of their sense partner(s) at either transcriptional or post-transcriptional level.[12] A recent work described that a rice *cis*-natural antisense RNA acts as a translational enhancer for its cognate mRNA.[13] SAS pairs in plants have the potential to become substrates for the ribonuclease III-like enzyme Dicer to produce short interfering RNAs (siRNAs) and natural antisense microRNAs (nat-miRNAs) with regulatory potential.[14,15] Many studies have confirmed that abiotic or biotic stresses induce production of the so-called nat-siRNA (natural-small interfering RNA) from *cis*-NATs.[14,16,17]

Plants used in the present study (*Trifolium repens,* cv Apis) were grown hydroponically on a standard nutrient solution[18] for 2 weeks (23 °C night / 26 °C day; 80% relative humidity; 14 h photoperiod; photosynthetic active irradiation of 200 μmol m^{-2} s). Some of them were subjected to 72 h treatment with PEG (100 g PEG-6000 added to 1 L nutrient solution), and others were transferred on standard nutrient medium supplemented with 1 μM ABA[19] or 75 mM NaCl[20] and were grown for additional 14 d. Total RNA extracted from differently treated plants was reversely transcribed in sense and in antisense direction, with oligo(dT$_{23}$) (Sigma-Aldrich) and forward gene specific primers (Y_nK_n F: ATGAATATGGAAACCCAGTG

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Physiology

Identification and expression of different dehydrin subclasses involved in the drought response of *Trifolium repens*

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ABSTRACT

Reverse transcribed RNAs coding for Y_nK_n, Y_nSK_n, SK_n, and KS dehydrin types in drought-stressed white clover (*Trifolium repens*) were identified and characterized. The nucleotide analyses revealed the complex nature of dehydrin-coding sequences, often featured with alternative start and stop codons within the open reading frames, which could be a prerequisite for high variability among the transcripts originating from a single gene. For some dehydrin sequences, the existence of natural antisense transcripts was predicted. The differential distribution of dehydrin homologues in roots and leaves from a single white clover stolon under normal and drought conditions was evaluated by semi-quantitative RT-PCR and immunoblots with antibodies against the conserved K-, Y- and S-segments. The data suggest that different dehydrin classes have distinct roles in the drought stress response and vegetative development, demonstrating some specific characteristic features. Substantial levels of YSK-type proteins with different molecular weights were immunodetected in the non-stressed developing leaves. The acidic SK_2 and KS dehydrin transcripts exhibited some developmental gradient in leaves. A strong increase of YK transcripts was documented in the fully expanded leaves and roots of drought-stressed individuals. The immunodetected drought-induced signals imply that Y- and K-segment containing dehydrins could be the major inducible Late Embryogenesis Abundant class 2 proteins (LEA 2) that accumulate predominantly under drought.

Introduction

Climate modeling studies implying changes in average temperatures predict substantial increase of heat and summer drought frequency in Central Europe (Della-Marta et al., 2007; Hansen et al., 2012). Crop yield is intensely affected by adverse environmental conditions, and an understanding of physiological mechanisms that plants have developed to withstand environmental stress will facilitate the selection of suitable genotypes and also will contribute to the introduction of novel molecular breeding approaches in agricultural practice.

Legumes are unique among cultivated plants for their ability to carry out endosymbiotic nitrogen fixation with rhizobial bacteria (Bissuel-Belaygue et al., 2002a,b). Their representative clovers are usually preferred as a cover crop in organic crop rotations. When sown in the summer, clover may experience problems with the drought that often occurs in these later plantings. Extreme environmental events are likely to shift the adaptive response of forage species, including clover, in the long term. Therefore, the identification of suitable diagnostic markers related to abiotic stress will contribute to the development of agronomic strategies to maintain and enhance productivity of grassland crops.

The majority of published data on the effects of drought in clover focus on productivity (Bissuel-Belaygue et al., 2002a, b; Sanderson et al., 2003), morphological parameters (Grieu et al., 1995; Annicchiarico and Piano, 2004) or compatible solutes and photosynthetic pigments (Kim et al., 2004; Lee et al., 2009). Dehydrin (DHN) accumulation under water stress is a well-established phenomenon, but DHN research in white clover, which is a major legume crop, is very limited (Vaseva et al., 2011; Singh et al., 2012). Drought-inducible DHNs may serve as indicators for alteration in plant water status. The present study aimed to monitor the differential accumulation of DHN types under normal and stress conditions and to elucidate DHN spatial distribution in the aboveground parts and roots.

Abbreviations: DHN, dehydrins; LEA 2, Late Embryogenesis Abundant proteins group 2.

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Short communication

Dehydrin expression as a potential diagnostic tool for cold stress in white clover

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ABSTRACT

Cold acclimation is important for crop survival in environments undergoing seasonal low temperatures. It involves the induction of defensive mechanisms including the accumulation of different cryoprotective molecules among which are dehydrins (DHN). Recently several sequences coding for dehydrins were identified in white clover (*Trifolium repens*). This work aimed to select the most responsive to cold stress DHN analogues in search for cold stress diagnostic markers. The assessment of dehydrin transcript accumulation via RT-PCR and immunodetection performed with three antibodies against the conserved K-, Y-, and S-segment allowed to outline different dehydrin types presented in the tested samples. Both analyses confirmed that Y_nK_n dehydrins were underrepresented in the controls but exposure to low temperature specifically induced their accumulation. Strong immunosignals corresponding to 37–40 kDa with antibodies against Y- and K-segment were revealed in cold-stressed leaves. Another 'cold-specific' band at position 52–55 kDa was documented on membranes probed with antibodies against K-segment. Real time RT-qPCR confirmed that low temperatures induced the accumulation of SK_n and Y_nSK_n transcripts in leaves and reduced their expression in roots. Results suggest that a Y_nK_n dehydrin transcript with GenBank ID: KC247805 and the immunosignal at 37–40 kDa, obtained with antibodies against Y- and K-segment are reliable markers for cold stress in white clover. The assessment of SK_n (GenBank ID: EU846208) and Y_nSK_n (GenBank ID: KC247804) transcript levels in leaves could serve as additional diagnostic tools.

1. Introduction

White clover (*Trifolium repens* L.) is one of the most important pasture legumes in temperate regions which could be used also for erosion control cover on moist fertile soils. Its growth initiates at 10 °C and develops optimally at 20–25 °C (Frame, 2004). *T. repens* aerial parts produce less biomass at temperatures below 7–5 °C (Davies and Evans, 1982). The minimum and optimum temperatures for growth of white clover are higher than those of their companion grasses (Murray et al., 2000; Collins et al., 2001; Humphreys and Lawless, 2006) and low temperature could be a limiting factor for the optimal biomass productivity of mixed swards (Goulas et al., 2003; Humphreys and Lawless, 2006).

Cold stress responses involve numerous genes coding for proteins associated with various biochemical pathways, physiological processes and regulatory networks (Hannah et al., 2005). Previous studies on white clover subjected to low temperature have focused on cold-induced changes in morphogenesis, nitrogen reserve accumulation (Goulas et al., 2003) and secondary metabolism (Rasmussen et al., 2006). It has been documented that cold stress triggers the increase of dehydrin content in plant vegetative organs (Wisniewski et al., 1999; Nylander et al., 2001; Renaut et al., 2004). Dehydrins (or LEA 2) are intrinsically disordered proteins (IDPs) (Tompa, 2009) which accumulate late in embryogenesis and in response to stress leading to cell dehydration (e.g. drought, low temperature and salinity) (Reyes et al., 2005). LEA 2 have at least one conserved, lysine-rich 15-amino acid domain,

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Abbreviations: DHN, dehydrin; IDPs, itrinsically disordered proteins; LEA 2, late embryogenesis abundant group 2; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction.

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Extreme climatic events: impacts of drought and high temperature on physiological processes in agronomically important plants

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Climate models predict more frequent and more severe extreme events (e.g., heat waves, extended drought periods, flooding) in many regions for the next decades. The impact of adverse environmental conditions on crop plants is ecologically and economically relevant. This review is focused on drought and heat effects on physiological status and productivity of agronomically important plants. Stomatal opening represents an important regulatory mechanism during drought and heat stress since it influences simultaneously water loss via transpiration and CO_2 diffusion into the leaf apoplast which further is utilized in photosynthesis. Along with the reversible short-term control of stomatal opening, stomata and leaf epidermis may produce waxy deposits and irreversibly down-regulate the stomatal conductance and non-stomatal transpiration. As a consequence photosynthesis will be negatively affected. Rubisco activase—a key enzyme in keeping the Calvin cycle functional—is heat-sensitive and may become a limiting factor at elevated temperature. The accumulated reactive oxygen species (ROS) during stress represent an additional challenge under unfavorable conditions. Drought and heat cause accumulation of free amino acids which are partially converted into compatible solutes such as proline. This is accompanied by lower rates of both nitrate reduction and *de novo* amino acid biosynthesis. Protective proteins (e.g., dehydrins, chaperones, antioxidant enzymes or the key enzyme for proline biosynthesis) play an important role in leaves and may be present at higher levels under water deprivation or high temperatures. On the whole plant level, effects on long-distance translocation of solutes via xylem and phloem and on leaf senescence (e.g., anticipated, accelerated or delayed senescence) are important. The factors mentioned above are relevant for the overall performance of crops under drought and heat and must be considered for genotype selection and breeding programs.

Keywords: drought, heat, abiotic stress, stomates, protein pattern, leaf senescence, xylem, phloem

INTRODUCTION

Besides the general temperature increase global change models predict more frequent and more severe extreme events such as drought periods, heat waves or flooding (Easterling et al., 2000; Schar et al., 2004; Fuhrer et al., 2006; Wehner et al., 2011; Mittal et al., 2014). These regional climatic extremes (Gilgen and Buchmann, 2009) are ecologically and economically relevant for agriculture and forestry (IPCC, 2012; Smith and Gregory, 2013; Nair, 2014). The susceptibility to abiotic stresses may differ considerably among species or varieties of a crop (Yordanov et al., 2000; Simova-Stoilova et al., 2009; Vassileva et al., 2011; Chen et al., 2012; Wishart et al., 2014). Therefore, the selection of suitable genotypes and breeding of less susceptible varieties could reduce negative effects of extreme climate events on plant productivity (Neumann, 2008; Mir et al., 2012; Jogaiah et al., 2013), which is particularly important for the annual crops.

The apparent significance of stress period for the crop productivity does not rule out the fact that subsequent recovery stages are equally crucial for a proper evaluation of the overall performance (Subramanian and Charest, 1998; Gallé and Feller, 2007; Gallé et al., 2007; Vassileva et al., 2011). The progression and duration of stress, plant developmental stage and other biotic and abiotic factors may influence the stress response. For example certain species may be affected at early developmental stage, but still be capable to recover and finally to survive. Others could cope with suboptimal conditions comparatively well at the beginning of the stress period remaining still quite productive. Later on their surviving potential could be exhausted leaving the plants irreversibly damaged. A comprehensive evaluation of plant stress response includes the overall characterization of plant physiological behavior and survival. Here we summarize some of the major physiological parameters which characterize stress response reactions and which could be implemented as tools for evaluation of stress effects.

The impact of drought and heat on physiological status and productivity of agronomically important plants will become even

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SOLUTE TRANSPORT VIA XYLEM AND PHLOEM IN TWO WHEAT GENOTYPES DIFFERING IN DROUGHT SUSCEPTIBILITY

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Summary: More frequent and more severe extreme events (e.g. flooding, drought, heat waves) were predicted from climate models for the next decades. Impacts of such events are relevant for crop productivity. Effects on a whole-plant level are the focus of this study. The influence of an extended drought period on solute allocation via the phloem was compared in two Bulgarian wheat varieties differing in drought susceptibility: "Sadovo" (drought-sensitive) and "Katya" (drought-tolerant). Young plants grown hydroponically on standard nutrient solution were transferred at the beginning of the experiment to fresh medium (controls) or to medium with polyethylene glycol 6000 (artificial drought). Radiolabeled solution (^{57}Co, ^{109}Cd, ^{134}Cs) was introduced via a flap into the lamina of the third or fourth leaf of young plants (youngest fully expanded leaf before stress treatment). Export and allocation of the radiolabel were monitored by gamma spectrometry. ^{57}Co, ^{109}Cd and ^{134}Cs are suitable radionuclides, since they are not metabolized and not released in gaseous form. The exported radionuclides were mainly found in the roots (30-50%) and in the younger leaves (30-60%), while only traces reached the older leaves indicating that only small quantities were transported back from the roots to the shoot via the xylem. A comparable percentage was transported from leaf 4 to leaves 5 and 6 in both varieties under control conditions. A much higher percentage was found in leaf 5 than in leaf 6 of "Katya" subjected to drought, while "Sadovo" was affected in the opposite direction. The results suggest that adaptations in the source/sink network are important for the drought susceptibility of wheat varieties.

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Key words: Drought; phloem transport; wheat varieties; *Triticum aestivum* L.

Abbreviations: PEG – polyethylene glycol; cpm – counts per minute.

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SEMI-QUANTITATIVE RT-PCR ANALYSIS OF SELECTED PROTEASE INHIBITORS IN DROUGHT-STRESSED *TRITICUM AESTIVUM*

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Summary: Proteases and their specific inhibitors are ubiquitously distributed and play a key regulatory role in many biological processes. Gene expression and activity of certain proteases has been shown to increase in *Triticum aestivum* L. leaves under drought, with a major contribution of cysteine proteases, especially in sensitive wheat varieties. However, little is known about the stress response of protease inhibitors (PIs) and their role in the regulation of intracellular proteolysis. In this study the changes in transcript abundance of some protease inhibitors (belonging to cystatin and serpin classes) were evaluated by semi-quantitative RT-PCR in leaves and roots of winter wheat seedlings from two varieties with differing tolerance. The expression of two cysteine proteases in the same samples was also assessed. The expression of the studied genes was compared in the tolerant variety “Katya” and the more susceptible to water deprivation variety “Sadovo”, applying severe but recoverable soil drought. Growth inhibition and stress related parameters confirmed the relatively higher drought sensitivity of variety “Sadovo”. Serpin transcript abundance in control roots was higher than in the leaves. An opposite trend was documented for cystatins – the level of their expression was stronger in the non-treated leaves compared to roots. Drought stress inhibited PI expression in roots, while varying effects on the transcript levels were detected in the leaves of water deprived plants. The levels of the two cysteine protease transcripts under drought exhibited organ-specific response – they declined in roots, and increased in leaves. Further detailed studies using more sensitive methods are necessary to evaluate the potential of protease inhibitors as biochemical markers for drought tolerance.

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Keywords : Cystatin; drought; leaves; roots; serpin; wheat.

Abbreviations: EL – electrolyte leakage; FW – fresh weight; MDA – malondialdehyde; PI - protease inhibitors; WD – water deficit; Ta – annealing temperature.

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Chapter 9

CHANGES IN 2-DE PROTEIN PROFILE OF WHITE AND RED CLOVER LEAVES IN RESPONSE TO WATERLOGGING STRESS AND RECOVERY

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ABSTRACT

Legumes are widely used forage crops often grown in flooding prone areas but data about proteome changes in waterlogged plants are scarce. In the present study leaf 2-DE protein profiles of white (*Trifolium repens* L. cv. Haifa) and red (*Trifolium pratense* L. cv. Start) clovers, differing in waterlogging tolerance, were compared. Flooding was imposed on 21-day-old plants for a period of 14 days, followed by a 21 days of recovery. Plant physiological status was assessed by changes in leaf area, water content, photosynthetic parameters and total soluble protein. Maximum (Fv/Fm) and the actual (Φ_{PSII}) PSII efficiency were not significantly affected in both cultivars. However, non-photochemical quenching (NPQ) increased substantially in waterlogged white clover. Following 2-DE separation (pI 5-8 and 12% SDS-PAGE), 90 variable protein spots were identified using MALDI-TOF/TOF MS, resulting in reliable hits for 22 individual proteins in red and 26 - in white clover, 17 of them being common for both clovers. In both varieties a strong diminution under stress was observed in Rubisco subunits, ATP synthase subunits α and β, oxygen-evolving enhancer protein and other chloroplastic proteins. Cytochrome b6-f complex iron-sulfur subunit exhibited opposite trends under waterlogging stress - decrease in red and increase in white clover. Several proteins

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RESEARCH ARTICLE

AMINOPEPTIDASE ACTIVITIES IN ROOTS AND LEAVES OF DROUGHT STRESSED WINTER WHEAT SEEDLINGS

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Summary: In order to evaluate the role of aminopeptidases (APs) in drought response and their potential as protein markers to distinguish between stress tolerant and sensitive varieties, various AP activities were studied in roots and leaves of winter wheat seedlings, subjected to severe but recoverable soil drought stress. Two varieties with contrasting drought tolerance – Yantar (drought tolerant) and Miziya (sensitive) were compared. Activity changes under severe water stress and subsequent recovery were related to changes in the pools of the major redox buffers ascorbate and glutathione, changes in protein profiles and total proteolysis in roots and leaves. Glutathione was responsive to drought both in roots and leaves, with increased total pool and transient rise in the oxidized form; stronger response in the roots of Yantar was observed. The sensitive variety had higher ascorbate content in leaves under stress. Severe drought led to reversible changes in protein profiles and increase in major protease bands in leaves but not in roots. AP activities were partly independent from the predominant endoprotease activities. Highest activities in roots were detected with substrates releasing terminal leucine, lysine and metionine. In stressed leaves AP activities toward most of the substrates increased under drought, without clear differences comparing varieties. Activities tested with Gly-pNA were raised in leaves only in recovery from stress. In roots, the tolerant variety Yantar presented increased AP activities under stress with most of the substrates used except Leu-pNA and Phe-pNA, whereas the sensitive variety Miziya had almost unchanged AP activities. Based on activity profile changes, at least two different AP enzymes should exist in wheat. It remains to be established which activities towards different substrates reflect distinct aminopeptidases.

Keywords: Aminopeptidase; drought; recovery; ascorbate; glutathione; *Triticum aestivum L.*

Abbreviations: AP – aminopeptidase; DMSO – dimethylsulfoxide; DTNB – dithionitrobenzoic acid; DTT – dithiotreitol; EDTA – ethylendiaminetetracetic acid; FW – fresh weight; MDA – malondialdehyde; PAR – photosynthetic active radiation; pNA – para-nitroanilide; PMSF – phenylmethylsulfonylfluoride; ROS – reactive oxygen species; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA – trichloroacetic acid; TW – weight at full turgidity; WD – water deficit.

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Transcript Profiling of Serine- and Cysteine Protease Inhibitors in *Triticum aestivum* Varieties with Different Drought Tolerance

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A high number of protease inhibitors (PI) have been identified in diverse plant species but information about their role in plant stress responses is still fragmentary. Transcript profiling of six published serine and cysteine protease inhibitor sequences in water-deprived plants from four winter wheat *(Triticum aestivum)* varieties with varying tolerance was performed in order to outline PIs predominantly accumulating under drought. Expression was analyzed by real time RT-qPCR. Considerable transcript accumulation of Bowman–Birk type PI WALI3 (BBPI) was detected in drought stressed leaves suggesting an important regulatory role of BBPI in adjustment of protein metabolism in leaves under dehydration. Serpin transcripts were less represented in water-deprived plants. Transient accumulation of cystatin transcripts revealed organ-specificity. Under drought cystatin and serpin expression in the leaves of the most drought tolerant variety “Katya” tended to preserve relatively stable levels close to the controls. This preliminary data will serve for future detailed study of regulation of proteolysis in winter wheat subjected to unfavorable environmental factors for development of molecular-based strategies for selection of tolerant varieties.

Keywords: Bowman–Birk protease inhibitors, cystatin, drought, serpin, wheat

Introduction

Changes in protein composition, expression and post-translational modifications are substantial part of plant development and response to various abiotic and biotic stresses. Proteases are the principal enzymes controlling cellular protein complement and the steady state level of individual proteins through selective protein breakdown (López-Otín and Bond 2008). Endogenous protease inhibitors (PIs) play role in regulation of the proteolytic activity and are ubiquitously distributed in animals, plants and microorganisms (Mosolov and Valueva 2005; Kidrič et al. 2014). Plant PIs have a major role in the defense against insect and pathogen attack. Accumulating evidence point out their active involvement in abiotic stresses (Kidrič et al. 2014) and recently stress responsive elements were identified in the promotor region of some PIs (de Almeida et al. 2012).

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REVIEWS

Cell Type Specificity of Plant Hormonal Signals: Case Studies and Reflections on Ethylene[1]

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Abstract—-In the light of increasing evidence that plant growth and development depend on signals perceived in distinct cell types where hormonal inputs are transformed into orchestrated responses triggering a plethora of physiological processes, we reflect on the case of ethylene signaling. Experimental approaches to address cell type-specificity of the ethylene response are discussed and future challenges in ethylene signaling studies are outlined.

Keywords: higher plants, auxins, brassinosteroids, cell type specificity, ethylene, plant hormone, signaling

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TISSUE-SPECIFIC REGULATION OF PLANT HORMONE RESPONSES: CASE STUDIES

Regulation of plant growth and development depends on the coordinated action of multiple plant hormones. Plant hormone action is traditionally viewed as organ specific, considering inter-organ communication and in certain cases also intra-organ tissue-specificity. Cell type specificity has long been neglected. In recent years however, a lot more attention is paid to how individual cell types respond to hormones. Several studies revealed that the spatial regulation of hormone synthesis and signaling is an important aspect in the control of specific processes related to organ growth, including cell proliferation, expansion, and differentiation [1]. Recent studies have shown that the site of action for elongation regulating hormones can mainly be accounted for by single cell types: the endodermis for gibberellins (GA) [2, 3], the epidermis for auxins [4] and brassinosteroids (BR) [5, 6]. Dello Ioio et al. [7] have demonstrated that the depletion of cytokinins in the vascular tissue specifically at the root transition zone (TZ) was sufficient for decreasing the differentiation rate of all the other tissues. All these findings were possible because of the availability of cell and tissue-specific promoters [8–10].

The previously published studies using cell type-specific promoters for identification of hormonal signal sites governing certain hormone responses are listed in the table. In most of the cases dominant negative alleles of key signal transduction components were placed under control of cell type-specific promoters, thus blocking perception or signaling in particular tissues or cells. This approach represents a powerful method to demonstrate the importance of particular cell types in the response to a hormonal signal which governs a given growth or developmental process. By cell type-specific expression of the auxin influx transporter AUX1 Swarup et al. [4] demonstrated that the root gravitropic response is regulated by basipetal (shootward) flow of auxins through the lateral root cap (LRC) and epidermis of the root elongation zone (LZ). In the case of cytokinins Dello Ioio et al. [7] have used targeted expression of the enzyme cytokinin oxidase to deplete the content of cytokinin in particular root tissues (table). Hacham et al. [6] have demonstrated that BR signaling in the root epidermis controls root meristem size by maintaining cell cycle activity and cell expansion. Recently translatome analyses presented an evidence for opposing effects of BR signals on root meristem size, depending on the site of action [11]. The authors demonstrated that BR signals in the inner tissues, endodermis, quiescent center (QC), and stele, had no growth-promoting effect and that the BR-repressed genes were autono-

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Abbreviations: ACC—1-aminocyclopropane-1-carboxylic acid; AUX1—Auxin Resistant 1; BR—brassinosteroid; BRI—Brassinosteroid Insensitive; Col-0—Columbia-0 ecotype; EBF—EIN3 binding factor; EDZ—elongation/differentiation zone; EIN—Ethylene Insensitive; CTR1—Constitutive Triple Response 1; EIL1—Ethylene Insensitive Like 1; EZ—elongation zone; GA—gibberellic acid; GAL4—Galactose-induced 4; GFP—green fluorescent protein; LRC—lateral root cap; NASC—The Nottingham Arabidopsis Stock Centre; PIN2—Pin-Formed 2; QC—quiescent centre; RAM—root apical meristem; TF—transcription factor; TZ—transition zone; UAS—upstream activation sequence.