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Enhanced cytokinin degradation in leaf primordia of transgenic *Arabidopsis* plants reduces leaf size and shoot organ primordia formation

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ABSTRACT

The plant hormone cytokinin is a key morphogenic factor controlling cell division and differentiation, and thus the formation and growth rate of organs during a plant's life cycle. In order to explore the relevance of cytokinin during the initial phase of leaf primordia formation and its impact on subsequent leaf development, we increased cytokinin degradation in young shoot organ primordia of *Arabidopsis thaliana* by expressing a cytokinin oxidase/dehydrogenase (*CKX*) gene under control of the *AINTEGUMENTA* (*ANT*) promoter. The final leaf size in *ANT:CKX3* plants was reduced to ~27% of the wild-type size and the number of epidermal cells was reduced to ~12% of the wild type. Kinematic analysis revealed that cell proliferation ceased earlier and cell expansion was accelerated in *ANT:CKX3* leaves, demonstrating that cytokinin controls the duration of the proliferation phase by delaying the onset of cell differentiation. The reduction of the cell number was partially compensated by an increased cell expansion. Interestingly, *ANT:CKX3* leaf cells became about 60% larger than those of *35S:CKX3* leaves, indicating that cytokinin has an important function during cell expansion as well. Furthermore, *ANT:CKX3* expression significantly reduced the capacity of both the vegetative as well as the generative shoot apical meristem to initiate the formation of new leaves and flowers, respectively. We therefore hypothesize that the cytokinin content in organ primordia is important for regulating the activity of the shoot meristem in a non-autonomous fashion.

Introduction

Shoot lateral organs, such as leaves and floral primordia, are generated from cells at the periphery of the shoot apical meristem (SAM). Organization of the SAM is complex, dynamic and requires communication between different domains (Barton, 2010). The central zone on the summit of the SAM contains a group of slowly dividing pluripotent stem cells. Their descendant cells are displaced into the peripheral zone and then recruited into the differentiation and growth programs of the lateral organs. A balance between perpetual differentiation and self-renewal of the stem cell population is vital for indeterminate SAM activity and is controlled by a local regulatory transcriptional network, by plant hormones and by external signals. A central regulatory module is the WUS/CLV pathway (Tucker and Laux, 2007).

Leaf primordia can be first recognized as a group of initial cells with a distinct transcriptional profile. The activity of meristem-specifying genes, such as *KNOTTED1*-like homeobox transcription factors, is suppressed and expression of a number of genes that control early stages of lateral organ development including *AINTEGUMENTA* (*ANT*; APETALA2-like transcription factor) is activated in organ initials (Jackson et al., 1994; Elliott et al., 1996; Long and Barton, 2000). A distinct number of initial cells change the plane and pattern of division and expansion, causing the formation of a morphologically distinct primordium (Poethig and Sussex, 1985). Whereas the initial phase of organ growth is promoted by extensive cell division, during organ maturation, proliferation ceases and cells differentiate. During leaf expansion, cell proliferation shows a strong longitudinal gradient and progressively declines in an apical–basal direction (Donnelly et al., 1999). The shoot forms lateral organs of determinate size and the rate and extent of cell division and expansion define their final size. The processes of cell proliferation, differentiation and expansion are closely connected, as suggested, for example, by compensation reaction, in which an enhanced cell growth is triggered by a decrease in cell number (Tsukaya, 2008).

The plant hormone cytokinin is an important factor controlling plant morphogenesis (Mok and Mok, 2001; Werner and Schmülling, 2009). Strong shoot growth defects in plants with lowered cytokinin status demonstrated that the proliferative activity of the SAM and developing leaves is dependent on cytokinin (Werner et al., 2001; Werner et al., 2003; Higuchi et al., 2004; Nishimura

Abbreviations: *ANT*, *AINTEGUMENTA*; CKX, cytokinin oxidase/dehydrogenase; SAM, shoot apical meristem.

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et al., 2004; Miyawaki et al., 2006). Cytokinin is synthesized by the SAM, at least in part, in an autonomous fashion (Kurakawa et al., 2007) and the cytokinin pathway closely interacts with transcriptional factors regulating SAM activity through mutual regulation on the transcriptional level (Jasinski et al., 2005; Leibfried et al., 2005; Yanai et al., 2005; Gordon et al., 2009). Further downstream targets mediating the cytokinin activity in the SAM and lateral organs involve factors controlling cell cycle progression (Zhang et al., 2005; Dewitte et al., 2007) and/or functional differentiation of meristematic cells (Werner et al., 2008). As plant growth depends on the supply of photoassimilates, it is interesting to note that cytokinin also controls the metabolic status of heterotrophic cells and thus the sink capacity of the shoot apex (Werner et al., 2008).

Although it is well established that cytokinin is essential for cell proliferation in the SAM and developing lateral organs, the understanding of which specific tissues and cell types are relevant for cytokinin-mediated control of shoot development and growth is not complete. In this study, we address this question by investigating the developmental consequences of ectopic expression of the cytokinin oxidase/dehydrogenase gene *CKX3* in young shoot organ primordia.

Material and methods

Plasmid construction

The 35S promoter was removed from the vector pBinSMGFP (Werner et al., 2003) with *Eco*RI and *Kpn*I and a synthetic double-stranded oligonucleotide (link-plus 5′-CGGAATTCCTAGGCTTCTGCCCGGGCTTCTGGGTACCCC-3′ and link-minus 5′-GGGGTACCCAGAAGCCCGGGCAGAAGCCTAGGAATTCCG-3′) containing *Eco*RI-*Avr*II-*Sma*I-*Kpn*I sites was inserted. A *CKX3* (At5g56970) genomic fragment was subcloned via *Kpn*I/*Xho*I restriction sites from pBS-AtCKX3 (Werner et al., 2001) to generate an N-terminal fusion with GFP. A 4.2 kb promoter fragment of the *ANT* gene (At4g37750) was amplified by PCR from plasmid MD65 (Schoof et al., 2000) using the forward primer ANT-5′-AvrII (5′-GACCTAGGCTCCTTCGGGTAATTATGTGA-3′) and the reverse primer ANT-3′-KpnI (5′-GCGGTACCGGTTTCTTTTTTTGGTTTCTG-3′). The fragment was cloned in *Avr*II/*Kpn*I sites upstream of the *CKX3-GFP* fusion gene, resulting in vector pANT:CKX3-GFP, which was used for plant transformation.

Plant transformation and growth conditions

Arabidopsis thaliana accession Col-0 was transformed by the floral dip method (Clough and Bent, 1998) and >40 independent *ANT:CKX3* transgenic lines were selected *in vitro* on half-strength MS medium containing 1% sucrose and 15 mg/L hygromycin. The analyses were performed with plants of two independent homozygous lines of the T3 generation (ANT:CKX3-5 and ANT:CKX3-6), which showed the strongest shoot growth reduction among the selected lines. Plants were grown in a greenhouse under 16 h light/8 h dark cycles, at 21 °C (light period) and 18 °C (dark period).

Gene expression analysis

Total RNA was isolated from six-day-old plants and used for semiquantitative RT-PCR as described previously (Brenner et al., 2005). For the detection of the chimeric *CKX3-GFP* transcript, forward primer CKX3-A5 (5′-GAAATAACATCACTAGCGGTCCT-3′) and reverse primer GFP-618 (5′-GGCAGATTGTGTGGACAGGTAA-3′) were used. For detection of the total *CKX3* transcript level, the gene-specific primers 5′-CTCGGCTAAAGACGGAGTTG-3′ (forward) and 5′-TCAAAAGCCTCCCAATTGTC-3′ (reverse) were used. *ANT* transcripts were amplified using primers 5′-GAGGTAGAGGAGGTAGAGAAGC-3′ (forward) and 5′-CCAGTGATGCAGCTAGATTG-3′ (reverse), and *Actin 2* (At5g09810) was amplified using primers 5′-TACAACGAGCTTCGTGTTGC-3′ (forward) and 5′-GATTGATCCTCCGATCCAGA-3′ (reverse). 10 μL of the reactions were loaded on a 1% agarose gel, electrophoretically separated and stained with ethidium bromide.

Kinematic analysis of leaf growth

Seeds were sown on soil, incubated at 4 °C for 3 days and grown under greenhouse conditions. Kinematic analysis was performed according to De Veylder et al. (2001) with the following modifications. The abaxial epidermis of the sixth rosette leaves was analyzed at 3-day intervals. The starting point of the analysis (t_0) was determined individually for each plant when a leaf length of 2 mm was reached. The leaves were harvested and fixed in ethanol:acetic acid (3:1), cleared in chloral hydrate/glycerol/water (8:1:2) and analyzed by a stereomicroscope (SZX12; Olympus, Tokyo, Japan) or a microscope (Axioskop 2 plus with AxioCam ICc3 camera; Zeiss, Jena, Germany). Image analysis was performed with Scion Image software (http://www.scioncorp.com). We measured the whole leaf blade area, and determined the number of pavement and guard cells per defined area at three different positions: at 25%, 50% and 75% distance between the base and the tip of the leaf blade, amid the leaf margin and the main vein. From these data, the number of cells per leaf, average cell size, cell division rate, and stomatal index were calculated.

Results

Ectopic expression of CKX3 in shoot organ primordia under the control of the ANT promoter

In order to explore the relevance of cytokinin activity during the early phase of shoot organ primordia formation and to study how this early activity impacts on subsequent leaf growth, the *Arabidopsis CKX3* gene was expressed under the control of the *AINTEGUMENTA* (*ANT*) promoter. *CKX3* is normally expressed in the central zone of the meristem (Gordon et al., 2009; Bartrina et al., 2011) but not in leaf primordia. The *ANT* gene, which encodes a transcription factor of the *APETALA2* domain family, is already expressed in precursors of cotyledon primordia in the globular-stage embryo (Elliott et al., 1996). During postembryonic development, *ANT* expression is excluded from the meristematic cells and the *ANT* promoter becomes first active in differentiating cells forming primordia of leaves, flowers and all floral organs, as well as in developing ovules (Elliott et al., 1996; Grandjean et al., 2004). *ANT* expression was also detected in procambial cells prior to and during vascular differentiation in very young leaves (Kang et al., 2007) and in procambial cells of developing stems, filaments and gynoecia (Elliott et al., 1996). In contrast to the 35S promoter, which is expressed during the lifespan of the leaf, *ANT* gene expression is highest in young leaves.

More than 40 independent *ANT:CKX3* transgenic lines were generated and two homozygous T3 lines, ANT:CKX3-5 and ANT:CKX3-6, were selected for further analysis. In these plants, semiquantitative RT-PCR analysis revealed strong expression of the *ANT:CKX3* transgene, an increase of the total *CKX3* transcript level in comparison to the wild type, and no influence of the transgene expression on *ANT* gene expression (Fig. 1A). Growth and development of *ANT:CKX3* transgenic *Arabidopsis* shoots were reduced in comparison to the wild type but not as strongly as in *35S:CKX3* transgenic plants (Fig. 1B). The rosette leaves of *ANT:CKX3* plants formed smaller leaf blades and shorter petioles (Fig. 1C). The rosette diameter was reduced by 52–45% at 28 DAS (days after sowing)