Expression of cell cycle regulatory genes and morphological alterations in response to salt stress in Arabidopsis thaliana

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Abstract. Hyperosmotic stress severely affects plant growth and development. To examine the effect of salt stress on cell cycle activity in Arabidopsis thaliana (L.) Heynh., the transcriptional regulation of a cyclin-dependent kinase, CDC2aAt, and two mitotic cyclins, Arath;CycB1;1 and Arath;CycA2;1, was studied by using the β-glucuronidase (gus) reporter gene. Moreover, the mRNA abundance of these cell cycle genes as well as CDC2bAt were monitored during salt stress. Upon NaCl treatment, the promoter activities and transcript levels of all cell cycle genes diminished initially in the shoot apex and were subsequently induced during salt-stress adaptation. Additionally, the promoter activities of CDC2aAt and CycA2;1 decreased in the vascular cylinder of the root in correlation with reduced lateral root formation. In the root tips, a regression of CDC2aAt, CycA2;1, and CycB1;1:gus expression was observed, concomitant with a shrinkage of the root meristem and inhibition of root growth. Our data indicate that salt stress interferes with cell cycle regulation at the transcriptional level, resulting in an adaptive growth response.

Key words: Arabidopsis (cell cycle) – Cyclin-dependent kinases – β-Glucuronidase – Mitotic cyclins – Salt stress

Introduction

Plant growth is mainly the result of cell division and elongation (Clark 1997; Cosgrove 1997; Kerstetter and Hake 1997; Meyerowitz 1997; Schiefelbein et al. 1997). Inhibition of elongation upon salt stress or water deficit has been reported in leaves (McCree and Davis 1974; Neumann 1993), and in roots (Sharp et al. 1988; Frisch and Hsiao 1994; Couto-Gastelier and Vartanian 1995). Also a diminution of cell numbers, mitotic activity or cell division rates has been described in several plant species either in leaves (Leceur et al. 1995; Schuppler et al. 1998), roots (Robertson et al. 1990; Bitonti et al. 1991; Bracale et al. 1997; Sacks et al. 1997), or the subapical part of the shoot meristem (Edelman and Loy 1987) upon osmotic stress.

The major checkpoints of the eukaryotic cell division cycle are situated at the G1-to-S and G2-to-M transitions. Progression through these boundaries is catalyzed by cyclin-dependent kinases (CDKs) the activity of which is regulated by phosphorylation and dephosphorylation events and by binding to a catalytic subunit, a cyclin. During the past decade, many key cell cycle regulators have been characterized in plants, including CDKs, cyclins, CDK inhibitors, and a suppressor of the cell cycle block/CKS protein (for reviews, see Burssens et al. 1998; Mironov et al. 1999).

In Arabidopsis thaliana, two main classes of CDKs have been identified so far. The CDC2aAt gene of A. thaliana belongs to the A-type class and is constitutively expressed during the cell cycle (Hemelryk et al. 1993; Segers et al. 1996). The activity of CDC2aAt histone H1 kinase is maximal at the G1-to-S and G2-to-M transitions (J.-P. Reichheld, personal communication), implying a functional involvement at both checkpoints. The gene CDC2bAt, a member of the B-type class, is predominantly transcribed in the S and G2 phases and, in contrast to CDC2aAt, is expressed in a patchy pattern in meristematic tissues. The expression of both genes can, however, be associated with division as well as with competence to divide (Martinez et al. 1992; Hemelryk et al. 1993; Segers et al. 1996). As in other plants, multiple cyclins have been characterized in A. thaliana that are classified into A, B, and D types, mainly based on sequence similarity (Renaudin et al. 1996; De Veylder et al. 1999; Sorrell et al. 1999). The
Arath; CycB1;1 and Arath; CycA2;1 genes of *A. thaliana* are both mitotic cyclins. The transcription of *CycB1;1* is associated with the G2 and M phases and is a marker for active cell division (Ferreira et al. 1994; Shaul et al. 1996). The promoter activity of *CycA2;1* increases upon entry into S phase and decreases at the entry to mitosis (Shaul et al. 1996). Expression of *CycA2;1* during plant development reflects cell division and also competence to divide (Burssens et al. 2000).

Little is known about how various stress conditions affect the cell cycle. Schuppler et al. (1998) demonstrated that dehydration imposed on wheat leaves reduced the CDK activity, probably as a result of an inhibitory phosphorylation. Furthermore, the transcription of the *A. thaliana* CDK inhibitor, ICK1, was shown to be induced by abscisic acid (Wang et al. 1998).

The objective of this study was to analyze morphological alterations to *A. thaliana* as a consequence of salt stress in relation to the transcriptional regulation of cell cycle regulatory genes. For this purpose, expression analyses of promoter-β-glucuronidase (gus) fusions were performed with *CycB1;1*, *CycA2;1*, and *CDC2aAt* in transgenic *A. thaliana* plants (Hemerly et al. 1993; Ferreira et al. 1994; Burssens et al. 2000) during growth in a saline environment. Moreover, semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed on *CycB1;1*, *CycA2;1*, *CDC2aAt*, and *CDC2bAt* of salt-stressed *A. thaliana* shoots.

**Materials and methods**

**Growth in saline conditions**

Transgenic Arath; CycB1;1:gus (Ferreira et al. 1994), Arath; CycA2;1:gus (Burssens et al. 2000) and *CDC2aAt:gus* Arabidopsis thaliana (L.) Heynh. plants (Hemerly et al. 1993) were grown in sterile conditions on K1 germination medium (Valvekens et al. 1988). Ten days after germination, the plants were transferred to the same medium without or with 1% or 1.5% NaCl. Samples were taken from non-stressed (control) and stressed plants after 0 h, 12 h, 36 h, 4 d, 1 week, and 2 weeks, and subsequently whole-mount GUS assays were performed.

**Anatomy and measurements**

For anatomical analysis, plant material (root tips, shoot apices, cotyledons, third and fifth leaves) was collected from *A. thaliana* plants that had been transferred to solid K1 medium without (control) or with 1% NaCl for 2 weeks. The leaves were numbered starting from the first rosette leaf that emerged after the cotyledons. The samples were dehydrated and embedded in Technovit 7100 resin (Hereaues Kulzer, Wehrheim, Germany) according to the manufacturer’s protocol. Transverse or longitudinal sections (2 μm) were cut at the center of the leaf blades and shoot apex, and through root tips of non-stressed (control) and stressed plants with a rotating micromere (Microm HM 360; Heidelberg, Germany). The sections were stained with a 0.05% (w/v H2O) toluidine blue solution (Merck, Darmstadt, Germany). Micrographs of the sectioned material were taken under bright-field illumination with a Diaplan microscope (Wild-Leitz, Wetzlar, Germany).

Measurements of epidermal cell diameter of the fifth leaves were obtained from digitized camera-lucida drawings made from the adaxial leaf surface of the third leaf, using differential interference contrast optics on a Diaplan microscope (Leitz, Wetzlar, Germany). A monolayer of epidermal cells was visualized in whole-mounted leaves that had been fixed in 100% methanol and cleared in 88% lactic acid (UCB, Leuven, Belgium). Leaf blades were divided into three equal segments: basal, middle, and top. In each leaf, a sector (0.125 mm²) was defined in the center of the middle segment, the number of epidermal cells of which was defined. Image analyses were performed with the public domain Image program (version β-3b; Scion Corporation, Frederick, Md., USA).

For the quantification of lateral root number, lateral root primordia were included as indicated by the presence of *CycB1;1* expression (Ferreira et al. 1994). Measurements of the length of the main root meristem were based on the zone in the root tip that showed gus expression for *CycB1;1*. All measurements and quantifications were performed for at least 15 samples with a stereoscope (STEMI SV11; Zeiss, Jena, Germany). The data of measurements or quantifications were incorporated by using the Excel Program (Microsoft) into histograms showing the mean values ± SE.

**Histochemical GUS assays**

Whole-mount GUS staining was performed as described by Beeckman and Engler (1994) and photographed with differential interference contrast optics. Thin sections of GUS-stained material were prepared according to Peleman et al. (1989) with modifications (Hemerly et al. 1993).

**Semi-quantitative RT-PCR**

Total RNA was prepared with TriZol reagent (Amersham, Aylesbury, UK) from shoots of *A. thaliana* seedlings that were non-stressed or had been stressed with 1% NaCl for 12 h, 36 h, and 4 d as described above. For the cDNA synthesis from 1 μg of RNA, the Superscript Preamplification System ( Gibco/BRL, Gaithersburg, Md., USA) was used according to the manufacturer’s protocol. Aliquots of 2 μl of cDNA were submitted to PCR using 200 ng of each gene-specific primer in a standard reaction of 50 μl with Taq polymerase (Pharmacia, Uppsala, Sweden). Primers were 5’-AAGTAGTGGAGGAGGGTTGATG-3’ and 5’-TCAGTGCGGTATTCCAAAACA-3’ for *PSS* ( Savouré et al. 1993), 5’-GCTCCAGTCTACCTGTGTCATGCTTAC-3’ and 5’-CCGGTGGAAGGCCAATAGGGATGG-3’ for *CycA2;1* ( Ferreira et al. 1994), 5’-CCAGACCGCCCCACTACCTAGGACTT-3’ and 5’-CGGGTTAAGGCTGAAATGGAATTC-3’ for *CycB1;1* (Hemerly et al. 1992), 5’-GCCACTCTCTGAGTCAGGGTTTCCCATCG-3’ and 5’-GGCAGCTCCTCCAAGTCTTGAAGT-3’ for *CDC2a* (Hirayama et al. 1991), and 5’-GGTGCTTGGGCTTTAGCTTG-3’ and 5’-CCAAGACGATGACAAAAATGTC-3’ for *CDC2b* (Imajuku et al. 1992). The PCR reactions were performed with one denaturation cycle of 2 min at 94 °C and 13 (for *PSS* and *CDC2a*) or 18 (for *CycA2;1*, *CycB1;1*, and *CDC2b*) cycles of denaturation at 94 °C for 30 s, of annealing at 56 °C for 30 s, and of extension at 72 °C for 60 s. Subsequently, 10 μl of the amplification mix was separated electrophoretically on a 0.9% agarose gel and transferred to a nitrocellulose filter (Hybond-N-+; Amersham) by the DNA gel blot method (Southern 1975). The DNA was fixed on the membrane by UV cross-linking. Hybridizations were performed with gene-specific probes, generated from *A. thaliana* seedlings by PCR with the primer combinations as described above. After detection with the Gene Image CDP-star prime labeling module (Amersham), the intensity of the signals was estimated by densitometry on autoradiographs using the program Image Master (Pharmacia).