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EFFECTOR OF TRANSCRIPTION2 is involved in xylem differentiation and includes a functional DNA single strand cutting domain

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Dedicated to the 65th birthday of Prof. Dr. Ulrich Wobus.

Abstract

EFFECTORS OF TRANSCRIPTION2 (ET) are plant-specific regulatory proteins characterized by the presence of two to five C-terminal DNA- and Zn-binding repeats, and a highly conserved cysteine pattern. We describe the structural characterization of the three member *Arabidopsis thaliana ET* gene family and reveal some allelic sequence polymorphisms. A mutation analysis showed that *AtET2* affects the expression of various *KNAT* genes involved in the maintenance of the undifferentiated state of cambial meristem cells. It also plays a role in the regulation of *GA5* (gibberellin 3-beta-dioxygenase) and the cell-cycle-related *GASA4*. A correlation was established between *AtET2* expression and the cellular differentiation state. AtET–GFP fusion proteins shuttle between the cytoplasm and nucleus, with the *AtET2* product prevented from entering the nucleus in non-differentiating cells. Within the nucleus, AtET2 probably acts *via* a single strand cutting domain. A more general regulatory role for ET factors is proposed, governing cell differentiation in cambial meristems, a crucial process for the development of plant vascular tissues.

Keywords: Gene regulation; Xylem differentiation; Transcription factors; Single strand cutting; GIY-YIG domain

Introduction

Plant growth is determined by the action of a small number of cells present at the shoot and root apical meristems. The vascular cambium is a secondary meristem, derived from the shoot apex. Although these meristems differ in function, a growing body of evidence suggests that their regulation shares many common principles and related genes (Groover, 2005). One of the products of cambial activity is the xylem, which develops towards the centre of the stem. The differentiation of xylem cells from the cambium is characteristically accompanied by a gradual accumulation of lignin, which therefore serves as a useful indicator of the progression of xylem cell differentiation. This process is controlled by the activity of several factors, including the phytohormone gibberellin (GA) and transcription factors of the *KNOTTED1*-like homeobox *KNAT* family (Hake et al., 2004; Scofield and Murray, 2006).

The plant hormone gibberellin (GA) is essential for the differentiation of the vascular tissues. Experiments in poplar, hybrid aspen and tobacco (Israelsson et al., 2003; Biemelt et al., 2004) have demonstrated that transgenic plants which ectopically express the biosynthetic gibberellin 3 beta dioxygenase encoding gene (*GA5*) exhibit significantly increased levels of xylem lignification. On the contrary, depletion of active GA by the ectopic expression of a gene which encodes the GA degrading enzyme *GA2-oxidase* inhibits lignin accumulation in tobacco (Biemelt et al., 2004). Furthermore, expression profiling in hybrid aspen showed an induction of GA-regulated genes in the early stages of cell differentiation near the cambial meristem (Hertzberg et al., 2001a).

Members of the *KNAT* gene family act as major regulators of several GA-mediated functions by inhibiting both the biosynthesis of and the meristematic response to GA. The *KNAT* genes *BREVIPEDICELLUS* (*BP*) and *SHOOTMERISTEMLESS* (*STM*) act redundantly to repress the transcription of *Arabidopsis thaliana GA5* (gibberellin 3-beta-dioxygenase) (Hay et al., 2002). In addition, the tobacco protein NTH15 represses the expression of

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the GA20 oxidase gene *NTC12* by interacting directly with an element in its promoter (Sakamoto et al., 2001a). *KNAT* gene expression in the cambium is essential for the control of xylem differentiation and lignin formation (Smith and Hake, 2003; Brown et al., 2005; Ehlting et al., 2005). In particular, *BP* activity prevents cambium-derived cells from differentiating into lignified xylem tissue (Mele et al., 2003). Other class I *KNAT* genes such as *At STM* and *KNAT2* and poplar *KNAP2* have similar activity (Israelsson et al., 2003; Ko and Han, 2004; Schrader et al., 2004a; Groover, 2005; Demura and Fukudo, 2007).

In addition to *KNAT* genes, the previously characterized members of the EFFECTOR OF TRANSCRIPTION (ET) family including the barley protein HORDEUM REPRESSOR OF TRANSCRIPTION (HRT) are also involved in GA-mediated processes of xylem differentiation (Raventos et al., 1998; Ellerstrom et al., 2005). Originally, ET factors have been isolated as DNA-binding proteins by south-western screens from *Hordeum vulgare*, *Brassica napus* and *Vicia faba*. They represent strictly plant-specific proteins characterized by one (*Physcomitrella patens*), two (*V. faba*), three (*H. vulgare*), four (*B. napus*, *A. thaliana*) or five (*Populus trichocarpa*) highly conserved cysteine-containing structural domains with a $CX_{8-9}CX_{10}CX_2H$ consensus sequence, designated as ET repeats. These repeats have been shown to bind zinc in a stoichiometric ratio of close to 1:1, although the cysteine pattern differs greatly from classical zinc finger motifs. The HRT protein interacts with gibberellin response elements of various promoters, whereas *B. napus* ET – although it is able to strongly interact with DNA – does not show a clear sequence specificity as it was shown by an ELISA-based binding assay (Mönke et al., 2004 and Mönke et al., unpublished). Barley HRT is targeted to the nucleus and functional tests in plant cells indicated that HRT can regulate the activity of certain GA-responsive promoters, including two α-amylase gene promoters. Northern hybridizations indicate that *HRT* transcripts accumulate to low levels in various tissues and a role for HRT in mediating developmental and phytohormones-responsive gene expression have been discussed (Raventos et al., 1998). Recently, we have described a dicot ET factor from *B. napus* (BnET) providing evidence for its role in gibberellin signaling modulation and cell differentiation. BnET is also targeted to the nucleus and its ectopic expression in either *Arabidopsis* or tobacco causes a pleiotropic phenotype including dwarfism due to shorter internodes and late flowering, reduced germination rate, increased anthocyanin content and reduced xylem lignification as a marker for terminal cell differentiation. Transient expression in protoplasts and transcript analysis support the notion that this is most likely due to a transcriptional repression of GA-controlled genes. In contrast to other GA-deficient mutants, the shorter internodes were due to fewer but not smaller cells, suggesting a function of BnET in GA-mediated cell division control (Ellerstrom et al., 2005).

In this paper, we present the initial characterization of the ET family in *Arabidopsis*. A T-DNA insertion in the *AtET2* gene leads to defects in xylem differentiation as detected by distortions of lignification. Array hybridization and RT-PCR analysis demonstrate altered expression of several GA-related genes and members of the *KNAT* family. Two of the three *AtET* genes are specifically up-regulated in differentiating cells and their regulation involves post-transcriptional control of their nuclear localization, preventing the AtET1 and AtET2 factors from entering the nucleus in non-differentiating cells. The molecular function of ET proteins as regulators of transcription most likely involves the activity of a functional single strand cutting domain. The data suggest a novel function of ET factors in the regulation of cell differentiation in cambial meristems.

Materials and methods

Molecular cloning

PCR, restriction digestion and DNA ligation were performed according to standard protocols (Molecular Cloning Third Edition, eds. Sambrook J. and Russel D., CSH Laboratory Press).

CAPS marker for the mutated AtET1 allele

A 1244-bp genomic fragment spanning the mutation site and an *Eco*RI cleavage site was amplified from the *AtET1* sequence, using primers 5′-ATGTTCAAGAGAGACGACTACA and 5′-ATCCTCGCATCGTTTTCTCC. The amplicon was digested with *Eco*RI (Amersham) and sized by agarose gel electrophoresis. The wild-type allele produced a 1082-bp fragment, whereas the frame-shifted allele produced a 897-bp one.

Plant transformation

The Ws-2 ecotype was transformed by vacuum infiltration as described (Bechthold et al., 1993).

ProAtET2-driven GUS expression

A 1.5-kbp upstream region of *AtET2* was placed ahead of the GUS reporter gene in the plasmid pMDC162 (Curtis and Grossniklaus, 2003), using GATEWAY cloning technology (Invitrogen). GUS activity was assayed in 2- to 4-week-old homozygous T3 plants following standard histological procedures, and the signal was visualized with a “Axioplan 2 imaging mot” (Zeiss, Jena, Germany) light microscope or a “StereoLumar V12” (Zeiss, Jena, Germany) binocular microscope.

In situ hybridization

Segments from the basal 5 mm of stem were fixed for 3 h, following vacuum infiltration with 2% paraformaldehyde, 0.2% glutardialdehyde, 0.01% Triton X100 in 0.5 M cacodylic acid buffer, pH 6.8. After two washing steps of 30 min each in the same buffer, the specimens were dehydrated in a graded ethanol series (10%, 30%, 50%, 70%, 90%, twice 96%, 100%) for at least 30 min per step. All buffers were prepared with ddH_20 treated with 0.05% DEPC. The embedding method was adapted from Tiedemann et al. (2000), using reduced incubation times. Samples were taken from plants subjected either to short days (8 h light) until bolting, or maintained under long days (16 h light). Hybridization was with *in vitro* transcribed riboprobes as described previously (Tiedemann et al., 2001) with the following modifications: hybridization conditions were 16 h at 50 °C, and the sections were washed (2 × 1 h) in 50% formamide in 0.5× SSC. Following RNase-A digestion (20 μg/ml, 5 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5), the sections were subjected to an additional stringent wash (50% formamide in 0.1× SSC, 50 °C) for a further 30 min. The primers used for the gene-specific probe synthesis were

T7 promoterAtET1 (antisense probe): 5′-AAACGACGGCCAGTGAATTG TAATACGACTCACTATAGGGCGAGTGACAACCAAACCGAAGAG;