Gene 522 (2013) 177–183

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Stage-specific regulation of four HD-ZIP III transcription factors during polar pattern formation in *Larix leptolepis* somatic embryos

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ARTICLE INFO

Article history:
Accepted 22 March 2013
Available online 6 April 2013

Keywords:
Somatic embryo
HD-ZIP III
Polar auxin transport
NPA
Larix leptolepis

ABSTRACT

Polar auxin transport provides a developmental signal for cell fate specification during somatic embryogenesis. Some members of the HD-ZIP III transcription factors participate in regulation of auxin transport, but little is known about this regulation in somatic embryogenesis. Here, four *HD-ZIP III* homologues from *Larix leptolepis* were identified and designated *LaHDZ31*, *32*, *33* and *34*. The occurrence of a miR165/166 target sequence in all four cDNA sequences indicated that they might be targets of miR165/166. Identification of the cleavage products of *LaHDZ31* and *LaHDZ32 in vivo* confirmed that they were regulated by miRNA. Their mRNA accumulation patterns during somatic embryogenesis and the effects of 1-N-naphthylphthalamic acid (NPA) on their transcript levels and somatic embryo maturation were investigated. The results showed that the four genes had higher transcript levels at mature stages than at the proliferation stage, and that NPA treatment down-regulated the mRNA abundance of *LaHDZ31*, *32* and *33* at cotyledonary embryo stages, but had no effect on the mRNA abundance of *LaHDZ34*. We concluded that these four members of *Larix HD-ZIP III* family might participate in polar auxin transport and the development of somatic embryos, providing new insights into the regulatory mechanisms of somatic embryogenesis.

1. Introduction

The somatic embryo is a useful tool for studying the molecular mechanisms underlying pattern formation in plant embryos (von Arnold et al., 2002). During embryogenesis, polar auxin transport (PAT) plays a central role in regulating apical–basal pattern formation (Weijers et al., 2005). Disturbing PAT often results in abnormal embryos (Friml et al., 2003; Jeong et al., 2011). To increase the production of somatic embryos, it is important to investigate the precise role of the regulators involved in PAT.

The direction of auxin transport is determined by the asymmetric membrane localization of efflux carriers known as PIN proteins. Changes in the expression or localization of PINs cause defects in embryo specification (Moller and Weijers, 2009). The suppression of auxin flow by auxin transport inhibitors also results in abnormal embryonic pattern formation. For example, treatment with 1-N-naphthylphthalamic acid (NPA) during the formation of somatic embryos of Norway spruce resulted in abnormal cell division, decreased programmed cell death, and apical and basal abnormalities (Larsson et al., 2008).

PAT is a complex process modulated by the precise spatial and temporal expression of regulatory genes. A loss of function of some members of the HD-ZIP III family results in abnormal *PIN1* expression and subsequent changes in auxin transport (Izhaki and Bowman, 2007; Zhong and Ye, 2001). HD-ZIP III proteins are transcription factors unique to plants that play key roles in many developmental aspects, including shoot apical meristem (SAM) formation and maintenance, the adaxial identity of lateral organs (Byrne, 2006; Emery et al., 2003; Kim et al., 2008; McConnell et al., 2001) and vascular patterning and differentiation (Ilegems et al., 2010). The apical regions of *phb phv rev* (three members of the *HD-ZIP III* family) embryos consist of a single radial cotyledon and lack both bilateral symmetry and the SAM, indicating that *HD-ZIP III* genes are required to properly pattern the apical region of the globular embryo (Emery et al., 2003; Prigge, 2005). However, the relationship between HD-ZIP III and PAT during somatic embryogenesis is largely unknown.

Members of the *HD-ZIP III* family are regulated at the transcript level by miR165/166 and at the protein level by LITTLE ZIPPER proteins (Wenkel et al., 2007). Transgenic *Populus* expressing a miR165/166-resistant form of PRE, a member of the *HD-ZIP III* family, showed abnormal formation of cambia within the cortical parenchyma, suggesting that PRE plays an important role in the initiation of the cambium and in regulating the patterning of secondary vascular tissues (Robischon et al., 2011). In embryogenesis, miR165/166 regulation of

Abbreviations: PAT, polar auxin transport; PIN, PIN-FORMED protein; NPA, 1-N-naphthylphthalamic acid; PCD, programmed cell death; HD-ZIP III, Class III Homeodomain Leucine Zipper proteins; PEM, proembryogenic mass; FAA, formalin–alcohol–acetic acid; SAM, shoot apical meristem; EST, expressed sequence tag; RACE, *rapid amplification of cDNA ends*; RLM-5′ RACE, RNA ligase-mediated amplification of cDNA ends; HD, DNA-binding Homeodomain; Zip, leucine zipper motif; RAM, root apical meristem.

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http://dx.doi.org/10.1016/j.gene.2013.03.117

HD-ZIP III transcription factors functions in the formation of the SAM (Juarez et al., 2004; Kim et al., 2005; Tang et al., 2003; Williams et al., 2005).

In our previous research, three mature miR166 sequences and two mRNA fragments of *HD-ZIP III* homologs were identified from *Larix leptolepis* somatic embryos, and cleavage products of these two mRNA fragments were detected (J. Zhang et al., 2012), indicating that miR165/166 may regulate *HD-ZIP III* transcription factors during *Larix* somatic embryogenesis. Subsequently, we supposed that the spatial and temporal expression of *HD-ZIP III* and miR165/166 might be modulated by PAT during polar pattern formation in *L. leptolepis* somatic embryos.

Based on the roles of HD-ZIP III proteins in plant embryogenesis and their involvement in regulating PAT, here we identified four full-length cDNA sequences of *HD-ZIP III* homologs from *L. leptolepis* and studied their mRNA accumulation patterns during somatic embryogenesis and the effects of NPA on their transcript levels and somatic embryo maturation.

2. Materials and methods

2.1. Plant materials and NPA treatment

Embryogenic tissues were generated from immature zygotic embryos of *L. leptolepis* in S basal medium (Ewald et al., 1995) supplemented with glutamine (450 mg L^{-1}), casein hydrolysate (500 mg L^{-1}), inositol (1000 mg L^{-1}), 2,4-D (2.2 mg L^{-1}), and BA (0.8 mg L^{-1}). Proembryogenic masses (PEMs) were maintained on S + B basal medium (Ewald et al., 1997) supplemented with glutamine (450 mg L^{-1}), casein hydrolysate (500 mg L^{-1}), inositol (1000 mg L^{-1}), 2,4-D (0.11 mg L^{-1}), and BA (0.04 mg L^{-1}), and subcultured in the same fresh medium every three weeks. To develop the somatic embryos further, about 100 mg of fresh PEMs was transferred directly to maturation medium (S + B + 450 mg L^{-1} glutamine + 500 mg L^{-1} casein hydrolysate + 100 mg L^{-1} inositol + 20 mg L^{-1} ABA + 7.5% w/v PEG4000). Somatic embryos were observed at different stages of somatic embryogenesis on maturation medium using a stereomicroscope and harvested at seven consecutive stages (Fig. 1).

To study the effects of PAT during somatic embryogenesis, 1-N-naphthylphthalamic acid (NPA; Sigma-Aldrich, Germany) was added to the maturation medium at a final concentration of 3 mg L^{-1}, as described previously (Hakman et al., 2009; Larsson et al., 2008). NPA-treated embryos were isolated at different developmental stages, defined primarily according to the sizes of phenotypically normal control embryos and not according to age (Larsson et al., 2012).

2.2. Histological analysis

Somatic embryos were fixed in formalin–alcohol–acetic acid (FAA), dehydrated in a series of ethanol, embedded in Spurr's resin (SPI, USA), longitudinally sectioned at 5 μm thick on an ultramicrotome (Leica EM UC7, Germany), and stained with toluidine blue O solution as described previously (Li et al., 2009). The sections were observed and photographed under an AXIOIMAGER A1 microscope (Zeiss, Germany) equipped with a computer-assisted AxioCam MRc5 camera (Zeiss). The pictures were further processed with Adobe Photoshop CS3.

2.3. Isolation and molecular characterization of LaHDZ31–34

To identify *HD-ZIP III* orthologs from *L. leptolepis*, we searched the ESTs in the *L. leptolepis* transcriptome (Y. Zhang et al., 2012) using *HD-ZIP III* of *Arabidopsis thaliana* as a query and 10 ESTs were found. After assembly with the ContigExpress Project software, four non-redundant contigs were identified. Based on these sequences, gene-specific primers for 5′ or 3′ RACE were designed (Supplemental Table S1) and *rapid amplification of cDNA ends* (RACE) experiments were performed with a SMARTer™ RACE cDNA amplification kit (Clontech, USA), following the manufacturer's instructions. The full-length cDNA sequences were submitted to GenBank, and designated *LaHDZ31*, *LaHDZ32*, *LaHDZ33* and *LaHDZ34*.

The HD-ZIP III amino acid sequences were predicted by searching the public databases at NCBI (http://www.ncbi.nlm.nih.gov/). HD-ZIP III amino acid sequences were aligned using the ClustalX program (version 1.83). A phylogenetic tree was constructed based on a full-length protein alignment with the neighbor-joining method using MEGA5. Bootstrap values at the branch points were calculated from 1000 replicates.

2.4. 5′ RNA ligase-mediated amplification of cDNA ends to identify the cleavage site

To identify cleavage of *LaHDZ31* and *LaHDZ32* by miRNA *in vivo*, RNA ligase-mediated amplification of cDNA ends (RLM-5′ RACE)

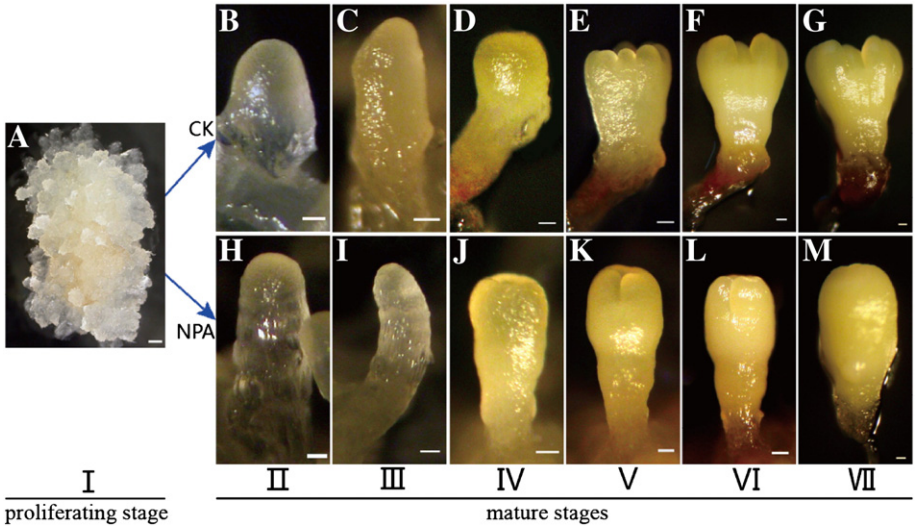

Fig. 1. Development of control and NPA-treated somatic embryos of *L. leptolepis*. A, stage I, proliferating proembryogenic masses (PEM) after 15 days of sub-culture in new proliferation medium. NPA was added to the cultures when PEMs were transferred to maturation medium. For B–M, number ranges indicate days after PEMs were transferred to maturation medium. B and H, stage II, early single embryos, 5–7 d. C and I, stage III, middle single embryos, 8–14 d. D and J, stage IV, late single embryos, 15–21 d. E and K, stage V, early cotyledonary embryos, 22–28 d. F and L, stage VI, middle cotyledonary embryos, 29–35 d. G and M, stage VII, fully mature embryos, 36–42 d. Scale bars, 100 μm (B–M); 1 mm (A).