Available online at www.sciencedirect.com
ScienceDirect
Gene 401 (2007) 28–37
GENE
www.elsevier.com/locate/gene

Comparative analysis of the SBP-box gene families in *P. patens* and seed plants

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Received 20 March 2007; received in revised form 14 June 2007; accepted 20 June 2007
Available online 10 July 2007
Received by G. Theissen

Abstract

To come to a better understanding of the evolution and function of the SBP-box transcription factor family in plants, we identified, isolated and characterized 13 of its members from the moss *Physcomitrella patens*. For the majority of the moss SBP-box genes, clear orthologous relationships with family members of flowering plants could be established by phylogenetic analysis based on the conserved DNA-binding SBP-domain, as well as additional synapomorphic molecular characters. The *P. patens* SBP-box genes cluster in four separable groups. One of these consists exclusively of moss genes; the three others are shared with family members of *Arabidopsis* and rice. Besides the family defining DNA-binding SBP-domain, other features can be found conserved between moss and other plant SBP-domain proteins. An AHA-like motif conserved from the unicellular alga *Chlamydomonas reinhardtii* to flowering plants, was found able to promote transcription in a heterologous yeast system. The conservation of a functional microRNA response element in the mRNA of three of the moss SBP-box genes supports the idea of an ancient origin of microRNA dependent regulation of SBP-box gene family members.

As our current knowledge concerning the roles of SBP-box genes in plant development is scarce and the model system *P. patens* allows targeted mutation, the material we isolated and characterized will be helpful to generate the mutant phenotypes necessary to further elucidate these roles.

Keywords: Land plant evolution; Rice; *Arabidopsis*; Transcription factor; AHA-motif; miR156

1. Introduction

In plants, as in all living organisms, transcription factors represent an important level of gene regulation. They enable plants to appropriately regulate growth, differentiation and metabolism, and to respond to endogenous and environmental cues. Particular transcription factors are able to directly interact with DNA in a sequence specific manner. Evolutionary conservation of the respective DNA-binding domains, allow to define over thirty such transcription factor families in the seed plant model *Arabidopsis thaliana*, half of which appear to be unique to plants (Riechmann et al., 2000; Iida et al., 2005). One of these families consists of SBP-domain proteins (Klein et al., 1996) and is in *Arabidopsis* represented by the 17 members of the *SPL* gene family (Cardon et al., 1999).

The SBP-domain encompasses *ca.* 74 amino acid (aa) residues, harbors a nuclear localization signal at its C-terminus and is sufficient to bind DNA involving two zinc-fingers of unusual structure (Klein et al., 1996; Yamasaki et al., 2004, 2006; Birkenbihl et al., 2005).

The corresponding genes, carrying the SBP-domain encoding SBP-box, have found to be highly conserved in green plants, from unicellular algae (Kropat et al., 2005) to mono- and dicotyledonous angiosperms (Cardon et al., 1999; Xie et al., 2006).

Our current knowledge of the regulatory roles SBP-box genes may play in plant development is rather superficial and largely based on a few identified mutant phenotypes. In maize, for instance, mutations of the SBP-box genes *LG1* and *TGA1* uncovered roles in

Abbreviations: aa, amino acid(s); cDNA, DNA complementary to RNA; CFP, cyan fluorescent protein; CRR1, Copper response regulator1; EST, expressed sequence tag; LG1, Liguleless1; miRNA, microRNA; MRE, microRNA responsive element; Myr, million years; RE, responsive element; SBP, SQUAMOSA promoter binding protein; SPL, SQUAMOSA promoter binding protein like; TGA1, teosinte glume architecture1; UTR, untranslated region; YFP, yellow fluorescent protein.

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doi:10.1016/j.gene.2007.06.018

leaf and glume development, respectively (Moreno et al., 1997; Wang et al., 2005). In *A. thaliana*, fertility is reduced upon *SPL8* loss-of-function and a role for *SPL8* as a local regulator of GA-mediated signalling has been suggested (Unte et al., 2003; Zhang et al., 2007). Stone et al. (2005) discovered that a reduction of *SPL14* expression increased resistance to the fungal toxin fumonisin B1. In addition, the *spl14* mutant displayed elongated petioles and enhanced leaf serration. Constitutative over-expression of *SPL3* and related SBP-box genes may cause earliness in transgenic *Arabidopsis* lines suggesting a role for these SBP-box genes during the floral transition (Cardon et al., 1997; Wu and Poethig, 2006). Interestingly, these latter genes represent a subfamily of SBP-box genes targeted by the highly similar miRNAs miR156 and miR157 (Rhoades et al., 2002; Schwab et al., 2005; Xie et al., 2006; Wu and Poethig, 2006; Gandikota et al., 2007). This interaction between SBP-box genes and miR156 seems to be of ancient origin as it could also be detected in mosses (Arazi et al., 2005). In addition to the phenotypes obtained from genetic alterations in SBP-box genes, it has recently been shown that an epigenetic mutation of an SBP-box gene causes the Colorless non-ripening phenotype in tomato (Manning et al., 2006). Finally, the only non-seed plant SBP-box gene mutant described to date concerns the *COPPER RESPONSE REGULATOR1* (*CRR1*) in *C. reinhardtii* required for both activating and repressing target genes of a copper- and hypoxia-sensing pathway (Kropat et al., 2005).

In general, the mutant phenotypes obtained so far suggest more physio-developmental roles for SBP-box genes. Furthermore, SBP-box genes in seed plants appear in moderately sized families and with sufficient degrees of similarity between different members, functional redundancy is to be expected. Together, this may lead to less obvious mutant phenotypes, especially under optimal growth conditions.

In order to reduce these restrictions in recognizing and elucidating the molecular genetic mechanisms underlying SBP-box gene actions in plant development, we decided to choose the moss *Physcomitrella patens* as a model system.

In contrast to flowering plants where a reversed situation is encountered, the life cycle of mosses is dominated by a haploid and photoautotrophic gametophyte that supports the diploid sporophytic generation (see Reski, 1998; Cove et al., 2006 for review). As most important difference to flowering plant molecular genetic models, *P. patens* offers the possibility of efficient gene targeting via homologous recombination (reviewed by Schaefer, 2002) as well as the possibility to make double or triple gene knockouts in one step (Hohe and Reski, 2003). Moreover, *P. patens* can fully complete its life cycle when grown *in vitro* and is thus easily accessible for manipulation in different environmental growth conditions.

Mosses and flowering plants are believed to be of monophyletic origin and evolutionary separated for around 500 Myr (Kenrick and Crane, 1997; Nickrent et al., 2000). Although mosses follow relatively simple developmental patterns, they do share many basic morphological features and physiological responses with other land plants, which make them interesting subjects for comparative evolutionary studies.

Here we report the molecular cloning and first characterization of 13 new SBP-box genes from the moss *P. patens*.

Phylogenetic reconstruction based on the conserved DNA-binding domain as well as comparison of additional synapomorphic molecular characters established clear orthologous relationships between the majority of the moss SBP-box genes and those of flowering plants.

2. Materials and methods

2.1. Plant material

P. patens ssp *patens* B.S.G. was grown under standard conditions as described by D.G. Schaefer (http://www.unil.ch/lps/docs/Ppprotocols2001.pdf).

2.2. Construction and screening of cDNA and genomic DNA library

We screened a RAGE (Rapid Analysis of Gene Expression)-pool of digested genomic DNA linked to adapter sequences as described by Henschel (2002) (kindly provided by K. Münster, MPIZ, Cologne, Germany) with the nested RAGE adapter-primers PAP1 (GTAATACGACTCACTATAGGGC) and PAP2 (ACTATAGGGCACGCGTGGT) and SBP-box primers SH65 (GATTACCATCGGCGGCAYAARGTNTG) and SBP1 (CATMGNTTCTGCCAGCAGTG). Based on the isolated sequences, primers were generated and a genomic library of *P. patens* was screened.

For the isolation of cDNAs representing SBP-box genes a library of cDNAs cloned in the phage vector λNM1149 was screened under stringent (2xSSC, 0.1% SDS; hybridization 68 °C, washing 60 °C) and moderate conditions (5xSSC, 0.1% SDS; hybridization 55 °C, washing 58 °C). The library represented all stages of the *P. patens* life cycle including protonema, young and mature gametophores and sporophytes of different stages.

Two SBP-box sequences encompassing only the SBP-box previously identified from a genomic library, and two EST's pp020005015 and pp15003060 from the Freiburg EST collection (Lang et al., 2005), were used for the screen. A segment of all four sequences was amplified by PCR using the following primer pairs: SH191 (TTGGGAAAGAGACATCGGGCAGG) and SH77 (CGGCAGCTTCGTTTGCCCTCGTC) for *PpSBP1*; SH143 (CGGCAGCTTCGTTTGCCCTCGTC) and SH79 (GCTTGATCCTCAACTCGAGGTGTCG) for *PpSBP2*; MR07 (CAAATTGCCGCAGTGAACTTGAGGACG) and MR09 (TGAGGAGCCCGACGAAGATTTG) for *PpSBP3*; SH178 (CATCGTCGACACAAAGTGTGTGAGC) and MR01 (GTCTTAACGCTTCATATCTTGCGAG) for *PpSBP4*. The PCR-fragments were radioactively labelled with α-^{32}P dCTP by a Klenow fill-in reaction and used as probes. A mixture of the labelled PCR-fragments of *PpSBP2* and *PpSBP4* were used in the heterologous screening of the cDNA library.

From the stringent screen we identified the full-length cDNA's of *PpSBP1*, *PpSBP3* and *PpSBP4* and the 5′ part of *PpSBP2*. Screening under moderate conditions identified the 3′ part of *PpSBP7*.

The cDNA was amplified sequenced after sub-cloning into plasmid vector pCR2.1 TOPO using the TOPO TA cloning kit