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Abstract of Ph.D thesis

Characterization of LOB-domain transcription factor family of *Brachypodium distachyon* and testing protein interactions regarding to two members of the family

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Introduction

Basic architecture of higher plants consists of stems, lateral branches, leaves and flowers emerging on the stems and root system which is built up from primary root and lateral roots. Organ development of plants is almost entirely postembryonic which means that plants – in contrast to animals - are able to produce new organs and tissues continuously during their entire lifespan. This ensures not only extremely flexible ontogeny and indeterminate growth for plants but enables adaptability to continuously changing environmental conditions, too.

Plasticity of plants can be traced back to two main sources: partly maintenance of pluripotent stem cell activity in meristems and partly competence of differentiated cells to form new meristems are responsible for the open and indeterminate plant development. However, in order to develop a proper and proportional architecture, cell division and differentiation of daughter cells must be coordinated precisely both in space and time in the case of plants as well. Establishment borders between meristems and emerging organ primordias plays an important role in this process. According to our recent knowledge, meristems to organ boundaries are discrete physical borders consisting of specialized cells rather than philosophical decision between "being a meristem or being a part of an organ". Boundary cells are required not just for restriction of meristematic stem cell niche but also for maintaining connection between meristems and developing organs. Although it is not known how they regulate plant organogenesis in details, some genes determining function and identity of boundary cells have already been characterized. Genes typical of boundary cells are mainly transcription factors such as CUC (CUP-SHAPED COTYLEDON), PTL (PETALLOS), or BOP (BLADE ON PETIOLE) which form complex gene regulatory networks in order to keep the balance between cell division and differentiation.

The firstly characterized LBD (LATERAL ORGAN BOUNDARIES DOMAIN) gene was originally discovered as a result of an enhancer trap experiment in *Arabidopsis thaliana* as one of these boundary cell identity genes, about 15 years ago. Name of the family comes from this characteristic expression pattern: founding member of the family is active preferentially at meristem to lateral organ boundaries. Common feature of LOB-domain transcription factors is that they have an approximately 100 amino acid long, highly conserved domain motif – termed as LOB-domain (or DUF260 – domain of unknown function) - at their N-terminal. This domain contains three different regions: a cysteine-rich motif ($CX_2CX_6CX_3C$) or C-block, central glycine-serine-alanine residues containing "GAS-block" and a reminiscent of a leucin-

zipper motif ($LX_6LX_3LX_6L$). Based on literature C-block has DNA-binding activity while the leu-zip like motif renders protein-protein interactions. LOB-domain motif can be found in 43 *Arabidopsis* proteins and in average LBD family consists of few dozens of members in higher plants but LBD transcription factors are found from *Carophytae* green algae to higher plants in every plant species. They are highly plant-specific suggesting plant specific roles to LOB-domain proteins and seem to be evolutionary conserved.

Known functions of LBDs are mainly related to regulation of developmental processes and organogenesis. It has been proven several times, that LBDs - besides establishing meristem to organ borders – control highly diverse aspects of plant development: e.g. they are involved in regulation of photomorphogenesis, leaf formation, lateral root emergence, floral development, microsporogenesis, callus formation and *in vitro* plant regeneration. Most of the LBDs direct development of several different organs simultaneously and it is reported frequently that different combinations of LBDs are responsible for these diverse functions. Despite LBDs have highly diverse roles in plant development it seems to be common that they are necessary for conversation of environmental stimuli and plant hormonal signals (e.g. auxin, cytokine or brassionosteroids) to developmental program. Although detailed function of LBDs at cellular and molecular level is mainly unrevealed, it is suggested that LOB-domain transcription factors direct organogenesis in close relation to the cell cycle. Phenotypic abnormalities caused by LBD mutation can be traced back often to cell division and differentiation abnormalities. For instance lack of AtLBD6 causes asymmetric leaf morphology as a consequence of dedifferentiation and enhanced cell proliferation of cells at the adaxial site of the leaf lamina. Deficiency of orthologous gene to AtLBD6 in maize (*Zea mays*) (ZmIg1 - Indeterminate gametophyte1) affects among others embryo sac development and cause appearance of extra synergids and egg cells in the ovule which can be explained by the extended proliferative phase of female gametophyte. A similar example is AtLBD27/SCP (SIDECAR POLLEN) that regulates the orientation and timing of microspore cell division *in Arabidopsis*.

We have hardly any information about mechanisms which links LBDs to cell division but previous results of our laboratory suggests that direct connection might exist between LBDs and cell cycle regulating machinery. About 20 years ago we were looking for interacting partners of alfalfa (*Medicago sativa*) A2 cyclin via Y2H (yeast two-hybrid) experiments. As a results we found a protein named MtCPP1 (Cyclin Partner Protein1) which later on proved to be a LOB-domain transcription factor (unpublished). Interaction between *Medicago* A2 cyclin

and MtCPP1 suggested us that LBDs themselves might be directly regulated by core regulators of cell division and few years ago it brought LBD gene family to our attention.

Aims

Owning to their diverse but important role in organogenesis, LBD genes belongs to intensively studied gene families and literature related to their characterization is broadening remarkably. Revealing the functions of LOB-domain transcription factors contributes not just to better understanding of molecular mechanisms prevailing at meristem to organ boundaries but comprehensive analysis of LBD gene family extended to several plant species helps us to better understand the evolution of plant ontogeny. However, our knowledge on LBDs are mainly comes from results of *Arabidopsis* experiments. LBDs in other species are poorly studied. For this reason one of our goals was comprehensive characterization and analysis of LBD gene family in purple false brome (*Brachypodium distachyon*), the broadly used model organism of grasses and cereals. We had three main scopes related to LBD gene family characterization:

- Identification of LOB-domain transcription factor coding genes in *Brachypodium* genome.
- Comparative phylogenetic analysis of *Brachypodium* LBDs.
- Detailed expression pattern analysis of *Brachypodium* LBD genes to determine their tissue and organ specific activity and comparison of their expression profiles to expression patterns of LBDs in other species.

Although there are no concrete evidences proving the hypothesis that LBDs keep the balance between cell division and differentiation but several studies supports this idea. Hereafter we focused on two of *Brachypodium* LBDs which sowed the highest homology to *Medicago* MtCPP1. According to our previous Y2H experiments MtCPP1 might be one of the interacting partners of *Medicago* A2 cyclin. Regarding the known function of *Medicago* A2 cyclin in pericycle cell reactivation during root development, we suppose that interaction between *Medicago* A2 cyclin and MtCPP1 must be important in this process. We were interested in testing the presence of similar interactions in *Brachypodium*. In order to this we aimed

- Y2H testing of potential interactions between *Brachypodium* homologues of MtCPP1 and *Brachypodium* A2 cyclin, as the closest counterpart of *Medicago* A2 cyclin.

Materials and methods

In silico analysis of Brachypodium LOB-domain transcription factors

We used two different approaches for identification of *Brachypodium* LOB-domain transcription factors. First, we performed BLAST analysis against *Brachypodium distachyon* proteome (source: Phytozome v12 portal - http://phyzozome.org) using known amino acid sequence of *Arabidopsis* LOB-domain motif as a query. Second, we analyzed the outcomes of BLAST analysis with Pfam (http://pfam.xfam.org) and SMART (http://smart.embl-heidelberg.de) programs for detecting conserved domain structures within the sequences. We took into consideration those proteins having DUF260 domain and belonging to PF03195 LOB-domain protein family according to the results of the above mentioned sequence analysis for further experiments.

Phylogenetic analysis of *Brachypodium* LBD proteins was carried out by MEGA6 software (http://www.megasoftware.net/) using the following methods: BLOSUM62 protein substitution matrix combined with NJ (Neighbor-Joining) phylogenetic tree reconstructing method. For protein tree reconstruction we used conserved domain sequences of *Brachypodium* LOB-domain transcription factors. In order to study phylogenetic relationships between *Brachypodium* LBDs and LBDs of other plant species we analyzed phylogenetic relationships between LOB-domain proteins of *Brachypodium*, maize (*Zea mays*), rice (*Oryza sativa*) and *Arabidopsis* similar to the above mentioned methods using multiple sequence alignment of LOB-domain motif sequences as a template.

Whole genome synteny of *Brachypodium* genome and gene duplication analysis regarding to *Brachypodium* LBD genes was carried out based on data deposited on Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication).

Expression pattern analysis of Brachypodium LBDs

For expression analysis we used seedlings of Bd21 *Brachypodium distachyon* inbred line, the accession with fully sequenced and annotated reference genome. After 4 days of

stratification (darkness, 4 ^{o}C) seedlings were grown in sand:perlite 2:1 mixture under normal growth conditions (photoperiod: 18h light/6h darkness, light intensity: 250 μmol*m^{-2}*s^{-1}, temperature: 21-22 ^{o}C, relative humidity: 60-65%). To maintain optimal water content and nutrient supply, plants were irrigated every day with 0.5% Hoagland solution up to 80% field capacity of sand:perlite (100% relative filed capacity of sand:perlite is 260-265 g/kg). We collected 37 different plant parts at three different developmental stages: 25 vegetative plant parts (6 different root segments from root tip to lateral root nodes, 5 different nodes from shoot, 2 different internodes, leaf blades, ligules and leaf seats from leafs at 4 different developmental stages) were collected from 14 DAG (day after germination) old seedlings, 9 plant parts (flag leaf, 4 different parts of the inflorescence, flowers at 3 different developmental stages, anthers) were collected from 28 DAG old plantlets and seeds representing 3 different developmental stages were harvested from 40-45 DAG old plants. Approximately 200 plants were dissected precisely to collect at least 50-100 mg plant material for total RNA isolation.

Transcript amount of *Brachypodium* LBD genes was determined by quantitative real-time PCR relative to average expression of two reference genes. As templates we used cDNA synthesized from total RNA amount. For PCR reactions we used primer pairs specific to *Brachypodium* LBD genes. Detection of PCR amplification was carried out by standard settings of ABI Prism 7900 HT-Fast Real-Time detection system.

Materials and methods used for yeast two hybrid tests

We identified the closest homologues of MtCPP1 (MTR_5g083960) and alfalfa A2 cyclin/ MsCYC-A2 (CAB46038) in *Brachypodium* by TBLASTX analysis. Based on homology searching we selected *Brachypodium* A2 cyclin/BdCYC-A2 (Bd4g06827) and two LBD genes - BdLBD1C-1 (Bd2g34520) and BdLBD1C-2 (Bd2g53690) for Y2H experiments. For testing protein interactions we created the following constructs by classical molecular cloning methods using Y2H vectors of two different vector systems (Clontech): BdCYC-A2 was cloned to bait vectors (pGBK-T7 and pGBK-T9) and *Brachypodium* LBD genes were cloned to prey vectors (pGAD-T7 and pGAD-424), respectively. We transformed different combinations of the above mentioned vector constructs into yeast competent cells besides using empty vectors for testing auto activity and pBD-GAL4-MsCYC-A2 and pAD-GAL4-MtCPP1 in combination as a positive control.

For Y2H experiments we used two different *Saccharomyces cerevisiae* strains: AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-

GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1 UAS-MEL1TATA-lacZ, MEL1) and PJ69-4A (MATa trp1-Δ901, leu2-3,112 901, ura3-52, his3-Δ 200, gal4Δ, gal8Δ, GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ). For preparation and chemical transformation of yeast competent cells we used protocols published in Yeast Protocols Handbook (Clontech, 2001). Interaction of proteins was tested by two different methods: 1) testing auxotrophic complementation by culturing transformed yeasts on minimal SD-WLHA media and 2) measurement of β-galactosidase enzyme activity *in vitro* in protein extracts isolated from transformed yeasts grown on SD-WL media.

Results and discussion

We identified 28 LOB-domain transcription factor coding gene in *Brachypodium* genome. According to phylogenetic analysis, *Brachypodium* LBD proteins can be classified in two main classes (class I and class II) and members of class I can be divided into five further subclasses (A-E). Based on this classification we established an unambiguous nomenclature for *Brachypodium* LBD proteins (e.g. BdLBD1A-1) where unique name consists of three components: number after abbreviations of scientific name of *Brachypodium* and LBD gene family marks the main class while letter following it indicates subclass classification and the last number identifying a given BdLBD protein refers to gene order on the phylogenetic tree (protein diverged the earliest is marked with "1" while protein diverged the latest is ranked with the highest number within a given subclass). This classification of BdLBDs is consonant to classification of LBD proteins of other species. Moreover, comparative phylogenetic analysis of *Brachypodium*, rice, maize and *Arabidopsis* LBD proteins revealed that there is no LBD protein unique to *Brachypodium* nor monocot specific lineages can be found among LBD family members. However, there are some characteristic differences between monocots and dicots regarding the complexity of some subclasses: subclass IB seems to be enlarged in monocots while subclass IA is more expanded in dicots. Results of surveys investigating evolutionary origin of LBD genes indicate that whole genome duplication events and gene dispersion in combination with gene preservation had crucial role in diversification of LBD gene family. Our *in silico* synteny analysis of *Brachypodium* LBD genes also supports this idea. By our estimates approximately 40% of BdLBD genes originated from gene duplication events.

High functional divergence typical of LOB-domain transcription factors can be traced back definitely to this evolutionary history and extremely various tissue specific expressions of

LBD genes reflects their functional diversity. Detailed expression pattern analysis of *Brachypodium* LBD genes by qRT-PCR measurements revealed that tissue specific activity of LBD genes differs from each other not just regarding to different subclasses but transcript profiles of LBDs within a same subgroup show high heterogenity, too. Moreover, we observed several times that expression of closely related LBD genes differs from each other dramatically. Because of high variances of expression profiles it is incredibly difficult - and there is no sense - to assign one general function to an LBD subclass or a gene. Nevertheless, some general features can be recognised: e.g. members of subclass IA are active in parts of the leaves and in the inflorescence, while members of subclass IB can be characterized with root specific expression in general and genes in subclass IE are expressed mainly in anthers. Differing to them, members of subclass IC and ID have highly diverse expression profiles. E.g. among members of subclass IC there is a gene which is active only in vegetative plant parts, another one is expressed in parts of the root and in generative organs, and another one is expressed only in root segments, mainly in root tip. Regarding to subclass ID, LBD1D-1 is active in specific parts of the leaf such as ligules and sheaths, while LBD1D-2 is active in almost all vegetative plant parts we tested. Expressions of LBDs in Class II do not exhibit tissue specificity. The highly diverse expression patterns of LBDs is not only typical of *Brachypodium* but our results also correspond to expression dates of LBDs from other species and expression of each BdLBD genes show high similarity to the expression pattern of their counterparts in other species such as *Arabidopsis*, maize or rice. This suggests not just great functional diversity of LBD gene family but also suggests that diverse functions might be evolutionary conserved among different plant species.

We started to reveal function of BdLBD1C-1 and BdLBD1C-2 based on the hypothesized functional conservation of LBD genes. According to our previous yeast two-hybrid (Y2H) studies, A2;2 type cyclin of alfalfa (*Medicago staiva*) interacts with MtCPP1 (cyclin partner protein1), a LOB-domain transcription factor of *Medicago truncatula*. Based on homology of BdLBD1C-1 and LBD1C-2 to MtCPP1, we hypothesize that both of the *Brachypodium* LBDs might have direct connection to cell cycle regulation. In order to confirm this hypothesis we attempted to reproduce the original Y2H experiment with the usage of *Brachypodium* homologues of MtCPP1 and alfalfa A2 cyclin. Although we tested potential interaction between BdCYC-A2 and BdLBD1C-1, BdLBD1C-2 respectively with different vector combinations and we used two different yeast strains, our Y2H experiments did confirm this hypothetical interaction neither by testing auxotrophic complementation nor by measuring β-

galactosidase enzyme activity. According to our experiences these outcomes might be explained by general limitations of Y2H method rather than by de facto lack of interaction between BdCYC-A2 and BdLBD1C-1, BdLBD1C-2, respectively. (Our observations that presence of BdCYC-A2 hindered the normal growth of yeast cells support this idea).

However, we observed that *Brachypodium* A2 cyclin was able to interact with MtCPP1 and both BdLBDs were able to interact with alfalfa A2 cyclin in Y2H tests. This means for us that both LBD1C-1 and LBD1C-2 have the potential to interact with cyclin(s) and *Brachypodium* A2 cyclin also might be able to interact with LOB domain transcription factors, indicating direct connection of LOB-domain transcription factors to cell cycle regulation. Moreover, results of *in vitro* kinase assay strongly support this hypothesis: both LBD1C-1 and LBD1C-2 is phosphorilated *in vitro* by total cyclin-cyclin dependent kinase (CDK) complexes purified from *Brachypodium* cell suspension culture (Zoltán Zombori, Gábor Horváth, unpublished). However, based on these observations we can't conclude far-reaching statements regarding the relationships between BdLBD1C-1, BdLBD1C-2 and cell division. Yet there are more questions than answers in this aspect. We don't know which cyclin-CDK complex is involved in this process. We don't know which developmental processes are coordinated by cell cycle dependent phosphorilation based regulation of BdLBD1C-1 and BdLBD1C-2. It is questionable too, whether there are other LOB-domain transcription factors regulated by cyclin-CDK complexes. The effect of phosphorilation on the protein function also needs to be elucidated and we don't know whether this regulation mechanism is conserved and typical of other plant species too or not. Answering these basic questions requires further experiments and it provides opportunity to reveal a completely unknown but exciting aspect of plant ontogenesis and leads to better understanding of the relationship between cell division and differentiation.

Conclusions

Results of comprehensive analysis of *Brachypodium* LOB-domain transcription factors demonstrated that:

- Phylogeny of LOB domain transcription factor family of *Brachypodium* consisting of 28 members shows similarities to phylogeny of LBDs of other monocot and dicot species regarding to classification, structure of subclasses and distribution of

proteins among main groups. This can be explained with conserved evolutionary history of LBD protein family.

- Consonant to the well-established functional divergence of LOB-domain transcription factor family, *Brachypodium* LBD genes showed huge heterogeneity in tissue or plant part specific expression and in relative expression level. Due to the heterogeneous expression patterns no unambiguous functions can be associated with one to each subclass. However, expression patterns of BdLBD genes show high similarity to expression patterns of their homologues in other species suggesting functional conservation.

- Although knowledge of transcript profiles is not enough for understanding the role of LBD genes, but expression pattern analysis is an essential and integral part of functional characterization of a gene family and provides a solid base for further studies.

We started to study relationships between LOB-domain transcription factors and cell cycle regulation on the basis of previous Y2H experiments. As a part of this project we tested the potential interaction between BdLBD1C-1, BdLBD1C-2 (closest homologues of *Medicago* MtCPP1) and *Brachypodium* A2 cyclin (counterpart of alfalfa A2 cyclin) in Y2H system. Although Y2H tests didn't confirmed interactions between *Brachypodium* counterparts of MtCPP1 and MsCYC-A2 but the outcomes indicated that:

- BdLBD1C-1 and LBD1C-2 have the potential to interact with cyclin(s) since both of them showed interaction with alfalfa A2 cyclin.

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List of publications

Publications related to the Ph.D thesis

- Gombos, M., Zombori, Z., Szécsényi, M., Sándor, G., Kovács, H., & Györgyey, J. (2017). **Characterization of the LBD gene family in Brachypodium: a phylogenetic and transcriptional study**. Plant Cell Reports, 36(1), 61-79. IP$_{2016}$: 3.088

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Conference abstracts

János Györgyey, Mária Secenji, Zoltán Zombori**,** Magdolna Gombos, Mátyás Cserháti, Dénes Dudits: **Towards understanding of links between drought adaptation and root architecture of cereals.**FESPB/EPSO Plant Biology Congress, Freiburg 2012

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J. Györgyey Z. Zombori, M. Gombos, M. Szécsényi: **Use of *Brachypodium distachyon*, the model plant of temperate cereals to study root architecture during drought adaptation.** "The 26th Even-Ari Memorial Conference" Israeli-Hungarian scientific workshop on sustainable agriculture, 26-27 April, 2015, Sede Boqer, Israel

János Györgyey, Magdolna Gombos, Gábor V. Horváth, László Facskó, Györgyi Sándor, Mária Szécsényi, Zoltán Zombori: **LOB-domain transcriptional factors are not only key components of plant root development but may link cell division and differentiation in Brachypodium distachyon**. Plant Biology Europe EPSO/FESPB 2016 Congress, Prague (June 26-30, 2016)

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Mária Szécsényi, Zoltán Zombori, Magdolna Gombos, Györgyi Sándor, János Györgyey**: Root architecture of Brachypodium – morphological, molecular and genetic approaches.** 1st International Brachypodium Conference 2013, Modena, Italy

Zoltán Zombori, Mária Szécsényi, Magdolna Gombos, János Györgyey: **Improved *Agrobacterium*-mediated transformation method in *Brachypodium distachyon* for the characterization of LOB-domain transcription factors.** Plant Biology Europe FESPB/EPSO Congress 2014, Dublin, Ireland, 2014

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Conflict of interest

I myself as corresponding author of the following publications declare that authors have no conflict of interest and Magdola Gombos Ph.D candidate had a great contribution to the published results. Results discussed in her thesis are regarded as outcomes of her own scientific work.

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