Transcription factors MhyFIL1 and MhyFIL3 (Monotropa hypopitys) determine the asymmetric development of above-ground lateral organs in plants


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It is believed that the complete mycoheterotroph pinesap Monotropa hypopitys adaptively evolved from a photosynthetic mycorrhizal ancestor, which had lost its photosynthetic apparatus and vegetative organs (stem and leaves). The aerial part of the plant is a reproductive axis with sterile bracts and inflorescence with a flower type canonical for higher plants. The origin of leaves and leaf-like lateral organs is associated, among other factors, with the evolution of the YABBY genes, which are divided into “vegetative” and evolutionarily recent “reproductive” genes, with regard to their expression profiles. The study of the vegetative YABBY genes in pinesap will determine whether their functions (identification of cell identity on the abaxial surface of the lateral organs) are preserved in the leafless plant. In this study, the structural and phylogenetic analysis of the pinesap vegetative genes MhyFIL1 and MhyFIL3 is performed, the main conserved domains and motifs of the encoded proteins are characterized, and it is confirmed that the genes belong to the vegetative clade YABBY3/FIL. The effect of heterologous ectopic expression of the MhyFIL1 and MhyFIL3 genes on the phenotype of transgenic tobacco Nicotiana tabacum is evaluated. The leaves formed by both types of plants, 35S::MhyFIL1 and 35S::MhyFIL3, were narrower than in control plants and were twisted due to the changed identity of adaxial surface cells. Also, changes in the architecture of the aerial part and the root system of transgenic plants, including aberrant phyllostaxis and arrest of the shoot and root apical meristem development, were noted. Some of the 35S::MhyFIL1 and 35S::MhyFIL3 plants died as early as the stage of the formation of the first leaves, others did not bloom, and still others had a greatly prolonged vegetation period and formed fewer flowers than normal ones. The flowers had no visible differences from the control except for fragile pedicles. Thus, the absence of structural changes from the M. hypopitys flower in comparison to autotrophic species and the effect of MhyFIL1/3 heterologous expression on the development of tobacco plants indicate the preservation of the functions of the vegetative YABBY genes by the MhyFIL1/3 genes in pinesap. Moreover, the activity of YABBY transcription factors of the FIL clade in M. hypopitys is not directly related to the loss of the ability of pinesap to form leaves during the evolutionary transition from autotrophic nutrition to heterotrophy.

Key words: Monotropa hypopitys; mycoheterotroph; heterologous gene expression; abaxial-adaxial asymmetry; transcription factors; YABBY; "vegetative" YABBYs; FILAMENTOUS FLOWER.


Факторы транскрипции MhyFIL1 и MhyFIL3 (Monotropa hypopitys) определяют асимметричное развитие боковых органов надземной части растения

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Считается, что полный микогетеротроф, подъельник Monotropa hypopitys, адаптивно эволюционировал из фотосинтезирующего микоризного предшественника, потеряя при этом аппарат фотосинтеза и вегетативные органы (стебель и листья). Надземная часть растения представляет собой цветонос со стерильными прицветниками и соцветием с каноническим для высших растений типом цветка. У растений происхождение плоского листа и других листолиственных латеральных органов связаны с эволюцией генов YABBY, которые, в зависимости от профиля экспрессии, разделяются на «вегетативные» и эволюционно более поздние «репродуктивные» гены. Изучение «вегетативных» генов YABBY подъельника позволит выяснить, сохранились ли их функции (определение идентичности клеток абаксиальной по-
Vertically asymmetric organs (e.g., leaves, flowers) are a basic and universal feature of life. Recent advances in comparative genomics have provided the opportunity to study the evolution of leaf morphology and its implications for plant survival. The study of leaf evolution in Monotropa hypopitys, a member of the Monotropoideae subfamily of the Ericaceae family (Leake, 1994), is of particular interest due to its unique life strategy. Monotropa hypopitys is an achlorophyllous plant that develops a mycorrhizal relationship with pinesap, which allows it to thrive in environments where other plants are unable to do so. The absence of chlorophyll and the reliance on mycorrhizal partenogenesis have led to significant modifications in the plant's morphology and gene expression profiles.
M. hypopitys. The study of homologs of genes determining leaf asymmetry in higher plants in a complete mycotroph can expand the understanding of the evolution of the YABBY transcription factor family in the course of dramatic adaptive rearrangement of the plant.

Materials and methods
We invoked data from the transcriptome analysis of M. hypopitys roots, sterile bracts, and flowers (at the stage of anthesis) (Beletsky et al., 2016). To amplify and clone the coding sequence of the pinesap YABBY genes MhyFIL1 and MhyFIL3, primers were designed on the basis of previously identified gene transcripts (Shchennikova et al., 2018): forward – 5'-catcatgtcctcctcaaattctt-3' (for both genes), and reverse – 5'-ctctttggattagggggacaca-3' (MhyFIL1) and 5'-ctctttggattagggggacaca-3' (MhyFIL3). Total RNA was isolated from pinesap flowers, where the expression of the MhyFIL genes was highest (the RNeasy Plant Mini Kit, QIAGEN, USA), and used for cDNA synthesis (Reverse Transcription System, Promega, USA). The complete coding sequences of the MhyFIL1 and MhyFIL3 genes were amplified at the following PCR conditions schedule: denaturation 95 °С 5 min; 30 cycles of denaturation (94 °C, 30 s), annealing (55 °С, 30 s) and elongation (72 °С, 1 min); extension (72 °С, 7 min).

Amplificates of the expected size were purified (MinElute Gel 30 s) and elongation (72 °С, 1 min); extension (72 °С, 7 min). 30 cycles of denaturation (94 °С, 30 s), annealing (55 °С, 30 s) and elongation (72 °С, 1 min); extension (72 °С, 7 min).

Results
Structural and phylogenetic analyses of MhyFIL proteins (Shchennikova et al., 2018) by NCBI-BLAST, NCBI-CDD and MEGA 6.0 confirm that MhyFILs belong to the YABBY3/FIL clade (Fig. 1). MhyFIL3 is closer to the ancestor than two other proteins, MhyFIL1 and MhyFIL2. As expected, the closest relatives of MhyFIL are representatives of the YABBY3/FIL clade in species of the Ericales order (basal Asterids), which include pinesap (see Fig. 1). Members of the YABBY3/FIL clade of other asterids form a sister subcluster (see Fig. 1). Inside the clade YABBY3/FIL, proteins of rosids (Arabidopsis thaliana) form a basal subcluster to analyzed asterid proteins (see Fig. 1). Analysis of putative conserved motifs (MEME 5.0.1) in the analyzed proteins reveals two sequences characteristic of all YABBY transcription factors and corresponding to the zinc finger and YABBY domains (Bartholmes et al., 2012). YABBY3/FIL proteins differ from members of other clades by the presence of six clade-specific (interdomain and C-terminal) motifs, and proteins of asterids, including Ericales, have an N-terminal motif, which is absent from A. thaliana YABBY3/FIL proteins (Rosids) (see Fig. 1). According to the conserved motif scheme obtained, all three MhyFILs are structurally closer to FIL than to YABBY3 (A. thaliana) (see Fig. 1).

For functional analysis of transcription factors MhyFIL1 and MhyFIL3, transgenic N. tabacum plants with individual constitutive expression of the cDNA of each of the MhyFIL1 and MhyFIL3 genes were obtained. Independent transgenic regenerants T_0 35S::MhyFIL1 (3 plants) and 35S::MhyFIL3 (12 plants), which rooted and formed true green leaves, were adapted to greenhouse conditions and then compared with the control (nontransgenic tobacco).

In contrast to the control, the obtained tobacco plants, 35S::MhyFIL1 and 35S::MhyFIL3, developed the bushy structure (instead of a single stem), had a significantly longer vegetation (on average, 282 days vs. the control 48 days), and formed abaxially twisted leaves (with an altered identity of the adaxial surface), and a strongly thickened and shortened root with abnormal leaf-like outgrowths (instead of an extensive root system) (Fig. 2).

Reproductive axes that developed on one of the shoots of the bushy 35S::MhyFIL1 and 35S::MhyFIL3 plants produced flowers outwardly similar to wild flowers, but often with rotting/brittle pedicles. Seeds were obtained from only one bushy plant 35S::MhyFIL3 formed a wild-type shoot that blossomed and gave seeds. The obtained seeds germinated, but the seedlings were characterized by abnormal development of roots (severe shortening and arrest in development) and shoot meristems (maximum shoot height 1.5–3.0 cm, bushiness, early development stop), which led to the death of the seedlings. In this regard, further analysis of the transgenic phenotype was impossible.

A microscopic analysis of the leaf surface of transgenic plants in comparison with the control confirmed that the cell identity on the adaxial side was partially changed as a...
result of heterologous transgene expression. There appeared stomata-like structures, which normally should not be on the upper surface of the leaf. Probably, they were the cause of leaf twisting.

**Discussion**

It is believed that mycoheterotrophic plants adaptively evolved from photosynthetic mycorrhiza lines, and the growth of such plants at poor insolation led to the inactivation and loss of the photosynthesis apparatus (Bidartondo, 2005; Buchanan-Wollaston et al., 2005; Zhang, Zhou, 2013; Ravin et al., 2016). In pinesap *M. hypopitys*, this was probably the cause of the subsequent disappearance of the unnecessary aboveground vegetative structures, including leaves (Wallace et al., 1975; Merckx et al., 2013). The achlorophyllous pinesap reproductive axis is often mistaken for a stem with leaves. However, the presence of MADS-box gene transcripts homologous to *APETALA3*, *TM6* and *SEPALATA3* in sterile bracts (“leaves”), whereas in higher plants these genes are expressed only in flowers, is one of the signatures of the reproductive nature of the *M. hypopitys* aerial part (Shulga et al., 2018).

The origin of asymmetric leaves and their further transformations, including the emergence of asymmetric flowering organs, as mentioned above, are associated, in part, with the evolutionary duplication and diversification of plant-specific *YABBY* genes (Eckardt, 2010; Mathews, Kramer, 2012). The structure and function of these genes are described in detail in a photosynthetic plants, model and other species (Bowman, 2000; Bowman et al., 2002; Finet et al., 2016; Strable et al., 2017). In complete mycoheterotrophs, *YABBY* genes are also present and transcribed (Shchennikova et al., 2018). It is not clear, however, whether the functions of the vegetative *YABBY* genes are preserved in these leafless plants.

In this study, we investigated possible functions of two “vegetative” *YABBY* genes of pinesap, *MhyFIL1* and *MhyFIL3*, by obtaining and characterizing two types of transgenic tobacco plants with overexpression of each of the analyzed genes.

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**Fig. 1.** Phylogenetic tree based on the alignment of 24 amino acid sequences of the YABBY transcription factors of pinesap and other plant species. Analysis was performed in MEGA 6.0 using the maximum likelihood method based on the JTT model (Tamura et al., 2013). The Pinus taeda YAB sequence was used as an outgroup. The lengths of the branches estimated as the genetic distance (the number of substitutions per site), and the essential bootstrap values for 1000 replicates are shown at the base of the branches. The NCBI accession numbers are given against the names of proteins. To the right of the dendrogram – a scheme of conserved motifs of the analyzed proteins obtained as a result of the MEME 5.0.1 (http://meme-suite.org/tools/meme) analysis is represented. Below are the sequences of two motifs corresponding to the zinc finger (ZF) and YABBY domains.
It is known that the simultaneous knockout of all “vegetative” YABBY genes in an *A. thaliana* plant leads to the formation of narrow twisted or radially symmetric leaves, since all leaf cells become adaxial (Stahle et al., 2009). Theoretically, in case of overexpression of such genes, the formation of radially symmetric leaves should also be expected, the only difference being their abaxial identity. The observed narrowing and curling of leaves in 35S::MhyFIL1/3 plants confirm this assumption.

Interestingly, the effects described above also occurred with the heterologous overexpression of the FIL genes *BraYAB1*−702 (*Brassica rapa*) and *TaYAB1* (*Triticum aestivum*) in transgenic *A. thaliana* plants (Zhao et al., 2006; Zhang et al., 2013). Both species (*B. rapa* and *T. aestivum*) are photosynthetic autotrophs; therefore, the similarity of the effect of constitutive expression of the *BraYAB1*−702 and *TaYAB1* genes in *A. thaliana* with the effect of the overexpression of MhyFIL1/3 in transgenic tobacco plants indicates the preservation of the ancestral role of the FIL genes in determining the identity of cells of the abaxial leaf surface.

It is also known that the correct morphogenesis of the meristem depends on the correct activity of the FIL genes (Bartholmes et al., 2012). For instance, *A. thaliana* with a double mutation, *fil yab3*, among other defects, demonstrates aberrant phyllotaxis (Goldshmidt et al., 2008). It is shown that transcription factor FIL nonautonomously and consistently affects the phyllotaxis and growth of lateral organs, coordinating the expression of markers (WUSCHEL, CLAVATA3 (CLV3)) of the central zone of the shoot apical meristem (Goldshmidt et al., 2008). The ectopic expression of *SrGRAM* (FIL-like gene in *Streptocarpus rexii*) completely suppressed the development of the *A. thaliana* shoot meristem (Tononi et al., 2010). The disturbance of the aboveground architecture of the 35S::MhyFIL1/3 transgenic plants and the resulting protracted vegetation may thus be caused by aberrant phyllotaxis up to the arrest of the shoot apical meristem development caused by ectopic MhyFIL1/3 overexpression.

It is worth highlighting the dramatic changes in the root structure of 35S::MhyFIL1/3 plants. In previously published papers, there was no information about what happens to the roots of such plants. The researchers may have omitted this aspect, since normally YABBY genes are expressed only in leaves and flowers, and therefore their functions are associated exclusively with these organs (Siegfried et al., 1999; Sarojam et al., 2010). Indeed, various combinations of yabby-mutations in *A. thaliana* do not affect root development (Boter et al., 2015). It is known that the apical meristems of the root and shoot are supported in a similar way, and CLV3 and WUSCHEL-RELATED HOMEOBOX 5 (WOX5) genes are markers of the quiescent center of the root meristem (Fiers et al., 2005; Stahl et al., 2009; Chu et al., 2013). Hence, it is reasonable to assume that the root phenotype in 35S::MhyFIL1/3 plants is a result of suppression of the apical root meristem development due to the interference of the MhyFIL1/3 transcription factor in the regulation of the expression of *N. tabacum* genes homologous to CLV3 and WOX5.

**Conclusion**

The obtained results may indicate that, despite the absence of aboveground vegetative organs from pinesap, the function of the MhyFIL1/3 genes as “vegetative” YABBY genes is preserved. In *M. hypopitys*, transcription factors FIL1 and FIL3 still determine the asymmetric development of the lateral organs of the plant aerial part, which follows from the normal structure of pinesap floral organs, as well as the characteristics of the influence of heterologous MhyFIL1/3 gene expression on the development of tobacco, in particular, its leaves. Thus, the activity of the MhyFIL1/3 genes is not
directly related to the loss of the pinsap ability to produce leaves during the evolutionary transition from autotrophic nutrition to heterotrophy.

References


