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 abaxial surface cells. Also, changes in the architecture of the aerial part and the root system of transgenic plants, including aberrant phylotaxis 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.

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Считается, что полный микогетеротроф, подъельник *Monotropa hypopitys*, адаптивно эволюционировал из fotosинтезирующего микоризного предшественника, теряя при этом аппарат фотосинтеза и вегетативные органы (стебель и листья). Надземная часть растения представляет собой цветонос со спиральными прицветниками и соцветием с каноническим для высших растений типом цветка. У растений происхождения плоского листа и других листоподобных латеральных органов связывают с эволюцией генов YABBY, которые, в зависимости от профиля экспрессии, делятся на "вегетативные" и эволюционно более поздние "репродуктивные" гены. Изучение "вегетативных" генов YABBY подъельника позволит выяснить, сохранились ли их функции (определение идентичности клеток абаксиальной по-
verhodnosti lateral’nykh organov) u rastenia bez li syev. V nastoichem isledovanii proveden strukturno-filogeneticheskiy analiz genov podyelnika MhyFIL1 i MhyFIL3, oхарактеризovannye osnovnye konservativnye domeny i motivy kodiruemых imi belkov i podtverdenna prinadлежnost genov k ‘vegetativnuyu’ klade YABBY3/FIL. Prowadena oshka y влияния гетерологической эктопической экспрессии genov MhyFIL1 i MhyFIL3 na fenotip tranzhennych rastenii tabaka Nicotiana tabacum. Pokazano, chto oba tipo rastenii, 35S::MhyFIL1 i 35S::MhyFIL3, formiruyu listya bol’ye uziy, chto v norme, i skryuchenyy za cht et izmenennoy identichnosti klakot adakxiialnoy povrchnosti. Vysavleny takie izmeneniya arhitekturny nadzamnoy chasti i kornevoy sistemy rastenii, vkluchaya aberrantnyy filiaktoss i podvalenya razvitia apikal’nykh meristam podbera i kor’ja. Chast rasteniya 35S::MhyFIL1 i 35S::MhyFIL3 pogibala yash na stadiy formirovaniya pervykh listya, chast ne cvela, ostalnye imeli silyu vykhenniy period vega teyi i chto cvenyami formirovali messy menee listyev, chto v norme. Cvetki ne imeli vidimykh otlichiy ot kontrola, za isklucheniyom lomkix cvenyonkix. Takim obrazom, ostatnie izmeneniya v strekne veka podyelnika v sverennom s avtophonymi vidami, i takoe osobennosti vliyniya heterologicheskoy ekspres ciy genov MhyFIL1/3 na razvitii rastenii tabaka govorят o soschrenii genami podyelnikiya MhyFIL1/3 funktsii ‘vegetativnykh’ genov YABBY. Pri etom ui M. hypopitys aktivnost YABBY-faktorov tranzhennoy gruppy FIL napravnu ne svyazana s poterей sposobnosti formirovati listya pri evolytsionnom pereshhe podyelnika ot avtoptofogo pitania k yeterofit. 
Kluchevye slova: Monotropa hypopitys; mikogeterotrof; yeterologicheskaya ekspresiya gena; adakxiialno-adakxiialna asimetriya; transkriptionnye faktory YABBY; ‘vegetativnaya’ YABBY; FILAMENTOUS FLOWER.

Introduction
The most significant event in plant evolution is considered to be the emergence of photosynthesis, due to which most modern plants are autotrophs and only about 1 % of flowering plants are heterotrophic. Among the latter, a special place is occupied by complete mycoheterotrophs, which, in the course of adaptation to adverse environmental conditions, have acquired the ability to obtain nutrients through mycorrhiza (a symbiotic association of roots with fungi). The range of adaptation consequences due to photosynthesis incapability includes the degradation and rearrangement of the genome, large-scale loss of functional genes, etc. (Wicke et al., 2016; Graham et al., 2017).

The monotropoid type of mycorrhiza is characteristic only for members of the subfamily Monotropoideae of the Ericaceae family (Leake, 1994), including Monotropa hypopitys (syn. Hypopitys monotropa). Compared with the related photosynthetic species Pyrola rotundifolia, aclorophyllous M. hypopitys is characterized by considerable structural rearrangements in the genome, an increased rate of accumulation of nucleotide substitutions in the genes, a significant reduction in the plastome, and a loss of the photosynthesis apparatus from both the plastome and the nuclear genome (Ravin et al., 2016; Graham et al., 2017). Such changes often lead to degradation and/or modification of vegetative structures (Graham et al., 2017). Thus, pinesap is not only deprived of chlorophyll but it does not form aboveground vegetative organs. The reproductive axis bearing sterile bracts and inflorescence develops bypassing the vegetative stage, from adventitious buds in the pinesap mycorrhiza root system (Wallace, 1975; Merckx et al., 2013).

The development of photosynthesis is closely related to the evolution of the leaf, which changed from radially symmetric to asymmetric, thus increasing the insolation of its surface (Stewart, Rothwell, 1993; Cronk, 2001; Bowman et al., 2002; Beerling, Fleming, 2007). It is believed that the asymmetric leaf of seed plants originated in part due to the duplication and diversification of YABBY genes (Eckardt, 2010). The evolution of the ancestral YABBY gene produced a family of genes with different specializations, which could be associated with further transformations of the leaf and the emergence of other asymmetric organs that formed the flower (Mathews, Kramer, 2012).

The abaxial-adaxial asymmetry of all lateral organs is characteristic of most extant plants. One of the main factors determining the identity of the abaxial surface of organs is the family of YABBY transcription factors (Bowman et al., 2002; Bartholmes et al., 2012). In angiosperms, this family is divided into five subfamilies: three “vegetative” – YABBY1/ YABBY3 (FILAMENTOUS FLOWER (FIL)), YABBY2/ FASCIATED (FAS) and YABBY5, and two “reproductive” – CRABS CLAW (CRC) and INNER NO OUTER (INO) (Yamada et al., 2011; Bartholmes et al., 2012; Finet et al., 2016). “Reproductive” YABBYs have a narrow specialization, while “vegetative” YABBYs are involved in determining the polar development of vegetative and reproductive organs and are also important for proper organization and phyllotaxis of the shoot apical meristem (McConnell, Barton, 1998; Bartholmes et al., 2012). Thus, the “vegetative” YABBY genes preserve the expression profile of the ancestral gene, although they cannot completely replace the “reproductive” YABBYs (Yamada et al., 2011; Bartholmes et al., 2012).

The study of the YABBY genes of the complete mycoheterotroph M. hypopitys could clarify the possibility of preserving the ancestral functions by the “vegetative” YABBYs upon loss of the vegetative organs. The YABBY5 (MhyYAB5) and YABBY3/FIL (MhyFIL1, MhyFIL2, and MhyFIL3) genes with opposite expression patterns have been identified in pinesap (Shchennikova et al., 2018). In bracts, which are evolutionarily closer to leaves than to floral organs, only trace amounts of MhyFIL2 transcripts are observed, and the expression levels of MhyFIL1 and MhyFIL3 are 5–10 times lower than that of MhyYAB5 (Shchennikova et al., 2018). In the absence of leaves in pinesap, the reduced MhyFIL1 and MhyFIL3 expression in bracts suggests a loss of part of the “vegetative” YABBY function.

In this study, we perform a functional analysis of the vegetative YABBY genes, MhyFIL1 and MhyFIL3, in leafless pinesap...
M. hypopitys. The study of homologs of genes determining leaf asymmetry in higher plants in a complete mycoterotroph 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'-catcatgccctcctcaatttctt-3' (for both genes), and reverse – 5'-ctccttgactagggggacaca-3'-3' (MhyFIL1) and 5'-ctccttgatagggggacg-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 °С, 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 Extraction Kit; QIAGEN, USA), cloned into the pGEM-T Easy (Promega, Madison, WI, USA), and sequenced (Core Facility “Bioengineering”, FRC “Fundamentals of Biotechnology” RAS). Sequence analysis of the fragments confirmed the cloning of the MhyFIL1 and MhyFIL3 cDNAs. The nucleotide and amino acid sequences were analyzed with the following software: Clone Manager 7.11 (http://clonemanager-professional.software.informer.com/), NCBI-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and MEGA 6.0 (Tamura et al., 2013). For phylogenetic analysis, NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) and MEGA 6.0. (Tamura et al., 2013) were applied with tree generation by the maximum likelihood method based on the JTT model (Zuckerkandl, Pauling, 1965). To analyze the function of the MhyFIL1 and MhyFIL3 transcription factors, two types of transgenic Nicotiana tabacum plants with constitutive expression of the cDNA of each of the MhyFIL1 and MhyFIL3 genes were obtained. Independent transgenic regenerants T0 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 35S::MhyFIL3 (two 35S::MhyFIL1 plants). In the T1 generation, changes in plant morphology increased. 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.

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 obtain 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 pinesap ability to produce leaves during the evolutionary transition from autotrophic nutrition to heterotrophy.

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