



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 phyllotaxis 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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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 микорризного предшественника, потеряв 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 происхождения плоского листа и других листовидных боковых органов связывают 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-

верхности латеральных органов) у растения без листьев. В настоящем исследовании проведен структурно-филогенетический анализ генов подбельника *MhyFIL1* и *MhyFIL3*, охарактеризованы основные консервативные домены и мотивы кодируемых ими белков и подтверждена принадлежность генов к «вегетативной» кладе *YABBY3/FIL*. Проведена оценка влияния гетерологичной эктопической экспрессии генов *MhyFIL1* и *MhyFIL3* на фенотип трансгенных растений табака *Nicotiana tabacum*. Показано, что оба типа растений, *35S::MhyFIL1* и *35S::MhyFIL3*, формируют листья более узкие, чем в норме, и скрученные за счет измененной идентичности клеток адаксиальной поверхности. Выявлены также изменения архитектуры надземной части и корневой системы растений, включая aberrантный филлотаксис и подавление развития апикальных меристем побега и корня. Часть растений *35S::MhyFIL1* и *35S::MhyFIL3* погибала еще на стадии формирования первых листьев, часть не цвела, остальные имели сильно увеличенный период вегетации и при цветении формировали меньше цветков, чем в норме. Цветки не имели видимых отличий от контроля, за исключением ломких цветоножек. Таким образом, отсутствие изменений в строении цветка подбельника в сравнении с автотрофными видами, а также особенности влияния гетерологичной экспрессии генов *MhyFIL1/3* на развитие растений табака говорят о сохранении генами подбельника *MhyFIL1/3* функции «вегетативных» генов *YABBY*. При этом у *M. hypopitys* активность *YABBY*-факторов транскрипции группы *FIL* напрямую не связана с потерей способности формировать листья при эволюционном переходе подбельника от аутотрофного питания к гетеротрофии.

Ключевые слова: *Monotropa hypopitys*; микогетеротроф; гетерологичная экспрессия гена; абаксиально-адаксиальная асимметрия; транскрипционные факторы *YABBY*; «вегетативные» *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*, achlorophyllous *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/FASCATED* (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 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'-catcatgtcctctcaaatctt-3' (for both genes), and reverse – 5'-cttcttgattagtagggggacaca-3' (*MhyFIL1*) and 5'-cttcttgattagtagggggagacc-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 °C 5 min; 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and elongation (72 °C, 1 min); extension (72 °C, 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://clone-manager-professional.software.informer.com/>), NCBI-CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and MEME 5.0.1 (Bailey, Elkan, 1994). Alignment of sequences of genes and proteins encoded by them was performed using ClustalX (Larkin et al., 2007) 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 (Zuckerandl, 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 *MhyFIL1* and *MhyFIL3* genes were obtained. Two binary vectors were constructed based on the pBin19 plasmid, containing the cDNA (*MhyFIL1/MhyFIL3*) expression cassette in sense orientation under the control of the 35S CaMV promoter and NOS terminator. *Agrobacterium tumefaciens* AGL0 strains carrying the constructed plasmids were used for tobacco leaf disc transformation with further regeneration according to the previously described protocol (Goloveshkina et al., 2012). Regenerants were analyzed for the transgene presence in the genome by PCR with primers specific for the 3'-sequence of each gene (see above) and the 35S promoter (5'-caatccactatccttcgcaagacc-3'). The phenotypes of plants that gave a positive PCR signal were compared with the control (nontransgenic tobacco). The following parameters were assessed: the vegetation period (from planting in the greenhouse to budding), the structure

of the aboveground vegetative part of the plant, and the phenotypes of the roots, leaves, and floral organs.

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 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 six 35S::*MhyFIL3* and 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

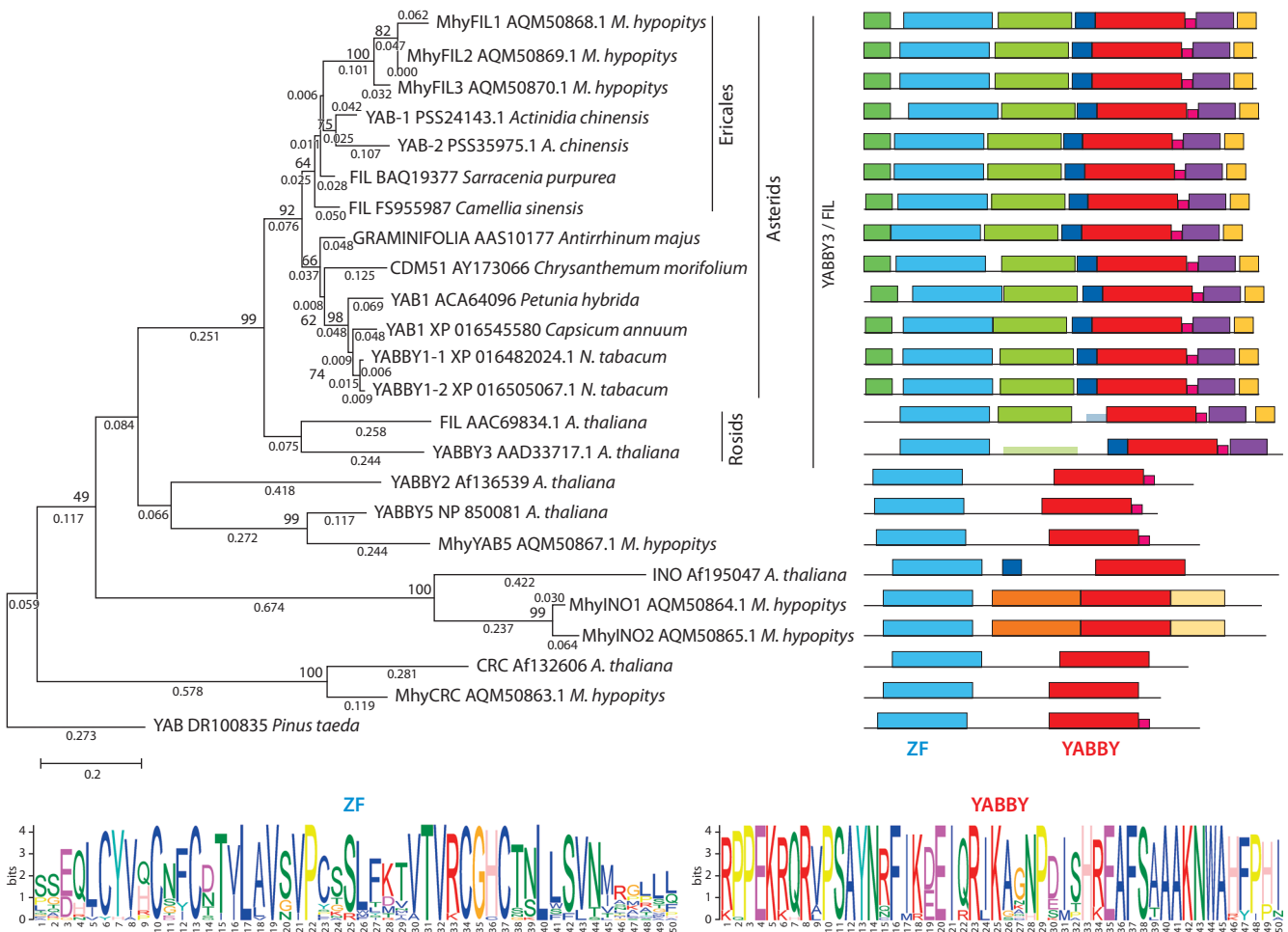


Fig. 1. Phylogenetic tree based on the alignment of 24 amino acid sequences of the YABBY transcription factors of pinesap and other plant species. Analysis was performed in MEGA 6.0 using the maximum likelihood method based on the JTT model (Tamura et al., 2013).

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.

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 *SEPALLATA3* 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.

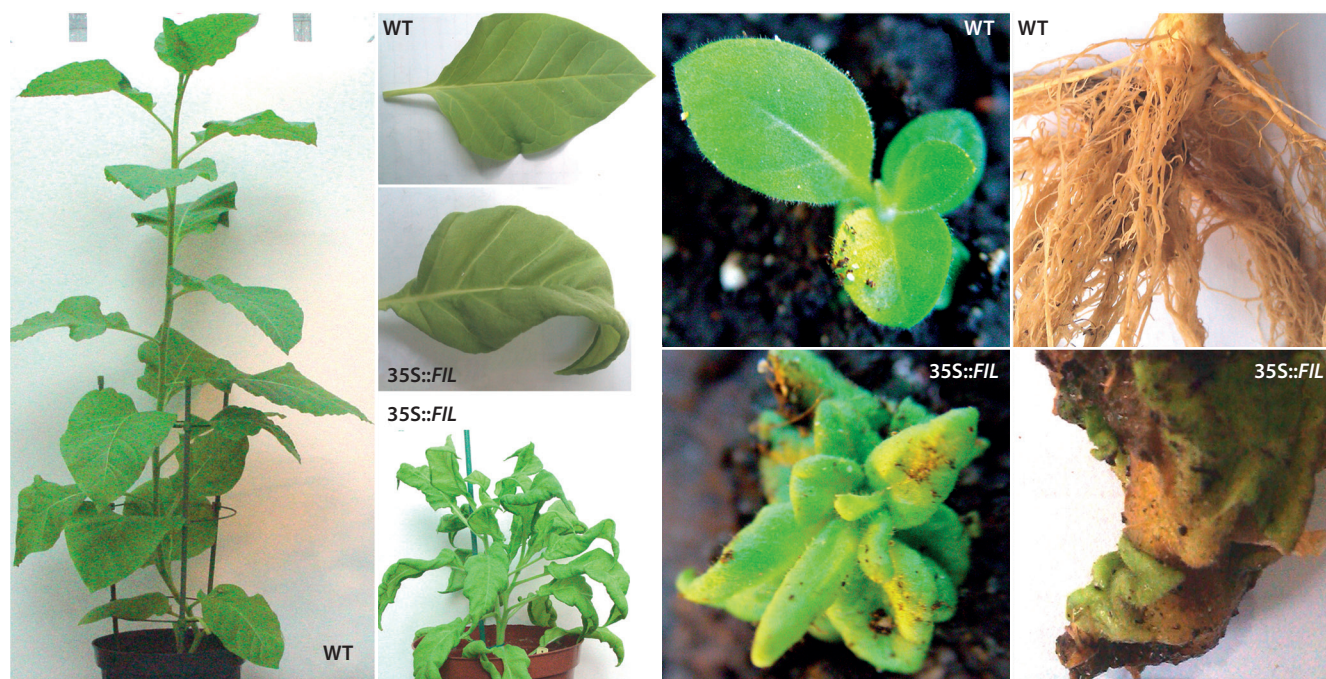


Fig. 2. Transgenic tobacco plants 35S::FIL1/3 (indicated as 35S::FIL) in comparison with the control nontransgenic plant *N. tabacum* (WT).

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 HOMEBOX 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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