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Identification and Expression Analysis of Chitinase Genes in Parasitic Plant *Monotropa hypopitys*

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Abstract—Genes encoding six chitinases, five of which belong to classes I (MhCHI3 and MhCHI4), IV (MhCHI1), V (MhCHI5), and VII (MhCHI2), were identified in the transcriptome of the parasitic mixoheterotrophic plant *Monotropa hypopitys*. The transcription level of MhCHI5 and MhCHI1 was low; however, in the leaves (bracts) and roots it was higher than in flowers. MhCHI4 transcripts were detected primarily in the flowers and were almost absent in the roots, whereas the expression level of MhCHI3 was relatively high in all organs but maximum in the leaves (bracts).

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Chitinases belong to the class of hydrolytic enzymes that catalyze the degradation of chitin, the second most abundant carbohydrate in nature after cellulose, to simple sugars [1]. Plant chitinases are considered primarily as proteins involved in response to phytopathogens, because their activity is induced in response to fungal, viral, and bacterial infections [2]. In addition, chitinases are involved in the response to abiotic stress and in many physiological and morphological processes, including embryogenesis, flowering, and seed ripening [3, 4].

Plant chitinases are a diverse group of proteins combined by the presence of catalytic glycoside hydrolase (GH) domain but substantially differing in primary sequence, domain composition, and cellular localization. Today, seven classes of plant chitinases are distinguished [5]. Chitinases of classes I, II, IV, and VII are characterized by the presence of the GH19 glycoside hydrolase domain, whereas the enzymes of classes III, V, and VI contain the GH18 domain. In addition to the catalytic domain, chitinases of classes I and IV, unlike the enzymes of classes II and VII, contain the N-terminal chitin-binding domain (CBD1) and a proline-glycine-rich linker peptide between these domains, which varies both in length and in composition [1].

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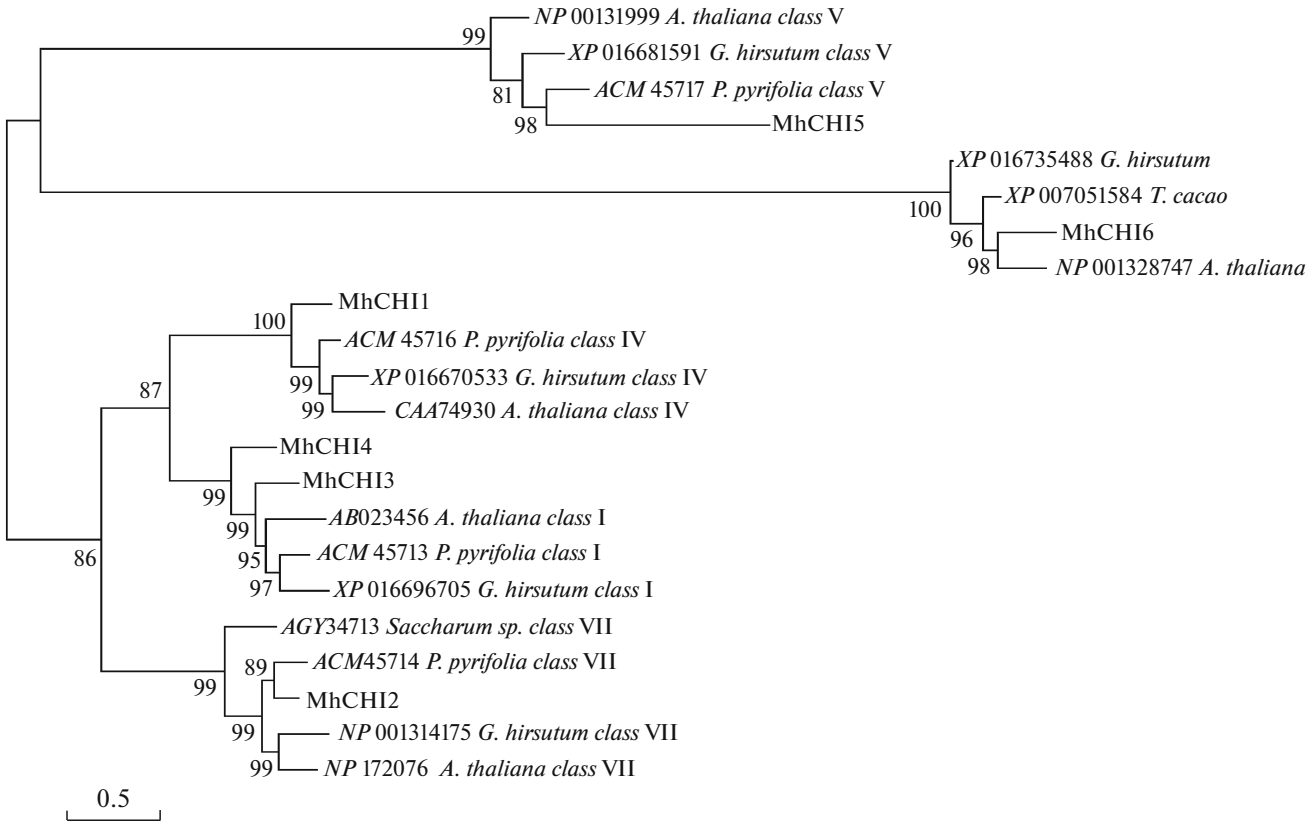
The pinesap *Monotropa hypopitys* (subfamily Monotropoideae, family Ericaceae) is a chlorophyll-free mycoheterotrophic plant parasitizing on the mycorrhiza formed by fungi of the class Agaricomycetes and coniferous trees [6, 7]. Most of the year, *M. hypopitys* exists in the form of a branched underground rhizome, on which chlorophyll-free generative shoots composed of leaves (bracts) and inflorescences are formed at the beginning of July. Through mycorrhiza, the fungus connects the pinesap roots and the root system of nearby trees. As a result, the pinesaps obtain nutrients and minerals not only from fungi but also from trees [6]. *M. hypopitys* roots are actively colonized by the hyphae of fungi, which can penetrate into the cell as well as ascend through intercellular spaces of the ground part of the plant to the flowers [8]. The dependence of the pinesap on fungi is also confirmed by the fact that the germination of seeds and the development of seedlings of the pinesap is observed only in the presence of fungal hyphae [6]. Previously, an important role of chitinases in the regulation of symbiosis with mycorrhizal fungi in autotrophic plants has been shown [3]. However, data on chitinases in mycoheterotrophic parasitic plants are absent.

Therefore, the aim of this study was to identify and characterize the chitinases of *M. hypopitys* and to analyze their expression in the above-ground and underground parts of the plant.

The search for chitinase transcripts was performed in the transcriptomes of roots, leaves (bracts), and flowers of individual plants *M. hypopitys*, which were sequenced by us earlier [9]. To identify the sequences of chitinases and determine the domain structure, we used the NCBI database (http://blast.ncbi.nlm.nih.gov/) and the MEME 4.11.2 software (http://meme-suite.org/



Comparison of *M. hypopitys* chitinases with known classes of chitinases of other plants.

tools/meme). N-terminal signal peptides were identified using the SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP). Comparative analysis of amino acid sequences was performed and dendrograms were built using the MEGA7.0 software [10]. The expression level of genes was calculated as the number of transcriptome reads mapped on their sequence per million reads, divided by the gene length.

As a result of *M. hypopitys* transcriptome analysis, we identified six transcripts homologous to sequences of other plant chitinases (table). NCBI database search and comparison with known chitinase proteins allowed us to classify the identified transcripts into four (I, IV, V, and VII) of the seven currently known [2, 11] classes of chitinases (figure).

The predicted protein products of MhCHI1, MhCHI2, MhCHI3, and MhCHI4 transcripts contained the GH19 domain characteristic of plant chitinases, whereas MhCHI5 and MhCHI6 contained the GH18 domain, which is found in chitinases of all organisms [2, 3]. The amino acid sequences of all chitinases except MhCHI6 contained N-terminal signal peptides, which can provide their export from the cell.

The results of comparative analysis of the amino acid sequences of MhCHI3 and MhCHI4 allowed us to classify them with class I chitinases (figure). In MhCHI3 and MhCHI4 sequences we identified the chitin-binding domain (38 amino acid residues) and the GH19 domain (232 amino acid residues), which are characteristic of class I chitinases. In the latter, we identified the functional motifs MLXXR, XFYTYX, AFXXAA, FXTTGX, and TSH, which are characteristic of this class of chitinases [12]. The MhCHI1 polypeptide, unlike MhCHI3 and MhCHI4 polypeptides, contains truncated chitin-binding domain (27 amino acid residues) and glycoside hydrolase domain (200 amino acid residues), which allowed us to classify it with class IV chitinases. In the GH19 domain of MhCHI1, we identified three motives DPLIAFKTALW, GFGETIRAIN, and TVNARVEYYIEYCNQLGV, which are characteristic of this class of chitinases [12]. The MhCHI2 sequence also contained the GH19 domain and was homologous to the sequences of chitinases of *Arabidopsis thaliana* (NP_172076), *Gossypium hirsutum* (NP_001314175), *Pyrus pyrifolia* (ACM45714), and *Saccharum* sp. (AGY34713), which belong to class VII [11].

MhCHI5 and MhCHI6, containing the GH18 domain, belong to another group of chitinases. The length of this domain in MhCHI5 was 340 amino acid residues. Comparative analysis of the amino acid sequence of MhCHI5 (figure) showed similarity with chitinases of class V of *Arabidopsis thaliana*

Chitinases identified in the *M. hypopitys* transcriptome and their expression level in rhizome and inflorescence

Transcript	Polypeptide length, amino acid residues	Domains	Class	Expression level					
				plant 1		plant 2		roots 1	roots 2
				inflorescence		inflorescence			
				leaf	flower	leaf	flower		
MhCHI1	275	CBD1 + GH19	IV	22.63	2.16	41.41	5.32	17.34	18.68
MhCHI2	321	GH19	VII	98.04	133.34	308.67	199.71	15.77	30.21
MhCHI3	323	CBD1 + GH19	I	1939.26	874.69	5247.28	479.67	168.96	350.44
MhCHI4	326	CBD1 + GH19	I	58.87	2209.00	142.00	2815.39	1.41	1.65
MhCHI5	390	GH18	V	16.12	3.88	20.42	1.43	8.74	14.45
MhCHI6	437	GH18	–	1.61	2.23	3.33	3.24	0.42	0.51

(NP_001319999), *Gossypium hirsutum* (XP_016681591), and *Pyrus pyrifolia* (ACM45717). In addition, in the sequence of this domain we identified the functional motif DGVDLDWEF, which is characteristic of this class of chitinases [12].

The search for and comparative analysis of the amino acid sequence of the MhCHI6 transcript showed that it contains the GH18 domain. However, this fact did not allow us to assign it to any of the known classes of chitinases. Sequences homologous to MhCHI6 were found in *Arabidopsis thaliana* (NP_001328747), *Gossypium hirsutum* (XP_016735488), and *Theobroma cacao* (XP_007051584) (figure).

Differences in the expression levels of chitinase gene in different organs and tissues were shown earlier for many plants [1]. In the case of pinesap, expression levels of chitinases in different organs greatly differed, except MhCHI6, for which a sufficiently low transcriptional activity was detected in all organs studied (table). The transcription level of MhCHI5 and MhCHI1 was low; however, in the leaves (bracts) and roots it was higher than in the flowers.

Of greatest interest were chitinases MhCHI3 and MhCHI4, which are characterized by a very high transcription level compared to other pinesap chitinases. MhCHI4 transcripts were detected primarily in the flowers and were practically absent in the roots. The MhCHI3 expression level was high in all organs and maximum in the leaves (bracts). Interestingly, it was the only chitinase analyzed for which a high transcriptional activity in the roots was shown (table). Thus, it can be assumed that this chitinase may be involved in the degradation of the cell walls of the fungus during the interaction of pinesap root haustoria with fungal hyphae.

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