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Proteomic analysis of secreted protein induced by a component of prey in pitcher fluid of the carnivorous plant *Nepenthes alata*

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ABSTRACT

The *Nepenthes* species are carnivorous plants that have evolved a specialized leaf organ, the 'pitcher', to attract, capture, and digest insects. The digested insects provide nutrients for growth, allowing these plants to grow even in poor soil. Several proteins have been identified in the pitcher fluid, including aspartic proteases (nepenthesin I and II) and pathogenesis-related (PR) proteins (β-1,3-glucanase, class IV chitinase, and thaumatin-like protein). In this study, we collected and concentrated pitcher fluid to identify minor proteins. In addition, we tried to identify the protein secreted in response to trapping the insect. To make a similar situation in which the insect falls into the pitcher, chitin which was a major component of the insect exoskeleton was added to the fluid in the pitcher. Three PR proteins, class III peroxidase (Prx), β-1,3-glucanase, and class III chitinase, were newly identified. Prx was induced after the addition of chitin to the pitcher fluid. Proteins in the pitcher fluid of the carnivorous plant *Nepenthes alata* probably have two roles in nutrient supply: digestion of prey and the antibacterial effect. These results suggest that the system for digesting prey has evolved from the defense system against pathogens in the carnivorous plant *Nepenthes*.

1. Introduction

Plants usually absorb nutrients such as nitrogen, phosphorus, potassium, and other minerals from the soil via their roots. However, carnivorous plants growing in nutrient-poor soils obtain nutrients from trapped insects using special organs to capture and digest them. This was first reported by Charles Darwin in *Insectivorous Plants* [1]. There are five types of trapping mechanisms in carnivorous plants: 1) pitfall traps, e.g. in *Nepenthes*, *Heliamphora*, *Sarracenia*, *Darlingtonia*, *Cephalotus*, and *Brocchinia*; 2) flypaper traps, e.g., in *Pinguicula*, *Drosera*, *Drosophyllum*, and *Triphyophyllum*; 3) snap traps, e.g. in *Dionaea* and *Aldrovanda*; 4) bladder traps, e.g., in *Daphnia* and *Utricularia*; and 5) lobster-pot traps, e.g. in *Genlisea*.

Nepenthes, which is commonly known as the tropical pitcher plant, includes more than 100 species. Within this genus, the greatest species diversity is in Borneo and Sumatra. *Nepenthes* species are widely distributed in the tropics, such as India, Sri Lanka, Australia, New Caledonia, Madagascar, and the Seychelles. The 'pitcher' bud forms at the end of the leaf and gradually expands into a pot-shaped trap. The rim of the trap, called the peristome, is slippery and often very colorful to attract insects. The inside wall is also slippery and waxy to prevent escape. The bottom of the trap contains a fluid, which has long been thought to contain digestive enzymes.

To elucidate the mechanisms by which the plant obtains nutrients from its prey, analyses on the pitcher fluid composition have been conducted. Studies on the enzymes in this fluid

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have been reviewed in detail by Frazier [2]. The most abundant constituent of pitcher fluid is "nepenthesin", an enzyme that shows stable activity up to 60 °C and appears to cleave peptides preferentially at the carboxyl side of aspartic acid residues [3]. Athauda et al. purified the enzymes nepenthesins I and II from *Nepenthes distillatoria* and the partial sequences were determined [4]. They cloned the cDNAs and deduced the complete amino acid sequences of the enzymes from *Nepenthes gracilis* [4]. These enzymes are major components of the pitcher fluid; however, it was suggested that other, minor enzymes are also present. Recently, we reported the proteome analysis on the pitcher fluid of *Nepenthes alata* [5]. We newly identified homologs of β-D-xylosidase, β-1,3-glucanase, class IV chitinase, and a thaumatin-like protein, most of which are pathogenesis-related (PR) proteins. PR proteins are a heterogeneous group of proteins induced in response to pathogen infection in plants [6]. We concluded that they may inhibit the proliferation of putrefying bacteria on prey undergoing digestion in the pitcher to ensure sufficient nutrients for plant growth [5].

To identify all the proteins involved in the digestion system, we collected a large amount of pitcher fluid so that we could identify minor proteins. In addition, we attempted to identify the protein(s) secreted in response to trapping the insect. To make a similar situation in which the insects falls into the pitcher, we added chitin (a linear polymer of β-1,4-linked *N*-acetylglucosamine) in suspension into the pitcher. The exoskeleton of insects consists of a hard cuticle composed of protein and chitin. The addition of chitin into closed pitchers of *Nepenthes khasiana* induced the synthesis of new proteins, including those with chitinase activity, in the pitcher fluid [7]. However, the identities of those proteins remain unknown.

The complete sequence data are not yet available for *Nepenthes*, although we are planning the genome project of *N. alata* to advance our research. However, enlarging plant protein databases and advancing bioinformatics tools allow us to identify proteins in plants with as-yet unsequenced genomes. The recent rapid developments in genomics, including next-generation sequencing technology, have resulted in the full sequencing of genomes of several plant species, including *Arabidopsis thaliana* [8], rice (*Oryza sativa*) [9], grape (*Vitis vinifera*) [10], papaya (*Carica papaya*) [11], and soybean (*Glycine max*) [12]. These data greatly improve our ability to analyze the plant proteome. Often, the amino acid sequence of an unknown protein matches a protein of a closely related species, as determined using identification software (Mascot) and tandem mass spectrometry (MS/MS) spectral data. Using this information, we can identify candidate genes that are homologous to the target proteins. In this study, we used this approach to answer the century old question of how carnivorous plants obtain nutrients from their prey.

2. Materials and methods

2.1. Sample preparation

N. alata plants were grown in a greenhouse under ambient light at 25–30 °C. Pitcher fluid samples (8–10 mL) were collected from newly opened pitchers 48 h after addition of distilled water (500 μL) or 1 mg chitin [500 μL of 2 mg/mL chitin from crab shells, suitable for analysis of chitinase, purified powder (Sigma-Aldrich Co., St Louis, MO, USA)]. To avoid contamination by organisms, mouths of the pitchers were filled with cotton. Collected samples were filtered through a 0.22 μm cellulose acetate membrane. To concentrate the proteins in these solutions, the samples were subjected to ultrafiltration [Amicon Ultra 10K, molecular weight cutoff (MWCO), 10 kDa; Millipore, Eschborn, Germany]. They were mixed with one-fifth volume of 5×SDS loading buffer, boiled for 5 min, and then separated by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue (CBB) or MS-compatible silver stain (ProteoSilver™; Sigma-Aldrich), according to the instruction manual.

2.2. In-gel digestion and mass spectrometric analysis

In-gel digestion with trypsin was performed according to a previously described protocol [5] with a modification to the destaining step. Silver-stained gel bands were destained using a 1:1 mixture of 30 mM potassium ferricynide/100 mM sodium thiosulfate solution until the brown color disappeared, and then washed with 400 μL distilled water until the yellow color disappeared. They were dehydrated with 100 μL acetonitrile by gentle shaking. The remaining steps were performed as described previously [5]. The digested peptides were subjected to LC-MS/MS analysis performed on a Q-Tof hybrid mass spectrometer (Q-Tof2: Micromass, Manchester, U.K.) interfaced with a capillary reversed-phase LC system (Micromass CapLC system). A 90 min linear gradient from 5 to 45% acetonitrile in 0.1% formic acid was produced and was split in a 1:20 ratio. The gradient solution was then injected into a nano-LC column (PepMap C18, 75 μm×150 mm; LC Packings, Sunnyvale, CA) at 100 nL/min. The eluted peptides were sprayed directly into the mass spectrometer. The MS/MS data were acquired with MassLynx (version 4.0; Micromass) using automatic switching between MS and MS/MS modes and converted to a single text file (containing the observed *m/z* value of the precursor peptide, the fragment ion *m/z* values, and intensity values) with ProteinLynx (version 2.0; Micromass).

2.3. Protein identification

The converted files were analyzed with the Mascot MS/MS Ions Search (version 2.2.0; Matrix Science Ltd., London, U.K.) to assign them to the NCBI nonredundant database (NCBInr 20090624; 9164896 sequences). The parameter settings in Mascot were as follows: parent mass error tolerance, ±0.1 Da; fragment mass error tolerance, ±0.1 Da; enzyme, trypsin (one missed cleavage site was allowed); post-translational modifications, oxidation (Met), carbamidomethylation (Cys), and propionamidation (Cys). For protein identification, the criteria were as follows: (1) Mascot scores above the statistically significant threshold ($P<0.05$) and (2) at least one top-ranked unique peptide matching the identified protein.

2.4. cDNA cloning of *N. alata* genes

Total RNAs were isolated from whole pitchers 48 h after the addition of chitin from crab shells (Sigma-Aldrich), as described by Kim and Hamada [13]. The RNAs were purified