The Characteristic of a Novel Metalloproteinase from *Puccinia Helianthi*

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1   **The characteristic of a Novel Metalloproteinase from *Puccinia helianthi***

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Abstract

Objective In phytopathogenic fungi, several metalloproteinase have been implicated in virulence, but pathogenesis roles in rust are largely unknown. Here, we identified a M36 family metalloproteases from *Puccinia helianthi* genome, designated PhMEP1, aimed to reveal the biological characteristics related to pathogenicity.

Result Bioinformatics analysis showed that PhMEP1 contains some of the conserved zinc-binding and active site motifs characteristics of metalloproteinase which might well be a new fungalysin-like peptidase in the M36 family. The PhMEP1 protein was over expressed as inclusion bodies in *Escherichia coli*. The recombinant expression products of *PhMEP1* could hydrolyze azocasein and the hydrolytic activity was suppressed by metal ion chelating agents such as 1,10-phenanthroline and ethylene diamine tetraacetic acid (EDTA). The subcellular localization of PhMEP1 was detected by confocal laser scanning microscopy of the leaves from a 2-day-old *Nicotiana benthamiana* seedlings expressed *PhMEP1-GFP* fusion that showed green fluorescent protein (GFP) signal accumulated in the plasma membrane of epithelial cells.

Conclusion PhMEP1 may serve as a pathogenicity factor contributing to *P. helianthi* penetration and infection.

Keywords Metalloproteinase · *Puccinia helianthi* · M36 family · Hydrolytic activity · Subcellular localization

Introduction

1 Sunflower rust, which is one of the major diseases of sunflower globally, spreads in
2 the sunflower planting area triggering a heavy loss. *Puccinia helianthi*, the causal
3 agent of sunflower rust, is a macrocyclic and autoecious pathogen. To develop
4 resistant plants, a better understanding of rust virulence factors, is needed. Exogenous
5 absorption of macromolecular nutrients is a common strategy of fungal growth, which
6 relies on the secretion of hydrolases including cutinases, cellulases, pectinases, and
7 proteases. Of these proteins, metalloproteinases are a type of protease that contain
8 conserved zinc-binding sites and HEXXH common sequences (Tallant et al. 2006;
9 Zhou et al. 2018).

10 Metalloproteases of pathogens were reported have roles in virulence (Jia et al. 2000;
11 Sanz-Martín et al. 2016). For instance, Avr-Pita, a zinc metalloprotease from the rice
12 blast fungus *Magnaporthe oryzae*, recognized by a cytoplasmic resistance
13 gene-encoded protein Pi-ta that triggers a signalling cascade leading to host rice
14 resistance (Jia et al. 2000). Recently we have identified a M36 domain-containing
15 metalloproteinase in *P. helianthi*, designated PhMEP1 (GenBank ID: KU994904) ,
16 investigated its expression pattern strongly up-regulated during the early stages of
17 infection (Jing et al. 2017; Li et al. 2019).

18 The first M36 metalloprotease was described from the opportunistic human
19 pathogen *Aspergillus fumigatus* is involved in the degradation of proteinaceous
20 structural barriers during invasion of the host lung (Monod et al. 1993). M36
21 metalloproteases from *Fusarium* fungi secrete fungalysins, that truncate maize class
22 IV chitinases, in order to stop the chitin mediated activation of the plant immune

1 system (Naumann et al. 2011). M36 metalloproteinase from *Trichophyton*
2 *mentagrophytes* is involved in the degradation of keratinase during invasion of the
3 host skin (Zhang et al. 2014). M36 metalloproteinase comes from *Aspergillus*
4 *fumigatus* (Silva et al. 2013), *Coprinopsis cinerea* (Lilly et al. 2008), *Perenniporia*
5 *fraxinea* (Kim et al. 2008), *Trichophyton* sp. (Jousson et al. 2004), *Microsporum* sp.
6 (Jousson et al. 2004), *Nomuraea atypicola* (Ueda et al. 2013), *Coccidioides posadasii*
7 (Sharpton et al. 2009), *Pyrenophora tritici-repentis* (NCBI protein XP 001940970.1),
8 and *Arthroderma gypseum* (NCBI protein XP 003173813.1). In the present study, we
9 analyzed the phylogenetic relationship of this metalloproteinase, and described the
10 expression of the mature proteinase of PhMEP1 in *Escherichia coli*. Moreover, the
11 hydrolytic activity of this enzyme against azocasein was tested, and the subcellular
12 localization of PhMEP1 was detected.

13

14 **Materials and methods**

15

16 Sequences analyses of full-length sequence of PhMEP1

17 The cDNA and genomic DNA was analyzed using BLAST
18 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). SOPMA

19 (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) was used to
20 analysis the second structure and Phyre2 to predict the three-dimensions structure.

21 The genome sequences and their corresponding annotated proteins database of the 20

22 MEPs are available from UniProt (www.uniprot.org), GenBank

1 (<http://www.ncbi.nlm.nih.gov/genbank/>) and the Fungal Genome Initiative at the
2 BROAD Institute (<http://www.broad.mit.edu/annotation/fungi/fgi>) databases. The
3 phylogenetic tree was constructed by Mega 7.0 using neighbor-joining (parameters:
4 1000 bootstraps).

5 Cloning and heterologous expression of PhMEP1

6 To express the mature PhMEP1 in *E. coli*, MaxCodon™ Optimization Program (V13)
7 was used to optimize the amino acid sequence of mature PhMEP1. Gene *PhMEP1*
8 was introduced to *NdeI* site and a *HindIII* site at the 5' and 3' ends, respectively, and a
9 Met start codon was created by the *NdeI* site at the N terminus. Total genes were
10 synthesized and inserted into expression vector Pet30a (His-tag at the N terminus).
11 The clones with correct insertion were identified by enzyme digestion and sequencing.
12 Pet30a-*PhMEP1* was transformed into expression host strain *E. coli* BL21 (DE3).
13 Positive colonies were identified by PCR and double digestion with *NdeI* and *HindIII*.
14 The primers were designed with Primer Premier 5.0 program and synthesized by
15 Invitrogen.

16 *PhMEP1*-F: 5'-CGCCCATATGATGTCCTATCGCGTCTTTCCCTGGTCG-3'

17 *PhMEP1*-R: 5'-CCCAAAGCTTTCAGTTCTCGCAAACACCTCTTGG-3'

18 PCR was carried out in 25 µL reaction system. The amplification protocol consisted
19 of the denaturation at 95 °C for 5 min followed by 35 cycles with 30 s at 94 °C, 30 s at
20 65.3 °C, and 1 min at 72 °C. A final elongation was done at 72 °C for 10 min. The
21 cDNA obtained after the total RNA amplification was separated on a 1.5% agarose
22 gel. A single colony of transformant clones containing Pet30a-*PhMEP1* was then

1 inoculated in 4 mL Luria-Bertani (LB) broth containing 50 $\mu\text{g mL}^{-1}$ Kanamycin in a
2 shaking incubator. When cells reached the log growth phase (OD_{600} 0.5–0.8),
3 isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1
4 mM to induce production of T7 RNA polymerase and thus the synthesis of the
5 PhMEP1, and cells were harvested after 16 h of growth at 15 °C or 37 °C. The culture
6 was then used for PhMEP1 expression analysis by Sodium Dodecyl Sulphate Poly
7 Acrylamide Gel Electrophoresis (SDS-PAGE).

8 The transformant was cultured in 3 L LB medium. When cells reached the log
9 growth phase (OD_{600} 0.8), IPTG was added to induce Mep expression and the final
10 concentration of IPTG was 1mM. The cells were harvested after 16 h of growth at
11 15 °C.

12 Purification of PhMEP1

13 The cells were harvested by centrifugation (6000 rpm for 5 min at 4 °C). The bacterial
14 pellets were resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1
15 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 20 mM
16 imidazole containing 1% Triton X-100]. The bacterial cell pellets were lysed on ice
17 with the aid of ultrasonic cell disruptor. At the same time, Ni-IDA affinity column
18 was equilibrated with the buffer [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM
19 imidazole]. The column was then eluted by the equilibrium buffer with different
20 concentrations of imidazole (50 and 500 mM) to obtain the target protein. Elution
21 components were collected for SDS-PAGE electrophoresis analysis.

1 The inclusion body was washed in buffer containing 50 mM Tris-HCl (pH 8),
2 300 mM NaCl containing 1% Triton X-100, 2mM ethylene diamine tetraacetic acid
3 (EDTA) and 5 mM DTT, and then resuspended in buffer containing 50 mM Tris-HCl
4 (pH 8), 300 mM NaCl, 8 mM Urea, and 20 mM imidazole. At the same time, Ni-IDA
5 affinity column was equilibrated with this buffer. The column was then eluted by the
6 equilibrium buffer with gradient concentration of imidazole (50 mM, 100 mM and
7 300 mM). The purification efficiency of protein was evaluated by 12% SDS-PAGE.
8 Protein complexes were purified by molecular-sieve chromatography (SEC) at 4 °C
9 on Superdex-200 pre-grade column 16/100 (Pharmacia, GE Healthcare Bio-sciences,
10 Pittsburgh, PA, USA), in 50 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl, 8
11 M Urea (running buffer) at a flow rate of 1.0 mL min⁻¹, and the elution profiles were
12 continuously monitored with an online detector at a wave length of 280 and 260 nm.
13 The target protein fractions were collected and protein purity was evaluated by
14 SDS-PAGE.

15 After purification by affinity chromatography, the relatively high purity of the
16 target fraction was pooled and loaded in dialysis bag. The purified recombinant
17 protein was renatured at 4 °C in buffer containing 1×PBS (Phosphate Buffered Saline,
18 pH 7.4), 4 mM GSH, 0.4 mM GSSG, 2 mM EDTA, 0.4 mL Arginine, and 2 M Urea.
19 After renaturation, fractions containing the protein were dialyzed against 1×PBS (pH
20 7.4) storage buffer for 6–8 h.

21 The dialyzates were separately loaded in tubes, freeze-dried, and then stored at
22 –80 °C; resulting protein powders were designated as the PhMEP1 fraction.

1 Immunoblot analysis

2 The separated proteins were electroblotted onto a polyvinylidene difluoride (PVDF)
3 membrane, which was blocked with 5% non-fat milk in PBS buffer with 0.05%
4 Tween 20 for 1 hour. The membrane was incubated overnight at 4°C with Anti-His tag
5 mouse monoclonal antibody (1:5000 dilution in blocking buffer), then washed, and
6 incubated with HRP-conjugated goat anti-mouse antibody (1:5000). The proteins
7 were detected using the chemiluminescence (CL) of luminol–H₂O₂ system.

8 Protease assay analysis

9 The enzyme activity was determined by the method of Ueda et al. 2013 with minor
10 modification. The Mep activity was detected using azocasein. The reaction system is
11 1 mL. The mixture of 2% (w/v) azocasein solution, 350 µL of 0.1 M Tris-HCl buffer
12 (pH 8.5) and 100 µL enzyme solution were incubated at 37 °C for 10 min. 500 µL of
13 10% trichloroacetic acid was then added to stop the reaction. The solution was left to
14 stand at room temperature for 1 hour, then centrifuged with 12000×g for 10 min. One
15 unit (U) of protease activity was defined as the amount of enzyme that will increase in
16 absorbance of 0.001 at 280 nm min⁻¹.

17 Inhibition experiment of metal ion chelating agent

18 The enzyme was incubated with an inhibitor (1,10-phenanthroline or EDTA) in 0.1 M
19 Tris-HCl buffer (pH 8.5) at 25 °C for 1 hour. The inhibitors and their concentrations in
20 the reaction system were as follows: EDTA (1 mM, 5 mM, 40 mM) and
21 1,10-phenanthroline (1 mM, 5 mM, 40 mM). The detection of the residual activity of
22 Mep after interaction with the inhibitors was based on the method of section above.

The activity observed in the absence of inhibitor (control) was set to 100% (Ueda et al. 2013).

Subcellular localization of *PhMEP1*

Solutions of *Agrobacterium tumefaciens*-carrying pCambia1302-eGFP-*PhMEP1* were infiltrated into leaf pavement cells of 4-week-old *N. benthamiana* plants. At two days post-infiltration (dpi), water-mounted slides of leaf tissue from agro-infected leaves were visualized by confocal microscopy. *A. tumefaciens*-carrying pCambia1302-eGFP was infiltrated into leaf cells as a control. Leaves were observed under a Nikon A1 confocal laser scanning microscopy.

Results and discussion

Sequence analyses of *PhMEP1* gene

The full-length cDNA sequence (3569 bp) of *PhMEP1* was obtained through 2 round 3'-RACE PCRs from *P. helianthi* isolate SY and deposited in the GenBank database (GenBank accession no. KU994904). The whole sequence of protease was analyzed by ORF Finder, and a sequence of 2781bp was obtained. The ORF region of the whole protease sequence was further analyzed by Interpro. The predicted PhMEP1 contained a signal peptide sequence (at 1–22aa) and a M36 metalloprotease domain (at 483–928 aa). The M36 peptidase domain is responsible for catalytic activity and contains the active site motif HEXXH (at 697–702 aa) characteristic of Zn metalloproteases (Fig. 1A). Further secondary structure analysis revealed that PhMEP1 were composed of 27.14% alpha helix, 13.19% extended strand, 4.86% beta turn and 54.81% random coil, and random coil was major part (Fig. 1B). The

1 predicted model of three-dimensional structure shows that PhMEP1 contained three
2 residues are important Zn binding sites: His697, Glu727 and His788 (Fig. 1C). These
3 results indicated that PhMEP1 should be a zinc metalloprotease in M36 family. A
4 phylogenetic tree of MEPs from 20 representative species was constructed, revealing
5 that PhMEP1 is most closely related to *Puccinia graminis* f. sp. *Tritici*
6 (KAA1133194.1) (Fig. 1D).

7 Fig. 1

8 Identification of PhMEP1 from *Puccinia helianthi*

9 The full-length ORF sequence of *PhMEP1* was amplified from the urediniospores of
10 *P. helianthi* isolate SY. The newly formed vector was named pET30a- *PhMEP1* and
11 identified by colony PCR (Fig. 2A) and double-enzyme digestion with *NdeI* and
12 *HindIII* (Fig. 2B).

13 Fig. 2

14 Expression and purification of *PhMEP1* in *E. coli*

15 PhMEP1 was expressed successfully in *E. coli* strain BL21 (DE3) after induction of
16 expression by IPTG for 16 h. The Mep fusion protein was obtained as expected (Fig.
17 3A).

18 The expressed fusion protein was harvested by ultrasonically lysing the *E. coli*.
19 After centrifugation, bacterial supersonic lysates and culture supernatants were tested.
20 Insoluble material from the lysates contained target protein (about 50 kDa).
21 Supernatant obtained from the lysed *E. coli* cell contained some protein with similar
22 molecular weight to the target protein and couldn't be adsorbed by Ni-IDA column

1 and eluted after incubation with Ni-IDA. No target protein was found in eluting
2 fractions after elution with 50 or 500 mM imidazole. Expressed fusion proteins
3 existed in the form of inclusion body in *E. coli* by SDS-PAGE.

4 After dissolution of inclusion bodies in binding buffer containing 8 M Urea, the
5 fusion protein was purified with metal affinity chromatography column. Eluted
6 fractions containing target proteins were obtained with different concentration
7 imidazole

8 By size-exclusion chromatography (SEC), the protein/DNA complexes were eluted
9 in a single peak and high purity protein was achieved. Fractions containing target
10 protein were shown to be homogeneous by SDS-PAGE (Fig. 3B). Relatively high
11 purity protein in lane 6–10 was pooled to be dialysed, and eventually concentrated to
12 a final protein concentration of 0.6 mg mL⁻¹ (before freeze drying).

13 Purified protein followed by SDS-PAGE revealed a band at about 50 kDa. This 50
14 kDa band represented the *P. helianthi* PhMEP1 further showed strong immunological
15 cross-reactivity with anti-His mouse monoclonal antibody prepared against the His
16 fusion protein of *P. helianthi* PhMEP1 (Fig. 3C).

17 Fig.3

18 Protease activity of PhMEP1

19 The results of azocasein hydrolysis showed that PhMEP1 could hydrolyze azocasein
20 substrate. The specific activity was 1560 U mg⁻¹.

21 PhMEP1 activity was suppressed by 1,10-phenanthroline and EDTA. The activity of
22 PhMEP1 was decreased step by step with the increasing inhibitor concentration.

1 When the concentration of 1,10-phenanthroline and EDTA is 1 mM, the activity of
2 purified Mep was reduced by 49% and 40% respectively. When the concentration of
3 1,10-phenanthroline and EDTA is 5 mM, the activity of purified Mep was reduced by
4 90% and 80% respectively. When the concentration of 1,10-phenanthroline and
5 EDTA is 40 mM, the activity of purified PhMEP1 was reduced by 99% and 96%
6 respectively (Table 1).

7 Table 1

8 The subcellular localization of PhMEP1

9 To determine the subcellular location of PhMEP1 , the full coding sequence of
10 PhMEP1 without the stop codon was subcloned in fusion to the N-terminus of GFP,
11 and the pCambia1302-eGFP-*PhMEP1* fusion-expressing vector was generated. The
12 resulting pCambia1302-eGFP-*PhMEP1* and the control pCambia1302-eGFP were
13 individually introduced into *N. benthamiana* leaf sell. These fluorescent proteins were
14 transiently expressed and observed via a confocal laser scanning microscopy. The
15 fluorescent images in *N. benthamiana* leaf sell showed that PhMEP1-GFP might be
16 distributed at the plasma membrane, but not in chloroplast, cell nucleus and cytoplasm,
17 whereas GFP protein alone was distributed in the plasma membrane and nucleus.

18 Fig. 4

19 In this research, we performed prokaryotic expression and described the
20 characteristics of a fungalsin-like metalloprotease from *P. helianthi*. As far as we are
21 concerned, this is the first time a fungalsin-like metalloprotease has been identified
22 from *P. helianthi*. Generally speaking, little is known about the function, specificity,

1 and modulatory mechanism of the extracellular proteases of fungus, so it is difficult to
2 speculate the roles of the individual M36 fungal proteases in *P. helianthi*. However,
3 the extensive understanding of this gene family provides a good model system for
4 learning the roles of the similar individual peptidases in the extracellular condition.

5 Extracellular proteases play a vital role as hydrolases that maintain and adjust the
6 nutritional requirements of microorganisms, and are widely accepted in the food and
7 pharmaceutical industries (Adekoya et al. 2009). The pathogen's extracellular
8 proteases are essential for regular growth and in favor of multiple ways of bacterial
9 infection. Among the secreted proteases, elastase and alkaline protease of
10 *Pseudomonas aeruginosa* are the two most recognized and understood
11 metalloproteases (Matsumoto 2004). Structurally, Mep active centers contain one or
12 two metal ions, most of which are zinc-containing proteins. In fact, many
13 zinc-containing bacterial proteases and fungal proteases are found throughout the
14 world within microorganisms (Chen et al. 2015; Ishii et al. 2014; Wu et al. 2011; Li et
15 al. 2014), such as the AprX extracellular metalloproteinases of many fluorescent
16 pseudomonads (Dufour et al. 2008; Zhang et al. 2009), members of the *Bacillus*
17 neutral protease family (Wu et al. 2011) the SmP metalloprotease (serralysin) of
18 *Serratia marcescens* (Chen et al. 2015), the RpA metalloprotease (serralysin) of
19 *Ralstonia pickettii* and alkaline protease of fungi . Extracellular Meps are known to be
20 important virulence factors related to bacterial and fungal pathogenicity (Zhang et al.
21 2017). Because of the great medical, physiological and commercial potential of these
22 enzymes in practical applications, it is essential to isolate and screen various

1 microbial strains.

2 Along with the results of the bioinformatic, the activity of *P. helianthi* PhMEP1 that
3 was suppressed by 1,10-phenanthroline and EDTA strongly suggested that the product
4 of the cloned gene was a metalloprotease. The localization of PhMEP1 in planta can
5 help us investigate if PhMEP1 associates with host molecules for interaction.

6 Taken together, our results suggest that PhMEP1 likely manipulates plants
7 molecules by targeting plasma membrane of epithelial cells, and degrading cellular
8 constituent via protein-binding, which is possibly a pathogenicity factor contributing
9 to penetration and infection by rust pathogen.

10 *P. helianthi* is an obligate pathogen of sunflower. The enzyme PhMEP1 secreted by
11 this pathogen would assist the invading fungus to penetrate through host barriers and
12 thus adding pathogen virulence. Since the use of extracellular enzymes to degrade the
13 structural barriers in the host appears to be a commonly used strategy of fungal
14 pathogens (Mercer et al. 2019; Podder et al. 2019), therapeutic approaches targeting
15 against the major virulence factors would be a highly desirable alternative to prevent
16 and treat sunflower rust.

17 **Conclusion**

18 We reported the characterization of an extracellular PhMEP1 gene from *P. helianthi*,
19 which is a causal agent of sunflower rust. Its molecular mass was about 50 kDa. The
20 Mep activity could be suppressed by 1,10-phenanthroline and EDTA. It could interact
21 with the cell wall, or the plasma membrane of host plant. It is possibly a pathogenicity
22 factor contributing to penetration and infection by rust pathogen.

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4   **Declaration**

5   **Conflict of interest** The authors declare no conflicts of interests.

6

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1   **Table 1.** Effect of protease inhibitors on enzyme activity

Inhibitors	Concentration (mM)	Relative activity (%)
Control		100
1,10-phenanthroline	1	51
	5	10
	40	1
EDTA	1	60
	5	20
	40	4

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Figure legends

Fig. 1 Bioinformatic analysis of PhMEP1 protein. **A:** The structural domain sites in PhMEP1 protein. **B:** The secondary structure of PhMEP1. **C:** The predicted model of PhMEP1 protein was made with the Phyre2 server. The residues implicate Zn binding sites are show in blue. **D:** Phylogenetic analysis of 20 MEP proteins.

Fig. 2 Cloning and heterologous expression of PhMEP1. **A:** Colony PCR of pET30a-*PhMEP1* transformant in *E. coli*, using primers synthesized on the basis of the amino acid sequences of the mature protein. Lanes: M, 15000 DNA Marker; 1–6, production of PCR. Target PCR product about 1300 bp is pointed with black arrowheads. **B:** Restriction analysis of recombinant plasmid pET30a-*PhMEP1*. Two separate bands at about bp 5200 and 1300 could be seen. Lanes: M, DNA Marker; 1, plasmid DNA; 2, fragments generated from pET30a-*Mep* digested by *NdeI* and *HindIII*

Fig. 3 Expression and purification of PhMEP1. **A:** SDS-PAGE of cell lysates of *E. coli* transformants containing the proteinase expression vector. Lanes: M, SDS-PAGE protein marker; 1, uninduced control; 2, induced with IPTG for 16 h at 15 °C; 3, induced with IPTG for 16 h at 37 °C. Target protein about 50 kDa is pointed with black arrowheads **B:** SDS-PAGE analysis of purified proteins by SEC. Lanes: M, protein marker; 1, purified sample; 2,3, fractions collected after purified by SEC. Target protein about 50 kDa is pointed with black arrowheads. **C:** Western blot analysis. Western hybridization of 2 µg His fusion protein with anti-His mouse monoclonal antibody in 1:5000 dilution. Lanes: 1, BSA (1.5 µg); 2, PhMEP1 protein (2 µg); M, Western blot marker. Target protein is about 50 kDa pointed with black arrowheads

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Fig. 4 Subcellular localization of the PhMEP1. The mature coding sequences of PhMEP1 was cloned in frame with GFP. Live cell imaging using confocal microscope of epidermal cells of 2-days-old *N. benthamiana* transient expression *Mep-GFP* plantlets. Bar=20 μ m

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1   **Fig. 2**

1 **Fig. 3**

1 Fig. 4

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