

Distribution of Legume Arabinogalactan Protein-Extensin (AGPE) Glycoproteins in Symbiotically Defective Pea Mutants with Abnormal Infection Threads

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Received February 11, 2008

Abstract—The interface between the host cell and the microsymbiont is an important zone for development and differentiation during consecutive stages of Rhizobium-legume symbiosis. Legume root nodule extensins, otherwise known as arabinogalactan protein-extensins (AGPEs) are abundant components of infection thread matrix. We have characterized the origin and distribution of these glycoproteins at the symbiotic interface of root nodules of symbiotically defective mutants of pea (*Pisum sativum* L.) by using immunogold localization with MAC265 an anti-AGPE monoclonal antibody. For mutants with defective growth of infection threads, the AGPE epitope was abundant in the extracellular matrix surrounding infected host cells in the central infected tissue of the nodule, as well as in the lumen of Rhizobium-induced infection threads. This seems to indicate a mistargeting of AGPE as a consequence of abnormal growth of the infection threads. Furthermore, mutants in the gene *sym33* showed reduced labeling with MAC265 and, in some infection threads and droplets, the label was completely absent, a phenomenon that is not observed in wild-type nodules. This suggests an alteration in the composition of the infection thread matrix for *sym33* mutants, which may be correlated to the absence of endocytosis of rhizobia into the host cytoplasm.

Key words: plant-microbe interactions, Rhizobium-legume symbiosis, symbiotic mutants, symbiotic nodule, infection thread, immunocytochemical analysis, garden pea (*Pisum sativum* L.), Pumilio family proteins

DOI: 10.1134/S1990519X09010131

Abbreviations used: ABB, Aurion blocking buffer; AGP, arabinogalactan protein; AGPE, arabinogalactan protein-extensin; BSA, bovine serum albumin; BSA-C, acetylated BSA; LPS, lipopolysaccharide; PBS, phosphate buffered saline; PUF, Pumilio protein family; RNE, root nodule extensin; 3'-UTR, 3'-untranslated region

INTRODUCTION

The development of Rhizobium-legume symbiosis involves the integration of two main processes, i.e., host cell invasion and nodule morphogenesis. The former process involves the formation of symbiotic compartments containing rhizobial cells (inter- and intracellular infection threads and symbiosomes), while the latter process involves the differentiation of metabolically specialized host cells and tissues (Provorov et al., 2002). These processes are controlled and integrated by the plant genetic system and apparently involves the differential expression of 800–900 genes (Udvardi et al., 2004), including the *nod* and *sym* genes.

The infection of legume host cells begins when rhizobia attach to the tip of the root hair cell. The colonization of the plant root by rhizobia occurs via the formation of a transcellular tunnel-like structure called the infection thread that grows through the cytoplasm by the apical deposition of the cell-wall material of a plant (Brewin, 1991, 2004). During the development of the legume nodule, the surface components of both plant and rhizobia undergo profound modifications associated with the molecular interactions between symbiotic partners (VandenBosch et al., 1989a; Brewin, 2004; Gage, 2004).

Bacteria in infection threads are topologically located in a transcellular tunnel inside the host cell and the infection thread wall is a continuation of the host cell wall, which comprises esterified and deesterified pectins, xyloglucans, and cellulose fibrils (Rae et al., 1992). A major component of the matrix inside the infection thread lumen that contains material similar to that of the intercellular matrix is plant glycoproteins, which seem to be extremely dynamic components of plant surfaces (see reviews Cassab, 1998; Brewin,

List of symbiotic mutants of the pea (*Pisum sativum* L.)

Line	Phenotype	References
SGE	Wild-type line	Kosterin, Rozov, 1993
SGEFix ⁻ -1 (<i>sym40</i>)	Abnormal (hypertrophied) infection droplet formation (Idd ⁻) ^a , leaky phenotype	Tsyganov et al., 1994, 1998
SGEFix ⁻ -2 (<i>sym33</i>)	No endocytosis of bacteria (Itn ⁻) ^b , leaky phenotype	Tsyganov et al., 1994, 1998
RBT3 (<i>sym33</i> , <i>sym40</i>)	No endocytosis of bacteria (Itn ⁻) ^b	Borisov et al., 1997a
RBT4 (<i>sym33</i> , <i>sym42</i>)	No endocytosis of bacteria (Itn ⁻) ^b	Borisov et al., 1997a
Finale	Wild-type line	Engvild, 1987
RisFixV (<i>sym42</i>)	Early senescence of nodule parenchyma, maturation of infection threads (Nop) ^c	Engvild, 1987; Morzhina et al., 2000

Note: ^a Idd, infection droplet differentiation; ^b Itn, infection thread growth in nodule, ^c Nop, nodule persistence.

2004). Arabinogalactan proteins (AGPs) are a class of hydroxyproline-rich glycoproteins that are located in infected tissues of certain legume root nodules, in cyanobacterial stem gland symbioses, and in arbuscular-vesicular mycorrhizae (Cassab, 1998). The infection-specific expression of AGPs suggests that these molecules play an essential role in symbiotic tissue, most likely at the symbiotic interface, in each of these plant-microbe associations (Perotto et al., 1991; Berry et al., 2002). Legumes are unique in that they synthesize a complex copolymer that contains alternating AGP and extensin motifs (Rathbun et al., 2002; Brewin, 2004). These tyrosine-rich glycoproteins, called AGP-extensins or AGPEs, are closely associated with the infection process.

Intensive genetic studies of the nodule formation process in pea have resulted in the identification of several plant genes that control the development of infection threads (Morzhina et al., 2000, Tsyganov et al., 2002). By using these mutants in combination with monoclonal antibodies that react with components of the plant-bacteria interface in infection threads, it has become possible to analyze surface interactions between the symbiotic partners.

The main goal of the present work was to study the growth and development of the infection thread during the morphogenesis of the nodule using monoclonal antibodies to analyze components of the plant cellular surface in a series of pea (*Pisum sativum* L.) symbiotic mutants blocked at different stages of infection-thread development. These studies have revealed that the targeting and post-translational modification of legume AGPEs in these symbiotically defective mutants are different.

MATERIALS AND METHODS

Plant material. Fix⁻-mutants of pea (*Pisum sativum* L.) blocked at different stages of infection thread

development, as well as their parental lines, were used in this study (table).

Growth conditions. Plants were grown in growth cabinets HeraeusVötsch HPS2000 (day : night 16 : 8 h, temperature 21 : 19°C, relative humidity of 75%, illumination of 490 mE/m²/s). Sterile vermiculite was used as a substrate. Seeds were surface sterilized with concentrated sulphuric acid for 30 min at room temperature and inoculated at the time of sowing with 1 ml of aqueous suspension of *Rhizobium leguminosarum* bv. *viciae* 3841 (Wang et al., 1982) (10⁸–10⁹ cells/ml). Plants were watered with a nitrogen-free nutrient solution as described previously (Borisov et al., 1997b). Nodules were collected 14 days after inoculation. Ten plants per variant were used.

Monoclonal antibodies. Monoclonal antibodies used for immunofluorescence and immunogold labeling were as follows: MAC57 recognizing *Rhizobium* 3841 lipopolysaccharide (Bradley et al., 1988); MAC265, recognizing legume arabinogalactan protein-extensin (VandenBosch et al., 1989a,b; Brewin, 2004) from the John Innes Centre collection, Norwich, United Kingdom.

Immunofluorescence and confocal microscopy. Nodules were harvested from roots and transferred directly into fixative. The whole nodules were fixed in 2.5% (v/v) glutaraldehyde (Sigma-Aldrich, United States) in 0.5 M sodium cacodylate, pH 7.2. A glancing cut on one side of a nodule was made to achieve better penetration of the fixative. After vacuum infiltration, floating nodules were discarded and the fixative was replaced with fresh solution. After overnight incubation at room temperature, nodules were dehydrated in an ascending ethanol series at -35°C and infiltrated and embedded in London Resin White (Sigma-Aldrich, United States) using benzoin methyl ether as a catalyst for UV polymerization at -20°C.

0.5- μm -thick, resin-embedded sections were cut with a glass knife and collected on IPN multiwell slides. Specimens were stained in 5% (w/v) Toluidine blue in 0.1 mM sodium borate for light microscopy. For immunofluorescence microscopy, the sections were incubated in a blocking solution containing 5% (w/v) dried milk in PBS (2.48 g/l NaH_2PO_4 , 21.36 g/l Na_2HPO_4 , 87.66 g/l NaCl, pH 7.4) for 1 h at room temperature. The sections were incubated with primary antibody for 1 h at 37°C, diluted 1 : 50 in 3% (w/v) bovine serum albumin, BSA (Sigma-Aldrich, United States), in PBS. The sections were rinsed several times in 3% (w/v) BSA in PBS, and incubated with secondary antibody (Alexa Fluor 488 goat anti-rat IgG, Molecular Probes, the Netherlands) for 1 h at 37°C or overnight at 4°C (diluted 1 : 100 in 3% (w/v) BSA in PBS). After washing several times in 3% (w/v) BSA in PBS, the sections were mounted with Vectashield (anti-fade, Vector Lab Inc., United Kingdom) and examined using fluorescent filters FITS (465–495 nm and 515–555 nm) on a Nikon Eclipse 800 with a Nikon Coolpix 995 digital camera (Japan).

For confocal study, 0.5- μm -thick, resin-embedded sections were blocked in 3% (w/v) BSA in PBS for 20 min at room temperature. Then, the sections were incubated with primary antibody (diluted 1 : 50 in 3% BSA in PBS) for 1 h at 37°C or overnight at 4°C. After washing in 3% BSA in PBS, the sections were incubated with secondary antibody, anti-rat antibody conjugated with colloidal gold (5 nm in diameter) (Amersham International, United Kingdom) diluted 1 : 200 for 1 h at 37°C and washed in 3% BSA in PBS for 20 min. The sections were postfixed in 0.5% (v/v) glutaraldehyde in PBS for 3 min to cross link antibodies in order to achieve better stability and washed in deionized water. After drying, the sections were incubated with silver staining solution using a silver enhancing kit (AGAR—R14115, BBI International, United Kingdom) at room temperature for 3 min in compliance with the manufacturer's instructions. Slides with sections were rinsed with running (medium flow) tap water for 10–20 min. Then, the sections were mounted using glycerol or Vectashield and examined in a Leica DMR (Germany) upright confocal microscope using reflection optics for the silver and broad fluorescence for the autofluorescent outline.

Electron microscopy. For transmission electron microscopy, 90–100-nm-thick ultrathin sections were collected on gold grids coated with 4% (w/v) pyroxilin and carbon. After blocking with 50 mM glycine in PBS for 15 min and in ABB (5% BSA, 0.1% cold water fish skin (CWFS) gelatin, 5–10% normal goat serum (the same species as the secondary antibody), 15 mM NaN_3 in PBS, pH 7.4; Aurion, the Netherlands) for 30 min, the sections were washed by passing through five drops of 0.1% BSA-C (Aurion, the Netherlands) in PBS and incubated overnight at 4°C with primary antibody

(diluted 1 : 50 or 1 : 100 in 0.1% BSA-C). After washing, the sections were passed through five drops of 0.1% BSA-C, incubated with secondary antibody, goat anti-rat conjugated with 10-nm colloidal gold (Amersham International, United Kingdom), and diluted 1 : 50 in 0.1% BSA-C in PBS for 4 h at room temperature. The grids containing sections were then washed three times in PBS for 20 min, twice in water for 30 min, and counterstained in 2% (w/v) aqueous uranyl acetate for 1 h followed by lead citrate for 1 min. All solutions were filtered before use and filter-sterilized deionized water was used throughout the experiment. The tissue of nodules was examined and photographed in a JEM-1200 EM (JEOL Corp., Japan) transmission electron microscope at 80 kV.

RESULTS

Histological and ultrastructural organization of root nodules. Nodule sections of the parental lines SGE and Finale had histological and ultrastructural organizations typical of the root nodules of garden pea (Newcomb, 1976; Brewin, 1991). Plants of mutant lines SGEFix⁻¹ (*sym40*), RisFixV (*sym42*), and SGEFix⁻² (*sym33*) formed ineffective nodules that showed alterations in histological and ultrastructural differentiation as described previously (Morzhina et al., 2000; Tsyganov et al., 1998, 2003). In most aspects the double mutants RBT3 (*sym33*, *sym40*) and RBT4 (*sym33*, *sym42*) showed the same histological and ultrastructural organization as the single mutant SGEFix⁻² (*sym33*) (Tsyganov et al., 2003).

Immunolocalization of AGPE. For laboratory lines SGE and Finale, immunofluorescent, confocal, and immunogold studies showed the presence of rhizobial lipopolysaccharide labeled with MAC57 in bacteria within infection threads and in bacteroids within symbiosomes (Figs. 1a, 2a). The immunofluorescent and confocal studies also identified MAC265 antigen (AGPE) as a major component of the infection thread lumen (Figs. 1b, 2b, 3a). Within wild-type nodules, the antibody recognized infection structures in the invasion zone, as well as remnant threads in the mature zone (Fig. 3a).

Single mutant SGEFix⁻¹ (*sym40*). After labeling with MAC57, immunofluorescence and confocal microscopy showed localization of rhizobial cells like bacteria in infection threads and like bacteroids in symbiosomes (Figs. 1c, 2c). The MAC265 antigen was recognized as a major component of the infection thread lumen and infection droplet matrix, like in wild-type nodules (Figs. 1d, 2-d). However, in contrast to wild-type nodules, label of MAC265 was also observed in intercellular spaces of the nodule infected tissue by both confocal (Fig. 2d) and immunogold (Fig. 3b) microscopy.

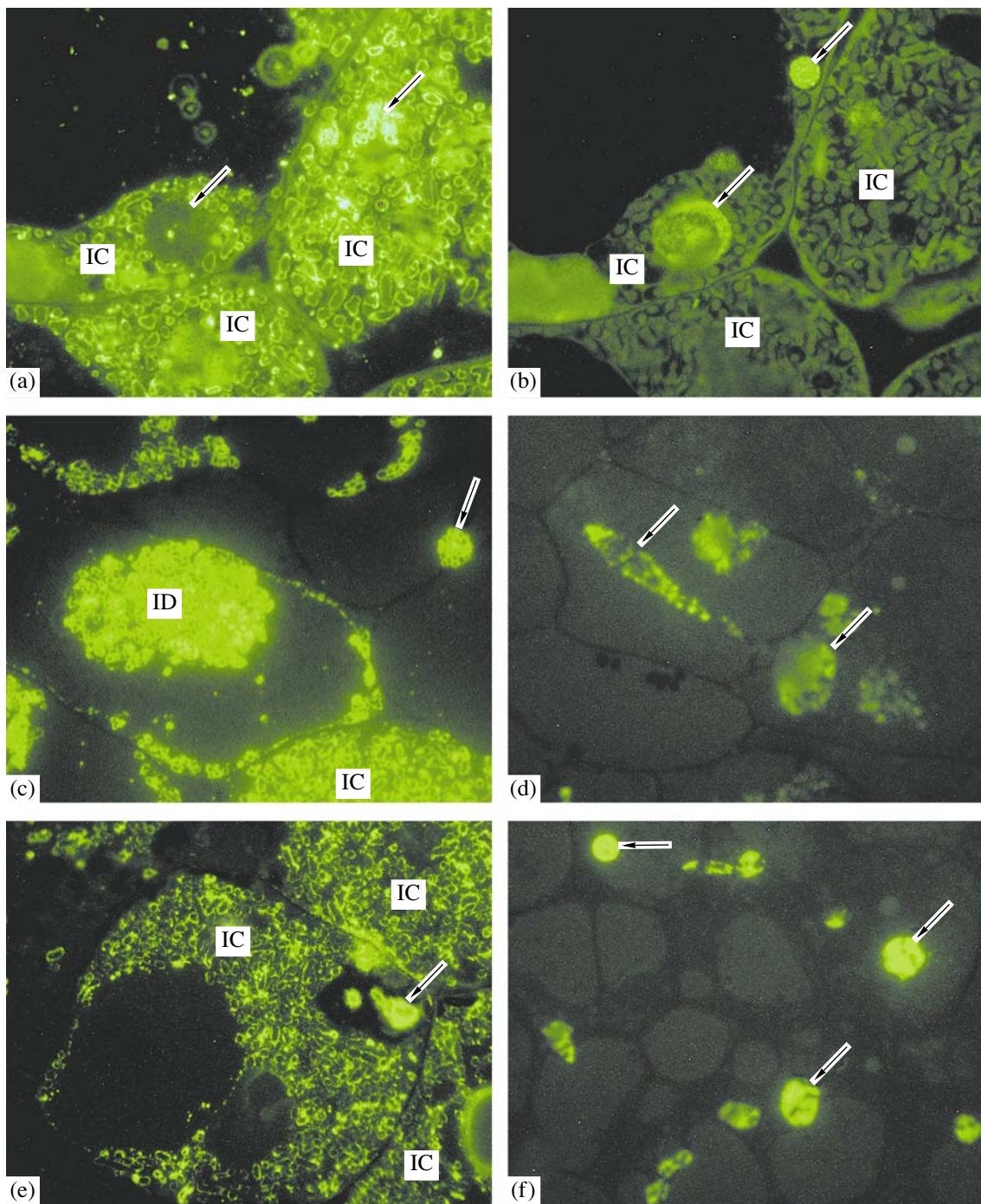


Fig. 1. Immunofluorescent localization of rhizobial lipopolysaccharide (LPS) (a, c, e, g, i, k) of bacteria inside infection threads, bacteroids inside infected cells and legume AGPE (b, d, f, h, j, l) of the infection thread matrix. Identification by using monoclonal antibody MAC57 for LPS of *R. leguminosarum* bv. *viciae* 3841 and MAC265 for AGPE.

(a, b) Parental laboratory line SGE; (c, d) mutant line SGEFix⁻¹ (*sym40*); (e, f) mutant line RisFixV (*sym42*); (g, h) mutant line SGEFix⁻² (*sym33*); (i, j) double mutant line RBT3 (*sym33, sym40*); (k, l) double mutant line RBT4 (*sym33, sym42*); arrows indicate infection threads, ID infection droplet, IC, infected cell. Obj.: $\times 100$.

Single mutant RisFixV (*sym42*). The rhizobial lipopolysaccharide recognized by MAC57 antibody was located in bacteria inside the infection threads and in bacteroids inside the host cell cytoplasm (Fig.

1e). In mutant RisFixV, immunofluorescent and immunogold studies revealed the presence of MAC265 antigen in the lumen of both types of infection threads (in normal infection threads and in infec-

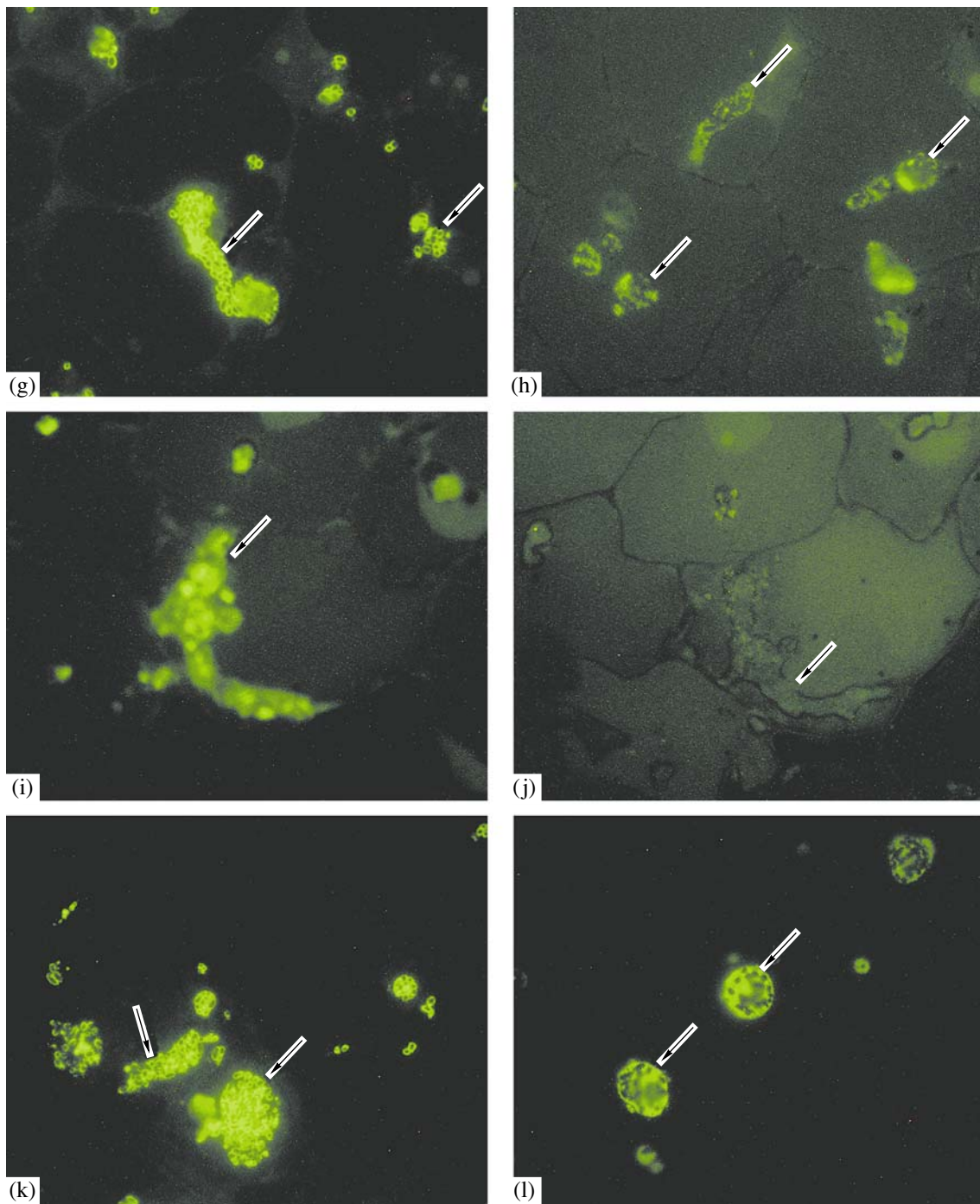


Fig. 1. (Contd.)

tion threads with abnormally thick cell walls) (Fig. 1f). However, in some infection threads, the MAC265 labeling was absent from part of the infection thread matrix (Fig. 3f).

Single mutant SGEFix⁻² (*sym33*) and double mutants RBT3 (*sym33, sym40*) and RBT4 (*sym33, sym42*). The immunofluorescence study of localization

of bacterial lipopolysaccharide showed the presence of MAC57 antigen inside infection threads (Figs. 1g, 1i, 1k, 2e). In these mutants (but not in wild-type nodules), the MAC265 epitope was identified in the intercellular spaces of infected nodule parenchyma using both silver staining and confocal microscopy techniques (data not shown). The immunofluorescence study also showed the MAC265 antigen signal in the lumen of infection

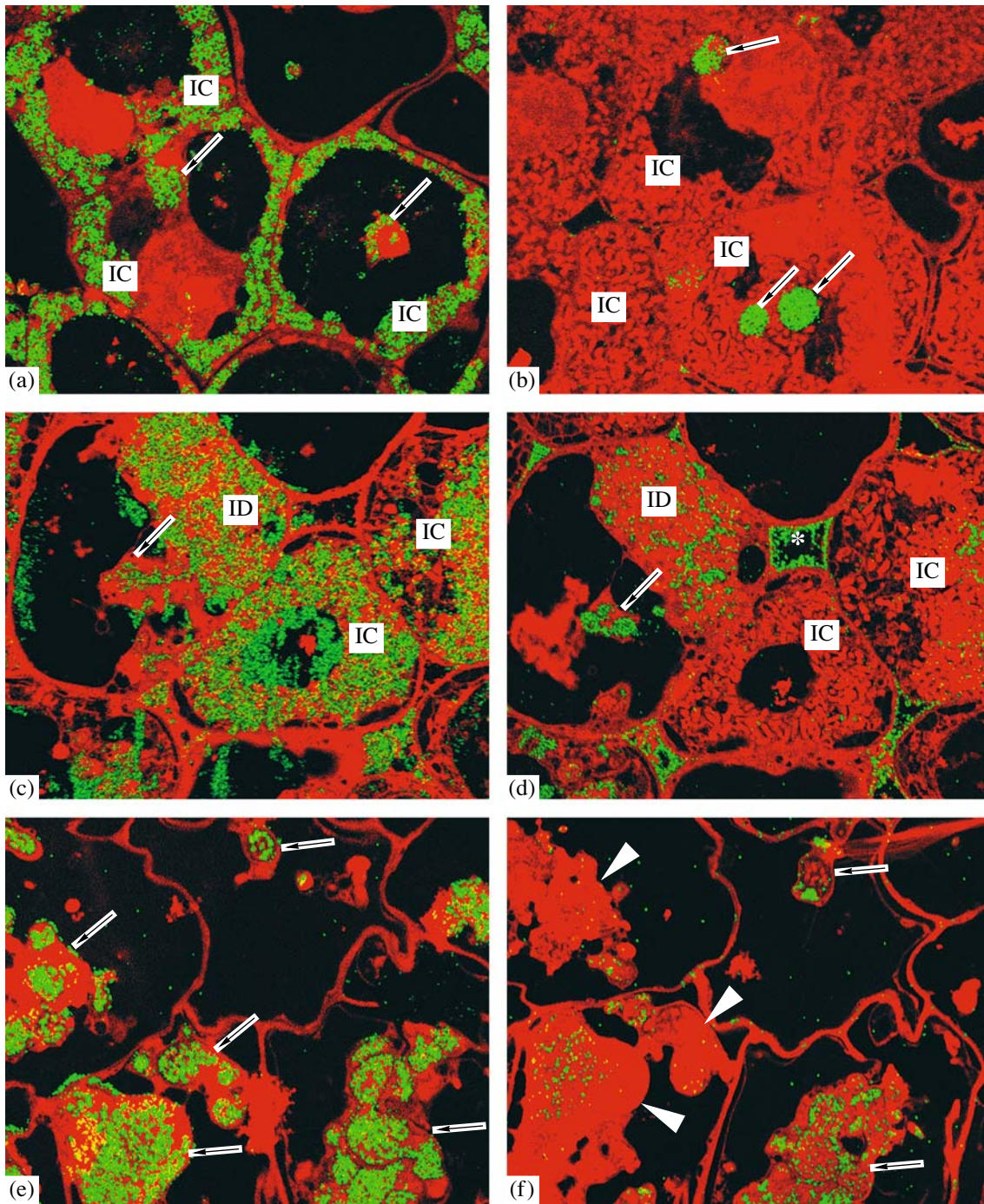


Fig. 2. Localization of LPS (a, c, e) of bacteria inside infection threads and droplets, bacteroids inside infected cells and AGPE (b, d, f) of the infection thread matrix in the fixation zone of pea nodules. Identification by using monoclonal antibody MAC57 for LPS of *R. leguminosarum* bv. *viceae* 3841 and MAC265 for AGPE and confocal microscopy.

(a, b) Parental line SGE; (c, d) mutant line SGEFix⁻¹ (*sym40*); (e, f) double mutant line RBT3 (*sym33*, *sym42*); arrows indicate infection threads; arrowheads indicate the relative absence of AGPE labeled MAC265 in infection threads of mutant RBT3, asterisk - localization of AGPE labeled MAC265 in the intercellular spaces of the central infected host tissue of mutant SGEFix⁻¹. Obj.: ×40.

threads (Figs. 1h, 1j, 1l, 2f). After the immunogold staining of nodule tissue, MAC265 antigens were localized in the lumen of infection threads and in the matrix of infection droplets (Figs. 3d, 3e). The MAC265 anti-

gen label was absent in some infection threads with electron dense matrix in the lumen, a phenomenon not observed in wild-type nodules (Fig. 3c). Features of degradation of rhizobia were observed inside these

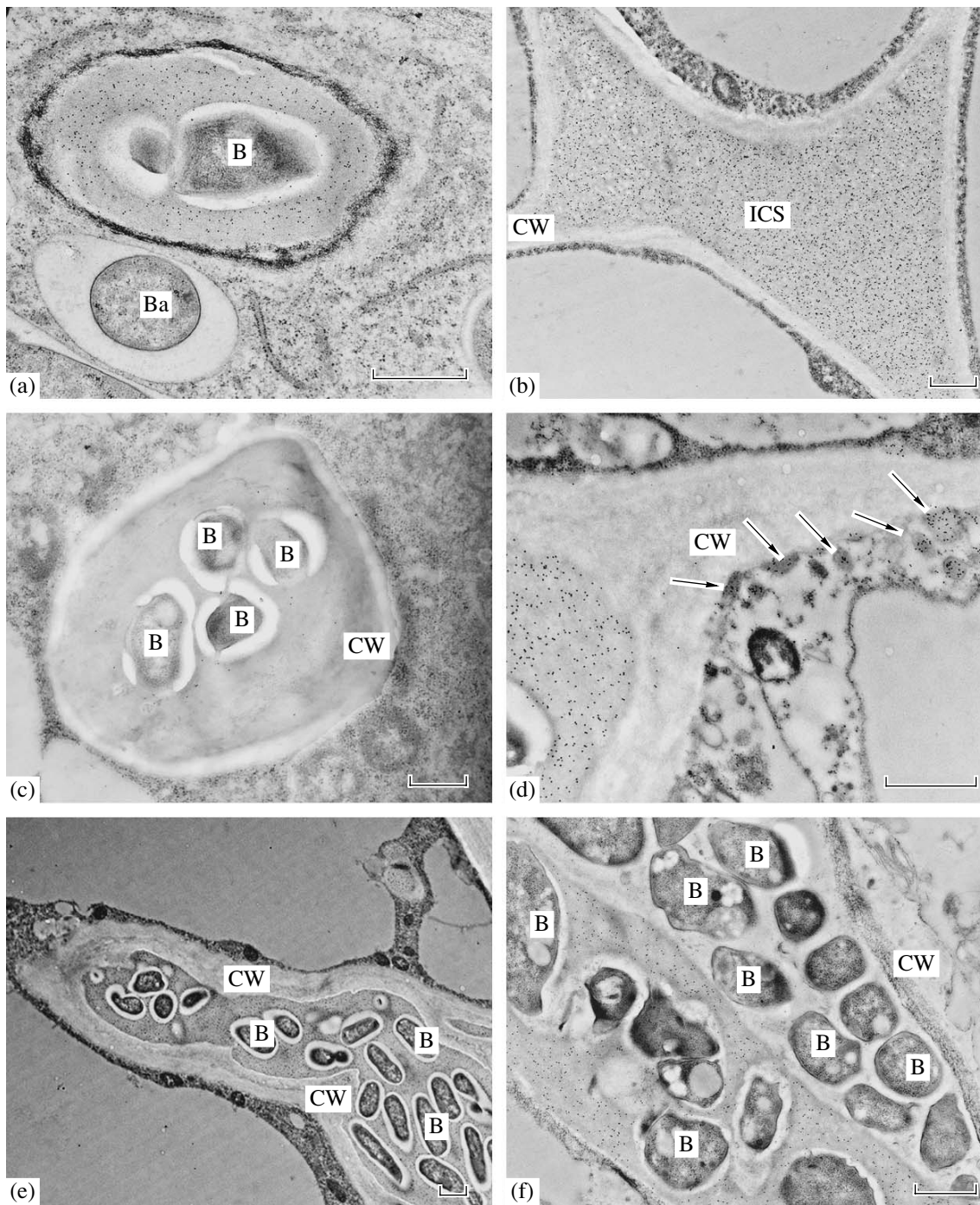


Fig. 3. Immunogold localization of AGPE labeled MAC265 in infection threads from the fixation zone of pea nodules.

(a) Localization AGPE in the lumen of wild-type infection thread of parental line SGE; (b) localization of AGPE in the intercellular spaces of mutant line SGEFix⁻¹ (*sym40*); (c) the absence of AGPE labeled MAC265 in the lumen of infection thread of mutant line SGEFix⁻² (*sym33*) with thick infection thread wall; (d) transport vesicles carrying AGPE labeled MAC265 near the plasma membrane of infected cell of mutant RBT3 (*sym33*, *sym40*); (e) localization of AGPE in the matrix of infection thread of mutant RBT4 (*sym33*, *sym42*); (f) the absence of AGPE labeled MAC265 from the part of infection thread matrix in mutant RisFixV (*sym42*). B, bacterium, Ba, bacteroid, CW, cell wall, ICS, intercellular space; arrows indicate transport vesicles. Bar: 0.5 μ m.

infection threads. In mutant RBT3 (*sym33*, *sym40*), some small cytoplasmic vesicles carrying MAC265 antigen were also seen near the plasma membrane (Fig. 3d).

DISCUSSION

Legume AGP-extensins (AGPEs), which were previously known as “root nodule extensins” (RNEs) are

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UAGAUAGCCC AUAUUUGGCA UCACACCAUA UGGAUCUAAA 40
CAGAAAGUGU GAAAGCAAAG AAAGCCAUGC ACAAAGAAGA 80
UCAAGGGGAU UCUUCACAU AUAUUUCGU CAGCAUUUGA 120
UUAAGAUUGU UUAGAGGGCU CCGUUUAUUU GUAUUUAAU 160
UGUGUUUUUG UUGUAUUAUC CACAGCUGUA AGGCACUGCC 200
CCAAUAUGCA UGUGUCUGCA GUUGUUUUCA UGCAUUGUAA 240
UUUUCAAAGU UCGGCUUUGU AUCAAGCUGA CUCACAAACU 260
AAAUAAUAU AUAUCCCCUA UUAUAGUAA AAAAAAAAAA 300
AAAAAAAA

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Fig. 4. 3'-Untranslated Region of mRNA of *MtN12 Medicago truncatula* encoding legume AGP-extensin (according to Gamas et al., 1996). The presence of seven UUGU motifs is highlighted in bold.

highly glycosylated plant glycoproteins (Rathbun et al., 2002); they were first identified as soluble components of pea root nodule extracts (Bradley et al., 1988). These glycoproteins migrated to SDS polyacrylamide gels in the molecular weight range of 900–110 kDa and could be recognized on western blots by the monoclonal antibody MAC265 (VandenBosch et al., 1989a, b). Immunocytological analysis with the monoclonal antibody MAC265 has revealed corresponding antigens located in the lumen of Rhizobium-induced infection threads where individual encapsulated bacterial cells appeared to be embedded in a matrix material containing the plant glycoprotein (Rae et al., 1992). In the present study, this antibody was shown to recognize new infection threads in the infection zone and mature infection threads in the nitrogen-fixing zone of root nodules of wild-type and mutant plants, which agreed with previous investigations (VandenBosch et al., 1989a; Rae et al., 1992; Rathbun et al., 2002). However, in these symbiotically defective Fix⁻mutants, the MAC265 antigen was also observed in intercellular spaces of infected nodule tissue (Fig. 4b). In the double mutant RBT3 (*sym33*, *sym40*), small cytoplasmic vesicles carrying MAC265 antigen were also found to have accumulated near the plasma membrane of infected cells (Fig. 3d). The appearance of the MAC265 label in intercellular spaces could be due to mistargeting of AGPE as a result of a blockage of normal infection thread development. This possibility is consistent with the observed accumulation of cytoplasmic vesicles carrying the MAC265 antigen (Fig. 3d). The targeted secretion of the matrix glycoprotein seems to be closely correlated with the process of infection thread growth (Rae et al., 1992).

These observations of abnormal targeting for AGPE in symbiotically defective lines could be associated with the possible existence of a plant locosome that controls the polar growth of infection threads. Rathbun et al. (2002) noted the 3'-untranslated region of mRNA of AGP-extensin from pea to be extremely conservative as compared with that in *MtN12*, the homologous gene from *Medicago truncatula*. As seen in the presented sequence (Fig. 4), there are multiple UUGU binding

sites for proteins of the Pumilio family in the 3'-UTR of mRNA of *MtN12*.

Members of the Pumilio (PUF) protein family are often involved in achieving cell polarity, e.g., in budding yeast (Puf6p/*ASH1* mRNA; Puf3p/*Mdm12p* mRNA) (Gu et al., 2004; Garcia-Rodriguez et al., 2007) and *Drosophila* embryo: (Pumilio/*nanos* mRNA) (Wreden et al., 1997); however, their role in plant cells has not yet been demonstrated. PUF proteins bind to the 3'-UTR sequence of target mRNA that carry the UUGU motif (Gerber et al., 2004). Prior to their release from the nucleus, the RNA-protein dimer becomes associated in a larger ribonucleoprotein complex. In budding yeast, this complex is called the "locosome" and contains She2p, She3p, and Myo4p components that repress protein translation and promote translocation of RNA along the polarized actin cytoskeleton towards the predetermined region of the cell (Gu et al., 2004). In the case of the Rhizobium-legume symbiosis, the PUF association could account for the polarized apical growth of infection threads. Thus, in mutants in which the development of infection threads is reduced or abnormal, vesicles carrying AGPE (Fig. 3d) may be diverted towards the extracellular matrix rather than towards the lumen of infection threads.

In the present study, we also revealed another immunocytological phenomenon that was not reported previously in wild-type nodules. In single and double mutants, the labeling of MAC265 antigens in lumen of some infection threads was absent (Fig. 3c). These infection threads showed features of bacterial degradation in the lumen, such as the appearance of the polyoxybutyrate in bacterial cells, swelling of the periplasmic space, and lysis of bacteria. In addition, immunogold labeling in the single mutant RisFixV (*sym42*) indicated that the MAC265 labeling was sometimes absent in a part of the matrix in some infection threads (Fig. 3f), which might suggest that the growth and development of infection structures are complex and nonuniform processes in these symbiotically noneffective mutants.

These observations indicate the importance of the plant cell wall remodeling in the *Rhizobium* infection process, as well as the need for a systematic analysis of the structure and development of infection threads using molecular probes that recognize components of the plant extracellular matrix. Studies of the process by which AGPE is targeted in the lumen of the infection thread could also help reveal how the architecture of the cell wall and the orientation of infection thread growth are connected to the cytoskeletal orientation and control of the cell cycle (Kistner and Parniske, 2002; Brewin, 2004).

ACKNOWLEDGMENTS

This work was supported by the INTAS (YSF 04-83-3196), the Federal Science Agency of Russia (state contract No. 02.442.11.7130), the Russian Foundation for Basic Research (RFBR) (05-04-49105-a), the United Kingdom Biotechnology and Biological Research Council, joint grants RFBR with the Netherlands' Organization for Scientific Research (06-04-89000-NVOTs-a), and U.S. Civilian Research & Development Foundation (CRDF) with the Russian Ministry of Education and Science (RUXO-012-ST-06, DP2M12).

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