

Microscopy of the interaction of *hrp* mutants of *Pseudomonas syringae* pv. *phaseolicola* with a nonhost plant

Abstract

Pseudomonas syringae pv. *phaseolicola* is a pathogen of green bean and induces localized, rapid plant cell death (the hypersensitive response or HR) when placed into leaves of the nonhost plant tobacco. The *hrp* genes control both pathogenicity and the ability to cause the HR. In this study we examined the interaction of the wild-type parent strain (NPS3121) and three independent, prototrophic *hrp* mutants (NPS4000, NPS4003, NPS4005) with tobacco by use of light and transmission electron microscopy. Even though the *hrp* mutants did not induce macroscopic symptoms on leaves by 48 h postinoculation, localized disruption of parenchyma cells did occur. The rapidity and severity of microscopic symptoms was related to the relative abilities of the mutants to cause macroscopic symptoms on green bean. Cells of the *hrp* mutants exhibited extensive surface blebbing. The increased surface blebbing of the mutants may reflect either outer membrane disorganization and/or a reduced ability to cope with an hypoosmotic apoplastic environment by production of periplasmic glucans. Unlike induction of a macroscopic HR, induction of more subtle responses by nonhost plant cells is not dependent on the presence of an intact *hrp* cluster.

Keywords: *Pseudomonas syringae*; *hrp*; Microscopy; Hypersensitive response; Tobacco

1. Introduction

Plant pathogenic bacteria often induce a rapid and severe defense response when inoculated onto leaves of nonhost plant species or resistant host cultivars. This phenomenon is called the hypersen-

sitive response (HR) and is characterized by rapid, highly localized death of leaf cells where bacteria are introduced. The HR is thought to restrict the growth and spread of plant pathogenic bacteria (for a review see Goodman and Novacky [1]). Tissue necrosis visible to the naked eye (a macroscopic HR) can result if bacterial concentrations of approximately 5×10^6 colony-forming units per ml or greater are used for inoculation.

By the use of molecular biological techniques, several research groups have generated so called

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hrp (hypersensitive reaction and pathogenicity) gene mutants of plant pathogenic bacteria which are no longer capable of inducing the HR on nonhost plants and are also of reduced virulence or nonpathogenic towards normally susceptible plants (for a review see Willis et al. [2]). The *hrp* gene clusters appear to be involved in the secretion of bacterial products encoded by *hrp* genes or avirulence (*avr*) genes (*avr* genes are involved in the induction of cultivar specific HR) such as proteinaceous or nonproteinaceous elicitors which determine both pathogenicity and/or host range [3–8]. The *hrp* gene clusters also contain regulatory genes which respond to the host environment and possibly to, as yet undefined, plant signal molecules [9–13].

The first set of *hrp* mutants was reported in 1986 by Lindgren et al. [14]. Using Tn5 mutagenesis, they generated a set of eight independent prototrophic *hrp* mutants of the bean pathogen *Pseudomonas syringae* pv. *phaseolicola*. This pathogen causes halo blight disease on susceptible bean cultivars characterized by the formation of water-soaked lesions on leaves and induces the HR on resistant bean cultivars as well as nonhost plants. Seven of the eight Tn5 insertions are physically linked and the mutants were placed in seven different *hrp* genes/operons (*hrpL*, *hrpAB*, *hrpC*, *hrpD*, *hrpE*, *hrpF* and *hrpRS*) by complementation analysis [15,16]. The seven contiguous operons are found in a 22-kb cluster.

The defect(s) leading to the Hrp phenotype of *P.s.* pv. *phaseolicola* have not been identified for the majority of mutants. The *hrpS* gene product acts along with alternative sigma factor 54 to control the operons in the *hrp* cluster along with HrpL and HrpR. HrpR shows strong sequence similarity to HrpS and both are members of the NtrC family of regulatory proteins [17]. Strain NPS4005 contains the unlinked locus designated as *hrpM* [18]. This locus is comprised of two functional open reading frames and was found to have greater than 97% sequence homology to the *hrpM* locus previously cloned from *P.s.* pv. *syringae* [19,20]. The second open reading frame (ORF2) is inactivated in strain NPS4005. Recently, Loubens et al. [21] reported high sequence homology of *hrpM* from *P.s.* pv. *syringae* and *mdoGH* from *Escherichia*

coli. The *mdoGH* operon is required for the synthesis of membrane-derived oligosaccharides found in the periplasmic space in response to low osmolyte environments. Two additional *hrp* genes (*hrpT* and *hrpQ*) were recently discovered and appear to control transcriptional activation of *hrpS* in planta [17].

To date, there have been no light or electron microscopic studies reported on the interaction of *hrp* mutants and host or nonhost plants. While unable to induce macroscopic symptoms on nonhost plants, it is not yet known if *hrp* mutants are able to induce disruption of individual plant cells. Such studies may also provide valuable clues as to the possible functions of *hrp* genes such as in outer membrane synthesis, assembly and/or stability. Thus, we undertook a study of the interaction of *hrp* mutants of *P.s.* pv. *phaseolicola* reported by Lindgren et al. [14] with the nonhost plant tobacco. One mutant strain representative of each of the three phenotypic mutant classes (based on host and nonhost reactions) [14] were selected for use in this study. Strain NPS4005 was described above. Strain NPS4000 has a mutation in *hrpA* and NPS4003 has a mutation in *hrpF* [16]. These mutations are linked to each other and to the other six *hrp* loci except the locus mutated in NPS4005 [14].

2. Materials and methods

2.1. Bacterial strains

Cultures of *P.s.* pv. *phaseolicola* wild-type strain NPS3121 as well as Tn5-generated *hrp* mutants were kindly supplied by Dr Peter Lindgren and Dr Nickolas Panopoulos. The relevant characteristics of these strains are given in Table 1. Bacteria were routinely cultured on *Pseudomonas* agar F (Difco) (PAF) containing the appropriate antibiotics [14]. Long-term storage was by lyophilization in double-strength skim milk.

2.2. Plant material and inoculations

Seed of tobacco cv. Turk was obtained from Dr Peter Lindgren and plants were cultivated in a potting mixture of Baccto potting soil (Michigan Peat Company, Houston, TX) and vermiculite (2:1) contained in clay pots in an environmentally controlled growth chamber at 24°C day, 20°C night, 75% relative humidity. Fluorescent and incandes-

Table 1
Strains of *Pseudomonas syringae* pv. *phaseolicola* used in this study

Designation	Relevant characteristics ^a	References
NPS3121	race 2, rif ^r , Hrp ⁺	[14,39]
NPS4000	NPS3121 <i>hrpA</i> ::Tn5, rif ^r , km ^r , Hrp ⁻	[14,39]
NPS4003	NPS3121 <i>hrpF</i> ::Tn5, rif ^r , km ^r Hr ⁻ p [±]	[14,39]
NPS4005	NPS3121 <i>hrpM2</i> ::Tn5, rif ^r , km ^r Hr ⁻ p [±]	[14,39]

^arif, rifampicin; km, kanamycin; ^r, resistant; Hrp⁺, induces a hypersensitive response (HR) in tobacco and is fully virulent on bean; Hrp⁻, unable to induce a HR response and nonpathogenic on bean; Hr⁻p[±], unable to induce a HR response in tobacco and attenuated in virulence on the bean cultivar Red Kidney. Strain NPS4005 is more highly attenuated in virulence on bean than is strain NPS4003 [14,31].

cent bulbs provided 1.1×10^4 lux on a 13-h photoperiod.

Plants were 2–3 months old when inoculated. Inoculum was prepared by suspending cells from bacterial cultures grown overnight on PAF plus appropriate antibiotics at 28°C in sterile water to an OD_{600nm} of 1.0 (approximately 7×10^8 cfu/ml based on a standard curve of OD_{600nm} versus cell concentration generated using standard dilution plating techniques). Intervial areas of leaves positioned near the middle of the plants were inoculated with bacterial suspensions by injection using a 1 cc syringe fitted with a 26 gauge, 3/8 inch needle. Inoculated plants were returned to the growth chamber after the first sampling time at 15 min postinoculation.

2.3. Bacterial populations in planta

Tobacco plants were grown, inoculum prepared and plants inoculated as described above. Immediately after inoculation and before each sampling time inoculated leaves were rinsed with running tap water and blotted dry with paper towels. Bacterial populations in planta were followed by removal of leaf discs from inoculated areas using a cork borer, triturating in buffer, and determining the number of bacteria by standard dilution plating techniques using PAF plus the appropriate antibiotics as described previously [22]. The experiment was done twice.

2.4. Microscopy

For microscopy, leaf tissue (1–2 cm²) was excised at 4, 24 and 48 h postinoculation and vacuum-infiltrated with 4% glutaraldehyde in 0.1 M sodi-

um cacodylate buffer, pH 7.4. Water-sprayed tissue was processed as a control. Tissue was fixed for 3–4 h at 4°C, rinsed overnight at 4°C in the same buffer plus 1.5% sucrose, minced into 2-mm² pieces, and post-fixed for 4 h in 2% osmium tetroxide in 0.05 M veronal-acetate-HCl buffer, pH 7.0 [23]. Fixed tissue was dehydrated by a graded water/acetone series (50%, 70%, 2 × 90%, 3 × 100% acetone, 10 min each) and embedded in Spurr's low viscosity resin. Plastic sections (1 μm) for light microscopy were stained with aqueous 1% methylene blue in 1% sodium borate [24] and observed with an Olympus BH light microscope. Ultrathin (60- to 70-nm) sections for transmission

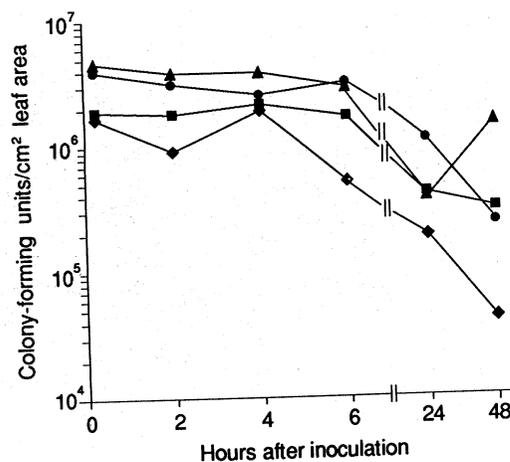


Fig. 1. Populations of *P.s.* pv. *phaseolicola* wild-type strain NPS3121 (▲) and the Tn5-induced *hrp* mutants NPS4000 (●), NPS4003 (◆) and NPS4005 (■) after injection into leaves of the nonhost plant tobacco.

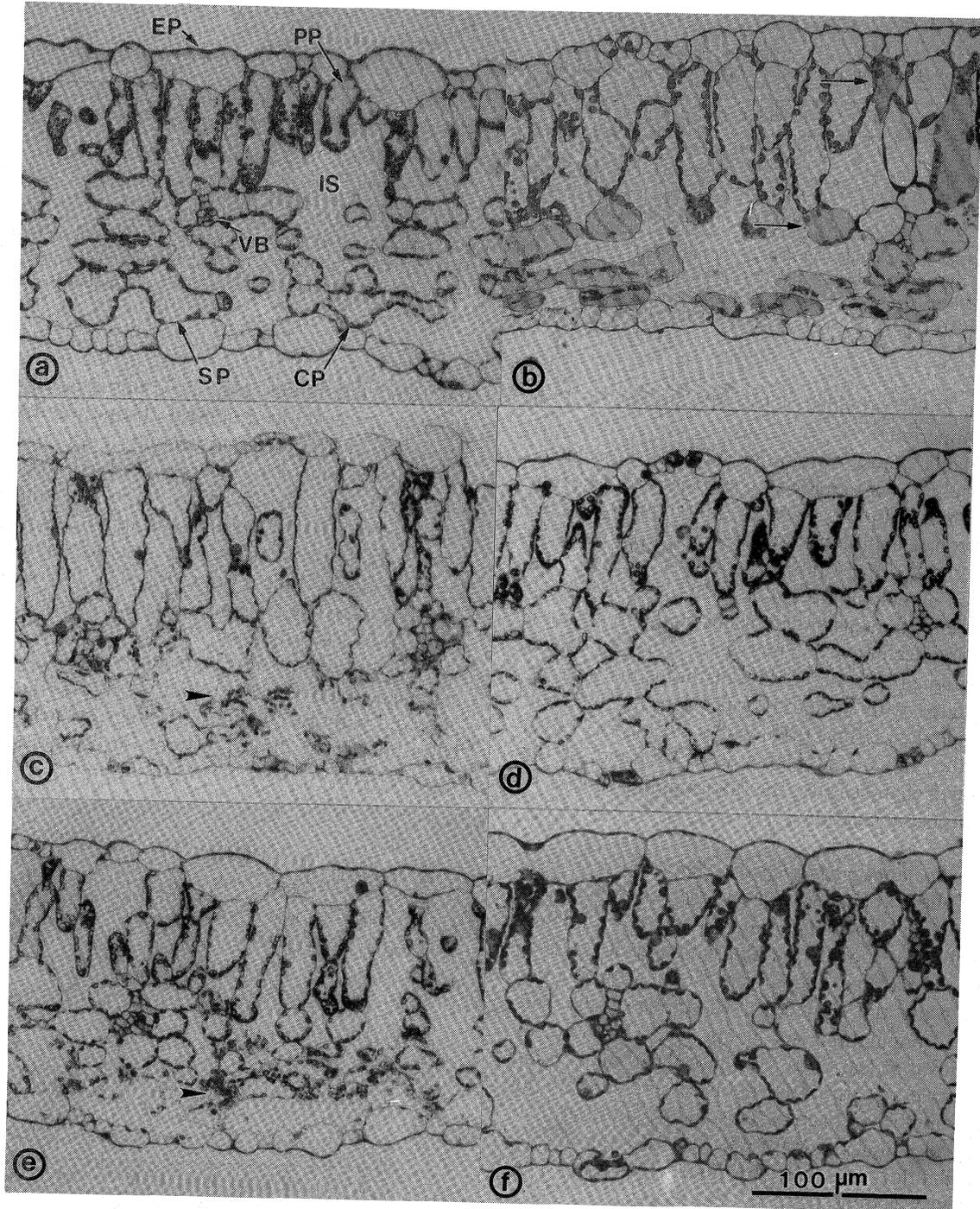


Fig. 2. Histology of tobacco leaves injected with *P.s. pv. phaseolicola*. (a) Control leaf 24 h after injection with sterile distilled water (CP, chloroplast; EP, epidermis; IS, intercellular space; PP, palisade parenchyma; SP, spongy parenchyma; VB, vascular bundle). (b) Four hours after injection with wild-type strain NPS3121. Some parenchyma cells showed uptake of stain (methylene blue) and disorganization (arrows). (c) Twenty-four hours after injection with mutant strain NPS4003. Some parenchyma cells were disrupted (arrow). (d) Four hours after injection with mutant strain NPS4005. No plant cell disruption was evident. (e) Twenty-four hours after injection with strain NPS4005. Disrupted parenchyma cells were noted (bold arrow). (f) Forty-eight hours after injection with mutant strain NPS4000. No plant cell disruption was evident. (Light micrographs are of equal magnification. See micron marker in f).

electron microscopy (TEM) were stained with aqueous 5% uranyl acetate for 1 h and Reynold's lead citrate [25] for 30 min. Sections were observed in a Zeiss EM 10B TEM at an accelerating voltage of 60 kV.

3. Results

3.1. Symptomology and bacterial growth in planta

As reported by Lindgren et al. [14] only the wild-type parent strain NPS3121 induced a hypersensitive response on tobacco cv. Turk. Inoculated tobacco tissue was flaccid by 4 h postinoculation, the HR induction period (the time at which the HR cannot be reversed by introduction of antibiotics into the inoculated area) reported for the *P.s. pv. phaseolicola*/tobacco interaction [26]. Confluent necrosis of the inoculated area was visible with the naked eye by 24 h. The three mutant strains did not cause any macroscopic symptoms on tobacco by 48 h postinoculation.

The bacterial strains showed stable (wild-type strain NPS3121 and mutant strains NPS4000 and NPS4005) or slightly declining populations (mutant strain NPS4003) during the first 6 h postinoculation. The data from one representative experiment are shown in Fig. 1. All four strains declined in population from 6 to 48 h with the apparent exception

of strain NPS3121. This strain gave increased cell counts per cm² leaf area between 24 and 48 h.

3.2. Microscopy

The histology and ultrastructure of tobacco leaves of tobacco cv. Turk injected with sterile water are shown in Figs. 2a and 3.

Observations using LM indicated that wild-type strain NPS 3121 induced alterations in selected leaf cells by 4 h postinoculation (Fig. 2b). The affected plant cells were located primarily in the spongy parenchyma, but some palisade parenchyma cells were also affected. The affected cells took up the dye methylene blue indicative of possible alteration of plasma membrane permeability and cell death [27,28]. Some of the cells which took up the dye also had chloroplasts displaced away from the cell wall. No collapsed leaf cells were noted at this time. No LM observations of this interaction were made at later times. Observations by TEM demonstrated that strain NPS3121 induced severe parenchyma cell organelle disruption as well as dissolution of the plasmalemma, tonoplast and release of vacuolar contents by 4 h (Fig. 4a,b). At 24 h disruption of the parenchyma cells was very severe (Fig. 6a,b) with cell collapse and darkly staining cell contents.

At 4 and 24 h postinoculation, the majority of cells of strain NPS3121 were not attached to the cell

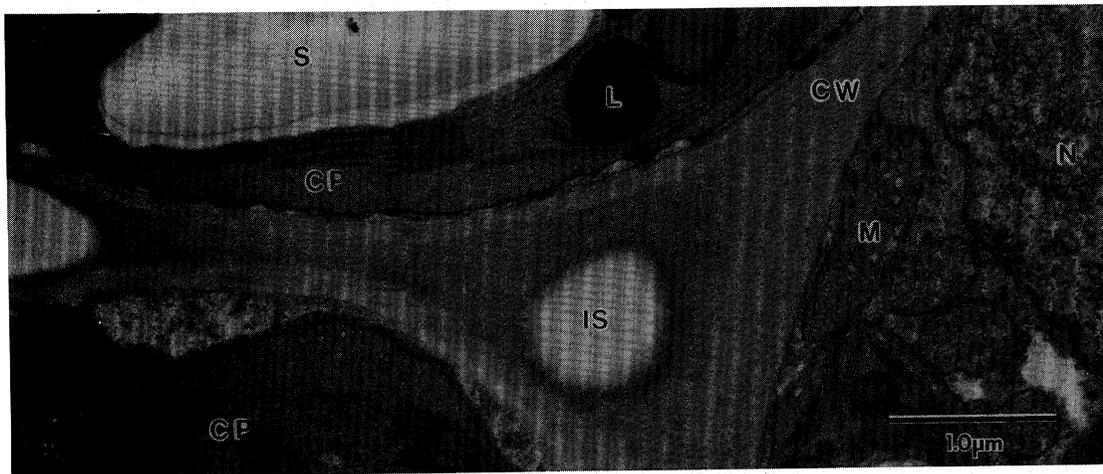


Fig. 3. Transmission electron micrograph of a control tobacco leaf 24 h after injection with sterile distilled water. Parenchyma cells contained well-defined organelles. No cell disruptions away from the injection sites were noted. (CW, cell wall; L, lipid body; M, mitochondrion; N, nucleus; S, starch granule.)

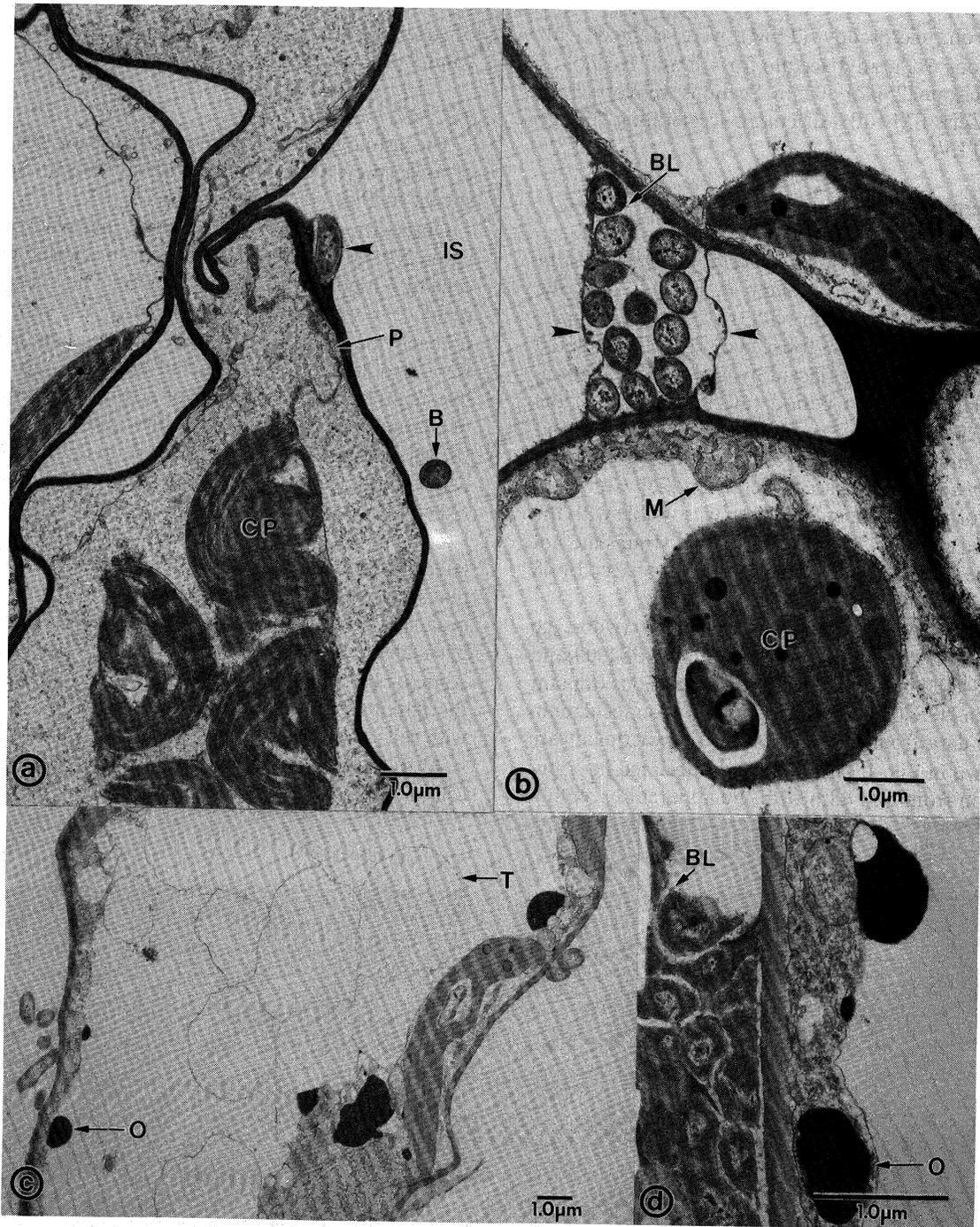


Fig. 4. Transmission electron micrographs of tobacco leaves 4 h after inoculation with *P.s. pv. phaseolicola*. (a,b) Injected with wild-type strain NPS3121. Tobacco cells were severely disorganized (a). Bacteria were present as single-cells either free in the intercellular space (a) or in microcolonies (b). Occasional bacterial cell surface blebs were noted (b). Some individual bacterial cells as well as microcolonies were covered or delineated by thin films of electron-dense material (bold arrows, a,b; B, bacterial cell; BL = bleb; P, plasmalemma). (c,d) Injected with mutant strain NPS4003. Localized tobacco cell disruption was evidenced by vesiculation of the tonoplast and the presence of electron dense deposits in the plant cells (c). Bacteria were either free in the intercellular spaces (c) or were present in microcolonies (d). Blebs emanated from the surface of bacterial cells present in microcolonies (d). (O, electron dense deposits; T, tonoplast.)

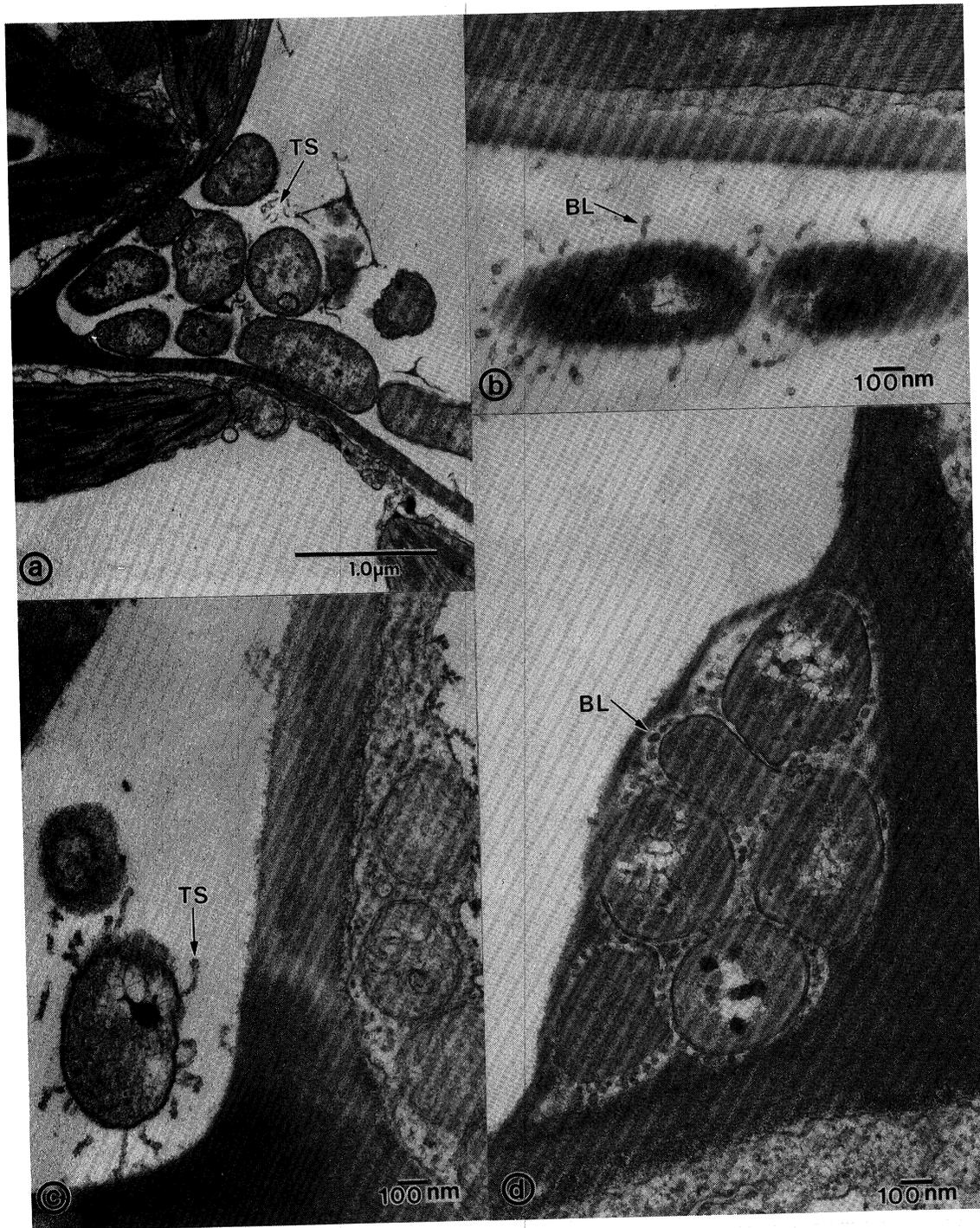


Fig. 5. Transmission electron micrographs of tobacco leaves 4 h after injection with *P.s. pv. phaseolicola*. (a,b) Leaf injected with mutant strain NPS4005. Tubular structures (a) and surface blebs (b) were on or near bacteria (TS, tubular structures). (c,d) Leaf injected with mutant strain NPS4000. Tubular structures (c) and surface blebs (d) emanating from bacterial cells were evident.

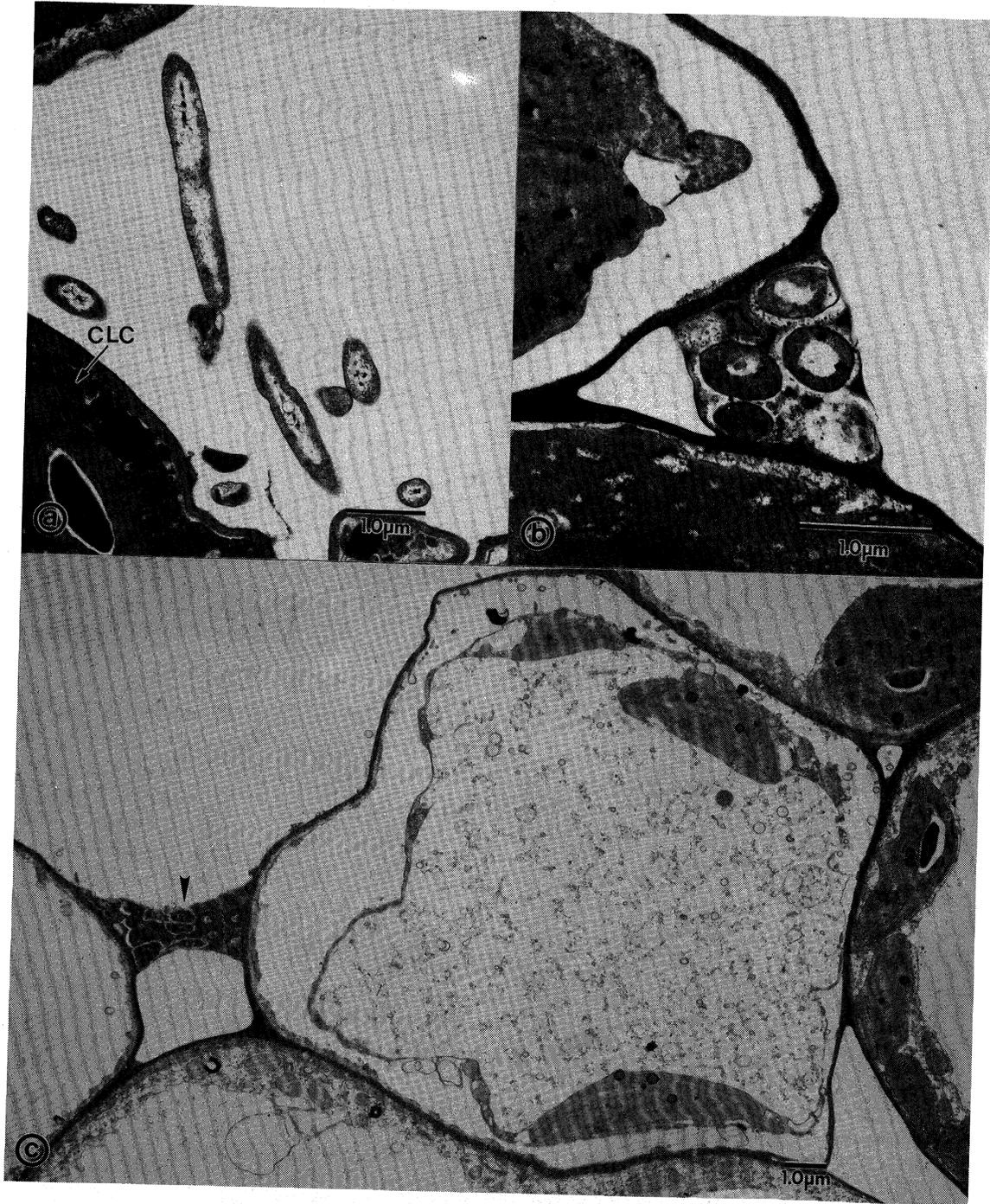


Fig. 6. Transmission electron micrograph of tobacco leaves 24 h after injection with *P.s. pv. phaseolicola*. (a,b) Leaf injected with wild-type strain NPS3121. Leaf cells were completely disorganized. Bacteria were present either free in the intercellular spaces (a) or in microcolonies (b; CLC, collapsed leaf cell). (c) Leaf injected with mutant strain NPS4003. Leaf cells in the vicinity of a bacterial microcolony (bold arrow) were severely disrupted.

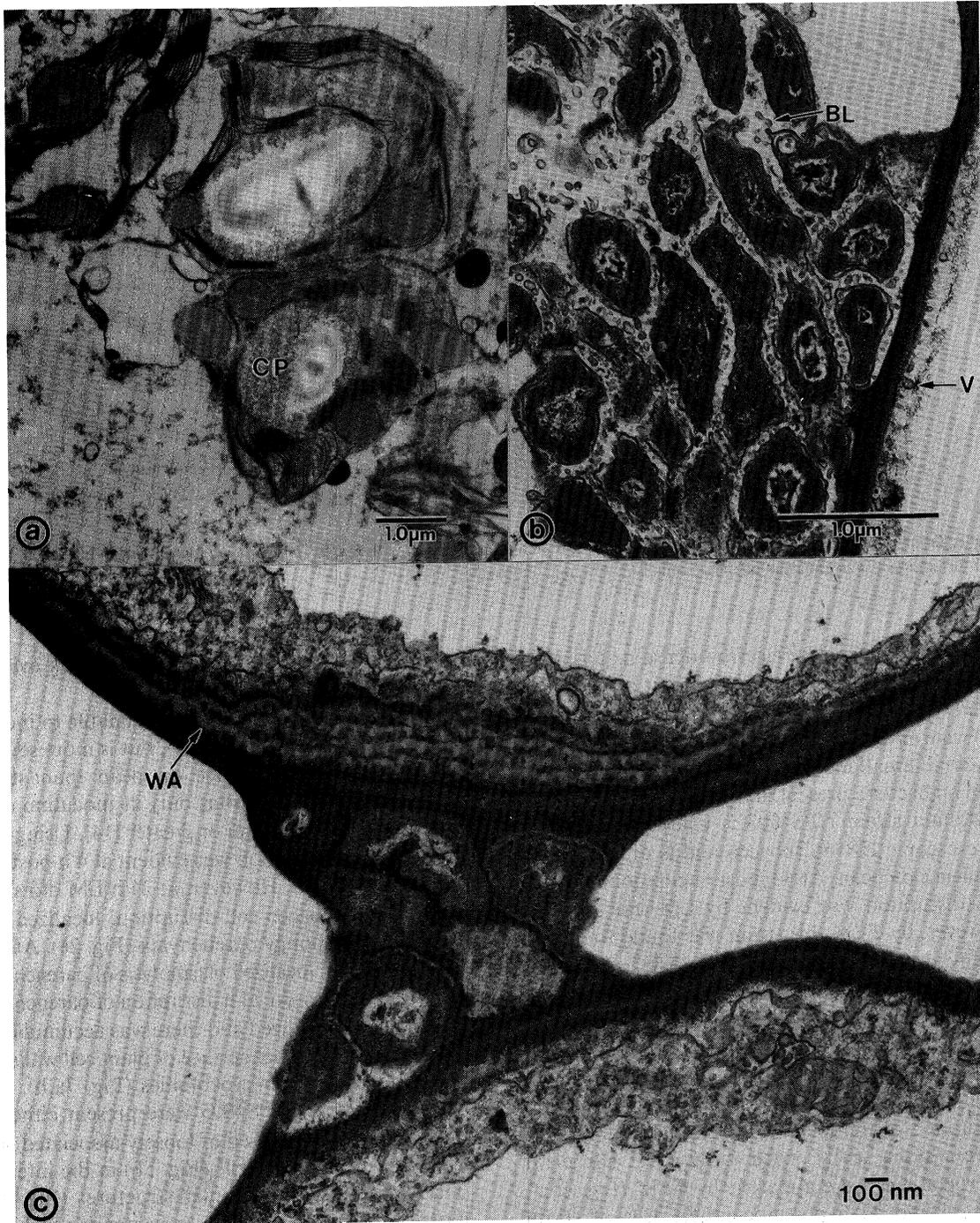


Fig. 7. Transmission electron microscopy of tobacco leaves injected with *P. s. pv. phaseolicola*. (a,b) Leaf 24 h after injection with mutant strain NPS4005. Localized disruption of leaf cells was evident (a). Bacteria in microcolonies showed extensive surface blebbing (b). Vesicles accumulated at the inner surface of the plant cell wall near bacterial microcolonies (b; V, vesicles). (c) Leaf 24 h after injection with mutant strain NPS4000. Ornate, multilayered plant cell wall appositions were present near bacterial microcolonies. All microcolonies were composed of very tightly appressed bacterial cells (WA, wall apposition).

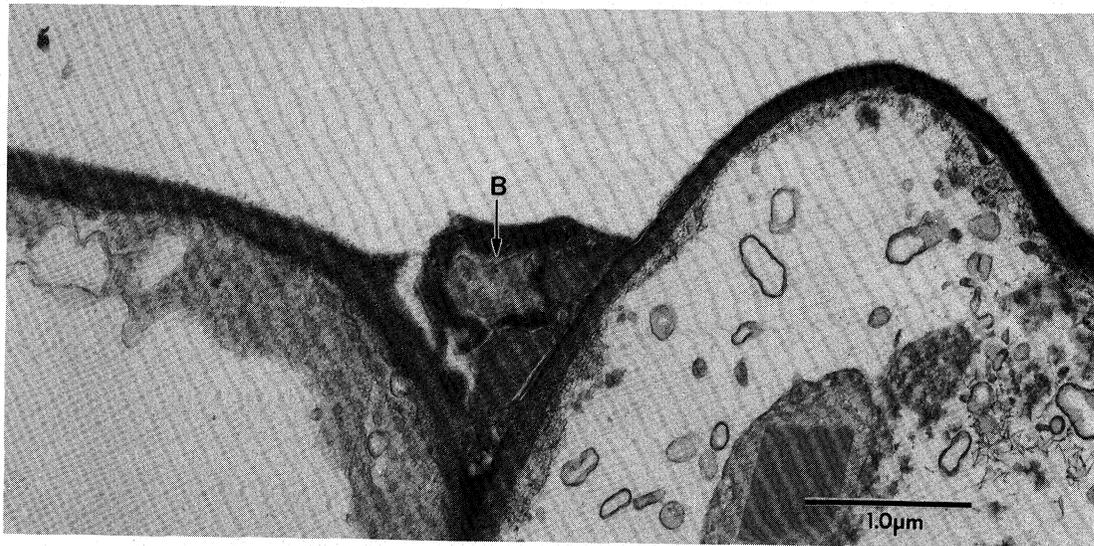


Fig. 8. Transmission electron microscopy of a tobacco leaf 48 h after injection with *P.s. pv. phaseolicola* mutant strain NPS4000. Plant cell disruption is evident near bacterial cells.

walls or enveloped by electron-dense materials in the intercellular spaces (Fig. 6a), but a few single cells (Fig. 4a) and bacterial microcolonies (Figs. 4b, 6b) were enveloped by thin layers of electron-dense material. Small numbers of cell surface blebs were seen on bacteria present in microcolonies (Fig. 4b).

Mutant strain NPS4003 is unable to induce a macroscopic HR on tobacco and is of attenuated virulence on bean. At 4 h postinoculation no plant cell disruption was evident by LM (not shown), however, by TEM this strain was found to induce highly localized effects (Fig. 4c,d). The localized effects included plasmolysis, disruption of the plasmalemma and tonoplast (Fig. 4c) and the presence of electron dense deposits (Fig. 4c,d). At 24 h, LM showed parenchyma cell disruption primarily of spongy parenchyma cells (Fig. 2c). Plasmolysis and disruption of tonoplast and plasma membrane of parenchyma cells with adhering bacterial microcolonies were apparent by TEM (Fig. 6c).

At 4 and 24 h postinoculation cells of strain NPS4003 were present in intercellular spaces either in microcolonies interspersed with electron dense material (Figs. 4d, 6c) or as single cells not associated with any electron dense material (Fig. 4c). At 24 h most bacteria cells were present in microcol-

onies and bacterial surface blebbing was evident (Fig. 4d).

Mutant strain NPS4005 is also unable to induce a macroscopic HR on tobacco, but is more severely attenuated in virulence on bean than strain NPS4003 giving symptoms only at inoculum concentrations of 10^7 cfu/ml or greater [14]. Using LM no plant cell disruption was evident at 4 h postinoculation (Fig. 2d). However, at 24 h LM did show evidence of tobacco cell disruption, localized primarily in the spongy parenchyma (Fig. 2e). At 4 h, TEM indicated localized plasmolysis of parenchyma cells (not shown). At 24 h disruption of parenchyma cells was still localized and there was accumulation of vesicles at the inner surface of plant cell walls adjacent to bacterial microcolonies (Fig. 7a,b).

Cells of strain NPS4005 were present either in microcolonies composed of loosely-associated cells (Fig. 5a) or as single cells (Fig. 5b) in the intercellular spaces at 4 h. Tubular structures (Fig. 5a), possibly of bacterial origin, and extensive blebbing of the bacterial cell surfaces (Figs. 5b, 7b) were noted at 4 and 24 h. At 24 h almost all bacterial were present in microcolonies (Fig. 7b).

Mutant strain NPS4000 does not induce an HR on tobacco and is nonpathogenic towards bean [18].

No plant cell disruption was evident by LM up to 48 h (Fig. 2f). Using TEM, parenchyma cells appeared normal at 4 and 24 h except that at 24 h large, ornate, multi-layered parenchyma cell wall appositions were apparent near all bacterial microcolonies (Fig. 7c). More severe disruption of isolated tobacco cells was evident at 48 h (Fig. 8).

At 4 h cells of strain NPS4000 were present in the intercellular spaces either as single cells (Fig. 5c) or as microcolonies composed of loosely-associated cells (Fig. 5d). Extensive tubular structures and cell surface blebs emanated from the surface of the bacteria (Fig. 5c,d). At 24 h almost all bacteria were present in microcolonies and these microcolonies now consisted of very tightly packed bacterial cells (Fig. 7c).

4. Discussion

Inoculation of tobacco leaves with *P.s. pv. phaseolicola* wild-type parent strain NPS3121 resulted in rapid tobacco tissue and cell disruption evident both macroscopically and microscopically as is typical of the HR of tobacco towards many incompatible plant pathogenic bacteria [29]. The covering of some single cells and microcolonies of the wild-type strain by thin-layers of electron-dense material evident as early as 4 h postinoculation may indicate an active response by the host [1] or a passive phenomenon due to solubilization of plant cell wall polymers during the infiltration of bacteria followed by the subsequent evaporation of the introduced water [30].

The three Tn5-induced *hrp* mutant strains did not cause any macroscopic symptoms on tobacco as previously reported by Lindgren, et al. [14]. All three mutant strains as well as the parent strain were unable to increase their overall numbers in tobacco leaves by 24 h postinoculation. Between 24 and 48 h postinoculation, only strain 3121 appeared to increase in viable numbers per area leaf tissue, but this increase was most likely more apparent than real due to the severe desiccation and shrinkage of the infiltrated tissue undergoing the HR rather than actual bacterial growth. The mutants were unable to grow in nonhost leaf tissue even in the absence of a macroscopic HR with accompanying severe tissue desiccation.

Even though the three mutant strains did not cause any macroscopic symptoms when inoculated into tobacco at high cell concentrations, examination of inoculated tissues by LM and TEM indicated that the mutant strains were able to induce highly localized tobacco cell disruption. The rapidity and severity of this disruption seen in this study appeared directly related to the ability of the mutant strains to cause symptoms on bean [14,31]. The ability of *hrp* mutants to induce nonhost plant cell metabolic responses was previously demonstrated for *hrp* mutants of *P.s. pv. tabaci* which activated the transcription of defense-related genes in the nonhost plant bean [32].

Only mutant strain NPS4000 induced the formation of ornate, multi-layered tobacco cell wall appositions to form near bacterial microcolonies. The cell wall appositions formed between 4 and 24 h postinoculation as no incipient cell wall appositions were seen at 4 h. Politis and Goodman [33] were the first to report that an incompatible bacterium (*P.s. pv. pisi*) could induce the formation of tobacco leaf cell wall appositions. Their study showed the presence of appositions composed of loosely-associated fibrils opposite bacterial microcolonies at 3 h postinoculation. By 4 h the appositions induced by *P.s. pv. pisi* were well-formed and striated, but were not of the electron density or as elaborate as those induced by mutant strain NPS4000 at 24 h. Politis and Goodman [33] did not report on the appearance of the cell wall appositions induced by *P.s. pv. pisi* at later times.

In light of the recent evidence for Hrp proteins of other plant pathogenic bacteria being located in the bacterial outer membrane [4,34] and that NPS strain 4005 may be defective in periplasmic glucan synthesis [21], it is interesting to note that the formation of blebs at the surface of bacterial cells in planta occurred primarily for the *P.s. pv. phaseolicola hrp* mutant strains. Wild-type strain NPS3121 exhibited very few surface blebs. The more extensive surface blebbing of the *hrp* mutants may be due to either disorganization of the outer membrane and/or to a reduced ability to respond to a hypo-osmotic environment upon introduction into the apoplast by the formation of periplasmic glucans. It is not known for certain if invading bacteria undergo osmotic shock in the leaf intercellular space

environment as the osmolarity of leaf intercellular space fluids at various stages of plant-bacteria interactions remains undefined. However, in support of a low osmolyte concentration in planta at the time of inoculation, the *hrp* genes of *P.s. pv. phaseolicola* are optimally expressed in vitro in minimal medium with a low osmolyte concentration [14]. Osmotic shock brought on by a sudden reduction in osmolarity is known to lead to bacterial outer membrane blebbing [35]. The blebs may be composed of lipopolysaccharide [36,37] or protein-lipopolysaccharide complexes [38].

In summary, by use of light and electron microscopy we have demonstrated that *hrp* bacterial mutants which have lost the ability to induce a macroscopic HR on nonhosts, still retain the ability to induce nonhost plant cell responses visible at the microscopic level. This finding indicates that unlike induction of a macroscopic HR, induction of more subtle responses by nonhost plant cells is not dependent on the presence of an intact *hrp* cluster.

Acknowledgments

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