Phytochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The role of cell wall-based defences in the early restriction of non-pathogenic *hrp* mutant bacteria in Arabidopsis

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Basal defence
Cell wall alterations
Apoplast
Arabinogalactan protein
Immunocytochemistry
Electron microscopy
Reactive oxygen species
Effector proteins

ABSTRACT

We have investigated the cause of the restricted multiplication of *hrp* mutant bacteria in leaves of Arabidopsis. Our focus was on early interactions leading to differentiation between virulent wild-type and non-pathogenic *hrpA* mutant strains of *Pseudomonas syringae* pv. *tomato*. An initial drop in recoverable bacteria detected 0–4 h after inoculation with either strain was dependent on a functional FLS2 receptor and H_2O_2 accumulation in challenged leaves. Wild-type bacteria subsequently multiplied rapidly whereas the *hrpA* mutant was restricted within 6 h. Despite the early restriction, the *hrpA* mutant was still viable several days after inoculation. Analysis of intercellular washing fluids (IWFs), showed that high levels of nutrients were readily available to bacteria in the apoplast and that no diffusible inhibitors were produced in response to bacterial challenge. Histochemical and immunocytochemical methods were used to detect changes in polysaccharides (callose, two forms of cellulose, and pectin), arabinogalactan proteins (AGPs), H_2O_2 and peroxidase. Quantitative analysis showed very similar changes in localisation of AGPs, cellulose epitopes and callose 2 and 4 h after inoculation with either strain. However from 6 to 12 h after inoculation papillae expanded only next to the *hrp* mutant. In contrast to the similar patterns of secretory activity recorded from mesophyll cells, accumulation of H_2O_2 and peroxidase was significantly greater around the *hrpA* mutant within the first 4 h after inoculation. A striking differential accumulation of H_2O_2 was also found in chloroplasts in cells next to the mutant. Ascorbate levels were lower in the IWFs recovered from sites inoculated with the *hrp* mutant than with wild-type bacteria. The critical response, observed at the right time and place to explain the observed differential behaviour of wild-type and *hrpA* mutant bacteria was the accumulation of H_2O_2, probably generated through Type III peroxidase activity and in chloroplasts. It is proposed that H_2O_2 and apoplastic peroxidase cross-link secreted glycoproteins and polysaccharides to agglutinate the *hrp* mutant. Generation of H_2O_2 has been identified as a likely target for effector proteins injected into plant cells by the wild-type bacteria.

1. Introduction

The timing of early events occurring after the inoculation of leaves by *Pseudomonas syringae* is critical for the success or failure of invading bacteria to colonise the challenged tissue. In the bacterium, the conditions in the plant activate expression of genes encoding the type III secretion system (T3SS) and effector proteins that are delivered through the Hrp pilus, composed of HrpA subunits, into plant cells (Boureau et al., 2002; Li et al., 2002). The speed of delivery of effectors can be gauged from the induction time required for the hypersensitive reaction (HR) to be induced if bacterial protein synthesis is arrested with antibiotics. For example a period of only 1.5 h is needed for effectors to be delivered and the HR to progress in Arabidopsis challenged with *P. syringae* pv. *tomato* (*Pst*) expressing the avirulence gene *avrRpm1* (Grant et al., 2000). Exposure to bacterial MAMPs (microbe associated molecular patterns) such as flagellin, can induce transcriptional responses in plant cells within 30 s following binding to the receptor protein complex containing FLS2 (Boller and Felix, 2009). The relative speed of effector delivery and MAMP induced responses are factors

Abbreviations: Col-0, Arabidopsis ecotype Columbia; cfu, colony forming unit; FLS2, flagellin sensitive 2; hai, hours after inoculation; HR, hypersensitive response; *hrp*, hypersensitive response and pathogenicity; MAMP, microbe-associated molecular pattern; *pmr*, powdery mildew resistant; POX, peroxidase; *Pst*, *Pseudomonas syringae* pv. *tomato*; ROS, reactive oxygen species; TEM, transmission electron microscopy.

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http://dx.doi.org/10.1016/j.phytochem.2014.07.015
0031-9422/

that may tip the balance in favour of the plant or pathogen (de Torres et al., 2006).

In the absence of effector delivery, *hrp* mutant bacteria fail to multiply in wild-type Arabidopsis accessions such as Columbia (Col) and are unable to trigger the HR or demonstrate pathogenicity (Lindgren et al., 1986). The restriction of multiplication of *hrp* mutants has often been reported to occur within the first day after challenge, results confirmed by microscopy and colony counts from homogenized tissues (for example see Soylu et al., 2005). The formation of callose-rich papillae adjacent to bacterial colonies is a well characterised plant response. Papilla formation is more pronounced next to *hrp* mutants than pathogenic Pseudomonads or Xanthomonads and the suppression of callose accumulation in a range of plants has emerged as a common property of bacterial effector proteins (Hauck et al., 2003; Ham et al., 2007; Xin and He, 2013). In bean, pepper and lettuce, papillae have been found to contain callose and cellulose, and the altered wall at reaction sites to be modified by the incorporation of proteins such as hydroxyproline rich glycoproteins and also phenolics (Bestwick et al., 1995; Brown et al., 1998; Keshavarzi et al., 2004, O'Connell et al., 1990). The biochemical nature of the additions to Arabidopsis cell walls in leaves challenged by *hrp* mutant bacteria was studied by Forcat et al. (2010), who extended the analysis of tissue undergoing the HR carried out by Hagemeier et al. (2001). Forcat et al. (2010) identified more rapid linkage of indole carboxylic acid, syringaldehyde and *p* hydroxybenzaldehyde occurring within 12 h of inoculation with *hrpA* mutant compared with wild-type *Pst* bacteria. Cross linking of phenolic and indolic derivatives was identified as a component of basal defence against *hrp* mutant bacteria.

Although callose deposition is recognised as a common feature of MAMP-induced defences the precise role of the glucan in resistance to either bacteria or fungi has been questioned. For example, the callose synthase deficient mutant of Arabidopsis, *pmr4-1*, was identified based on its enhanced resistance to powdery mildew (Nishimura et al., 2003). Papillae are still produced in *pmr4-1* as shown by Soylu et al. (2005) and the restriction of non-pathogenic bacteria occurs as in wild-type plants.

An intriguing feature of cell wall alterations occurring adjacent to bacteria is the localised accumulation of reactive oxygen species (ROS, Bestwick et al., 1998; Soylu et al., 2005). The role of ROS in the orchestration of plant defences was first put forward by Lamb and colleagues. Their initial experiments focused on the role of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase systems in plants analogous to their involvement in generation of the mammalian oxidative burst (Bradley et al., 1992; Torres et al., 2002). The NADPH dogma was challenged by Bolwell and colleagues who demonstrated that alternative sources of ROS had an important role in plants. In particular, the ability of Class III peroxidases to produce H_2O_2 at certain pHs and in the presence of suitable reductants was clearly demonstrated (Bindschedler et al., 2006; Bolwell et al., 2002; Daudi et al., 2012).

Recently, additional enzymes have been reported to be involved in pathogen-elicited oxidative bursts. An aspartate oxidase was shown to be required for the RBOHD-triggered ROS burst in Arabidopsis (Macho et al., 2012), and the photorespiratory enzyme

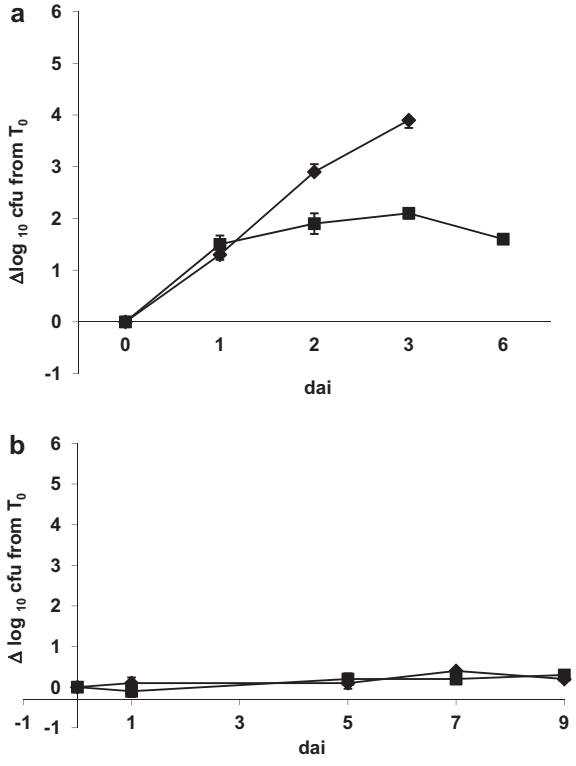

Fig. 1. Long term population dynamics of (a) Wild-type *Pst* DC3000 and (b) the *hrpA* mutant in Col-0 (diamonds) and *pmr-4.1* (squares). Changes in colony forming units recovered from the initial inoculation with *Pst* DC3000 at 2.5×10^5 cells ml^{-1} or the *hrpA* mutant at 5×10^7 cells ml^{-1}. Error bars ± SD are given.

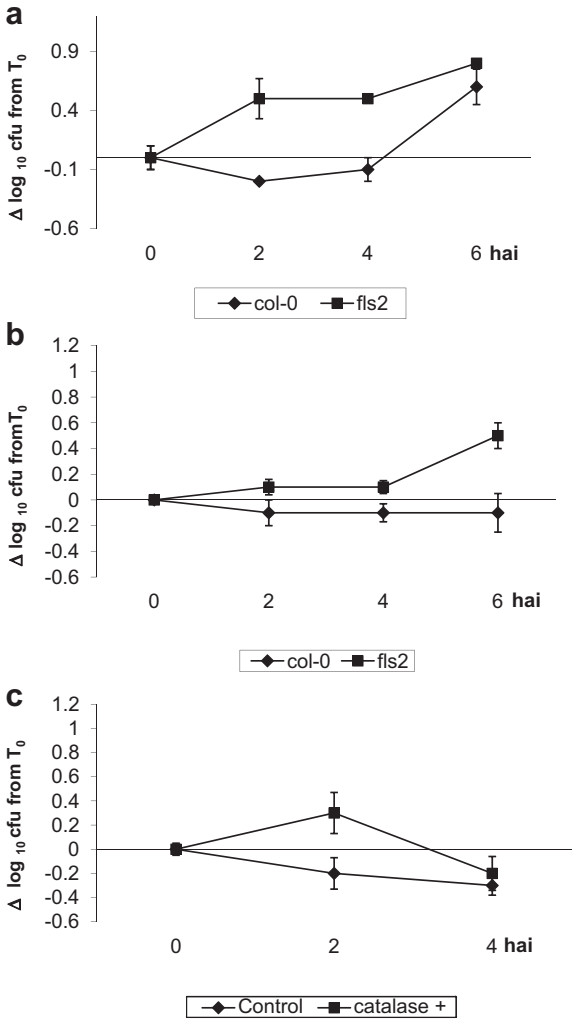

Fig. 2. Investigation of the early population decrease in wild-type *Pst* DC3000 and the *hrpA* mutant ($\Delta\log_{10}$ from T_0). (a) *Pst* DC3000; (b) *hrp* A mutant bacteria. (c) Col-0, after inoculation with *Pst* in 0.5 μg/ml catalase. Data from 4 replicates of each treatment (inoculum density = 5×10^7 cells ml^{-1}). Experiments were repeated with similar results, error bars ± SD.

Please cite this article in press as: Mitchell, K., et al. The role of cell wall-based defences in the early restriction of non-pathogenic *hrp* mutant bacteria in Arabidopsis. Phytochemistry (2014), http://dx.doi.org/10.1016/j.phytochem.2014.07.015