



Profiling the extended phenotype of plant pathogens

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Profiling the extended phenotype of plant pathogens

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Abstract

One of the most fundamental questions in plant pathology is what determines whether a pathogen grows within a plant? This question is frequently studied in terms of the role of elicitors and pathogenicity factors in triggering or overcoming host defences. However, this focus fails to address the basic question of how the environment in host tissues acts to support or restrict pathogen growth. Efforts to understand this aspect of host-pathogen interactions are commonly confounded by several issues, including the complexity of the plant environment, the artificial nature of many experimental infection systems, and the fact that the physiological properties of a pathogen growing in association with a plant can be very different from the properties of that pathogen in culture. It is also important to recognise that the phenotype and evolution of pathogen and host are inextricably linked through their interactions, such that the environment experienced by a pathogen within a host, and its phenotype within that host is a product both of its interaction with its host and its evolutionary history, including its co-evolution with host plants. As the phenotypic properties of a pathogen within a host cannot be defined in isolation from the host it may be appropriate to think of pathogens as having an “extended phenotype” that is the product of their genotype, host interactions and population structure within the host environment. This paper reflects on the challenge of defining and studying this extended phenotype, and considers how knowledge of the phenotype of pathogens in the host environment could be used to improve disease control.

25 **Questions:**

26 What determines whether a pathogen grows within a plant?

27 What aspects of pathogen biology should be considered in describing the extended phenotype of a
28 pathogen within a host?

29 How can we study the extended phenotype in ways that provide insight into the phenotypic
30 properties of pathogens during natural infections?

31 **Introduction**

32 The development of biotic plant diseases depends on the interaction of plant, pathogen and
33 environment, a combination commonly referred to as “the disease triangle” (McNew, 1960). Disease
34 develops when a virulent pathogen infects a susceptible host and environmental conditions favour
35 disease development. Disease is prevented or reduced when a host is resistant to infection by a
36 specific pathogen or when environmental conditions are unfavourable for disease development.
37 However, the mystery that lies at the centre of the disease triangle is what determines whether a
38 pathogen thrives, persists or dies inside host tissues?

39 For some obligate biotrophic pathogens, such as fungal or oomycete pathogens that establish
40 haustorial feeding structures inside plant cells, disease resistance can be explained in part as denial
41 of access to nutrients when the establishment of such structures is blocked, or when the infected
42 plant cell dies due to elicitation of the hypersensitive response (HR) (Hückelhoven et al., 1990). In
43 other cases resistance can be explained in terms of anti-microbial chemicals that are toxic to
44 invading pathogens (Jeandet et al., 2013; Schmelz et al., 2011). However, for many pathogens, such
45 as bacterial pathogens, there is no clear link between the development of specialised cell types and
46 the outcome of infection, and resistance arises through the combined effect of multiple defence
47 mechanisms. In these cases, pathogenesis and disease resistance must be understood holistically in
48 terms of a pathogen’s phenotype within the host, including its ability to suppress, evade or tolerate

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49 host defences while growing in the environment within plant tissues (e.g. Mishina and Zeier, 2007;
50 Macho, 2016; Ökmen and Doehlemann, 2014; Win et al., 2012; Bozkurt et al., 2012; Doehlemann
51 and Hemetsberger, 2013).

52 **The extended phenotype and niche construction**

53 The phenotype of a pathogen inside the host is in turn dependent on the environment provided by
54 that host, the genotype and population structure of the pathogen within that host, and the impact
55 of the pathogen and other environmental factors on the host. The definition of genotype can be
56 extended to include epigenetically inherited features (Na and Gijzen, 2016); while the definition of
57 “environmental factors” in this context includes abiotic factors such as light, temperature and soil
58 composition, biotic factors such as endophytes, herbivores and rhizosphere bacteria, as well as
59 neighbouring plants and agricultural practices. The pathogen phenotypes on which natural selection
60 acts, and towards which disease control measures need to be directed, can only be fully understood
61 in the context of host-environment-pathogen interactions, and can perhaps best be understood as
62 extended phenotypes (Dawkins, 1982), which include both a pathogen’s interaction with its host and
63 its phenotypic properties within the host environment (Hunter, 2009).

64 During infection cycles, within which a single infective spore or cell may give rise to thousands of
65 descendants, and over evolutionary timescales, the impact of pathogen-host-environment
66 interactions on pathogen evolution can also be conceived as being akin to an evolutionary process
67 known as “niche construction” that is sometimes encapsulated within the concept of the extended
68 phenotype, or considered as a distinct process (Odling-Smee et al., 2003; Hunter, 2009). Although a
69 detailed discussion of the conceptual differences between the extended phenotype and niche
70 construction is beyond the scope of this article (readers interested in this topic are directed to
71 articles by Hunter (2009) and Scott-Phillips et al., 2014); both present useful frameworks for
72 reflecting on the processes affecting pathogen evolution. A niche-constructing organism’s activities
73 alter its own environment, and potentially the environment experienced by its descendants, thereby

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3 74 having consequences, either positive or negative, for the survival and evolution of both an individual
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5 75 organism and its descendants (Figure 1). In the case of plant pathogens, examples of positive niche
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7 76 construction might include that of the tumorigenic bacterium *Agrobacterium tumefaciens*, which
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9 77 engineers plant tissues to provide an enhanced nutritional environment for itself and its
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11 78 descendants (Schell et al. 1979); or of bacterial pathogens such as *Pseudomonas syringae* that
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13 79 suppress plant immune mechanisms, enabling bacterial growth within the plant apoplast (Lindeberg
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15 80 et al., 2012; Xin et al., 2013). Examples of negative niche construction include interactions that
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17 81 induce local and systemic responses, or even volatile signals transmitted within and between plants,
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19 82 which result in the priming or induction of defences that subsequently limit pathogen growth (Fu
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21 83 and Dong, 2013; Scala et al., 2013). To understand pathogen phenotypes and their evolution, we
22
23 84 need to understand the niche-constructing activities or extended phenotypes of pathogens not only
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25 85 at a single point in time, but throughout the infection cycle and over evolutionary timescales.
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30 86 However, experimental analyses of the extended phenotypes and niche-constructing activities of
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32 87 plant pathogens are commonly confounded by a number of issues, including (i) the complex and
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34 88 multifactorial nature of the environment within plant tissues, and of pathogen interactions with
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36 89 plant hosts and the external environment; (ii) the fact that many physiological and developmental
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38 90 properties of pathogens are only expressed during host interactions and not *in vitro*; (iii) the artificial
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40 91 nature of many laboratory-based infection systems; and (iv) the difficulty of distinguishing host and
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42 92 pathogen processes. In addition, we currently have very little understanding of the impact of
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44 93 selective processes occurring during plant-pathogen interactions on pathogen phenotypes,
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46 94 particularly when considered in relation to their action throughout the whole of a pathogen's
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48 95 lifecycle, which can include growth in very different environments early in infection, late in infection,
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50 96 or during survival outside the host (Morris et al., 2009). Such analyses are complicated further if a
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52 97 pathogen is capable of infecting multiple host species or different tissues within a host.
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98 This article considers the challenges that the study of the extended phenotype presents and briefly
99 highlights some of the opportunities presented by emerging technologies to address these
100 challenges. For simplicity examples are mainly drawn from interactions involving foliar bacterial
101 pathogens, such as the model pathogen *Pseudomonas syringae*, which colonises the apoplast of
102 plant leaves. However, it should be noted that the issues discussed below are broadly applicable
103 across a wide range of host-pathogen interactions.

104 **The apoplastic phenotype of *Pseudomonas syringae***

105 The interaction of the foliar pathogen *P. syringae* with the leaves of its host plants is one of the best-
106 studied examples of plant-pathogen interactions, and therefore an ideal model in which to explore
107 the challenge of defining the extended phenotype of plant pathogens (Preston, 2000; Xin et al.,
108 2013). Strains of *P. syringae* are also able to colonise a range of different plant tissues, including
109 seeds, seedlings, fruit and bark parenchyma (e.g. Getz et al., 1983; Hirano and Upper, 1999;
110 Scortichini et al., 2012), as well as persisting in non-host environments (Morris et al., 2010; Diallo et
111 al., 2012; Monteil et al., 2013; Monteil et al., 2014). However, it is their growth within and on leaves
112 that has been best characterised to date.

113 In a natural setting *P. syringae* gains access to the apoplastic compartment of plant leaves through
114 natural openings and wounds, and subsequently colonises the surfaces of plant cells within the leaf
115 mesophyll (Badel et al., 2002; Godfrey et al., 2010). The major structural component of the
116 apoplastic compartment is the cell wall, which is surrounded by an apoplastic fluid containing
117 proteins, metabolites, and inorganic ions, which act as the primary source of nutrients for the
118 pathogen (Sattelmacher 2001; O’Leary et al., 2016). The composition of the apoplastic fluid depends
119 on transport processes within the xylem, phloem and symplasm of adjacent cells, as well as
120 metabolic processes within the apoplast and the activities of endophytic microorganisms; which in
121 turn are affected by physiological conditions including nutrition, light and abiotic and biotic stress
122 (López-Millán et al., 2000a; Sattelmacher 2001; Grignon & Sentenac 1991).

P. syringae has been widely studied for its ability to produce a protein secretion system, known as a type III secretion system (T3SS), which can deliver proteins, known as effectors, to the cytoplasm of an adjacent plant cell (Xin et al., 2013). These effectors act in conjunction with other virulence factors, such as low molecular weight toxins and pathogen-derived phytohormones, to suppress the host immune system and to modulate host physiology to favour infection (Lindeberg et al., 2012; Xin et al., 2013). The pathogenicity and virulence mechanisms of *P. syringae* have been extensively reviewed and will not be discussed in depth here. Instead we will consider the challenges involved in defining the extended phenotype, and specifically the “apoplastic phenotype” of *P. syringae*, with a particular emphasis on the phenotypic features of *P. syringae* that are likely to be of particular value in understanding the links between the extended phenotype and the outcome of infection.

Lenses on the apoplastic phenotype

The major determinants of apoplast colonisation by *P. syringae* are summarised in Figure 2. These determinants can be broadly grouped into four classes, or lenses, through which plant-pathogen interactions can be viewed: (i) identity; (ii) physiology; (iii) regulation and (iv) pathogenicity and virulence mechanisms. A fifth class of determinants emerges when one considers the lifecycle of pathogens such as *P. syringae*, where infection results in the establishment of microcolonies and biofilms within which (v) social interactions have a significant impact on pathogen phenotypes and evolution. The apoplastic environment and pathogen interactions with the apoplastic environment have a significant impact on the expression and functional properties of each class of determinants, and thus on the extended phenotype of *P. syringae*, as outlined below.

(i) Identity. Determinants of identity include surface features, secreted effectors and pathogen activities; including secreted factors that are subject to recognition as a consequence of their interaction with host factors, such as enzymes that act to degrade host molecules and release damage associated molecular patterns (DAMPs) (Boller and Felix, 2009). These determinants often play a pivotal role in determining whether pathogens elicit or suppress host defences, as well as

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148 functioning in processes such as adhesion, motility, pathogenicity, stress tolerance, transport and
149 social interactions (Boller and Felix, 2009; Thomma et al., 2011; Mott et al., 2016). Thus, they often
150 need to be viewed through multiple lenses, including that of pathogenicity and virulence, to fully
151 understand their contribution to infection. However, it is their role as potential elicitors of host
152 defences that is central to their classification as determinants of identity.

153 Identity determinants are frequently studied in a purified or functionally isolated form in terms of
154 their structure and activity, which has yielded valuable insights into their recognition by host
155 defence mechanisms, but has the potential to overlook some factors that could affect their activity
156 *in planta*. For example, the dosages used in experiments involving purified molecules may be higher
157 than those observed in nature, or molecules may have an altered distribution within the apoplastic
158 compartment than achieved when determinants are produced locally by plant pathogens. We
159 currently have a relatively limited understanding of how the biophysical, structural and biochemical
160 properties of identity determinants, including features such as pathogen membrane and cell wall
161 composition, are affected by and interact with the biochemical and biophysical properties of the
162 apoplastic environment, including apoplastic metabolites, proteins, pH, temperature, light, cell wall
163 constituents and ionic composition.

164 The contribution of identity determinants to pathogen phenotypes is intrinsically dependent on
165 whether they are expressed *in planta*, and our ability to link *in vitro* studies, or studies using purified
166 determinant to *in planta* effects is also dependent on whether they are expressed in a structurally
167 identical form to that observed *in vitro*. There is increasing evidence that some surface-associated
168 and secreted proteins are regulated, post-translationally modified or degraded by pathogens in a
169 manner that affects their recognition by host defences (e.g. Tasset et al., 2010; Bardoel et al., 2011;
170 Hirai et al., 2011; Chiku et al., 2013). For example, Schreiber and Desveaux (2011) observed that
171 both motility and the production of flagellin, a major elicitor of host immune responses, were
172 reduced when *P. syringae* is incubated in apoplast-mimicking conditions, potentially assisting *P.*

173 *syringae* in evading immune recognition. Similar behaviour has been observed in other pathogens,
 174 including *A. tumefaciens*, where a reduction in flagella biosynthesis was observed in response to
 175 acidic, plant-mimicking conditions and in response to light (Yuan et al., 2008; Oberpichler et al.,
 176 2008). Bacterial cell surface remodelling to facilitate host colonisation has been observed in a range
 177 of symbiotic, commensal and pathogenic bacteria (Henderson et al., 2016), but has not been
 178 intensively studied in plant pathogens.

179 An additional challenge to consider when attempting to understand the contribution of identity
 180 determinants to the outcome of infection, is that these determinants are not produced in isolation,
 181 but instead are produced and act in combination with other molecules, introducing both synergistic
 182 and antagonistic effects into their interaction with host plants (e.g. Crabill et al., 2010; Kakkar et al.
 183 2013; Tsuda et al., 2013; Schenk et al., 2014; Mine et al., 2014). For, example in the study discussed
 184 above by Schreiber and Desveaux (2011), down-regulation of flagellin biosynthesis was associated
 185 with increased synthesis of the T3SS, and of the extracellular polysaccharide alginate, which both act
 186 to affect plant immune responses. Engl et al. (2014) subsequently identified a diguanylate cyclase
 187 that was implicated in suppressing flagellin synthesis and increasing polysaccharide synthesis in *P.*
 188 *syringae* pv. tomato DC3000.

189 **(ii) Physiology.** Physiological determinants of pathogen growth *in planta* include the uptake and
 190 utilisation of plant nutrients, the allocation of resources between replication and other cellular
 191 processes, and the ability of pathogens to adapt to stress, for example through the uptake or
 192 synthesis of compatible solutes to adapt to osmotic and water stress (Chen et al., 2013), the use of
 193 metabolic processes and membrane transport mechanisms to adapt to pH stress (Yuan et al., 2008),
 194 or the use of membrane transport proteins to import or extrude plant antimicrobials (Burse et al.,
 195 2004; Stoitsova et al., 2008; Vargas et al., 2011). Many of the determinants within this class are
 196 intrinsically dependent on factors present in the apoplastic environment, including apoplastic

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197 metabolites, ions and transport processes, which are in turn modulated by the impact of
198 environmental factors and host-pathogen interactions (e.g. Chen et al., 2010; O’Leary et al., 2016).
199 One relatively understudied aspect of pathogen physiology in the apoplast that warrants further
200 investigation is the ability of pathogens to use apoplastic metabolites as sources of carbon, nitrogen,
201 sulphur and phosphate, and to acquire essential mineral ions. Comparative analysis of *P. syringae*
202 metabolism *in vitro* and in apoplast extracts has demonstrated that this pathogen has a wide range
203 of nutrient utilisation capabilities, but preferentially uses specific apoplastic metabolites, including
204 glucose, organic acids and certain amino acids when growing in apoplast extracts (Rico and Preston,
205 2008; Rico et al., 2011; O’Leary et al., 2016; McCraw et al., 2017). Some metabolites and minerals
206 are notable for their low availability or absence in plant tissues; for example, plant tissues lack the
207 cobalt-containing co-factor cobalamin (vitamin B12) required by the methionine biosynthetic
208 enzyme MetH, and the apoplast generally contains very little cobalt or apoplastic methionine. Thus,
209 pathogens such as *Ralstonia solanacearum* use the cobalamin-independent methionine biosynthetic
210 enzyme MetE, which is co-regulated with virulence gene expression and upregulated *in planta*, for
211 methionine synthesis *in planta* (Plener et al., 2012).
212 A related question to that of pathogen nutrient utilisation is whether pathogens manipulate plant
213 metabolism and physiology to increase the availability of limiting nutrients in the apoplast. This
214 raises the particular challenge of distinguishing pathogen activities that have evolved to increase
215 apoplastic nutrient availability from pathogen activities that have evolved to suppress plant
216 defences, and from plant defence responses that alter apoplast composition. It is of course
217 conceivable that some pathogen activities have multiple benefits, for example pathogen-mediated
218 suppression of photosynthesis may have the dual benefits of modifying chloroplast-mediated
219 defences and mobilising nutrients to the site of pathogen infection (Bonfig et al., 2006; Berger et al.,
220 2007; Rodríguez-Herva et al. 2012). Similarly, toxin-mediated inhibition of nitrogen metabolism by *P.*
221 *syringae* toxins such as tabtoxin and phaseolotoxin may compromise defensive processes and alter

222 nutrient availability (Bender et al., 1999; Arrebola et al., 2011; Turner and Mitchell, 1985; Turner et
223 al. 1986). Chen et al. (2010) have presented evidence that multiple pathogens, including *P. syringae*,
224 can manipulate the expression of a group of *Arabidopsis thaliana* sugar transporters known as
225 AtSWEETs, although interestingly, of 7 AtSWEETs observed to be differentially regulated following
226 infection, only 3 were found to be altered through the activity of the T3SS. Evidence of the
227 functional significance of this altered sugar transport still awaits definitive experiments
228 demonstrating whether the pathogen benefits from increased nutrients in the apoplast.

229 **(iii) Regulation.** The apoplastic phenotype cannot be fully defined without an in depth
230 understanding of pathogen regulatory processes and their activity in pathogens during apoplast
231 colonisation. The majority of studies of gene expression in *P. syringae* have focused on the
232 regulation of its pathogenicity and virulence factors, and have found that relatively subtle changes in
233 the composition of the apoplastic environment can have a significant impact on virulence gene
234 expression and on the outcome of infection (e.g. Park et al., 2010; Anderson et al., 2014; McCraw et
235 al., 2017). They have also shown that regulation of virulence gene expression is intrinsically linked
236 with physiological processes such as nutrient stress and growth phase through global regulators such
237 as (p)ppGpp, GacS and Lon protease activity (Chatterjee et al., 2003; Lan et al., 2007; Chatnaparat et
238 al., 2015).

239 However, many studies of the regulation of pathogenicity and virulence gene expression focus on
240 phenotypes observed in minimal media designed to provide an “apoplast-mimicking” environment,
241 (e.g. Huynh et al., 1989; Kim et al., 2009; Rahme et al. 1992). Such studies, while useful for
242 genetically investigating the major regulators involved in virulence gene regulation, frequently start
243 from an assumption that is not commonly challenged or evaluated, which is that pathogens are
244 adapted to maximally express their pathogenicity and virulence genes in the host environment. This
245 assumption is challenged by studies such as that of Yu et al. (2014), who observed that the type III

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246 regulator HrpL is upregulated in the apoplastic compartment, but to a lesser degree than in a
247 nitrogen-limited minimal medium.

248 *In vitro* studies also commonly rely on liquid media that do not capture the biochemical or spatial
249 complexity of the apoplastic environment, which includes a complex cocktail of ions, metabolites,
250 proteins and cell wall constituents; local osmotic potential and water availability; the spatial
251 anatomy and biophysical nature of apoplastic surfaces and airspaces; gases and volatile organic
252 compounds within the apoplastic airspace; and dynamic changes in apoplast composition resulting
253 from interactions between host, pathogen and the external environment (Sattelmacher 2001;
254 Sattelmacher and Horst, 2007; O’Leary et al., 2016). Such changes can include the production of
255 plant metabolites that act to induce or repress pathogenicity and virulence gene expression,
256 including, in the specific case of *P. syringae* compounds such as GABA, flavonoids, flavonoid
257 glycosides, shikimate and quinate (Mo et al., 1995; Li et al., 1998; Park et al., 2010; Vargas et al.,
258 2013; McCraw et al. 2017). Discrepancies between virulence gene regulation *in vitro* and *in planta*
259 were noted early on in work by Xiao et al. (1992), who observed that the T3SS of *P. syringae* was
260 induced to a higher level following inoculation into tobacco leaves than when bacteria were
261 incubated in tobacco sap or sap supplemented with a tobacco cell wall extract. More recently, Yu et
262 al. (2014) noted that GacS regulation of T3SS expression in *P. syringae* pv. *syringae* B728a could only
263 be detected *in planta*, and not in minimal medium, demonstrating that some regulatory processes
264 governing pathogenicity gene expression can only be observed *in planta*.

265 To address these issues, an increasing number of studies, including the aforementioned study by Yu
266 et al. (2014), have begun to apply global gene analysis approaches to pathogens *in planta*. The
267 results from these analyses are already beginning to provide rich insights into pathogen biology.
268 However, such studies commonly rely on bulk analysis of RNA extracted from pathogen populations,
269 which may display a significant degree of heterogeneity in gene expression and regulation within the
270 host environment. Additionally, RNA-based studies inevitably fail to capture post-translational

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5 272 work by Little et al. (2016) has demonstrated that post-translational modification of ribosomal
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7 273 proteins may have a significant impact on the phenotype of *P. syringae in planta*. It remains of great
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9 274 interest to determine more precisely, and in relation to the spatial distribution of pathogen
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11 275 populations within plant tissues, the organisation and activity of the regulatory networks that act to
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13 276 modulate gene and protein expression and activity *in planta*.

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17 277 **(iv) Pathogenicity and Virulence.** The apoplastic phenotype associated with pathogenicity and
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19 278 virulence mechanisms is intrinsically linked with their interaction with the specific host in which a
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21 279 pathogen is growing. Many pathogenicity and virulence mechanisms act to induce plant responses
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23 280 that modify the apoplastic environment in ways that enhance or restrict pathogen growth (Asai and
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25 281 Shirasu, 2015; Hagemeyer et al., 2010; Rajniak et al., 2015; O’Leary et al., 2016). Some of these
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27 282 effects may be highly localised, others may generate signals that act systemically throughout the leaf
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29 283 or the host (e.g. Simon et al., 2010; Stahl et al., 2016), raising questions as to the sphere of influence
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31 284 of the extended phenotype, particularly since pathogen-induced signals may be transmitted to
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33 285 adjacent hosts and other plant-associated organisms as volatile or diffusible signals in air or water
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35 286 (Rudrappa et al., 2008; Scala et al., 2013; Baetz and Martinoia, 2014; Mommer et al., 2016). A
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37 287 particularly intriguing aspect of pathogen biology in terms of the extended phenotype is the ability
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39 288 of some pathogens to produce molecules that are identical to or which mimic plant hormones such
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41 289 as indole-acetic acid and indole-acetonitrile, ethylene and methyl jasmonate (Mazzola and White,
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43 290 1994; Weingart et al., 2001; Howden et al., 2009; Geng et al. 2014). The effects of pathogen
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45 291 produced phytohormones are not fully understood, but are thought to include the ability to modify
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47 292 plant physiology to promote nutrient transport to and alter apoplast composition at the site of
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49 293 infection, as well as modulating defence mechanisms to inhibit or redirect plant defences, or even to
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51 294 attract insect vectors (Howden and Preston, 2009; Geng et al., 2014; Groen et al., 2016; Ma and Ma,
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53 295 2016). There is also evidence of a role for certain phytohormones, whether of pathogen or plant
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296 origin, in modulating pathogen gene expression (Mazzola and White, 1994; Aragón et al., 2014; Yang
297 et al., 2007)

298 **(v) Social interactions.** A final element that needs to be captured in descriptions of the apoplastic
299 phenotype are social interactions between individual pathogen cells. The most widely studied and
300 best understood of these interactions are quorum sensing-based interactions, in which
301 microorganisms secrete, sense and respond to low molecular weight chemical signals that provide
302 information on the abundance, identity and activity of neighbouring cells (Atkinson and Williams,
303 2009). Such interactions enable pathogens to exhibit coordinated, density dependent behaviour for
304 traits such as motility, gene transfer and pathogenicity (von Bodman et al., 2003; Quiñones et al.,
305 2005), and can be strongly influenced by the environment, as illustrated in work by Dulla and Lindow
306 (1998), which examined the impact of water availability on quorum sensing by *P. syringae* in the leaf
307 environment. There is limited knowledge regarding the production, stability and availability of
308 quorum sensing molecules in the apoplastic environment, although the most widely studied
309 quorum-sensing molecules, acyl homoserine lactones (AHLs), are relatively stable at acidic pH (Yates
310 et al., 2002). Quorum sensing signals may also be actively degraded by lactonases produced by
311 plants or other microorganisms. In addition to producing and responding to quorum sensing signals,
312 endophytic organisms may also secrete and exchange a wide range of other small molecules,
313 proteins and even electrical signals that act to regulate behaviour and survival within local
314 communities (Prindle et al., 2015; Hazan et al., 2016).

315 A second aspect of social interactions that requires further study is the extent to which pathogens
316 enter into indirect social interactions through their niche constructing abilities. It has been shown
317 that avirulent “cheats” can persist in plant tissues when co-inoculated with pathogenic bacteria, as
318 the defence-suppressing activity of the pathogenic bacteria create an environment in which both
319 non-pathogenic bacteria can grow (Macho et al., 2007; Barrett et al., 2011). Similarly, although the
320 delivery of effectors that are recognised by the plant immune system can trigger plant defences,

when such bacteria are in a minority they may persist in a population of pathogenic bacteria (Arnold et al., 2011; Lovell et al., 2011). Further analysis of these phenomena requires more detailed study of pathogen populations *in planta* and in relation to their local environment and population structure.

A related issue is that many laboratory studies favour gnotobiotic systems, in which plants interact with a limited range of carefully controlled microorganisms, thereby excluding plant and pathogen interactions with the diverse population of epiphytes, endophytes and rhizobacteria that may be associated with plants and affect plant-pathogen interactions in a field setting (Lamichhane and Venturi, 2015). An example of the complexity of pathogen populations in naturally infected hosts is described in work by Moore et al. (1997), who characterised both opine production and bacterial diversity in naturally-infected tumours diagnosed as being likely to be caused by *A. tumefaciens*. Epidemiologically, the galled plants within small geographic areas were identified as being caused by diverse Ti plasmids responsible for production of different opines, indicative of a significant degree of diversity in local pathogen populations. Galls producing specific opines were found to be colonised by diverse bacteria, including agrobacteria and pseudomonads with opine-utilising abilities that did and did not correspond to the tumour from which they were isolated. They also found examples of agrobacteria that appeared to be avirulent towards the host from which they were isolated, despite being able to exploit opines produced by the tumour from which they were isolated. This is clearly indicative of a natural situation in which both related and unrelated bacteria can benefit from the extended phenotype or “niche-constructing” activity of a founding population. Similarly, researchers have observed that the knots established during olive knot disease caused by *Pseudomonas savastanoi* pv. *savastanoi* harbour communities of pathogenic and commensal organisms that interact to modulate disease severity (Passos da Silva et al., 2014; Buonauro et al., 2015), and that natural populations of *A. thaliana* are colonised by a mixture of *P. syringae* strains that are pathogenic, non-pathogenic or capable of eliciting host defence responses in the host they are resident in (Kniskern et al., 2011).

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347 Additionally, the existence of competing and cooperating organisms within the plant environment
348 presents an alternative explanation for the production of some factors commonly regarding as plant
349 pathogenicity or virulence factors. Many of the toxins produced by plant pathogens such as *P.*
350 *syringae* are non-selective toxins, which act on plant cells, but can also act on other microorganisms
351 (Völksch and Weingart, 1998; Bender et al., 1999). Further work is needed to examine whether the
352 production of such toxins is driven to a greater degree by their role in plant-pathogen interactions,
353 antagonistic interactions with competing microorganisms, or even by their ability to confer a positive
354 benefit to the host in terms of protection against other organisms (Kusari et al., 2012).

355 **Natural infection systems**

356 The preceding section considered the various determinants that are likely to be important
357 components of the extended phenotype of pathogens *in planta*. An important additional element to
358 consider when attempting to define and describe the extended pathogen phenotype is that many
359 commonly used infection methods and laboratory-based assays fail to mimic natural infection
360 mechanisms in ways that may affect these determinants and our understanding of pathogen
361 phenotypes in a natural setting. For example, for pathogens such as *P. syringae* artificial apoplastic
362 infection is commonly performed by inoculating a relatively high density of a clonal population of
363 bacteria directly into the apoplastic compartment, suspended in water or an aqueous solution to
364 initiate a synchronous and reproducible response to infection (Weigel and Glazebrook, 2009). This
365 approach is highly effective for a wide range of experimental purposes, where it is desirable to
366 generate rapid and reproducible plant responses, or to bypass interactions with other aspects of the
367 infection cycle such as epiphytic growth or interactions with stomata in order to focus on cellular
368 interactions within plant leaves. However, such inoculation methods have a number of potential side
369 effects, including mechanical and osmotic perturbation of the apoplastic compartment, and where
370 solutes or buffers are used, the potential for that solute to alter the phenotype of the interaction
371 (Lohaus et al., 2001; Boudart et al., 2005). For practical reasons some researchers have developed

assays using detached plant parts or seedlings (e.g. Ishiga et al., 2011; Schreiber et al., 2008). These systems have proven effective in isolating major pathogenicity or resistance determinants, and are useful for studying plant-pathogen interactions that are difficult to study in controlled conditions such as tree diseases, but cannot be considered to fully mimic natural infections in terms of host physiology and pathogen phenotypes

A secondary consequence of infection systems that use the infiltration approach, or other inoculation methods that are used to introduce high densities of inoculum into plant tissue, is that high inoculum density can elicit a more rapid and pronounced plant defence response (or a more overwhelming assault by pathogens on plant defences) than that observed in a natural system where infection may be initiated by a single infective spore or cell, but asymptomatic pathogen populations may persist for extended periods of time following infection. Such methods can also favour social interactions between pathogens at an early stage of infection, altering infection dynamics and pathogen persistence and survival.

An additional factor that potentially affects a wide range of infection systems is that the physiological state of a pathogen within the host, and thus its extended phenotype *in planta*, may be determined in part by its culture and treatment prior to infection. For example, incubation in laboratory media may enable a pathogen to accumulate stores of mineral nutrients, reducing dependency on apoplastic nutrients (Kim et al., 2009). Alternatively, the degree to which stress tolerance or pathogenicity mechanisms are induced prior to inoculation may affect the ability of a pathogen to adapt to the plant environment or express its virulence genes in a timely manner. One area that has attracted recent interest is the ability of pathogens to sense and respond to light, with the light environment experienced prior to or during infection being reported to significantly alter pathogen interactions with their hosts (Wu et al., 2013; Moriconi et al., 2013; Río-Álvarez et al., 2014).

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396 An optimal infection system in terms of a holistic understanding of the extended pathogen
397 phenotype might therefore use inoculum that has been obtained from a natural infection source,
398 inoculated via a natural infection mechanism at a relatively low inoculum density into a host plant in
399 a similar physiological and developmental state, with a similar microbiome, and under similar
400 environmental conditions to that associated with infection in the field or in a commercial
401 greenhouse setting. Such a system would however face the technological challenges associated with
402 achieving reproducible infection rates and infection outcomes, and in generating the samples
403 needed for experimental analysis of pathogen phenotypes using many current methodologies.

404 **Transformative technologies**

405 Some potential technological solutions to the challenges posed above may lie in three overlapping
406 areas: (i) the use of advanced microscopy, imaging and sensor techniques to study the plant
407 microenvironment and the phenotypic properties of individual pathogen cells within plant tissues; (ii)
408 the use of cell-specific analytical approaches, and (iii) systems biology approaches capable of
409 providing insight into complex pathogen activities such as metabolism, gene expression and protein
410 synthesis within the context of host environment. Significant progress has already been made in
411 understanding pathogen activities *in planta* using approaches such as transcriptomics and
412 proteomics (e.g. Yu et al., 2014; Jacobs et al., 2012; Ailloud et al., 2016), with ongoing developments
413 in proteomic technology providing the potential for greater insight into regulation of pathogen
414 activities *in planta* and of post-translational modification of pathogen regulatory proteins and
415 surface proteins (Semanjski and Macek, 2016). Work by Ailloud et al. (2016) illustrates the
416 importance of understanding the extended phenotype in the context of specific host-pathogen
417 interactions, by comparing gene expression *in vitro* and *in planta* in the Moko (banana) and NPB (not
418 pathogenic to banana) strain groups of *R. solanacearum*, which are closely related but are adapted
419 to distinct hosts. Among the genes observed to be differently regulated in the two strains were
420 those involved in microaerobic metabolism, which were distinctively up-regulated in the NPB strain

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3 421 during melon pathogenesis, but down-regulated in the Moko strain during banana pathogenesis,
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5 422 from which they inferred that the NPB strain may be subject to greater restriction of oxygen
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7 423 availability during host colonisation.
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10 424 The identity and abundance of the metabolites and proteins present in the apoplastic compartment
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12 425 have been specifically studied by using the infiltration-centrifugation approach to extract apoplastic
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14 426 fluid (Baker et al., 2012). Proteomic and functional analyses specifically targeting apoplastic proteins
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16 427 and inhibitors has proven particularly valuable in achieving greater understanding of the role of
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18 428 proteins and inhibitors acting at the interface between plant and pathogen (Kołodziejek and van der
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20 429 Hoorn, 2010; Feussner and Polle, 2015; Morimoto and van der Hoorn, 2016). However, it should be
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22 430 noted that the need to achieve a sufficiently robust response for analysis encourages the use of high
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24 431 inoculum densities, which raises issues in terms of failing to mimic natural infection systems, while
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26 432 approaches based on homogenisation of plant tissues or extraction of apoplastic fluid are unable to
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28 433 provide detailed information on the spatial structure of the plant environment and of localised
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30 434 pathogen activities within that environment. Potential strategies to address this include the use of
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32 435 microprobe and microsampling techniques, with the latter benefiting from ongoing improvements in
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34 436 the sensitivity of analytical techniques. In one recent study Dalsing and collaborators (2015) were
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36 437 able to use an oxygen microprobe to demonstrate that the level of O₂ was reduced in the xylem sap
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38 438 of tomato plants infected with *R. solanacearum*. Further progress may also be made by linking
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40 439 multiple approaches, for example using technologies focused on specific functional aspects of
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42 440 pathogen biology that can be monitored *in situ* together with 'omics approaches.
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47 441 A particularly exciting prospect to address these issues is that offered by emerging technologies for
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49 442 single cell-specific and cell-type specific analysis of gene and protein expression (Hodne and
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51 443 Weltzien, 2015; Wang et al., 2015; Willison and Klug, 2013; Handley et al., 2015). Metabolic labelling
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53 444 technologies can be combined with expression analyses to provide new insight into protein or cell
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55 445 wall and cell membrane synthesis and dynamics *in vivo* (Hatzenpichler et al., 2014; Siegrist et al.,
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2015; Agarwal et al., 2015). Questions about how pathogen cells or biomolecules can best be isolated from plant tissues for single cell analysis remain to be addressed, but there are a number of technologies, such as optical tweezers and laser induced forward transfer (LIFT) that could be developed to achieve this aim (Wang et al., 2013).

Advanced microscopy techniques can be used for imaging and analysis of the local chemical, ionic, structural and biochemical environment within plant tissues, and of the identity, physiology and functional properties of pathogens within the plant environment. In addition to the metabolic labelling studies highlighted above, recent studies of note include work by Ryffel et al. (2016), which used quantitative nuclear magnetic resonance (NMR) and imaging high-resolution mass spectrometry (IMS) to identify *A. thaliana* leaf surface compounds and their possible involvement in the epiphytic lifestyle of three leaf epiphytes, including *P. syringae* pv. tomato, by monitoring relative changes in compound pools. Moore et al. (2014) were able to use synchrotron X-ray fluorescence (S-XRF) and high-resolution secondary ion mass spectrometry (NanoSIMS) to observe the cellular and subcellular distribution and speciation of elements in plant tissues (reviewed in Zhao et al. 2014). Spatial proteomics integrating ultra-high speed MALDI-TOF with IMS to observe the distribution of proteins within tissues is also a rapidly emerging methodology (Spraggins et al. 2016). An increasing number of studies have begun to use IMS to monitor metabolic and social interactions between bacteria and fungi *in vitro* (Watrous et al., 2013; Baig et al., 2015; Spraker et al., 2016) and it will be interesting to extend these analyses to observe social interactions within the host environment.

A full discussion of relevant technologies is beyond the scope of this article, but in the context of this article it is worth briefly discussing some of the challenges that still remain in achieving the aim of profiling the extended phenotype using imaging approaches. One challenge that relates specifically to some of the issues discussed above is that most current imaging methods rely on manipulation and control of the light environment, either through keeping tissues dark for precise imaging of low

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3 471 intensity emitted light, or local illumination of tissues with light at specific wavelengths. Light can
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5 472 have an effect on the behaviour of both pathogen and host (e.g. de Wit et al., 2013; Griebel and
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7 473 Zeier, 2008; Wu et al., 2013; Kraiselburd et al., 2012; Roden and Ingle, 2009) and the potential
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9 474 effects of such light or dark treatments on pathogen, host and the extended phenotype of the
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11 475 pathogen in the host therefore need to be considered when interpreting results. A second challenge
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13 476 is that for some research questions it is highly desirable to monitor the extended phenotype over
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15 477 time, tracking dynamic changes in plant-pathogen interactions. This requires the maintenance of
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17 478 imaging specimens for extended periods of hours or days, a challenge which is currently being
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19 479 addressed by using perfusion approaches to support prolonged viability of host-pathogen systems
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21 480 (Littlejohn et al., 2014). Such approaches show significant potential, but still introduce an unnatural
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23 481 element into the study of host-pathogen systems. Thirdly, for a wide range of research questions,
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25 482 including those based on forward and reverse genetics, or screens of chemical libraries, it is highly
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27 483 desirable to perform high-throughput screens. The availability of automated microscopy makes it
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29 484 possible use cellular imaging for large-scale applications. However, there are significant challenges in
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31 485 achieving accurate quantitative annotation, while mimicking natural infection conditions as closely
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33 486 as possible, and therefore assays need to be carefully developed.

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38 487 Despite these challenges, some of the most exciting opportunities for future progress in exploring
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40 488 the extended phenotype include the integrative use of imaging techniques with cell-type specific or
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42 489 single cell technologies. Such techniques are already being exploited in relation to the metabolism,
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44 490 development and physiology of multicellular organisms and the analysis of unculturable
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46 491 microorganisms and complex microbial communities, and can be applied in future studies for *in situ*
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48 492 imaging of the phenotypic properties of individual pathogen cells. Compatible imaging technologies
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50 493 can be used to simultaneously detect properties of the local plant environment, such as apoplastic
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52 494 pH or cellular responses to infection. Once a pathogen cell of interest has been identified, single cell
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54 495 isolation techniques and cell-specific analytical techniques can permit the recovery of a specific cell
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496 or cell type for further analysis of properties such as gene and protein expression, genotype, or
497 other biochemical and biophysical properties.

498 **Summary**

499 There are many challenges associated with defining and studying the extended phenotype of
500 pathogens within host tissues, beginning with our definition of what aspects of pathogen biology
501 should be included in its extended phenotype, and how far-reaching this phenotype is. In reflecting
502 on these challenges it is appropriate to return to the question of why a deeper understanding of the
503 extended phenotype could enhance our understanding of pathogen interactions. This can best be
504 illustrated by considering the questions that greater knowledge of the extended phenotype would
505 allow us to address. Firstly, consider the question of resistance mechanisms and the durability of
506 resistance. At present, analyses of host range or resistance for pathogens such as *P. syringae* are
507 commonly restricted to relatively simple analyses of pathogen growth and symptom development,
508 sometimes in conjunction with a limited analysis of defence mechanisms (e.g. Monteil et al., 2013;
509 Fernández-Sanz et al., 2016). Extended phenotype analyses of pathogen physiology *in planta* using
510 natural infection methods would help to address additional questions that are of particular
511 relevance for pathogen evolution and population dynamics, such as whether a pathogen remains
512 viable in, or actively grows in host tissues for a period of time following natural infection, thereby
513 providing a window of opportunity for natural selection to favour lineages that overcome plant
514 defences.

515 Analyses of pathogen physiology and gene expression *in planta* could also be used to systematically
516 explore which mechanisms are acting to restrict pathogen growth, with the potential to consider the
517 long term durability of disease resistance; or to investigate potential risks such as the ability of
518 pathogens and endophytes to modify the local plant environment to support the growth or
519 evolution pathogens within non-host tissues. In considering virulent interactions with diverse hosts,
520 nutrient utilisation analyses would help researchers to understand diagnostic traits, such as carbon

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3 521 utilisation patterns, in the context of growth within the host environment, or growth in wild plants
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5 522 and other environments that act as reservoirs for infection of crop species. Metabolic pathways
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7 523 identified as having central roles in pathogen growth within the plant environment could provide
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9 524 new targets for disease control.
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12 525 Alternatively, consider the commonly observed scenario in which amendment of a crop species with
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14 526 a fertiliser enhances its yield, but also enhances its susceptibility to disease (Fagard et al., 2014).
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17 527 Why? It is possible to propose several non-exclusive explanations for this phenomenon, including
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19 528 firstly, that application of fertiliser results in the suppression of plant defence mechanisms that are
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21 529 normally effective in limiting disease development; secondly that fertiliser application modifies
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23 530 apoplast composition in a way that enhances pathogen nutrition or stress tolerance, or thirdly, that
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25 531 it modifies pathogen gene expression in a way that enhances the effectiveness of pathogenicity
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27 532 mechanisms, thereby resulting in more effective suppression of defence mechanisms. Systematic
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29 533 analysis of the apoplastic phenotype of the pathogen during infection, including its pathogenicity
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31 534 and virulence mechanisms, metabolism and stress responses, together with analyses of the local
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33 535 apoplastic environment can be used to understand the consequences of fertiliser treatment for the
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35 536 local environment surrounding the pathogen, and facilitate targeted investigation into whether crop
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37 537 or fertiliser improvement can be used to separate benefit and loss.
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41 538 In each of these scenarios, and others, a more complete understanding of the extended phenotype
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43 539 of pathogens within host tissues would bring us significantly closer to understanding why pathogens
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45 540 thrive or die..

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965 **Figures**

966 **Figure 1:** Niche construction in the plant apoplast. Infection is initiated when a pathogen (yellow
967 oval) invades the apoplastic compartment (A). As the pathogen proliferates a combination of plant-
968 microbe and microbe-microbe interactions modulate host defences and alter the composition of the
969 apoplast (B). Interactions where pathogens successfully suppress host defences or increase nutrient
970 availability to support pathogen growth can be regarded as a form of positive niche construction, in
971 which a pathogen’s descendents benefit from its niche-constructing activity (C). Interactions
972 resulting in the induction of host defence mechanisms alter apoplast composition in ways that
973 restrict pathogen growth, a form of negative niche construction (D). In the early stages of infection
974 natural selection favours pathogens that have extended phenotypes that include the ability to
975 survive in the apoplastic fluid of healthy leaves and suppress host defences, while in later stages of

infection, or following induction of host defences, pathogens are under selection for growth and survival in the modified apoplast.

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Figure 2: Determinants of the apoplastic phenotype of *Pseudomonas syringae*. This schematic diagram summarises some of the major determinants of the extended phenotype of *P. syringae* in the plant apoplast. The determinants shown can be grouped into five major classes: **Identity (yellow); Physiology (dark blue); Regulation (red), Pathogenicity (pink), Social (orange)**. Host determinants are shown in green. **Identity determinants** include surface features (1), secreted proteins (5,6) and pathogen activities that are recognised by the plant immune system through PAMP-triggered immunity (PTI), DAMP-triggered immunity (DTI) and effector-triggered immunity (ETI). **Physiological determinants** include cellular processes such as DNA replication (3), metabolism and nutrient uptake (12), as well as mechanisms used to resist antimicrobials (8) and to adapt to stress (11). Stresses encountered in the apoplastic environment include osmotic and oxidative stress, inhibition of cellular processes by molecules such as cyanide, and damage caused to proteins, membranes and DNA by molecules such as reactive oxygen species (11). Pathogens adapt to oxidative stress through detoxification of reactive oxygen species and cellular repair mechanisms, and to osmotic stress through the synthesis or uptake of compatible solutes. Adaptation to stress, temperature and the apoplastic environment can also include changes in membrane composition (2), which can affect transport and sensory processes as well as tolerance to anti-microbials. **Regulatory determinants** include sensors that recognise apoplastic signals, as well as the complex regulatory networks that operate at a transcriptional and post-transcriptional level to regulate cellular processes, adaptation to stress and pathogenicity and virulence gene expression. **Pathogenicity and virulence determinants** include proteins secreted to the apoplastic compartment (5) and to the host cell cytoplasm (6), as well as small secreted molecules that act as toxins, inhibitors of plant defence mechanisms or interact with plant hormone signalling mechanisms. **Social determinants** include small molecules such as quorum sensing signals (4) that provide

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1002 information on the local environment and population density within that environment, as well as

1003 determinants that cooperatively or antagonistically affect neighbouring organisms. **Host**

1004 **determinants** include receptors that recognise pathogen elicitors or activities, triggering PTI, DTI or

1005 ETI, leading to defence activation, including the production of antimicrobials and volatile and

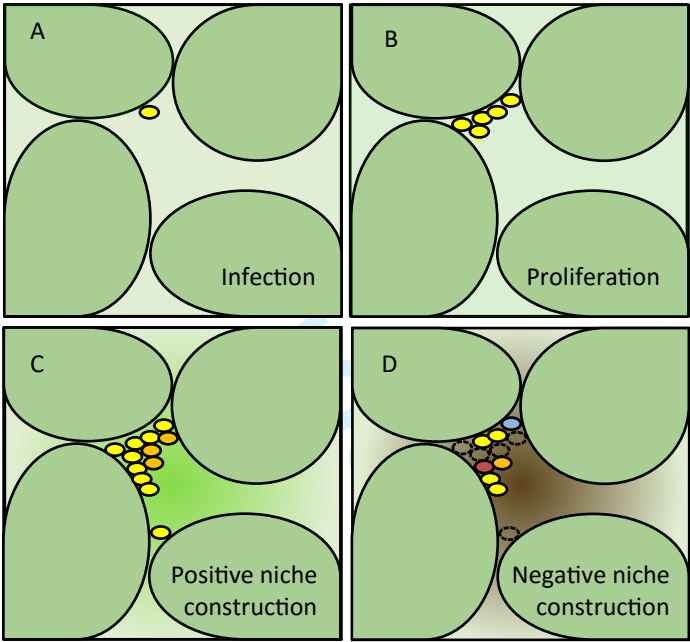
1006 reactive chemicals (5), and apoplastic enzymes that target pathogens (10). They also include features

1007 that are exploited by pathogens to suppress defences (4), loss of membrane integrity during ETI or

1008 disease (6) and the integrated impact of metabolic, physiological, environmental, developmental and

1009 transport processes on apoplast composition (6).

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