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Infection of *Arabidopsis thaliana* by *Phytophthora parasitica* and identification of variation in host specificity

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SUMMARY

Oomycete pathogens cause severe damage to a wide range of agriculturally important crops and natural ecosystems. They represent a unique group of plant pathogens that are evolutionarily distant from true fungi. In this study, we established a new plant–oomycete pathosystem in which the broad host range pathogen *Phytophthora parasitica* was demonstrated to be capable of interacting compatibly with the model plant *Arabidopsis thaliana*. Water-soaked lesions developed on leaves within 3 days and numerous sporangia formed within 5 days post-inoculation of *P. parasitica* zoospores. Cytological characterization showed that *P. parasitica* developed appressoria-like swellings and penetrated epidermal cells directly and preferably at the junction between anticlinal host cell walls. Multiple haustoria-like structures formed in both epidermal cells and mesophyll cells 1 day post-inoculation of zoospores. Pathogenicity assays of 25 *A. thaliana* ecotypes with six *P. parasitica* strains indicated the presence of a natural variation in host specificity between *A. thaliana* and *P. parasitica*. Most ecotypes were highly susceptible to *P. parasitica* strains Pp014, Pp016 and Pp025, but resistant to strains Pp008 and Pp009, with the frequent appearance of cell wall deposition and active defence response-based cell necrosis. Gene expression and comparative transcriptomic analysis further confirmed the compatible interaction by the identification of up-regulated genes in *A. thaliana* which were characteristic of biotic stress. The established *A. thaliana–P. parasitica* pathosystem expands the model systems investigating oomycete–plant interactions, and will facilitate a full understanding of *Phytophthora* biology and pathology.

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INTRODUCTION

The oomycete pathogens were originally classified as fungi as a result of their fungus-like morphology, physiology and strategies for attacking plants. However, the phylogenetic detachment of fungi and oomycetes is reflected in radical differences in their biochemistry, cell structure and development (Money *et al*., 2004). They belong to the separate kingdom Stramenopila, and are believed to form a monophyletic group with the Hyphochytriomycota and Labyrinthulomycota (Barr, 1992; Roetschi *et al*., 2001). The genus *Phytophthora* represents typical oomycete pathogens, and consists of over 100 species that damage a wide range of agriculturally and ornamentally important crops, forests and natural ecosystems. *Phytophthora infestans* is one of the most notorious plant pathogens and caused the Great Irish Famine in the 1840s. *Phytophthora ramorum*, discovered in the last decade, caused sudden oak death in the west coast of the USA (Rizzo *et al*., 2002; Tyler *et al*., 2006).

Arabidopsis thaliana has been used extensively as a model plant to elucidate plant–pathogen interactions with a wide variety of pathogens, including bacteria (Debener *et al*., 1991; Deslandes *et al*., 1998; Meyer *et al*., 2005; Quirino and Bent, 2003), fungi (Adam and Somerville, 1996; van Baarlen *et al*., 2007; Bohman *et al*., 2004; Chen *et al*., 2006; Narusaka *et al*., 2004; Urban *et al*., 2002) and oomycetes (Bittner-Eddy and Beynon, 2001; Koch and Slusarenko, 1990; McDowell *et al*., 2000; Roetschi *et al*., 2001). The accomplishment of its genome sequence has provided a huge amount of bioinformatic information for genetic and molecular studies (Redman *et al*., 2004). Several positive attributes of *Arabidopsis* include its small genome size, the ease of performance of mutagenesis, the extensive mutant collections and other available genetic and genomic resources, and various well-developed research tools. These make it ideally suited to serve as a model plant to investigate the sophisticated interactions between plant and pathogen. Since

the 1990s, *A. thaliana* and its natural associations with downy mildew and other fungal pathogens have provided a rich and fascinating model for cellular, physiological and molecular studies, as well as the genetics of plant–pathogen interactions. To date, there have been only a few instances involving the infection of *Arabidopsis* by *Phytophthora* pathogens. These have included the compatible pathogen *P. brassicae* (Roetschi *et al*., 2001) and incompatible *P. infestans* (Huitema *et al*., 2003), *P. palmivora* (Daniel and Guest, 2006), *P. sojae* (Takemoto *et al*., 2003) and *P. cinnamomi* (Robinson and Cahill, 2003). Very recently, Attard *et al*. (2010) described the root infection of *A. thaliana* by *P. parasitica* and characterized mutants with impaired salicylic acid, jasmonic acid and ethylene signalling pathways, concluding that these mutants were more susceptible than the wild-type to root infection by *P. parasitica*. Similar to the well-studied model species *P. infestans* (Pieterse *et al*., 1992) and *P. sojae* (Tyler *et al*., 2006), *P. brassicae* has a limited host range (Roetschi *et al*., 2001).

Our current knowledge on the molecular interactions between plants and oomycetes has been derived mainly from research on *Hyaloperonospora parasitica*, *P. sojae* and *P. infestans*. However, *H. parasitica* is an obligate foliar pathogen and genetically intractable, and the majority of agronomically important oomycetes are soil-borne pathogens with a broad-spectrum host range. *Phytophthora infestans* is a foliar pathogen, and both *P. infestans* and *P. sojae* have narrow host ranges. Most other *Phytophthora* species are soil-borne, root-infecting and are pathogens that infect a broad spectrum of host plants.

In this study, we describe the compatible interaction between *Arabidopsis* and *P. parasitica*, and its use as a model pathosystem. The soil-borne oomycete pathogen *P. parasitica* Dastur, also called *P. nicotianae* Breda de aan, is the causal agent of root and stem rot of many plant species. One of the most important diseases, it causes black shank of tobacco, which is a destructive disease of flue-cured tobacco in major tobacco-producing areas worldwide (Erwin and Ribeiro, 1996). Losses can occur at all stages of growth, and range from minor injury to complete destruction of the tobacco plant. *Phytophthora parasitica* has a wide spectrum of hosts, including both herbaceous and woody hosts, in a range of over 60 different plant families, notably the Solanaceae and other cultivated crops of worldwide importance (Erwin and Ribeiro, 1996). *Phytophthora parasitica* is heterothallic and has two mating types, A1 and A2. Both mating types are required to produce numerous sexual spores, called oospores. This is different from the homothallic *P. brassicae* which has been demonstrated to be a pathogen of *A. thaliana* (Roetschi *et al*., 2001).

Phytophthora parasitica is emerging as a model oomycete species for several reasons: (i) the increasing amount of genetic and genomic resources, including the generation of expressed sequence tag (EST) sequences during pathogen development and plant infection (Kebdani *et al*., 2010; Le Berre *et al*., 2008; Panabieres *et al*., 2005; Rosa *et al*., 2007; Shan *et al*., 2004; Skalamera *et al*., 2004); (ii) a large-insert genomic library that has allowed the confirmation of the *P. parasitica* genome size as 95.5 Mb, which is similar to that of *P. sojae* (Shan and Hardham, 2004); (iii) its genetic tractability (Bottin *et al*., 1999; Gaulin *et al*., 2002); and (iv) the availability of genomic tools. Over 80% of the EST sequences obtained were also found within the *P. sojae*, *P. ramorum* or *P. infestans* genomes. However, 10%–15% did not match any available sequence data for *Phytophthora* species (Le Berre *et al*., 2008). These sequences are likely to be species specific and are probably involved in the capacity of *P. parasitica* to infect a wide host range. The available draft genome sequences of *P. sojae*, *P. ramorum* and *P. infestans* (Haas *et al*., 2009; Tyler *et al*., 2006) will aid in the identification of the genes that determine the molecular interaction between *Phytophthora* and plants.

The infection process of *A. thaliana* by *P. parasitica* was investigated cytologically by scanning electron microscopy and interference contrast microscopy. The development of water-soaked lesions and numerous haustoria-like structures in colonized plant tissues and the production of asexual sporangia demonstrated the compatible interaction between *A. thaliana* and *P. parasitica*. Gene expression analysis further confirmed the compatible interaction and identified 19 *A. thaliana* genes significantly up-regulated in response to *P. parasitica* infection. An analysis of 25 *A. thaliana* ecotypes and six *P. parasitica* strains indicated the presence of a natural variation in specificity among plant ecotypes and pathogen strains, making this a significant step in the establishment of *P. parasitica* and *A. thaliana* as a model pathosystem for the investigation of fundamental questions, such as the genetic basis of host specificity, in this broad host range oomycete pathogen.

RESULTS

Arabidopsis thaliana is susceptible to *P. parasitica*

Phytophthora parasitica has a wide range of host plants. To test whether the model plant *A. thaliana* was a potential host plant, detached leaves of ecotype Col-0 were drop inoculated with zoospores of *P. parasitica* strain Pp016, which was originally isolated from diseased tobacco plants. Water-soaked lesions formed within 3 days post-inoculation of zoospores (Fig. 1A), suggesting a compatible interaction between *A. thaliana* and *P. parasitica*. Under our experimental conditions, the detached *A. thaliana* leaves remained green and showed no obvious differences from those of live plants within 10 days.

Microscopic characterization showed that most of the inoculated zoospores remained motile in the initial 30 min and encysted within 1 h post-inoculation. Shortly after forming

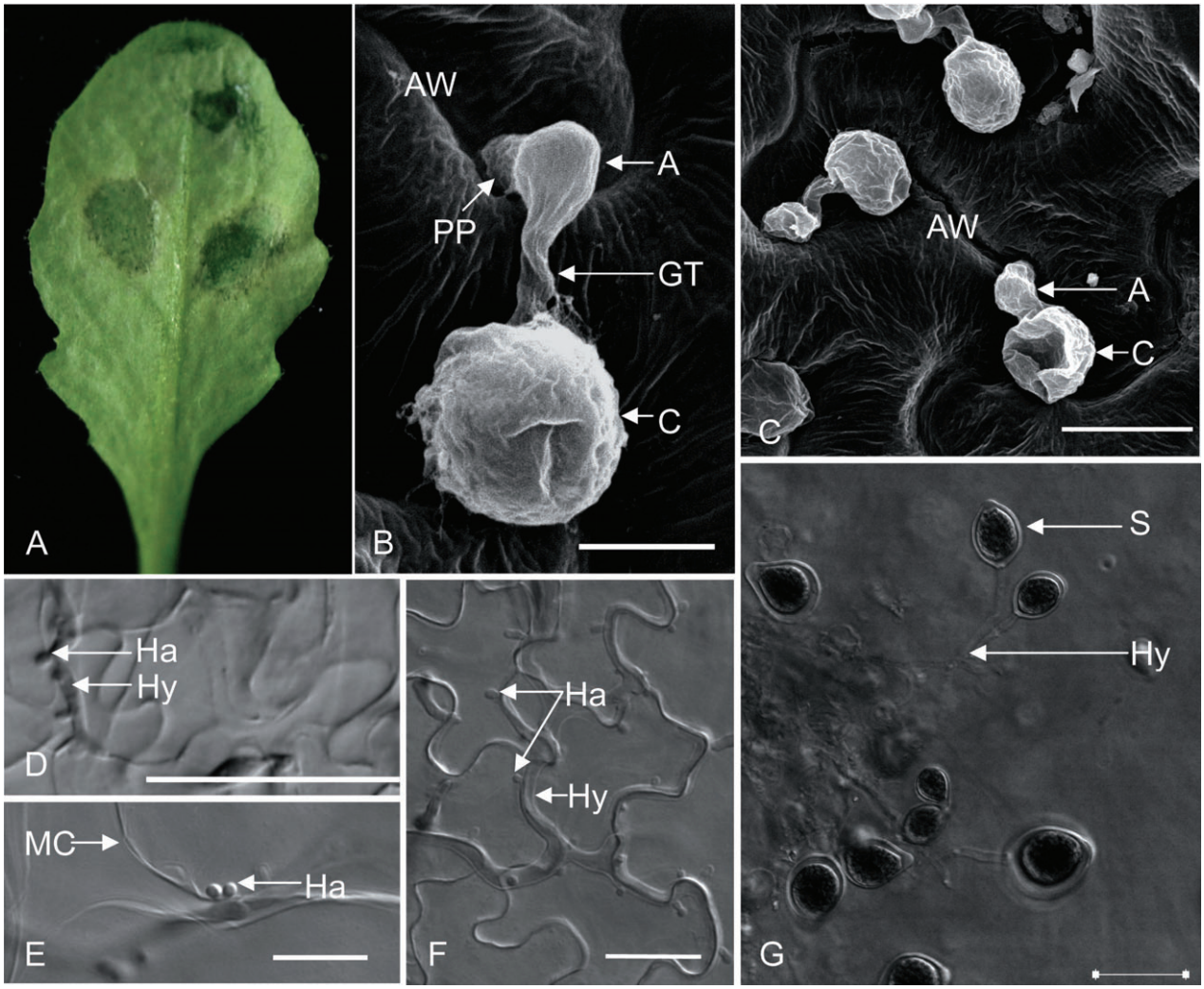

Fig. 1 Infection of *Arabidopsis thaliana* leaves by *Phytophthora parasitica*. Four-week-old leaves of *A. thaliana* were drop inoculated with zoospores of *P. parasitica* strain Pp016. Inoculated leaves were collected at different time points post-inoculation and stained with trypan blue before being subjected to microscopic characterization. (A) Water-soaked lesions developed 3 days post-inoculation (dpi) of strain Pp016 zoospores. (B) Germ tube (GT) differentiated from cyst (C), appressorium (A) developed and penetration peg (PP) formed to invade the plant at the anticlinal walls (AW) of adjacent plant epidermal cells (bar, 5 μm). (C) Appressorium-like structures (A) mostly differentiated at the anticlinal walls (AW) of epidermal cells (bar, 10 μm). (D) Haustoria-like structures (Ha) formed by intercellular hyphae (Hy) in epidermal cells 6 h post-inoculation (bar, 50 μm). (E) Haustoria (H) developed on invasive hyphae in mesophyll cells (MC) (bar, 20 μm). (F) Infection hyphae (Hy) with multiple branches, as well as abundant haustoria (Ha), were evident in both epidermal cells and mesophyll cells (bar, 20 μm). (G) Sporangia (S) were abundant on the leaf surface within 4 dpi (bar, 20 μm).

encysts, the cysts germinated by means of germ tubes (Fig. 1B) on the leaf epidermis. Cysts usually produced one germ tube and, in rare instances, two or more germ tubes. The germ tubes grew in random directions and with different lengths. Appressoria usually formed on the tips of the germ tubes (Fig. 1B), but some were produced directly by the cysts. Most appressoria formed over, or in close proximity to, the anticlinal walls of adjoining epidermal cells (Fig. 1C), but some developed on the epidermal cells. The penetration peg was differentiated from the appressorium to invade the host epidermis.

Within 3 h post-inoculation of zoospores, invasive hyphae were observed to be predominant at the anticlinal walls between the epidermal cells (Fig. 1C). Yet, some hyphae entered through the stomata. Appressoria were also found to form on the guard cells, but the frequency was very low. At this time point, most cysts were empty, indicating that the cytoplasm had already been moved into the penetration hyphae that extended into the epidermal cells.

Within 6 h post-inoculation of zoospores, haustoria-like structures were visible in the epidermal cells (Fig. 1D) and, by 8 h, in

the mesophyll cells. By this time point, mild reactions of plant cells and cell wall deposition were occasionally visible on the plant epidermal cells in response to the pathogen.

Typically, one haustorium was observed in each mesophyll cell from the intercellular penetrating hyphae 24 h post-inoculation. By 48 h post-inoculation, intercellular hyphae with multiple branches were evident, and abundant haustoria, two or more in each mesophyll cell (Fig. 1E) or epidermal cell (Fig. 1F), were dominant.

By 60 h post-inoculation, sporangia were visible and the number increased remarkably with the extensive growth and expansion of infection hyphae. The intercellular hyphae appeared to grow predominantly through the stomata and the junction between adjoining epidermal cells to the outside of the leaves. By 4 days post-inoculation, abundant sporangia (Fig. 1G) had formed on the leaf surface.

Infection and colonization of root tissues

Phytophthora parasitica is a typical root and stem rot pathogen of many plants. To check whether the root tissue of *A. thaliana* was susceptible to *P. parasitica* infection, as observed for leaf tissue, 2-week-old seedlings were dip inoculated with zoospores and characterized under a microscope. Compared with the water control (Fig. 2A1), the inoculated seedlings wilted and collapsed 4 days post-inoculation (Fig. 2A2). The microscopic results showed that pathogen penetration and hyphal development were similar to those on leaf tissues. The infection process also started with the attachment of zoospores to the root surface, followed by encystment and germination. The number of germinated zoospores increased dramatically by 1.5 h post-inoculation, and approximately 90% of zoospores had developed germ tubes within 6 h after inoculation. Appressoria formed at the tips of the germ tubes were visible by 1.5 h post-inoculation. Pathogen penetration into the root epidermal cells also occurred mainly at the junction between anticlinal walls. In general, the pathogen reached the cortex after invasion of the epidermis, and cortical cells were essential for successful colonization of the pathogen. Infection hyphae grew intercellularly and intracellularly in the cortex and developed haustoria in the cortex cells. By 16 h post-inoculation, the apex of the root was colonized by branched invasive hyphae, present both inside the cells and in the intercellular spaces, and no microscopic cell death was observed, as determined by microscopic characterization. Multiple haustoria were visible in the cortex 24 h post-inoculation (Fig. 2B) and, by 48 h post-inoculation, sporangia were visible (Fig. 2C).

Whole seedlings were also inoculated with pathogen mycelial agar cultures. Inoculation of *A. thaliana* ecotype Col-0 with *P. parasitica* strain Pp016 revealed that the seedling hypocotyls were constricted, the growth of the plants was restrained, and the leaves turned a yellowish colour 5 days post-inoculation. Whole seedlings wilted within 7–10 days post-inoculation.

Natural variation in specificity between *A. thaliana* and *P. parasitica*

To investigate whether there was host specificity between *P. parasitica* and *A. thaliana*, as with tobacco, five additional *P. parasitica* strains were tested for pathogenicity on ecotype Col-0. These strains were selected because of their opposite mating type (A2) to Pp016, which facilitates the genetic analysis of host specificities in the pathogen, and because they potentially contain multiple avirulence genes according to a brief bioassay on a set of 12 tobacco cultivars (data not shown). Similar to Pp016, strains Pp014 and Pp025 caused water-soaked lesions on detached leaves, whereas strains Pp008 and Pp009 showed no visible symptoms on both detached leaves and whole live plants when either zoospores or mycelial pieces were used as inocula. Strain Pp005 ranged from weakly virulent to avirulent on the tested ecotypes (Table 1).

Microscopic characterization revealed remarkable differences between Pp009 and Pp016 when interacting with Col-0 leaves. Single germ tubes were usually differentiated from cysts of Pp009 with, occasionally, two germ tubes formed, and one or both developed mature appressoria, as with Pp016. However, the development of secondary appressoria (SA), which are usually a result of a failed attempt to penetrate host cells, was frequent for Pp009 (Fig. 3D). Cell wall deposition at the attempted penetration sites of appressoria on epidermal cells (Fig. 3A1, A2) was observed quite frequently with inoculation by Pp009. Staining with aniline blue revealed the presence of callose, which can be stained and detected by epifluorescence microscopy under UV light.

In addition to the deposition of host material at an early stage of infection, hypersensitive host epidermal cell death (Fig. 3B) was evident adjacent to the infection site, suggesting that active defence responses were responsible for the incompatible interaction between *A. thaliana* Col-0 and *P. parasitica* Pp009. Occasionally, the pathogen was able to penetrate into the mesophyll, but this was quickly stopped by the blue-staining mesophyll cells next to the infection hyphae (Fig. 3C). As a result, no haustoria or subsequent sporangia were observed in this interaction. Similarly, ecotype Col-0 showed no obvious susceptible symptoms when inoculated with strain Pp008.

Further pathogenicity assays of 24 additional *A. thaliana* ecotypes, including Ler, with the same set of six *P. parasitica* strains showed the presence of a variation in host specificity in *A. thaliana* against *P. parasitica*. Most *Arabidopsis* ecotypes, except Zu-1, exhibited severely susceptible symptoms and were fully colonized by the pathogen when inoculated with strains Pp014, Pp016 and Pp025. Restricted chlorosis developed at the

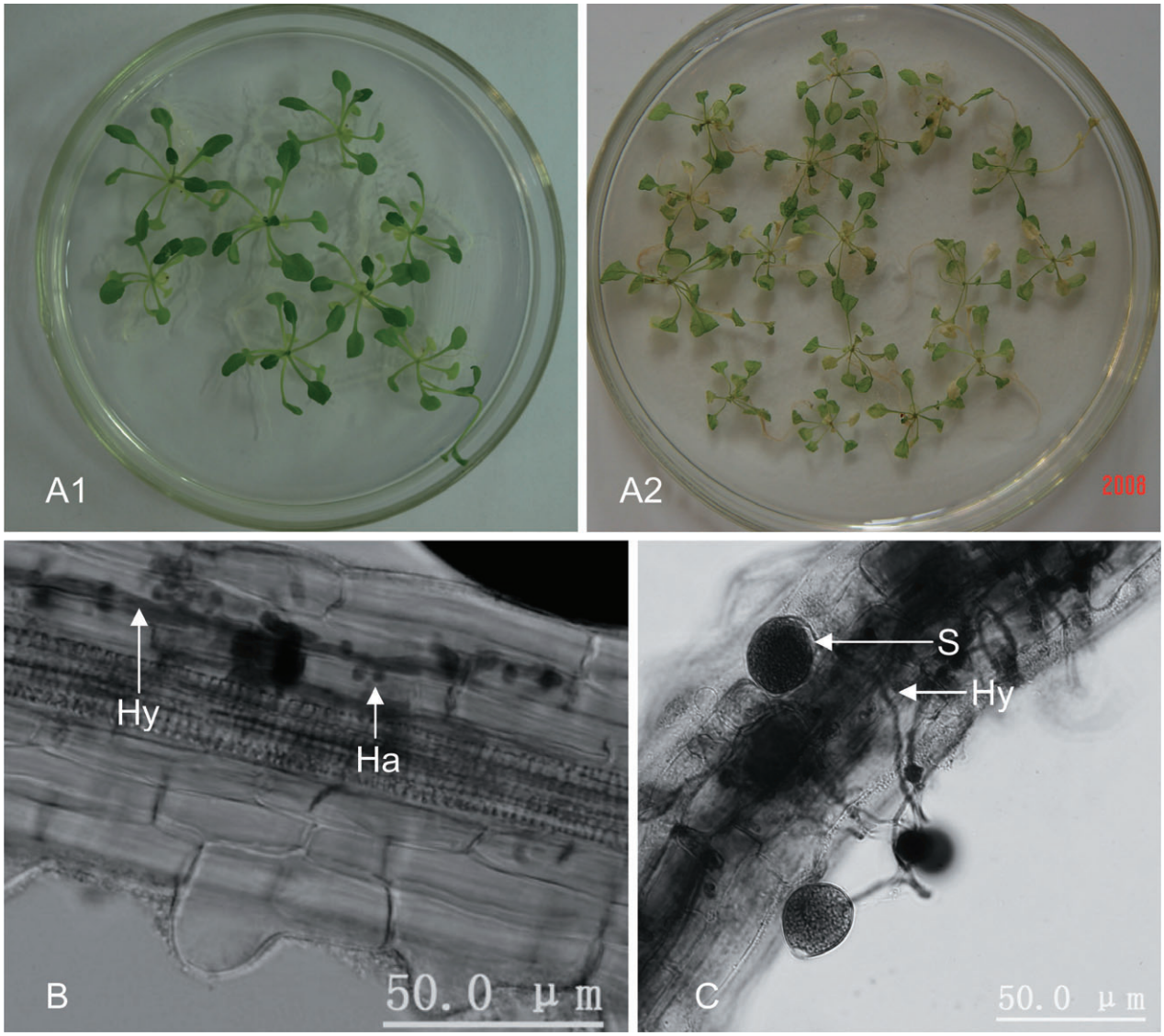

Fig. 2 Root infection and colonization of *Arabidopsis thaliana* by *Phytophthora parasitica*. Live root tissues were inoculated by dipping into a zoospore suspension (10^5 spores/mL) for 5 s, followed by removal of excess solution on sterile Whatman paper and transfer to Petri dishes containing half-strength Murashige and Skoog (MS) medium without sugar. (A) Wilt and collapse of whole plant seedlings 4 days post-inoculation of strain Pp016 zoospores (A2); (A1) is the water control plate. (B) Haustoria-like structures (Ha) developed by intercellular hyphae (Hy) in the cortex 24 h post-inoculation of zoospores (bar, 50 μm). (C) Heavy colonization of root tissues as shown by the dramatic increase in pathogen biomass by 48 h post-inoculation of zoospores; Hy, hyphae; S, sporangia.

inoculation site, but no lesion development, in Zu-1. In contrast, ecotype Nd-0 was highly susceptible to infection by strain Pp016.

Pathogenicity assays showed that strains Pp008 and Pp009 were both incompatible with all 25 *Arabidopsis* ecotypes. For example, ecotype Sap-0 showed no symptoms 7 days post-inoculation when both detached leaves and whole live plants were inoculated with Pp009, whereas seedlings infected by Pp016 showed near-collapse or severe constriction in their hypocotyls (Fig. 4). Pp005 was weakly virulent to avirulent in all 25 ecotypes examined, except Sap-0, on which pathogen growth was significantly restrained and whole plants wilted within 7 days post-inoculation.

Gene expression and comparative transcriptomic analysis in *A. thaliana* infected with *P. parasitica*

ESTs, which provide the most comprehensive evidence for gene expression, were employed to examine the response of *Arabidopsis* genes during the stress of *P. parasitica* infection. We

Table 1 Interaction phenotypes of 25 *Arabidopsis thaliana* ecotypes following inoculation with six *Phytophthora parasitica* strains.

	Phytophthora parasitica strains					
	Pp005	Pp008	Pp009	Pp014	Pp016	Pp025
Arabidopsis ecotypes	WS/DL	WS/DL	WS/DL	WS/DL	WS/DL	WS/DL
Bs-1	A/—	A/—	A/—	V/—	V/V	V/—
Bsch-0	M/—	A/—	A/—	V/—	V/—	V/—
Co-1	A/—	MA/—	A/—	V/—	V/V	V/—
Col-0	A/—	A/—	A/A	V/V	V/V	V/V
Di-1	M/—	MA/—	A/—	V/—	V/M	V/—
Er-0	M/—	MA/—	A/—	V/—	V/V	V/—
Fe-1	V/—	MA/—	A/—	V/—	V/V	V/—
Gd-1	V/—	MA/—	A/—	V/—	V/—	V/—
Is-0	A/—	A/—	A/—	V/—	V/M	V/—
Ler	A/—	A/—	A/A	V/V	V/V	V/V
Lo-1	A/—	A/—	A/—	V/—	V/V	V/—
Mc-0	M/—	A/—	A/—	V/—	V/M	V/—
Ms-0	A/—	MA/—	A/—	V/—	V/V	V/—
Mt-0	MA/—	A/—	A/—	V/—	V/V	V/—
Mz-0	MA/—	A/—	A/—	V/—	V/—	V/—
Nc-1	M/—	A/—	A/—	V/—	V/—	V/—
Nd-0	A/—	A/—	A/A	V/V	V/V	V/V
Old-1	A/—	A/—	A/—	V/—	V/V	V/—
Pla-2	M/—	A/—	A/—	V/—	V/V	V/—
Sap-0	V/—	MA/—	A/—	V/—	V/V	V/—
Sf-1	V/—	MA/—	A/—	V/—	V/V	V/—
Sg-1	M/—	A/—	A/—	V/—	V/V	V/—
Sorbo	M/—	A/—	A/—	V/—	V/V	V/—
Ta-0	V/—	A/—	A/—	V/—	V/—	V/—
Zu-1	A/—	A/—	A/A	V/A	V/A	V/A

All 25 *Arabidopsis* ecotypes were assessed for susceptibility by inoculation of whole seedlings (WS) with pathogen mycelial pieces, and the results were scored 10 days post-inoculation. Selected ecotypes were further evaluated by inoculation of detached leaves (DL) with zoospores, and the results were scored 3 days post-inoculation.

A, avirulent, as defined by healthy seedlings (WS) or leaves (DL) with no visible disease symptoms; V, virulent, as defined by seedling collapse (WS) and water-soaked lesions on leaves with numerous haustoria formed (DL); M, moderately virulent, as defined by the development of yellowish leaves in the lower parts of seedlings for the whole-seedling assays (WS) and restricted lesion expansion (less than one-third of leaf size) and a yellowish colour at the inoculation site with occasional visible haustoria for detached leaf assays (DL); MA, moderately avirulent, as defined by restricted seedling development (in size), but no disease symptoms, for whole-seedling assays (WS); —, not determined.

therefore constructed a Gateway CloneMiner-based cDNA library using mRNAs isolated from leaves of *P. parasitica*-infected *A. thaliana*. The titre of the library was 6.34×10^6 colony-forming units (cfu)/mL, which was sufficient to meet the quality standard. Primary library sequencing led to a total of 3021 ESTs, 2256 of which, corresponding to 1692 unigenes, were of plant origin (GenBank accession numbers HO206545–HO208800). Up to 75.0% of these plant ESTs were unigenes, further confirming the high level of quality of the library. Over 25.3% ESTs (765) were of *P. parasitica* origin, indicating heavy plant infestation by the pathogen.

To obtain a general view of the genes expressed, we annotated, using the Functional Catalogue (FunCat) scheme (Ruepp *et al*., 2004), the generated ESTs and five additional non-normalized, non-subtracted *Arabidopsis* cDNA libraries downloaded from the DFCI Arabidopsis Gene Index Build 14.0 (AtGI).

The results of FunCat annotation (Fig. 5) showed that the expression levels of the genes involved in cell rescue, defence and virulence (in yellow) were much higher in *P. parasitica* (P)- and *Erysiphe cichoracearum* (E)-infected leaf tissues. This was similar to hormone-treated *A. thaliana* callus (H) (Castelli *et al*., 2004) and leaves undergoing senescence (L) (Guo *et al*., 2004). However, leaf senescence could be distinguished from other development stages and stresses by the expression of metabolism-associated genes (green). All six libraries, except the leaf tissues at the senescence stage, show a relatively stable expression level of metabolism genes, indicating reduced metabolism activity during leaf senescence.

Genes involved in the interaction of *Arabidopsis* with the environment (red) and cellular communication/signal transduction (light blue) were also up-regulated during infection with *P. parasitca* (P), suggesting active responses of the plant to biotic

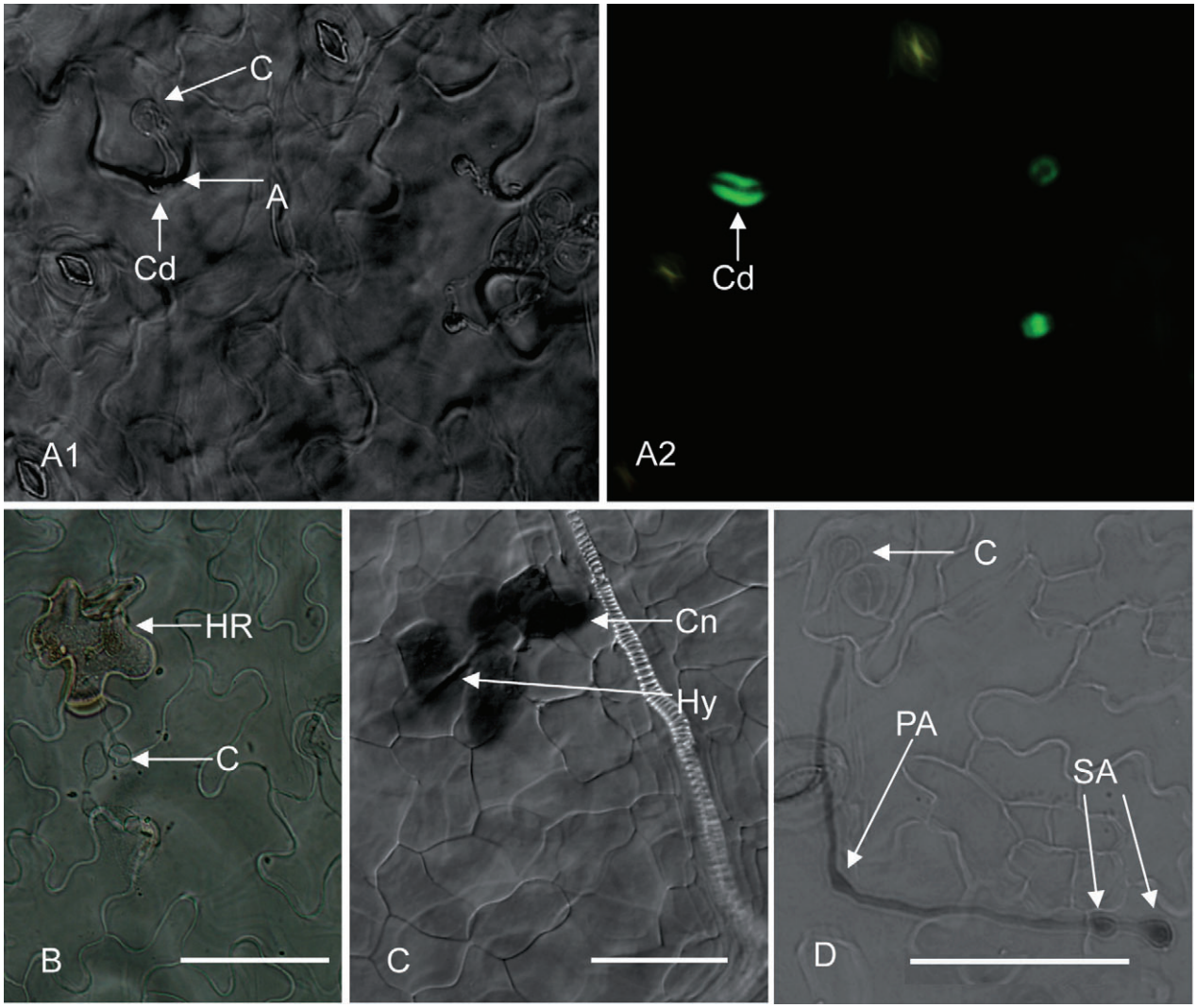

Fig. 3 Microscopic characterization of the incompatible interaction between *Arabidopsis thaliana* Col-0 and *Phytophthora parasitica* Pp009. (A) Cell wall deposition (Cd) at the attempted infection site of epidermal cells by strain Pp009, as revealed by aniline blue staining and examination under UV light, in which the green fluorescence indicates the accumulation of callose (A2); (A1) is the same image under visible light to show the infection site; A, appressorium; C, cyst. (B) Rapid hypersensitive response (HR) of a Col-0 epidermal cell in response to penetration by strain Pp009; C, cyst. (C) Penetration of mesophyll cells by progressive hyphae (Hy) is stopped by the cell undergoing HR. The leaf was stained with trypan blue and deeply blue-stained tissues indicate cell necrosis (Cn). (D) Germinated cyst (C) with development of secondary appressoria (SA); PA indicates primary appressorium.

stresses. Similarly, genes involved in protein synthesis (blue) were actively expressed in response to biotic stresses, as also observed in flower and bud tissues, whose developmental phases were significantly different from those of leaf and root tissues, and were likely to be undergoing accelerated protein synthesis.

Identification of up-regulated genes in *A. thaliana* infected with *P. parasitica*

To compare the relative abundance of gene transcripts in cDNA libraries, the log-likelihood ratio *R*, developed by Stekel *et al*. (2000), was employed. *R* was derived from the partitioning of genes among cDNA libraries to measure the extent to which the differences in gene expression correspond to specific or random. Up to 72 genes ($R > 12$) were identified to be specifically expressed significantly ($P = 0.005$) (Table S1, see Supporting Information). A matrix of 72×6, by clustering of genes according to their relative abundance in various EST libraries and expression patterns across various tissues and different stresses (Fei *et al*., 2004), was generated, and genes with similar patterns were grouped (Fig. 6).

The clustering results identified few consensus genes that were abundant in the leaf development stages/treatments or

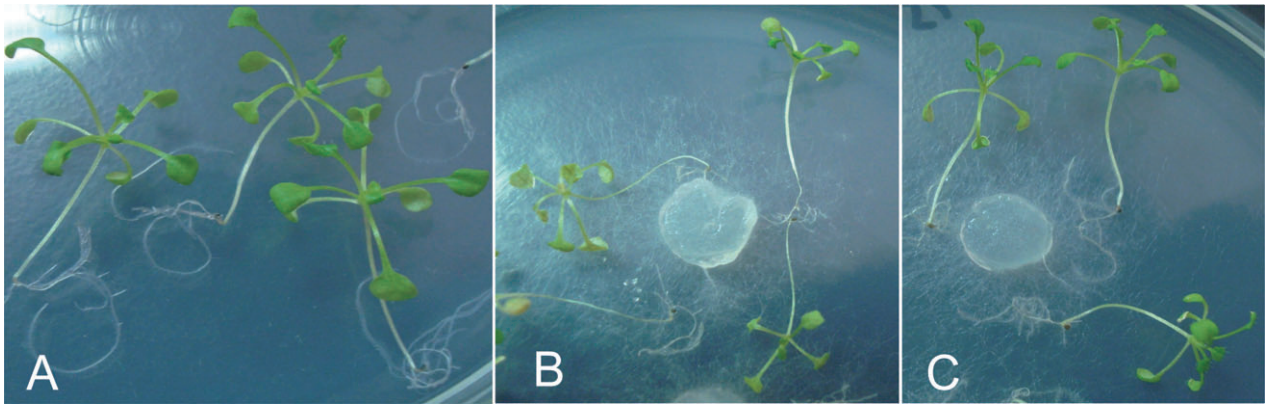

Fig. 4 Host specificity in *Arabidopsis thaliana* Sap-0 to infection by *Phytophthora parasitica*. Two-week-old sterile Sap-0 seedlings were inoculated with *P. parasitica* mycelial cultures and the results were scored 7 days post-inoculation. (A) Water agar plug control. (B) Constricted seedling hypocotyls inoculated with strain Pp016. (C) No visible symptoms appeared in hypocotyls in seedlings inoculated with Pp009.

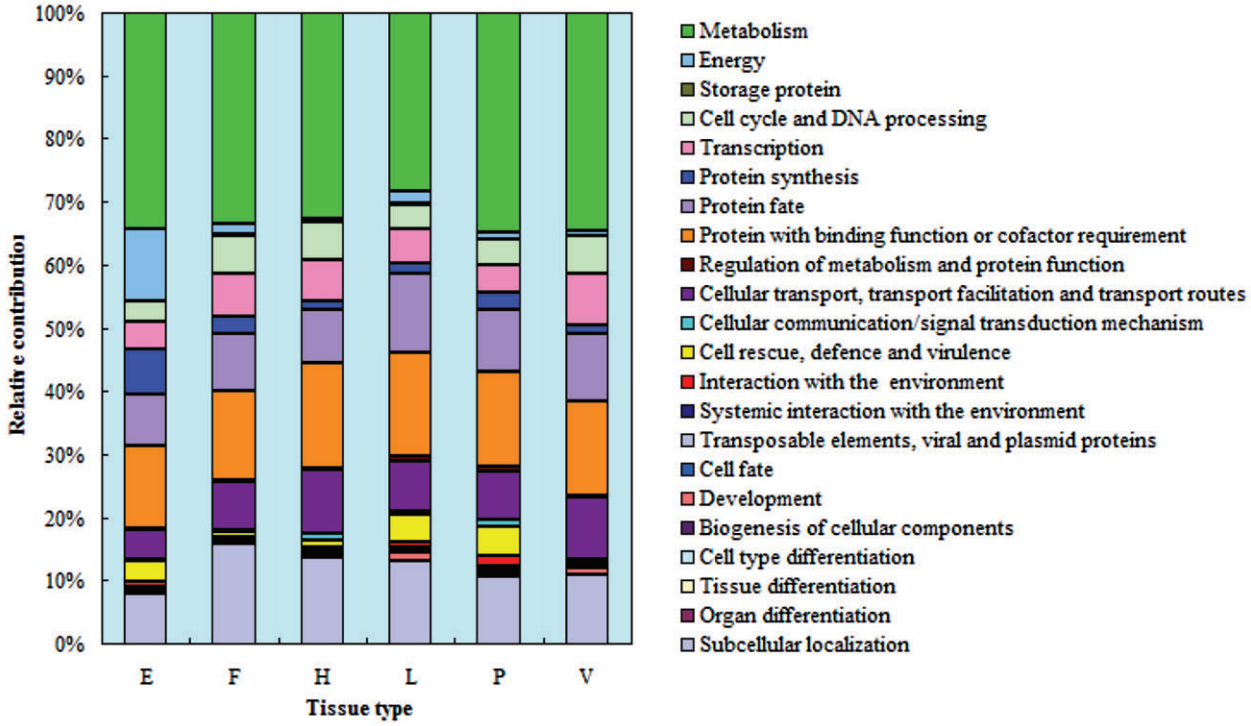

Fig. 5 A comparison of Functional Catalogue annotation of six cDNA libraries representing different developmental stages and biotic and abiotic stresses. Five non-normalized, non-subtracted *Arabidopsis thaliana* cDNA libraries were downloaded from DFCI Arabidopsis Gene Index Build 14.0 (AtGI) and compared with expressed sequence tags (ESTs) derived from *Phytophthora parasitica* Pp016-infected *A. thaliana* Col-0 leaf tissues. The libraries were derived from *A. thaliana* adult vegetative tissues of Col-0 (Castelli *et al*., 2004) (AtGI cat #F0G, V), *A. thaliana* leaf at senescence stage (Guo *et al*., 2004) (AtGI cat #DGL, L), *Erysiphe cichoracearum*-infected *A. thaliana* leaf tissues (AtGI cat #BKG, E), hormone-treated *A. thaliana* Col-0 callus (Castelli *et al*., 2004) (AtGI cat #F0H, H) and *A. thaliana* Col-0 flowers and buds (Castelli *et al*., 2004) (AtGI cat #F0J, F).

tissues (Fig. 6). The number of genes that were specifically expressed in the development stage (F, V) and during abiotic stress (H, hormone-treated *A. thaliana* callus) were quite limited, whereas genes expressed during biotic stresses (P and E) and leaf senescence (L) were more diverse. The presence of some overlapping genes between *P. parasitica*-infected *A. thaliana* leaf tissues (P) and leaves undergoing senescence (L) suggested that they might share some common mechanisms. Genes

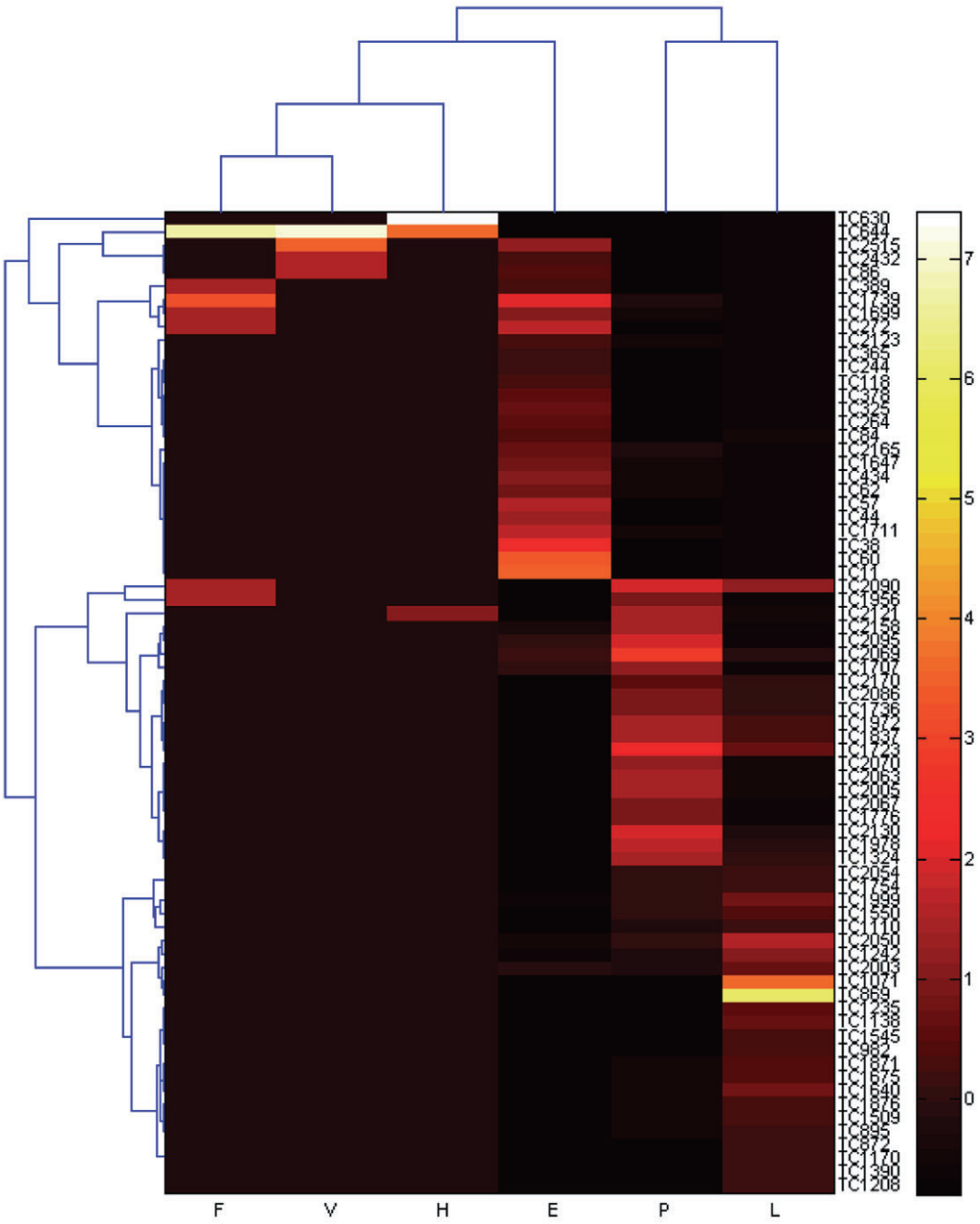

Fig. 6 Differential gene expression profiles in the six *Arabidopsis thaliana* cDNA libraries. The cDNA libraries represent different plant developmental stages and biotic and abiotic stresses. Genes with a log-likelihood ratio *R* of over 12 were included in the figure. *R*, which measures the extent to which the differences in gene expression correspond to specific or random, was derived from the entropy of partitioning of genes among cDNA libraries. Colours from dark to bright represent low to high levels of gene expression in the corresponding libraries, respectively.

expressed in both *E. cichoracearum* (E)- and *P. parasitca* (P)-infected leaf tissues and leaf tissues at the senescence stage (L) were notable. These were quite different from those expressed in hormone-treated *A. thaliana* callus (H), suggesting that plants respond differently to biotic and abiotic stresses. The gene expression profile of *P. parasitca*-infected leaf tissues (P) was significantly different from that of *E. cichoracearum*-infected leaf tissues (E), suggesting that *Phytophthora* may possess different genetic and molecular mechanisms for the infection of plants.

Table 2 *Arabidopsis thaliana* genes up-regulated in response to *Phytophthora parasitica* infection.

TC sequence	Function description
TC2069	TCTP (translationally controlled tumour protein)
TC1723	Similar to SAG21 (senescence-associated gene 21)
TC2095	1-Aminocyclopropane-1-carboxylic acid (ACC) oxidase
TC2130	Integral membrane family protein
TC1978	BCB (blue copper-binding protein)
TC1972	FAD-binding domain-containing protein
TC1837	Thioredoxin h
TC1324	Light-regulated protein, putative
TC2005	Xyloglucan endo-1,4-β-D-glucanase precursor
TC2063	Calcium ion binding
TC2158	Cysteine proteinase RD21A
TC2121	60S ribosomal protein L10
TC1707	CAT3 (catalase 3)
TC2070	Lactoylglutathione lyase family protein
TC1736	Peroxidase
TC2086	Putative trypsin inhibitor
TC1776	3-Ketoacyl-CoA thiolase
TC2067	GASA1 (gast1 protein homologue1)
TC2170	NSP5 (nitrile specifier protein 5)

TC, tentative consensus.

Of the 72 TCs, nearly one-third of plant genes expressed in response to *P. parasitica* infection (P) were up-regulated and highly specific, as their transcripts were not detected in the cDNA libraries derived from flower and bud tissues (F), adult vegetative tissues (V) and hormone-treated callus (H). In addition, their transcript abundance was much lower in *E. cichoracearum* (E)-infected tissues and leaf tissue at the senescence stage (L). In combination with the results of comparative analysis of transcript abundance ($R > 12$), a set of 19 genes was identified to be up-regulated (Table 2).

DISCUSSION

We have described in this study a compatible interaction between the model plant *A. thaliana* and the broad host range oomycete pathogen *P. parasitica*. Our pathogenicity assay and microscopic investigation of the infection process showed that *P. parasitica* was capable of infecting and colonizing both leaf and root tissues of *A. thaliana*, which is highly coincident with the description of the compatible interaction between oomycetes and plants (Hardham, 2007).

Several pieces of evidence pointed to a compatible interaction. First, the inoculated *A. thaliana* developed water-soaked lesions within 3 days post-inoculation of *P. parasitica* zoospores.

Second, the microscopic investigation of the plant infection process by *P. parasitica* revealed pathogen development and plant responses that were characteristic of a compatible interaction. Cytological characterization showed that cell wall deposition and mild cell death adjacent to the infection sites of the leaves were occasionally observed in the interaction between Pp016 and Col-0, but these did not have a significant influence on the infection process. The deposition of dense materials and the hypersensitive response (HR) are considered to be resistance responses of the plant to limit the growth of the pathogen. The penetrated pathogen grew intracellularly and intercellularly, and formed haustoria-like structures in both epidermal and mesophyll cells. Haustoria-like structures facilitate the absorption by pathogens of nutrients from the host cells, and are critical to their further expansion. The formation of haustoria in host plant cells by the pathogen is indicative of a compatible interaction. In the late stages of infection, infection hyphae grew out of the stomata or anticlinal walls of adjoining epidermal cells and produced abundant sporangia. In a compatible interaction, successful colonization of the host typically culminates in sporulation, with the development of asexual sporangia on the plant surface (Hardham, 2007).

Third, the infection and colonization of *A. thaliana* by *P. parasitica* was further confirmed by gene expression and comparative transcriptomic analysis. Sequencing of the infection cDNA library showed that over 25.3% of ESTs (765 of 3021) were of *P. parasitica* origin (data not shown), indicating that *A. thaliana* was heavily colonized, even at the early stages of infestation by the pathogen. Comparative transcriptomic analysis showed genes expressed in both *E. cichoracearum* (E)- and *P. parasitca* (P)-infected leaf tissues (Fig. 6). The gene expression profile of *P. parasitca*-infected leaf tissues (P) was significantly different from that of leaves challenged with the fungal pathogen *E. cichoracearum* (E), suggesting that *Phytophthora* and *Erysiphe* may have different genetic and molecular mechanisms for the infection of plants.

Among the 19 genes significantly up-regulated in *A. thaliana* in response to *P. parasitica* infection, signature sequences characteristic of biotic stresses were notable. For example, a homologue of TC1736, which encodes a putative peroxidase, was identified to be up-regulated in papaya challenged with *P. palmivora* (Porter *et al*., 2009). The homologues of TC2158, TC2086 and TC1736 were reported to be up-regulated by twofold or more in soybean infected with *P. sojae* (Moy *et al*., 2004).

Up-regulated genes were also identified in plants challenged with fungal pathogens. A homologue of TC1978, which is predicted to encode the blue copper-binding protein, was identified in barley infected with powdery mildew pathogen (Jansen *et al*., 2005). A catalase homologue of TC1707 appeared to be amongst the 10 most abundantly expressed genes in blast-infected rice leaves (Matsumura *et al*., 2003). Up-regulated TC2095 is predicted to encode a 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase which is responsive to both abiotic and biotic factors. A gene silencing experiment indicated that ACC oxidase is involved in the length of the biotrophic phase of *Colletotrichum orbiculare* in *Nicotiana benthamiana* (Shan and Goodwin, 2006).

The rare presence of cell wall deposition, as well as HR, the rapid expansion of the infection hyphae and the development of haustoria, the production of abundant sporangia, and the identification of signature transcripts during plant infection indicated that the broad host range oomycete pathogen *P. parasitica* is capable of infecting and interacting compatibly with the model plant *A. thaliana*.

Phytophthora parasitica showed significant variations in host specificity when six *P. parasitica* isolates were applied to 25 *Arabidopsis* ecotypes. Strains Pp014, Pp016 and Pp025, of different origins and mating types, were highly virulent to nearly all of the 25 tested *Arabidopsis* ecotypes, and produced disease symptoms on both leaf tissues and whole seedlings, except for ecotype Zu-1, which was resistant by detached leaf assay, but susceptible by whole-seedling root inoculation, to *P. parasitica* infection.

Multiple resistance phenotypes were observed to underlie the incompatible interaction. For instance, both rapid cell wall deposition and cell necrosis (HR) at the infection sites of the epidermal cells were frequently observed in *A. thaliana* Col-0 in response to infection by incompatible strain Pp009. Moreover, occasionally, HR extended well into the mesophyll, which resulted in the cessation of the further extension of invasive hyphae as well as the subsequent development of haustoria and sporangia. Similarly, no susceptible symptoms developed on *A. thaliana* tissues inoculated with strain Pp008.

The presence of specificity is a significant step in establishing *P. parasitica* and *A. thaliana* as a model pathosystem for the investigation of fundamental questions, such as the genetic basis of the compatible interactions between oomycete pathogens and host plants. *Phytophthora parasitica* can be cultured *in vitro* and is amenable to transformation (Bottin *et al*., 1999), and gene silencing protocols have been developed (Gaulin *et al*., 2002; W. X. Shan and A. R. Hardham, unpublished results). Whole-genome sequencing for *P. parasitica* is underway (B. Tyler, Virginia Bioinformatics Institute, USA, personal communication), and will allow comparative genomic analyses to decipher the common molecular mechanisms for all *Phytophthora* species, as well as the specific functions that are required for the infection of a large range of host plants by *P. parasitica*.

The established pathosystem will also facilitate an understanding of the molecular mechanisms involved in the compatibility between oomycete pathogens and host plants, which has attracted attention in recent years (O'Connell and Panstruga, 2006). The identification of the plant genes required for successful infection by bacterial (Yang *et al*., 2006), fungal (Vogel *et al*., 2002) and oomycete (van Damme *et al*., 2009) pathogens has revealed that host plants contribute significantly to the creation of an environment that favours pathogen colonization and disease development.

EXPERIMENTAL PROCEDURES

Phytophthora parasitica strains and culture conditions

A collection of six *P. parasitica* strains, originally isolated from diseased tobacco plants in Melbourne (Pp005) and Queensland (Pp008, Pp009, Pp014 and Pp016), Australia, and from *Dendrobium candidum* in Zhejiang (Pp025), China (Li *et al*., 2008), were used in this study. All strains are A2 mating type, except Pp016 which is an A1 strain.

Phytophthora parasitica was routinely maintained on 5% (v/v) cleared carrot juice agar (CA) medium supplemented with 0.002% (w/v) β-sitosterol and 0.01% (w/v) $CaCO_3$. *Phytophthora parasitica* zoospores were prepared as follows: six discs (~1 cm in diameter) from a culture on CA plates were grown on 15–20 mL of 5% (v/v) cleared carrot broth in a 90-mm Petri dish at 25 °C in the dark for 2–3 days. The carrot broth was decanted and the cultures were washed twice with sterile distilled water before being covered with 20 mL of Petri broth [$Ca(NO_3)_2$, 0.4 g; KH_2PO_4, 0.15 g; $Mg(NO_3)_2$, 0.15 g; $CaCl_2$, 0.06 g; each 1000 mL) and incubated for another 3–5 days at 25 °C. To release zoospores, cultures with numerous sporangia were chilled by washing twice with cold sterilized distilled water and placing (10 mL/plate) at 4 °C for 0.5 h. Numerous zoospores were released within 1 h after returning to 25 °C. The zoospores were filtered through one layer of Miracloth and the concentration was adjusted to 1×10^5 spores/mL.

Arabidopsis thaliana ecotypes and pathogenicity assay

A collection of 25 *A. thaliana* ecotypes (Bs-1, Bsch-0, Co-1, Col-0, Di-1, Er-0, Fe-1, Gd-1, Is-0, Ler, Lo-1, Mc-0, Ms-0, Mt-0, Mz-0, Nc-1, Nd-0, Old-1, Pla-2, Sap-0, Sf-1, Sg-1, Sorbo, Ta-0 and Zu-1) was used in this study. The maintenance of seeds and the plant culture conditions followed a standard protocol (Salinas and Sanchez-Serrano, 2006). The temperature range for growth was 20–25 °C, and the photoperiod was 12 h day/night.

Three efficient inoculation methods were developed. For detached leaf inoculation, fully expanded apical leaves from 4-week-old *Arabidopsis* plants were excised and thoroughly rinsed with sterile water. The leaves were placed on moist filter paper in a plastic tray and a *P. parasitica* zoospore suspension of the appropriate concentration was applied as droplets to the abaxial side of the leaf; sterile water was used as a control.

For whole-seedling inoculation, *A. thaliana* seeds were sterilized with 75% ethanol for 1 min, rinsed three times with sterile distilled water, and further sterilized with 1% (w/v) NaClO for

10 min. The treated seeds were then rinsed three times with sterile distilled water. The imbibing seeds were sown on 0.8% agar plates containing half-strength Murashige and Skoog (MS) nutrient solution in 150-mm Petri dishes, and subsequently vernalized in the dark at 4 °C for 3 days. The plates were kept in a growth chamber under a 12-h photoperiod condition at 22 °C for 2 weeks. The seedlings with about six expanded leaves were inoculated by dipping the seedling roots into a *P. parasitica* zoospore suspension for approximately 5 s, followed by the transfer of the seedlings onto Petri plates containing half-strength MS medium without sugar. Sterile water was used as a control. The inoculated seedlings were incubated for an initial 12 h in the dark and a further 2 days with a photoperiod of 12 h day/night at 22 °C. The susceptibility of the seedlings to pathogen infection was evaluated and scored 3 days post-inoculation.

To perform plant inoculation with pathogen mycelia, agar discs (1 cm in diameter) from the colony edges of 1-week-old *P. parasitica* cultures were transferred onto half-strength MS agar plates (without sugar). Two-week-old sterile seedlings were placed next to the pathogen culture. The Petri dishes were kept in an illumination incubator under alternate duration of light and dark at 22 °C. The inoculated seedlings were evaluated for susceptibility 7–10 days after inoculation.

Microscopic characterization of the infection process

Leaves of 4-week-old *A. thaliana* seedlings were detached, drop inoculated with *P. parasitica* zoospores and incubated at 25 °C in a humid container with a photoperiod of 12 h dark/light. The inoculated tissues were collected 0.5, 1.0, 1.5, 3.0, 6.0, 8.0, 10, 12, 24, 36, 48, 72 and 120 h post-inoculation. The entire experiment was repeated at least three times.

Samples were treated following the trypan blue staining protocol as described by the Mauch group (http://commonweb.unifr.ch/biol/pub/mauchgroup/staining.html) with the following modification. Infected tissues were transferred into test tubes with a lid and covered with diluted trypan blue solution (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 10 mL water and 10 mg of trypan blue). The tubes (lid slightly unscrewed) were treated in a heated water bath and boiled for 4 min. After cooling to room temperature, the samples were destained by replacing the staining solution with chloral hydrate solution (5 g chloral hydrate/2 mL water) for 24 h. The samples were finally mounted in distilled water and viewed under an Olympus BX51 (Shinjuku-ku, Tokyo, Japan) microscope with differential interference contrast optics.

To determine the presence of cell wall deposition, the infected leaves were treated in aniline blue solution (0.05%, w/v) prepared in 0.1 mol/L phosphate buffer (pH 9.0). The treated samples were destained overnight in 96% ethanol in a Petri dish. After discarding the destaining solution, the samples were covered with an appropriate volume of aniline blue solution and heat treated in a water bath for 1 min. The samples were immediately observed under a fluorescence microscope after discarding the aniline blue solution.

Scanning electron microscopy

To observe the early stages of plant infection, inoculated leaves were fixed in glutaraldehyde solution (4%, v/v) prepared in phosphate buffer (100 mmol/L, pH 6.8) for 14 h at 4 °C. The fixed samples were washed six times with phosphate buffer (100 mmol/L, pH 6.8) and dehydrated with a gradual series of ethanol (30%, 70%, 80%, 90% and 100%). The samples were then treated twice with isoamyl acetate, each for 0.5 h, before being dried with CO_2. The samples were mounted with conductive lithium paint and sputter coated with platinum for observation and photography on a JEOL-6360 (Akishima, Tokyo, Japan) scanning electron microscope at 15 kV.

cDNA library construction and sequencing

A *P. parasitica* infection cDNA library was constructed using mRNAs isolated from *A. thaliana* Col-0 leaf tissues infected with *P. parasitica* Pp016. The detached leaves were inoculated with zoospores and the freshly developed water-soaked tissues were collected 2 days post-inoculation. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as described by Shan *et al*. (2004). Poly(A+) RNA was purified using the Oligotex-dT30 (Super) mRNA Purification Kit (TaKaRa, Dalian, China). Five micrograms of mRNA were used to synthesize first- and second-strand cDNA, followed by adaptor ligation, using a Gateway CloneMiner Kit (Invitrogen, USA) according to the manufacturer's protocol. The cDNA was size fractionated using Sephacryl S-500 HR, and eluted fractions were pooled until 200 ng of cDNA was obtained, which was used to construct a directional plasmid cDNA library in *Escherichia coli* DH10B cells. Plasmid DNA isolated from random colonies was sequenced (Beijing Genomics Institute, China) using M13 forward primer.

Comparative gene expression analysis

To compare the ESTs with other cDNA libraries, five non-normalized, non-subtracted *Arabidopsis* cDNA libraries were downloaded from DFCI *Arabidopsis* Gene Index Build 14.0 (AtGI), including different plant developmental stages, pathogen infection phases, and biotic and abiotic stresses: *A. thaliana* adult vegetative tissues of ecotype Col-0 (Castelli *et al*., 2004) (AtGI cat #F0G, 2631 sequences) cDNA library (V), which was employed as a control or background; *A. thaliana* leaf senescence cDNA library (Guo *et al*., 2004) (AtGI cat #DGL, 7041 sequences) (L); *E. cichoracearum*-infected *A. thaliana* leaf tissues (AtGI cat #BKG,

3985 sequences) (E); hormone-treated callus of *A. thaliana* ecotype Col-0 (Castelli *et al*., 2004) (AtGI cat #F0H, 2440 sequences) (H); and the flower and bud tissues of *A. thaliana* ecotype Col-0 (Castelli *et al*., 2004) (AtGI cat #F0J, 1847 sequences) (F).

To compare the differences between the six libraries, the libraries were annotated by the FunCat scheme (Ruepp *et al*., 2004) to obtain a general view of the genes expressed, and were profiled by assembling ESTs to tentative consensus (TC) sequences to screen specific genes responsive to plant infection by the pathogen.

FunCat is a hierarchically structured, organism-independent, flexible and scalable controlled classification system enabling the functional description of proteins from any organism (Ruepp *et al*., 2004). Each *Arabidopsis* protein was assigned a catalogue number (CatNum) and, by BLAST against *Arabidopsis* proteins (e-value cut-off = 1e-5), we passed the CatNum to the query EST through hash, which is a data structure used in Perl programming. The proportion of each catalogue in the six libraries was calculated individually. All of the annotations were performed by Perl script FunCat.pl.

A statistical analysis method developed by Stekel *et al*. (2000) was used, in which a log-likelihood ratio R, derived from the entropy of partitioning of genes among cDNA libraries, was employed to compare the abundance of gene transcripts in the cDNA libraries. The libraries were assembled into TC sequences to estimate the relative expression level of each gene. TC sequences with five or more constituent ESTs were extracted (Ewing *et al*., 1999; Fei *et al*., 2004) to construct the expression profiles. Genes with $R > 12$ (Stekel *et al*., 2000) were categorized to be specifically expressed significantly ($P = 0.005$). By clustering of the genes according to their relative abundance in the EST libraries, the expression patterns of genes across various tissues were generated and genes with similar patterns were grouped (Fei *et al*., 2004). The data were centred and scaled using the Z score (Schmid *et al*., 2005) to improve clustering. Pairwise distances of both gene expression profiles and tissue similarities were calculated by correlation coefficient.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 The nucleotide sequences of 72 TCs (Tentative Consensus sequences) differently expressed significantly in the six libraries of *Arabidopsis thaliana*.

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