Turk J Agric For
26 (2002) 125-132

Microscopic Evaluation of Interactions between Varieties of *Arabidopsis thaliana* Challenged by *Peronospora parasitica*

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Received: 17.09.2001

Abstract: *Peronospora parasitica* (Pers ex Fr.) Pers. is an obligate biotrophic pathogen that causes downy mildew in *Arabidopsis thaliana* (L.) Heynh. In this study, cotyledons of four *A. thaliana* varieties were inoculated with the Cala2 isolate of *P. parasitica* and the degree of susceptibility was observed under the microscope 1, 2, 3 and 7 days after inoculation (DAI). Microscopic examination of infected tissues revealed that early restriction of the pathogen was accompanied by a hypersensitive response (HR) characterised by autofluorescence under UV or blue light excitation. All haustoria, if visible at all, formed in the cells that underwent the HR in both Ws-3 and Oy-0 at all time points tested. Susceptibility was characterised by extensive pathogen growth in intercellular spaces with the formation of haustoria in both epidermal and mesophyll cells without any host response. In the Nd-1/Cala2 interaction, 100% of the haustoria formed in unresponsive cells 1 DAI; 5% of the haustoria was, however, ensheathed with callose deposits by 3 DAI in the same host, presumably because of haustorial ageing. Sexual and asexual sporulations of *P. parasitica* begun approximately 3 DAI. In the intermediate interaction, the deposition of callose around the haustorial body and delayed cell death were identified as the main mechanisms of resistance in the Col-0 variety.

Key Words: *Peronospora parasitica, Arabidopsis thaliana*, disease resistance, compatibility, hypersensitive response

Peronospora parasitica ile İnokule Edilen *Arabidopsis thaliana* Varyeteleri Arasındaki İlişkinin Mikroskop Altında İncelenmesi

Özet: *Peronospora parasitica* (Pers ex Fr.) Pers. bir obligat biyotrof Oomycetes üyesi olup *Arabidopsis thaliana* (L.) Heynh.'da mildiyö hastalığına sebep olmaktadır. Bu çalışmada, dört *A. thaliana* varyetesinin kotiledonları, *P. parasitica*'nın Cala2 izolatı ile inokule edilmiş ve bitkilerin duyarlılık derecesi inokulasyondan 1, 2, 3 ve 7 gün sonra mikroskop altında incelenmiştir. İnfekte edilmiş bitki dokularının mikroskop altında incelenmesi sonucu, patojenin doku içinde gelişmesinin hipersensitif reaksiyon (HR) ile sınırlandığı, bu hücrelerin UV veya mavi ışık altında incelenmesiyle ortaya çıkarılmıştır. Ws-3 ve Oy-0 varyetelerinde, patojenin hücrelerde oluşturduğu ve görülebilen bütün haustoryumlar sonuçta o hücrenin hipersensitif reaksiyona uğramasına sebep olmuştur. Duyarlı bitkilerde patojenin hiç bir konukçu savunmasına karşılaşmadan intersellüler boşluklarda hifsel olarak geliştiği ve haustorium oluşturduğu gözlenmiştir. Nd-1/Cala2 ilişkisinde, inokulasyondan 1 gün sonra, haustoryumların oluştuğu hiçbir hücre tepki göstermezken, muhtemelen hastoryumun yaşlanmasına bağlı olarak, yaklaşık haustoryumların %5'i kallus maddesi ile kaplanmıştır. Seksüel ve aseksüel üreme inokulasyondan yaklaşık 3 gün sonra başlamıştır. Kısmi dayanıklı olan Col-0'da, haustorium çevresinde kalloz oluşumu ve geç kalmış hücre ölümü temel dayanıklılık mekanizması olarak gözlenmiştir.

Anahtar Sözcükler: *Peronospora parasitica*, *Arabidopsis thaliana*, hastalıklara dayanıklılık, uyumluluk, hipersensitif reaksiyon

Introduction

Microbial plant parasites obtain their nutrients either biotrophically from living cells or necrotrophically from cells that they have killed (Lewis, 1973). Many destructive plant pathogenic fungi (for example, *Botrytis* spp. and *Pythium* spp.) and bacteria (for example, *Xanthomonas* spp. and Erwinia spp.) have a necrotrophic mode of nutrition. Some necrotrophic parasites can also live as saprophytes on plant debris or healthy plant surfaces, whereas others are facultative parasites. Although necrotrophic pathogens clearly employ toxins and cell-wall-degrading enzymes to kill plant cells, the molecular mechanisms that distinguish biotrophic and necrotrophic lifestyle remain elusive (Crute et al., 1994). Organisms that are traditionally considered biotrophic fungal parasites include those causing plant diseases of leaf curl, rust, smut and powdery mildew, in addition to Oomycetes, which cause downy mildew and white rust disease. However, Oomycetes are no longer considered to be closely related to the true fungi (Health and Skalamera, 1997). *Peronospora parasitica* is a species in the family Peronosporales that causes downy mildew in some species of Cruciferae (Lucas et al., 1995).

Arabidopsis thaliana (mouse-ear grass) has become an important model for the investigation of many aspects of higher plant biology (Holub and Beynon, 1997). An obvious interest is in understanding the genetic control of plant recognition and response to pathogens in this model system, and potentially some of the genes isolated from *A. thaliana*, to eventually benefit crop plants (Dangl et al., 1992). Genetic variation among *A. thaliana* varieties has been demonstrated for resistance to bacterial (*Pseudomonas syringae* and *Xanthomonas campestris*), fungal (*Erysiphe cruciferarum*) and oomycete (*P. parasitica*) pathogens (Baker et al., 1997). One advantage of *A. thaliana*/ *P. parasitica* interactions has been that they enable one to discern quite subtle differences among different interaction phenotypes.

Microscopic analysis of pathogen growth in resistant and susceptible plants could help to define the timing of resistance and the relative degree of penetration by the parasite in four wild-type varieties of *A. thaliana* following inoculation with *P. parasitica*. The aim of this investigation was to evaluate the response of various varieties of *A. thaliana* when challenged by the Cala2 isolate of *P. parasitica* at the microscopic level.

Materials and Methods

Inoculum and inoculation method. Cala2 isolate of *P. parasitica* was used in this investigation. Because *P. parasitica* is an obligate biotrophic pathogen, it is maintained in susceptible live plants. The conidium suspension was adjusted to 4-5X104 conidia/ml. One-week-old seedlings were drop-inoculated on the upper surface of the cotyledon and incubated in a growth room at 18-20ºC with a 10-hour photoperiod at a photon density of 150-350 µE m-2 s-1. Samples were taken daily until 3 DAI and then 7 DAI.

Plant materials. Four different varieties of *A. thaliana*, Col-0, Nd-1, Oy-0 and Ws-3, were assessed microscopically. The seeds were sown in pots containing a compost mixture of commercial peat-based seedling compost, vermiculite and fine sand. The pots were put in a tray, covered with a transparent lid and sealed to prevent cross-contamination with other microorganisms.

Phenotypes of the interactions. A compatible interaction (susceptible) was characterised by heavy sporulation of the pathogen. The conidiophores were visible on both sides of the cotyledons beginning 3 DAI and abbreviated as EH (early heavy sporulation). In incompatible interactions (resistant), host responses and the sporulation level of the pathogen were assessed. The degree of macroscopically observed cell death associated with the hypersensitive response was recorded as minute flecks (FN) or more expanding cavities (CN) with no sporulation of the pathogen. FR referred to flecking chlorosis on the host and rare sporulation of the pathogen (less than 1 conidiophore per cotyledon). The interaction phenotypes of the varieties of *A. thaliana* and the Cala2 isolate of *P. parasitica* are given in the Table.

Light and fluorescence microscopy. Microscopy was carried out using a Nikon Optiphot Microscope equipped with Differential Interface Contrast (DIC) and epifluorescence optics at the Imperial College at Wye, University of London, UK. Auto-fluorescence of phenolic compounds induced following infection was determined in cleared, unstained tissue with UV or blue light excitations.

Tissue clearing for microscopy. The samples were put in absolute methanol and incubated over night at room temperature. The samples were then transferred into chloral hydrate solution (2.5g/ml) for at least 8 h to soften and clear the tissue. The seedlings were placed on glass microscope slides and about 5 µl of 50% glycerol was dropped onto the seedlings. A cover-slip was placed over the drops to produce semi-permanent preparations.

Callose staining. Aniline blue stain was used to detect callose (β, 1-3 glucan) in infected tissues. Cleared cotyledons were incubated in 0.05% (w/v) aniline blue made up in 0.1 M phosphate buffer at pH 8.0, overnight at room temperature. The cotyledons were remounted in 50% glycerol and viewed by epifluorescence microscopy under UV or blue light irradiation (O'Brien and McCully, 1981).

Results

Microscopic examination of the interaction between an isolate of *P. parasitica* and four variesties of *A. thaliana*. The percentage of infection sites where the parasite penetrated was influenced by host genotype, isolate of the parasite and their interaction. Following inoculation, the isolate penetrated the tissues of Nd-1 variety directly where it broke a hole between the junctions of two adjacent cells (Figure 1.a). After entering the host, the hyphae expanded and grew initially into

Table. Development of *Peronospora parasitica* isolate Cala2 in infected tissues of the *Arabidopsis thaliana* varieties (Col-0, Ws-3, Oy-0 and Nd-1).

a Interaction phenotypes (IPs): The phenotypes were recorded macroscopically 7 dai. N: No sporulation of the parasite; R: Rare sporulation (<1 conidiophore per cotyledon); H: Heavy sporulation (>20 conidiophores per cotyledon). Flecking chlorosis (F) and more dominant cavities (C) were seen 7 DAI as macroscopic response of the host to the pathogen. The macroscopic examinations were performed 7 dai.

b Days after inoculation. More than 30 infection sites selected randomly from 5 cotyledons of *A. thaliana* per host-isolate combination were examined 1, 2, 3 and 7 dai.

c Not all infection sites had haustoria. The numbers state if any haustoria were observed, all were in cells that underwent the HR.

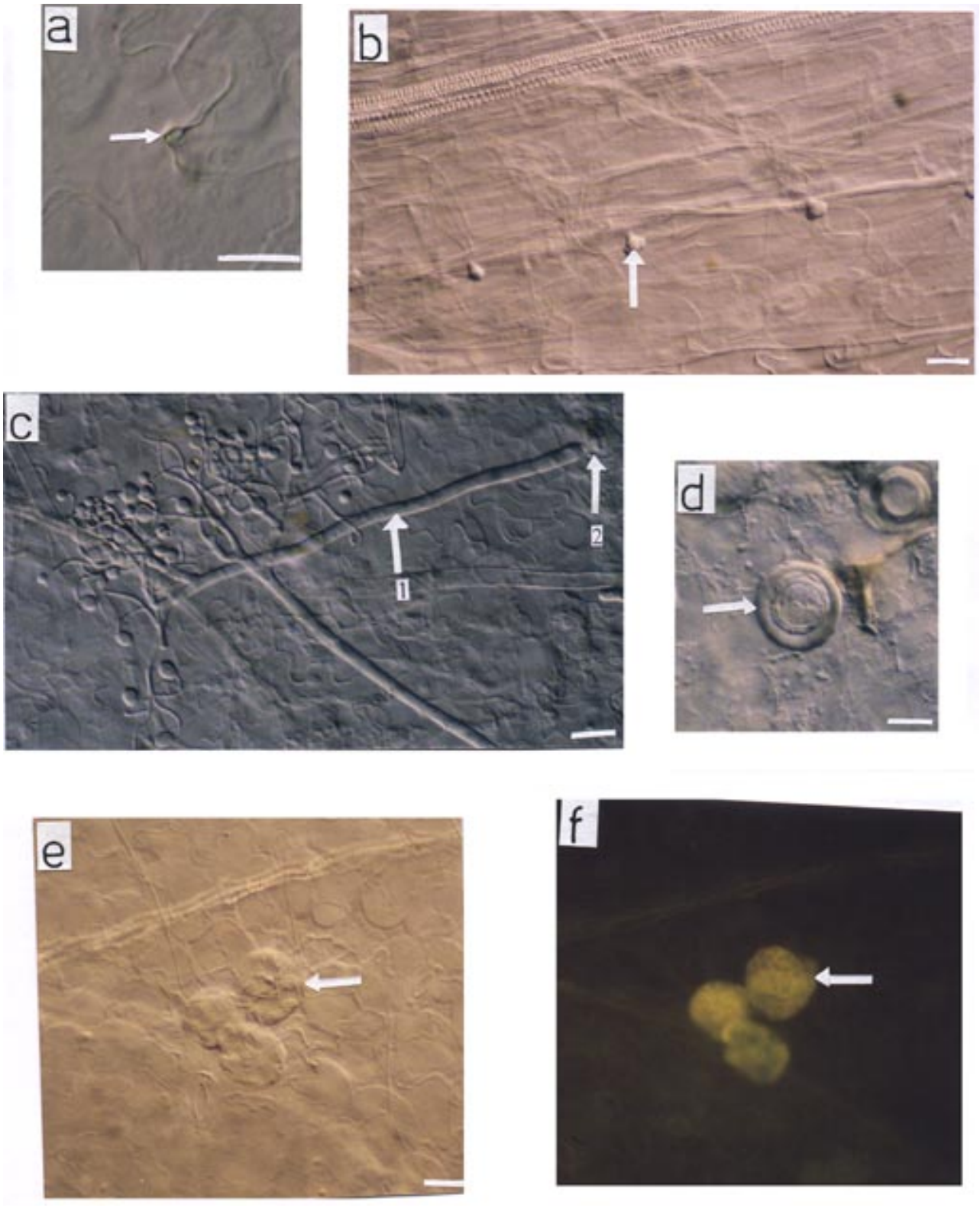
Varieties of Arabidopsis thaliana	IPs 7 DAI[a]	% of infection sites with visible haustoria			% of infection site with cells undergoing the HR			% of haustoria											
								No cellular reaction			in HR cells			with callose			callose in dead cells		
		1[b]	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Nd-1	EH	83.3	86.7	100	0	0	0	100	98	95	0	0	0	0	2	5	0	0	0
Ws-3	CN	6	2	1	82	88	94	0	0	0	100[c]	100	100	0	0	0	0	0	0
Oy-0	FN	5	0	0	85	91	97	0	0	0	100	100	100	0	0	0	0	0	0
Col-0	FR	60	73.3	70	13.3	13.3	20	76	35	15.8	16	26.5	30.4	8	32	32.3	0	6.5	6

epidermal cells. Haustoria formed as branche from the intercellular hyphae within host cells. Single, sometimes twin or triplet haustoria had formed at infection sites by 24 hours after inoculation (HAI). By 48 and 72 HAI, the number of infection sites with visible haustoria had increased dramatically; for example, the parasite had formed haustoria in up to 100% of the infection sites in Nd-1 by 3 DAI (Table). Extensive hyphal growth allowed more haustoria to form (Figure 1.b). No cell death was observed at any time point tested in Nd-1. Hyphal length was occasionally over one mesophyll cell length 1 DAI, and had increased to over 6 mesophyll cell length by 2 DAI and over 9 mesophyll cell lengths by 3 DAI.

Callose ensheathment of haustoria was observed, beginning 2-3 DAI. However, the number of ensheathed haustoria was very small; approximately 5% of the haustoria were ensheathed 3 DAI. The occurrence of such deposits during compatible interactions is presumably the result of haustorial ageing.

Extensive growth of fungal hyphae was followed by the development of conidiophores apparently differentiated from the intercellular hyphae (Figure 1.c). The intercellular hyphae appeared to grow profusely beneath the epidermis cells and grew predominantly through the stomata to the outside of the host tissue, with formation of conidiospores beginning 3 DAI. Young oospores were evident by 3 DAI and fully grown, mature oospores were dominant in the Nd-1 variety by 7 DAI (Figure 1.d). The parasite growth in intercellular tissues resulted in massive oospore formations.

The response of both Oy-0 and Ws-3 varieties to the Cala2 isolate was different from that observed in Nd-1. Holub et al. (1994) reported that the Cala2 isolate of *P. parasitica* is recognised by the *RPP1* resistance gene in Ws-3 and by *RPP2* in Oy-0 varieties characterised by cavities (CN) and flecking chlorosis (FN) in the host, respectively, with no sporulation. Penetration of both varieties was followed by a progressive increase in the intensity of fluorescence and the appearance of amorphous aggregates of fluorescing material within the penetration cells 1 DAI characterised by hypersensitive response (HR). The cells where the penetration occurred underwent the HR in Oy-0 (Figures e and f), but more spreading responsive cells were characteristic in Ws-3. The number of fluoresced cells was restricted to 1-2 cells in Oy-0 1 DAI, while 4-5 host cells underwent the HR in Ws-3 during the same time interval (Figures g and h). The number of fluoresced cells increased over time in both varieties, reaching 4 and 10 cells by 3 DAI in Oy-0 and Ws-3, respectively. The infection sites with visible haustoria decreased dramatically over time, and it was not visible at all by 3 DAI (Table). Hyphal growth without host response was not observed at any stage of the microscopic examination in either Nd-1 or Ws-3. No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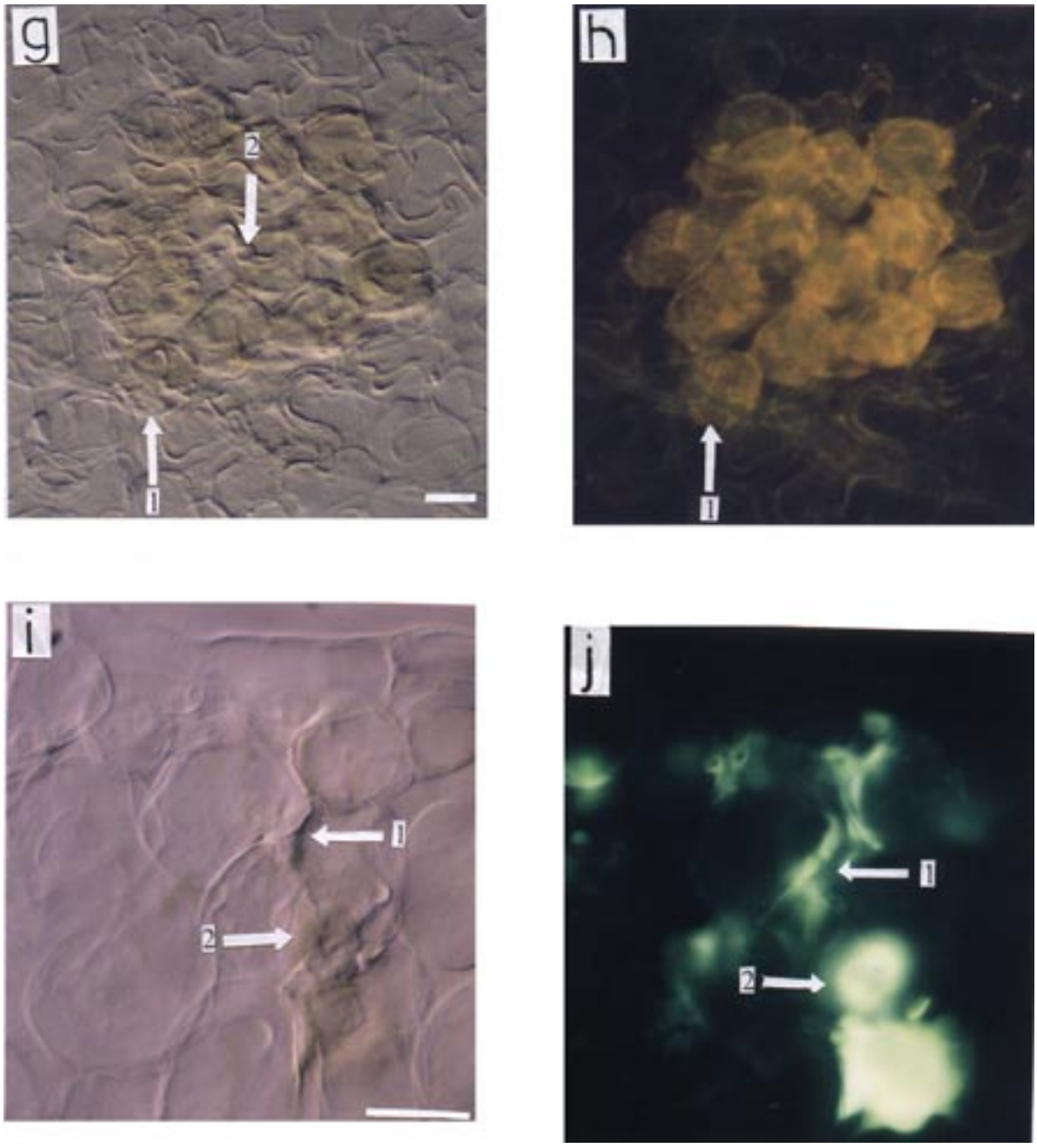

Figure 1. Microscopic development of *Peronospora parasitica* isolate Cala2 in four varieties of *Arabidopsis thaliana*. (a) penetration hole (arrowed) between two epidermal cells; (b) haustorium in stem tissues of susceptible line, Nd-1; (c) conidiophores (arrow no. 1) commonly emerged from stomata (arrow no. 2) starting 3 DAI in Nd-1; (d) oospores were mature by 7 DAI in Nd-1 (arrowed) and occasionally in Col-0. (e) and (f) show infection in resistant line Oy-0 in light and blue light microscopy, respectively. Note only a few cells underwent the HR (arrowed); (g) and (h) infection in resistant variety Ws-3 under light and blue light microscopy. Note that radial expansion of fluoresced cells (arrow no. 1). No. 2 arrow shows the infection hole where the pathogen penetrated the host. (i) callose deposition around the host cell (arrow no. 1) and haustoria (arrow no. 2) wall was proved by aniline blue staining; (j) is the same picture of (i) but under UV light. Scale bar: 50 µm.

callose deposition was detected around the haustoria. Sexual or asexual reproduction of the parasite was not seen in this interaction.

RPP2-mediated downy mildew resistance in Col-0 to the isolate Cala2 was characterised macroscopically by the delayed (approximately 7 DAI) and rare level of asexual sporulation of the pathogen (Holub et al., 1994). An intermediate interaction was also observed between Cala2 and Col-0 in the microscopic examination. The isolate penetrated into the host by the same way as observed in the compatible interaction; however, differences in parasite growth were observed, beginning 1 DAI, but common 2 DAI. The number of penetration sites with visible haustoria increased over time, but the growth of the parasite seemed to be slower than those observed in the compatible interaction between Nd-1 and Cala2. The hyphal growth of the parasite on Col-0 was approximately 2.1 mesophyll cell length by 2 DAI, and 3.6 by 3 DAI. The hyphal growth was restricted because of host responses. Callose ensheathment of the haustoria and delayed cell death were common responses to this isolate (Table). The ensheathing materials were found to include callose as indicated by fluorescence under UV or blue light after aniline blue staining (Figures i and j). However, despite the ensheathment of the haustoria, the parasite continued to spread into neighbouring cells, forming more haustoria. The callose deposition around the haustorial body and host cell walls began 1 DAI, and frequency increased to 32.3% of the total number of haustoria produced in the cotyledons of Col-0. Occasionally the cells with ensheathed haustoria appeared to undergo cell death 2 or 3 DAI in some infection sites. The occurrence of a delayed cell death in infected cells was the other characteristic observed in these interactions. More than 13.3% of the infection sites in Col-0 fluoresced under UV or blue light 2 DAI and approximately 20% of them by 3 DAI following inoculation with Cala2 suggesting infected cells had undergone cell death. This type of cell death seems to be distinct from the HR as the speed of cell death was not as rapid as the HR.

No conidiophore formation was observed 3 DAI on Col-0, but some conidiophore and oospore formations by 7 DAI were visible, suggesting that the pathogen could still complete its life-cycle in the intermediate interaction.

Discussion

Microscopic analysis of *A. thaliana* has shown that resistance genes are associated with phenotypically and microscopically distinct isolate-specific recognition of *P. parasitica*. Resistance or susceptibility was correlated with the development of the pathogen in the infected tissues. Three types of interactions were observed between *A. thaliana*/*P. parasitica*: compatible, incompatible and intermediate interactions. Compatible interactions were typically characterised by the absence of host cell necrosis, the rapid spread of the intercellular hyphae, a high frequency of haustorium formation and production of conidia and oospores. Similarly, rapid biothrophic growth has been reported for other fungal pathogens of *A. thaliana*, e.g., powdery mildew (Adam and Somerville, 1996). Formation of haustoria by *P. parasitica* appeared to be an essential step in the establishment of large intercellular hyphae or vice versa. It is possibile that the establishment of haustoria provides an additional or different source of nutrients not available to the parasite growth in the intercellular spaces.

No differences were observed between susceptible and resistant plants in rates of spore germination and penetration. Rapid cell death (HR) was, however, indicated by the autofluorescence of responding cells. Host responses to infection were clearly visible 1 DAI. Fluorescence was more obvious in mesophyll cells than in epidermal cells, especially 2 and 3 DAI. Two types of resistance response were observed in the wild-type varieties of *A. thaliana* inoculated with *P. parasitica*. (1) Extensive cell death radiated from the site of penetration to neighbouring cells; this was associated with the macroscopic phenotype, CN, e.g., Ws-3 inoculated with Cala2. (2) Only penetrated cells underwent the HR and this was associated with the FN phenotype which was characteristic in Oy-0 following inoculation with Cala2.

The role of the HR in resistance to fungal pathogens has been a major source of controversy among plant pathologists. Some researchers (Kiraly et al., 1997; Mayama et al., 1975; Prusky et al., 1980) have suggested that cell death may occur as a consequence, not a cause, of resistance, as a result of the release of fungal substances and/or death of the haustorium. However, there are a number of interactions in which plant cell death clearly precedes fungal death (Miller and Maxwell, 1984; Bennett et al., 1996). Mansfield (1990) pointed out that death of the penetrated cell should itself prevent

haustorium-forming biotrophic pathogens, such as rust and mildews, which are strictly dependent on living cells, from which they absorb sufficient nutrients to allow colonisation.

Pathogen development in the host tissues was slightly different in intermediate interactions (Col-0/Cala2). The pathogen penetrated into the epidermal cells; first and occasionally second haustoria were often observed at the epidermal cells. However, host cell death and/or more dominantly callose deposition were visible beginning, 1 DAI, and often 2 and 3 DAI. It seems that the occurrence of these responses did not stop the growth of the pathogen by the time of their expression, because the pathogen had already infected neighbouring cells. The number of haustoria in this interaction was smaller than that observed in compatible interactions. This may suggest that although haustoria seem to be an important organelle for nutrient uptake, they are not essential, as intercellular hyphae seem to compensate for this need. Forming fewer haustoria may be another way by which the parasite overcomes the plant's defence, enabling it to complete its life cycle.

Callose deposition around the haustorial body and cell wall has been implicated in plant defence in many studies (Allen and Friend; 1983; Skalamera et al., 1997). Deposition of callose was often reported around the haustoria of rust fungi, downy mildew and powdery mildew (Heath and Heath, 1971; Heath, 1982; Parker et al., 1993; Adam and Somerville, 1996; Bennett et al., 1996). It is thought that callose functions as a mechanical barrier to limit pathogen growth by conferring greater strength to the host cell wall (Heath, 1981) and it may block nutrient uptake in encased haustoria (Heath, 1982). Callose deposition is particularly important in *mlo*-determined race non-specific resistance in barley, which confers resistance to all known isolates of *Erisiphe graminis* f. sp. *hordei* (Görg et al., 1993). The role of callose deposition in the *A. thaliana*/*P. parasitica* interaction, however, appeared to be different in studies in which the early appearance of callose was coincident with a failure of fungal penetration (Bayles et al., 1990). Apparently, accumulation of callose during intermediate interactions does not act as an effective resistance mechanism since such deposits did not completely restrict parasite growth and haustorium formation as proposed for rust infections (Heath, 1982). However, pathogen growth was much slower than that observed in compatible interactions. Hence, this observation may suggest that callose plays an important role in the limitation of the pathogen.

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