Penetration resistance to *Erysiphe pisi* in pea mediated by *er1* gene is associated with protein cross–linking but not with callose apposition or hypersensitive response.

R Iglesias–García[1], D Rubiales[1], S Fondevilla[2]

[1] *Institute for Sustainable Agriculture, CSIC, Apdo.4084, 14080 Córdoba, Spain.*

[2] *University of Frankfurt, Institute for Molecular Bioscience Max–von Laue Str. 9, D–60438 Frankfurt am Main, Germany.*

*For correspondence E-mail: rbkig@ias.csic.es

Phone: +44 (0) 7597811178

Fax: +44 (0) 1206872592

ABSTRACT

Powdery mildew caused by *Erysiphe pisi* DC is an important pea disease which causes high yield and quality losses. The most efficient way to control this disease is the use of resistant cultivars. Three genes of resistance are known so far in pea, namely *er1*, *er2* and *Er3*. The most widely deployed gene in pea cultivars worldwide is *er1*. Resistance conferred by *er1* is complete and considered to be durable, being recently demonstrated that *er1* is in fact a member of the *mlo* gene family. Resistance is caused by colony abortion albeit the responsible cellular mechanisms causing this colony abortion are not known. In this study, the presence of different mechanisms related with colony abortion, such as callose apposition, protein cross–linking and hypersensitive response was quantified in a resistant pea *er1* line compared to a susceptible check. Our histological studies showed that protein cross–linking, plays a determinant role in the penetration resistance conferred by *er1*, whereas callose apposition or hypersensitive response played little role.

Keywords: *MLO* gene family, Powdery mildew, Protein cross–linking, Resistance

INTRODUCTION

Erysiphe pisi DC, causing powdery mildew in pea, is a widespread pathogen of *Pisum sativum* L. all over the world. This disease causes up to 50% yield losses and reduces total biomass, plant height, node number, pod number per plant, seeds per pod, pod quality and seeds weight (Fondevilla and Rubiales, 2012).

The infection process begins when asexual spores of *E. pisi* dispersed by wind land and germinate on a suitable host, forming a single germ tube that elongates before differentiating into an appressorium (Smith *et al*., 1996). An infection peg then attempts to breach the plant epidermal host cell through the cuticle and cell wall. If successful, a biotrophic haustorium is invaginated within the epidermal cell that extracts nutrients to support growth of ectophytic secondary hyphae (Singh and Singh, 1983; Smith *et al*., 1996). Secondary appressoria are then formed in secondary hyphae from which secondary haustoria will be produced, allowing successful establishment of colonies that sporulate to complete the infection cycle.

The most efficient strategy to control this biotrophic fungus is the use of resistant cultivars. Disease control through inherent plant resistance eliminates dependence on costly fungicides, with their potential and perceived threats to the environment and consumers. Therefore, host resistance to powdery mildew is a vital component in sustainable pea crop production.

Only three genes conferring resistance to *E. pisi* in pea, named *er1*, *er2* and *Er3* have been described so far (Fondevilla et al. 2011). Unlike the other two genes, gene *er1* is widely used in pea breeding programmes. Resistance conferred by this gene has been proven to be stable and is caused by a low success in colony establishment. In *er1* lines the vast majority of *E. pisi* conidia germinate and form appressoria. However, the pathogen is stopped soon after, and no secondary hyphae are formed. In contrast, resistance governed by *er2* and *Er3* is mainly expressed as a post–penetration Hypersensitive Response (HR) that stops the colony growth (Fondevilla et al., 2006a, 2006b, 2007, 2008, 2011).

The *er1* gene has recently been proven to be a member of the *MLO* (*Mildew Resistance Locus* O) gene family and called *PsMLO1* (Humphry et al., 2011). Loss–off function mutations of *MLO* genes confer highly effective broad–spectrum powdery mildew resistance through penetration resistance (Stolzenburg *et al*., 1984; Gold *et al*., 1986; Russo and Bushnell, 1989; Bayles *et al*., 1990; Wolter *et al*., 1993; Lyngkjær *et al*., 1998; Consonni *et al*., 2006; Bai *et al*., 2008). *Mlo* resistance was originally discovered in barley (*Hordeum vulgare* L.) (Freisleben et al., 1942) and has been successfully employed

in agriculture for more than 40 years (Lyngkjaer et al., 2000). Later on, *mlo* resistance to powdery mildew has also been reported in the dicotylenous plants *Arabidopsis thaliana* L. and tomato (*Solanum lycopersicum* L.), indicating that *mlo*–based powdery mildew resistance was not a barley–specific phenomenon and was probably existing in many higher plant species (Consonni *et al*., 2006). Nowadays, *MLO* genes have been identified in other plant species such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), grapevine (*Vitis vinifera* L.), wheat (*Triticum aestivum* L.) or soybean (*Glycine max* L. Merr.) (Büschges *et al*., 1997; Consonni *et al*., 2006; Bai *et al*., 2008; Liu *et al*., 2008; Konishi *et al*., 2010; Shen *et al*., 2012). In barley, *Arabidopsis* and tomato, penetration resistance conferred by *MLO* mutations is associated with the formation of papillae in the penetration sites. Papillae are formed by the deposition of a callose matrix containing various inorganic and organic constituents and constitute a physical and chemical barrier to pathogen penetration (Hückelhoven, 2007).

In addition to callose apposition, another mechanism that can delay or stop colony growth is the formation of protein cross–linking in the host cell walls that acts as a physical barrier. This mechanism has proved to be an effective and fast defensive response against pathogens, through the insolubilization of proteins (extensins and other glycoproteins rich in hydroxyproline or HRGPs), which would take place before callose apposition and increase cell wall resistance only a few minutes after pathogen attack (Bradley et al., 1992; Showalter, 1993; Brisson et al., 1994; Hammond–Kosack and Jones, 1996; Brown et al., 2002).

HR, based on death of the invaded epidermal cell after penetration of the host, is a common mechanism of resistance against biotrophic pathogens. This defensive reaction is characterized by the accumulation of reactive oxygen species (ROS), antimicrobial proteins and phytoalexins, culminating in a local cellular suicide (Huckelhoven, 2007). HR typically occurs after the pathogen has penetrated the plant cell wall and has started to produce a haustorium (Aist and Bushnell, 1991). Cell death can occur very fast so no haustorium can be observed, or rather slow, allowing formation of some haustoria and secondary hyphae (Prats et al., 2007). HR is frequently described in resistance to powdery mildews (Boyd *et al*., 1995; Niks and Rubiales, 2002) and is the main mechanism of resistance in *er2* and *Er3* lines of pea (Fondevilla *et al*., 2006b).

A low success in colony establishment has been reported in *er1* pea lines (Fondevilla et al. 2006a), but the cellular mechanism responsible for this colony abortion is not known. The objective of this work was to identify the mechanism that avoids colony establishment in *er1* lines. With this aim, the

presence of different mechanisms that could cause colony abolishment such as formation of callose apposition, HR and protein cross–linking was studied in a *er1* line compared to a susceptible check.

MATERIAL AND METHODS

Plant and fungal material

The resistant pea accession JI2302 (*er1er1*) (Heringa et al., 1969), kindly provided by Jonh Innes Centre (Norwich, UK), and the susceptible pea cv. Messire (*Er1Er1*) were used in this study.

The *E. pisi* isolate CO–01 was used in the inoculations. This isolate, avirulent in absence of a functional *Er1* gene, was obtained from a population collected from naturally infected pea plants in a field at Córdoba (Spain) in 2001 and maintained on the susceptible pea cv. Messire (Fondevilla et al., 2006b).

Inoculation and incubation

A total amount of 16 plants per line was grown in pots, one plant per pot, containing 250 cm3 of 1:1 sand-peat mixture in a growth chamber at 20 ± 0.5°C with a threshold photons flux density at plant level around 250 µmol m^{-2} s^{-1}. The fourth leaf (two leaflets) of four plants per line at the fifth leaf stage was excised and carefully laid in Petri dishes containing a medium consisting of technical agar (4 g/l) and benzimidazole (62.5 mg/l) (Rubiales et al., 1993). Leaves were inoculated using a settling tower to obtain an inoculum density of about 5 conidia mm^{-2}. Two independent inoculations, each containing 8 leaves per line were performed. After inoculation, lids were fitted to the Petri dishes, which were placed back in the same growth chamber and conditions. Incubation lasted 48 hours and was initiated with a 6 h light period, followed by cycles of 12 h darkness/12 h light.

Histological studies

Leaflets were sampled at 48 hours after inoculation and subjected to different stains. Two leaflets from each of the two independent inoculations (considered replicates) were examined per line to measure the incidence of each of the resistance mechanisms studied.

To identify the presence of protein cross–linking in cell walls underlying *E. pisi* appressoria, we used the method described by Mellersh et al. (2002) with some modifications. Thus, fresh leaflets from each replicate were submerged in 1% sodium dodecyl sulphate (SDS) for 20 hours at 75 ºC to remove soluble proteins. After that, they were left for 3–5 min in 0.1% Coomassie blue in 4:1 (v/v) ethanol: acetic acid to stain proteins covalently linked, rinsed in a solution of 4: 1 ethanol: acetic acid, and kept in

distilled water till they were finally mounted in lactoglycerol. Using this method, the presence of protein cross–linking is marked with a deep blue colour. One hundred epidermal cells underlying *E. pisi* appressoria per leaflet and genotype were examined for the presence of protein cross–linking. In addition, it was also scored whether a colony had been established (spores showing secondary hyphae) or not (absence of secondary hyphae). In order to stain fungal structures a drop of Uvitex staining (Ciba) was placed over the leaflets.

To assess host cell death, indicating a HR as a result of pathogen attack, leaflets were laid, adaxial surface up, on filter paper moistened with a 1:3 (v/v) mixture of glacial acetic acid:absolute ethanol for fixation. When bleached, leaflets were transferred to filter paper moistened with distillate water, left for 1 h to soften the tissues and then transferred to filter paper moistened with lactoglycerol (1:1:1, lactic acid:glycerol:water, v/v) until cleared (2 h) and for storage (Rubiales and Carver, 2000). The epidermal cell underlying 100 appressoria per leaflet and line were examined. Leaflets were observed using bright field and differential interference contrast (DIC) microscopy. The walls and contents of dead cells were discoloured yellow or brown by bright field microscopy, and by DIC the cell contents appeared granular and disorganised. To stain fungal structures and score whether a colony was established or not as described above, a drop of aniline blue in lactoglycerol (0.1%) was deposited on the leaves.

To detect the presence of callose, leaflets were stained with aniline blue and with the aniline blue fluorocrom pure (Underwood and Somerville, 2008). Aniline blue in lactoglycerol (0.1%) was applied by spraying the stain over the samples, which were observed under visible wavelengths. In the case of the aniline blue fluorocrom pure the samples were stained by depositing a drop of a solution of 0.1% aniline blue fluorochrom in water and waiting for 15–30 min before observations (Bordallo et al., 2002). After staining samples were observed under UV fluorescence (340–380 nm) to avoid blue staining and preserve fluorescence of callose.

Statistical analyses

Analysis of variance was performed according to a completely randomized block design, considering each inoculation as a block. Statistical analyses were performed using Statistix 8.0 statistical package (Analytical Software, Tallahase, USA).

RESULTS

Most sporelings (95.5%) succeeded in establishing a colony on the susceptible cv. Messire (*Er1Er1*) (Table 1). No penetration attempts were associated with protein cross–linking (Fig.1. a, c) and very few (11%) with HR.

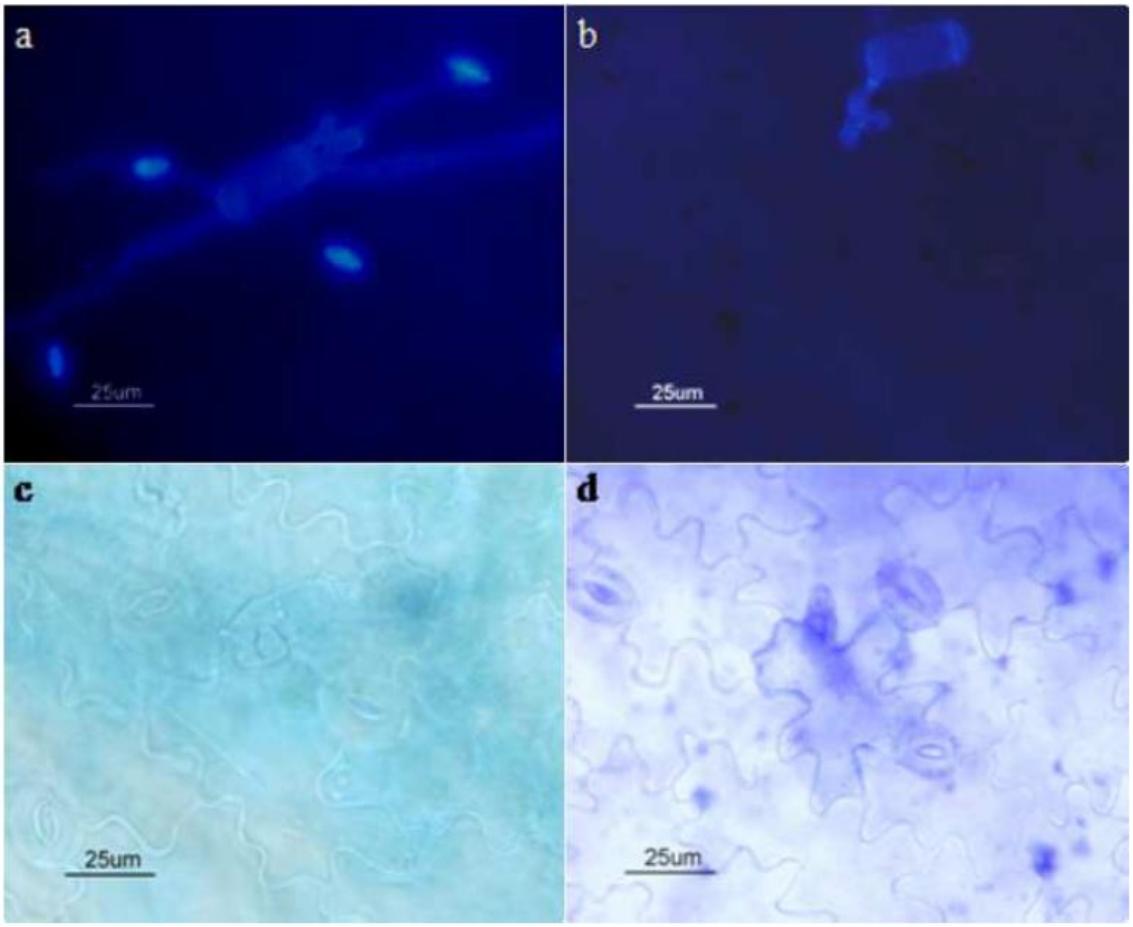

Figure 1. Leaflets of cv. Messire (a, c) and JI2302 (b,d) infected by *Erisiphe pisi* and stained with Uvitex (a, b) or Coomassie blue (c, d) 48 hai. External fungal structures show autofluorescence under UV–light excitation after staining by Uvitex. Protein cross–linking, stained blue by Coomassie blue, can be observed in JI2302 around the penetration point and slightly on the epidermal cell wall, whereas in Messire there is not such staining.

In contrast, very few (2.3%) sporelings succeeded in establishing a colony in JI2302 (*er1er1*), with 63% of the invaded epidermal cells showing protein cross–linking in the point of penetration (Fig.1. b, d). Both HR and protein cross–linking resulted in the abolishment of colony establishment.

Table 1. Mean percentages for infection progress of *E. pisi* (isolate CO-01) and host defence responses on pea accessions JI2302 and cv. Messire 48 hai.

Genotype	% Infection units successfully establishing a colony	% Infection units triggering protein cross-linking	% Infection units triggering protein cross-linking resulting in aborted colony	% Infection units triggering Hypersensitive Response	% Infection units triggering Hypersensitive Response resulting in aborted colony
JI2302	2.3 *[a]	63.0 *	100.0 *	21.3	100.0
Messire	95.5	0	0	11.0	83.2

[a] Values with * by columns differs significantly (ANOVA $p < 0.05$).

No statistical differences were found between the analyzed genotypes for the percentage of spores with appressorium which triggered HR (ANOVA $p > 0.05$).

Callose deposition was not observed in any of the lines with the staining techniques used.

DISCUSSION

Resistance conferred by *er1* has been reported to be associated with low success in colony establishment (Fondevilla et al., 2006a). However, the cellular mechanism behind this colony abortion was not fully understood. In this study we performed a detailed quantification of the presence of three mechanisms that could cause this colony abortion: callose apposition, protein cross–linking and HR.

In our previous histological study (Fondevilla et al., 2006a), haustoria were not observed in *er1* lines, suggesting a penetration resistance. However, a fast hypersensitive response, immediately after penetration, when the haustoria have just started to be formed, could also stop haustoria development so soon that these structures could not be observed under the appressoria. The so–called HR is a common mechanism of resistance against biotrophic pathogens that need to get their nutrients from living cells. This mechanism has also been reported to be the main cause of the resistance to *E. pisi* mediated by *er2* and *Er3* pea genes (Fondevilla et al., 2006a; 2007; 2011). In the present study, in order to do an accurate and better estimation of the role of HR in *er1* lines, we scored the percentage of penetration attempts associated with HR (no matter whether resulted in aborted or established colonies) in an *er1* line and a susceptible one. Results showed that *circa* 20 per cent of the epidermal cells that were attacked by *E. pisi* died in the *er1* genotype studied. Our present work confirms the effectiveness of HR, since the vast majority of host cells showing this response resulted in aborted colonies of the fungus in both the resistant

and the susceptible lines. However, while most spores were not able to establish colonies in the *er1* genotype, the percentage of infected epidermal cells presenting HR was low and did not differ from that observed in the susceptible cv. Messire. Therefore, although HR is an effective mechanism against *E. pisi* on pea, in this study we confirm that HR is not the primary cause of colonies abortion mediated by *er1*.

Resistance to powdery mildew penetration conferred by mutations in the *MLO* genes is characterized in other plant species by formation of papillae, complex structures which can even be seen using visible light microscopy and formed among other compounds by callose apposition. In Fondevilla et al., 2006a using light miscroscopy papilla were not observed in *er1* lines. However, no sstaining was applied to specifically detect the components of papilla. In this study, to definitively discard or confirm the presence of papilla, we applied dyes that specifically stain callose, the main component of papillae. Finally, we did not observe the presence of callose in JI2302 or Messire albeit using specific stains. These results suggested that callose apposition is not associated with the resistance to *E. pisi* conferred by *PsMLO1* loss of function. Interestingly, Consonni et al. (2010) found that resistance against the powdery mildew pathogen *Golovinomyces orontii* conferred by the *MLO* mutant allele *mlo2* in *Arabidopsis* is independent of callose apposition, but not of papilla formation. Thereby, *mlo2* lines defective in callose formation remained resistant to powdery mildew. Similarly, Perumalla and Health (1989) outlined that callose deposition was not a prerequisite for other cell wall modifications observed in the bean (*Phaseolus vulgaris* L.) and cowpea rust (*Uromyces vignae* Barcl.) interactions, supporting the fact that callose is not a requirement for successful penetration resistance of the host plant (Jacobs *et al*., 2003; Nishimura *et al*., 2003). Our results support these hypotheses.

Probably the best studied example of prehaustorial resistance is the mlo–based resistance of cultivated barley to *B. graminis* f.sp. *graminis* (Lyngkjær et al., 2000). Other major genes for prehaustorial resistance, with more quantitative effects, occur in wheat against *Puccinia triticina*, i.e. the *Lr34* gene, associated with reduced haustorium formation that was not associated with papillae neither with hypersensitivity (Rubiales and Niks, 1995).

Instead of callose apposition, a high percentage of infected epidermal cells showed protein cross–linking on the *er1* line JI2302. This reaction was not observed in the susceptible cv. Messire, indicating that protein cross–linking is associated with *PsMLO1* mutation mediated resistance. Protein cross–linking has been observed to be a defence mechanism in pea against *Didymella pinodes* (Carrillo *et al*., 2013) or parasitic plants such as broomrape (Pérez–de–Luque *et al*., 2006). In our study, the presence of

protein cross–linking was in all cases associated with colony abortion, which pointed towards protein cross–linking as the effective mechanism to avoid penetration by *E. pisi* in pea *Psmlo1* lines.

As we have previously mentioned, *MLO* genes codify membrane proteins. Although its precise function has not been unravelled yet, it is thought that these genes act as suppressors of defence mechanisms against powdery mildew (Consonni *et al*; 2006). In *mlo* mutants this defensive reaction is not repressed and mildew attack would result in the activation of mechanisms of resistance that could differ between plant species. Thus, in some plant species the activation of the defensive response could lead to callose apposition while in pea it could result in protein cross–linking.

In conclusion, our study demonstrates that *E. pisi* resistance mediated by loss of function in *Er1* gene is associated with protein cross–linking formation in the attacked host epidermal cells but not with callose apposition or HR. To our knowledge this is the first report of a *mlo* type resistance not associated with callose apposition. This study increases our knowledge about *MLO* genes and suggests that the mutation in these genes may result in different cellular mechanisms of resistance depending on the plant species.

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