A NOVEL BROAD SPECTRUM RESISTANCE GENE (*RPW8*) CONTROLS POWDERY MILDEWS

Özer ÇALIŞ
Gaziosmanpaşa Üniversitesi Ziraat Fakültesi Bitki Koruma Bölümü 60240 Taşlıçiftlik Kampusu-TOKAT

Abstract

Plant disease resistance (*R*) genes govern the recognition of specific pathogens and activate subsequent defense responses. The recently cloned *RPW8* gene controls resistance to powdery mildew pathogens. To characterise *RPW8* specificity, a range of powdery mildew pathogens was examined for virulence on *Arabidopsis thaliana* Col-0 containing the *RPW8* transgene. All the tested *Erysiphe* pathogens were avirulent on the *RPW8* containing plants showing the *RPW8* gene was a broad-spectrum powdery mildew resistance gene. The resistance conferred by *RPW8* gene was associated with a hypersensitive response (HR). This was characterized by timing of hydrogen peroxide (H_2O_2) accumulation in plant epidermal cells penetrated by the pathogen. The powdery mildew attacked cells accumulated H_2O_2 producing rapid cell death that restricts growth of the pathogen in the plant cells.

Keywords: Plant disease resistance, *RPW8* gene, powdery mildew pathogens, hypersensitive response, hydrogen peroxide accumulation.

Geniş Spektrumlu Yeni Bir Gen (*RPW8*) Külleme Hastalıklarını Kontrol Ediyor

Özet

Bitki dayanıklılık (*R*) genleri spesifik patojenlere karşı dayanıklılığı kontrol ederek onlara karşı aktif savunma oluştururlar. Son zamanlarda klonlanan *RPW8* geni külleme etmeni patojenlere karşı dayanıklılığı sağlamaktadır. *RPW8* genini karakterize edebilmek için *RPW8*-transgenini içeren *Arabidopsis thaliana* bitkileri külleme patojenleri ile test edilmiştir. Test edilen tüm *Erysiphe* patojenleri *RPW8* geni içeren bitkilerde hastalık oluşturmadığı için (avirulent), *RPW8* geninin geniş spektrumlu bir gen olduğu ortaya konmuştur. *RPW8* tarafından ortaya konan dayanıklılık hypersensitif response (HR) ile ilgilidir. Bu olay bitki hücrelerinde hidrojen peroksit (H_2O_2) oluşumunun patojenin penetrasyonu ile eş zamanda gerçekleşmesi ile ortaya konmuştur. Külleme etmeni tarafından saldırıya uğrayan hücrelerde H_2O_2 birikimi artmış ve hızlı hücre ölümleri gerçekleşmiştir ki bu da patojenin bitki hücrelerinde gelişimini sınırlandırmıştır.

Anahtar Kelimeler: Bitki dayanıklılık geni, *RPW8* geni, külleme patojenleri, hypersensitif tepki, hidrojen peroksit oluşumu

1. Introduction

A majority of known plant species are potential hosts for various bacterial, fungal and viral pathogens. However, plant disease is the exception rather than the rule; most encounters of plants with pathogens do not lead to a disease (Agrios, 1997). Most interactions between plants and pathogens are of the 'nonhost' type, where the pathogen is not pathogenic on a given plant. 'Host' type interactions are between plants and their pathogens. Not all 'host' type of interactions result in disease. The expression of defense responses in the host to a particular pathogen is conditional on the pathogen genotype. A molecular recognition of pathogens by plants is known as the 'gene-for-gene' interaction (Flor, 1971). In this gene-for-gene interaction a plant resistance (*R*) gene encodes the *R* product, that appears to act as a receptor and recognises a product of the corresponding avirulence (*Avr*) gene from the pathogen, inducing defense responses (De Wit, 1992; Hammond-Kosack and Jones, 1996; Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001).

Pathogen defense in plants has been well studied, particularly with the goal ofidentifying target genes for engineering long lasting, broad spectrum resistance in crops (Greenberg *et al*., 1996; Cao *et al*., 1998). Many crops rely on *R* genes for resistance to specific pathogens, but resistance fails in the presence of strains of

the pathogens that lack the corresponding *avr* genes. Broad-spectrum disease resistance is therefore desirable, and has been achieved through the use of recessive mutations (Baschges *et al*., 1997); a challenge is to develop broad-spectrum resistance with dominant *R* genes (Rommens and Kishore, 2000).

A novel resistance gene *RPW8* governs resistance to all tested isolates of the four species of powdery mildew pathogens of *Arabidopsis thaliana*, a small brassica plant. *RPW8* contains two dominant *R* genes *RPW8.1* and *RPW8.2* encode small, probable membrane proteins with possible coiled-coil domain and no homology to other known proteins (Xiao *et al*., 2001).

Plant incompatible responses to their pathogens frequently result in the appearance of necrotic flecks containing dead cells at sites of attempted entrance. This rapid collapse of the challenged host cells is called the hypersensitive response (HR) and is deployed as a battery of inducible defences in both the challenged and surrounding cells (Hammond-Kosack and Jones, 1996; Levine *et al*., 1994). Multiple biochemical events are associated with HR in which one of the most effective resistance reactions is the production of reactive oxygen species (ROS) termed the 'oxidative burst' (Frye and Innes, 1998; Del Pazo and Lam, 1998, Rajasekhar, *et al*., 1999).

A majority of the characterised *A. thaliana R* genes mediate resistance mechanisms that involve a HR, including induced H_2O_2 formation. With respect to the broad-spectrum mildew resistance in *A. thaliana* mediated by *RPW8* how does the gene operate to limit pathogen development? Is the *RPW8* resistance mechanism triggering the HR response similar to other *R* genes? Furthermore, Adam and Somerville (1996) reported that they did not associate powdery mildew resistance genes with the rapid hypersensitive necrosis response observed in many pathosystems. In order to address all the above questions, a detailed time course experiment was designed to investigate H_2O_2 induction in the physiological mechanism of the *RPW8*-mediated hypersensitive response.

2. Materials and Methods

2.1. Plant and Fungal Materials

Homozygous *RPW8* transgenic (TB6) plants, and wild ecotype Ms-0 and Col-0 *Arabidopsis* plants were infected with *Erysiphe cruciferarum* UEA1, *E. cichoracearum* UCSC1 and *Oidium lycopersici* Oxford (See details in chapter 2). Plant inoculations were conducted as described in Adam *et al*. (1999).

2.2. Plant maintenance and growth conditions

All the *Arabidopsis thaliana* accessions were obtained from the Nottingham *Arabidopis* Centre, Nottingham University, England. Courgette, cucumber and tomato seeds were purchased commercially from E. W. Kings Co. Ltd. (Colchester, UK). The Brassica and Oil-seed Rape Department, John Innes Centre, Norwich Research Park, UK, supplied *Brassica napus* cv. W10 seeds.

Arabidopsis seeds were sown in a sterilised soil mixture, consisting 2 volumes (v) John Innes compost No:3 (Gem Gardening Ltd, Lancashire, UK), 2 v horticultural grit (Gem Gardening Ltd, Lancashire, UK), 2 v peat (Shamrock, Newbridge, Ireland) and 1 v vermiculate (Vermiperl, Lincoln, UK). The seed sown pots were covered with Clingfilm to maintain high humidity and vernalised for 2 days at 4°C to produce homogenous seedling germination. Germinated seedlings were kept in a room with an 8 h day, 16 h night cycle at 20°C; this growth room had 110 μmol m^{-2} s^{-1} illumination provided by broad-spectrum lamps (400W fluorescent metal halide, Kolor Arc). The Clingfilm was removed a few days after germination and the plants were grown until they had produced a pair of cotyledons and a pair of true real leaves. The seedlings were then transplanted into fresh soil to provide enough nutrients for the remainder of the tests. The plants were grown to the 5-8 leaf stage before powdery mildew inoculation and transferred to a 16 h day and 8 h night photoperiod regime at 22°C.

Arabidopsis plants were usually contaminated with polyphagus aphids (*Myzus persicae*). To control the aphids, the plant surfaces were sprayed with Nicotine (1 ml l^{-1}) which was not only effective against the aphids but also decomposed quickly after application. In addition to the insecticide control, yellow sticky traps were used for catching mushroom flies. Biocontrol agents (Ambly-line Cu, Novartis, Colchester, UK) containing predators of Thrips were also used.

2.3. Detection of H_2O_2

Detection of H_2O_2 was performed by endogenous, peroxidase-dependent, *in situ* histochemical staining with diamino benzidine as described in Thordal-Christensen *et al*. (1997). *Arabidopsis* primary leaves were cut and placed in a solution of 1 mg ml^{-1} DAB for 12 h under light conditions as described by Thordal-Christensen *et al*. (1997) before examination and subsequently immersed in a clearing solution for 2 days. DAB is a commercially available chemical (Sigma-Aldrich, UK) and soluble in water at low pH, 3.8. It reacts with H_2O_2, and polymerises to produce a stable brown-reddish colour. To prevent auto-oxidation of DAB, the staining solution was freshly prepared and staining was performed under a light (~110 µmol m^{-2} s^{-1} at 22°C). The DAB-stained leaves were laid on a slide and further stained with trypan blue. The powdery mildew spores and dead plant cells become visible when they uptake the trypan blue stain. H_2O_2 accumulation was commonly observed as a brown reaction product in epidermal and mesophyll cells penetrated by the pathogen which was also observed as blue in colour.

2.4. Epifluorescence microscopy

The fluorescent dye 3,3'dihexyloxacarbocynin iodide (DiOC6) was prepared as a stock solution in ethanol

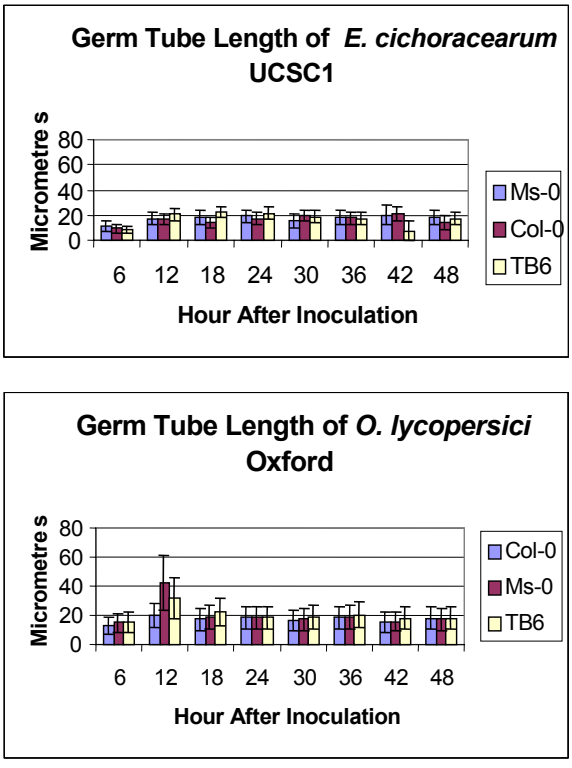

Figure 1. Comparisons of germ tube lengths for two different fungi. Resistant Ms-0, TB6 and susceptible Col-0 plants were inoculated with **A.** *E. cichoracearum* UCSC1 and **B.** *O. lycopersici* Oxford. Plant leaves were stained with DAB and trypan blue at indicated time points. Germinated tube lengths of conidia spores were measured. Error bars show standard deviations of 10 replicates on different plant leaves.

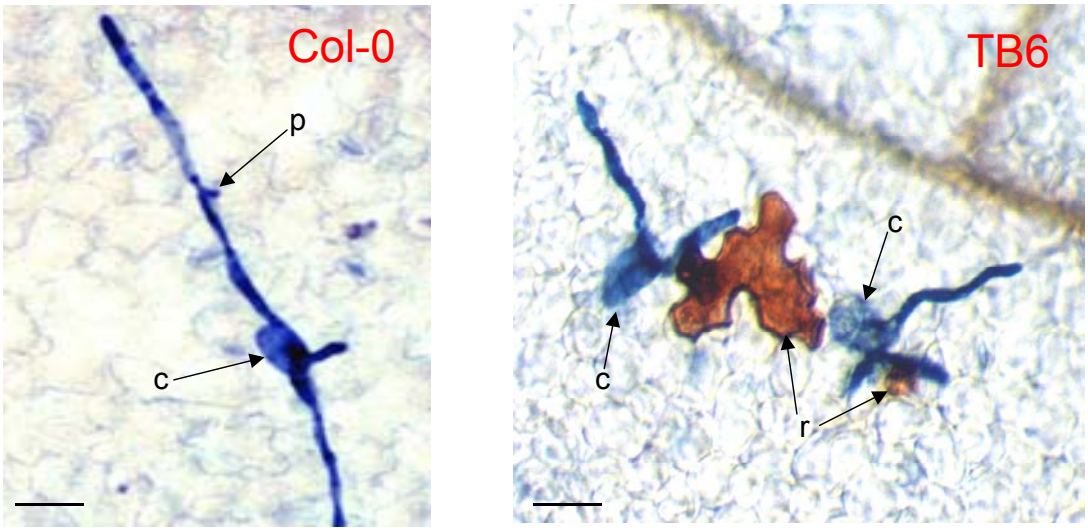

Figure 2. Germinated conidia (c) spore on surfaces of leaves 30 hours after inoculation. Hydrogen peroxide formation was detected with diamino benzidine as a brown reaction product(r) in TB6 epidermal cells penetrated by the pathogen, but could not be detected in Col-0 epidermal cells cells penetrated (p) by the pathogen. Bar, 30 μm.

(0.5 mg ml^{-1}) and stored at -20°C as described by Duckett and Read (1991). Plant leaves were immersed in freshly prepared aqueous solution of DiOC6 at 50 μg ml^{-1} where the stock solution was diluted ten times in distilled water. Following 2-3 minutes of exposure, the samples were placed on slides and the excess solution was removed with tissue. The samples were analysed in a Nikon Optiphot-2 light microscope system fitted with an epifluorescence filter B2A (450-490 nm excitation filter and a 520 nm barrier filter) and a Plan X 10 Phl/D1 objective camera. Fungal hyphae stained bright yellow and conidiospores stained bright green to yellow in colour depending on either age of spores or exposure time with the DiOC6 solution. Healthy plant cells exhibited a deep red colour due to the autofluorescence of chloroplasts whereas hypersensitive plant cells appeared dark green to dark brown in colour.

3. Results

To provide more detailed information about *RPW8*-mediated resistance to powdery mildews, Col-0, Ms-0 wild type accessions and transgenic TB6 plants were inoculated and observed by DAB-staining every 6 hours from 0 to 48 hai. The primary germ tube emerged from the end of the conidium at 2 hour after inoculation (hai). The spore germ tube lengths did not show any significant differences during the study (Figure 1). The primary germ tube length is independent from plant genotypes. Prolonged germ tube growth is dependent upon successful penetration, even unsuccessful penetrations will continue to produce secondary and tertiary germ tubes.

The appressorial germ tube produced a penetration peg that often penetrated successfully into plant cells to establish a haustorium. This penetration was often associated with H_2O_2 formation, which was detected with DAB as a brown reaction product, in *RPW8* containing resistant plant epidermal cells (Figure 2) but could not be detected in Col-0 epidermal cells penetrated by the pathogen (Figure 2). Histochemical staining for H_2O_2 with DAB produced a brown reaction product in the epidermal cells of *RPW8* resistant plants. However, the conidia spore penetration in Col-0 plant cells could produce a brown speck just underneath the penetration peg, this brown speck was different from H_2O_2 formation in

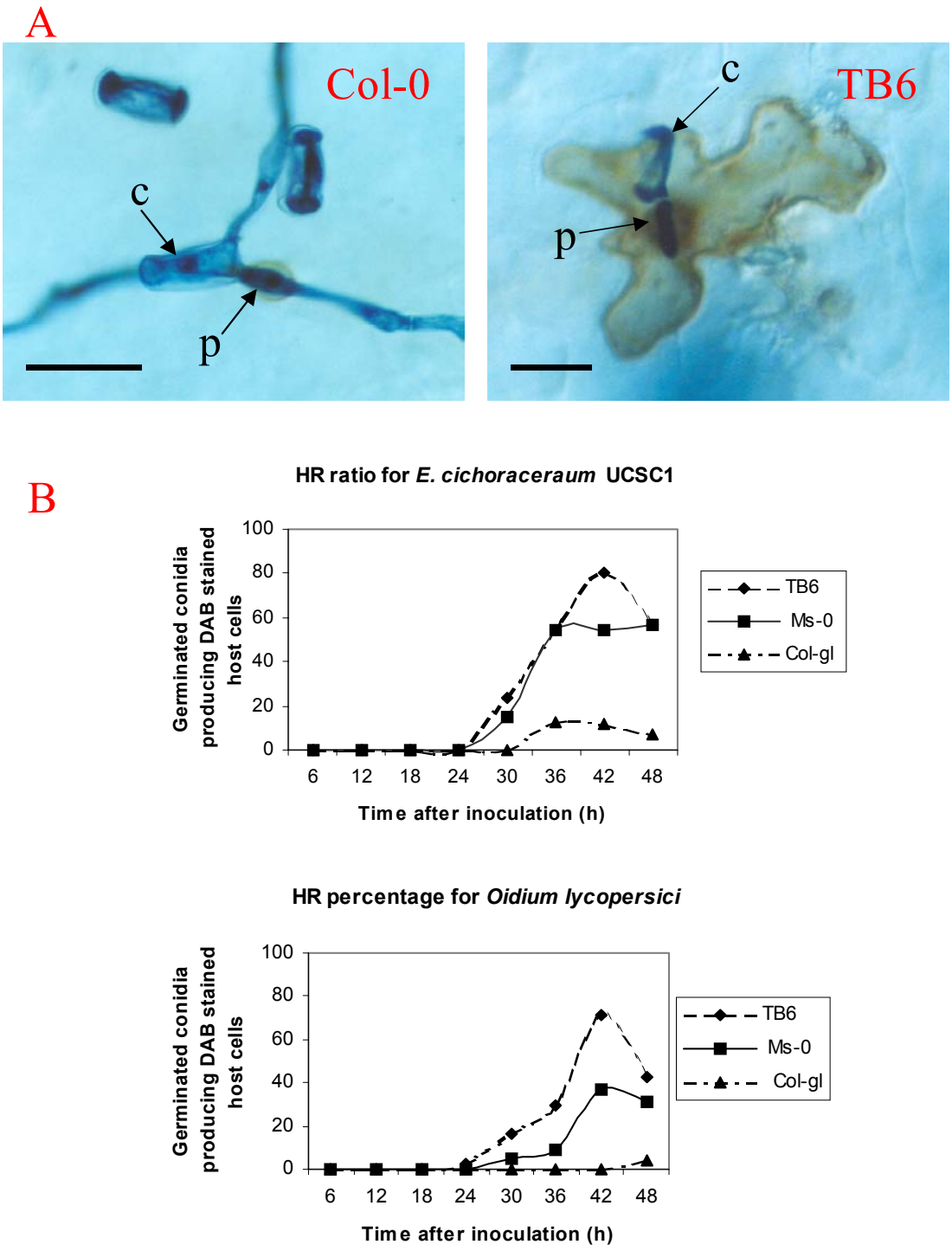

Figure 3. The course of H_2O_2 induction and physiological analysis of *RPW8*-mediated hypersensitive response. (**A**) Germinated conidia (c, blue) on a leaf surface 48 hours after inoculation. A brown speck was observed in Col-0 (left) epidermal cells penetrated by the pathogen but the brown speck was different from hydrogen peroxide formation detected with diamino benzidine as a brown reaction in TB6 (right) epidermal cells penetrated by the pathogen. (**B**) Time-course of cellular HR, detected by diaminobenzidine-staining, in Ms-0, Col-0, and T-B6 leaf cells. Upper: *E. cichoracearum* UCSC1 inoculated, Lower: *O. lycopersici* inoculated.

the *RPW8* resistance mechanism (Figure 3A right). The brown speck did not extend from underneath the penetration peg to the whole cell (Figure 3A left).

The progression of different powdery mildew diseases could be morphologically distinguished on plant leaf surfaces with microscopy techniques. This visual inspection was combined with measurements of H_2O_2 induction in time course experiments with *E. cruciferarum* UEA1, *E. cichoracearum* UCSC1 and *O. lycopersici* Oxford inoculated plants. There was no H_2O_2 accumulation detected in Col-0, Ms-0 and TB6 plant cells until germ tube penetration occurred (Figure 3B). The *E. cruciferaum* UEA1 and *O. lycopersici* Oxford germ tube penetrations started at 20-22 hai but there was no germ tube penetration observed before 26-28 hai for *E. cichoracearum* UCSC1. The H_2O_2 formation, which was detected as a brown reaction product, was highest in TB6, less in wild type Ms-0 and very little (as determined brown speck beneath the penetration peg) in Col-0 epidermal cells penetrated by the pathogens (Figure 3B). These results clearly suggest that germ tube penetration is directly associated with accumulation of H_2O_2, producing rapid cell death in *RPW8*-mediated resistance.

The time course results also revealed that earlier penetrated (primary) hyphae and secondary and even tertiary germ tubes that attempted to penetrate the host epidermis, also resulted in H_2O_2 induction in *RPW8*-mediated resistance but not in the Col-0 epidermal cells (Figure 4A). The hyphae on Col-0 penetrated cells develop sporangia at 48 hai. The rapid accumulation of H_2O_2 was produced during HR and the attacked cells collapsed soon after the H_2O_2 induction. The H_2O_2 accumulated in cells appeared as a dark black colour whilst the healthy plant cells were a deep red colour with the epifluorescent dye DiOC6 (Figure 4B). This effective HR resistance response in *RPW8* resistant plants possibly controls resistance by arresting fungal penetration and limiting the intruders' nutrient supply.

4. Discussion

The majority of characterised *A. thaliana R* genes mediate resistance mechanisms that involve an HR, induced H_2O_2 formation and expression of the pathogenisis-related (PR) genes. Defense responses in TB6 plants could be detected 20 hai with *E. cruciferarum* UEA1 or *O. lycopersici* Oxford and 30 hai with *E. cichoracearum* UCSC1, as the formation of H_2O_2 in epidermal cells penetrated by the pathogens (Figure 3). The penetrated cells subsequently collapsed, forming microscopic lesions characteristic of the HR and there was no further growth of the pathogen. By contrast there was no evidence of cellular H_2O_2 when Col-0 epidermal cells were penetrated by the pathogens, which grew to form masses of white mycelia and conidia on the leaf surface in 10 days.

The time course results clearly explain why *RPW8* is a broad-spectrum mildew resistance gene. Germinated conidia attempt at penetration is directly associated with H_2O_2 production in epidermal cells producing a HR in *RPW8* resistant plants. This suggests that the *RPW8* gene product works in a pathway that recognizes the fungal penetration and activates a rapid, transient H_2O_2 burst producing cell death. Activation of cell death is controlled by both internal and external factors, with exquisite co-ordination of the various chemical events (Hengartner and Bryant, 2000). The *RPW8*-mediated resistance could employ a similar resistance mechanism in which the external stimulus was the germ tube penetration directly or indirectly recognized by the *RPW8* gene product which then activated H_2O_2 release, producing the HR in effected plant cells. The occurrence of the transient increase of H_2O_2 production is very rapid but could vary depending on the time of penetration of the powdery mildew pathogen. First, second and third hyphae penetration attempts occurred at somedistance from the first penetration attempt and also resulted in induction of H_2O_2 producing the HR. Furthermore, neighboring epidermal cells also accumulated H_2O_2 (Figure 4), this result may indicate that H_2O_2 was a diffusible

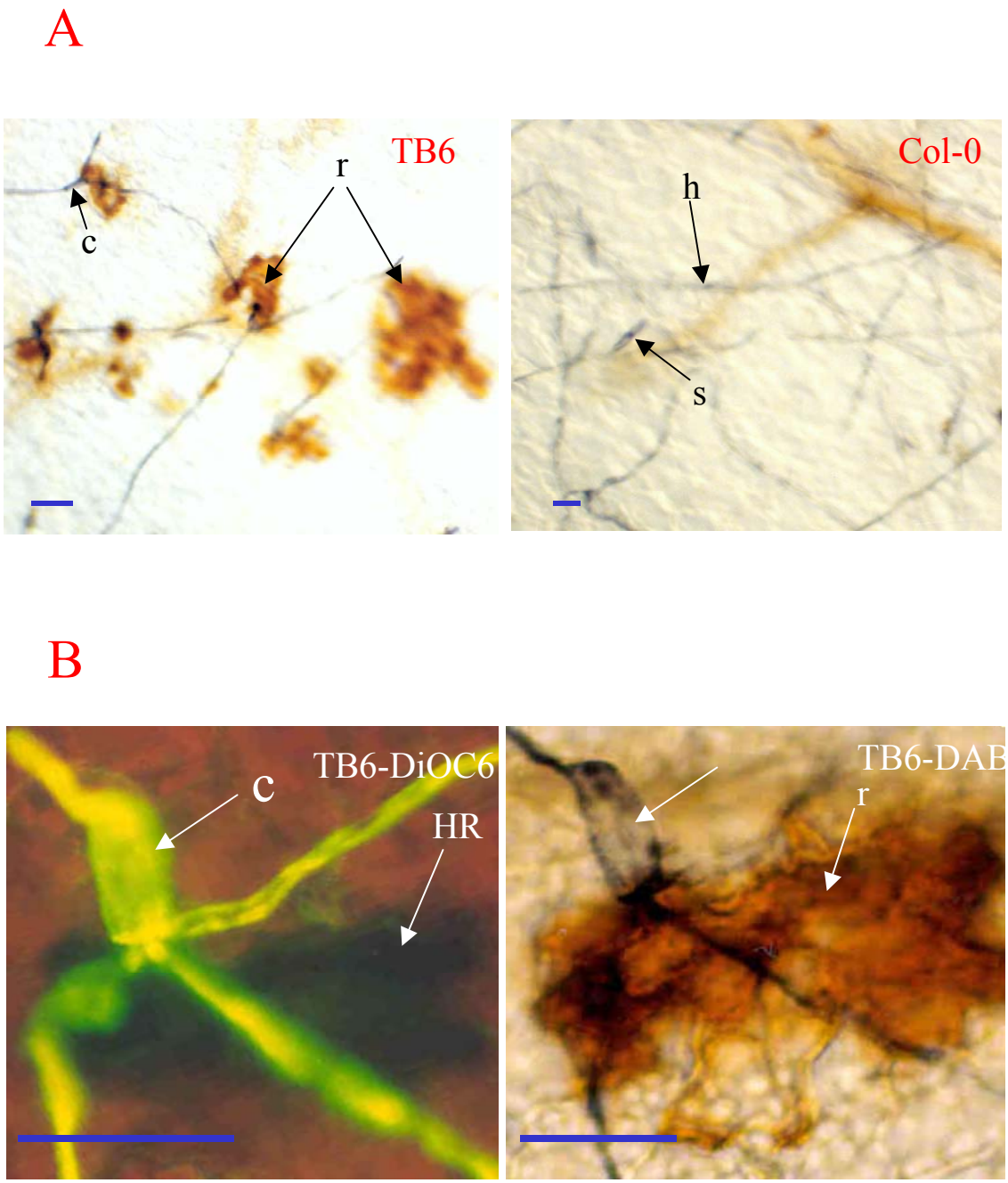

Figure 4. Histochemical analysis of *RPW8*-mediated hypersensitive response in TB6 plants.(A) Germinated conidia on surfaces of leaves 48 hai. H_2O_2 formation (r) was detected with every penetration attempts in (TB6) epidermal cells but was not detected in Col-0 epidermal cells penetrated by the pathogen which grew to produce hyphae (h) and sporangia (s). (B) Disease reactions of TB6 leaves 48 hai. Fungal structures (c) fluorescent yellow or green, whereas living host epidermal cells are red due to chlorophyll autofluorescence, penetrated conidia overlying necrotic (HR) epidermal cells (left). Same area showing H_2O_2 formation with DAB (right). Bar: 30 µm.

signal for the induction of cellular protectant genes in surrounding cells (Levine *et al*., 1994; Hammond-Kosack Jones, 1996). Similarly, Yun and Loake (2002) were reviewed that H_2O_2 had multiple functions in the establishment of plant disease resistance

consisting: the signal activity, the oxidative cross linking of cell wall structural proteins, and direct microbial toxicity allowed host cell death at the penetration sides.

The *RPW8*-mediated resistance mechanism involves an HR, and H_2O_2 production, similar to other *A. thaliana R* genes. This result contradicts previously published data by Adam and Somerville (1996). They reported that no rapid cell death (≤2 dpi) was detected in plants inoculated with *E. cichoracearum* UCSC1. Additionally, they did observe limited fungal growth 7 dpi. However, in this study, a rapid hypersensitive necrosis response was observed immediately on penetration of the leaf surface by the fungal appressorium consistently resulting in fungal growth arrest. These observations were directly correlated with *RPW8*-mediated resistance. This study shows that induction of H_2O_2 is associated with *RPW8* resistant gene.

Germinating conidia spores could grow on the leaf surface without interacting with plant defense systems. This could be a reason that the germ tube length did not show any significant difference on Ms-0, Col-0 and TB6 leaf surfaces. Although the germ tubes of *O. lycopersici* Oxford inoculated on Ms-0 and TB6 plants were longer than Col-0 germ tubes at 12 hai, this could be due to a sampling error in which leaf surfaces or germinated spore numbers might be unequal. Furthermore, time course experiments showed that germinated spore percentages (=germinated spore numbers/total spore numbers) were different on *E. cichoracearum* UCSC1 *and O. lycopersici* Oxford inoculated Col-0, Ms-0 and TB6 plant leaf surfaces (data not presented).

A comprehensive time course study has recently confirmed the importance of H_2O_2 in plant resistance systems where H_2O_2 triggers sensing and early signal transduction and orchestrates the molecular response during both biotic and abiotic stresses (Vandenabeele *et al*., 2003). In our study, germinated spore percentages could indicate that plant defense systems somehow interfere with the conidia spore germination rate. Consistent with this, penetration acts as a stimulus to activate resistance responses mediated through the *RPW8* gene. Although the biosynthesis reaction steps have been described and identified in previous works, the mechanisms for the biochemical conversion of H_2O_2 from molecular oxygen is still unclear.

References

Adam, L. and Somerville, S. C., 1996. Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana*. *The Plant Journal* 9: 341-356.

Agrios, G. N. (1997). Plant Pathology. Academic Press, San Diego.

Baschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., Van Daelen, R., Van der Lee, T. *et al*., (1997). The barley *Mlo* gene: a novel control element of plant pathogen resistance. *Cell* 88: 695-705.

Cao, H., Li, X. and Dong, X. (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proceeding of the National Academy of Sciences of the U.S.A.* 95: 6531-6536.

Dangl, J. L. and Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411: 826-833.

De Wit, P. J. G. M. (1992). Molecular characterisation of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. *Annual Reviews of Phytopathology* 30: 391-418.

Del Pozo, O. and Lam, E., 1998. Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Current Biology* 8: 1129-1132

Duckett, J. G. and Read, D. J. (1991). The use of the fluorescent dye 3,3'dihexyloxacarbocyanin iodide, for selective staining of ascomycete fungi associated with liverworth rhizoids and ericoid mycorrhizal roots. *New Phytologist* 118: 250-272.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annual Reviews of Phytopathology* 9: 275-296.

Frye, C. A. and Innes, R. W., 1998. An *Arabidopsis* mutant with enhanced resistance to powdery mildew. *The Plant Cell* 10: 947-956.

Greenberg, J. T. (1996). Programmed cell death: a way of life for plants. *Proceeding of the National Academy of Sciences of the U.S.A.* 93: 12094-12097.

Hammond-Kosack, K. E. and Jones J. D. G., 1996. Resistance gene-dependent plant defense responses. *The Plant Cell* 8: 1773-1791.

Hammond-Kosack, K. E. and Jones, J. D. G. (1997). Plant Disease resistance genes. *Annual Reviews of Plant Physiology and Plant Molecular Biology* 48: 575-607.

Hengartner, M. O. and Bryant, J. A. (2000). Apoptic cell death: from worms to wombats… but what

about the weeds? In *Programmed Cell Death in Animals and Plants* (Bryant, J.A., Highes, S. G. and Garland, J. M. eds.) pp.1-12 Bios Scientific Publishers Oxford.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C., 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583-593.

Rajasekhar, V. K., Lamb, C. and Dixon, R. A., 1999. Early events in the signal pathway for the oxidative burst in soybean cells exposed to avirulent *Pseudomonas syringae* pv *glycinea*. *Plant Physiology* 120: 1137-1146.

Rommens, C. M. and Kishore, G. M., 2000. Exploiting the full potential of disease-resistance genes for agricultural use. *Current Opinion in Biotechnology* 11: 120-125.

Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D. B., 1997. Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley powdery mildew interaction. *Plant Journal* 11: 1187-1194.

Yun, B-W. and Loake, G. (2002). Plant defence responses: current status and future exploitation. *Journal of Plant Biotechnology* 4: 1-6

Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M. and Turner, J. G. (2001). Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by *RPW8*. *Science* 291: 118-120

Vandenabeele, S., Van Der Kelen, K., Dat, J., Gadjev, I., Booneferaes, T., Morsa, S., Rottiers, P., Slooten, L., Van Mantagu, M., Zabeau, M., Inze, D. and Van Breusegem, F. (2003). A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. *Proceeding of the National Academy of Sciences of the U.S.A.* 100: 16113-16118.