European Journal of Plant Pathology **109:** 327–330, 2003.

Screening for differential gene expression in *Atropa belladonna* leafy gall induced following *Rhodococcus fascians* infection

El Hassan Nouar[1], Danny Vereecke[2], Koen Goethals[2], Mondher Jaziri[1,*] and Marie Baucher[1]
[1]*Laboratory of Plant Biotechnology, Université Libre de Bruxelles, Chaussée de Wavre 1850, B-1160 Brussels, Belgium;* [2]*Plant Systems Biology, Vlaams Interuniversitair Instituut voor Biotechnologie, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium;* **Author for correspondence (Phone: +32 2 650 9181; Fax: +32 2 650 9175; E-mail: jaziri@ulb.ac.be)*

Accepted 27 November 2002

Key words: differential display, phytopathogenic bacteria, PR proteins

Abstract

To better characterise at the molecular level the nature of plant responses to infection by *Rhodococcus fascians* PCR-based differential display patterns of *Atropa belladonna* leafy gall (LG) and non-infected plant tissues were compared. Six differentially expressed genes were identified and their altered expression was confirmed by RT-PCR. Three of them corresponded to up-regulated genes which encode proteins involved in plant defence. The three remaining cDNA fragments which correspond to down-regulated genes in LG, encoded proteins with similarity to a multicystatin, a miraculin and a methallothionein-like protein, respectively. Upon elimination of the bacteria from infected plant tissue, the expression of up-regulated genes was maintained, whereas expression of down-regulated genes resumed suggesting a potential role of these up-regulated genes in plant growth and development.

The phytopathogenic Gram-positive bacterium *Rhodococcus fascians* infects a wide range of monocotyledonous and dicotyledonous plants causing several types of malformations (Vereecke et al., 2000). The most typical symptom is the initiation of multiple shoot primordia at the site of infection that has been associated with the *de novo* cortical cell division (de O. Manes et al., 2001). The suppression of outgrowth of these shoots results in the formation of leafy galls (LGs) (Vereecke et al., 2000). The relationship between *R. fascians* and host plants is unique among plant pathogens. In *R. fascians*–plant interactions, a hypersensitive response has not been described and the persistence of the symptom is associated with the presence of the bacteria within the gall tissue (Goethals et al., 2001). The particular property of *R. fascians* to induce shoot multiplication is useful for the establishment of routine regeneration programme for a wide range of host plants, including several recalcitrant plant species (Jaziri et al., 1998). Despite the potential biotechnological application for plant propagation and its biological significance, no attempts have been made to explore the host gene expression in response to *R. fascians* infection.

To characterise LGs at the molecular level, a PCR-based technique of differential display (DDRT-PCR) was used (Liang et al., 1994). *Atropa belladonna* plants were infected with *R. fascians* strain D188 (Vereecke et al., 2000). Total RNA was extracted from one-month-old non-infected *in vitro* propagated plants (aerial part) and LGs using an RNAeasy kit (Qiagen, Germany). Forty-five primer combinations were performed and the cDNA subpopulations from non-infected plant and from LG material were compared on denaturating polyacrylamide gels. Visual analysis allowed the identification of 150 differential bands on the gels. An independent experiment confirmed the differential expression for 28 cDNA fragments which were excised from the gels, cloned and sequenced. Sequence similarity to known genes was found for 14 of the 28 cDNA fragments isolated. RT-PCRs using sequence specific primers were

performed and the differential expression patterns were confirmed for 6 of the 28 cDNA fragments (LG1–6) in a second independent experiment (Figure 1 and Table 1).

Transcripts LG1–3 correspond to up-regulated genes in LG tissue and the predicted peptides showed homology to proteins related to those associated with stress responses (Table 2). The polypeptides are similar to the pathogenesis-related (PR) protein NtPRp27 from *Nicotiana tabacum*, a putative chitinase-like protein from *Arabidopsis thaliana* and a β-1,3-glucanase from *N. tabacum*, respectively. Because *R. fascians* interacts with many plant species it is not surprising that a common defence machinery is involved.

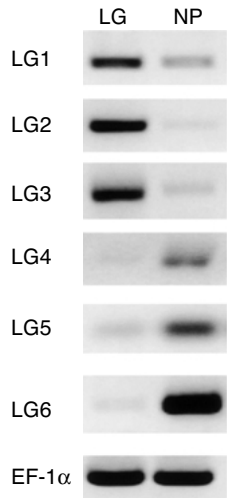

Figure 1. A. belladonna gene expression response to *R. fascians.* Total RNA extracts (0.5 µg) from one-month-old LG tissue or normal plant (NP) tissue was used as template and PCRs were performed using specific primers for each cDNA fragment (LG1–6). The constitutively expressed EF1-α gene was used as control. The RT reaction was carried out at 42 °C for 60 min. Conditions for the PCR reaction were 2 min denaturation at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C and a final elongation step of 7 min at 72 °C. The sequences of gene-specific primers used are shown in Table 1.

Transcripts LG4–6 correspond to down-regulated genes in LG tissue and the derived peptides were homologous to multicystatin (MC) (a class of proteinase inhibitors) from *Solanum tuberosum*, to miraculin (MIR) (a flavourless protein that causes sour substances to be perceived as sweet) from *Lycopersicon esculentum* and to a metallothionein-like (MT-like) protein type 2 (a small cystein-rich proteins with strong binding capacity for heavy metals) from *L. esculentum*, respectively (Table 2). Two hypotheses could explain the down-regulation of these genes in LG in comparison to normal plant (NP) tissue. *R. fascians* could specifically inhibit the expression of specific plant genes as part of a strategy for colonisation, as was described after infiltration of bean with *Pseudomonas syringae* (Jakobek et al., 1993). Alternatively, the down-regulation of these genes could be linked to the suppression of the shoot outgrowth that is a characteristic of the LG structure. In this respect, earlier work demonstrated that elimination of the bacteria from a LG leads to the release of the inhibition imposed on the shoots and results in the development of a number of normal shoots (Jaziri et al., 1998; Vereecke et al., 2000). Therefore, the expression pattern of the six-cDNA fragments was analysed after 1 and 3 weeks upon elimination of the bacteria from the LG by Claforan®. Shoot elongation from LG structure was visible after one week and well-developed shoots with expanded leaves were observed 3 weeks after antibiotic treatment.

As shown in Figure 2, expression of up-regulated genes (LG1–3) was maintained until week 3 after antibiotic treatment. In contrast, the expression of down-regulated genes (LG4–6) resumed one week after antibiotic treatment. Consequently, gene

Table 1. Sequences of gene-specific primers used for RT-PCR analysis shown in Figures 1 and 2

cDNA fragment	Forward primer	Reverse primer
LG1	5′-GATTATGTGAGGCTCAAAGCTGGTCT-3′	5′-TTCAACAACACCGTGACACAAGAGTA-3′
LG2	5′-ATGCTAAGAAGAATGGATTGCTTGG-3′	5′-AGTACATCACATGGCTTTTGCTGTTG-3′
LG3	5′-AGCCTGGGCCTATTGAGACCTATTTA-3′	5′-GCCAATATATCATTTGGGTCTCTGGT-3′
LG4	5′-GAAGGAATGGGAGAACTTCAAGGAA-3′	5′-GTCAGTGACATTGGCATCAAGGTTGT-3′
LG5	5′-GTCAGTGACATTGGCATCAAGGTTGT-3′	5′-ACACACAGTGGAAAGGATCTAGAAATCG-3′
LG6	5′-ATGTCKTGCTGTGGAGGAAACTGT-3′	5′-CTTCAGCCATAACCTCTGCTCTGTA-3′
EF1-α	5′-TGCTACCACCCCCAAGTACTCC-3′	5′-TAAAGCTGGCAGCACCCTTAGC-3′

Table 2. Protein homology of cDNA fragments corresponding to both up- and down-regulated genes in *A. belladonna* LG tissue

cDNA fragment (accession number)	Fragment size (bp)	Protein similarity (accession number)	Origin	Similarity (%)	BLASTX (probability)[a]	References
Up-regulated genes						
LG1 (AJ309384)	597	PR protein NtPRp27 (BAA81904)	*N. tabacum*	90 (199/217 aa)	1 e–109	Okushima et al. (2000)
LG2 (AJ309376)	421	Putative chitinase (CAA19698)	*A. thaliana*	75 (39/51 aa)	5 e–13	De Haan et al. (1998)[b]
LG3 (AJ309385)	691	Prepro-β-1,3-glucanase precursor (AAA34082)	*N. tabacum*	84 (245/289 aa)	1 e–129	Shinshi et al. (1988)
Down-regulated genes						
LG4 (AJ309374)	349	MC precursor (P37842)	*S. tuberosum*	84 (28/33 aa)	2 e–10	Waldron et al. (1993)
LG5 (AJ309386)	769	MIR homologue (T07871)	*L. esculentum*	34 (70/203 aa)	5 e–16	Brenner et al. (1998)
LG6 (AJ309387)	544	MT-like protein type 2B (Q40158)	*L. esculentum*	92 (76/82 aa)	5 e–39	Whitelaw et al. (1997)

[a]The probability of finding these homology by chance. [b]Direct submission.

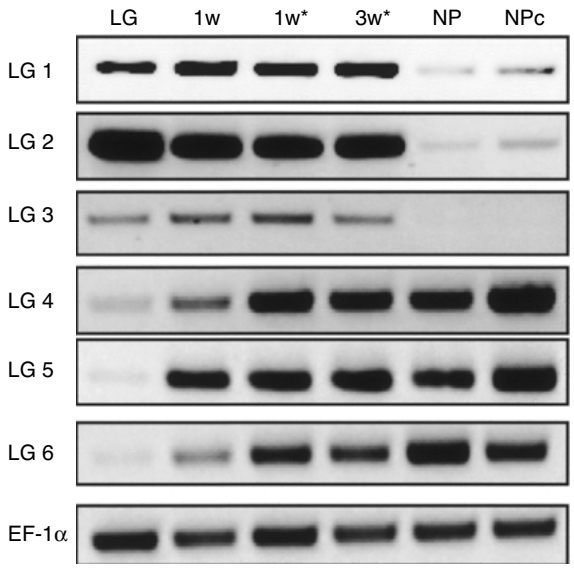

Figure 2. Expression pattern of LG 1–6 during the elimination of *R. fascians* from LG tissue as determined by RT-PCR. One-month-old LGs were collected from infected plants and cultured on medium supplemented with 500 mg/l Claforan® as previously described (Vereecke et al., 2000). After one-week culture two groups of LGs were identified: LGs that do not show shoot development (1w) and those with emerging shoots (1w*). In LGs that were treated with Claforan® for 3 weeks, numerous shoots had developed (3w*). *In vitro* propagated NPs and NP treated with Claforan® for one week (NPc) were used as controls. RT-PCR conditions are as in Figure 1 and the constitutively expressed EF1-α gene was used as control.

down-regulation in LG seems to be associated with the presence of viable bacteria in infected tissue. This result is not consistent with a role for these genes in defence.

Although literature survey indicates that MC-, MIR- and MT-like proteins may be mainly involved in plant defence, a role in plant developmental processes has been suggested.

Whereas some members of the MC family were up-regulated in response to bacterial pathogen attack (Pautot et al., 1991), the expression of other MC members was down-regulated during apple fruit development (Ryan et al., 1998), seed germination (Botella et al., 1996) or programmed cell death in plants (Solomon et al., 1999). These observations suggest that the down-regulated subset of the MCs has an endogenous function rather than a protective role against pathogen attack. Similarly, the observation that nematodes induce the expression of a tomato MIR gene (*LeMir*) indicates that the protein is involved in plant defence (Brenner et al., 1998). *LeMir* was excreted into the rhizosphere and it was suggested that it may interact with soil-borne microorganisms for defence assistance. However, spatial and temporal gene expression patterns allowed the identification of other members of the MIR gene family which are differentially expressed during corn development (de-Castro et al., 1992) or during reproductive development (Milligan and Gasser, 1995).

MT-like gene expression is up regulated by metals (Zhou and Goldsbrough, 1995), by wounding and pathogen stress (Choi et al., 1996), or during leaf senescence (Hsieh et al., 1995). The expression of

other MT-like genes has been correlated with plant growth and development, i.e., fruit developmental stage (Moriguchi et al., 1998) or during embryogenesis (Jin-Zhou and Dunstan, 1996).

In conclusion, this study demonstrates that plants can modulate their gene expression in response to *R. fascians*, a Gram-positive phytopathogenic Actinomycete. Particularly, *R. fascians* induces defence responses that are commonly triggered by other biotic stress as well as responses that appear to be specific to the type of symptom (LG) resulting from its interaction with plant species. The differential screening of plant gene expression during the earlier stage of *R. fascians*-interaction would help identifying markers associated with activation of cell division and with adventitious shoot organogenesis.

Acknowledgements

The authors thank Marcelle Holsters for critical reading of the manuscript. M.B. is a Research Associate of the Fonds National de la Recherche Scientifique (Belgium).

References

Botella MA, Xu Y, Prabha TN, Zhao Y, Narasimhan ML, Wilson KA, Nielsen SS, Bressan RA and Hasegawa PM (1996) Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. Plant Physiology 112: 1201–1210

Brenner ED, Lambert KN, Kaloshian I and Williamson VM (1998) Characterization of LeMir, a root-knot nematode-induced gene in tomato with an encoded product secreted from the root. Plant Physiology 118: 237–247

Choi D, Kim HN, Yun HK, Park JA, Kim TW and Bok SH (1996) Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa* L. and its induction by wounding and tobacco mosaic virus infection. Plant Physiology 112: 353–359

de-Castro LA, Carneiro M, Neshich DC and de Paiva GR (1992) Spatial and temporal gene expression patterns occur during corn development. Plant Cell 4: 1549–1559

De Haan M, Maarse AC, Grivell LA, Bancroft I, Newes HW, Mayer K, Schueller C and Bevan M (1998) EU *Arabidopsis* sequence project. Direct submission: CAA19698

Goethals K, Vereecke D, Jaziri M, Van Montagu M and Holsters M (2001) Leafy gall formation by *Rhodococcus fascians*. Annual Review Phytopathology 39: 27–52

Hsieh HM, Liu WK and Huang PC (1995) A novel stress-inducible metallothionein-like gene from rice. Plant Molecular Biology 28: 381–389

Jakobek JL, Smith JA and Lindgren PB (1993) Suppression of bean defence responses by *Pseudomonas syringae*. Plant Cell 5: 57–63

Jaziri M, Goethals K and Van Montagu M (1998) Plant micropropagation and germplasm storage. Patent WO 98/36635

Jin-Zhou D and Dunstan DI (1996) Expression of abundant mRNAs during somatic embryogenesis of white spruce (*Picea glauca* (Moench) Voss). Planta 199: 459–466

Liang P, Zhu W, Zhang X, Guo Z, O'Connell RP, Averboukh L, Wang F and Pardee B (1994) Differential display using one-base anchored oligo-dT primers. Nucleic Acids Research 22: 5763–5764

Milligan SB and Gasser CS (1995) Nature and regulation of pistil-expressed genes in tomato. Plant Molecular Biology 28: 691–711

Moriguchi T, Kita M, Hisada S, Endo-Inagaki T and Omura M (1998) Characterization of gene repertoires at mature stage of citrus fruits through random sequencing and analysis of redundant matallothionein-like genes expressed during fruit development. Gene 211: 221–227

de O Manes CL, Van Montagu M, Prinsen E, Goethals K and Holsters M (2001) *De novo* cortical cell division triggered by the phytopathogen *Rhodococcus fascians* in tobacco. Molecular Plant–Microbe Interactions 14: 189–195

Okushima Y, Koizumi N, Kusano T and Sano H (2000) Secreted proteins of tobacco cultured BY2 cells: Identification of a new member of pathogenesis-related proteins. Plant Molecular Biology 42: 479–488

Pautot V, Holzer FM and Walling LL (1991) Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding. Molecular Plant–Microbe Interactions 4: 284–292

Ryan SN, Laing WA and McManus MT (1998) A cysteine proteinase inhibitor purified from apple fruit. Phytochemistry 49: 957–963

Shinshi H, Wenzler H, Neuhaus JM, Felix G, Hofsteenge J and Meins FJ (1988) Evidence for N- and C-terminal processing of a plant defence-related enzyme: The primary structure of tobacco prepo-beta-1,3-glucanase. Proceeding of the National Academy of Science USA 85: 5541–5545

Solomon M, Belenghi B, Delledonne M, Menachem E and Levine A (1999) The involvement of cysteine proteinase and protease inhibitor genes in the regulation of programmed cell death in plants. The Plant Cell 11: 431–443

Vereecke D, Burssens S, Simon-Mateo C, Inzé D, Van Montagu M, Goethals K and Jaziri M (2000) The *Rhodococcus fascians*–plant interaction: Morphological traits and biotechnological applications. Planta 210: 241–251

Waldron C, Wegrich LM, Merlo PA and Walsh TA (1993) Characterization of a genomic sequence coding for potato multicystatin, an eight-domain cystein proteinase inhibitor. Plant Molecular Biology 23: 801–812

Whitelaw CA, Le-Huquet JA, Thurman DA, Tomsett AB (1997) The isolation and characterization of type II metallothionein-like genes from tomato (*Lycopersicon esculentum* L.). Plant Molecular Biology 33: 503–511

Zhou J and Goldsbrough PB (1995) Structure, organisation and expression of the metallothionein gene family in *Arabidopsis*. Molecular and Generel Genetics 248: 318–328