

Gene expression profiling and protection of *Medicago truncatula* against a fungal infection in response to an elicitor from green algae *Ulva* spp

S. CLUZET,^{1,2} C. TORREGROSA,¹ C. JACQUET,¹ C. LAFITTE,¹ J. FOURNIER,¹ L. MERCIER,^{1,*} S. SALAMAGNE,² X. BRIAND,² M.-T. ESQUERRÉ-TUGAYÉ¹ & B. DUMAS¹

¹UMR CNRS-UPS 5546 'Surfaces Cellulaires et Signalisation chez les Végétaux', Pôle de Biotechnologie Végétale, 24, chemin de Borde-Rouge, BP17 Auzeville, 31326 Castanet-Tolosan, France and ²SECMA Biotechnologies Marines, BP65, 22260 Pontrieux, France

ABSTRACT

Elicitors are molecules known to trigger plant defence responses against pathogens. In a search for new sources of eliciting compounds from marine algae, an extract was prepared from green algae, *Ulva* spp., and its elicitor activity was established on the model legume, *Medicago truncatula*. When infiltrated into plant tissues or sprayed onto the leaves, this extract induced the expression of the defence-related marker gene *PR10* without provoking necrosis. Spraying a solution at 500 µg mL⁻¹ was sufficient to obtain maximum induction of *PR10* after 2 d. Using a cDNA array enriched in genes potentially involved in plant defence, the expression of 152 genes was monitored after one or two consecutive treatments. A broad range of defence-related transcripts was found to be up-regulated, notably genes involved in the biosynthesis of phytoalexins, pathogenesis-related proteins and cell wall proteins. In contrast, the expression of primary metabolism-related genes did not change significantly. Consistent with its effect on defence gene expression, it was found that prior treatment of *M. truncatula* with the *Ulva* extract protected the plants against subsequent infection by the pathogenic fungus *Colletotrichum trifolii*.

Key-words: *Colletotrichum*; disease; environment; fungi; plant defence; transcriptome.

INTRODUCTION

The ability of plants to defend themselves against pathogens depends on several mechanisms that detect invaders through the perception of signal molecules, also called elicitors. Elicitor perception triggers various signalling pathways, usually beginning with an influx of calcium and an oxidative burst, followed by the synthesis of signal mole-

cules such as salicylic acid, jasmonic acid and ethylene. Defence-related genes leading to reinforcement of plant cell walls, accumulation of antimicrobial compounds such as phytoalexins, and synthesis of proteins with hydrolytic or inhibitory activities towards microbes are then induced (Kombrink & Somssich 1995). A wide variety of molecules can act as elicitors, including oligo- and polysaccharides, peptides, proteins and lipids (Boller 1995; Côté *et al.* 1998).

Elicitors have been considered as alternative tools for disease control in agronomic crops. Marine algae represent an abundant, naturally occurring source of potential elicitors. Improved seed germination, higher yields and increased resistance to diseases were recorded upon treatment of various plants with algal extracts (Hankins & Hockey 1990; Jolivet, Langlais-Jeannin & Morot-Gaudry 1991). Due to their activity as plant protectants, it was proposed that algal extracts, which contain a variety of unique polysaccharides (Kloareg & Quatrano 1988), might act as elicitors of plant defence responses. Laminarin, a linear β-1,3 glucan polymer, and sulphated fucans from brown algae induce the formation of antifungal compounds in alfalfa cotyledons (Kobayashi *et al.* 1993) and several defence responses in tobacco cell suspension cultures (Klarzynski *et al.* 2000, 2003). Carrageenans, which form a family of sulphated linear galactans found in the cell walls of many red algae, are potent elicitors of defence in tobacco plants (Mercier *et al.* 2001).

For the potential use of elicitors as plant protectants, it is important to select extracts which: (1) trigger a large array of defence responses; (2) do not strongly disturb plant primary metabolism; and (3) ensure protection against diseases. The development of new genomic tools offers the opportunity to obtain an integrated view of elicitor effects on the plant transcriptome. Recently, *Medicago truncatula*, a close relative to alfalfa, has emerged as a model legume because it has a small diploid genome, is self-fertile and is easily transformed (Cook 1999). The range of genomic and genetic tools available in *M. truncatula* is rapidly expanding and diversifying (Oldroyd & Geurts 2001). For example, the large-scale sequencing of *M. truncatula* ESTs (Fedorova *et al.* 2002; Journet *et al.* 2002) gives the opportunity to

Correspondence: Dr Bernard Dumas. Fax: +33 5 6219 3502; e-mail: dumas@scsv.ups-tlse.fr

*Present address: Moët et Chandon, Laboratoire de Recherches, 6, rue Croix de Bussy, 51200 Epernay, France.

study variation of the plant transcriptome in response to various stimuli.

This article reports on the elicitor activity of an extract from green algae, *Ulva* spp., in *M. truncatula* plants. Macroarray analyses showed that treatment with the extract induced changes in the expression of a large number of plant defence genes. Protection of treated plants against the fungal pathogen *Colletotrichum trifolii* is also reported.

MATERIALS AND METHODS

Biological material

Seeds of *Medicago truncatula* line F83 005.5 (provided by J.M. Prosperi, ENSAM, INRA Montpellier, France) were surface-sterilized for 5 min in sulphuric acid, rinsed several times with sterile water and germinated on Petri dishes for 3 d at 15 °C in the dark. Seedlings were transferred to soil and cultivated in a growth chamber (16 h light at 25 °C, 8 h dark at 20 °C). Green algae *Ulva* spp., a mixture of several *Ulva* species (mostly *U. armoricana*), were harvested on the north Brittany coast (France) at Archipel de Bréhat. *Colletotrichum trifolii* Bain and Essary race 1, which was kindly supplied by M.B. Dickman (University of Nebraska, USA), was routinely grown on ANM medium (malt extract 2%, bactopectone 0.1%, glucose 2%, agar 2%) in the dark at 24 °C in Petri dishes. The conidia were produced on a solid medium containing glucose 0.28%, MgSO₄·7 H₂O 0.13%, KH₂PO₄ 0.27%, pancreatic peptone 0.2%, yeast extract 0.01%, agar 2% at 24 °C (Bannerot 1965). Conidia were collected from 7-day-old cultures in sterile water and their concentration was adjusted to 10⁶ conidia cm⁻³ in a solution containing Tween 20 (0.01% v/v).

The elicitor of the oomycete pathogen, *Phytophthora parasitica* var. *nicotianae*, hereafter called P₂, was prepared as described previously (Roux *et al.* 1994). Briefly, it consisted of the dialysed ethanol-soluble fraction of an extract obtained by autoclaving isolated *P. parasitica* var. *nicotianae* cell walls.

Preparation of the *Ulva* extract

Ulva spp. algae (100 g fresh weight) were autoclaved for 2 h at 110 °C (1.97 atm) in 1 L distilled water and the resulting extract was filtered through nylon mesh (80 µm porosity) and on a fritted glass funnel with the porosity G2. The filtrate was concentrated to 150–200 mL under vacuum with a Büchi rotary evaporator (Büchi, Flawil, Switzerland). The soluble compounds were precipitated with 2.5 volumes of ethanol for a period of 48 h at –20 °C. The precipitated compounds were recovered by filtration and lyophilized. The dry fraction was weighed and dissolved in water.

Chemical analysis of the *Ulva* extract

The protein content of the extract was estimated according to the method of Lowry (Lowry *et al.* 1951) using bovine serum albumin as a standard protein.

Sugars analysis of the *Ulva* extract was performed with a DIONEX high-performance liquid chromatography system (Dionex, Sunnyvale, CA, USA) by anion-exchange chromatography as described by Boudart *et al.* (1995). The sugar residues were detected by amperometry with the Dionex AI450 package, identified according to their retention time and quantified by reference to commercial standards (Sigma, Saint-Quentin Fallavier, France). The uronic acid content was quantified by the meta-hydroxybiphenyl assay (Blumenkrantz & Absoe-Hansen 1973).

Mineral element analysis was performed by inductively coupled plasma-atomic emission spectrometry (Jobin Yvon Ultima, Longjumeau Cedex, France; Montaser & Golightly 1992).

Soluble phenolic compounds were quantified by a colorimetric assay (Singleton & Ross 1965).

Biological assays

Each experiment was carried out at least three times independently. The *Ulva* extract (UE, 1 mg mL⁻¹), the P₂ elicitor (0.03 mg mL⁻¹) or water was infiltrated into leaves of 1-month-old plants with a syringe without a needle. Macroscopic observations of the treated leaves were made before harvesting them for subsequent RNA extraction.

For spraying experiments, a surfactant, Silwett L-77 (OSI Specialities, Danbury, CT, USA), was added to a final concentration of 0.01% to improve the spreading and wetting properties of the sprayed solutions. Routinely, the UE and the control solution (water + Silwett) were sprayed on 10 1-month-old *M. truncatula* plants (250 µL plant⁻¹; 1 mg mL⁻¹). To perform a single treatment, plants were treated with UE, water or left untreated (controls). Treated plants were incubated for various periods, and then the leaves were harvested, immediately immersed in liquid nitrogen and subsequently used for RNA extraction. To perform two consecutive treatments, a single UE treatment was applied and the plants were allowed to recover for 3 d before a second treatment with UE or water.

For the protection assays, UE treatments were performed on 1-month-old *M. truncatula* plants as described previously. Two days after the last treatment, plants were inoculated by spraying a spore suspension of *C. trifolii* race 1 (1 mL plant⁻¹), covered with a plastic film (Saran; Dow Chemical Company, Paris-Roissy, France) for 3 d and transferred to a growth chamber (Firlabo, Meysieu, France) with 22 °C/16 h day and 20 °C/8 h night at saturated humidity atmosphere. First symptoms were observed at 7 d post-inoculation (dpi) and photographs were taken with a digital camera at 15 dpi. One month later, aerial parts of the plants were harvested and weighed. Statistical analyses were conducted with the STATLETS software which uses ANOVA method to test for significant differences between means (<http://www.mrs.umn.edu/~sungurea/statlets/statlets.htm>).

Total RNA extraction

Total leaf RNA was extracted with 2:1 (v/v) extraction buffer (0.5 M Tris-HCl, pH 8.2, 0.25 M ethylenediaminetet-

raacetic acid, and 5% sodium dodecyl sulphate): phenol solution, followed by two extractions with chloroform and overnight precipitation with LiCl (3 M final concentration). The RNA pellet was dissolved in H₂O and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The pellet was rinsed with 70% ethanol, air-dried, and dissolved in H₂O. RNA concentrations were measured spectrophotometrically at 260 nm.

RNA gel blot analysis

Each experiment was carried out three times independently. Samples (15 µg total RNA) were denatured at 65 °C for 10 min in 5 × RNA buffer (Sambrook, Fritsch & Maniatis 1989) prior to electrophoresis in a 1.2% (w/v) agarose-formaldehyde gel and transfer overnight to a nylon membrane (Hybond N⁺; Amersham Biosciences, Orsay, France). Equal loading of the gel was checked by visualization under UV light ($\lambda_{254\text{nm}}$) of ribosomal RNA on the membrane. Probes were *M. truncatula* cDNA clones encoding an arabinogalactan protein (TC86688), a glutathione S-transferase (TC85451), an isoflavone reductase (TC85477), a *PR10-1* protein (TC76513) and a ribonuclease (TC77019). Probes were labelled with digoxigenin DIG-dUTP (Roche Diagnostics, Meylan, France) by polymerase chain reaction (PCR) using T3 and T7 as primers and the DNA of interest as a template. Hybridization and detection of DIG-labelled probes were performed according to the manufacturer's instructions. Quantification of hybridization signals was achieved using IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA, USA).

Macroarray preparation

The selection of *M. truncatula* ESTs was based essentially on their putative involvement in plant defence using the TIGR (<http://www.tigr.org>) and the MENS databases (<http://medicago.toulouse.inra.fr/Mt/EST>). A complete list of the genes could be supplied upon request. 165 ESTs belonging to 144 *M. truncatula* tentative consensus sequences (TC) were recovered from MtBA and MtBB (Gamas, de Billy & Truchet 1998) and MtBC (Journet *et al.* 2002) libraries. Eight genomic fragments (TC76726, TC77277, TC77910, TC77988, TC78214, TC85619, TC85687

and TC85808) were amplified by PCR using *M. truncatula* genomic DNA as template and specific primers as indicated in Table 1. These eight ESTs were then cloned in pGEM-T Easy vector (Promega, Charbonnières, France) and verified by sequencing. The 173 DNA fragments were amplified by PCR using universal primers complementary to vector sequences flanking both sides of the DNA insert. Amplimers were analysed by electrophoresis for size, quality and quantity and were adjusted to 0.2–0.5 µg µL⁻¹ with dimethyl sulphoxide 50% (Sigma) and spotted in duplicate onto positively charged nylon membranes (Hybond N⁺ Amersham) using a Eurogridder spotting robot (Plateforme de Génomique Fonctionnelle, Génomôle Toulouse, France).

RNA preparation, radioactive probes synthesis and hybridization

Total RNA was extracted as described above and RNA quality and quantity were assessed with the OD₂₆₀/OD₂₈₀ measurement and gel electrophoresis. Reverse retrotranscription was performed from 40 µg of total RNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies Cergy Pontoise Cedex, France) according to the manufacturer's instructions using 500 mCi mmol⁻¹ of [α -³³P]dCTP (Amersham Pharmacia Biosciences) to generate radioactive probes. Macroarray pre-hybridization, hybridization and washing were performed as described on the Génomôle Toulouse web site (<http://genopole.toulouse.inra.fr>). Subsequently, membranes were exposed to a Phosphor Screen (Molecular Dynamics) for at least 48 h and scanned by a Storm 840 PhosphorImager (Molecular Dynamics).

Data acquisition and analysis

Data acquisition and quantification were performed with IMAGEQUANT software (Molecular Dynamics). To calculate the signal intensities for each spot, a grid was overlaid on the array image and the total intensity of all pixels within each grid was determined.

Normalization procedures were based on the following criteria: (1) subtraction of local background; (2) adjustments of spots lower than background to two times back-

Table 1. Primers used to amplify specific TC from the genomic DNA

TC TIGR ^a	Forward primer (5'-3')	Reverse primer (5'-3')
TC76726	TCAAATTCTCAATTGCCAAGG	CAGTCACTTGTCACCAACCATACC
TC77277	TGTGACAACAAGACAGAATTGG	GCTCTCTAGCTTCTTCTCTGG
TC77910	ACAGAATTCAAAGGGTCATCG	AGGGTCAAACCTCTGGTAACTCC
TC77988	TATCTGAGGGCTTGGGAACG	GCCATTGAGGAGTAGAAATCG
TC78214	CATTCCAATGAGATTTCAGG	GTTGATGCAGAACAAAGCTAACG
TC85619	CCACATGGCCCTAAAAGTAGC	CTAGAACAAGGACTTTAGTGACC
TC85687	ATTAGGGCTCTCTGACTTGG	ACATGAGAAATGGAGTCTGAGG
TC85808	TTTACTGTGATGTGGTGGAACC	AGCAGAAAGCAATTAGCAAAGG

^aTC TIGR, tentative consensus no. according to The Institute of Genome Research

ground value; (3) signal averaging of the two duplicate spots of the same EST and of the spots of ESTs included in the same TC, if the values did not differ by more than 5%; (4) adjustments for differences in the exposure background between membranes; and (5) normalization based on the spots of seven house-keeping genes (TC51283, TC76508, TC84558, TC85141, TC85160, TC86260, TC86577) to remove labelling differences and to control the effects of environmental conditions.

Quality assessments were done to compare the reproducibility of the result by statistical algorithms described by Beissbarth *et al.* (2000). Within a given experiment (duplicate spots of the same EST), the plot of log-2 expression ratios corresponding to the treated and control plants (LR) obtained from the duplicate spots was produced and it was found that 95% of the values fell inside the standard deviation equivalent to LR 0.65 (± 1.6 -fold) so the data were reproducible. The same strategy was used to test the variability between experiments (three independent experiments): 75% of the signals were within LR 0.65. The data from independent experiments using RNA from plants grown in the same conditions and harvested over a period of 1 year showed greater variability. Similar variations have been observed in other microarray experiments (Kawasaki *et al.* 2001; Liu *et al.* 2003). For this reason, the average for the spots from the three independent experiments was not calculated. Accordingly, the ratio of the adjusted signal in treated/control plants for each gene was calculated separately in each independent experiment. To be considered significantly induced or repressed (ratios over 1.5-fold or under 0.5-fold, respectively), a gene had to be differentially expressed in the same way in at least two out of the three independent experiments.

Hierarchical clustering was performed using LR by HIERARCHICAL CLUSTERING EXPLORER 2.0 software (Seo & Shneiderman 2002), available at <http://www.cs.umd.edu/hcil/hce>, using complete linkage. A table of all up-regulated genes (>1.5-fold) is provided in the Appendix.

RESULTS

The *Ulva* extract triggers the induction of a defence-marker gene

To prepare the *Ulva* extract (UE), fresh *Ulva* spp. algae were autoclaved in water, and the resulting solution was filtered and concentrated. The soluble material was precipitated with ethanol, lyophilized and dissolved in water. Total proteins, sugars, mineral elements and phenolics were measured. This preparation contained mainly sugars (43% neutral sugars and 17% uronic acids). Rhamnose was the most abundant neutral sugar residue but xylose and glucose were also present. The sugar composition of the UE showed typical features of a green algal polysaccharide, named ulvan, whose main constituent is a disaccharide unit, β -D-glucuronosyluronic acid (1 \rightarrow 4) L-rhamnose 3 sulphate (Paradossi *et al.* 1999). Low amounts of proteins and phe-

nolics were detected. Analysis of mineral elements revealed the presence of sulphite ions (20%) and sulphur (8%).

As a first approach to look for elicitor activity, the UE at a concentration of 1 mg mL⁻¹ was infiltrated into *M. truncatula* leaflets or sprayed onto plants. An elicitor fraction (P₂), prepared from the tobacco pathogen *Phytophthora parasitica* var. *nicotianae* (Rickauer *et al.* 1990) served as control in the bioassay. Both the UE and P₂ similarly induced the expression of the pathogenesis-related marker gene *PR10* (TC76513; Fig. 1a). However, only the elicitor P₂ produced necrosis, whereas the UE, similar to water, did not show any phytotoxic activity even when infiltrated into the leaflets (Fig. 1b).

The effect of spraying dilutions of UE onto *M. truncatula* plants was followed by analysing *PR10* gene expression. As shown on Fig. 2a, the response of *PR10* was dose and time dependent. A UE concentration of 0.1 mg mL⁻¹ was sufficient to slightly induce the expression of *PR10*. Induction of *PR10* lasted for 8 d at least and was still observable 15 d after treatment with the UE at concentrations of 0.25, 0.5 and 1 mg mL⁻¹.

To investigate whether prior treatment with the UE could potentiate the response to a further treatment, the UE was first sprayed on the leaves of 1-month-old plants, and then again 3 d later, on the same leaves. A solution containing 0.01% Silwett L-77 was sprayed as a control (S). The leaves were harvested 2 d after the last treatment.

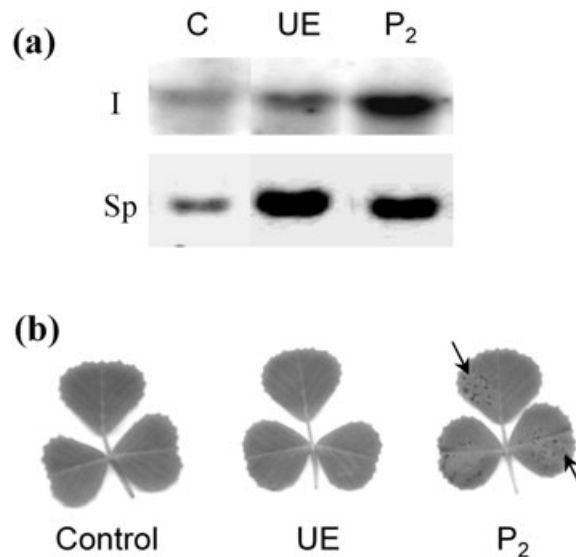


Figure 1. The *Ulva* extract (UE) induced expression of a pathogenesis-related gene (*PR10*) without necrotizing activity. (a) Northern blot analysis of *PR10* (TC76513) was performed 2 d post-treatment of *M. truncatula* plants. UE (UE, 1 mg mL⁻¹), P₂ elicitor (P₂, 0.03 mg mL⁻¹) or water were either infiltrated into leaves (lane I) or sprayed (lane Sp) onto leaves. (b) 15 μ L of UE, P₂ or water (control) were infiltrated into leaflets at a concentration of 1 mg mL⁻¹ and 0.03 mg mL⁻¹, respectively. The photographs were taken 7 d after infiltration. Representative data of three independent experiments are shown. Necrotic lesions induced by the elicitor P₂ are shown by arrows.

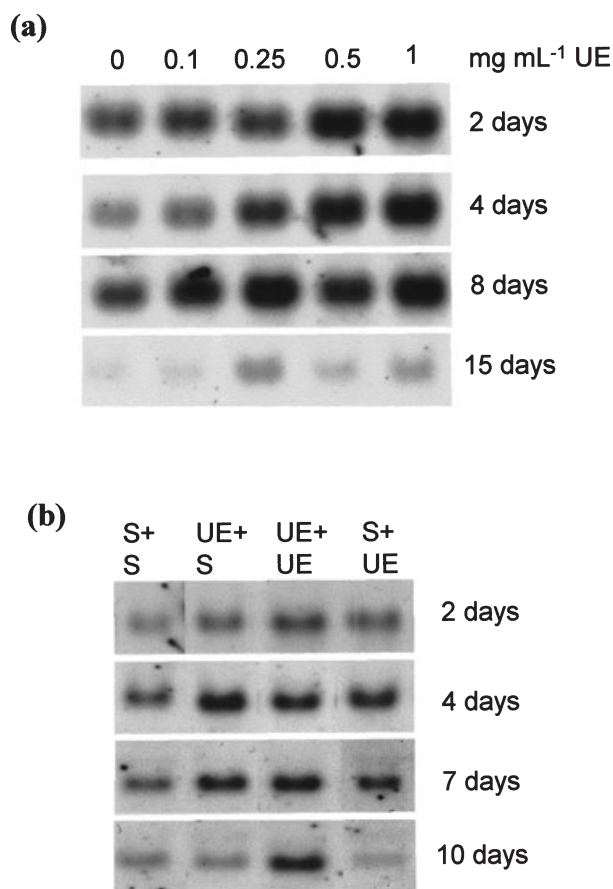


Figure 2. Effect of *Ulva* extract (UE) at increasing concentrations on *PR10* gene expression. One-month-old *M. truncatula* plants were treated with UE at different concentrations. Northern blot analysis of *PR10* (TC76513) was performed on leaves harvested: (a) 2, 4, 8 and 15 d after a single treatment; and (b) 2, 4, 7 and 10 d after two consecutive treatments. In the latter case, UE was sprayed at 1 mg mL⁻¹ and the second treatment was performed 3 d after the first one, resulting in: two UE treatments (UE + UE); one UE and one control treatment (UE + S); one control and one UE treatment (S + UE), and two control treatments (S + S).

Figure 2b shows that expression of *PR10* was strongly induced by either one or two consecutive treatments with the UE compared with two consecutive treatments with the control solution alone. No differences in signal intensity were noticed between one or two treatments with UE. However, whereas the expression of *PR10* started to decrease after 7 d in the case of one treatment, it was prolonged at least up to 10 d when the UE was sprayed twice.

The *Ulva* extract induces a broad range of defence-related genes

Expression profiling of *M. truncatula* ESTs related to defence, signalling and primary metabolism was studied through microarray analysis. A total of 165 ESTs originated from three cDNA libraries made from mRNA of *M. truncatula* roots (Journet *et al.* 2002) and eight DNA fragments were amplified with specific primers from *M. trunca-*

tula genomic DNA. This set of ESTs represented 152 tentative consensus sequences (TC) encoding proteins involved in different aspects of plant defence (phytoalexin biosynthesis, cell wall proteins, PR proteins, oxylipin and ethylene pathway, senescence-HR and abiotic stresses) and proteins involved in nitrogen and carbohydrate metabolism, house-keeping proteins and also nodulins. The leaves of 1-month-old plants were treated once or twice with the UE as described above. Total RNA was extracted from at least 10 plants. Data acquisition and analysis were performed as indicated in Materials and Methods.

A global representation of the changes detected on microarrays is depicted in Fig. 3. Compared to the control treatment, one and two consecutive UE treatments clearly induced the expression of several genes to a similar extent. A single UE treatment (UE) induced genes in all functional categories (Table 2) except one (ethylene), and two consecutive UE treatments (UE + UE) induced slightly more genes than one UE (29 versus 25%). About half of the genes induced by a single UE (UE + S) were still induced 5 d later. Only a few genes, namely the chitinase TC85427, the ascorbate peroxidase TC76384, the lipoxygenase TC85619 and the ribonuclease TC77019 were repressed. Interestingly, UE treatment induced several genes encoding enzymes involved in primary metabolism; these included key enzymes involved in nitrogen metabolism, such as the nitrite reductase, and in carbohydrate metabolism, such as the citrate synthase.

To identify groups of genes with similar expression patterns in UE-treated plants, the microarray data were clustered via hierarchical clustering explorer (Fig. 4). From this analysis, it emerged that a large group of transcripts assembled in cluster I was up-regulated by one and two consecutive UE treatments. This cluster comprises genes from all selected functional categories, notably many genes involved in phytoalexin biosynthesis and genes encoding the PR proteins. Interestingly, in a few cases, genes belonging to the same functional family were tightly co-clustered as illustrated for the five genes related to lipid metabolism comprising one phospholipase D (TC76357), three lipoxygenases (TC85148, TC85171 and TC84245) and one desaturase (TC85814). Likewise, three genes involved in phenylpropanoid and phytoalexin biosynthesis, namely phenylalanine ammonia-lyase (TC85501), chalcone synthase (TC85169) and chalcone isomerase (TC85633) were also co-clustered. Cluster II comprises genes induced by a single treatment but that did not respond to a second UE treatment. This might reflect a desensitization effect of UE treatment on some genes. In contrast, a potentiation effect of UE was illustrated by the genes of cluster III whose induction occurred only after two consecutive treatments. Finally, from the clustering analysis it appeared that within a given treatment, the data from three independent experiments all grouped together, confirming that despite some variability, the overall results were highly similar.

Changes in gene expression revealed by microarray analyses were validated for selected genes by northern blot analysis. Expression of five *M. truncatula* cDNAs

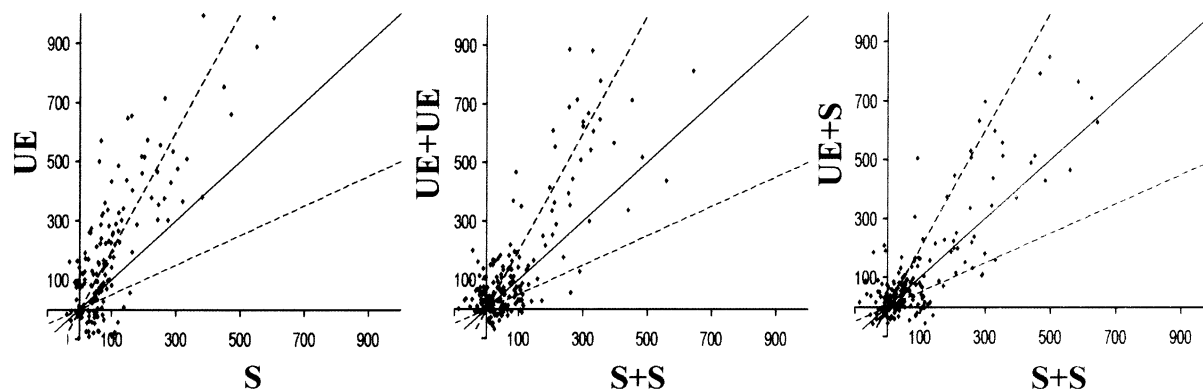


Figure 3. Global transcript changes upon *Ulva* extract (UE) treatment represented as a scatter plot of signal intensities (SI). Comparison of transcript levels from UE treated plants: one UE treatment (UE), two UE treatments (UE + UE), one UE and one control treatment (UE + S), one control treatment (S), and two control treatments (S + S). Each data point represents the SI of an individual gene within a representative experiment (in this case the third experiment). Labelling of axes is in arbitrary units. Dotted diagonal lines represent 1.5-fold induction/repression ratio cut-offs relative to the best fit line through the normalized data. The relative x/y positions in the scatter plots indicate the respective transcript level. A location along the diagonal denotes a similar level of transcript expression in treated and control samples. Data points significantly above or below the diagonal line denote up- and down- regulation as compared to controls.

(TC86688, TC85451, TC85477, TC76513 and TC77019) was followed after UE, UE + UE and UE + S treatments (Fig. 5). The results showed an expression pattern very similar to the profile obtained through macroarray analysis. For example genes encoding PR10-1 (TC76513) and isoflavone reductase (TC85477) were induced by the three treatments (UE, UE + UE, UE + S) in both macroarray analyses and northern blots. Likewise, the gene encoding glutathione *S*-transferase (TC85451) was shown to be only induced after two consecutive UE treatments (UE + UE) both in macroarray analyses and northern blots. Moreover,

a good correlation was found between treated/control ratio values obtained by northern blot and macroarray analyses (Fig. 5 and Appendix).

The *Ulva* extract protects *M. truncatula* plants against the anthracnose fungus *C. trifolii*

The question as to whether induction of defence reactions might protect *M. truncatula* plants against a potential pathogen was then investigated with *C. trifolii*, the causal agent of *Medicago* anthracnose (Barnes *et al.* 1969).

Gene family	Number of TC ^a	Treatment ^b		
		UE ^c	UE + UE ^d	UE + S ^e
Phenylpropanoid pathway	8	4	2	1
Phytoalexin pathway	10	5	6	4
Cell wall protein	17	6	7	2
Oxidative stress	8	2	3	2
Defence	20	5	5	2
Senescence-HR	3	1	1	1
Ethylene	2	0	0	0
Oxylipin pathway	23	1	7	0
Nitrogen pathway	6	2	4	0
Carbohydrate pathway	19	3	2	1
Abiotic stress	3	1	0	0
Signal transduction	8	4	0	0
Nodulin	6	0	1	0
Others	8	1	3	3
Total	141	35	41	16

Table 2. Number of genes induced by UE treatment

^aThese values correspond to the number of TC (TIGR Tentative Consensus sequence) in each gene family. ^bValues are means of three independent treatments, corresponding to the number of induced genes from *Ulva* extract (UE)-treated plants. Only genes induced (ratio > 1.5) in two independent experiments were included. ^cOne UE treatment (UE). ^dTwo UE treatments (UE + UE). ^eOne UE and one control treatment (UE + S).

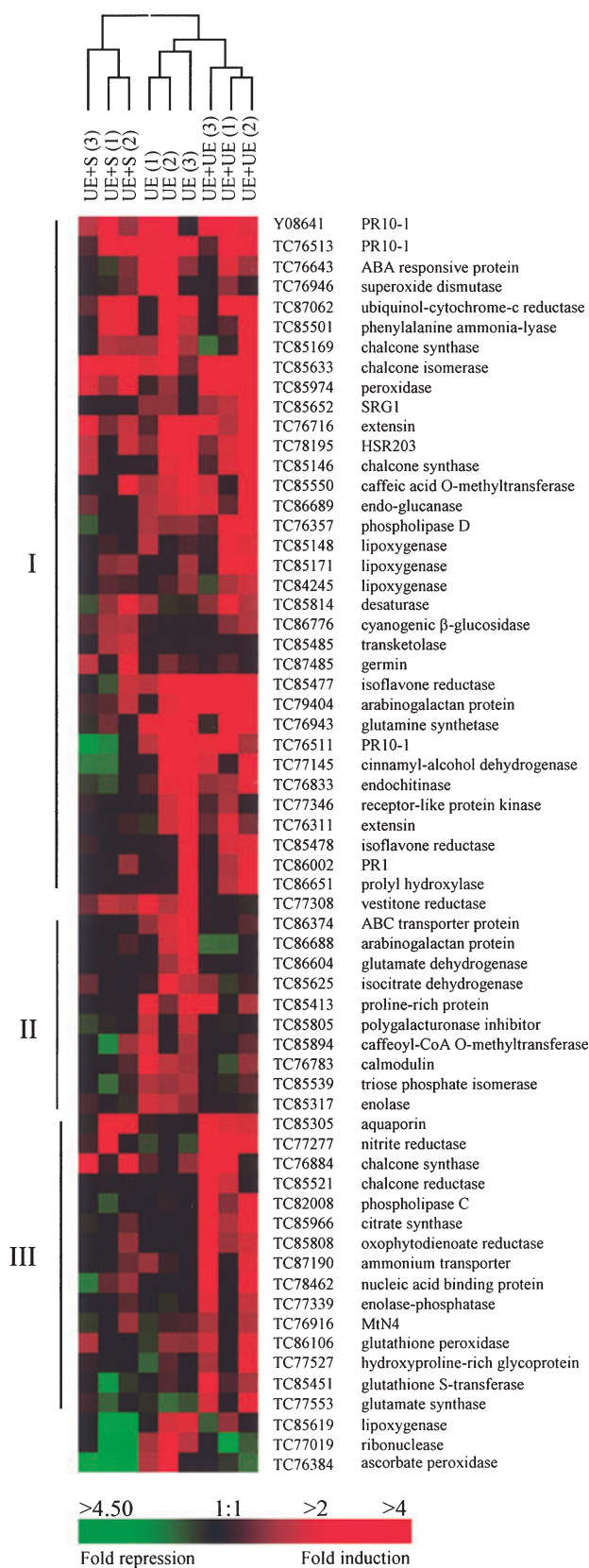


Figure 4. Hierarchical clustering of *M. truncatula* genes after the *Ulva* extract (UE) treatment. One-month-old *M. truncatula* plants were submitted to one UE treatment (UE), two consecutive treatments (UE + UE) or one UE treatment followed by the control solution (UE + S). The treated/control ratios were calculated. For simplicity, only the genes (63) for which the transcript levels changed substantially in at least two replicates were included. Genes were ordered using the HCE clustering program (see Methods) using the complete linkage method. Each gene is represented by a single row of coloured boxes, and each treatment is represented by a single column. Induction (or repression) ranges from pale to saturated red (or green). The results of three independent experiments are shown for each treatment. Cluster I includes genes induced by one and two UE treatments, cluster II comprises genes specifically induced by a single UE treatment, cluster III includes genes only induced by two UE consecutive treatments. TC number and putative function of the genes are indicated on the right of the figure.

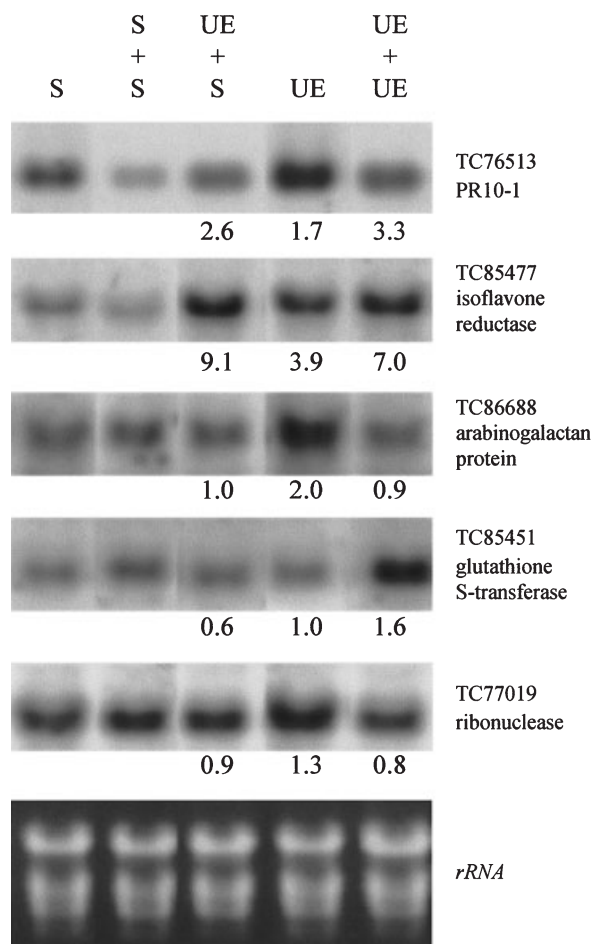


Figure 5. Expression pattern of five *M. truncatula* genes after the *Ulva* extract (UE) treatment. Northern-blot analyses were carried out to verify macroarray results on five selected genes. One single treatment with the elicitor (UE) or the control solution (S), and two consecutive treatments with the elicitor (UE + UE) or the control solution (S + S) were performed. Leaves were harvested 48 h after the last treatment. The ratios of treated/control signals were quantified as described in Materials and Methods, and are provided at the bottom of each blot. Equal loading was checked by visualization under UV light ($\lambda_{254\text{nm}}$) of ribosomal RNA stained with ethidium bromide on the membrane.

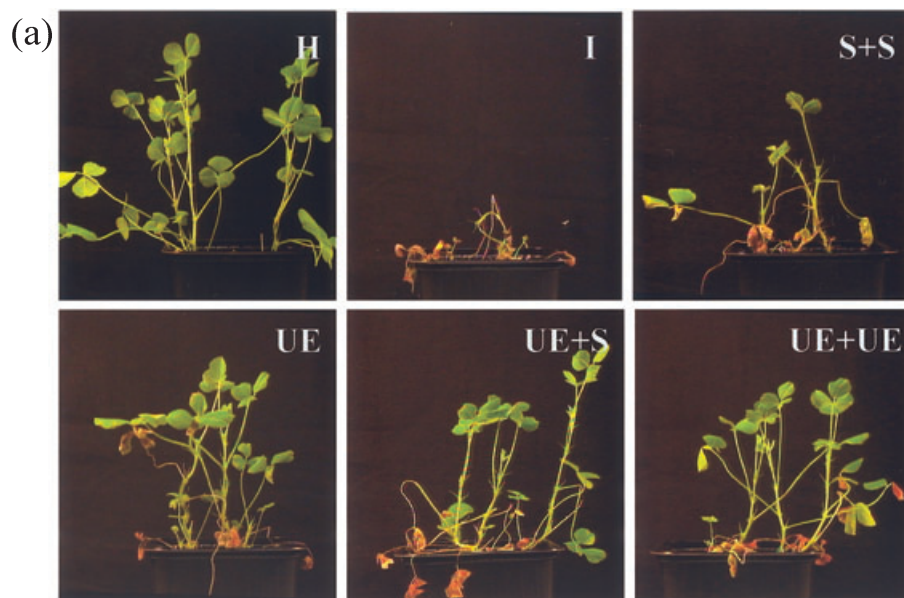
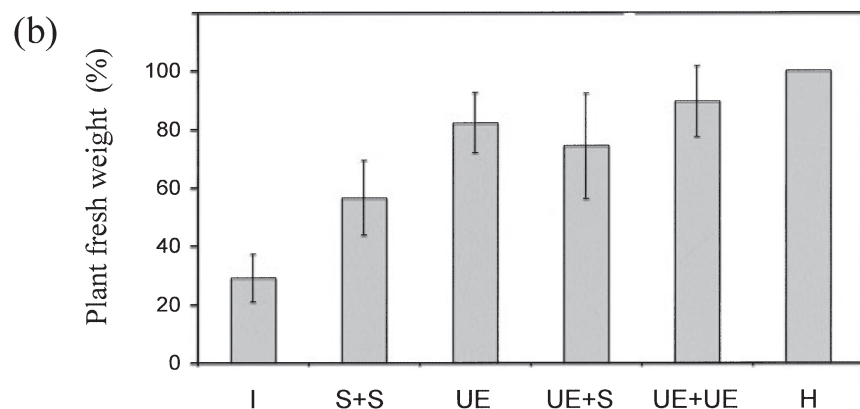


Figure 6. Protection of *M. truncatula* plants against *Colletotrichum trifolii* in response to *Ulva* extract (UE) treatment. UE was sprayed (one or two consecutive treatments) onto 1-month-old plants and 48 h later, plants were either inoculated with a suspension of conidia of *C. trifolii* race 1 or left healthy. H, healthy plants; I, inoculated and untreated; UE, one single treatment with the *Ulva* extract; UE + UE, two consecutive UE treatments; UE + S, one UE treatment followed by the control solution; S + S, two control treatments. (a) Photographs were taken 15 d post-inoculation and are representative of three independent experiments. (b) One month post-inoculation, the aerial part of the plants was harvested and weighed to estimate water loss. The data are expressed as percent fresh weight of healthy unsprayed plants and are the average of three independent experiments. Bars indicate \pm mean deviation. Differences between UE (UE, UE + UE) and control treatment (S + S) are statistically significant at the 95% confidence level as determined by the ANOVA method. The UE + S treatment gave an intermediate result which is not significantly different from the control treatment.



A compatible interaction was observed between *C. trifolii* race 1 and the *M. truncatula* F83005.5 line. The effect of one and two UE treatments on this interaction was evaluated. Plants were inoculated 2 d after the last UE treatment. Results representative of three independent experiments are shown in Fig. 6. Fifteen days after inoculation, the aerial parts of untreated plants were totally necrotic and most plants died (Fig. 6a, I). Control treatments (S + S) led to some decrease of symptom severity. However, a nearly complete protection was obtained after one (UE) or two consecutive (UE + UE) treatments with UE. A UE treatment performed 5 d before inoculation (UE + S) did not confer a significant protection compared to control treatment (S + S).

Consistent with macroscopic observations, untreated and control inoculated plants had lost about 70 and 50%, respectively, of their fresh weight in comparison with healthy plants, whereas only 20 and 10% losses were observed after one or two UE treatments, respectively (Fig. 6b).

DISCUSSION

Elicitation of defence mechanisms is assumed to be a powerful approach for controlling plant diseases and to be an alternative to environmentally undesirable chemical control. The aim of this work was to find a new elicitor extract from green algae and to investigate its effect on an array of plant genes closely related to defence. Although it was already known that brown and red algae contain polysaccharide elicitors such as laminarins, carrageenans and sulphated fucans (Kobayashi *et al.* 1993; Mercier *et al.* 2001; Klarzynski *et al.* 2003), the presence of elicitors in green algae has not been reported. Our extraction protocol was designed to obtain soluble cell wall components of *Ulva* spp. and was very similar to the one recently reported by Paradossi *et al.* (1999) developed to purify ulvans. Accordingly, analysis of the UE revealed the presence of the characteristic soluble sugars of this alga (rhamnose, uronic acids, xylose). The absence of fucose was expected, since green algae, unlike brown algae, do not contain fucans.

To study the elicitor activity of the UE, the model legume *M. truncatula* was chosen. A macroarray mainly composed of defence-related genes was constructed and expression profiling studies on these genes were undertaken after direct spraying of UE on *M. truncatula* leaves. Induction of the *PR10-1* marker gene was significant at 0.1 mg mL⁻¹ and saturated at 0.5 mg mL⁻¹ of UE. The concentration of 1 mg mL⁻¹ that we routinely used was in the same range as those previously reported for other elicitors such as laminarin and carrageenan (Mercier *et al.* 2001; Aziz, Heyraud & Lambert 2003). A significant proportion of defence-related genes was up-regulated after one UE treatment, i.e. 25 and 11% at 2 and 5 d post-treatment, respectively. Among them, genes encoding key enzymes involved in phytoalexin and phenylpropanoid biosynthesis (phenylalanine ammonia-lyase, chalcone synthase, isoflavone reductase, caffeic acid *O*-methyltransferase) as well as several PR genes (chitinase, *PR1* and *PR10-1*) were particularly induced. These effects are shared with other algal compounds. Linear β -1,3 glucans (Klarzynski *et al.* 2000), sulphated fucan oligosaccharides (Klarzynski *et al.* 2003) and carrageenans (linear galactans; Mercier *et al.* 2001) induce similar responses in tobacco. In addition it was shown that UE treatment up-regulated other defence genes whose induction by marine algal elicitors was not previously reported, notably genes encoding cell wall proteins, calmodulin, ribonuclease, aquaporin and *HSR203*. Previous reports have indicated that these genes respond to elicitor treatments and/or pathogen attack (Oppermann, Taylor & Conkling 1994; Chappell *et al.* 1997; Galiana *et al.* 1997; Garcia-Muniz, Martinez-Izquierdo & Puigdomenech 1998; Ali *et al.* 2003).

Induction of defence might proceed at the expense of other forms of metabolism. However, after UE treatment, genes involved in carbon and nitrogen metabolism were not down-regulated. Some genes such as those encoding nitrite reductase and citrate synthase were up-regulated. These observations suggest that upon elicitation with the UE, *M. truncatula* plants did not reduce primary metabolism.

Consistent with its strong elicitor activity, the UE triggered the protection of *M. truncatula* plants against the fungal pathogen *C. trifolii*. Since an *in vitro* assay showed that the UE had no effect on *C. trifolii* development (S. Cluzet, unpublished results), it can be assumed that the protection was only due to the elicitor activity of the UE. This acquired protection is consistent with the observation that β -glucans isolated from brown algae protect wheat against *Septoria tritici* and *Erysiphe graminis*, and reduce tobacco infection by the soft rot pathogen *Erwinia carotovora* ssp. *carotovora* (Joubert *et al.* 1998; Klarzynski *et al.* 2000). Two consecutive UE treatments protected plants more efficiently than one single treatment, in agreement with the fact that *PR10-1* gene expression persisted 7 d after one single UE treatment and at least 10 d after two consecutive UE treatments. It is worth mentioning that two consecutive UE treatments induced several genes that were not affected by a single treatment, particularly genes related to the oxylipin pathway and genes encoding a glu-

tathione *S*-transferase and an isoflavone reductase. This capacity to respond to a pre-treatment is known as potentiation or priming (Conrath, Pieterse & Mauch-Mani 2002). Necrotizing pathogens or chemical inducers of resistance (Ryals *et al.* 1996) as well as non-pathogenic rhizobacteria (van Loon, Bakker & Pieterse 1998) have been shown to trigger potentiation.

This work reports for the first time the use of macroarray analyses to characterize the elicitor activity of a marine algal extract. The results show that the UE is an efficient elicitor of multiple defence responses in *M. truncatula* plants. Moreover, a nearly complete protection against *C. trifolii*, the causal agent of anthracnose disease, was obtained. Since UE is not phytotoxic and does not seem to alter the primary plant metabolism, it may constitute a new source of elicitors for cost-effective, durable, and environmentally friendly disease control.

ACKNOWLEDGMENTS

This research was partly supported by SECMA Biotechnologies Marines and by a grant from 'Région Midi-Pyrénées'. We thank Dr Pascal Gamas for kindly providing the *Medicago truncatula* EST collections, Dr Jean-Marie Prossperi for supplying seeds of *M. truncatula* F83005.5. We are grateful to the 'Plateforme de Génomique Fonctionnelle' (INSA Toulouse) for access to the Eurogridder spotting robot and to Nathalie Ladouce for technical help in robot utilization. We also thank Dr Martin B. Dickman for kindly supplying the *Colletotrichum trifolii* strain and Dr Richard O'Connell for critical reading of the manuscript.

REFERENCES

- Ali G.S., Reddy V.S., Lindgren P.B., Jakobek J.L. & Reddy A.S. (2003) Differential expression of genes encoding calmodulin-binding proteins in response to bacterial pathogens and inducers of defense responses. *Plant Molecular Biology* **51**, 803–815.
- Aziz A., Heyraud A. & Lambert B. (2003) Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against *Botrytis cinerea*. *Planta* **14**, 14.
- Bannerot H. (1965) Résultats de l'infection d'une collection de haricots par six races physiologiques d'anthracnose. *Annales de l'Amélioration Des Plantes* **15**, 201–222.
- Barnes D.K., Ostazeske S.A., Shillinger J.A. & Hanson C.H. (1969) Effect of anthracnose (*Colletotrichum trifolii*) infection on yield, stand and vigor of alfalfa. *Crop Science* **9**, 344–346.
- Beissbarth T., Fellenberg K., Brors B., *et al.* (2000) Processing and quality control of DNA array hybridization data. *Bioinformatics* **16**, 1014–1022.
- Blumenkrantz N. & Absoe-Hansen G. (1973) New method for quantitative determination of uronic acids. *Analytical Biochemistry* **54**, 484–489.
- Boller T. (1995) Chemoperception of microbial signals in plant cells. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 189–214.
- Boudart G., Dechamp-Guillaume G., Lafitte C., Ricart G., Barthe J.P., Mazau D. & Esquerre-Tugaye M.T. (1995) Elicitors and suppressors of hydroxyproline-rich glycoprotein accumulation are solubilized from plant cell walls by endopolygalacturonase. *European Journal of Biochemistry* **232**, 449–457.
- Chappell J., Levine A., Tenhaken R., Lusso M. & Lamb C. (1997)

- Characterization of a diffusible signal capable of inducing defense gene expression in tobacco. *Plant Physiology* **113**, 621–629.
- Conrath U., Pieterse C.M. & Mauch-Mani B. (2002) Priming in plant–pathogen interactions. *Trends in Plant Science* **7**, 210–216.
- Cook D.R. (1999) *Medicago truncatula*—a model in the making!. *Current Opinion in Plant Biology* **2**, 301–304.
- Côté F., Ham K.S., Hahn M.G. & Bergmann C.W. (1998) Oligosaccharide elicitors in host–pathogen interactions. Generation, perception, and signal transduction. In *Subcellular Biochemistry*, Vol. 29: *Plant–Microbe Interactions* (ed. B. B. Ba. H. K. Das), pp. 385–432. Plenum Publishing Corp., New York, USA.
- Fedorova M., van de Mortel J., Matsumoto P.A., Cho J., Town C.D., VandenBosch K.A., Gantt J.S. & Vance C.P. (2002) Genome-wide identification of nodule-specific transcripts in the model legume *Medicago truncatula*. *Plant Physiology* **130**, 519–537.
- Galiana E., Bonnet P., Conrod S., Keller H., Panabieres F., Ponchet M., Poupet A. & Ricci P. (1997) RNase activity prevents the growth of a fungal pathogen in tobacco leaves and increases upon induction of systemic acquired resistance with elicitor. *Plant Physiology* **115**, 1557–1567.
- Gamas P., de Billy F. & Truchet G. (1998) Symbiosis-specific expression of two *Medicago truncatula* nodulin genes, *MtNI* and *MtNI3*, encoding products homologous to plant defense proteins. *Molecular Plant–Microbe Interactions* **11**, 393–403.
- Garcia-Muniz N., Martinez-Izquierdo J.A. & Puigdomenech P. (1998) Induction of mRNA accumulation corresponding to a gene encoding a cell wall hydroxyproline-rich glycoprotein by fungal elicitors. *Plant Molecular Biology* **38**, 623–632.
- Hankins S.D. & Hockey H.P. (1990) The effect of a liquid seaweed extract from *Ascophyllum nodosum* (Fucales, Phaeophyta) on the two-spotted red spider mite *Tetranychus urticae*. *Hydrobiologia* **204/205**, 555–559.
- Jolivet E., Langlais-Jeannin I. & Morot-Gaudry J.F. (1991) Les extraits d'algues marines: propriétés phytoactives et intérêt agronomique. *Année Biologique* **30**, 109–126.
- Joubert J.M., Yvin J.C., Barchietto T., Seng J.M., Plesse B., Klarzynski O., Kopp M., Fritig B. & Kloareg B. (1998) A β -1,3-glucan, specific to a marine alga, stimulates plant defence reactions and induces broad range resistance against pathogens. In *Proceedings of the Brighton Crop Protection Conference – Pests and Diseases*, pp. 441–448. BCPC Press, Farnham, UK.
- Journé E.P., van Tuinen D., Gouzy J., et al. (2002) Exploring root symbiotic programs in the model legume *Medicago truncatula* using EST analysis. *Nucleic Acids Research* **30**, 5579–5592.
- Kawasaki S., Borchert C., Deyholos M., Wang H., Brazille S., Kawai K., Galbraith D. & Bohnert H.J. (2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* **13**, 889–905.
- Klarzynski O., Descamps V., Plesse B., Yvin J.C., Kloareg B. & Fritig B. (2003) Sulfated fucan oligosaccharides elicit defense responses in tobacco and local and systemic resistance against tobacco mosaic virus. *Molecular Plant–Microbe Interactions* **16**, 115–122.
- Klarzynski O., Plesse B., Joubert J.M., Yvin J.C., Kopp M., Kloareg B. & Fritig B. (2000) Linear beta-1,3 glucans are elicitors of defense responses in tobacco. *Plant Physiology* **124**, 1027–1038.
- Kloareg B. & Quatrano R.S. (1988) Structure of cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. *Oceanography and Marine Biology: an Annual Review* **26**, 259–315.
- Kobayashi A., Tai A., Kanzaki H. & Kawazu K. (1993) Elicitor-active oligosaccharides from algal laminaran stimulate the production of antifungal compounds in alfalfa. *Zeitschrift für Naturforschung* **48**, 575–579.
- Kombrink E. & Somssich I.E. (1995) Defense responses of plants to pathogens. *Advances in Botanical Research* **21**, 1–34.
- Liu J., Blaylock L.A., Endre G., Cho J., Town C.D., VandenBosch K.A. & Harrison M.J. (2003) Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. *Plant Cell* **15**, 2106–2123.
- van Loon L.C., Bakker P.A.H.M. & Pieterse C.M.J. (1998) Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* **36**, 453–483.
- Lowry D.H., Rosenbrought N.J., Farr A.L. & Randall R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- Mercier L., Lafitte C., Borderies G., Briand X., Esquerré-Tugayé M.T. & Fournier J. (2001) The algal polysaccharide carrageenans can act as an elicitor of plant defence. *New Phytologist* **149**, 43–51.
- Montaser A. & Golightly D.W. (1992) *Inductively Coupled Plasmas in Analytical Atomic Spectrometry*, 2nd edn. VCH Publishers, New York, USA.
- Oldroyd G.E. & Geurts R. (2001) *Medicago truncatula*, going where no plant has gone before. *Trends in Plant Science* **6**, 552–554.
- Oppermann C.H., Taylor C.G. & Conkling M.A. (1994) Root-knot nematode-directed expression of a plant root-specific gene. *Science* **25**, 221–223.
- Paradossi G., Cavalieri F., Pizzoferrato L. & Liquori A.M. (1999) A physico-chemical study on the polysaccharide ulvan from hot water extraction of the macroalga *Ulva*. *International Journal of Biology Macromolecules* **25**, 309–315.
- Rickauer M., Fournier J., Pouéat M.L., Berthalon E., Bottin A. & Esquerré-Tugayé M.T. (1990) Early changes in ethylene synthesis and lipoxygenase activity during defense induction in tobacco cells. *Plant Physiology and Biochemistry* **28**, 647–653.
- Roux C., Mazau D., Rickauer M., Fournier J., Berthalon E., Bottin A. & Esquerre-Tugayé M.T. (1994) Hydroxyproline-containing fragments in the cell wall of *Phytophthora parasitica*. *Phytochemistry* **35**, 591–595.
- Ryals J.A., Neuenschwander U.H., Willits M.G., Molina A., Steiner H.Y. & Hunt M.D. (1996) Systemic acquired resistance. *Plant Cell* **8**, 1809–1819.
- Sambrook J., Fritsch E.F. & Maniatis T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
- Seo J. & Shneiderman B. (2002) Interactively exploring hierarchical clustering results. *IEEE Computer* **35**, 80–86.
- Singleton V.L. & Ross J.A.J. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* **16**, 144–153.

Received 11 December 2003; received in revised form 2 March 2004; accepted for publication 12 March 2004

APPENDIX

List of all up-regulated genes (1st part)

Class	TC TIGR ^b	Putative function	GB accession ^c	TC MENS ^d	Treatment ^a								
					UE ^e			UE + UE ^f			UE + S ^g		
					1	2	3	1	2	3	1	2	3
Phenylpropanoid pathway	TC85501	phenylalanine ammonia-lyase	AL372483	MtC00502_GC	NS	2.33	1.83	1.18	4.44	1.00	2.10	5.65	NS
	TC85550	caffeic acid <i>O</i> -methyltransferase	AL367074	MtD00040_GC	1.52	1.93	2.54	NS	4.33	1.90	NS	2.33	0.92
	TC85894	caffeoyl-CoA <i>O</i> -methyltransferase	AL368189	MtC10945_GC	2.03	0.67	1.57	NS	1.22	NS	0.64	1.48	NS
	TC77145	cinnamyl-alcohol dehydrogenase	AL372163	MtC30100_GC	1.00	5.47	4.33	1.03	3.40	2.01	0.70	NS	0.70
	TC76884	chalcone synthase	AL369218	MtC00212_GC	1.20	NS	1.45	4.92	0.96	4.01	1.06	2.66	2.35
	TC85146	chalcone synthase	AL368203	MtC10863_2_GC	1.00	2.09	2.34	2.00	NS	1.13	0.76	NS	1.80
	TC85169	chalcone synthase	AL370220,AL385833	MtC00785_GC	1.51	2.93	1.54	2.17	0.97	2.42	0.73	1.62	1.51
	TC85521	chalcone reductase	AL381630	MtC00294_GC	1.00	1.00	NS	1.85	1.00	2.95	1.00	1.00	NS
	TC85633	chalcone isomerase	AL381790	singleton	2.01	3.31	1.25	4.88	6.04	5.17	2.33	3.12	4.83
	TC85477	isoflavone reductase	AL384237	MtC00234_1_GC	1.68	3.61	2.55	3.41	2.25	5.99	0.81	1.85	5.36
Cell wall proteins	TC85478	isoflavone reductase	AL383870	MtC00234_2_GC	1.00	1.00	NS	1.99	2.09	1.00	1.00	1.00	1.08
	TC77308	vestitone reductase	AL383703, AL384920	MtC20122_GC	3.96	1.92	3.09	NS	1.00	1.00	1.00	2.47	1.15
	TC76311	extensin	AL381854	MtC00023_1_GC	1.00	2.76	1.79	2.00	2.34	1.47	2.23	4.56	1.37
	TC76716	extensin	AL373614	MtC00611_1_GC	1.15	3.08	2.59	1.64	3.51	2.12	1.30	1.79	2.22
	TC77527	hydroxyproline-rich glycoprotein	AL370995	MtC45475_GC	0.77	1.19	NS	NS	2.50	1.56	NS	NS	1.06
	TC79404	arabinogalactan protein	AL368602	MtC11007_GC	1.00	2.08	5.16	3.33	3.37	2.97	1.18	1.78	1.11
	TC86688	arabinogalactan protein	AL381434	MtC10757_GC	1.00	1.94	4.04	0.75	NS	0.74	0.54	1.14	NS
	TC85413	proline-rich protein	AL386974	MtC10169_GC	3.97	1.29	2.99	NS	1.60	2.59	NS	NS	NS
	TC86651	prolyl hydroxylase	AL367499	MtC20071_GC	1.00	1.00	3.30	1.69	2.50	1.00	1.00	2.90	1.00
	TC86689	endo-1,3- β -D-glucanase	AL387547	MtC00792_GC	1.62	1.89	2.09	1.19	2.51	2.00	NS	NS	1.28
Defence	TC76511	PR10-1	AL382676	MtC00219_GC	1.72	5.69	4.40	3.43	1.54	2.15	0.65	NS	0.58
	TC76513	PR10-1	AL373773	MtC00009_GC	2.14	12.14	2.69	3.29	3.04	1.18	2.41	2.85	1.15
	-	PR10-1	Y08641	MtC00009_GC	5.12	19.05	0.99	11.22	2.07	2.70	3.92	1.47	1.67
	TC76833	endochitinase	AL380364	MtC10306_GC	1.16	2.19	2.61	1.52	2.22	1.43	0.81	NS	0.89
	TC85652	SRG1	AL379718	MtC30088_GC	1.41	1.40	NS	1.71	3.50	1.48	NS	1.00	NS
	TC85805	polygalacturonase inhibitor	AL381114	MtC10369_GC	1.80	1.00	1.89	0.97	NS	1.00	0.97	NS	0.86
	TC86002	PR1	AL386306	MtC00352_GC	1.00	1.00	5.22	1.63	2.81	1.00	1.00	1.45	1.00
	TC76384	ascorbate peroxidase	AL367369	MtC00008_GC	1.60	2.40	1.00	0.90	0.74	NS	0.42	0.26	0.55
	TC85974	peroxidase	AL371851	MtC10717_GC	1.05	1.46	NS	2.77	5.38	2.69	1.47	3.30	2.05
	TC76946	superoxide dismutase	AL375556	MtC00676_GC	2.49	3.12	1.00	1.40	NS	1.00	0.95	1.29	0.98
Senescence-HR	TC86106	glutathione peroxidase	AL374155	MtC00308_GC	0.88	1.43	1.40	NS	2.08	1.00	NS	NS	1.61
	TC85451	glutathione <i>S</i> -transferase	AL368847	MtC10018_GC	1.00	1.16	1.00	1.15	1.55	3.01	NS	0.90	1.02
	TC87485	germin-like	AL373691	singleton	NS	1.32	1.10	1.16	NS	NS	1.00	2.55	1.75
	TC78195	HSR203	AL366024	MtC40065_GC	1.34	2.58	4.38	1.98	2.78	1.31	NS	1.85	1.74

Appendix Continued

Class	TC TIGR ^b	Putative function	GB accession ^c	TC MENS ^d	Treatment ^a								
					UE ^e			UE + UE ^f			UE + S ^g		
					1	2	3	1	2	3	1	2	3
Lipid-related signalling	TC76357	phospholipase D	AL383583,AL387293	MtC00743_GC	1.60	1.39	1.44	2.75	5.49	2.40	1.18	3.52	NS
	TC82008	phospholipase C	AL380498	MtC20027_GC	1.00	1.04	NS	1.37	4.17	3.45	0.80	NS	NS
	TC84245	lipoxigenase	AL371045,AL389771	MtC10274_GC	1.00	1.14	1.46	1.57	1.86	0.83	1.18	1.12	NS
	TC85148	lipoxigenase	AL370268,AL381315	MtC00630_GC	1.75	NS	1.11	2.04	2.03	1.03	1.09	NS	NS
	TC85171	lipoxigenase	AL378899,AL380164	MtC40012_GC	1.00	NS	1.53	2.27	2.75	NS	1.37	1.47	NS
Nitrogen pathway	TC85619	lipoxigenase			1.00	1.93	2.05	1.44	NS	0.78	0.47	0.47	NS
	TC85808	oxophytodienoate reductase			1.00	NS	1.00	1.49	1.92	4.42	1.00	1.45	NS
	TC85814	desaturase	AL367066,AL377575	MtC20093_GC	1.54	0.91	0.95	2.28	1.63	1.41	1.44	3.38	0.83
	TC76943	glutamine synthetase	AL366171	MtC00744_GC	2.19	4.33	4.06	2.88	2.82	0.94	1.28	NS	0.94
	TC77277	nitrite reductase			0.84	NS	0.83	3.56	2.20	2.34	2.83	1.00	1.10
Carbohydrate pathway	TC77553	glutamate synthase	AL366890	MtC20017_GC	0.92	0.74	0.82	NS	2.28	1.97	0.78	NS	1.09
	TC86604	glutamate dehydrogenase	AL372216	MtC20111_GC	1.00	1.51	2.21	1.00	NS	NS	1.01	NS	NS
	TC87190	ammonium transporter	AL370643	MtC30408_GC	1.47	1.07	NS	NS	2.09	2.54	NS	1.27	NS
	TC77339	enolase-phosphatase	AL378849	MtC10352_GC	1.00	0.93	NS	NS	1.79	2.09	NS	1.20	NS
	TC85317	enolase	AL367711	MtC10068_GC	1.93	1.75	1.43	NS	0.96	NS	NS	0.92	1.12
Abiotic stress	TC85485	transketolase	AL373090	MtC20051_2_GC	1.00	1.00	1.00	1.00	NS	1.00	1.62	1.90	NS
	TC85539	triosephosphate isomerase	AL365666	MtC00059_GC	1.61	1.47	1.61	0.94	1.23	NS	0.74	1.08	NS
	TC85625	isocitrate dehydrogenase	AL368524	MtC10579_1_GC	1.07	2.67	1.70	0.87	1.07	1.44	NS	NS	1.25
	TC85966	citrate synthase	AL374303	MtC30134_GC	1.00	NS	NS	1.59	2.44	2.24	NS	1.25	0.95
	TC77019	ribonuclease	AL371802	MtC10459_GC	1.52	2.72	1.67	0.47	0.80	NS	0.22	0.31	NS
Signal transduction	TC77346	receptor-like protein kinase	AL383027,AL384392	MtC00228_GC	1.66	1.44	4.89	1.28	NS	1.06	1.00	NS	1.03
	TC76783	calmodulin	AL378480	MtC00080_GC	2.40	1.71	1.52	0.82	1.46	NS	NS	1.17	NS
	TC76643	ABA responsive protein	AL373345	MtD22154_GC	3.27	2.60	1.42	2.04	1.48	NS	0.87	1.32	1.04
	TC86374	ABC transporter protein	AL365693	MtC00386_GC	1.44	1.98	2.71	NS	1.16	NS	NS	NS	NS
	TC76916	MtN4	AL376203	MtC10024_GC	0.89	1.11	1.14	NS	1.63	1.58	NS	1.48	0.88
Others	TC86776	cyanogenic beta-glucosidase	AL370555	MtC50294_GC	1.03	NS	1.00	1.38	1.65	1.00	1.56	1.64	1.19
	TC78462	nucleic acid binding protein	AL367624	MtC20082_GC	1.00	NS	NS	NS	3.41	3.30	1.23	1.47	0.71
	TC85305	aquaporin	AL370135	MtC00001_GC	1.04	NS	NS	2.02	1.93	2.61	3.17	2.58	0.96
	TC87062	ubiquinol-cytochrome-c reductase	AL386789	MtC00336_GC	NS	4.41	4.54	3.81	8.96	NS	2.12	6.17	1.21

^aRatios correspond to signal intensities from *Ulvax* extract (UE)-treated plants relative to control plants. Only genes induced (ratio > 1.5; bold type) in at least two independent experiments were included. When we compared the three replicate experiments, the ratio of a single gene has not to be induced in one replicate and repressed in at least one of the others, otherwise it was not considered significant (NS). ^bTC TIGR, Tentative Consensus No. according to The Institute of Genome Research. ^cGB, GeneBank No. accession. ^dTC MENS, Tentative Consensus No. according to Medicago EST Navigation System. ^eUE, one single UE treatment. ^fUE + UE, two consecutive UE treatments. ^gUE + S, one UE and one control treatment.