

# Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*

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## Summary

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Received: 24 April 2008

Accepted: 10 June 2008

- Upon appropriate stimulation, plants can develop an enhanced capacity to express infection-induced cellular defense responses, a phenomenon known as the primed state. Colonization of the roots of *Arabidopsis thaliana* by the beneficial rhizobacterial strain *Pseudomonas fluorescens* WCS417r primes the leaf tissue for enhanced pathogen- and insect-induced expression of jasmonate (JA)-responsive genes, resulting in an induced systemic resistance (ISR) that is effective against different types of pathogens and insect herbivores.
- Here the molecular mechanism of this rhizobacteria-induced priming response was investigated using a whole-genome transcript profiling approach.
- Out of the 1879 putative methyl jasmonate (MeJA)-responsive genes, 442 genes displayed a primed expression pattern in ISR-expressing plants. Promoter analysis of ISR-primed, MeJA-responsive genes and ISR-primed, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000)-responsive genes revealed over-representation of the G-box-like motif 5'-CACATG-3'. This motif is a binding site for the transcription factor MYC2, which plays a central role in JA- and abscisic acid-regulated signaling. MYC2 expression was consistently up-regulated in ISR-expressing plants. Moreover, mutants impaired in the *JASMONATE-INSENSITIVE1/MYC2* gene (*jin1-1* and *jin1-2*) were unable to mount WCS417r-ISR against *Pst* DC3000 and the downy mildew pathogen *Hyaloperonospora parasitica*.
- Together, these results pinpoint MYC2 as a potential regulator in priming for enhanced JA-responsive gene expression during rhizobacteria-mediated ISR.

**Key words:** *Arabidopsis thaliana*, defense signaling, ISR, jasmonic acid, microarray, MYC2, priming, *Pseudomonas fluorescens*.

*New Phytologist* (2008) **180**: 511–523

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doi: 10.1111/j.1469-8137.2008.02578.x

## Introduction

To survive, living organisms have evolved complex mechanisms to detect aggressors and to defend themselves. Resistance against pathogens and insect herbivores can be mediated through defenses that are constitutively present, or through defense mechanisms that are induced only upon attack. Induced

resistance is typically effective against a broad range of attackers and is often associated with the production of defensive compounds such as pathogenesis-related (PR) proteins with antimicrobial activity (Van Loon *et al.*, 2006b), proteinase inhibitors that affect insect feeding (Howe, 2004), or volatiles that attract parasitoids and predators of the herbivores that feed on the plant (Van Poecke & Dicke, 2004). Induced plant defenses are regulated by a highly interconnected signaling network in which the plant hormones jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) play central roles

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(Durrant & Dong, 2004; Pozo *et al.*, 2004; Van Loon *et al.*, 2006a; Asselbergh *et al.*, 2008). It is thought that spatio-temporal intensities of these alarm signals determine the specific nature of the defense response triggered, as marked by the activation of an attacker-specific set of defense-related genes (Reymond & Farmer, 1998; Rojo *et al.*, 2003; De Vos *et al.*, 2005; Mur *et al.*, 2006; Koornneef *et al.*, 2008). The outcome of the induced defense response seems to be finely tuned by cross-talk between the corresponding signaling pathways (Koornneef & Pieterse, 2008) and regulatory elements in the promoters of the defense-related genes, resulting in quantitative and/or kinetic effects on the induced resistance response (Katagiri, 2004).

The enhanced defensive capacity of plants expressing induced resistance often cannot be attributed to direct activation of defenses. Instead, induced resistance seems to be based on a faster and stronger activation of basal defense mechanisms upon exposure to pathogens or insect attack. It is therefore hypothesized that the broad-spectrum characteristic of induced resistance is largely based on potentiation of the tissue to react more effectively to a stress condition, rather than on direct activation of defenses (Conrath *et al.*, 2006; Frost *et al.*, 2008). Plant cells can be sensitized to react more rapidly and/or more strongly to environmental stresses upon appropriate stimulation. This phenomenon is called priming (Conrath *et al.*, 2002) and can be induced biologically by beneficial rhizobacteria, mycorrhizal fungi, pathogens, and insect herbivores, or chemically by, for example, exogenous application of low doses of SA, its functional analog benzothiadiazole (BTH), JA or  $\beta$ -aminobutyric acid (BABA) (Conrath *et al.*, 2006; Frost *et al.*, 2008). In primed plants, defense responses are not induced directly by the priming agent, but are activated in an accelerated manner following perception of biotic or abiotic stress signals, resulting in an enhanced level of resistance against the stressor encountered. Priming is a common feature of different types of induced resistance and may represent an important ecological adaptation to resist environmental stress (Heil & Silva Bueno, 2007; Pieterse & Dicke, 2007; Walters *et al.*, 2007). By studying the costs and benefits of priming in *Arabidopsis thaliana*, it was recently shown that the fitness costs of priming are lower than those of constitutively activated defenses, and that the costs of priming are outweighed by its benefits under conditions of disease pressure, suggesting that priming is a cost-effective strategy of plants to cope with environmental stress (Van Hulten *et al.*, 2006).

Priming in beneficial plant–microbe interactions has been studied most extensively in the interaction of plants with plant growth-promoting rhizobacteria. These nonpathogenic rhizobacteria are abundantly present on the surface of plants roots, where they utilize nutrients that are exuded by the roots. Selected rhizobacterial strains are capable of reducing disease incidence in above-ground plant parts through a plant-mediated defense mechanism known as induced systemic resistance (ISR) (Van Loon *et al.*, 1998). Rhizobacteria-mediated ISR has been

demonstrated in many plant species, and is effective against a broad spectrum of plant pathogens, including oomycetes, fungi, bacteria and viruses (Van Loon *et al.*, 1998), and even insect herbivores (Van Oosten *et al.*, 2008). In *A. thaliana*, ISR triggered by *Pseudomonas fluorescens* WCS417r is regulated by a JA- and ET-dependent signaling pathway (Pieterse *et al.*, 1998). In contrast to pathogen-induced systemic acquired resistance (SAR), WCS417r-ISR is not associated with a direct activation of genes encoding pathogenesis-related (PR) proteins (Pieterse *et al.*, 1996). Analysis of the *A. thaliana* transcriptome revealed that, locally in the roots, ISR-inducing WCS417r bacteria elicited a substantial change in the expression of almost 100 genes (Verhagen *et al.*, 2004; Léon-Kloosterziel *et al.*, 2005; Van der Ent *et al.*, 2008). However, systemically in the leaves, no consistent alteration in gene expression was observed, demonstrating that the onset of ISR is not associated with detectable changes in gene expression (Van Wees *et al.*, 1999; Verhagen *et al.*, 2004). In addition, no alterations in the production of either JA or ET were detected in the leaves of induced plants expressing WCS417r-ISR, suggesting that the state of ISR relies on an enhanced sensitivity to these plant hormones rather than an increase in their production (Pieterse *et al.*, 2000).

Analysis of the transcriptome of ISR-expressing *A. thaliana* leaves after challenge inoculation with the bacterial speck pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) revealed 81 genes with amplified expression patterns, indicating that the plants were primed to respond more rapidly and/or more strongly to pathogen attack (Verhagen *et al.*, 2004). The majority of these primed genes were predicted to be regulated by JA and/or ET, confirming earlier findings that colonization of the roots by WCS417r primed *A. thaliana* plants for potentiated expression of the JA- and/or ET-responsive genes *VEGETATIVE STORAGE PROTEIN2* (*VSP2*; At5g24770), *PLANT DEFENSIN1.2* (*PDF1.2*; At5g44420), and *HEVEIN-LIKE PROTEIN* (*HEL*; At3g04720) (Van Wees *et al.*, 1999; Hase *et al.*, 2003; Van Oosten *et al.*, 2008). Other ISR-inducing rhizobacteria, but also ISR-inducing mycorrhizal fungi and other beneficial fungi, have been demonstrated to enhance the defensive capacity of the plant by priming the plant for potentiated expression of defense-related genes (Van Wees *et al.*, 2008). This strongly suggests that priming for enhanced defense is a common mechanism in the immune response that is triggered by beneficial micro-organisms.

To gain more insight into how ISR-related priming is regulated, we used a genome-wide expression profiling approach to search for JA-responsive *A. thaliana* genes that show an augmented expression pattern in plants expressing WCS417r-ISR. Subsequently, we performed an *in silico* promoter analysis to identify motifs that are potentially involved in the regulation of the priming response. Here, we provide evidence that the motif CACATG is significantly enriched in the 1-kb promoter regions of the JA-responsive ISR-primed genes. The CACATG motif has previously been demonstrated to be a binding site

for the MYC-type helix-loop-helix transcription factor MYC2. We show that mutants *jin1-1* and *jin1-2*, which are impaired in the *JASMONATE-INSENSITIVE1/MYC2* gene (At1g32640), are blocked in their ability to express WCS417r-ISR, indicating that the MYC2 transcription factor is an essential regulator of WCS417r-ISR.

## Materials and Methods

### Growth conditions for rhizobacteria and plants

Nonpathogenic *Pseudomonas fluorescens* WCS417r bacteria were used for the induction of ISR. WCS417r was grown for 24 h at 28°C on King's medium B agar plates containing the appropriate antibiotics as described previously (Pieterse *et al.*, 1996). Bacteria were collected and re-suspended in 10 mM MgSO<sub>4</sub> to a density of 10<sup>9</sup> colony-forming units (cfu) ml<sup>-1</sup> (optical density (OD<sub>660</sub>) = 1.0) before being mixed through soil.

Seeds of wild-type *Arabidopsis thaliana* (L.) Heynh. accessions Columbia (Col)-0 and Col-5 (the *gl1* mutant of Col-0, carrying a mutation in *GLABROUS 1 (GL1)*; At3g27920) resulting in a trichomeless phenotype (Oppenheimer *et al.*, 1991), the *MYC2* mutants *jin1-1* and *jin1-2* (Col-0 background; Lorenzo *et al.*, 2004), the JA-insensitive mutant *coi1-16* (Col-5 background), which is impaired in the *CORONATINE-INSENSITIVE1* gene (At2g39940) (Ellis & Turner, 2002), and the ISR-minus mutant *npr1-1* (Col-0 background), which is impaired in the *NONEXPRESSOR OF PATHOGENESIS RELATED GENES1* gene (At1g64280) (Cao *et al.*, 1994; Pieterse *et al.*, 1998) were sown in quartz sand. Two-week-old seedlings were transferred to 60-ml pots containing a sand/potting soil mixture that had been autoclaved twice for 20 min at a 24-h interval. Before transfer of the seedlings, a suspension of ISR-inducing WCS417r bacteria (10<sup>9</sup> cfu ml<sup>-1</sup>) was mixed through the soil to a final density of 5 × 10<sup>7</sup> cfu g<sup>-1</sup>, as described previously (Pieterse *et al.*, 1996). Control soil was supplemented with an equal volume of 10 mM MgSO<sub>4</sub>. Plants were cultivated in a growth chamber with a 9-h day (200 µmol m<sup>-2</sup> s<sup>-1</sup> at 24°C) and a 15-h night (20°C) cycle at 70% relative humidity. Plants were watered twice a week with water and once a week with modified half-strength Hoagland nutrient solution.

### Methyl jasmonate treatment

Induction treatment with methyl jasmonate (MeJA) was performed by dipping 5-wk-old Col-0 or Col-5 plants in an aqueous solution containing 50 µM MeJA (Serva; Brunschwig Chemie, Amsterdam, the Netherlands) and 0.015% of the surfactant Silwet L-77 (Van Meeuwen Chemicals B.V., Weesp, the Netherlands), as described previously (Pieterse *et al.*, 1998). Leaf rosettes were harvested at 0, 1, 3, 6 and 12 h after induction treatment and immediately frozen in liquid nitrogen. Because the expression levels of the JA-responsive genes studied are

affected by light (data not shown), plants were kept under continuous light during the 12-h time-course to avoid disruption of the time-course by a dark period.

### Pathogen inoculations and ISR bioassays

The virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Whalen *et al.*, 1991) and the oomycete pathogen *Hyaloperonospora parasitica* strain WACO9 (Van Hulten *et al.*, 2006) were used for challenge inoculations. *Pst* DC3000 was grown overnight in liquid King's medium B at 28°C. Bacterial cells were collected by centrifugation and re-suspended to a final density of 2.5 × 10<sup>7</sup> cfu ml<sup>-1</sup> in 10 mM MgSO<sub>4</sub> containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV). Five-week-old plants were placed at 100% relative humidity 1 d before challenge inoculation. Plants were inoculated by dipping the leaves for 2 s in a *Pst* DC3000 suspension. To confirm expression of ISR in WCS417r-treated plants, ISR bioassays were performed as described previously (Pieterse *et al.*, 1996), using a subset of plants grown in parallel with the plants for the chemical treatment. Four days after challenge inoculation, disease severity was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when they showed necrotic or water-soaked lesions surrounded by chlorosis. The disease index was calculated for each plant (*n* = 20), based on the percentage of diseased leaves.

*Hyaloperonospora parasitica* bioassays were performed as described previously (Van Hulten *et al.*, 2006; Van der Ent *et al.*, 2008). Three-week-old plants were misted with an *H. parasitica* WACO9 spore suspension containing 7.5 × 10<sup>4</sup> sporangiospores ml<sup>-1</sup>. Inoculated plants were maintained at 17°C and 100% relative humidity for 24 h. Subsequently, humidity was lowered to 70% to reduce effects on plant development and to lower the chance of secondary infections by opportunistic pathogens. Seven days after challenge inoculation, humidity was raised again to 100% to induce sporulation. Disease symptoms were scored for ~250 leaves per treatment at 9 d after inoculation. Disease rating was expressed on the basis of symptom severity and pathogen sporulation on each leaf: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered by sporangia; IV, > 50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. In addition, 15 leaves per treatment were stained for quantification of callose deposition.

### Callose staining

Quantification of callose deposition was performed as described by Ton *et al.* (2005). Leaves were collected 2 d after *H. parasitica* inoculation and incubated overnight in 96% ethanol. Destained leaves were washed in 0.07 M phosphate buffer, pH 9, incubated for 15 min in 0.07 M phosphate buffer containing 0.005% Calcofluor (fluorescent brightener; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and 0.01% aniline-blue (water blue; Merck, Darmstadt, Germany), and then washed in 0.07 M

phosphate buffer containing only 0.01% aniline-blue to remove excess Calcofluor. Observations were performed with an epifluorescence microscope with a UV filter (band pass 340–380 nm; long-path 425 nm). Callose depositions were quantified by determining the percentage of callose-inducing spores per infected leaf.

### RNA gel-blot analysis

Total RNA was obtained by phenol/chloroform extraction and LiCl precipitation, as described by Sambrook *et al.* (1989). For RNA gel-blot analysis, 15 µg of total RNA was denatured in formamide, electrophoretically separated on 1.2% formaldehyde agarose gels and blotted onto Hybond-N<sup>+</sup> membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. Equal loading was visualized by ethidium bromide staining of rRNA. Templates for the preparation of gene-specific probes were prepared by PCR with primers based on the annotated gene sequences of *LIPOXYGENASE* (*LOX2*; At3g45140), a gene encoding a protein with β-glucosidase/myrosinase activity (*PYK10*; At3g09260), *NIM1-INTERACTING1* (*NIMINI*; At1g02450), and a gene encoding WRKY transcription factor 54 (*WRKY54*; At2g40750). DNA probes were labeled with α-<sup>32</sup>P-dCTP by random primer extension and hybridizations were carried out overnight at 42°C using Ultrahyb (Ambion, Huntingdon, UK). Blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, the Netherlands) with QUANTITY ONE software (BioRad).

### Quantitative real-time PCR (Q-PCR)

Gene expression analysis by Q-PCR was performed basically as described previously (Van der Ent *et al.*, 2008). To check for contamination with genomic DNA, a PCR with primers designed for *ETHYLENE-INSENSITIVE-LIKE2* (*EIL2*; At5g21120: EIL2<sub>F</sub>, 5'-TCT CGT GAG ACG GTC TAG AAG TT-3', and EIL2<sub>R</sub>, 5'-ATG AAA CCT AAT CTT CTC CAT TGC-3') was carried out. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT<sub>20</sub> primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and SuperScript<sup>TM</sup> III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by Q-PCR, using primers of the constitutively expressed gene *UBIQUITIN10* (*UBQ10*; At4g05320: UBQ10<sub>F</sub>, 5'-AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3', and UBQ10<sub>R</sub>, 5'-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3'). Based on the results, cDNA of each sample was diluted to obtain a *UBQ10* threshold cycle (*C<sub>T</sub>*) value of 18 ± 0.5. Gene-specific primers for *MYC2* (At1g32640: MYC2<sub>F</sub>, 5'-GAT GAG GAG GTG ACG GAT ACG GAA-3', and MYC2<sub>R</sub>, 5'-CGC TTT ACC AGC TAA TCC CGC A-3') were designed previously by Czechowski *et al.* (2004). Q-PCR reactions were performed in a volume of 20 µl,

containing cDNA, 0.5 µl of each of the two gene-specific primers (10 pmol µl<sup>-1</sup>), and 10 µl of 2 × IQ SYBR<sup>®</sup> Green Supermix reagent (BioRad, Veenendaal, the Netherlands). The following PCR program was used for all PCR reactions: 95°C for 3 min; 40 cycles of 95°C for 30 s, 59.5°C for 30 s, and 72°C for 30 s. *C<sub>T</sub>* values were calculated using OPTICAL SYSTEM SOFTWARE, version 1.0 for MyIQ<sup>TM</sup> (Bio-Rad). *C<sub>T</sub>* values were normalized for differences in dsDNA synthesis using the *UBQ10* *C<sub>T</sub>* values. Normalized transcript levels of each gene were compared in ISR-expressing plants and noninduced controls and the relative levels of transcription were calculated using the 2<sup>ΔΔCT</sup> method (Livak & Schmittgen, 2001). Melting curves were recorded after cycle 40 by heating from 55 to 95°C with a ramp speed of 1.9°C min<sup>-1</sup>.

### Sample preparation, microarray data collection, and transcript profiling

For isolation of RNA, whole leaf rosettes were harvested at 0, 1, 3 and 6 h after MeJA treatment and immediately frozen in liquid nitrogen. RNA was prepared from eight biological replicates that were pooled to reduce noise arising from biological variation. Total RNA was prepared as described above in the section 'RNA gel-blot analysis' and cleaned using RNeasy Plant Mini Kit columns (Qiagen Benelux BV, Venlo, the Netherlands). RNA samples were analyzed for quality by capillary electrophoresis using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo Alto, CA, USA). cRNA probe synthesis, hybridization to a GeneChip (Affymetrix, Santa Clara, CA, USA), and collection of data from the hybridized GeneChip were performed as described previously (De Vos *et al.*, 2005). Hybridizations with labeled cRNAs were conducted with *A. thaliana* ATH1 full-genome GeneChips (Affymetrix), containing a total number of 22 810 probe sets representing approx. 23 750 *A. thaliana* genes (Redman *et al.*, 2004). Probe preparations and GeneChip hybridizations were carried out by ServiceXS (Leiden, the Netherlands) and the Affymetrix service station of Leiden University Medical Centre (LUMC) where they passed all internal quality checks.

After hybridization, GeneChips were analyzed using the GENECHIP OPERATING SOFTWARE (GCOS) (Affymetrix) and GENESPRING 6.1 (Silicon Genetics, Redwood, CA, USA), as previously described (De Vos *et al.*, 2005). The *P*-values from the Pearson correlation tests for GeneChips that were hybridized with probes from samples from the same time-point ranged between 0.979 and 0.992. This is in good agreement with the high correlation coefficients previously reported for independent biological samples (Redman *et al.*, 2004) and indicates that the GeneChip hybridizations and microarray data collection were performed in a technically sound manner. Expressed genes were identified using GCOS, which uses statistical criteria to generate a 'present' (P-flag) or 'absent' (A-flag) call for genes represented by each probe set on the array. The average number of detectable genes with a 'present' call was 14 296 (62.7%),

which is in good agreement with the 60% previously reported by Redman *et al.* (2004). Expression values from each sample were normalized globally by GCOS using a target average intensity of 200 for global scaling so that hybridization intensity of all chips was equivalent. Default settings used during the data analyses were per chip = normalize to 50th percentile and per gene = normalize to specific samples. Genes with accurately detectable transcript levels were defined by probe sets showing averaged expression levels > 30 (95% of all probe sets with a 'present' call had a signal intensity above 30). Probe sets showing an expression value of < 30 were adjusted to 30 to reduce false positives. All microarray data have been deposited in NASC arrays (<http://www.affymetrix.arabidopsis.info>).

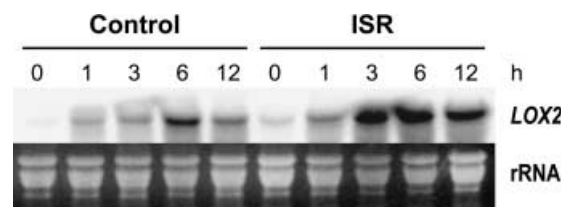
### Regulatory motif analysis of promoter regions

For the analysis of the frequency distribution of *cis*-acting elements in the promoters of selected groups of genes, 1 kilobase (kb) of genomic DNA sequences upstream from the inferred translational start sites was downloaded from The Arabidopsis Information Resource (<http://www.arabidopsis.org/tools/bulk/sequences/index.html>). To identify significantly over-represented putative regulatory sequences, the 1-kb regions of genes belonging to the selected groups were analyzed using the Athena database (<http://www.bioinformatics2.wsu.edu/Athena>) (O'Connor *et al.*, 2005). Once putative regulatory sequences were selected, the statistical significance of their differential distribution in selected clusters was verified using POBO (update 18 May 2005), a promoter bootstrapping program that allows a three-way comparison between two clusters and the background (Kankainen & Holm, 2004). POBO can be used to detect, compare and verify predetermined transcription factor binding site motifs in the promoters of one or two clusters of co-regulated genes. The program calculates the frequencies of the motif in the input promoter sets. A bootstrap analysis detects significantly over- or under-represented motifs. To perform the POBO analysis, the 1-kb promoter sequences of the selected MeJA-responsive and ISR-primed genes (in FASTA format) were uploaded separately in POBO. Subsequently, the frequency distributions of the regulatory motifs were analyzed using 'Arabidopsis\_thaliana\_clean' as the background organism. Other parameters used were: number of samples to generate: 1000; number of sequences to pick out: equal to the number of promoters in the smallest group to be compared.

## Results

### WCS417r-ISR primes the plant for enhanced MeJA-induced expression of *LOX2*

Previously, microarray analyses revealed that *A. thaliana* plants expressing WCS417r-ISR are primed for accelerated expression of genes that are activated upon attack by *Pst* DC3000



**Fig. 1** Priming for enhanced methyl jasmonate (MeJA)-responsive *LIPOXYGENASE2* (*LOX2*) gene expression in induced systemic resistance (ISR)-expressing plants. Results are shown of northern blot analysis of *LOX2* transcript levels in leaves of *Arabidopsis thaliana* wild-type Columbia (Col)-0 plants grown in soil with or without ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria. RNA was isolated at different time-points after exogenous application of 50  $\mu$ M MeJA. To check for equal loading, rRNA bands were stained with ethidium bromide. h, hours post treatment.

(Verhagen *et al.*, 2004). The majority of these primed genes were predicted to be regulated by JA signaling, suggesting that WCS417r-ISR is associated with an enhanced sensitivity of the induced tissues to JA that is produced upon pathogen attack. To test this hypothesis, the responsiveness of noninduced and WCS417r-ISR-expressing plants to exogenously applied MeJA was investigated. *Arabidopsis thaliana* Col-0 plants grown in soil with or without ISR-inducing WCS417r bacteria were treated with 50  $\mu$ M MeJA and harvested 0, 1, 3, 6 and 12 h later, after which the expression level of the JA-responsive marker gene *LOX2* was determined by northern blot analysis. Fig. 1 shows that in MeJA-treated control plants the *LOX2* transcript level already started to rise at 1 h after MeJA treatment and reached a maximum at 6 h. In WCS417r-ISR-expressing plants, *LOX2* transcript accumulated to higher levels at all time-points tested. Moreover, at 12 h after MeJA treatment *LOX2* mRNA levels were still high in WCS417r-ISR-expressing plants, whereas they had decreased to almost basal levels in MeJA-treated control plants. These results indicate that colonization of the roots by ISR-inducing WCS417r bacteria primes the leaf tissue for accelerated expression of JA-responsive genes such as *LOX2*.

### Identification of putative MeJA-responsive genes

To identify JA-responsive genes that, like *LOX2*, are primed during WCS417r-ISR, we analyzed the whole-genome transcript profile of control and WCS417r-ISR-expressing *A. thaliana* Col-0 plants at different time-points after MeJA treatment using whole-genome Affymetrix *A. thaliana* ATH1 GeneChips (Redman *et al.*, 2004). Because the primed expression pattern of *LOX2* was clear within the first 6 h after MeJA treatment (Fig. 1), we performed the transcript profiling with leaf material that was harvested at 1, 3 and 6 h after application. Expressed genes were identified and expression values for each sample were normalized globally using GCOS. To identify putative MeJA-responsive genes, we applied the following selection criteria. Firstly, the expression level had to

significantly exceed the background (P-flag generated by GCOS) and the hybridization intensity had to be  $> 30$  units at  $t = 0$  for down-regulated genes and at at least two of the later time-points for the up-regulated genes. Secondly, the expression level of the significantly up- or down-regulated genes had to change at least twofold in MeJA-treated leaves compared with that in control leaves (0 h). To reduce the number of false positives, we required the change to occur at at least two of the three time-points and in the same direction. Although the cut-off value of twofold is arbitrary, in combination with the additional timing and direction criteria this value has been demonstrated to yield a robust selection of gene sets (De Vos *et al.*, 2005). Probe sets corresponding to 1879 genes (955 up- and 924 down-regulated) met these criteria. These genes were considered putative MeJA-responsive genes and are listed in Supporting Information Table S1. The list includes well-characterized JA-responsive genes that are commonly used as markers for JA-dependent responses, such as *LOX2*, *PDF1.2*, *THIONIN2.1* (*THI2.1*; AT1g72260), *VEGETATIVE STORAGE PROTEIN1* (*VSP1*; At5g24780), *CORONATINE-INDUCED PROTEIN1* (*COR1*; At1g19670), *JASMONIC ACID RESPONSIVE1* (*JR1*; At3g16470), *JASMONIC ACID RESPONSIVE2* (*JR2*; At4g23600), and genes involved in the JA biosynthetic pathway, such as *ALLENE OXIDE SYNTHASE1* (*AOS1*; At5g42650), and *JASMONIC ACID CARBOXYL METHYLTRANSFERASE* (*JMT*; At1g19640). To validate the GeneChip data obtained, we compared the putative MeJA-responsive genes with those identified in other transcript profiling studies in *A. thaliana*. Sasaki *et al.* (2001) performed a similar time-course study of the *A. thaliana* response to MeJA treatment using a cDNA macroarray. Despite the different type of array used and experimental set-up, 80% of the MeJA-responsive genes described in their work also showed a more than twofold change in expression in our data (not shown).

### Identification of putative ISR-primed MeJA-responsive genes

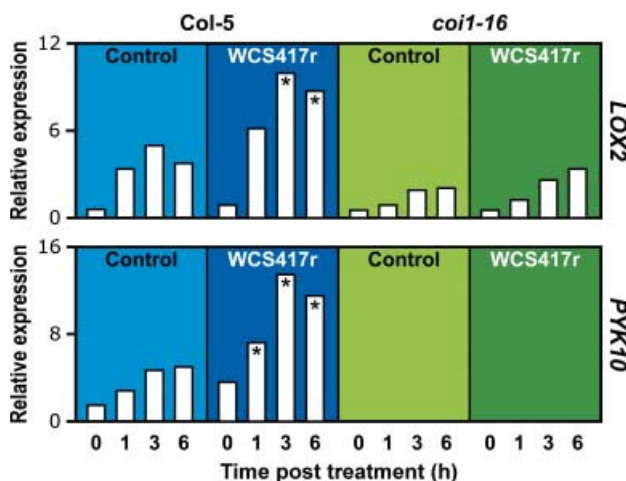
To identify genes that may be primed to respond more rapidly and/or with different amplitude to MeJA in WCS417r-ISR-expressing plants (so-called ISR-primed genes), we required the expression level of the putative MeJA-responsive genes listed in Table S1 to be  $> 1.5$ -fold different in MeJA-treated ISR-expressing plants versus MeJA-treated control plants. This priming cut-off value is based on previous quantitative expression data for genes that showed a robust primed expression pattern after pathogen attack (Verhagen *et al.*, 2004). A total of 442 genes met these selection conditions and are listed in Table S2. Although *LOX2* showed a clear ISR-primed expression pattern after MeJA treatment when northern blotting was used (Fig. 1), it is not listed in Table S2. The reason is that *LOX2* transcript levels could not be reliably compared in the microarray analysis, because *LOX2* was among the eight MeJA-responsive genes whose probe sets were saturated (expression levels  $> 5000$

units) after hybridization with all probes from MeJA-treated plants.

To further verify the microarray data, we selected the putative ISR-primed MeJA-responsive gene *PYK10* (At3g09260) and analyzed its expression along with that of *LOX2* in an independent experiment. *PYK10* encodes a  $\beta$ -glucosidase/myrosinase, which is implicated in JA-dependent plant defenses, and was recently demonstrated to be crucial for the beneficial interaction between *A. thaliana* and the endophytic fungus *Piriformospora indica* (Hara-Nishimura & Matsushima, 2003; Matsushima *et al.*, 2003; Sherameti *et al.*, 2008). The response of *LOX2* and *PYK10* to MeJA treatment was assessed at 0, 1, 3 and 6 h after treatment of the leaves of wild-type and JA-insensitive *coi1-16* mutant plants that were grown in soil with or without ISR-inducing WCS417r bacteria. In a parallel set of plants, expression of ISR was verified using *Pst* DC3000 as a challenging pathogen. Because mutant *coi1-16* is in the background of glabrous Col-5, we used Col-5 plants as a control. As expected, colonization of the roots by WCS417r bacteria induced systemic protection against *Pst* DC3000 in wild-type (Fisher's least significant difference (LSD) test;  $P < 0.001$ ) but not in mutant *coi1-16* plants (Fisher's LSD test;  $P < 0.368$ ) (data not shown). Northern blot analysis and quantification of the hybridization signals with a Phosphorimager (BioRad, Veenendaal, the Netherlands) showed that *LOX2* and *PYK10* mRNAs accumulated to high levels in MeJA-treated Col-5 plants, whereas the transcript levels of both genes were much lower or undetectable in MeJA-treated *coi1-16* plants (Fig. 2). Moreover, *LOX2* and *PYK10* mRNA levels were more than 1.5-fold higher in ISR-expressing Col-5 plants at at least two of the three time-points tested, confirming the ISR-primed and MeJA-responsive expression pattern of these genes.

### Selected ISR-primed MeJA-responsive genes also show a primed expression pattern upon pathogen attack

Because the ISR-primed genes were selected on the basis of their augmented expression after MeJA treatment, we anticipated that these genes would also show a primed expression pattern upon infection by a JA-inducing pathogen. Therefore, we analyzed the expression patterns of the ISR-primed MeJA-responsive genes *LOX2*, *PYK10*, *NIMIN1* and *WRKY54* in control and WCS417r-treated Col-5 and *coi1-16* plants at 0, 3, 6 and 24 h after challenge with *Pst* DC3000. Of the four selected genes, *LOX2* and *PYK10* were ISR-primed MeJA-responsive genes that showed an accelerated up-regulation in ISR-expressing plants after MeJA treatment (Fig. 2; Table S2), whereas *NIMIN1* and *WRKY54* were suppressed in ISR-expressing plants after MeJA treatment (Table S2). Expression of WCS417r-ISR against *Pst* DC3000 was confirmed for WCS417r-treated Col-5 plants, while the ISR minus mutant *coi1-16* did not mount a significant level of protection against *Pst* DC3000 (data not shown). *LOX2* and *PYK10* were induced in Col-5 after inoculation with *Pst* DC3000 (Fig. 3a) and



**Fig. 2** Priming for enhanced methyl jasmonate (MeJA)-responsive expression of *LIPOXYGENASE2* (*LOX2*) and a gene encoding a protein with  $\beta$ -glucosidase/myrosinase activity (*PYK10*) in *Pseudomonas fluorescens* WCS417r-treated Columbia (Col)-5 and *coi1-16* mutant plants. Results are shown of quantitative northern blot analysis of *LOX2* and *PYK10* transcript levels in the leaves of *Arabidopsis thaliana* wild-type Col-5 plants and jasmonate (JA)-insensitive *coi1-16* mutant plants grown in soil with or without induced systemic resistance (ISR)-inducing *Pseudomonas fluorescens* WCS417r bacteria. RNA was isolated at different time-points after exogenous application of 50  $\mu$ M methyl jasmonate (MeJA). Hybridization signals on the northern blots were quantified using a Phosphorimager and normalized using 18S rRNA signal intensities. Asterisks indicate that the ratio of the MeJA-induced expression level in WCS417r-treated plants to that in control plants was at least 1.5. The experiments were repeated with similar results.

showed a potentiated expression pattern in ISR-expressing Col-5 plants at at least two of the three time-points tested. By contrast, in the *coi1-16* mutant, *Pst* DC3000-induced expression and WCS417r-mediated priming of the genes were fully abolished (*LOX2*) or strongly diminished (*PYK10*). Also *NIMIN1* and *WRKY54* were induced in Col-5 upon inoculation with *Pst* DC3000 (Fig. 3b). However, in the ISR-expressing plants, expression of both genes showed a significantly lower level of induction after pathogen challenge, consistent with the WCS417r-mediated suppression of their expression in MeJA-treated plants. In *coi1-16* mutant plants, *Pst* DC3000-induced expression of *NIMIN1* and *WRKY54* was similar to that observed in Col-5 plants, indicating that the pathogen-induced expression of these genes is regulated in a JA-independent manner. Nevertheless, the WCS417r-mediated suppression of *Pst* DC3000-induced *NIMIN1* and *WRKY54* expression was blocked in *coi1-16* plants, indicating that the WCS417r-mediated priming of these genes was regulated in a JA-dependent manner. As both *NIMIN1* and *WRKY54* are SA-responsive genes (Weigel *et al.*, 2001; Wang *et al.*, 2006), their JA-dependent suppression in plants expressing WCS417r-ISR may be caused by antagonistic cross-talk between the SA and JA signaling pathways (Koornneef & Pieterse, 2008). Collectively, these results confirm that the selected ISR-

primed genes *LOX2*, *PYK10*, *NIMIN1* and *WRKY54* show a similarly primed expression pattern in ISR-expressing plants upon treatment with MeJA or inoculation with the pathogen *Pst* DC3000.

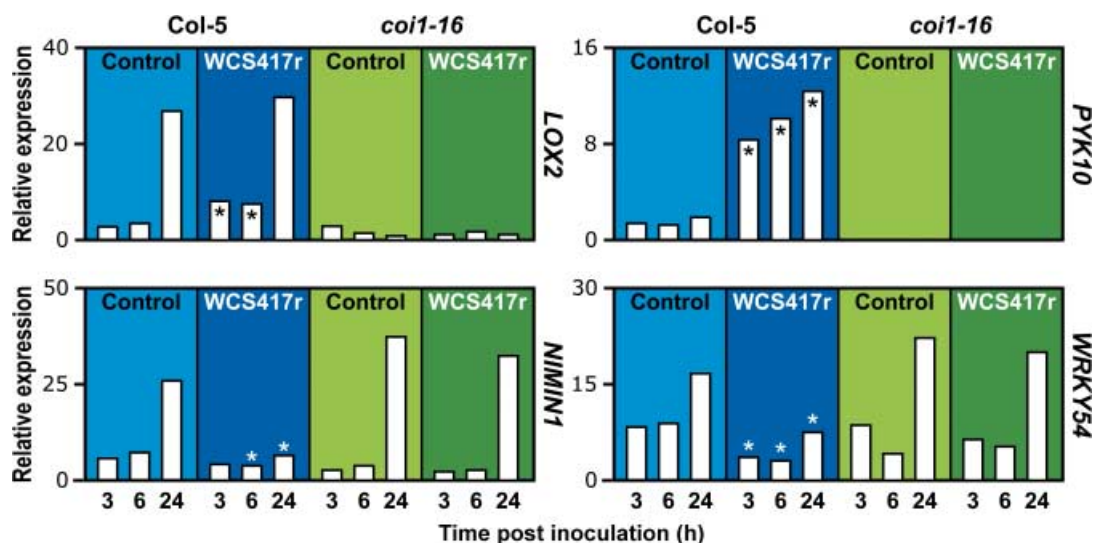
### Promoter analysis of ISR-primed genes

To gain further insight into the molecular mechanisms of priming for enhanced MeJA-responsive gene expression during WCS417r-ISR, we performed an *in silico* analysis of the promoter sequences of the selected ISR-primed genes to identify putative *cis*-regulatory elements. Functional *cis*-regulatory elements in plant promoters are typically found within the first 1 kb upstream of the ATG translation start site (Rombauts *et al.*, 2003). Therefore, we scanned the 1-kb regions upstream of the 5' untranslated regions (UTRs) of the genes listed in Tables S1 and S2, using the transcription factor binding site enrichment tool of the Athena database (<http://www.bioinformatics2.wsu.edu/Athena>) (O'Connor *et al.*, 2005), to identify statistically overrepresented *cis*-regulatory elements in ISR-primed versus nonprimed MeJA-responsive genes. Five motifs, including the G-box-related element 5'-CACATG-3', were enriched in the 1-kb promoter regions of the nonprimed and ISR-primed MeJA-responsive genes (Table S3). However, no unique motifs could be identified in the ISR-primed gene set, suggesting that the potentiated expression of these MeJA-responsive genes in ISR-expressing plants is caused by quantitative rather than qualitative differences in transcription factor activity.

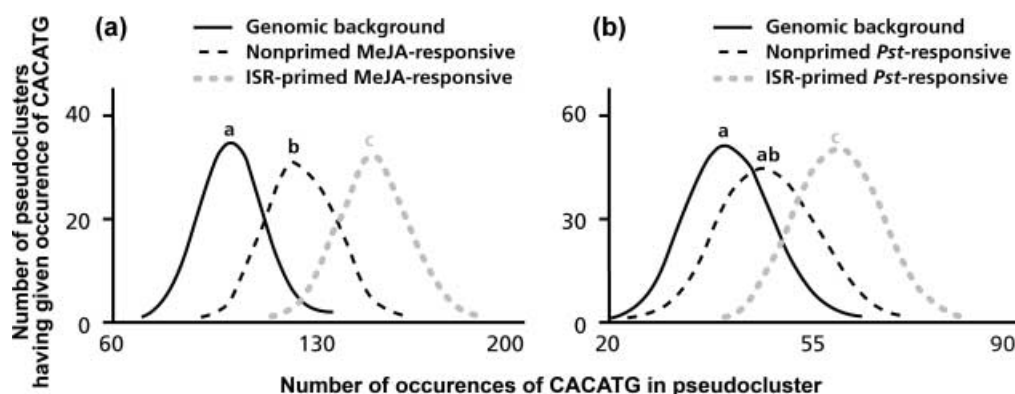
To search for motifs that are specifically enriched in ISR-primed over nonprimed genes, we statistically analyzed the frequency distribution of the identified transcription factor binding motifs in the promoters of the nonprimed and the ISR-primed MeJA-responsive genes. To this end, we used *POBO*, a promoter bootstrapping program that allows a three-way comparison between two clusters of co-regulated genes and the genomic background (Kankainen & Holm, 2004). Of the overrepresented motifs in the MeJA-responsive genes (Table S3), the G-box-related motif CACATG stood out as being the only one that was significantly more overrepresented in the ISR-primed genes. Fig. 4(a) shows that the promoters of the MeJA-responsive genes are significantly enriched in CACATG motifs in comparison to the genomic background. Yet, the CACATG motif was significantly more overrepresented in the promoters of the ISR-primed MeJA-responsive genes as compared with the nonprimed MeJA-responsive genes.

If the CACATG motif is indeed associated with the primed expression pattern of JA-responsive genes, it could be expected that the promoters of the previously identified ISR-primed *Pst* DC3000-responsive genes (Verhagen *et al.*, 2004) are also enriched for this transcription factor binding site. Indeed, *POBO* analysis of the 1-kb promoter region of the 81 ISR-primed *Pst* DC3000-responsive genes from this study revealed that the CACATG motif is significantly overrepresented in these genes (Fig. 4b). Together, these results suggest a functional





**Fig. 3** Primed expression patterns of *LIPOXYGENASE2* (*LOX2*), a gene encoding a protein with  $\beta$ -glucosidase/myrosinase activity (*PYK10*), *NIMIN1*, and a gene encoding WRKY transcription factor 54 (*WRKY54*) in *Pseudomonas fluorescens* WCS417r-treated Columbia (Col)-5 and *coi1-16* plants after inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Results are shown of quantitative northern blot analysis of *LOX2*, *PYK10*, *NIMIN1* and *WRKY54* transcript levels in the leaves of *Arabidopsis thaliana* wild-type Col-5 plants and jasmonate (JA)-insensitive *coi1-16* mutant plants grown in soil with or without induced systemic resistance (ISR)-inducing WCS417r bacteria. RNA was isolated at different time-points after inoculation with *Pst* DC3000. Hybridization signals on the northern blots were quantified using a Phosphorimager and normalized using 18S rRNA signal intensities. Asterisks indicate that ratio of the *Pst* DC3000-induced expression level in WCS417r-treated plants to that in control plants was at least 1.5. The experiments were repeated with similar results.



**Fig. 4** Frequency distribution of G-box-related CACATG motif in the promoter sequences of nonprimed and induced systemic resistance (ISR)-primed *Arabidopsis thaliana* genes. The occurrence of CACATG motifs was quantified in the 1-kb sequences upstream of the 5' untranslated region (UTR) of the nonprimed and ISR-primed methyl jasmonate (MeJA)-responsive genes (a) and nonprimed and ISR-primed *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000)-responsive genes (b) using POBO bootstrapping analysis (Kankainen & Holm, 2004). The nonprimed and ISR-primed genes were compared with randomly selected promoter sequences from the *A. thaliana* genome. Nonprimed and ISR-primed *Pst* DC3000-responsive genes are taken from Verhagen *et al.* (2004). Different letters indicate statistically significant differences in the occurrence of the *cis*-acting element ( $\chi^2$  test;  $\alpha = 0.05$ ).

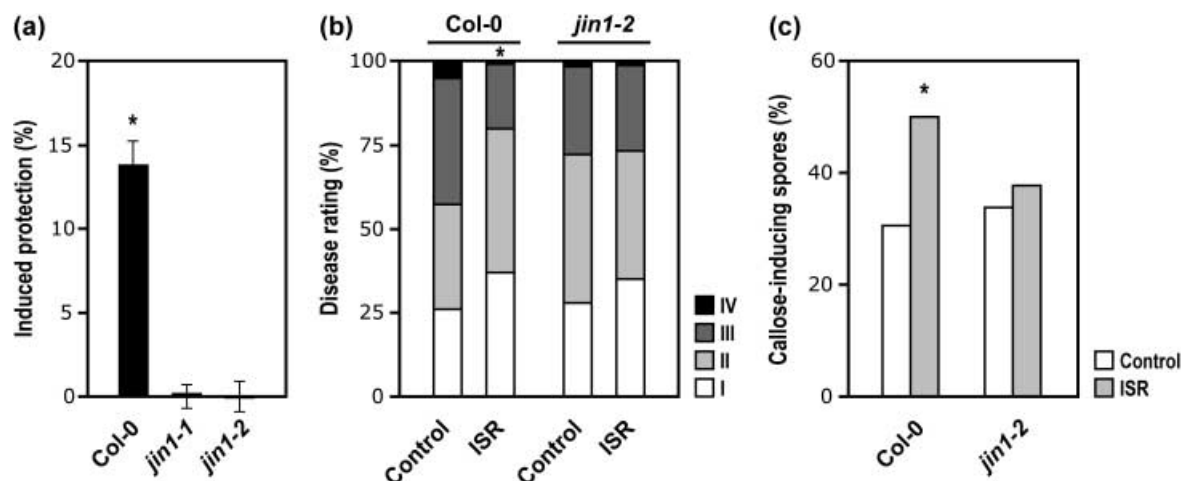
role for this motif in the priming for enhanced JA-responsive gene expression during WCS417r-ISR.

#### The MYC2 transcription factor is required for WCS417r-ISR

The CACATG motif was previously identified as a binding site for the MYC2 transcription factor in the promoter of

the ABA- and drought-responsive gene *RESPONSIVE TO DEHYDRATION 22* (*RD22*; At5g25610) (Abe *et al.*, 1997). The transcription factor MYC2 also plays an important role in the regulation of JA-responsive gene expression and defense against pathogen and insect attack (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Lorenzo & Solano, 2005; Dombrecht *et al.*, 2007). To investigate whether MYC2 is required for the expression of WCS417r-ISR, we tested the ability of the

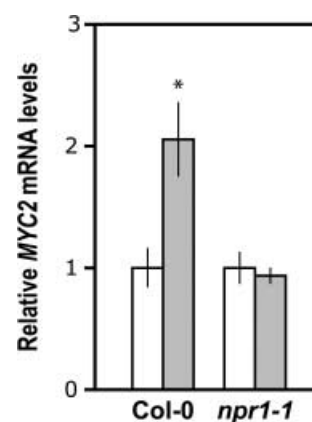




**Fig. 5** *Pseudomonas fluorescens* WCS417r-induced systemic resistance (ISR) against *Pst* DC3000 and *Hyaloperonospora parasitica* is blocked in MYC2-impaired *jin1* mutants. (a) Levels of induced protection against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) in *Arabidopsis thaliana* Columbia (Col)-0 and mutants impaired in the *JASMONATE-INSENSITIVE1/MYC2* gene (*jin1-1* and *jin1-2*). ISR was induced by growing the plants for 3 wk in soil containing ISR-inducing WCS417r bacteria. Five-week-old plants were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000. Four days after challenge inoculation, the percentage of diseased leaves was assessed and the level of induced protection calculated on the basis of the reduction in disease symptoms relative to challenged, noninduced plants. \*, statistically significant differences compared with noninduced control plants (Student's *t*-test,  $\alpha = 0.05$ ,  $n = 20$ ). (b) Quantification of ISR against *Hyaloperonospora parasitica* in Col-0 and *jin1-2*. ISR was induced by growing the plants in soil containing ISR-inducing WCS417r bacteria. Plants were challenge inoculated with *H. parasitica* when 3 wk old. Disease severity was determined 7 d after challenge. Disease ratings are expressed as the percentage of leaves ( $n = \sim 250$ ) in disease-severity classes: I, no sporulation; II, trailing necrosis; III, < 50% of the leaf area covered with sporangia; IV, > 50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. \*, statistically significantly different distributions of the disease severity classes compared with the noninduced control treatments ( $\chi^2$  test,  $\alpha = 0.05$ ). (c) Induced resistance against *H. parasitica* is associated with enhanced deposition of callose-containing papillae at sites of attempted penetration, resulting in a reduction of the number of spores that successfully penetrate into *A. thaliana* leaves. Two days after challenge with *H. parasitica*, the proportion of spores that induced the formation of callose-containing papillae around the infection site was quantified in leaves of Col-0 and *jin1-2* plants ( $\chi^2$  test,  $\alpha = 0.05$ ).

MYC2-impaired mutants *jin1-1* and *jin1-2* (Berger *et al.*, 1996; Lorenzo *et al.*, 2004) to express WCS417r-ISR against the pathogens *Pst* DC3000 and *H. parasitica*. Fig. 5(a) shows that WCS417r-ISR resulted in a significant level of protection of Col-0 plants against *Pst* DC3000. However, mutants *jin1-1* and *jin1-2* failed to develop ISR against this pathogen. Similarly, *jin1-2* failed to develop ISR against *H. parasitica* (Fig. 5b). Moreover, priming for enhanced deposition of callose-containing papillae at sites of attempted penetration of *H. parasitica*, a typical reaction of WCS417r-ISR-expressing Col-0 plants (Van der Ent *et al.*, 2008), was significantly reduced in WCS417r-treated *jin1-2* plants (Fig. 5c).

To investigate whether WCS417r-ISR is associated with an increase in the level of MYC2 mRNA, we analyzed MYC2 transcript levels in noninduced control and WCS417r-ISR-expressing Col-0 plants in five independent experiments in which significant levels of ISR were detected (data not shown). Q-PCR analysis revealed that MYC2 transcript levels were consistently raised 1.6- to 3.5-fold in WCS417r-ISR-expressing plants (Fig. 6). In *npr1-1* mutant plants that are unable to mount an ISR response (Pieterse *et al.*, 1998), the level of MYC2 mRNA was not elevated upon colonization of the roots by WCS417r (Fig. 6). Thus, colonization of the roots by ISR-inducing WCS417r bacteria results in a moderate, but



**Fig. 6** *Pseudomonas fluorescens* WCS417r-induced MYC2 expression. Results are shown of quantitative PCR analysis of MYC2 transcript levels in the leaves of 5-wk-old *Arabidopsis thaliana* Columbia (Col)-0 and induced systemic resistance (ISR)-impaired *npr1-1* plants (impaired in the *NONEXPRESSOR OF PATHOGENESIS RELATED GENES1* gene), the roots of which were treated (closed bars) or not (open bars) with ISR-inducing WCS417r bacteria. MYC2 transcript levels in noninduced control plants were set at 1. \*, statistically significant differences compared with the noninduced control (Student's *t*-test;  $\alpha = 0.05$ ).

consistently enhanced expression of the transcription factor gene *MYC2*. Together, these results demonstrate that *MYC2* is required for the expression of WCS417r-ISR.

## Discussion

In many interactions between plants and beneficial micro-organisms, induced resistance is not associated with direct activation of defense-related genes, but with potentiated expression of the defense responses that are activated upon pathogen or insect attack. This suggests that priming for enhanced defense is a common mechanism by which ISR-inducing beneficial organisms confer broad-spectrum resistance in plants (Van Wees *et al.*, 2008). This is not illogical, because activation of inducible defenses involves major costs that affect plant growth and reproduction (Heil, 2002), and this is inconsistent with the beneficial nature of these plant–microbe interactions. In this study, we identified a group of putative MeJA-responsive genes for which the expression kinetics showed a potentiated pattern in WCS417r-ISR-expressing plants. *In silico* analysis of the promoters of these ISR-primed MeJA-responsive genes revealed that, when compared with the nonprimed MeJA-responsive genes, the primed genes contain a significantly larger number of the G-box-related motif CACATG (Fig. 4), suggesting a role for this motif in WCS417r-mediated priming for enhanced JA-responsive gene expression.

The G-box-related motif CACATG was previously shown to function as a binding site for the basic helix-loop-helix (bHLH) domain-containing transcription factor *MYC2* (Abe *et al.*, 1997; De Pater *et al.*, 1997). In addition to binding to G-box-related sequences such as CACATG, *MYC2* has been demonstrated to preferentially bind to the G-box sequence CACGTG (Dombrecht *et al.*, 2007). The *MYC2* locus was first identified in a mutant screen for reduced sensitivity to JA (Berger *et al.*, 1996) and is allelic to *JASMONATE-INSENSITIVE1* (*JAI1/JIN1*) (Lorenzo *et al.*, 2004). In several studies, *MYC2* has been demonstrated to play an important role in the regulation of JA- and ABA-responsive genes (Abe *et al.*, 1997; Abe *et al.*, 2003; Anderson *et al.*, 2004; Boter *et al.*, 2004; Lorenzo *et al.*, 2004; Lorenzo & Solano, 2005). Recent advances in JA signaling research revealed that *MYC2* interacts with JASMONATE-ZIM-DOMAIN (JAZ) repressor proteins that, upon induction of the JA pathway, become targeted for SCF<sup>CO11</sup> E3 ubiquitin ligase-dependent degradation by the proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007), thereby relieving the JAZ-mediated suppression of the transcription factor activity of *MYC2*. Upon induction of the JA pathway, *MYC2* functions as a positive regulator of JA-responsive genes such as *LOX2* and *VSP2*, which are associated with the wound response (Boter *et al.*, 2004; Lorenzo *et al.*, 2004). In contrast, *MYC2* acts as a negative regulator of JA-responsive genes such as *PDF1.2* and *HEL*, both of which are associated with defense against pathogens

(Anderson *et al.*, 2004; Lorenzo *et al.*, 2004). Moreover, *MYC2* modulates the expression of JA-responsive transcription factor genes, thereby indirectly affecting the expression of a large number of downstream JA-responsive genes (Dombrecht *et al.*, 2007). Mutations in the *MYC2/JIN1* gene affect the level of resistance against the pathogens *Botrytis cinerea*, *Plectosphaerella cucumerina*, *Fusarium oxysporum* and *P. syringae* (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Nickstadt *et al.*, 2004; Laurie-Berry *et al.*, 2006) and the insect herbivore *Helicoverpa armigera* (Dombrecht *et al.*, 2007), highlighting the important regulatory function of this transcription factor in JA-dependent plant defense.

If *MYC2* is important for WCS417r-mediated priming for enhanced JA-responsive gene expression upon pathogen or insect attack, then one would expect that mutations in the *MYC2* gene will affect the ability to develop ISR. Indeed, *MYC2* mutants *jin1-1* and *jin1-2* were blocked in their ability to mount WCS417r-ISR against *Pst* DC3000 (Fig. 5), a pathogen that was previously shown to be affected by JA-dependent defenses (Pozo *et al.*, 2004). Furthermore, we demonstrated that ISR-expressing Col-0 plants accumulated higher levels of *MYC2* mRNA, whereas the ISR-impaired mutant *npr1-1* did not (Fig. 6), suggesting that elevated *MYC2* mRNA levels play a role in mediating the primed expression pattern of JA-responsive genes in pathogen-challenged, WCS417r-ISR-expressing plants. The observation that the WCS417r-induced expression of *MYC2* by itself is not sufficient for the activation of *MYC2*-dependent genes suggests that a second signal, induced, for example, upon pathogen or insect attack, is required for the expression of the primed defense response. This is corroborated by the observation that over-expression of *MYC2* does not lead to increased expression of JA- or ABA-responsive genes, but rather enhances the sensitivity of these plants to JA and ABA (Abe *et al.*, 2003; Boter *et al.*, 2004).

Our study also demonstrates that a mutation in the *MYC2* gene affects WCS417r-ISR against the biotroph *H. parasitica* (Fig. 5), a pathogen that is not very sensitive to JA-dependent defenses. So how can the role of *MYC2* in WCS417r-ISR against this pathogen be explained? Previously, we demonstrated that WCS417r-ISR against *H. parasitica* is associated with priming for enhanced deposition of callose-rich papillae at the sites of attempted spore penetration (Van der Ent *et al.*, 2008). This primed defense response has been demonstrated to depend on ABA signaling (Ton *et al.*, 2005; Van der Ent, 2008). Because *MYC2* is an important regulator of ABA-dependent defenses (Abe *et al.*, 1997; Abe *et al.*, 2003; Boter *et al.*, 2004), it is plausible that the *MYC2* dependence of WCS417r-ISR against *H. parasitica* is caused by the role of *MYC2* in ABA signaling.

In conclusion, our results suggest the following model for WCS417r-induced priming of JA-dependent defenses during ISR. Colonization of the roots by ISR-inducing WCS417r bacteria leads to a systemic induction of the expression of the *MYC2* transcription factor gene. In the absence of a JA-inducing

attacker, increased MYC2 levels do not result in enhanced defense-related gene expression, possibly because the action of MYC2 is suppressed by previously identified JAZ proteins (Chini *et al.*, 2007). Upon attack by a JA-inducing pathogen or insect, the JAZ repressor proteins are degraded via the proteasome. This clears the way for the accelerated expression of MYC2-regulated JA-responsive genes, resulting in enhanced resistance against the JA-inducing attacker encountered. This study provides novel insights into the molecular mechanisms of priming for enhanced defense and may be instrumental in developing novel strategies for durable crop protection.

## Acknowledgements

We thank Roberto Solano and Oscar Lorenzo, Centro Nacional de Biotecnología, CSIC, Madrid, Spain for providing *jin1-1* and *jin1-2* seeds, and John Turner, University of East Anglia, Norwich, UK, for providing the *coi1-16* seeds. We also wish to thank Bas van Breukelen and Coral del Val for assistance in the analysis of the GeneChip data and clustering. The research was supported by grant CBSG A4 of the Centre for BioSystems Genomics, VICI grant 865.04.002 of the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organisation of Scientific Research (NWO), and a post-doctoral EU Marie Curie Individual Fellowship (QLK5-CT-2001-52136).

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** Normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the putative methyl jasmonate (MeJA)-responsive genes

**Table S2** Normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the

putative induced systemic resistance (ISR)-primed methyl jasmonate (MeJA)-responsive genes

**Table S3** *P*-values (hypergeometric distribution) of over-represented motifs in promoters of induced systemic resistance (ISR)-primed methyl jasmonate (MeJA)-responsive genes, compared with those of nonprimed MeJA-responsive genes

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