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Hexanoic acid protects tomato plants against *Botrytis cinerea* by priming defence responses and reducing oxidative stress

IVAN FINITI[1], MARÍA DE LA O. LEYVA[1,†], BEGONYA VICEDO[2], ROCÍO GÓMEZ-PASTOR[1,‡], JAIME LÓPEZ-CRUZ[1], PILAR GARCÍA-AGUSTÍN[2], MARIA DOLORES REAL[3] AND CARMEN GONZÁLEZ-BOSCH[1,*]

[1]*Departamento de Bioquímica y Biología Molecular, Instituto de Agroquímica y Tecnología de los Alimentos (IATA, CSIC), Universidad de Valencia, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain*

[2]*Laboratorio de Bioquímica y Biotecnología, Área de Fisiología Vegetal, Departamento de Ciencias Agrarias y del Medio Natural, ESTCE, Universitat Jaume I, 12071, Castellón, Spain*

[3]*Departamento de Genética, Universidad de Valencia, Dr. Moliner 50, Burjassot, 46100 Valencia, Spain*

SUMMARY

Treatment with the resistance priming inducer hexanoic acid (Hx) protects tomato plants from *Botrytis cinerea* by activating defence responses. To investigate the molecular mechanisms underlying hexanoic acid-induced resistance (Hx-IR), we compared the expression profiles of three different conditions: *Botrytis*-infected plants (Inf), Hx-treated plants (Hx) and Hx-treated + infected plants (Hx+Inf). The microarray analysis at 24 h post-inoculation showed that Hx and Hx+Inf plants exhibited the differential expression and priming of many *Botrytis*-induced genes. Interestingly, we found that the activation by Hx of other genes was not altered by the fungus at this time point. These genes may be considered to be specific targets of the Hx priming effect and may help to elucidate its mechanisms of action. It is noteworthy that, in Hx and Hx+Inf plants, there was up-regulation of proteinase inhibitor genes, DNA-binding factors, enzymes involved in plant hormone signalling and synthesis, and, remarkably, the genes involved in oxidative stress. Given the relevance of the oxidative burst occurring in plant–pathogen interactions, the effect of Hx on this process was studied in depth. We showed by specific staining that reactive oxygen species (ROS) accumulation in Hx+Inf plants was reduced and more restricted around infection sites. In addition, these plants showed higher ratios of reduced to oxidized glutathione and ascorbate, and normal levels of antioxidant activities. The results obtained indicate that Hx protects tomato plants from *B. cinerea* by regulating and priming *Botrytis*-specific and non-specific genes, preventing the harmful effects of oxidative stress produced by infection.

**Correspondence*: Email: carmen.gonzalez@uv.es

Present addresses:

†Departamento de Biología Experimental, Universidad de Jaén, Campus Las Lagunillas s/n, 23071 Jaén, Spain.

‡Department of Pharmacology and Cancer Biology, Levine Science Research Center, Duke University, 308 Research Drive, Durham, NC 27710, USA.

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INTRODUCTION

On pathogen attack, plants activate defence mechanisms to limit pathogen invasion. This so-called basal resistance is regulated by a complex network of signal molecules and transcriptional regulators (Glazebrook *et al*., 2003; Robert-Seilaniantz *et al*., 2011). In addition, plants are able to develop induced resistance by activating an alarmed state, which enhances their defensive capacity against future pathogen attacks (van Loon *et al*., 1998). These systemic defences result from microbial elicitor recognition (systemic acquired resistance, SAR; Durrant and Dong, 2004) or rhizobacteria (induced systemic resistance, ISR; Ton *et al*., 2001). Resistance mechanisms can also be stimulated by chemical treatments, known as defence inducers. Some of the best characterized are 2,6-dichloro-isonicotinic acid (INA), benzo-(1,2,3)-thiadiazole-7-carbotionic acid *S*-methyl ester (BTH) and β-aminobutyric acid (BABA) (Conrath *et al*., 2002; Oostendorp *et al*., 2001). Several induced resistance processes are associated with enhanced capacity to express specific defence responses on attack by a pathogen, which is known as priming (Conrath *et al*., 2002). We have demonstrated recently that hexanoic acid (Hx) can act as a priming defence inducer by activating responses only after pathogen attack to protect tomato and Arabidopsis plants against *Botrytis cinerea* (Kravchuk *et al*., 2011; Leyva *et al*., 2008; Vicedo *et al*., 2009). In addition, a gene is considered to be primed by a chemical inducer treatment if treatment alone is unable to alter (positively or negatively) its expression. The gene boosting requires treatment plus infection, and is revealed only after pathogen inoculation (Conrath *et al*., 2002).

Botrytis cinerea is a pathogen with a broad host range that causes huge losses in crops, and also during the storage of fruit and vegetables (Elad and Evensen, 1995). The effective control of

this necrotrophic fungus requires several chemical treatments and often leads to unsatisfactory results (Leroux, 2004). Infection, colonization and suppression of host defences by *B. cinerea* is mediated by the delivery of lytic enzymes, toxins, necrosis-inducing factors, altered reactive oxygen species (ROS) levels and an array of secondary metabolites (Choquer *et al*., 2007; van Kan, 2006). Although salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) signalling pathways are known to be implicated in the interaction by complex interplays, tomato defence mechanisms against this pathogen still remain unclear (Asselbergh *et al*., 2007; Diaz *et al*., 2002; Flors *et al*., 2007).

In this work, we performed global transcription profiling in tomato plants 24 h after *B. cinerea* inoculation, and also in Hx-treated, and Hx-treated and infected plants. Our results reveal that fungal infection induces the expression of many defence-, hormone signalling- and oxidative metabolism-related genes. We also provide data indicating that hexanoic acid-induced resistance (Hx-IR) is based on an earlier and stronger activation of plant basal defence on *B. cinerea* infection. The inducer treatment also boosted a group of regulatory and oxidative stress-related genes that can participate in the restriction of *B. cinerea* expansion.

ROS play a major role in plant–pathogen interactions: the recognition of a pathogen by the plant rapidly triggers an oxidative burst, which may contribute to both the killing of biotrophic pathogens and/or activation of further defence reactions. Plants have also evolved an oxidative stress response (OSR) to scavenge high intracellular ROS levels that can damage plant integrity. These response mechanisms can be divided into non-enzymatic and enzymatic systems. The former involve soluble molecules that are oxidized by ROS, and therefore remove oxidants from solution. They include the major cellular redox buffer glutathione (GSH), as well as ascorbic acid, which is quantitatively the most important antioxidant compound to accumulate in plant cells (Foyer and Noctor, 2011; Smirnoff, 2007). GSH is a ubiquitous thiol-containing reductant that maintains intracellular redox homeostasis by reducing cellular disulphide bonds and by detoxifying damaging molecules. During the reaction, GSH is converted into its oxidized form, glutathione disulphide (GSSG). However, cells maintain not only a fairly high intracellular concentration of GSH, but also a high GSH to GSSG ratio, through the action of glutathione reductase (GR) (Apel and Hirt, 2004).

Enzymatic ROS scavenging mechanisms include superoxide dismutase (SOD) and various peroxidases, such as glutathione peroxidase (GPX), peroxiredoxin and catalase (CA). ROS scavenging systems are crucial for the suppression of toxic ROS levels in cells, and the OSR must be regulated very tightly. ROS play an important role in all fungus–plant interactions as signalling components. In plant–*Botrytis* interactions, an oxidative imbalance takes place. The fungus can produce ROS and contribute to this imbalance in plants, and takes advantage of the host's response (Heller and Tudzynski, 2011).

Here, we found that the inducer Hx alters the expression of the plant genes involved in *Botrytis*-specific and non-specific defences, including some related to the redox balance. We also showed that increased resistance against this necrotroph in treated plants is associated with a reduction in oxidative stress markers.

RESULTS

Transcriptome analysis of *Botrytis*-inoculated and Hx-treated tomato plants

We recently reported that preventive treatment with Hx protects tomato plants against *B. cinerea* through a priming mechanism, known as Hx-IR (Vicedo *et al*., 2009). Here, we performed a large-scale microarray gene expression study to obtain information about the molecular mechanisms underlying Hx-IR. This study also reports the defence gene expression pattern of *Botrytis*-inoculated tomato plants. Hx treatment was performed 48 h prior to *B. cinerea* spores or water (mock) inoculation. We analysed four conditions: untreated non-infected plants (Ctrl), untreated and *Botrytis*-infected plants (Inf), Hx-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf). Leaves from six plants per condition were sampled at 24 h post-inoculation (hpi) of *Botrytis* spores or water. The expression profiling experiments were performed using customised DNA microarrays based on a Cornell University TOM2 array (http://ted.bti.cornell.edu/cgi-bin/TFGD/array/home.cgi). Total RNA was hybridized and the expression data were obtained as described in the Experimental procedures. Only those hybridizations that gave $P \leq 0.05$ under all the conditions assayed were considered for further analysis. The fold change in gene expression was calculated by dividing the expression level of each sample by that of the Ctrl samples. A threshold of a two-fold difference when compared with Ctrl was assumed in order to consider a gene as being differentially expressed.

Venn diagrams (Fig. S1A, see Supporting Information) depict the number of differentially expressed transcripts from the *Botrytis*-infected tomato plants, and show the overlap between the Hx and Hx+Inf plants, as well as the number of differentially expressed genes which were unique for each condition.

At 24 hpi, when no lesions were visible, 116 genes were differentially expressed in Inf plants: 90 were induced and 26 were down-regulated. We refer to them as *Botrytis* differentially expressed genes (BDEGs). In the Hx+Inf plants, the number of differentially expressed genes rose to 163, with 114 induced and 49 repressed. Among them, 106 were BDEGs, but 57 were not altered by the fungus at this early time point. We refer to them as Hx+Inf-specific genes. The Hx samples showed 139 differentially expressed genes: 91 induced and 48 repressed. Eighty-five genes were commonly altered under all the conditions when compared with Ctrl plants, with 60 genes induced and 25 repressed.

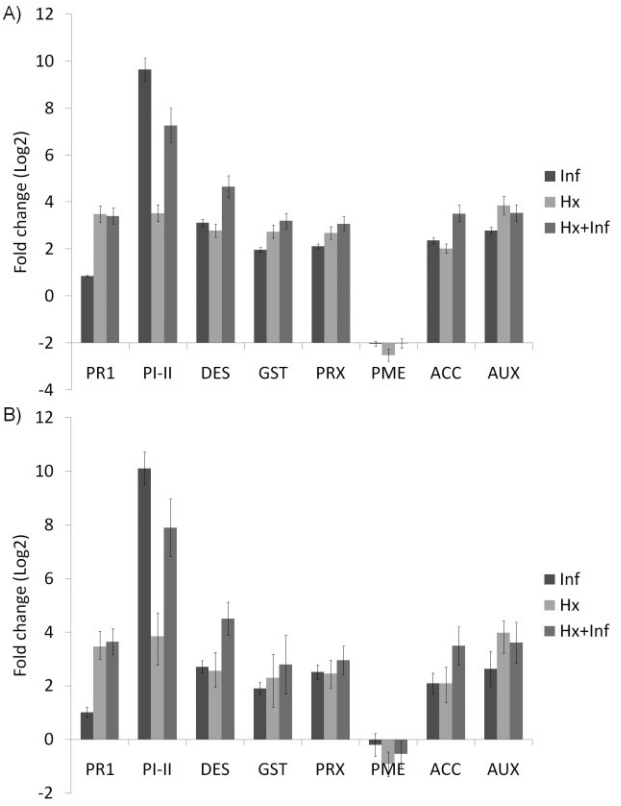

Fig. 1 Microarray data validation by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). (A) Fold change values obtained by RT-PCR. (B) Fold change values obtained by microarray hybridization. The data shown are the means of two independent experiments ± standard deviation (SD). Inf, untreated and *Botrytis*-infected plants; Hx, hexanoic acid (Hx)-treated and non-infected plants; Hx+Inf, Hx-treated and infected plants. ACC, aminocyclopropane-1-carboxylic acid synthase; AUX, AUX/IAA family gene; DES, divinyl ether synthase; GST, glutathione *S*-transferase; PI-II, proteinase inhibitor II; PME, pectin methylesterase; PR1, pathogenesis-related 1; PRX, peroxidase.

The global expression data were hierarchically clustered (Fig. S1B), showing almost the same pattern of induced and repressed genes under all the conditions relating to Ctrl. These data suggest that Hx treatment itself (Hx), and on infection (Hx+Inf), alters the gene expression similarly to the *B. cinerea* challenge.

Although microarray analysis is a widely applied, reliable technique, reverse transcription-polymerase chain reaction (RT-PCR) corresponding to eight differentially expressed genes was performed to validate the data (Fig. 1). Genes with different fold changes in the array were chosen and corroborated the data obtained in all cases.

Gene ontology (GO)

In order to group differentially expressed genes according to the GO classification, we used the Tomato Array GO Term Enrichment Analysis Tool (http://ted.bti.cornell.edu/cgi-bin/TFGD/array/home.cgi).

The data indicated that *Botrytis* infection induced an array of genes, which have been described in other plant–pathogen systems, and related mainly to the stress response (Fig. 2). The most represented categories were defence, stress response, wounding and response to chitin. Interestingly, Hx and Hx+Inf induced almost the same categories, except for chemical and biotic stimuli, which were only significantly represented in Hx+Inf.

Early response to both *Botrytis* and Hx treatment

To analyse the data obtained, differentially expressed genes were organized into functional groups. Putative gene functions are based on the tomato Solgenomics (http://solgenomics.net) and The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) annotation databases; a selection of the most relevant genes is shown in Table 1.

As mentioned earlier, Hx treatment altered many BDEGs at 24 hpi, when a priming response was shown that potentiated their expression in Hx+Inf plants; Hx application without further infection can activate some genes, but always at a lower level. These Hx primed genes are highlighted in italic in Table 1. The Hx-specific genes not induced by the fungus, but primed in Hx+Inf plants, are highlighted in bold italic. No negatively primed genes were found.

Defence response genes

Among the BDEGs belonging to the defence response category (Fig. 2, Table 1), those coding for putative patatin-like protein, *Tobacco mosaic virus* (TMV)-induced protein I, basic endochitinase and *N*-hydroxycinnamoyl transferase THT1-3 were primed by Hx treatment. Priming of a putative patatin-like precursor (*sgn-U217988*) could prove to be particularly interesting, given patatin's essential role in cell death execution in Arabidopsis, which affects the biosynthesis of oxylipins and resistance to pathogens (La Camera *et al*., 2009). Hx potentiation of *THT1-3* (*sgn-U227510*) is also noteworthy, as THT is involved in hydrocinnamic acid biosynthesis, a precursor of dopamine-derivative compounds with antimicrobial activities (von Roepenack-Lahaye *et al*., 2003).

The early induction of antifungal defence genes by Hx is particularly interesting in order to understand HX-IR against *B. cinerea*. These genes include *Pr1a* (*sgn-U212922*), hevein-related protein precursor (*sgn-U214651*), basic endochitinase (*sgn-U212883*), osmotin-like protein precursor (*sgn-U212927*) and glucan endo-1,3-β-glucosidase B (*sgn-U212829*). The hydrolytic activities of endochitinase and 1,3-β-glucosidase are involved in the degradation of fungal cell wall polymers, and are considered to be pathogenesis-related (PR) proteins (Bulcke *et al*., 1989; van Loon *et al*., 2006). The JA-regulated hevein-like (HEL) and osmotin-like precursor genes encode vacuolar proteins. The former display chitin-binding activity, whereas osmotin is an antimicrobial

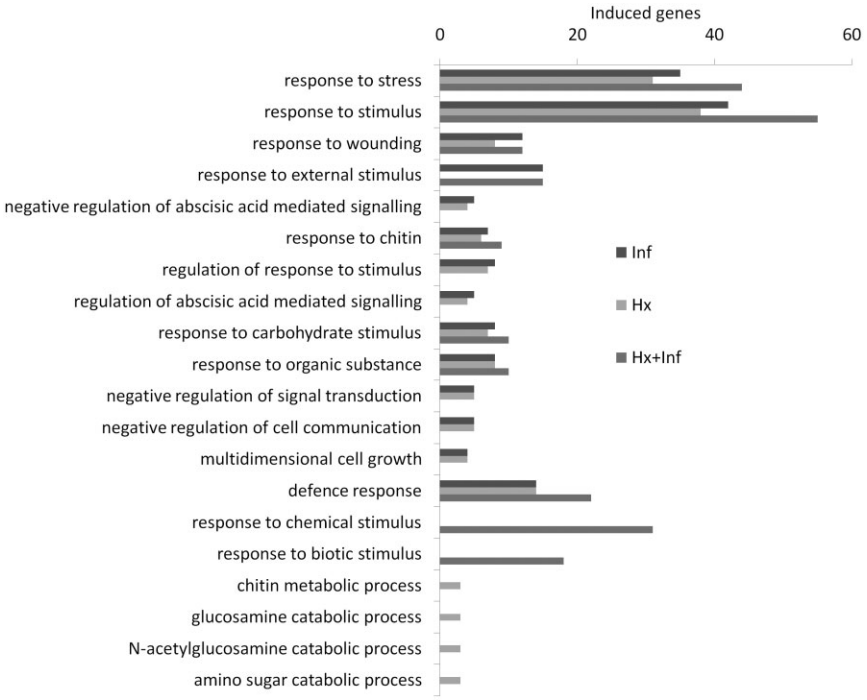

Fig. 2 Functional classification of genes induced in untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf). Bars show the number of induced genes in each gene ontology (GO) category according to the TFGD database.

protein. Both play key roles in disease resistance against phytopathogenic fungi in tomato (Lee *et al*., 2003; Rodrigo *et al*., 1993). Their induction, observed only in Hx and Hx+Inf plants, suggests the capability of Hx to activate specific defence and PR genes, which may contribute directly to limit fungal advance.

Protease inhibitor genes

Interestingly, we found that the most induced BDEGs encode protease inhibitors. These genes are activated in tomato and Arabidopsis after wounding and insect feeding (Ryan, 1990), and include cathepsin D inhibitor (*sgn-U212880*), proteinase inhibitors I and II (*sgn-U213021*, *AY129402*) and cysteine proteinase inhibitor (*sgn-U223902*). This result is concordant with the description of the *B. cinerea* early secretome, which includes a vast number of proteases (Espino *et al*., 2010). The Hx and Hx+Inf challenges activate these genes, but at lower levels than in Inf plants, and are an exception to the general pattern observed. According to this proteinase inhibitor activation, Hx and Hx+Inf down-regulated a gene encoding serine carboxypeptidase (*sgn-U223774*). *Botrytis cinerea* did not alter this gene at 24 hpi, and it is a specific target of Hx treatment. This gene is similar to *BRS1*, which is repressed after *B. cinerea* challenge in Arabidopsis (Kaschani *et al*., 2009).

Signalling and transcriptional regulation genes

We found BDEGs to be involved in the synthesis and signalling of various plant hormones and other defence-related metabolites, which have also been described in Arabidopsis during *Botrytis* infection (AbuQamar *et al*., 2006; Ferrari *et al*., 2007). We discovered that two genes were involved in the oxylipin biosynthesis pathway, which were clearly induced in Inf and Hx, and primed in Hx+Inf plants: *divinyl ether synthase* (DES) (*sgn-U214318*) and *α-dioxygenase1* (DOX1) (*sgn-U215223*). DES converts the hydroperoxides generated from fatty acid oxidation into divinyl ethers, a class of oxylipins. This gene has been reported to be inducible by pathogen infections in tomato leaves (Itoh and Howe, 2001). DOX1 catalyses the primary oxygenation of fatty acids converted into a poorly studied group of oxylipins, formed by aldehydes and hydroxides, which seem to protect plants from oxidative damage and cell death (Tirajoh *et al*., 2005). We also found that Hx does not prime *lipoxygenase D* (LoxD) (*sgn-U37840*), a gene induced early by the fungus, which encodes a chloroplast-targeted lipoxygenase that initiates JA synthesis and constitutes a marker gene of the JA pathway (Heitz *et al*., 1997; Zhao *et al*., 2003). We also observed the up-regulation of the ET-forming *ACC* oxidase gene (*sgn-U212786*) in response to *Botrytis* and Hx treatment. Its priming in Hx+Inf suggests the involvement of ET in Hx-IR.

An auxin-inducible AUX/IAA family member gene (*sgn-U219359*), very similar to Arabidopsis *IAA29*, was induced by *Botrytis*. In this case, Hx treatment greatly induced this gene in the absence of infection. In contrast, another auxin-inducible gene, *AIR12* (*sgn-U221902*), and auxin-binding factor *GER1* (*sgn-U215755*) were down-regulated on *Botrytis* infection and also by Hx treatment. Finally, we found a gene (*sgn-U231291*) similar to Arabidopsis *BRI1* that was induced by *Botrytis* and primed in Hx+Inf. BRI1 is a leucine-rich repeat receptor-like kinase located in

Table 1 Summary of most relevant genes differentially expressed in untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf).

Category	SGN ID	Probe ID	GB acc	Putative function*	Inf versus Ctrl	Hx versus Ctrl	Hx+Inf versus Ctrl
Defence	**U217988**	**LE8K05**	AAF98368	**Patatin-like protein**	**3.09**	**1.72**	**5.68**
	U217308	**LE15E11**	AAF63515	**TMV-induced protein I**	**2.80**	**3.97**	**5.69**
	U212884	**LE13O18**	P08252	**Basic endochitinase**	**2.17**	**2.32**	**3.73**
	U227510	**LE23I01**	**XP_002530925**	**N-hydroxycinnamoyl transferase THT1-3**	**2.02**	**3.15**	**4.53**
	U212922	***LE33C20***	**P04284**	***Pathogenesis-related protein PRIa (P4)***	***1.80***	***11.26***	***10.66***
	U214651	***LE20O16***	**AAB49688**	***(HEL) Hevein-related protein precursor***	***1.92***	***2.35***	***2.98***
	U212883	***LE24P06***	**Q05538**	***Basic endochitinase***	***1.91***	***3.29***	***4.20***
	U212927	***LE1K14***	**AAU95237**	***Osmotin-like protein precursor***	***1.86***	***2.92***	***3.25***
	U212829	***LE33A03***		***Glucan endo-l,3-β-glucosidase B***	***1.86***	***2.86***	***3.64***
	U216371	***LE23E12***	***XP_002516533***	***LLR receptor protein kinase***	***1.50***	***1.49***	***2.49***
Proteases inhibitors	U212880	LE31P14	CAC00536	Cathepsin D inhibitor	842.91	10.75	210.44
	U213021	LE29B20	P16231	Proteinase inhibitor I, trypsin inhibitor I	181.89	4.22	45.01
	AY129402	LE25H20	P05119	Proteinase inhibitor II gene	801.19	11.55	153.87
	U223902	LE26O04	P37842	Cysteine proteinase inhibitor	281.24	5.74	55.34
	U223774	LE12H24	CAC19488	Serine carboxypeptidase	−1.73	−2.78	−2.61
Hormone pathways	U37840	LE29M08	AAB65767	Lipoxygenase (Loxd)	2.59	2.29	2.00
Oxylipins	**U214318**	**LE19K07**	AAG42261	**Divinyl ether synthase**	**8.71**	**6.88**	**25.14**
	U215223	**LE8I15**	AAR05646	**DOX1 α-dioxygenase**	**2.17**	**4.89**	**6.73**
Ethylene	**U212786**	**LE10B24**	P05116	**ACC oxidase (ethylene-forming enzyme)**	**5.18**	**4.07**	**11.44**
Auxin	U219359	LE22A19	XP_002515504	AUX/IAA family	6.93	14.43	11.66
	U221902	LE15L20	XP_002533061	Auxin-induced protein (AIR12)	−2.46	−2.27	−2.66
	U215755	LE33N10	BAH15357	Germin-like protein (atgerl)	−2.91	−3.62	−3.19
Brassinosteroids	**U231291**	**LE4P20**	**XP_002514709**	**Protein kinase, similar to BRSI-1**	**2.16**	**1.65**	**3.32**
DNA binding	**U217978**	**LE24N20**	AAR88435	**No apical meristem (NAM)**	**4.79**	**1.93**	**7.37**
	U213218	LE8A24	ABK96797	No apical meristem (NAM)	2.76	2.65	2.69
	U213215	LE13C21	ABK96797	No apical meristem (NAM)	2.40	2.89	2.36
	U213219	LE30K03	ABI34386	NAC domain protein NAC2	2.17	2.05	2.06
	U214466	**LE19B22**	BAG50064	**AP2 domain transcription factor**	**2.46**	**2.26**	**4.44**
Wrky	**U213245**	**LE14B23**	ACF04195	**atWRKY40**	**5.42**	**4.92**	**11.01**
	U214599	**LE28N13**	ABI95141	**AtWRKY33**	**4.07**	**2.20**	**6.38**
	U226247	LE10K08	AAR92477	AtWRKY53	3.75	3.93	3.62
	U214107	**LE23G13**	ACJ04728	**AtWRKY 53**	**3.17**	**1.84**	**5.21**
	U219786	**LE23B10**	BAC23031	**AtWRKY75**	**7.28**	**4.32**	**15.73**
	U223447	LE31O23	XP_002532159	Helix–loop–helix DNA binding	2.62	5.55	5.12
Zinc	U213537	LE11E03	ABR68563	Salt-tolerance zinc-finger protein	3.66	3.58	3.45
	U224735	LE5N09	XP_002527502	C2h2 zinc-finger transcription factor	2.32	2.54	3.05
	U217330	LE33K02	XP_002516957	Zinc-finger protein similar to RING-H2	2.18	2.03	2.32
	U213848	LE1O12	AAU12056	Osmotic stress-induced zinc-finger	2.26	1.77	2.13
	U217330	LE33K02	XP_002516957	Zinc-finger protein	2.18	2.03	2.32
	U221112	**LE31P17**	ABK96800	**C3HC4-type zinc-finger protein**	**4.04**	**3.92**	**6.76**
	U221097	***LE8J01***	***NP_196245***	***B-type cyclin-related***	***1.50***	***1.70***	***2.70***
Heat shock	**U226141**	**LE24J09**	**XP_002522270**	**Heat-shock transcription factor family**	**3.16**	**3.52**	**5.37**
	U223342	**LE27O05**	**XP_002525885**	**Heat-shock transcription factor**	**3.14**	**3.49**	**5.05**
	U217418	***LE26M09***	P51819	***Heat-shock protein 81-1 (HSP81-1)***	***1.67***	***1.61***	***2.47***
Transport	**U223072**	**LE12N09**	ABM55247	**EDS5 calmodulin-binding family protein**	**2.96**	**1.49**	**5.49**
	U218455	**LE32G18**	**XP_002513573**	**Cyclic nucleotide-regulated ion channel I**	**3.67**	**1.93**	**8.89**
	U219700	**LE15C19**	**XP_002527859**	**Similar to Arabidopsis At2g38060 transp**	**3.03**	**−1.65**	**4.95**
Cell wall	U220494	LE9O13	AAS46243	Xyloglucan hydrolase	−2.58	−3.83	−3.46
	U213347	LE14P07	CAA96434	Pectin methylesterase	−3.19	−4.33	−3.47
	U213346	LE28J06	XP_002518523	Pectin methylesterase	−4.09	−5.74	−4.03
	U218509	LE30G15	ABG34280	Pectin acetylesterase family	−1.51	−2.02	−1.93
	U213442	LE10N09	Q9LW96	Myo-inositol 1-phosphate synthase	−3.85	−4.17	−3.55
	U214451	LE20J14	AAX20046	Pectin methylesterase inhibitor	2.65	1.53	2.19
Redox	**U213575**	**LE7B06**	**XP_002511189**	**PAP27 serine/threonine phosphatase**	**2.03**	**3.62**	**4.71**
	U213351	**LE14E03**	ACK57683	**Peroxidase 12**	**4.33**	**6.44**	**8.44**
	U226166	**LE14J03**	Q03664	**Glutathione transferase, auxin-induced**	**3.94**	**6.71**	**9.18**
	U215029	**LE23G02**	BAC81649	**Glutathione transferase**	**2.78**	**1.85**	**3.56**
	U212756	**LE6G19**	AAG16757	**Glutathione transferase**	**2.00**	**1.54**	**3.19**
	U212754	***LE8E06***	AAX20044	***Glutathione transferase***	***1.70***	***2.09***	***2.93***
	U217320	***LE20N03***	***XP_002509419***	***Glutaredoxin (thioltransferase)***	***1.76***	***4.17***	***3.57***
	U215934	**LE23B13**	**Q8H9D2**	**NADPH:quinone oxidoreductase**	**2.17**	**1.54**	**3.54**
	U213748	**LE3F13**	P37122	**Cytochrome P450 76A2**	**7.97**	**2.52**	**14.94**
	U215025	LE30I06	AAL54884	Cytochrome P450 fatty acid hydroxylase	2.09	2.01	2.94
	U222641	***LE11A05***	***XP_002333806***	***Cytochrome p450 hydroxylase***	***1.80***	***1.78***	***3.23***
	U222800	**LE24D04**	**XP_002310008**	**Cytochrome P450 84AI-hydroxylase**	**2.32**	**2.01**	**3.66**

*Annotation based on best match similarity according to Solgenomics annotation database (http://solgenomics.net).

Bold type, genes primed in Hx+Inf. Bold italic type, Hx-specific genes primed in Hx+inf and not induced by the fungus, but primed in Hx+Inf plants.

the plasma membrane, which participates in brassinolide perception (Li and Chory, 1997).

We searched for those BDEGs that encode putative DNA-binding proteins and found many putative transcription factor genes belonging to WRKY, non-apical meristem (NAM), APETALA2/ethylene response factor (AP2/ERF) and the zinc-finger family, which have been described previously in stress perception (AbuQamar *et al*., 2006; Jensen *et al*., 2010). It is noteworthy that most of the putative *WRKY* genes responding to *Botrytis* were induced by Hx and primed in Hx+Inf. These genes are similar to Arabidopsis *WRKY33* (*sgn-U214599*), *WRKY40* (*sgn-U213245*), *WRKY53* (*sgn-U214107*) and *WRKY75* (*sgn-U219786*), and have been described previously in the stress response.

The other DNA-binding factors induced after *Botrytis* infection were three putative NAM genes: *sgn-U217978*, *sgn-U213218* and *sgn-U213215*. They were all induced in Hx, but only the first was primed in Hx+Inf. An AP2 domain transcription factor (*sgn-U214466*) also responded to *Botrytis* and Hx, and was primed in Hx+Inf. Other BDEGs that encode putative transcription factors were helix–loop–helix DNA-binding factor *sgn-U223447*, which was primed in Hx+Inf, and six putative zinc-finger transcription factors: *sgn-U217330*, *sgn-U213537*, *sgn-U224735*, *sgn-U217330*, *sgn-U213848*, *sgn-U221112*. Finally, two BDEGs coding for putative heat-shock genes, *sgn-U226141* and *sgn-U223342*, were primed by treatment. Interestingly, *sgn-U217418*, another heat-shock gene, and *sgn-U221097*, which encodes a putative B-type cyclin, were not induced by either *Botrytis* or Hx itself, but were both primed in Hx+Inf plants.

Cell wall and membrane transport genes

We found that three BDEGs were involved in transport and primed in Hx+Inf plants: *sgn-U223072*, similar to Arabidopsis *EDS5*, *sgn-U218455*, similar to ion channel *CNGC1*, and *sgn-U219700*, which encodes a similar transporter to Arabidopsis *At2g38060*. Arabidopsis *EDS5* encodes a multi-drug and toxin extrusion transporter protein induced in response to pathogens. This gene has been highlighted by AbuQamar *et al*. (2006) in the Arabidopsis response to *Botrytis*.

The microarray data also indicated the down-regulation of genes involved in cell wall catabolism in Inf, Hx and Hx+Inf plants. These include a putative analogue of Arabidopsis xyloglucan endotransglucosylase-hydrolase (*XTH7*; *sgn-U220494*), two pectin methylesterases (*sgn-U213347*, *sgn-U213346*) and a pectin acetylesterase family member (*sgn-U218509*). We also found the induction of an inhibitor of the invertase/pectin methylesterase gene (*sgn-U214451*). These cell wall-related genes underwent similar regulation on *Botrytis*, Hx and Hx+Inf challenge. This agrees with the negative relationship between cell wall hydrolytic activities and pathogen defence (Cantu *et al*., 2008; Finiti *et al*., 2013; Flors *et al*., 2007).

Genes related to the redox environment

We identified genes involved in redox environment control: three glutathione *S*-transferases (GSTs) (*sgn-U226166*, *sgn-U215029*, *sgn-U212756*) and a peroxidase gene (*sgn-U213351*) induced by the fungus and Hx, and primed in Hx+Inf plants. Another GST (*sgn-U212754*) and a glutaredoxin (*sgn-U217320*) were Hx-specific genes not induced by the fungus, but primed in Hx+Inf plants. We also noted the induction of a NADPH:quinone reductase (*sgn-U215934*) and other genes involved in redox systems, such as some cytochrome P450 monooxygenase family members. A putative P450 hydroxylase (*sgn-U215025*) was induced similarly under all conditions, whereas another putative P450 hydroxylase (*sgn-U222641*), P450 76A2 (*sgn-U213748*) and P450 84A2 ferulate-5-hydroxylase (*sgn-U222800*) were clearly primed in Hx+Inf. These results suggest that Hx can modulate the plant redox metabolism by activating these genes. This may be particularly interesting, considering the massive ROS production which occurs in the early fungal infection stage (Heller and Tudzynski, 2011). To further investigate this hypothesis, we measured the accumulation of oxidative stress-related compounds and enzymatic activities in infected and treated plants.

Hx treatment alleviates oxidative stress

Hx treatment alters ROS accumulation on infection

We determined the effect of Hx treatment on oxidative stress and ROS by analysing the accumulation of the superoxide ion (O_2^-) and hydrogen peroxide (H_2O_2), respectively, by nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) staining, both at 72 h post-infection (Fig. 3).

The mock-treated plants infected with *Botrytis* (Fig. 3A,C) showed diffuse dark spots mainly around the infection site, but also systemic staining away from these areas. These are indicative of O_2^- (Fig. 3A) and H_2O_2 (Fig. 3C) local and systemic accumulation. Hx+Inf plants showed reduced dark areas in both locations, especially in the systemic zones (Fig. 3B,D).

Hx controls ascorbate and GSH steady-state levels

Ascorbate is quantitatively the most important antioxidant compound to accumulate in plant cells. One of its main functions is to scavenge the most dangerous forms of ROS and to participate in H_2O_2 detoxification, together with GSH (Foyer and Noctor, 2011). No differences were observed in the reduced ascorbate to oxidized dehydroascorbate (AsA/DAsA) ratio at 48 and 72 hpi between Ctrl and Inf plants (Fig. 4A). Interestingly, however, Hx+Inf plants showed a diminished total ascorbate content (Fig. S2, see Supporting Information) and oxidized DAsA (Fig. S3, see Supporting Information), which led to a significantly higher AsA/DAsA ratio (Fig. 4A).

We assayed the GSH content, the main soluble thiol compound in plant cells, which scavenges most ROS and participates in the

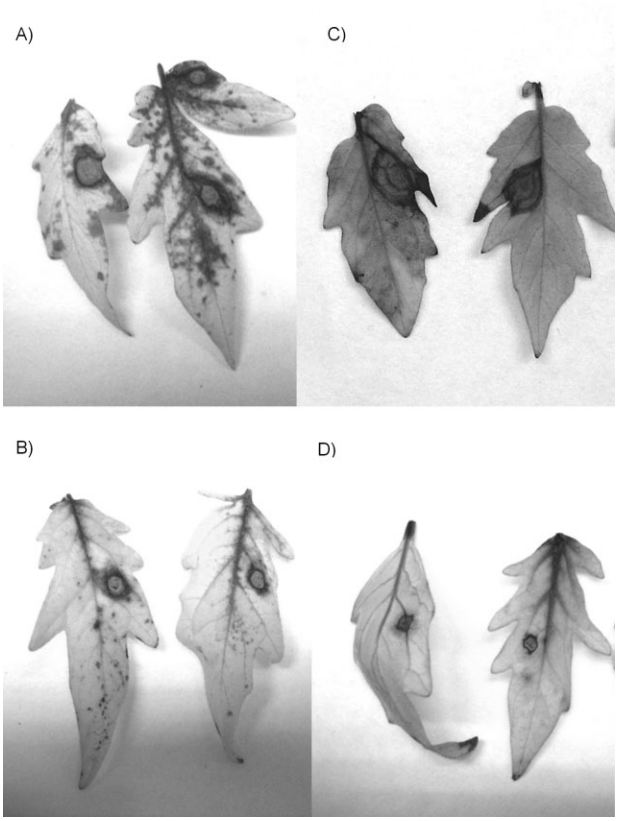

Fig. 3 Reactive oxygen species (ROS) accumulation in untreated plants (A, C) and hexanoic acid (Hx)-treated plants (B, D) at 72 h post-inoculation (hpi). (A, B) O_2^- was determined by nitroblue tetrazolium (NBT) staining. (C, D) H_2O_2 was visualized by 3,3'-diaminobenzidine (DAB) staining. Images are representative of three independent experiments.

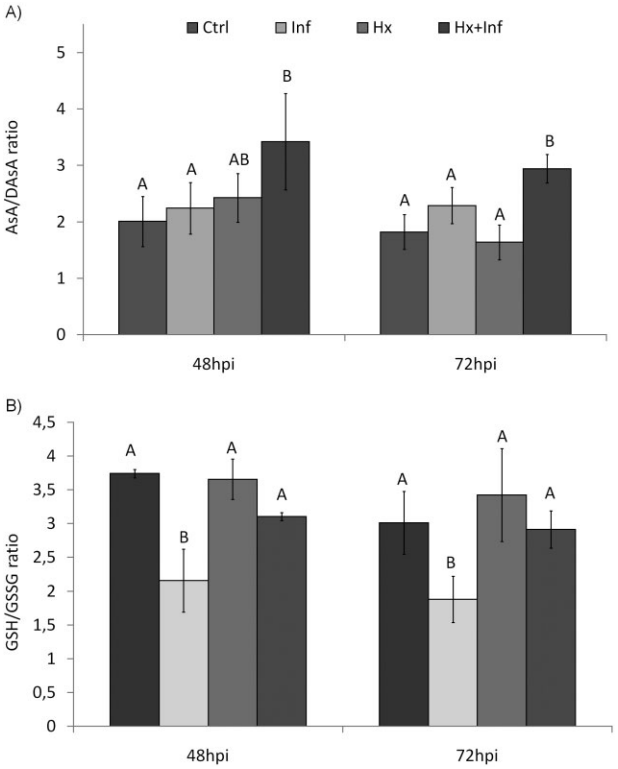

Fig. 4 Ascorbate and glutathione levels in the untreated non-infected plants (Ctrl), untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf) at 48 and 72 h post-inoculation (hpi). (A) Reduced ascorbate (AsA) and oxidized dehydroascorbate (DAsA) ratios. (B) Reduced (GSH) and oxidized (GSSG) glutathione ratios. The data shown are the means of three independent experiments ± standard deviation (SD). Different letters indicate Student's *t*-test significant differences at the 95% confidence level.

regeneration of ascorbate via dehydroascorbate reductase (DHAR), thus contributing to H_2O_2 detoxification (Smirnoff, 2007). At 72 hpi, total GSH content and its oxidized form (GSSG) increased significantly in Inf plants (Figs S4 and S5, see Supporting Information), leading to a significantly lower reduced/oxidized GSH/GSSG ratio than in Ctrl plants, which reflects an oxidized status (Fig. 4B). This agrees with the early oxidative burst which occurred on fungal inoculation. Hx and Hx+Inf plants showed total and oxidized GSH (GSSG) levels close to those of Ctrl plants (Figs S4, S5), with a normal GSH/GSSG ratio, similar to Ctrl plants (Fig. 4B). This suggests an alleviating effect of Hx treatment on the oxidative imbalance associated with *Botrytis* infection.

Hx treatment avoids alteration in antioxidant enzymatic activities

We measured two antioxidant enzymatic activities that relate closely to oxidative stress control: GR and CA activities. GR is an enzyme that reduces GSSG to the reduced sulphydryl form GSH, which is an important cellular antioxidant (Meister, 1988). CA catalyses H_2O_2 decomposition to water and oxygen, and is a very important enzyme that protects cells from oxidative damage by ROS (Mhamdi *et al*., 2010).

Our data show that GR activity decreased in *Botrytis*-infected plants at 48 hpi, but increased at 72 hpi (Fig. 5A). In Hx+Inf, treatment avoided GR activity fluctuations, whose level remained close to that of Ctrl plants at both 48 and 72 hpi.

CA activity rose in Inf plants at 72 hpi (Fig. 5B), but a less marked increase was noted in Hx+Inf plants, with levels closer to those of Ctrl.

These results indicate that the early gene expression alterations observed in Hx+Inf plants can prime redox control mechanisms to produce visible alterations after pathogen attack, leading to a less stressed environment during infection.

DISCUSSION

Hx-IR primes protection against *B. cinerea* in tomato plants. Our results indicate that Hx alters tomato gene expression in a similar manner to fungal infection, and that it provides more efficient

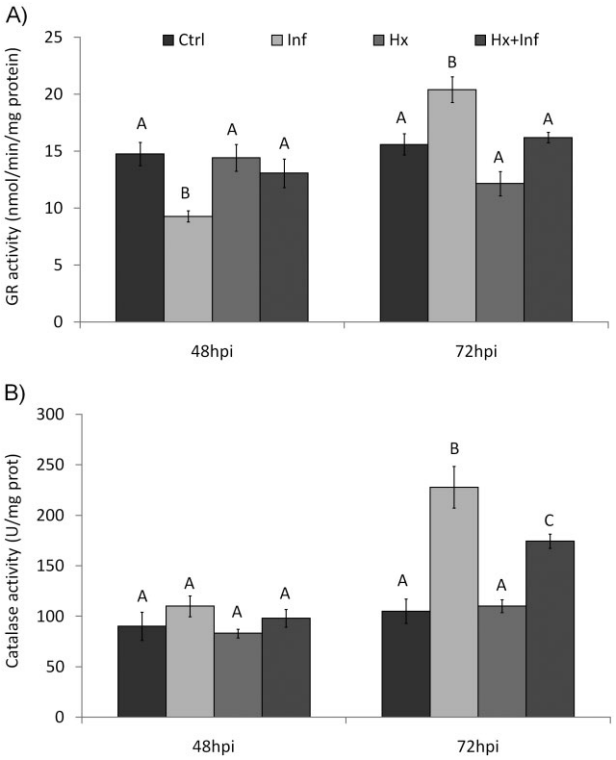

Fig. 5 Antioxidant enzymatic activity in the untreated non-infected plants (Ctrl), untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf) at 48 and 72 h post-inoculation (hpi). (A) Glutathione reductase activity. (B) Catalase activity. Quantifications were performed spectrophotometrically. The data shown are the means of three independent experiments ± standard deviation (SD). Different letters indicate Student's *t*-test significant differences at the 95% confidence level.

defence against this pathogen when applied as a preventive treatment. The analysis of the tomato transcriptome on *Botrytis* infection showed the activation of many defence-related, DNA-binding, hormonal regulation, cell wall metabolism, transport and oxidative stress genes. Interestingly, Hx treatment and Hx+Inf altered and primed many BDEGs. In addition, Hx and Hx+Inf activated other genes that were not altered by *Botrytis* at this early time point, which are considered to be Hx-specific genes.

The overlapping of the Inf, Hx and Hx+Inf expression patterns indicates that Hx-IR can rely on both the pre-activation of BDEGs and their boosting after pathogen attack. In addition, the pathogen-independent gene activation observed in Hx plants may contribute to explain the broad-spectrum protection conferred to Hx-treated plants recently reported in Scalschi *et al*. (2013).

Some BDEGs induced in Hx and boosted in Hx+Inf are related to the defence response, such as basic endochitinase and *N*-hydroxycinnamoyl transferase (*THT1-3*), and both have been reported previously in tomato fruits infected by *B. cinerea* (Cantu *et al*., 2009). The Hx priming of *THT1-3* may be important in Hx-IR against *Botrytis* when considering that transgenic tomato plants overexpressing *THT1-3* are more resistant to *Pseudomonas syringae* and show higher levels of *p*-cumaroyl curamine, an antimicrobial compound with very high antioxidant activity in leaves (von Roepenack-Lahaye *et al*., 2003). The treatment's capability to induce the expression of some defence-related genes not induced by *Botrytis* at 24 hpi is also noteworthy, e.g. *PR1a*, endo-1,3-β-glucanase, osmotin and basic endochitinase. This early induction of PR genes may represent an important advantage of Hx-treated plants to limit further infections.

Our data revealed the high induction of several genes encoding proteinase inhibitors in response to *B. cinerea* in Inf plants. These genes were also induced in Hx and Hx+Inf plants, but at lower levels than in Inf. There is no direct evidence for the importance of proteinases and proteinase inhibitors in tomato–*Botrytis* interaction. However, the release of fungal proteinases probably contributes to its colonization strategy (van Loon *et al*., 2006). The relevance of proteinases in fungal virulence is supported by the large number of aspartic and serine proteinases found in the early *B. cinerea* secretome (Espino *et al*., 2010). Hence, it may be hypothesized that the delivery of massive fungal degrading enzymes could be counteracted by plant proteinase inhibitors. This matches the considerable induction of these particular genes by *B. cinerea* described herein, and makes their previous activation in Hx-treated plants especially interesting, which might greatly contribute to the establishment of Hx-IR on fungal infection in tomato.

Our study also showed the slight repression of a plant serine carboxypeptidase gene on infection. In Arabidopsis, the negative regulation of several genes coding for serine proteinases on *Botrytis* challenge has also been reported (Kaschani *et al*., 2009). Interestingly, this repression increased in Hx and Hx+Inf plants. This result matches the induction of previously described proteinase inhibitors, which further indicates that the limitation of proteinase activities might form an active part of the plant defence strategy to limit fungal advance. Hx treatment may preventively activate these responsive mechanisms to provide better protection to further pathogen attacks, as observed in Hx+Inf plants.

Botrytis infection and Hx treatment also activated the genes involved in plant hormone synthesis and signalling. We have reported previously that Hx-IR requires the JA signalling pathway (Vicedo *et al*., 2009). Here, we found that the early activation of two genes is involved in the biosynthesis of different oxylipins. *DES* was induced in Inf and Hx plants, and was greatly boosted in Hx+Inf plants. It is known that DES converts hydroperoxides into divinyl ethers, and that it is involved in the biosynthesis of the antifungal toxin colneleic acid (Itoh and Howe, 2001). A similar pattern was displayed by *DOX1*, which was induced in Inf and Hx and primed in Hx+Inf plants. DOX1 is involved in plant protection from oxidative damage and cell death (Tirajoh *et al*., 2005). This gene is also induced on *P. syringae* infection in Arabidopsis (De Leon *et al*., 2002), on herbivorous attack in *Nicotiana attenuata*

(Steppuhn *et al*., 2010) and on *B. cinerea* inoculation in tomato fruits (Cantu *et al*., 2009). Therefore, these results extend our previous findings, and indicate that Hx can prime oxylipin-related genes linked to the defence response, probably by achieving the activation of broad protection against different pathogens.

LoxD, a gene that initiates the JA synthesis pathway, was also induced by *B. cinerea* at 24 hpi. Hx treatment did not prime *LoxD* at 24 hpi, suggesting that the early response in the Hx-IR might require the activation of other JA-independent genes, as demonstrated previously in other pathosystems (Prost *et al*., 2005; Vicente *et al*., 2012).

Another BDEG found herein encodes an ACC oxidase induced in Inf and Hx, and boosted in Hx+Inf plants. This suggests that ET synthesis forms part of the early plant response to fungal infection, and that Hx can promote and amplify this response. The role of ET in plant–pathogen interactions is complex; its contribution can be positive or negative, depending on the timing and relative levels of other hormones. According to Diaz *et al*. (2002), early activation of ET synthesis prior to pathogen attack can increase plant resistance to *B. cinerea*. Hence, the pre-activation and potentiation of the *ACC* gene found herein in Hx and Hx+Inf plants probably contributes to the increased resistance of Hx-treated tomato plants to this fungus.

In this study, we also describe the induction of many putative DNA-binding protein genes under all the conditions assayed. They belong mostly to the WRKY, NAM, AP2/ERF and zinc-finger transcription factor families. The Arabidopsis WRKY transcription factor family has been largely linked to the defence response, which makes the Hx capability of inducing WRKY genes in tomato particularly interesting. Among the induced WRKY factors found herein, some orthologues in Arabidopsis are *WRKY40*, *WRKY33*, *WRKY53* and *WRKY75*. The Arabidopsis *wyrky40* mutant is more susceptible to *B. cinerea* (Xu *et al*., 2006). Tomato *sgn-U213245* is closely related to Arabidopsis *WRKY40* and *WRKY18*, which are also involved in the defence response (Pandey *et al*., 2010; Xu *et al*., 2006). *sgn-U214599* is similar to *WRKY33*, which was been reported recently as a key transcriptional regulator of hormonal and metabolic responses against *B. cinerea* (Birkenbihl *et al*., 2012). *sgn-U214107* is similar to *WRKY53*, which has been reported previously to be up-regulated in *Arabidopsis thaliana* following *B. cinerea* infection (AbuQamar *et al*., 2006). In addition, it is especially noteworthy that Jaskiewicz *et al*. (2011) have recently reported this gene as a specific target of the priming effect of BTH inducer treatment. According to the authors, priming occurs through the changes in histone acetylation observed prior to pathogen inoculation. Hence, *WRKY53* gene overinduction in Hx+Inf tomato plants reinforces the possibility of Hx being a priming agent. We also noted the down-regulation of the genes involved in cell wall metabolism under all the conditions assayed, among them endotransglucosylase-hydrolase, pectin methylesterase, a probable pectin acetylesterase and a myo-inositol 1-phosphate synthase analogue. Furthermore, a gene coding for an inhibitor of invertase/pectin methylesterase was induced under all conditions. These data suggest that infected plants make attempts to restrict cell wall degradation by both reducing hydrolytic enzymes and releasing specific inhibitors. This agrees with previous data, which indicate that the impairment of cell wall-degrading enzymes can contribute to increased plant resistance (Finiti *et al*., 2013; Flors *et al*., 2007).

It is noteworthy that we observed that tomato responds to *Botrytis* by activating many redox status-related genes. The oxidative burst and ROS accumulation are critical factors in *Botrytis*–tomato and other pathosystems (Heller and Tudzynski, 2011). Pathogen infection promotes the oxidative burst in invaded plant cells, often followed by the activation of antioxidant and detoxifying enzymes, such as glutathione *S*-transferases (GSTs) and peroxidases (AbuQamar *et al*., 2006; Lamb and Dixon, 1997; Parisy *et al*., 2007). GSH- and redox equilibrium-controlling genes are essential for an appropriate defence response against *B. cinerea* (Chassot *et al*., 2008). However, the contribution of ROS to the plant–pathogen interaction is complex. They form part of the plant defence response, but *B. cinerea* is also able to stimulate their production for its own benefit (Temme and Tudzynski, 2009). The timing, duration and intensity of the ROS wave are critical factors for final infection outcome.

As mentioned earlier, genes involved in the plant's general redox system were induced in Inf plants, but, more interestingly, most were induced and potentiated in Hx and Hx+Inf plants: putative peroxidase, GR, glutathione transferase, NADPH:quinone reductase. This suggests that plants attempt to reduce early the oxidative burst occurring on *Botrytis* challenge. The early overinduction of detoxifying and redox balance-related genes by the inducer suggests that limitation of the oxidative burst on inoculation contributes to reduce fungal colonization in Hx+Inf plants. Indeed, a putative GST (*sgn-U212754*) and a putative glutaredoxin (*sgn-U217320*), which were not induced in Inf plants at 24 hpi, were activated in Hx and Hx+Inf plants. This indicates that at least part of the activation of redox-related genes is a direct target of the inducer treatment. Hence, we can rule out the possibility that the gene boosting observed in Hx+Inf plants might be an indirect effect of the reduced fungal invasion caused by Hx treatment. Therefore, these data indicate that part of Hx-IR relies on the capacity to activate detoxifying systems early in order to prevent the harmful effects of oxidative stress during later steps of the infection.

The analysis of the oxidative stress-related compounds in treated and untreated plants after *Botrytis* inoculation showed that O_2^- and H_2O_2 accumulation was reduced and more restricted around the infection site in Hx+Inf plants. According to the reduced oxidative damage observed in treated plants on infection, ascorbate and GSH reduced/oxidized ratios increased in Hx+Inf plants at 72 hpi, whereas the levels of GR and CA enzymatic activities remained closer to those of non-infected plants.

The early Hx transcriptional induction of redox-related genes, including GST and glutaredoxin, agrees with the alleviating effect observed on oxidative stress markers in Hx+Inf plants. This suggests that the inducer can act by damping the fluctuations of the redox equilibrium to provide a less stressed environment in infected plants. The priming of other genes, such as peroxidase and several GSTs, supports the direct effect of this inducer on this defensive mechanism. The fact that Hx treatment alone did not reveal significant changes in the redox environment control, which were expected for a priming agent, makes it difficult to clearly establish whether the redox environment control is a target in Hx-IR that limits fungal spread, or whether it is the result of reduced infection caused by Hx-IR. Although both effects probably contribute to the control of *B. cinerea* in Hx-treated plants, our data suggest that Hx primes the gene transcription to control the redox metabolism which, as the priming definition establishes, is fully activated and is observed only after pathogen recognition occurs (Conrath *et al*., 2002). Further experiments are underway to establish the contribution of limiting oxidative stress in Hx-IR.

In conclusion, the data obtained in this work have helped to identify many gene markers of the host response to *Botrytis* and Hx treatment. We found genes that help to characterize the Hx priming effect, especially those related to defence, the signalling network and oxidative stress control, which are boosted in Hx+Inf plants. Activation and priming of a large set of defence genes reflects the probable broad-spectrum action of this natural inducer, which is further supported by its effectiveness against *P. syringae*, as reported recently by Scalschi *et al*. (2013). In that work, the results indicated a positive relationship between SA and JA pathways in Hx-primed tomato plants. This agrees with the present concept that the effectiveness of the plant response against biotrophic and necrotrophic pathogens is much more complex than the classical dichotomy between SA and JA pathways. The timing and intensity of these signals, as well as their interaction, are critical for the final outcome of the infection. The effect of Hx treatment on both SA and JA pathways could activate broad-spectrum responses that would be reinforced by pathogen-specific responses in each particular case. Finally, we found that Hx priming of redox-related genes correlates with the antioxidant protective effect observed in treated plants at later steps of the infection. This result shows the importance of controlling oxidative stress and redox equilibrium to improve plant protection against this fungus and other necrotrophic pathogens.

EXPERIMENTAL PROCEDURES

Plant material, microbial strains and Hx treatment

Tomato (*Solanum lycopersicum*) cv. Ailsa Craig plants were grown in commercial peat in a glasshouse with 16 h of daylight for 4 weeks.

Botrytis cinerea CECT2100 (Spanish collection of type cultures) was cultured routinely on potato dextrose agar (PDA) (Difco, Franklin Lakes, NJ, USA; http://www.bd.com) at 24 °C. *Botrytis cinerea* spore collection and plant inoculation were performed as described by Flors *et al*. (2007). Hx (Sigma, Barcelona, Spain) was dissolved in water and applied to plants as described by Vicedo *et al*. (2009).

RNA extraction and microarray analysis

For each biological replicate, inoculated leaves from six different plants were harvested, pooled and immediately frozen in liquid nitrogen. Total leaf RNA was isolated as described by de Torres-Zabala *et al*. (2003), and was further purified following the RNAeasy kit cleanup protocol (Qiagen, Valencia, CA, USA). RNA was quantified by spectrometry (NanoDrop ND1000, NanoDrop Technologies, Wilminton, DE, USA) and RNA quality was confirmed with an RNA 6000 Nano Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) assay. Five hundred nanograms of control ('C' samples) versus infected ('I' samples) RNAs were used to produce cyanine 3-CTP- or cyanine 5-CTP-labelled cRNA with the Quick Amp Labelling Kit, Two-Color (Agilent p/n 5190-0444), according to the manufacturer's instructions. Following the Two-Color Microarray-Based Gene Expression Analysis protocol Version 5.0 (Agilent p/n G4140-90050), 1000 ng of 'C'- and 'I'-labelled cRNA products were co-hybridized with a Custom GE Oligo Microarray (Agilent p/n G2519F-027077) containing 11 339 probes (60-mer) obtained from Operon (Eurofins MWG Operon, Ebersberg, Germany; http://www.operon.com), plus 339 customised 60-mer probes designed from sequences of less than 50-mer Operon probes. All the probes were represented in triplicate. Fifty probes from the Agilent tomato array (022270) were used as internal replicated controls, with 10 replicates per probe. Arrays were scanned in an Agilent Microarray Scanner (G2565BA) according to the manufacturer's protocol.

For each condition assayed, two biological replicate RNA samples were used for hybridization. Eight hybridization experiments were performed. In each hybridization experiment, the normalized ratios of the three replicate spots for each probe were averaged. Only those genes with a final expression ratio of ≥ 2.0 or ≤ 0.5, calculated as the average ratio of the two biological replicates with $P \leq 0.05$ (analysis of variance, ANOVA) under all the conditions, were counted as differentially expressed.

Microarray data mining

Hierarchical clustering was performed by CLUSTER 3.0, and visualized with JavaTreeview V.1.1.4. Complete linkage using an uncentred Pearson correlation was applied to the normalized logged data.

Validation of expression data

The cDNA for the RT-PCR validation assay was generated from 2 μg of RNA processed with the Superscript III kit (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. RT-PCR was performed with the QuantiTect SYBRGreen kit (Qiagen) in a LightCycler480 (Roche Diagnostics, Mannheim, Germany).

Differential gene expression was determined by relative quantification. Tomato *18S* and *actin* gene expressions were used as internal standards with similar results. The efficiency (*E*) was fixed for each primer pair at 1.8.

The relative expression was determined by the $\Delta\Delta Ct$ method (Pfaffl, 2001). RT-PCR was performed in triplicate per gene for each biological replicate. In all instances, the RT-PCR expression levels (for the eight individual genes) corroborated the array expression data.

Genes and primer sequences are reported in Table S1 (see Supporting Information).

Statistical analysis

The statistical analysis was carried out by performing ANOVA for the microarray data, and Student's *t*-test at a 95% confidence level for the ROS assays.

The experiments for the microarray assays were repeated twice, and were repeated three times for ROS determination. All the experiments were performed with six plants for each condition. All the values shown are the average of independent experiments ± standard deviation.

Redox metabolite assays

H_2O_2 accumulation

H_2O_2 was determined by staining leaves with DAB (Sigma, D8001), as described by Orozco-Cardenas and Ryan (1999).

O_2^- accumulation

O_2^- was determined by NBT (Sigma, N6639) staining, as described by Carol *et al*. (2005).

GR activity

Samples (0.1 g of tissue) were homogenized in 0.5 mL of 50 mM 2-(*N*-morpholino)ethanesulphonic acid (MES)/KOH buffer, pH 6.0, and centrifuged at 10 000 ***g*** for 10 min at 4 °C. The supernatant was diluted (10–40 μL) in a final volume of 0.2 mL of 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (Hepes) buffer, pH 8.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA). GR activity was assayed in microplates and was measured spectrophotometrically as NADPH oxidation at 340 nm in the presence of 0.25 mM NADPH (Murshed *et al*., 2008). The GR reaction was started by the addition of 5 μL of 20 mM of oxidized GSH (GSSG) to each well. Activity was calculated from the extinction coefficient at 6.22 mM^{-1} cm^{-1}. GR activity was defined as 1 micromole of substrate consumed per minute per milligram of protein. Protein concentration was quantified in each extract by Bradford's procedure (Bradford, 1976) using the Bio-Rad (Bio-Rad, Hercules, CA, USA) protein assay.

CA activity

Samples (0.05 g of tissue) were homogenized in 0.2 mL of 50 mM phosphate buffer, pH 7.0, with a mix of protease inhibitors [200 μM phenylmethylsulphonyl fluoride (PMSF), 20 μM tosyl phenylalanyl chloromethyl ketone, 200 μM pepstatin A] and centrifuged at 10 000 ***g*** for 10 min at 4 °C. CA activity was assayed in microplates, as described by Jakubowski *et al*. (2000). Absorbance was measured at 240 nm and activity was calculated using an extinction coefficient of 43.66 M^{-1} cm^{-1}. CA activity was expressed as micromoles of H_2O_2 per minute per milligram of protein.

Glutathione

Total and oxidized GSH (GSSG) were assayed using 5,5-dithiobis-nitrobenzoic acid (DTNB), according to the method of Griffith (1980). Tissue samples (0.1 g) were homogenized in 1 ml of ice-cold 8 mM HCl, 1.3% (w/v) 5-sulphosalicylic acid. Samples were centrifuged at 10 000 ***g*** for 15 min at 4 °C, and the supernatants were used for GSH determination. For total GSH determination, the supernatant was diluted directly in 0.2 mL of 0.4 M MES, 0.1 M sodium phosphate, pH 7.4, 2 mM EDTA, pH 6.0. GSSG content was measured after having removed reduced GSH by 2-vinyl pyridine derivatization for 1 h at room temperature. A sample aliquot (0.2 mL) was mixed with 0.12 mL of NADP (0.4 mg/mL), glucose-6-phosphate (0.16 mg/mL), glucose-6-phosphate dehydrogenase (3 μg/mL), GR (1 mU) and 0.48 mL of 0.2 mM DTNB. The mixture was incubated at room temperature in the dark under agitation. After 20 min, the absorbance was measured at 412 nm. To calculate GSH content, a standard curve prepared with GSSG was used. GSH levels are expressed as micromoles per gram of fresh weight.

Ascorbate

AsA and DAsA were determined according to Kampfenkel *et al*. (1995). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA and the spectrophotometric detection of Fe^{2+} complexed with 2,2'-dipyridyl at 525 nm. DAsA was reduced to AsA by preincubating the sample with dithiothreitol (DTT). Excess DTT was removed with *N*-ethylmaleimide (NEM) and total AsA was determined by the 2,2'-dipyridyl method. Frozen leaves (0.1 g) were homogenized in 0.2 mL of 6% trichloroacetic acid (w/v). The mixture was incubated on ice for 15 min and centrifuged at 10 000 ***g*** for 10 min at 4 °C. Supernatants were assayed directly to measure AsA and DAsA. Concentrations were determined using a standard curve of ascorbic acid. Values are expressed as micromoles per gram of fresh weight.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Overlap of the early gene expression response on *Botrytis* infection (Inf), hexanoic acid treatment (Hx) and Hx treatment followed by infection (Hx+Inf). (A) Venn diagram showing the number of differentially expressed genes under each condition. (B) Expression profiles hierarchically clustered by CLUSTER 3.0.

Fig. S2 Total ascorbate levels in the untreated non-infected plants (Ctrl), untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf) at 48 and 72 h post-inoculation (hpi). Data are the means of three independent experiments ± standard deviation. Different letters indicate Student's *t*-test significant differences at the 95% confidence level. FW, fresh weight.

Fig. S3 Oxidized ascorbate levels (DAsA) in the untreated non-infected plants (Ctrl), untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf) at 48 and 72 h post-inoculation (hpi). Data are the means of three independent experiments ± standard deviation. Different letters indicate Student's *t*-test significant differences at the 95% confidence level. FW, fresh weight.

Fig. S4 Total glutathione (GSH) levels in the untreated non-infected plants (Ctrl), untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf) at 48 and 72 h post-inoculation (hpi). Data are the means of three independent experiments ± standard deviation. Different letters indicate Student's *t*-test significant differences at the 95% confidence level. FW, fresh weight.

Fig. S5 The oxidized glutathione (GSSG) levels in the untreated non-infected plants (Ctrl), untreated and *Botrytis*-infected plants (Inf), hexanoic acid (Hx)-treated and non-infected plants (Hx), and Hx-treated and infected plants (Hx+Inf) at 48 and 72 h post-inoculation (hpi). Data are the means of three independent experiments ± standard deviation. Different letters indicate Student's *t*-test significant differences at the 95% confidence level. FW, fresh weight.

Table S1 Primers used for reverse transcription-polymerase chain reaction (RT-PCR).