

## The *fou2* Gain-of-Function Allele and the Wild-Type Allele of *Two Pore Channel 1* Contribute to Different Extents or by Different Mechanisms to Defense Gene Expression in Arabidopsis

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The *fatty acid oxygenation up-regulated 2 (fou2)* mutant in *Arabidopsis thaliana* creates a gain-of-function allele in a non-selective cation channel encoded by the *Two Pore Channel 1 (TPC1)* gene. This mutant genetically implicates cation fluxes in the control of the positive feedback loop whereby jasmonic acid (JA) stimulates its own synthesis. In this study we observed extensive transcriptome reprogramming in healthy *fou2* leaves closely resembling that induced by treatment with methyl jasmonate, biotic stresses and the potassium starvation response. Proteomic analysis of *fou2* leaves identified increased levels of seven biotic stress- and JA-inducible proteins. In agreement with these analyses, epistasis studies performed by crossing *fou2* with *aos* indicated that elevated levels of JA in *fou2* are the major determinant of the mutant phenotype. In addition, generation of *fou2 aba1-5*, *fou2 etr1-1* and *fou2 npr1-1* double mutants showed that the *fou2* phenotype was only weakly affected by ABA levels and unaffected by mutations in *NPR1* and *ETR1*. The results now suggest possible mechanisms whereby *fou2* could induce JA synthesis/signaling early in the wound response. In contrast to *fou2*, transcriptome analysis of a loss-of-function allele of *TPC1*, *tpc1-2*, revealed no differential expression of JA biosynthesis genes in resting leaves. However, the analysis disclosed reduced mRNA levels of the pathogenesis-related genes *PDF1.2a* and *THI2.1* in healthy and diseased *tpc1-2* leaves. The results suggest that wild-type *TPC1* contributes to their expression by mechanisms somewhat different from those affecting their expression in *fou2*.

**Keywords:** Cation Flux — Jasmonic acid — 13-Lipoxygenase — Oxylipin — *TPC1*.

Abbreviations: AOS, allene oxide synthase; bHLH, basic helix–loop–helix; CAPS, cleaved amplified polymorphic sequences; 2-DE, two-dimensional electrophoresis; DTT, dithiothreitol; ET, ethylene; *fou2*, fatty acid oxygenation up-regulated 2; GO, gene ontology; IEF, isoelectric focusing; JA, jasmonic acid; LOX, lipoxygenase; MALDI-TOF MS; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry;

MeJA, methyl jasmonate; PME, pectin methylesterase; qPCR, quantitative PCR; SA, salicylic acid; *TPC1*, Two Pore Channel 1; WT, wild type.

The nucleotide sequences reported in this paper have been submitted to GenBank under the following accession numbers: *TPC1* (At4g03560), *LOX1* (At1g55020), *LOX2* (At3g45140), *eIF4A1 $\alpha$*  (At3g13920), *PDF1.2a* (At5g44420), *THI2.1* (At1g72260).

### Introduction

Jasmonic acid (JA) is one of the key mediators in plant responses to wounding, herbivory and diverse biotic stresses including pathogenesis. Attack stimulates the production of JA through a mechanism that involves a positive feedback loop where JA signaling activates the expression of JA synthesis genes. The two processes, JA synthesis and JA signaling, are thus intimately related (Sasaki et al. 2001, Schaller et al. 2005). Significant advances have been achieved in the identification of genes involved in JA biosynthesis in different plant species (Turner et al. 2002, Schilmiller and Howe 2005). The same can be said of JA signaling which leads to the control of diverse physiological processes (in addition to direct defense responses) such as growth, development and metabolic homeostasis (Ellis et al. 2002, Armengaud et al. 2004, Devoto et al. 2005, Ko et al. 2006, Mandaokar et al. 2006, Zavala and Baldwin 2006, Beveridge et al. 2007). It is known that most if not all JA-mediated cellular responses depend on signaling through CORONATINE INSENSITIVE 1 (COI1), an F-box protein that associates with other components to form an E3-ubiquitin ligase complex (Xie et al. 1998). The feedback loop to JA synthesis is no exception, being largely dependent on COI1. Attempts are now being made to understand other genetic elements controlling the positive feedback regulation of JA biosynthesis which is apparently essential for both basal and induced expression of JA biosynthesis genes. Mechanisms

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regulating this process are expected to be complex and could, theoretically, be exerted at all levels of gene expression.

With this in mind, we recently isolated the *fatty acid oxygenation up-regulated 2* (*fou2*) mutant which was identified based on its elevated lipoxygenase (LOX) and allene oxide synthase (AOS) activities in leaves. As a result, *fou2* has an increased capacity to accumulate jasmonates after leaf wounding and increased resistance to the necrotrophic pathogen *Botrytis cinerea* (Bonaventure et al. 2007). One of the most interesting features of the mutant is that its phenotype only becomes apparent at a vegetative phase transition state where the activity of the JA pathway in wild-type (WT) plants is naturally up-regulated (Bonaventure et al. 2007). The mutation in *fou2* was mapped to the *Two Pore Channel 1* (*TPC1*) gene. This gene is described as encoding a Ca<sup>2+</sup>-regulated non-specific cation channel that forms part of the slow-vacuolar (SV) channel in the tonoplast of Arabidopsis (Furuichi et al., 2001, Peiter et al. 2005). *fou2* carries an amino acid substitution in the TPC1 protein where an aspartate is replaced by an asparagine at residue 454 of the protein (TPC1<sup>D454N</sup>). This substitution generates a functional version of the TPC1 channel that has electrophysiological properties different from those of the WT channel (Bonaventure et al. 2007).

Whereas *fou2* showed elevated LOX activity levels and was more resistant than the WT to the fungus *B. cinerea*, a loss-of-function mutant of TPC1 (*tpc1-2*) showed no elevated LOX activity, no elevated LOX2 and AOS mRNA expression, no increased resistance to *B. cinerea* compared with WT, and a similar morphology to WT plants. These results suggested that the *fou2* mutation conferred a new function to the TPC1 channel different from its physiological function (Bonaventure et al. 2007). The *tpc1-2* mutant is, however, affected in its stomatal response to external calcium, and its seeds show a reduced sensitivity to ABA compared with the WT (Peiter et al. 2005). In other plant species, several studies have recently linked TPC1 to a diverse group of cellular responses related to defense, such as programmed cell death and expression of defense genes and antioxidant enzymes. For example, co-suppression of TPC1 in tobacco causes inhibition of cryptogein-induced cell death and defense-related gene expression (Kadota et al. 2004). Kurusu et al. (2005) showed that TPC1 contributes to the regulation of elicitor-induced defense responses and cell death in rice. These diverse cellular responses, ranging from the regulation of defense-related processes in tobacco and rice cells to the control of germination and stomatal dynamics in Arabidopsis, suggest that TPC1 may have a broad action in plants, contributing to the regulation of different cellular events.

Although the *fou2* mutation most probably generates a TPC1 channel with a novel function, we reasoned that the cellular mechanisms that act downstream of and become activated by the mutated channel (e.g. activation of cation responses) may represent physiological mechanisms acting early in the activation of oxylipin biosynthesis after wounding or biotic stresses in WT plants. Thus, to begin to understand the cellular mechanisms that are activated by the *fou2* allele of TPC1, we performed transcriptome and proteome analyses of healthy *fou2* leaves together with genetic studies utilizing *fou2* and several mutants implicated in hormone biosynthesis and signaling. In addition, to explore a potential role for WT TPC1 in the expression of biotic stress-responsive genes, transcriptome analysis was also performed with healthy *tpc1-2* leaves. The results demonstrated that the *fou2* allele induces a biotic stress response largely mediated by JA and similar to that induced by diverse pathogens and potassium starvation. In contrast, analysis of gene expression in *tpc1-2* suggests that WT TPC1 either does not or only weakly affects JA signaling in resting leaves and contributes to the expression of two defense genes (*PDF1.2a* and *THI2.1*) after pathogen infection.

## Results

### *Transcriptome analysis of fou2 leaves*

To investigate further the role of TPC1<sup>D454N</sup> in Arabidopsis, gene expression profiling analyses were conducted with the *fou2* mutant. For these experiments, gene expression was evaluated in rosette leaves of 4-week-old plants. This developmental stage was chosen because it coincides with the time-specific appearance of the *fou2* morphological and biochemical phenotypes (Bonaventure et al. 2007). Before 3 weeks of growth, *fou2* resembles WT plants both morphologically and at the level of LOX and AOS activities. Just at the beginning of the fourth week, the *fou2* phenotype becomes slowly apparent and is marked at the end of the same week. Because of this stage-specific onset of both the biochemical and morphological phenotypes in *fou2*, the developmental program of the mutant is not altered compared with the WT (Bonaventure et al. 2007) and ensures that most if not all changes in gene expression are the result of the specific genetic lesions rather than differences in the developmental stages of the genotypes used. For microarray analysis, we used the CATMA array containing 22,473 gene-specific tags (Hilson et al. 2004, Little et al. 2007). Differentially expressed genes in the mutant vs. the WT were obtained from three independent biological replicates, and significant changes were considered when: (i) the gene expression ratios of mutant vs. WT were  $\geq 2$  or  $\leq 0.5$ ; and (ii) the changes in expression were statistically significant when

**Table 1** List of selected genes up-regulated in leaves of *fou2* compared with the wild type

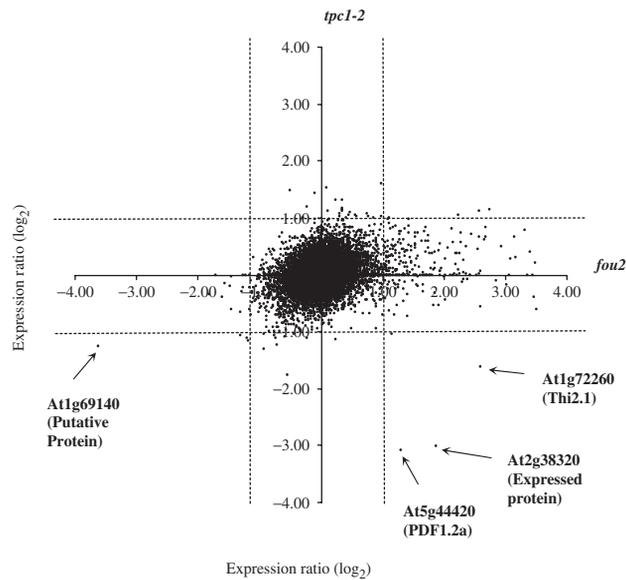
Description	Symbol	AGI	Log <sub>2</sub> (FC) <sup>a</sup>	P-value <sup>b</sup>
Defense-related				
Myrosinase-binding protein, putative	F-ATMBP	At1g52030	3.4	0.011
Pathogenesis-related protein 1	PR-1	At2g19990	3.1	0.022
Plant defensin protein	PDF1.2a	At5g44420	2.9	0.019
Plant defensin fusion protein	PDF1.1	At1g75830	4.5	0.040
Plant defensin fusion protein	PDF1.4	At1g19610	4.0	0.040
Protease inhibitor	DR4	At1g73330	2.9	0.008
Thionin	THI2.1	At1g72260	4.5	0.041
Vegetative storage protein 1	VSP1	At5g24780	2.4	0.003
Vegetative storage protein 2	VSP2	At5g24770	7.1	0.050
Hormone biosynthesis/metabolism				
12-Oxophytodienoate reductase/delayed dehiscence1	OPR3/DDE1	At2g06050	2.5	0.005
1-Aminocyclopropane-1-carboxylate oxidase	ACO	At1g05010	2.3	0.019
Allene oxide synthase	AOS	At5g42650	2.2	0.009
Phenylpropanoid pathway				
4-Coumarate-CoA ligase	4CL	At3g21230	2.2	0.033
Dihydroflavonol 4-reductase	DFR	At5g42800	2.8	0.005
Flavonoid 3'-hydroxylase	F3H	At5g07990	2.7	0.019
Phenylalanine ammonia-lyase 1	PAL1	At2g37040	4.4	0.042
5-Hydroxyferulic acid O-methyltransferase (OMT1)	OMT1	At5g54160	2.3	0.010
Cinnamic acid 4-hydroxylase	C4H	At2g30490	2.5	0.020
Cell wall metabolism				
Arabinogalactan-protein	AGP10	At4g09030	2.8	0.041
Expansin	EXP10	At1g26770	2.2	0.038
Polygalacturonase-inhibiting protein 1	PGIP1	At5g06860	4.1	0.014
Polygalacturonase-inhibiting protein 2	PGIP2	At5g06870	2.8	0.009
Polygalacturonase-inhibitor, putative	FLR1	At3g12145	2.9	0.011
Peroxidase 21	PER21	At2g37130	2.3	0.021
Peroxidase 64	PER64	At5g42180	2.5	0.003
Transcription				
AP2 domain-containing transcription factor	ABR1	At5g64750	2.3	0.011
Basic helix-loop-helix (bHLH) protein	MYC2	At1g32640	2.5	0.003
Heat shock transcription factor 4	HSTF4	At4g36990	2.5	0.001
WRKY family transcription factor	WRKY38	At5g22570	2.6	0.050
WRKY family transcription factor	WRKY28	At4g18170	2.4	0.033
WRKY family transcription factor	WRKY15	At2g23320	2.5	0.010
WRKY family transcription factor	WRKY18	At4g31800	3.0	0.012
WRKY family transcription factor	WRKY8	At5g46350	3.0	0.006
WRKY family transcription factor	WRKY6	At1g62300	2.7	0.007
WRKY family transcription factor	WRKY45	At3g01970	2.5	0.005
Zinc finger (C2H2 type) family protein	ZAT10	At1g27730	2.6	0.003
Zinc finger (C2H2 type) family protein	ZAT12	At5g59820	2.3	0.036

<sup>a</sup>FC, fold change.<sup>b</sup>The *P*-values from the Student's *t*-test are shown.

independently analyzed by two different methods (Student's *t*-test and the *q*-value method; see Materials and Methods for further details).

Results from gene expression analysis of healthy *fou2* leaves vs. healthy WT are summarized in Table 1,

Supplementary Table SI and Fig. 1. A total of 273 genes were identified as differentially expressed in *fou2* compared with the WT. Transcripts corresponding to 246 genes were up-regulated, whereas 27 were down-regulated in this mutant. Differentially expressed genes were classified



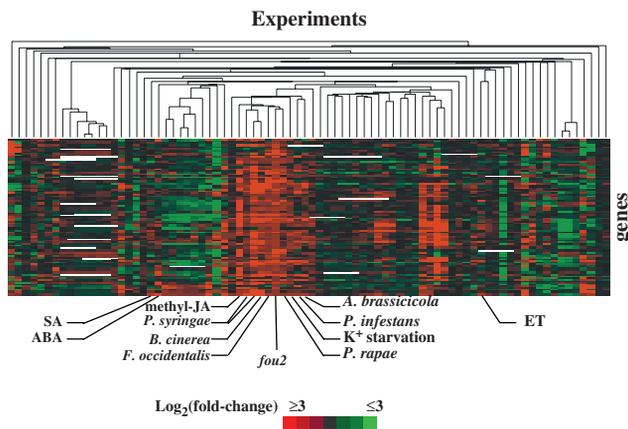
**Fig. 1** Transcriptome analysis of *fou2* and *tpc1-2* leaves. Dot plot representing the fold change in gene expression (transformed by  $\log_2$ ) in *fou2* (x-axis; *fou2* vs. WT) and *tpc1-2* (y-axis; *tpc1-2* vs. WT). Vertical and horizontal lines (dotted) intersect the axes at 1 and  $-1$ . Each dot represents a different gene, and dots with values  $\geq 1$  or  $\leq -1$  represent, respectively, genes up- or down-regulated in the mutants. Some relevant genes for this study are labeled with their AGI numbers and a short description.

according to their potential cellular function based on gene ontology (GO) terms (Provar and Zhu 2003, Berardini et al. 2004). The distribution of genes in different functional classes of known biological processes revealed a relatively high abundance of genes in defense- and stress-related responses, transcription, transport and cell wall metabolism (Table 1 and Fig. S1). Differential expression of 24 defense-related genes was observed in *fou2* (Tables 1, SI). Among the predicted herbivore-related defense genes, six encoded proteinase inhibitors, seven putative jacalin/lectin proteins, and three are implicated in glucosinolate metabolism (Tables 1, SI). All these genes are known JA-inducible genes in *Arabidopsis* (Reymond et al. 2004). Additionally, three plant defensins (*PDF1.2a*, *PDF1.1* and *PDF1.4*), two thionins (*THI2.1* and At1g66100), two chitinases (At3g12500 and At2g43590) and *PR-1* were also up-regulated in *fou2* (Tables 1, SI). A second group of 17 genes up-regulated in *fou2* corresponded to enzymes involved in phenylpropanoid metabolism and included chorismate mutase (At5g22630) and tyrosine aminotransferase (At2g24850). Up-regulated members of the phenylpropanoid pathway included phenylalanine ammonia lyase 1 (At2g37040), caffeic acid *O*-methyl transferase (At5g54160) and dihydroflavonol 4-reductase (At5g42800; Tables 1, SI; Rohde et al. 2004). Twenty genes belonging to different classes of transporters were differentially

expressed in *fou2*. The two classes more recurrent were MATE efflux family proteins and proton-dependent oligopeptide transport (POT) family proteins, with four and two members up-regulated, respectively (Table SI). Additionally, 12 genes in primary metabolism were up-regulated in *fou2* (Tables 1, SI). The latter group contained, among others, six enzymes involved in amino acid metabolism and two in starch and sucrose metabolism. Finally, a total of 44 genes of unknown function were up-regulated in *fou2*, with 17 of these genes (39%) being JA regulated (see below). An interesting and large group of genes up-regulated in *fou2* corresponded to proteins or enzymes involved in cell wall metabolism. Among them were six related genes encoding putative pectin methyl-esterases (PMEs; Tables 1, SI).

Transcripts encoding putative regulatory genes were up-regulated in *fou2* and included 17 genes encoding transcription factors, among which were seven WRKY (WRKY6, 8, 15, 18, 28, 38 and 45), four AP2 domain-containing factors, two basic helix-loop-helix (bHLH) proteins, two C2H2-type proteins (ZAT10 and ZAT12) and one myb-family protein. MYB28, on the other hand, was the only transcription factor down-regulated in this mutant (Tables 1, SI). WRKY6, WRKY15, ZAT10 and ZAT12 are induced after *B. cinerea* infection and may therefore participate in the activation of some defense genes (AbuQamar et al. 2006). One of the bHLH proteins up-regulated in *fou2*, AtMYC2, is a well-characterized regulator of JA/ethylene (ET)-responsive genes and ABA signaling in *Arabidopsis* (Lorenzo and Solano 2005).

In agreement with the increased LOX and AOS activities in *fou2* leaves and their enhanced capacity to accumulate jasmonates, the transcript levels for some JA biosynthesis enzymes were  $\geq 2$ -fold above WT levels in *fou2*. These included *AOS* and *OPR3* (*12-OXOPHYTODIENOATE REDUCTASE 3*, Table 1). The mRNA levels for other oxylipin biosynthesis enzymes were slightly increased in *fou2* but with fold changes  $< 2$ , including LOX2 (1.7-fold), LOX6 (1.8-fold), allene oxide cyclase (AOC; 1.5-fold) and LOX1 (1.4-fold, see Supplementary material). These results were in agreement with our previous observations based on quantitative PCR (qPCR), where slight up-regulation of most LOX transcripts was detected in *fou2* (Bonaventure et al. 2007). Changes in the expression of genes involved in the biosynthesis of other hormones that participate in biotic stress responses [e.g. salicylate (SA) and ABA] were not observed. Only the transcript levels for one enzyme in the ET biosynthesis pathway, 1-aminocyclopropane-1-carboxylate oxidase (ACO), were increased by  $\sim 2$ -fold (Table 1). Expression of this enzyme is induced by methyl jasmonate (MeJA) in *Arabidopsis* (Reymond et al. 2004). The levels of 27 transcripts were statistically below WT



**Fig. 2** Cluster analysis of gene expression responses in *fou2*. The expression of 265 genes differentially expressed in *fou2* leaves was clustered with available expression data from different treatments of WT plants within the GENEVESTIGATOR database (Zimmermann et al. 2004). Genes (rows) and experiments (columns) were clustered with the CLUSTER software utilizing Hierarchical Complete Linkage with an uncentered Pearson correlation. The cluster was visualized with Java TREEVIEW.

levels in *fou2* (Tables 1, SI). Among these, 10 genes (34%) corresponded to expressed or putative proteins. *EXPANSIN 11* (*EXP11*) was one of the most down-regulated mRNAs in this mutant (Table SI).

#### *The fou2* mutation induces a response similar to diverse biotic stresses and potassium starvation

The changes in expression of the genes differentially expressed in *fou2* were compared with their differential expression in experiments where WT plants were subjected to different treatments or stress conditions. For this purpose, gene expression data were retrieved from the GENEVESTIGATOR repository using Meta-Analyzer (www.genevestigator.ethz.ch; Zimmermann et al. 2004). The set of experiments selected corresponded to 88 different treatments of WT (Col-0) plants catalogued as stress responses in this database. In all the experiments selected for the analysis, green tissue was studied. A subset of the 273 genes differentially regulated in *fou2* was selected based on their signal intensity in the Affymetrix probe array and their presence on both Affymetrix and CATMA array platforms (see Materials and Methods). This selection resulted in 265 genes that fulfilled the criteria and were used for further analysis. The logarithm in base 2 ( $\log_2$ ) of the expression ratios between treated and untreated WT plants was used for all comparisons. Hierarchical cluster analysis showed that regulation of these genes was very similar in *fou2* and in WT plants treated with MeJA or challenged by diverse biotic stresses (Fig. 2). These results suggested that the changes in gene expression induced by the *fou2* mutation mimic a biotic-like stress response

similar to that induced during infection by different pathogens or insect feeding. These results agree with the induction of a large number of defense-related genes in *fou2* leaves and their jasmonate dependence. Furthermore, the gene response in *fou2* also clustered closely with a potassium ( $K^+$ ) starvation gene response (Fig. 2).

Available microarray data from GENEVESTIGATOR were also used to perform comparative analysis of the transcriptome of *fou2* with the transcriptome of WT plants after 1 h of MeJA treatment. The results showed that approximately 42% of the genes differentially regulated in *fou2* ( $0.5 \geq \text{fold changes} \geq 2$ ; *fou2*/WT) changed expression after MeJA treatment of WT plants ( $0.5 \geq \text{fold changes} \geq 2$ ; MeJA/control). Importantly, the response for each gene was similar between *fou2* and MeJA treatment, by either up-regulation or down-regulation in both conditions. However, the magnitude of the gene response was not always the same (Table SII). Additionally, the *fou2* transcriptome was compared with the transcriptomes of WT plants after 1 h of ABA, ET and SA treatments. These comparisons showed that a smaller number of genes were similar in their induction/repression between *fou2* and ABA (16%), ET (15%) and SA (10%) treatments (Table SII). These results suggested a larger contribution of jasmonate in the gene response of *fou2* leaves, but they also suggested additional contributions from JA-independent mechanisms.

#### *Proteomic comparison of soluble proteins in fou2 and WT leaves*

Two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry analyses was used to evaluate the differential expression of proteins in resting leaves of *fou2* and WT. For this purpose, soluble proteins from *fou2* and WT leaves were extracted, first separated by isoelectric focusing (IEF) on a non-linear pH range 3–11, further separated on 12.5% acrylamide SDS-PAGE gels, and stained with Coomassie G-250. Protein spot intensities were quantitated by high-resolution densitometric scanning. The normalized volume of the stained proteins (%Vol) was used to quantitate changes in protein amounts that were compared with a two-tailed and paired *t*-test. Differences in %Vol between *fou2* and WT protein spots with *P*-values  $\leq 0.05$  were considered statistically significant. Protein spots were compared among five independent biological replicates representing for each sample an average of five plants. Soluble protein extracts of WT and *fou2* leaves gave approximately 800 protein spots under the conditions used for 2-DE (Fig. S2). Among these 800 spots, seven were identified as differentially expressed in *fou2* leaves compared with the WT (Figs. S2, S3). For these seven spots, changes in protein accumulation ranged between 1.4- and 2.5-fold higher in *fou2* than in WT leaves (Fig. S3).

**Table 2** Protein spots identified with MALDI-TOF

Spot No.	AGI	Annotation	MALDI score	Theoretical mol. wt	Ratio <sup>a</sup> ( <i>P</i> -value)
1	At5g24780	Vegetative storage protein 1	289	30,399	ND <sup>b</sup>
2	At5g24770	Vegetative storage protein 2	186	29,824	2.3 (0.036)
3	At3g16530	Lectin family protein	218	30,547	1.5 (0.027)
4	At4g23600	Putative cystine lyase	870	47,408	2.5 (<0.01)
5	At5g25980	Myrosinase	329	61,885	1.4 (<0.01)
6	At4g08870	Putative arginase	227	37,957	2.0 (0.043)
7	At1g19570	Putative dehydroascorbate reductase	486	23,740	1.9 (<0.01)

Mass spectrometric identification of protein spots with differential accumulation in *fou2* leaves compared with the WT. Peptide mass fingerprints were compared with the UniProt protein databases using the Mascot software.

<sup>a</sup>The expression ratios were quantitated by calculating the %Vol (normalized expression volume of the stained proteins) in WT and *fou2* leaves. The differences (expressed as ratios, *fou2*/WT) in %Vol were compared with a two-tailed and paired *t*-test. Differences in %Vol with *P*-values  $\leq 0.05$  were considered statistically significant (see Supplementary Figs. S2 and S3 for further details).

<sup>b</sup>ND, not determined. The intensity of the corresponding protein spot in WT samples was similar to the background level.

The ratio for spot 1 could not be reliably calculated because the corresponding intensity in WT leaves was similar to background levels. Proteins were extracted from these seven spots and successfully identified using peptide mass fingerprints (Table 2). The identity of the differentially accumulated proteins was consistent with their JA-mediated induction in *fou2* leaves. In this regard, these seven proteins are induced at the mRNA level by biotic stresses and/or exogenous JA treatment in WT plants (Reymond et al. 2004, Zimmerman et al. 2004), and most have a defined role in defense and cell protection responses (see Discussion). Also consistent with the microarray data, most of these proteins presented up-regulated mRNA levels in *fou2* leaves. The transcript levels for vegetative storage proteins 1 and 2 (VSP1 and VSP2), a lectin family protein, a putative cystine lyase and a putative arginase were approximately 2.5-, 7.0-, 2.3-, 2.3- and 4.6-fold higher in *fou2* than in the WT, respectively (Tables 1, SI). In contrast, the transcript levels for a putative dehydroascorbate reductase were not statistically different between *fou2* and WT. Data for the mRNA levels of a putative myrosinase could not be obtained from our microarray study.

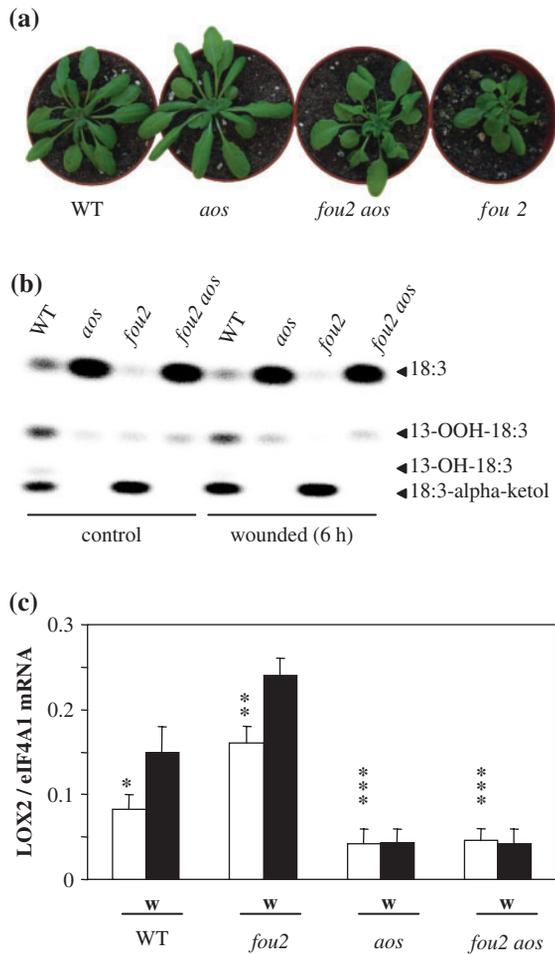
#### *Epistasis studies of fou2, aos, aba1-5, npr1-1 and etr1-1 alleles*

Recently, Peiter et al (2005) reported a link between TPC1 function and ABA-dependent inhibition of germination in Arabidopsis. Based on these previous reports and the present observations, we evaluated the dependence of the *fou2* phenotype on JA and ABA biosynthesis. For this purpose, *fou2* was crossed with the JA-deficient *aos* mutant (Park et al. 2002) and the ABA-deficient *aba1-5* mutant (Leon-Kloosterziel et al. 1996). This latter mutant accumulates 10 times less ABA than the WT and has a

strong dwarf phenotype. In contrast, the rosette morphology of *aos* is similar to that of the WT (Fig. 3a; Park et al. 2002). In addition, TPC1 function has been linked to SA-mediated responses in tobacco cells (Lin et al. 2005). To assess the dependence of the *fou2* phenotype on the NONEXPRESSER OF PR GENES (NPR1) and the ETHYLENE RESISTANT 1 (ETR1), signaling components of the SA and ET pathways, respectively, *fou2* was crossed with the *npr1-1* and *etr1-1* mutants (Chang et al. 1993, Cao et al. 1997). Similarly to *fou2*, all these mutants are in the Col-0 background.

#### *Elevated LOX activity in fou2 depends completely on JA biosynthesis*

We showed previously that in a *fou2 coi1-1* double mutant the higher rate of LOX activity present in *fou2* was completely suppressed, whereas its growth and epinastic leaf phenotype were partially suppressed (Bonaventure et al. 2007). Analysis of the *fou2 aos* double mutant showed that, similarly to *coi1-1*, the *aos* allele partially suppressed the rosette morphology of *fou2* (Fig. 3a). In this regard, the size of the rosette was intermediate between that of WT and *fou2*, the petioles were longer and the degree of leaf curvature and epinasty was less pronounced than in *fou2* (Fig. 3a). However, in contrast to *fou2 coi1-1*, the first 4–6 leaves of *fou2 aos* were morphologically similar to those of the WT. Thus, although the suppression of the *fou2* morphological phenotype in *fou2 aos* was not complete, it was more extensive than in *fou2 coi1-1*. The onset of the morphological phenotypes in *fou2 aos* still occurred after 3 weeks of growth. The in vitro oxygenation of linolenic acid (18:3) using fresh tissue extracts has been established as a reliable method to assess the levels of LOX and AOS activities (Caldelari and Farmer 1998,



**Fig. 3** Characterization of a *fou2 aos* double mutant. (a) Rosette morphology of 4-week-old plants. (b) Linolenic acid (18:3) oxygenation activity of leaf extracts from different genotypes before and 6 h after mechanical wounding. Leaf extracts were incubated for 2 min with [ $^{14}\text{C}$ ]18:3 and products separated by thin-layer chromatography. Each assay contained 15  $\mu\text{g}$  of total protein. The data are representative of two independent experiments. Radioactive bands were detected and digitalized with an optical scanner for radioactively labeled samples (Bonaventure et al. 2007). 13-OOH-18:3, 13-hydroperoxy-18:3; 13-OH-18:3, 13-hydroxy-18:3; 18:3-alpha-ketol, 12-oxo-13-hydroxy-18:3; (c) qPCR analysis of *LOX2* mRNA in leaves before and 6 h after mechanical wounding (w). White bars, control leaves; black bars, 6 h after wounding. Data are the average of three biological replicates ( $n=3$ ) and bars denote standard deviations ( $\pm\text{SD}$ ). Levels of *LOX2* mRNA were calculated relative to the abundance of *eIF4A1- $\alpha$*  mRNA (reference). \* $P < 0.05$  (*t*-test, WT control vs. WT wounded); \*\* $P < 0.05$  (*t*-test, *fou2* control vs. WT control); \*\*\* $P < 0.05$  (*t*-test, *aos* control vs. WT control; *aos fou2* control vs. WT control).

Bonaventure et al. 2007). Therefore, 18:3 oxygenation activity was assessed in unwounded and wounded leaves of *fou2 aos* and control genotypes. Analysis of extracts from unwounded leaves showed that the *fou2 aos* double mutant

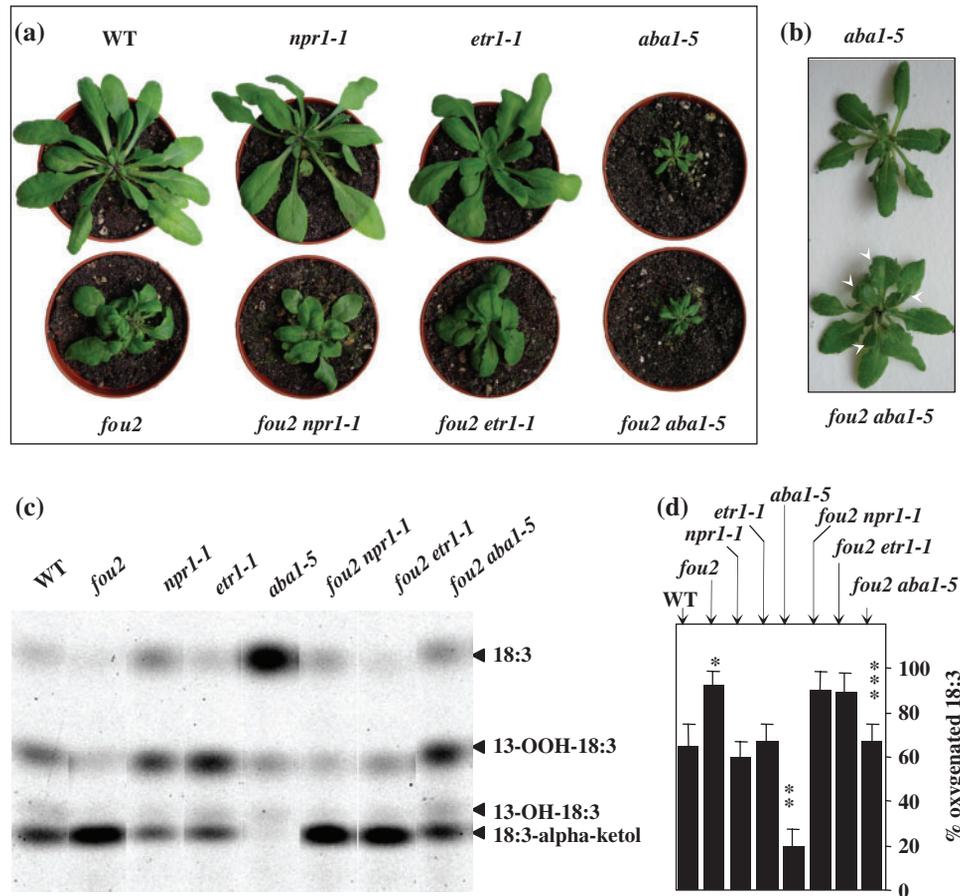
presented less basal 18:3 oxygenation activity than both the WT and *fou2*, and similar levels to those in *aos* (Fig. 3b). At 6 h after wounding, increased 18:3 oxygenation activity could be detected in both the WT and *fou2*, but not in *aos* and *fou2 aos*. Thus, the *aos* allele completely suppressed the elevated basal and wound-induced levels of 18:3 oxygenation activity in *fou2*. In agreement with these results, qPCR analysis of *LOX2* mRNA showed that control leaves of WT and *fou2* contained higher amounts of this transcript compared with *aos* and *fou2 aos* leaves. Moreover, after wounding, *LOX2* mRNA levels increased in WT and *fou2* but remained unchanged in *aos* and *fou2 aos* (Fig. 3c). *LOX2* in *Arabidopsis* plays a defined role in jasmonate biosynthesis (Bell et al. 1995).

#### *Elevated LOX activity in fou2 depends partially on ABA biosynthesis but is independent of NPR1 and ETR1 signaling*

The morphological analysis of *fou2 npr1-1* and *fou2 etr1-1* double mutants showed that their rosette morphologies were similar to that of *fou2*. In these two double mutants, the width of the rosettes was smaller than the width of those of the corresponding single mutants (*npr1-1* and *etr1-1*). The leaves also presented a marked epinasty with shorter petioles, and anthocyanin accumulation was heightened at the base of the petioles (Fig. 4a). However, in the *fou2 abal-5* double mutant, the reduction in size of its rosette compared with *abal-5* was not apparent. The presence of epinastic leaves with shorter petioles and anthocyanin accumulation was however evident in *fou2 abal-5* (Fig. 4a, b). Biochemical analysis of leaves from 4-week-old mutants showed that 18:3 oxygenation activity in *npr1-1* and *etr1-1* was similar to that in the WT; however, in *fou2 npr1-1* and *fou2 etr1-1* double mutants the activity was increased to levels similar to that in *fou2* (Fig. 4c, d). Biochemical analysis of *abal-5* plants revealed that the 18:3 oxygenation activity in this single mutant was substantially reduced compared with the WT. However, it was increased in the *fou2 abal-5* double mutant compared with *abal-5*, albeit at levels lower than in *fou2* (Fig. 4c, d). These results suggest that wild-type levels of ABA are necessary to reach the higher than wild-type levels of LOX and AOS activities observed in *fou2* (Fig. 4c, d).

#### *Gene expression profiling of tpc1-2 leaves*

Having established that a TPC1 gain-of-function mutant (*fou2*) results in strong effects on the transcription of defense-related genes, we investigated whether a loss-of-function TPC1 mutant also presented effects on defense gene expression. The *Arabidopsis tpc1-2* mutant carries a T-DNA insertion in exon 17 of *TPC1* that renders the gene product inactive (Peiter et al. 2005). Inactivation of TPC1 causes neither obvious morphological phenotypes nor shifts in the developmental program of the mutant



**Fig. 4** Characterization of *fou2 aba1-5*, *fou2 etr1-1* and *fou2 npr1-1* double mutants. (a) Rosette morphology of 4-week-old plants; (b) Detail of rosette morphology of *aba1-5* and *fou2 aba1-5*. Arrowheads point to epinastic leaves. (c) Linolenic acid (18:3) oxygenation activity of leaf extracts from different genotypes. Leaf extracts were incubated for 2 min with [ $^{14}\text{C}$ ]18:3 and products separated by thin-layer chromatography. The data shown are representative of two independent experiments. Each assay contained 15  $\mu\text{g}$  of total protein. Refer to the legend of Fig. 3b for chemical nomenclature; (d) Radioactive bands corresponding to fatty acids were quantitated using an optical scanner and the ImageQuant software. The percentage of oxygenated 18:3 corresponds to radiolabeled 13-OOH-18:3, 13-OH-18:3 and 18:3- $\alpha$ -ketol formed from the initial  $^{14}\text{C}$ -18:3 activity (100%). Data are the average of two biological replicates and bars denote standard deviations ( $\pm\text{SD}$ ). The conditions used for this experiment allowed the almost complete utilization of 18:3 by *fou2* and was designed to detect differences between leaves with high, medium and low 18:3 oxygenation activity. Activity corresponds to 15  $\mu\text{g}$  of total protein. \* $P < 0.05$  (*t*-test, *fou2* vs. WT), \*\* $P < 0.05$  (*t*-test, *aba1-5* vs. WT), \*\*\* $P < 0.05$  (*t*-test, *aba1-5 fou2* vs. *fou2*).

(Peiter et al. 2005, Bonaventure et al. 2007). Therefore, similarly to *fou2*, transcriptome analysis of *tpc1-2* leaves was conducted on 4-week-old plants. Statistical analysis of changes in gene expression in *tpc1-2* was conducted using the same criteria as for analysis of *fou2* microarray data (see Materials and Methods for details). The microarray results showed that a small number of transcripts (33) had altered levels in *tpc1-2* compared with the WT. Moreover, the fold changes in transcript levels were subtle (between 2- and 3-fold for most of the genes; Table SIII). Only the transcript levels for *PLANT DEFENSIN 1.2A* (*PDF1.2a*) and an expressed protein (At2g38320, encoding a plant-specific protein of unknown function) were reduced between 5- and 6-fold in this mutant. Five up-regulated

genes were associated with the formation/structure of the cell wall or the extracellular matrix. These included two genes encoding glycine-rich proteins (GRPs) and putative extracellular lipid transfer proteins (LTPs; Table SIII). Additionally, two MYB family transcription factors were increased in *tpc1-2* compared with the WT. Among the 13 down-regulated genes in *tpc1-2* were two defense-related genes, *PDF1.2a* and *THIONIN 2.1* (*THI2.1*). In contrast to their down-regulation in *tpc1-2*, the transcript levels for these two genes were elevated in *fou2* (~3-fold for *PDF1.2a* and ~4.5-fold for *THI2.1*; Fig. 1 and Table 1). Transcript levels for a putative transcription factor and a putative kinase were also down-regulated in *tpc1-2* (Table SIII).

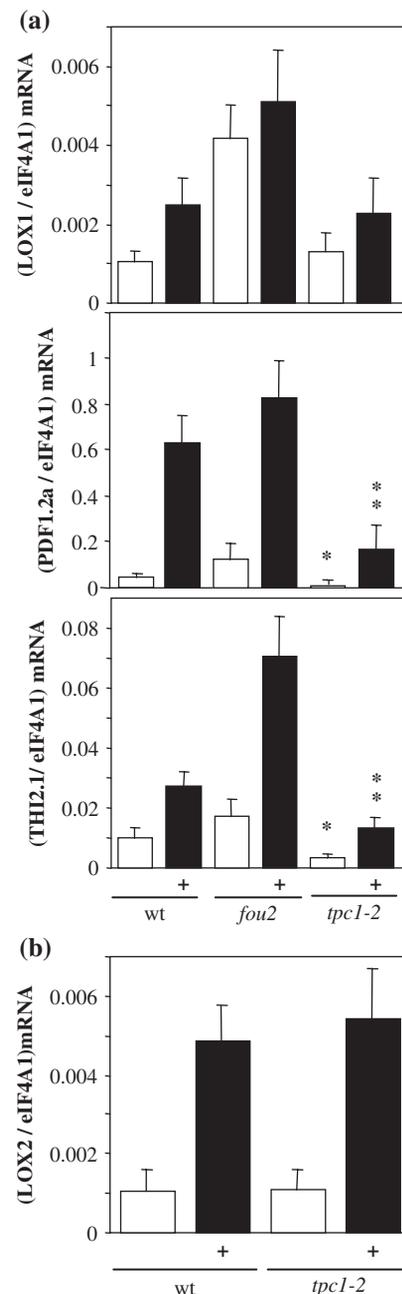
*Expression of PDF1.2a and THI2.1 mRNAs is compromised in tpc1-2 leaves after B. cinerea infection*

In WT leaves, *PDF1.2a* and *THI2.1* transcripts are rapidly induced after *B. cinerea* infection (AbuQamar et al. 2006). To explore the contribution of TPC1 to the expression of *PDF1.2a* and *THI2.1*, qPCR was used to analyze the mRNA levels of these two genes in leaves of WT, *fou2* and *tpc1-2*. For this experiment, WT and mutant plants were spray-inoculated with *B. cinerea*, and leaf material was analyzed after 24 h. This time is sufficient to induce *PDF1.2a* and *THI2.1* mRNA expression in WT plants with minimal tissue necrosis (AbuQamar et al. 2006). Control plants were mock-inoculated with dead spores. In addition, expression of *LOX1* and *LOX2* mRNAs was tested as control. These two transcripts are also induced by *B. cinerea* infection in the WT (AbuQamar et al. 2006). First, the results obtained with non-infected leaves corroborated the microarray data. In this regard, *tpc1-2* leaves contained significantly lower basal levels of *PDF1.2a* (4- to 5-fold) and *THI2.1* (2- to 3-fold) transcripts than WT, but similar levels of *LOX1* and *LOX2* transcripts. The levels of these four mRNAs were above those of the WT in *fou2* leaves (Fig. 5a, Bonaventure et al. 2007). Secondly, in infected *tpc1-2* leaves, the induction of *PDF1.2a* and *THI2.1* mRNAs was significantly attenuated (~3-fold) compared with the WT, whereas it was accentuated in *fou2* (Fig. 5a). In contrast, the levels of *LOX1* and *LOX2* mRNAs were similar in WT and *tpc1-2*-infected leaves (Fig. 5a, b). Expression of *PDF1.2a*, *THI2.1*, *LOX1* and *LOX2* mRNAs was also quantitated in WT and *tpc1-2* plants treated with MeJA. Similar transcript levels were observed for all transcripts in both genotypes after 6 h of MeJA treatment (Fig. S4). Likewise, 6 h after treatment of WT and *tpc1-2* with ethephon (an ET-releasing agent), similar levels of *PDF1.2a* and *THI2.1* transcripts were observed (data not shown).

## Discussion

### *The fou2 mutation triggers a JA-like response*

The first important results obtained with transcript and protein profiling of *fou2* leaves were that: (i) the response induced by TPC1<sup>D454N</sup> in resting leaves of this mutant extensively mimics a biotic stress response similar to that induced by different pathogens or herbivores; and (ii) this response is largely mediated by JA. In addition, analysis of soluble protein extracts by 2-DE and MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) identified seven proteins up-regulated in mature *fou2* leaves compared with the WT. The mRNAs for these seven proteins are induced after biotic stress responses and by JA (Reymond et al. 2004, Zimmermann et al. 2004). Vegetative storage proteins,



**Fig. 5** Analysis of gene expression after *Botrytis cinerea* infection of WT, *fou2* and *tpc1-2*. (a) Quantitative real-time PCR (qPCR) was used to assess *PDF1.2a*, *THI2.1* and *LOX1* mRNA levels in leaves of plants spray-inoculated with *B. cinerea*. Changes in gene expression were evaluated 24 h post-infection. Data are the average of three biological replicates ( $n=3$ ) and bars denote standard deviations ( $\pm$ SD). Levels of *PDF1.2a*, *THI2.1* and *LOX1* mRNAs were calculated relative to the abundance of *eIF4A1*- $\alpha$  mRNA (reference). +, *B. cinerea* infection. \* $P < 0.05$  ( $t$ -test, *tpc1-2* control vs. WT control), \*\* $P < 0.05$  ( $t$ -test, *tpc1-2* infected vs. WT infected). (b) qPCR analysis of *LOX2* mRNA levels in WT and *tpc1-2* leaves spray-inoculated with *B. cinerea*. Changes in gene expression were evaluated as in (a).

myrosinases and lectins have anti-insect roles (Kliebenstein et al. 2005, Liu et al. 2005). GSH-dependent dehydroascorbate reductase (GSHDAR) is an enzyme implicated in cell protection. Indeed, wounding is associated with an oxidative stress that generates reactive oxygen species (Fryer 1992). Interestingly, this putative GSHDAR (At1g19570) was up-regulated at the protein level but not at the transcript level in *fou2*, suggesting that expression of this enzyme is under post-transcriptional regulation. Cystine lyase [CS; also annotated as tyrosine aminotransferase-like (TAT-like)] may catalyze the breakdown of L-cystine to thiocysteine, pyruvate and ammonia. The CS gene is strongly induced upon JA and wounding treatments (Sandorf and Hollander-Czytko 2002); however, no defined role in plant defense or cell protection has been assigned thus far to this enzyme.

*The fou2 phenotype depends predominantly on JA biosynthesis but it is also affected by ABA*

In agreement with the central participation of JA in the *fou2* phenotype, analysis of a *fou2 aos* double mutant demonstrated that the elevated LOX activity and levels of *LOX2* mRNA observed in *fou2* were completely suppressed by the *aos* allele. In contrast, the morphology of *fou2* was only partially suppressed by the *aos* allele. These results were similar to those previously reported for the *fou2 coil-1* double mutant; however, the morphological suppression of the *fou2* phenotype in *fou2 aos* was more extensive compared with *fou2 coil-1* (Bonaventure et al. 2007). These results suggest an effect of JA (or its precursors) on growth-related processes independently of COI1 signaling. Interestingly, ZAT10 mRNA levels were up-regulated in *fou2*. Transcript levels for this putative transcription factor are transiently induced by wounding and 12-oxophytodienoic acid (OPDA) but not by JA (Taki et al. 2005). Thus, responses activated by OPDA but not by JA (Stintzi et al. 2001) may also be induced in *fou2* leaves.

Only a partial suppression of both the *fou2* morphological and biochemical phenotypes was achieved by combining the *fou2* with the *abal-5* allele. In this regard, the rosette size of *fou2 abal-5* was not substantially affected compared with *abal-5*, and the leaf epinasty, shorter petioles and anthocyanin accumulation typical of *fou2* were still evident in this double mutant. In addition, *fou2 abal-5* contained higher levels of LOX activity than *abal-5* although the levels were lower than in *fou2*. Together, the results indicate that the *fou2* phenotype is weakly affected by ABA levels and suggest that the levels of this hormone must remain within certain limits for *fou2* to manifest its full phenotype. In accordance with this conclusion, 18:3 oxygenation activity in leaves of a 4-week-old *aba2-1* mutant (which presents a less severe phenotype compared with *abal-5*; Leon-Kloosterziel et al.

1996) was similar to that in the WT (G.B. and E.E.F., unpublished results). The lower LOX activity in *abal-5* is in agreement with a recent report describing a role for ABA in modulating the JA response during pathogenesis (Adie et al. 2007). In contrast to the *abal-5* allele, analysis of *fou2 etr1-1* and *fou2 npr1-1* double mutants demonstrated that the *fou2* morphological and biochemical phenotypes were independent of ETR1 and NPR1 signaling. These two signaling components play central roles in the ET and SA signaling pathways, respectively (Chang et al. 1993, Cao et al. 1997).

*How do gene expression data correlate with the fou2 phenotype?*

Analysis of gene expression in *fou2* revealed higher than WT transcript levels for defense genes and transcription factors implicated in defense gene regulation and/or JA signaling. Other groups of genes up-regulated in *fou2* may provide information that will, in the future, relate the morphological phenotype of the mutant to early events in jasmonate signaling initiated by wounding or pathogenesis.

In plants, cell expansion is dependent on cell wall relaxation coupled to water influx into plant vacuolar compartments to increase cell turgor (McCann et al. 1993). Recent characterization of Arabidopsis mutants accumulating higher than wild-type levels of JA has begun to unveil an intimate connection between regulation of JA biosynthesis and cell wall/plasma membrane homeostasis, and consequently growth (Ellis et al. 2002, Ko et al. 2006). Perturbation of cell wall/plasma membrane homeostasis by mutations in either a cellulose synthase or a plasma membrane glycosylphosphatidylinositol (GPI)-anchored protein triggers JA biosynthesis and JA-mediated gene responses. In addition, a well-documented effect of JA application on WT plants is the arrest of growth and, in agreement with this phenomenon, a common phenotype of JA-overaccumulating mutants is their smaller size compared with the WT (Hilpert et al. 2001, Ellis et al. 2002, Jensen et al. 2002, Ko et al. 2006). Interestingly, six genes encoding PME were strongly up-regulated (>4-fold) in *fou2*. These enzymes catalyze the de-esterification of highly methylated homogalacturonans (HGAs) in the cell wall to facilitate binding of Ca<sup>2+</sup> to HGAs, thereby controlling cell wall rigidity. These enzymes are usually only expressed in mature but not in rapidly elongating tissue (Micheli 2001). Some of these PME genes are induced by JA treatment and biotic stresses including *B. cinerea* infection (AbuQamar et al. 2006). In addition, three genes encoding peroxidases were up-regulated in *fou2*. MeJA is known to induce the activity of cell wall-bound peroxidases, thereby promoting irreversible inhibition of root growth (Tsai et al. 1997). Genes encoding several other

cell wall-modifying enzymes were up-regulated in *fou2*, including *EXPANSIN 10 (EXP10)*, *ARABINOGLACTAN PROTEIN 10 (AGP10)*, two *GLYCINE-RICH PROTEINS (GRPs)* and two *POLYGALACTURONASE INHIBITING PROTEINS (PGIP1 and 2)*. Among the most down-regulated genes in *fou2* were *EXPANSIN 11 (EXP11)* and a pectate lyase family protein (At3g07010). Shorter petioles than WT is a feature of *fou2* and several mutants that overaccumulate JA (Hilpert et al. 2001, Jensen et al. 2002, Bonaventure et al. 2007). Levels of the *EXP11* mRNA might be related to this phenotype. This transcript is known to be highly expressed in petioles of developing seedlings and is down-regulated by MeJA treatment and some biotic stresses including fungi and bacteria (Zimmermann et al. 2004).

One of the striking features of the transcript profile in *fou2* is its resemblance to the  $K^+$  starvation transcriptome (Fig. 2). Interestingly,  $K^+$  starvation responses trigger JA biosynthesis, and it was proposed that JA plays a central role in the control of  $K^+$  homeostasis in Arabidopsis (Armengaud et al. 2004). Here, we raise the possibility that the alternative may also be true and that  $K^+$  homeostasis may affect JA responses. While the nature of the cation(s) responsible for the *fou2* phenotype is unknown and cannot be determined from our study, it is known, for example, that  $Ca^{2+}$  plays a critical role in the  $K^+$  starvation response (Xu et al. 2006). One possibility for the activation of JA biosynthesis in *fou2* is that deregulation of intracellular  $K^+$  fluxes, perhaps indirectly involving  $Ca^{2+}$  signaling (and maybe resembling those occurring during  $K^+$  starvation), may occur in *fou2*. It is possible that the genetic and physiological characterization of *fou2* may help unite electrophysiology with previous physiological studies of the wound response. Changes in turgor as a consequence of wounding have been discussed as contributing to wound signaling in previous studies (Hause et al. 1996, Stankovic and Davies 1998). Since  $K^+$  is a major contributor to cell turgor, a hypothesis that will need to be considered in future research is that JA synthesis in *fou2* is activated by altered cell turgor status in the mutant.

#### *Wild-type TPC1 contributes to the basal and induced expression of two pathogenesis-related proteins by JA-independent mechanisms*

Recently, TPC1 function in plants has been linked to the elicitation of programmed cell death and to the expression of defense genes and antioxidant enzymes (Kadota et al. 2004, Kurusu et al. 2005). Leaf gene expression profiling of the *tpc1-2* loss-of-function mutant indicated that a small number of genes (33, according to the statistical criteria applied) were differentially expressed in this mutant compared with the WT. Nineteen genes were up-regulated; however, no transcript was induced more

than 3-fold compared with the WT. The transcript levels for 14 genes were down-regulated in *tpc1-2* and, among these, only three, *PDF1.2a*, an expressed protein (At2g38320) and *THI2.1*, were decreased by  $\geq 3$ -fold (Table SIII). Thus, both up- and down-regulation of transcript levels in *tpc1-2* was subtle. The absence of substantial changes in gene expression in *tpc1-2* was consistent with its normal morphology and development.

Comparison of the changes in the transcriptome of healthy *fou2* and *tpc1-2* leaves revealed that transcripts corresponding to three genes showed opposite regulation in these two mutants. In this regard, *PDF1.2a*, *THI2.1* and At2g38320 mRNAs were up-regulated in *fou2*, whereas they were down-regulated in *tpc1-2* (Fig. 1). In addition, the results demonstrated that WT TPC1 does not affect expression of either JA biosynthesis genes or most JA-responsive genes in resting tissue. Furthermore, the induction of *PDF1.2a* and *THI2.1* mRNAs was not affected by MeJA treatment in *tpc1-2*, indicating that JA perception and signaling is not altered in this mutant. Based on these results and on our previous observations showing that the activity and expression of *LOX2* and *AOS* mRNAs are not affected in *tpc1-2* after wounding (Bonaventure et al. 2007), we conclude that the mechanisms involved in the differential expression of *PDF1.2a* and *THI2.1* transcripts in unwounded *tpc1-2* is probably independent of JA signaling (however, a weak effect on JA signaling in this mutant cannot be excluded) and may involve other cellular mechanisms. Whether these mechanisms are direct (i.e. cation mediated) or indirect remains to be elucidated. Importantly, however, the expression of these two genes is influenced by both loss- and gain-of-function mutants of TPC1 in Arabidopsis. They will provide useful targets for the further investigation of cation fluxes in the regulation of jasmonate and defense responses.

## Materials and methods

### *Plant material and treatments*

WT *Arabidopsis thaliana* (Col-0) and mutant plants were grown on soil at 22°C under white fluorescent light ( $100 \mu E m^{-2} s^{-1}$ ) in a 16 h light/8 h dark photoperiod. After sowing, seeds were stratified for 4 d at 4°C in the dark. For all the experiments, plants were grown for 4 weeks from the beginning of germination (4 weeks old). Conidial spores of *B. cinerea* cultures were resuspended in 0.6% (w/v) potato dextrose broth (Difco, St Louis, MO, USA) at  $1 \times 10^4$  spores  $ml^{-1}$ . The solution was sprayed on the upper surface of rosette leaves of WT and mutant plants. For controls, medium with spores was boiled for 10 min and sprayed on leaves as indicated above. Control and challenged plants were placed in sealed transparent boxes under a 12 h light/12 h dark photoperiod ( $100 \mu E m^{-2} s^{-1}$ ) at

22°C and >85% humidity. Leaf material was harvested after 24 h of infection and frozen in liquid nitrogen for total RNA extraction. For MeJA treatment, pots with WT and mutant plants were placed in transparent plexiglass boxes (air volume 15 liters). A cotton tip was placed in each box and 1 µl of 10% (v/v) MeJA (Sigma) in ethanol was applied on its surface. For controls 1 µl of ethanol was used. The boxes were closed with hermetic lids and placed under continuous fluorescent white light for 6 h at 22°C. Leaf material was harvested and frozen in liquid nitrogen for subsequent RNA extraction. Ethephon (Sigma) was reconstituted at 1 mM in aqueous 40% (v/v) acetone and 0.02% (v/v) Tween-20, and sprayed on plants until run-off. Control plants were sprayed with the solvent. Experimental conditions were the same as for MeJA treatment. For wounding experiments, all but one leaf (systemic) from the rosette were wounded. For this purpose, ('distal') from ~25% of the leaf area (tip) was mechanically crushed with sharp forceps. In all the experiments, the distal leaf was used for analysis.

#### *Microarray experiments and data analysis*

The Arabidopsis CATMA microarray was used for mRNA expression profiling (Hilson et al. 2004, Little et al. 2006). Total RNA isolation, preparation of fluorescent probes, hybridizations and washes were performed according to Reymond et al. (2004; <http://www.unil.ch/ibpv/microarrays.htm>). Microarray experiments were performed in triplicate with dye-swap design, and slides were scanned with a ScanArray 4000 (Packard BioScience, Zurich, Switzerland) using the same conditions as described (Reymond et al. 2004). The average fluorescence intensity for each fluor and for each gene was determined using the ImaGene program (BioDiscovery, Los Angeles, CA, USA). Data processing and analysis were performed using a relational database (NOMAD, University of California, San Francisco, <http://ucsf-nomad.sourceforge.net>) installed locally and adapted using custom Perl scripts (Reymond et al. 2004). To identify differentially expressed genes between samples, a Student's *t*-test (two-sample hypothesis, equal variance) was conducted between  $\log_2$ -transformed expression ratios from mutants vs. the WT. Genes with a *P*-value  $\leq 0.05$  were considered as differentially regulated (Reymond et al. 2004). In addition, the *q*-value method was used for data analysis. Data were computed with the QVALUE software as described (Storey and Tibshirani 2003) using the Limma package from Bioconductor (Gentleman et al. 2004). The *q*-value method computes a *q*-value for each gene using the distribution of *P*-values of all measurements and an estimated false discovery rate (FDR). Based on *q*-values, *P*-value cut-offs of 0.033 and 0.018 for an FDR of 5% were calculated and used to define significant changes in gene expression in *fou2* and

*tpc1-2*, respectively (Storey and Tibshirani 2003, Gentleman et al. 2004). Functional classes for GO analysis were created according to the TAIR and MIPS classifications generated as described (Provart and Zhu 2003, Bernardini et al. 2004). Hierarchical clustering was performed with CLUSTER (Eisen et al. 1998) and visualized with Java TREEVIEW (Saldanha 2004). Complete linkage using an uncentered Pearson correlation was applied to the  $\log_2$  of normalized expression ratios (treated/WT). Experimental data were retrieved from 88 stress response experiments present in the GENEVESTIGATOR database (the complete list of experiments is available at [www.genevestigator.ethz.ch](http://www.genevestigator.ethz.ch)) and analyzed with the Meta-Analyzer software. Only experiments where WT plants (Col-0) were tested and green tissue was analyzed were selected for analysis. Genes that were both present in the CATMA array and represented unique genes in the Affymetrix probe array were selected (Redman et al. 2004). In addition, only genes with overall signal intensity >200 in the Affymetrix probe array were used for analysis (Zimmermann et al. 2004).

#### *Soluble protein extraction*

Frozen leaf material (500 mg) from Arabidopsis WT and *fou2* plants was placed in a mortar chilled with liquid nitrogen. A 69 µl aliquot of Solution 1 [one tablet of proteinase inhibitors cocktail (*Complete*<sup>TM</sup> *mini*, Molecular Biochemicals Roche, Mannheim, Germany) dissolved in 2 ml of 100 mM KCl, 20% (v/v) glycerol, 50 mM Tris-HCl, pH 7.1] and 16.8 µl of Solution 2 [1 mM pepstatin A, 1.4 µM phenylmethylsulfonyl fluoride (PMSF) dissolved in ethanol] were placed onto the surface of the leaves and allowed to freeze. The leaves were then ground to a fine powder in the presence of liquid nitrogen. The homogenate was centrifuged for 30 min (430,000 × *g* at 4°C). The supernatant (0.45 ml) was recovered and proteins were precipitated by the successive addition of 1.8 ml of methanol, 0.45 ml of chloroform and 1.35 ml of water. The mixture was briefly agitated and centrifuged for 5 min (9,000 × *g* at 4°C). After centrifugation, the proteins were recovered from the interface of the aqueous and organic phases. Protein samples were precipitated with methanol (1.35 ml) and centrifuged for 5 min at 9,000 × *g* (4°C). The supernatant was removed and the pellet was air dried. The samples were resuspended in 150 µl of DIGE lysis buffer [DLB; 30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate (CHAPS), pH 8.5] and sonicated on ice five times for 5 s with 1 min intervals. Protein concentrations were quantified using the 2-D Quant Kit (Amersham Biosciences Corp., Piscataway, NJ, USA) according to the manufacturer's instructions.

### *Two-dimensional gel electrophoresis, gel staining and analysis*

For the first dimension, IEF, 24cm pH3-11 NL (non-linear) Immobiline DryStrips (GE Healthcare Bio-sciences AB, Uppsala, Sweden) were used. The strips were rehydrated overnight in 0.45 ml of DeStreak buffer (De Streak Rehydration Solution, GE Healthcare Bio-sciences AB) containing 0.5% (v/v) ampholites (IPG buffer pH 3–11NL, GE Healthcare Bio-sciences AB) and covered with mineral oil to prevent evaporation. The protein sample (300 µg) was mixed with 2 × DIGE lysis buffer [2 × DLB; 8 M urea, 130 mM 1,4-dithio-DL-threitol (DTT), 4% (w/v) CHAPS, 2% (v/v) ampholites] to a proportion of 1:1 (protein sample: 2 × DLB buffer). The sample mixture was vortexed for 10 min, centrifuged for 10 min at 9,000 × g and loaded onto the strip according to the manufacturer's instructions (GE Healthcare Bio-sciences AB). Samples were run with an Ettan™ IPGphor™ 2 IEF System (GE Healthcare Bio-sciences AB) using the following conditions: step 1, 3 h at 150 V; step 2, 3 h at 300 V; gradient 1, 6 h at 300–1,000 V (rate 117 V h<sup>-1</sup>); gradient 2, 4 h at 1,000–8,000 V (rate 1,750 V h<sup>-1</sup>); step 3, 4 h 30 min at 8,000 V. After the run, the strips were incubated first for 15 min in equilibration buffer 1 [0.5 M Tris-HCl, pH 6.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT] and secondly in equilibration buffer 2 [2.5% (w/v) iodoacetamide] for 15 min. The strips were soaked in 2 × SDS-PAGE running buffer [50 mM Tris, 500 mM glycine, 0.2% (w/v) SDS], placed on top of 12.5% polyacrylamide gels and sealed together with warm 0.5% (w/v) agarose prepared in 2 × SDS-PAGE running buffer. Electrophoresis was performed with an Ettan-DALT-Six-Gel system (GE Healthcare Bio-sciences AB) at 20°C using the following conditions: 15 min at 80 V and 17 h 30 min at 15 mA per gel. After electrophoresis, gels were placed in fixing solution [50% (v/v) ethanol, 3% (v/v) phosphoric acid] for 3 h. The gels were washed three times (30 min) with water and placed in equilibration solution [30% (v/v) methanol, 3% (v/v) phosphoric acid, 17% (v/v) ammonium sulfate] for 1 h. Gels were stained in equilibration solution containing 0.35 g l<sup>-1</sup> of Coomassie G-250 (Sigma, Buchs, Switzerland) according to the manufacturer's instructions. Gels were scanned with a calibrated densitometer (GS-800, Bio-Rad, Zurich, Switzerland). Spot detection, indexing, matching, normalization and quantitation were done with the Imagemaster Platinum 5.0 software (Amersham Biosciences, Uppsala, Sweden). For quantitation of protein spots, the normalized expression volume of the stained proteins (%Vol =  $[\text{Vol}/\sum_{s=1}^N \text{Vol}_s] \times 100$ , where Vol<sub>s</sub> represents the spot volume and N is the total number of spots) was used. The ratios (*fou2*/WT) of the relative spot quantities from *fou2* and WT leaves were compared with a two-tailed and paired *t*-test. The *P*-values were calculated with 2-DE images of five biological replicates (*n* = 5) for

both WT and *fou2*, and ratios with *P*-values ≤ 0.05 were considered statistically significant.

### *Protein digestion and MALDI-TOF analysis*

Protein identification was performed as described by Crettaz et al. (2004). Briefly, gel bands were excised from the SDS-polyacrylamide gel and transferred to 96-well plates (Perkin Elmer Life Sciences, Foster City, CA, USA). In-gel proteolytic cleavage with sequencing-grade trypsin (Promega, Madison, WI, USA) was performed automatically in the robotic workstation Investigator ProGest (Perkin Elmer Life Sciences) according to the protocol of Shevchenko et al. (1996). Digests were evaporated to dryness and reconstituted in 3 µl of α-cyano-hydroxycinnamic acid matrix [5 mg ml<sup>-1</sup> in 60% (v/v) acetonitrile/water], of which 0.7 µl were placed in duplicate on a target plate. MALDI-TOF MS analysis was performed on a 4700 Proteomics Analyser (Applied Biosystems, Framingham, MA, USA). Internal calibration was performed with trypsin autolysis peaks and, after subtraction of matrix peaks, the 10 most intense ion signals were selected for analysis. Non-interpreted peptide tandem mass spectra were used for direct interrogation of the Uniprot (Swissprot-TrEMBL) database using the Mascot 2.0 software (<http://www.matrixscience.com>). The mass tolerance for database searches was 50 p.p.m. The MASCOT software was set up to report only peptide matches with a score >14. With the parameters used, the threshold for statistical significance (*P* ≤ 0.05) corresponded to a total (protein) MASCOT score of 33. Proteins scoring >80 were considered automatically as valid, while all protein identifications with a total MASCOT score between 33 and 80 were manually validated. Validation included examination of the RMS (root mean square) mass error of individual peptide matches. MS peptide matches were validated only if at least an ion series of four consecutive *y* ions were matched, in addition to ions belonging to other series. Generally, only proteins matched by at least two peptides were accepted.

### *Linolenic acid oxygenation activity*

The assay for oxygenation of linolenic acid by fresh leaf extracts of *Arabidopsis* was performed as previously described (Bonaventure et al. 2007).

### *Quantitative real-time PCR (qPCR)*

Plants were mechanically wounded as indicated above and the distal leaf harvested and frozen in liquid nitrogen for total RNA extraction. For this purpose, 100 mg of frozen material was extracted using TRIzol® (Invitrogen, Merelbeke, Belgium) according to commercial instructions. Total RNA was cleaned up with the RNeasy extraction kit following commercial instructions (Qiagen, Zurich, Switzerland). A 5 µg aliquot of total RNA was copied to

cDNA with the SuperScript II First Strand Synthesis System (Invitrogen) and random hexamers. Prior to qPCR, the cDNA was treated with RNase H and RNase A according to the manufacturer's instructions and the cDNA purified with the Plant DNA extraction kit (Qiagen). Specific primers were designed to have a  $T_m$  of 60°C and to give amplicons between 200 and 300 bp (Table SIV). RT-qPCR analysis was performed in a final volume of 25 µl according to the FullVelocity™ SYBR® Green instruction manual (Stratagene, La Jolla, CA, USA). The Arabidopsis eukaryotic translation initiation factor 4A1- $\alpha$  (eIF4A1- $\alpha$ ) was used as the internal control. Quantitation of gene expression was based on the comparative threshold (CT) method as described in User Bulletin #2 (Perkin Elmer-Applied Biosystems, Foster City, CA, USA). PCR and data analysis were performed, respectively, in an Mx3000P™ spectrofluorometric thermal cycler and its corresponding software (Stratagene).

#### Generation and analysis of double mutants

Pollen from *fou2* was used to fertilize male-sterile *aos* plants. Pollen from *npr1-1*, *abal-5* and *etr1-1* mutants was used to fertilize *fou2* plants. The presence of *npr1-1*, *abal-5* or *etr1-1* alleles in the double mutants was confirmed by PCR amplification and CAPS (cleaved amplified polymorphic sequences) analysis (Chang et al. 1993, Cao et al. 1997, Audran et al. 2001). For *npr1-1*, the primers used were: 5'gaagctattggatagatg3' and 5'gttgagcaagtcaact3'; for *abal-5*, 5'gctgggtgtatcactggtgatcg3' and 5'ccattctcagataccacagtaacc3'; and for *etr1-1*, 5'catctccgatttctcat3' and 5'aaga tcaggaataatag3'. The *fou2* allele was confirmed by CAPS analysis (Bonaventure et al. 2007).

#### Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website [www.pcp.oxfordjournals.org](http://www.pcp.oxfordjournals.org).

The complete microarray data are available at the ArrayExpress database ([www.ebi.ac.uk/aerep/login](http://www.ebi.ac.uk/aerep/login); Experiment accession number E-MEXP-1095).

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