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Transcriptome analyses suggest a disturbance of iron homeostasis in soybean leaves during white mould disease establishment

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SUMMARY

Sclerotinia sclerotiorum is a serious pathogen of numerous crops around the world. The major virulence factor of this pathogen is oxalic acid (OA). Mutants that cannot produce OA do not cause disease, and plants that express enzymes that degrade OA, such as oxalate oxidase (OxO), are very resistant to *S. sclerotiorum*. To examine the effect of OA on plants, we infiltrated soybean leaves with 5 mM OA and examined the gene expression changes at 2 h post-infiltration. By comparing the gene expression levels between leaves of a transgenic soybean carrying an OxO gene (OxO) and its parent AC Colibri (AC) infiltrated with OA (pH 2.4) or water (pH 2.4 or 5.5), we were able to compare the effects of OA dependent or independent of its pH. Gene expression by microarray analysis identified 2390 genes that showed changes in expression, as determined using an overall *F*-test *P*-value cut-off of 0.001. The additional requirement that at least one pairwise *t*-test false discovery rate (FDR)-corrected *P* value should be less than 0.001 reduced the list of the most highly significant differentially expressed genes to 1054. Independent of pH, OA altered the expression levels of 78 genes, with ferritin showing the strongest induction by OA. The combination of OA plus its low pH caused 1045 genes (99% of all significant genes) to be differentially expressed, with many of the up-regulated genes being related to basal defence, such as genes of the phenylpropanoid pathway and various cytochrome P450s. RNA-seq was also conducted on four samples: OxO and AC genotypes infiltrated with either OA pH 2.4 or water pH 2.4. The RNA-seq analysis also identified ferritin paralogues as being strongly induced by OA. As the expression of ferritin, a gene that encodes for an iron storage protein, is induced by free iron, these results suggest that *S. sclerotiorum* benefits from the ability of OA to free iron from plant proteins, as this induces host cell death, and also allows the uptake and assimilation of the iron for its own metabolic needs.

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INTRODUCTION

Oxalic acid (OA) is deemed to be the main virulence factor of the plant pathogen *Sclerotinia sclerotiorum* (Marciano *et al*., 1983). Mutants of *S. sclerotiorum* deficient in the production of OA were first identified in screens of mycelia grown from ascospores after UV irradiation (Godoy *et al*., 1990). These mutants are unable to invade plant tissue, although they produce and secrete a full range of degradative enzymes. The exact mutation of these strains has never been identified and it is unclear whether other genes involved in virulence are also affected because of the random nature of the mutagenesis method. More recently, another mutant was obtained by site-directed mutagenesis of a single *S. sclerotiorum* gene, oxalate acetylhydrolase (Oah), a gene that catalyses the hydrolytic cleavage of oxaloacetate to acetate and OA (J. Rollins, University of Florida, Gainesville, FL, USA, personal communication). Like the UV-induced OA-deficient mutants, the Oah mutant is unable to either colonize healthy plant tissue or produce sclerotial bodies when growing on rich medium. The observation that Oah mutants can only infect a host via a wound, and that the spread of the mutant does not extend beyond the infection point, further confirms that OA should be classified as a virulence factor rather than a pathogenicity factor.

Although OA is the main virulence factor of *S. sclerotiorum*, its exact mechanism of action is not fully understood. Different studies have suggested that OA plays several roles, ranging from very obvious effects, such as acidification of the medium, to numerous subtle effects that aid the pathogen during infection and invasion. Marciano *et al*. (1983) proposed that OA chelates Ca^{2+} ions from pectin in the plant middle lamellae to facilitate the degradation of the plant cell wall by fungal pectin-degrading enzymes. OA also creates an acidic environment favourable to activation and increased expression of various exo- and endopolygalacturonases (Favaron *et al*., 2004; Riou *et al*., 1991). Guimaraes and Stotz (2004) demonstrated that OA can act at the cellular level to regulate the opening of stomata by disrupting the abscisic acid (ABA)-dependent stomatal closure, making the tissue more easily penetrable by fungal mycelia. Studies in soybean and

Nicotiana tabacum have shown that OA can act independently of calcium to inhibit the defence-associated oxidative burst, presum ably by restraining the production of hydrogen peroxide (Cessna *et al*., 2000).

Programmed cell death (PCD) can be a very effective plant defence mechanism against biotrophic pathogens that are generally race specific and able to elicit effector-triggered immunity (ETI) (Jones and Dangl, 2006). In contrast, *S. sclerotiorum* is a non-race-specific necrotroph that would possibly benefit from PCD. Kim *et al*. (2008) provided evidence supporting the hypothesis that OA induces PCD, allowing *S. sclerotiorum* to use the dead and dying cells to facilitate infection of the tissue. More recently, the same group used a green fluorescent protein (GFP)-tagged reporter construct (ro-GFP) capable of changing its excitation state according to the oxidation state of the cell environment. The ro-GFP reporter enabled a comparison of the oxidative states between leaves inoculated with either wild-type *S. sclerotiorum* or an OA-deficient mutant (Williams *et al*., 2011). The authors concluded that OA creates an initial reduced environment as early as 3 h after inoculation, which suppresses the oxidative burst, thus supporting the theory advocated by Cessna *et al*. (2000). Therefore, according to these studies, OA initially reduces the cell redox environment and suppresses the oxidative burst and reactive oxygen species (ROS) production, but later OA is involved in inducing ROS production and eliciting PCD. This scenario seems complex and it remains unclear as to the exact role of OA in these observed redox changes.

In the work presented here, we used transcriptome profiling with oligo microarrays and RNA-seq to analyse the changes in mRNA levels in leaves following infiltration of an OA solution. Infiltrations were carried out on both a transgenic soybean line expressing oxalate oxidase (OxO) and its parental line AC Colibri (AC), in an attempt to distinguish the effects of OA from other *S. sclerotiorum* virulence factors. Our findings confirm that OA is capable of modulating the oxidative stress response of the plant, and that this modulation appears to be fostered by the release of iron ions, as reflected by the high induction of ferritin and iron-associated genes.

RESULTS

Microarrays

OxO transgenic soybean plants and parental AC plants were infiltrated with 5 mM OA (pH 2.4), acidified water (pH 2.4) or water more reflective of plant physiological acidity (pH 5.5), and sampled 2 h following infiltration to examine differential gene expression using soybean oligo-based microarrays (Gonzalez and Vodkin, 2007). Pairwise hybridizations followed a loop design (Fig. 1). Analysis of the microarray data revealed a total of 2390 sequences which changed significantly in abundance between the transgenic and parent plants, or between treatments, at overall

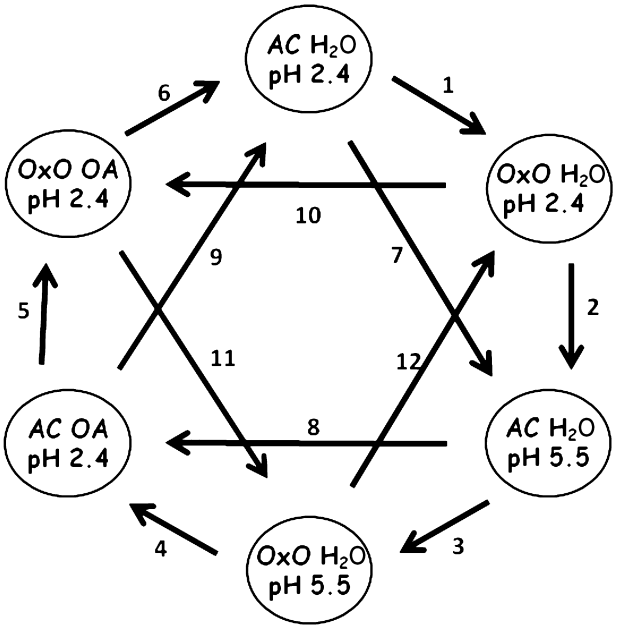

Fig. 1 Microarray slide hybridization layout showing the design used in the oxalic acid infiltration experiment. Arrows represent oligo microarray slides with two hybridized samples, arrowheads indicate cyanine 5 (Cy5)-labelled samples and the tails represent Cy3-labelled samples; circles show the treated samples. AC, AC Colibri parental soybean line; OA, oxalic acid; OxO, transgenic soybean line expressing oxalate oxidase.

F-test *P* values of <0.001 for genotype, treatment or genotype-by-treatment (Table S1, see Supporting Information). In addition to the *F*-tests, 13 pairwise *t*-tests between the least-square means estimated from analysis of variance (ANOVA) were run to estimate the effect of the combination of both OA and its acidic pH, OA independent of its pH, transgene and acidity (summarized in Table 1, detailed in Table S1). The list of significantly differentially expressed genes, as determined by overall *F*-test *P* values of <0.001, was further narrowed by selecting only genes that also had at least one specific pairwise *t*-test false discovery rate (FDR)-corrected *P* value of <0.001, giving a list of 1054 genes deemed to be the most highly significant (Table S2, see Supporting Information), and which serve as the focus of discussion for this article.

Effects of OA and its pH

Of the 1054 genes selected as being the most significant for this study, nearly all (1045) showed a change in expression as a result of a combination of OA and its low pH, as seen when comparing leaves infiltrated with OA pH 2.4 versus water pH 5.5 (Table S3, see Supporting Information). The vast majority of these changing genes (95.3%) showed the same direction of induction or repression in both plant genotypes, but AC showed stronger responses in general, reflective of a gradual OA removal by OxO enzyme activity in the transgenic line. The strongest effects of OA and its low pH were seen in AC; there were 46 oligos with at least three-fold

Table 1 Pairwise *t*-tests performed between the microarray experiment treatment factors. Each of the 13 tests aids in the understanding of one of the four possible effects. Detailed results are shown in Table S1.

Effect	Factor 1	Factor 2	(Factor 1/Factor 2) estimated value is:
Transgene	OxO	AC	Overall effect of the transgene
	OxO_H_2O_pH 5.5	AC_H_2O_pH 5.5	Effect of the transgene on water at physiological pH
	OxO_H_2O_pH 2.4	AC_H_2O_pH 2.4	Effect of the transgene on infiltration of acidic water
	OxO_OA_pH 2.4	AC_OA_pH 2.4	Effect of the transgene on infiltration of OA
pH	H_2O_pH 2.4	H_2O_pH 5.5	Overall effect of the pH change 2.4 vs. 5.5
	AC_H_2O_pH 2.4	AC_H_2O_pH 5.5	Effect of pH 2.4 vs. pH 5.5 on AC plants
	OxO_H_2O_pH 2.4	OxO_H_2O_pH 5.5	Effect of pH 2.4 vs. pH 5.5 on OxO plants
OA independent of its pH	OA_pH 2.4	H_2O_pH 2.4	Overall effect of OA infiltration independent of its pH
	AC_OA_pH 2.4	AC_H_2O_pH 2.4	Effect of OA infiltration in AC independent of OA pH
	OxO_OA_pH 2.4	OxO_H_2O_pH 2.4	Effect of OA infiltration in OxO independent of OA pH
OA and its pH	OA_pH 2.4	H_2O_pH 5.5	Overall effect of OA infiltration
	AC_OA_pH 2.4	AC_H_2O_pH 5.5	Effect of OA infiltration in AC plants
	OxO_OA_pH 2.4	OxO_H_2O_pH 5.5	Effect of OA infiltration in OxO plants

AC, AC Colibri parental soybean line; OA, oxalic acid; OxO, transgenic soybean line expressing oxalate oxidase.

increased expression, which can be summarized as being ferritins, members of the phenylpropanoid pathway, cytochrome P450s, glutathione S-transferases, peroxidases or pathogenesis-related (PR) proteins. The oligos that decreased by one-third or more in AC plants as a result of OA at pH 2.4 were related mainly to primary metabolism and redox, including several iron-associated, redox-related proteins, such as ferric reductase, iron superoxide dismutase and ferredoxin. Comparing the AC and OxO genotypes, of the 10 genes most reduced in AC versus OxO, six were unknowns or no hits, one was an aminotransferase, one was a ferric reductase and two were ferredoxins. The 10 oligos most induced in AC versus OxO reflected the activation of defences: three peroxidases, three no hits or unknowns, two to the same PR protein, one MLO10 and one β-glucosidase. The observed results point to a defensive response being elicited by the combined effect of OA and its pH, in addition to alterations in plant iron homeostasis, as indicated by the strong induction of ferritin.

Effects of OA, independent of pH

By screening the fold-change values in the comparisons between infiltrations with OA versus infiltrations with water at pH 2.4, we were able to identify genes that changed in expression as a result of OA independent of its acidic pH. Of the 1054 differentially regulated oligo spots selected for discussion, 78 showed a significant differential expression comparing OA infiltration versus water infiltration at pH 2.4 (Table 2). The direction of expression (induced or repressed) was the same for all 78 genes for both AC and OxO, but the strength of the differential expression tended to be stronger in AC, again presumably because of the removal of OA in the OxO-expressing plants. Only three genes showed a stronger change in OxO than AC in response to OA versus water at pH 2.4: Glyma01g26840.2, an unknown (2.13- versus 1.08-fold); Glyma10g32820.1, a protease inhibitor (2.60- versus 1.82-fold); and Glyma10g44170.2, a homogentisate phytylprenyltransferase (1.95- versus 1.70-fold). Notably, among the highest OA-induced microarray spots were eight genes coding for ferritin or ferritin subunits. These eight oligo spots correspond to seven predicted coding sequences, i.e. Glyma models (the computationally predicted set of coding sequences in the assembled soybean genome available at http://www.phytozome.net/soybean.php; Schmutz *et al*., 2010). These data show that ferritin is induced by the infiltration of OA, independent of the pH change. Many of the other genes induced were consistent with a defensive/stress response, such as genes of the phenylpropanoid pathway, a protease inhibitor, peroxidases, ethylene related and cytochrome P450s. The genes showing the greatest down-regulation in response to OA relative to water at pH 2.4 tended to be poorly characterized, but probably within chloroplast or mitochondria.

Effects of the OxO transgene

The overall effect of the OxO transgene across the three treatments (OA, pH 2.4 and pH 5.5) was statistically low, and there were no genes that met the strict selection criteria of an overall *F*-test *P* value of <0.001, as well as a *t*-test FDR-corrected *P* value of <0.001. Therefore, to identify the genes with the highest probability of fluctuating differently between OxO and AC in response to OA, we selected genes that had an *F*-test *P* value of 0.001 for genotype-by-treatment effect, as well as a *t*-test FDR-corrected *P*-value cut-off of <0.05 (Table 3). It should be noted that, of the 12 genes identified using these criteria, all but two showed higher expression in AC than OxO, and eight genes showed at least a 1.5-fold ($\log_2 > 0.56$) increase when AC was infiltrated with OA versus water at pH 2.4. None of these genes fluctuated in OxO. One gene, chloroplast ferredoxin, was expressed more strongly in OxO versus AC, but that was caused by the reduced expression ($\log_2 = -1.096$) in AC on OA treatment, with little effect ($\log_2 = -0.244$) on this gene in OxO. Genes induced in AC included (in addition to a few unknowns and no hits): a PR protein, regulator of gene silencing, NADPH isocitrate dehydrogenase, gibberellin 2-oxidase and an R2R3 Myb transcription factor (Table 3).

Table 2 Differentially regulated genes as a result of oxalic acid (OA) infiltration independent of its pH. Comparisons between infiltrations of OA in leaves of OxO (the transgenic soybean expressing oxalate oxidase) and its parent AC (AC Colibri).

	Log_2 ratio				
	OA/H_2O pH 2.4				
Oligo array ID	Overall	OxO	AC	Best match to Glyma models	Short annotation
GM18751P049N07	4.23	3.39	4.04	Glyma18g43650.1	Ferritin light chain
GM18681P049K09	4.00	2.98	4.20	Glyma18g43650.1	Ferritin
GM14987P040A11	3.49	2.72	3.62	Glyma03g06420.2	Ferritin 2
GM05373P014P21	2.41	1.71	2.40	Glyma11g35610.1	Ferritin 2
GM12232P032N16	2.32	1.74	2.28	Glyma01g31300.1	Ferritin 3
GM18577P049G01	1.90	1.18	2.01	Glyma14g06160.1	Ferritin 4
GM04737P013F09	1.79	1.42	1.91	Glyma02g43040.2	Ferritin 4
GM14944P039O16	1.72	1.13	1.52	Glyma18g02800.2	Ferritin 3
GM03715P010K19	1.40	1.60	1.65	Glyma09g25470.4	Putative acyl-CoA synthetase
GM09947P026O11	1.35	1.38	0.87	Glyma10g32820.1	Protease inhibitor 2
GM10142P027G14	1.15	0.75	1.13	Glyma07g02180.2	Long-chain fatty acid CoA ligase
GM04776P013G24	1.06	0.69	1.24	Glyma07g33780.2	Caffeoyl-CoA *O*-methyltransferase
GM07411P020E19	1.01	0.96	1.29	Glyma09g39390.1	No hits
GM04726P013E22	0.97	0.82	1.39	Glyma13g01080.2	4-Coumarate:coenzyme A ligase
GM10325P027O05	0.90	0.89	1.06	Glyma08g23560.2	Hydroxycinnamoyl transferase
GM18849P050B09	0.89	0.69	0.98	No match	Caffeic acid *O*-methyltransferase
GM12430P033F22	0.87	1.09	0.11	Glyma01g26840.2	Unknown
GM38358P100O06	0.87	0.98	1.17	No match	Phenylalanine ammonia-lyase
GM14959P039P07	0.87	0.86	1.08	Glyma10g06600.1	Phenylalanine ammonia-lyase
GM18945P050F09	0.86	0.68	0.83	No match	Caffeic acid *O*-methyltransferase
GM19041P050J09	0.86	0.71	0.95	No match	Caffeic acid *O*-methyltransferase
GM13565P036F05	0.85	0.83	1.19	Glyma13g20800.1	Phenylalanine ammonia-lyase
GM35930P094J02	0.85	0.55	0.92	Glyma13g06550.1	Quercetin 3-*O*-glucoside-6"-*O*-malonyltransferase
GM04128P011L24	0.84	0.69	0.97	Glyma14g38580.1	*trans*-Cinnamate 4-monooxygenase
GM01662P005F06	0.83	0.97	0.76	Glyma10g44170.2	Homogentisate phytylprenyltransferase
GM15500P041F20	0.83	1.08	1.27	Glyma01g33150.1	Putative cytochrome P450
GM31871P083P23	0.81	0.65	1.50	Glyma10g40750.1	Unknown
GM04366P012F22	0.81	0.61	1.22	Glyma11g15680.5	Ascorbate peroxidase
GM06501P017O21	0.78	0.71	0.86	Glyma13g20800.1	Phenylalanine ammonia-lyase
GM36437P095O05	0.78	0.10	1.26	Glyma14g05350.1	1-Aminocyclopropane-1-carboxylate oxidase
GM18406P048O22	0.77	0.48	1.24	Glyma12g07780.3	Ascorbate peroxidase 2
GM19137P050N09	0.76	0.63	0.87	No match	Caffeic acid *O*-methyltransferase
GM14124P037M12	0.75	0.69	1.31	Glyma01g32400.1	Protein kinase
GM38070P100C06	0.75	0.82	1.14	No match	Phenylalanine ammonia-lyase
GM12369P033D09	0.75	0.85	1.47	Glyma19g01470.1	α-Dioxygenase
GM38262P100K06	0.75	0.94	1.12	No match	Phenylalanine ammonia-lyase
GM19158P050O06	0.75	0.69	1.05	No match	Phenylalanine ammonia-lyase
GM07122P019I18	0.74	0.97	1.08	Glyma10g06600.1	Phenylalanine ammonia-lyase
GM18660P049J12	0.73	0.84	1.01	Glyma18g45260.1	2'-Hydroxydihydrodaidzein reductase
GM38166P100G06	0.69	0.73	1.12	No match	Phenylalanine ammonia-lyase
GM18870P050C06	0.66	0.72	0.77	No match	Phenylalanine ammonia-lyase
GM19150P050N22	0.66	0.77	0.91	No match	Cinnamoyl CoA dehydrogenase
GM19062P050K06	0.65	0.61	0.80	No match	Phenylalanine ammonia-lyase
GM18966P050G06	0.65	0.59	0.84	No match	Phenylalanine ammonia-lyase
GM04388P012G20	0.65	0.63	0.97	Glyma10g40870.3	Cinnamyl-alcohol dehydrogenase
GM18958P050F22	0.63	0.76	0.81	No match	Cinnamoyl CoA dehydrogenase
GM12707P034B11	0.60	0.57	0.87	Glyma0169s00210.1	Monodehydroascorbate reductase
GM03619P010G19	0.59	0.52	0.78	Glyma01g41450.1	Lipase class 3
GM18862P050B22	0.59	0.62	0.84	No match	Cinnamoyl CoA dehydrogenase
GM12288P032P24	0.57	0.71	0.93	Glyma13g36110.1	Putative cytochrome P450
GM35875P094G19	0.57	0.59	0.99	Glyma02g40550.1	2-Hydroxyphytanoyl-CoA lyase
GM31372P082L04	0.55	0.73	1.04	Glyma12g01780.1	Alcohol dehydrogenase
GM15220P040K04	0.48	0.36	0.58	Glyma01g36890.1	Dehydroquinate synthase
GM38242P100J10	0.47	0.40	0.84	No match	Cinnamoyl CoA reductase
GM10980P029J12	0.44	0.72	0.87	Glyma02g04120.2	ATP citrate lyase b-subunit
GM13944P037E24	0.44	0.52	0.85	Glyma15g15020.1	ATP citrate lyase a-subunit
GM19054P050J22	0.43	0.55	0.72	No match	Cinnamoyl CoA dehydrogenase
GM34640P091D08	0.39	0.41	1.04	Glyma16g01710.1	Zinc finger

Table 2 Continued.

Oligo array ID	Log_2 ratio OA/H_2O pH 2.4 Overall	OxO	AC	Best match to Glyma models	Short annotation
GM19138P050N10	0.36	0.33	0.54	No match	Cinnamoyl CoA reductase
GM05086P014D22	0.33	0.37	0.76	Glyma02g11740.1	No hits
GM04356P012F12	0.32	0.43	0.72	Glyma06g19820.3	Aldehyde dehydrogenase
GM03791P010N23	0.30	0.32	0.56	Glyma18g50940.1	frnE protein-like
GM12611P033N11	−0.16	−0.46	−0.73	Glyma01g42360.1	Unknown
GM07176P019K24	−0.28	−0.40	−0.65	Glyma05g02250.1	Inositol 1,3,4-trisphosphate 5/6-kinase
GM07686P021A06	−0.31	−0.50	−0.97	Glyma04g01750.2	Glyceraldehyde-3-phosphate dehydrogenase
GM11660P031F20	−0.32	−0.22	−0.41	Glyma07g13210.1	No hits
GM12224P032N08	−0.32	−0.49	−1.12	Glyma03g42240.3	Unknown
GM01627P005D19	−0.35	−0.27	−0.81	Glyma12g06920.2	PPF-1 protein
GM02011P006D19	−0.38	−0.62	−1.00	Glyma04g02100.1	FtsH protease
GM07366P020C22	−0.38	−0.60	−1.00	Glyma20g12740.1	Phosphate translocator
GM22715P060C11	−0.42	−0.44	−1.12	Glyma04g24430.1	Unknown
GM17124P045J12	−0.43	−0.51	−0.76	Glyma18g06510.1	Cinnamoyl CoA reductase
GM12235P032N19	−0.47	−0.46	−1.47	Glyma09g34410.1	Protein kinase
GM07160P019K08	−0.69	−0.80	−0.92	Glyma12g06760.2	Unknown
GM14246P038B14	−1.12	−0.66	−0.69	Glyma10g06620.1	RNA-binding protein
GM17482P046I10	−1.12	−0.86	−1.26	Glyma11g11350.3	Unknown
GM27219P071O03	−1.32	−1.15	−1.49	Glyma11g11350.3	Nodulin
GM25844P068E20	−1.46	−1.24	−1.79	Glyma12g03520.2	Unknown

Table 3 Differentially regulated genes in OxO versus AC, determined by selecting oligo spots with an overall *F*-test *P* value of <0.001 for genotype effect.

Oligo array ID	Genotype-by-treatment *P* value*	OxO/AC OA_pH 2.4 FDR-corrected *P* value†	Log_2 ratio	Best match to Glyma models	Short annotation
GM09502P025L22	6.919E-07	0.0133	−1.40	Glyma06g12890.3	Pathogenesis-related protein
GM04200P011O24	2.101E-06	0.0126	1.16	Glyma08g13360.1	Chloroplast ferredoxin
GM36357P095K21	4.480E-06	0.0050	−0.78	Glyma04g37040.1	Calmodulin-related protein
GM04742P013F14	1.011E-05	0.0289	−0.73	Glyma02g40820.4	NADPH-specific isocitrate dehydrogenase
GM12430P033F22	4.652E-05	0.0218	0.76	Glyma01g26840.2	Unknown, PAR domain
GM28544P075F08	5.330E-05	0.0245	−0.89	Glyma03g02600.1	No hits
GM06066P016M18	1.296E-04	0.0033	−0.42	Glyma20g02040.1	Serine carboxypeptidase S10 family
GM30894P081H06	1.429E-04	0.0362	−0.58	Glyma17g11610.1	No hits
GM11391P030K15	1.882E-04	0.0075	−1.01	Glyma15g39750.1	Gibberellin 2-oxidase
GM01974P006C06	5.961E-04	0.0075	−1.10	Glyma01g41290.2	No hits
GM26086P068O22	6.299E-04	0.0339	−0.59	Glyma07g06990.2	Unknown, PAR domain
GM24080P063L08	9.244E-04	0.0362	−0.64	Glyma12g32540.1	Transcription factor Myb protein

AC, AC Colibri parental soybean line; FDR, false discovery rate; OA, oxalic acid; OxO, transgenic soybean line expressing oxalate oxidase.

*Overall genotype-by-treatment *F*-test *P* value.

†Pairwise *t*-test FDR-corrected *P* value.

Effects of pH on the plant response

Relative expression changes between the treatments involving water at pH 2.4 (pH of the OA solution) versus water at pH 5.5 (pH of the apoplast) can aid in the understanding of how plants respond to pH changes, specifically the acidification of the apoplast, as well as the role of the acidic nature of OA on pathogenesis. Seven genes made our stringent significance cut-off (Table 4) and, in all cases, the OxO and AC lines showed the same direction of differential expression; AC showed a stronger expression level modulation for all seven genes, whether increasing or decreasing, than OxO. The most significant up-regulated genes were two unknowns, a cytochrome P450 (possibly an isoflavone synthase) and a protein disulphide isomerase. Three of the genes significantly affected by pH were down-regulated, and included a light-responsive gene, a proline-rich protein and a solanesyl diphosphate synthase.

Differentially expressed genes detected by RNA-seq

To increase gene coverage and to verify and validate the microarray data, four of the same RNA samples used in the microarray study were deep sequenced for RNA species (RNA-seq) via Solexa/Illumina technology. The four samples sequenced were individual replicates of OxO infiltrated with water at pH 2.4, OxO

Table 4 Differentially regulated genes as a result of pH change. Comparisons between infiltrations with water at pH 2.4 and water at pH 5.5 in OxO (the transgenic soybean expressing oxalate oxidase) and its parent AC (AC Colibri).

Oligo array ID	Ratio H_2O_pH 2.4/H_2O_pH 5.5			Best match to Glyma models	Short annotation
	Overall	OxO	AC		
GM04281P012C09	1.05	0.72	1.11	Glyma02g15430.1	Unknown
GM04288P012C16	0.88	−0.49	1.52	Glyma10g36650.1	Light stress responsive
GM06874P018O10	0.77	0.42	1.12	Glyma06g46570.1	Proline-rich family
GM19147P050N19	0.58	0.52	1.08	Glyma15g39070.1	Solanesyl diphosphate synthase 1
GM16856P044O08	−0.61	−0.17	−0.91	Glyma06g12090.3	Protein disulphide isomerase
GM09369P025G09	−0.83	−0.15	−1.02	Glyma17g14620.1	Unknown
GM07293P019P21	−1.08	−0.36	−1.23	No match	Cytochrome P450

Table 5 Summary of reads obtained by RNA-seq analysis. Sequenced RNA from OxO (the transgenic soybean expressing oxalate oxidase) and its parent AC (AC Colibri) after vacuum infiltration treatments with water, pH adjusted to 2.4 or oxalic acid (OA) (pH 2.4).

Library name	Soybean line	Total reads sequenced	Total number of uniquely aligned reads*
AC_H_2O	AC-Colibri parent line	34 633 488	26 882 986 (77.6%)
AC_OA	AC-Colibri parent line	41 734 185	32 735 158 (78.4%)
OxO_H_2O	80(30)-1 transgenic	30 066 023	23 311 016 (77.5%)
OxO_OA	80(30)-1 transgenic	31 037 297	24 306 468 (78.3%)

*Reads were quality checked and trimmed; trimmed reads of less than 30 bp were removed and the remaining reads were aligned to the soybean genome. Only reads that uniquely mapped to coding sequences with less than two mismatches every 36 bp are reported. See Experimental procedures for more details.

infiltrated with OA at pH 2.4, AC infiltrated with water at pH 2.4 and AC infiltrated with OA at pH 2.4. Each of the four libraries yielded between 30 and 42 million 100-bp reads (Table 5). After quality checking and trimming, reads were aligned to the publicly available soybean genome. About 88% of the reads from each library were successfully aligned; 82% aligned uniquely on the genome and about 78% aligned uniquely to a predicted coding region (Table 5). A total of 39 885 coding sequences, representing 71% of the predicted genes in the soybean genome, were matched by at least one sequence read in at least one of the four libraries. All further discussion of these RNA-seq data focuses on the set of reads uniquely aligned to predicted coding genes.

Four comparisons were performed by calculating the fold change (log_2 ratios of reads per kilobase per million, RPKM) between the different libraries for each of the reads, namely: OxO_H_2O_pH 2.4 vs. AC_H_2O_pH 2.4, OxO_OA vs. AC_OA, AC_OA vs. AC_H_2O_pH 2.4, and OxO_OA vs. OxO_H_2O_pH 2.4. The data were then filtered by selecting those reads having fold-change differences greater or equal to eight, or smaller or equal to 1/8 (log_2 of 3 or –3). Reads having less than 10 raw counts in all four libraries were removed from the analysis. A total of 936 predicted genes were selected as having the greatest changes in abundance between the plant lines or between the treatments (Table S4, see Supporting Information), and were classified into functional categories. Genes that changed in abundance the most were related directly to nucleic acids (DNA/RNA); of the 111 genes falling into this category, 96 were putative transcription factors, constituting 10% of all differentially regulated genes. The second largest category was signalling genes. Genes related to primary metabolism comprised 9.7% of the changing genes, whereas membrane and signalling-related genes each accounted for 9% of the total. Many of these genes were related to upstream physiological events, suggesting that we were observing early responses to OA and acidity. Of the 936 differentially expressed genes selected, 630 showed up-regulation in both AC and OxO lines infiltrated with OA, relative to the samples infiltrated with water, at pH 2.4. Once again, ferritin was one of the strongest genes induced by OA. Also, among this group of induced genes were 13 auxin-related genes, 20 genes involved in secondary metabolism, including seven genes involved in terpenoid biosynthesis, and six genes in the lignin biosynthesis pathway. Eight peroxidases and six heavy metal transport genes were also induced in both samples. Only 49 genes were down-regulated in both lines as a result of OA infiltration; among these repressed genes were several membrane transporters and disease resistance genes.

Cross-platform comparison: microarray versus RNA-seq

The RNA-seq results confirmed and validated the differential expression of genes between the OxO transgenic line and its parent identified by the oligo microarray assays. Of the 39 885 reads from RNA-seq that were successfully aligned to a predicted soybean coding sequence (Glyma model), 2158 matched one of the 2390 significantly differentially regulated sequences from the oligo-based microarray study which assayed the same RNA samples (Table S1).

To assess the degree of correlation between the two platforms, the Pearson correlation coefficient (ρ) was calculated for each of the 2158 genes on corresponding comparisons between the log_2 intensity ratios of the microarrays and the log_2 RPKM ratios of the RNA-seq data. A highly significant positive linear correlation ($P < 0.001$) was found in the four comparisons tested (Fig. 2). To verify

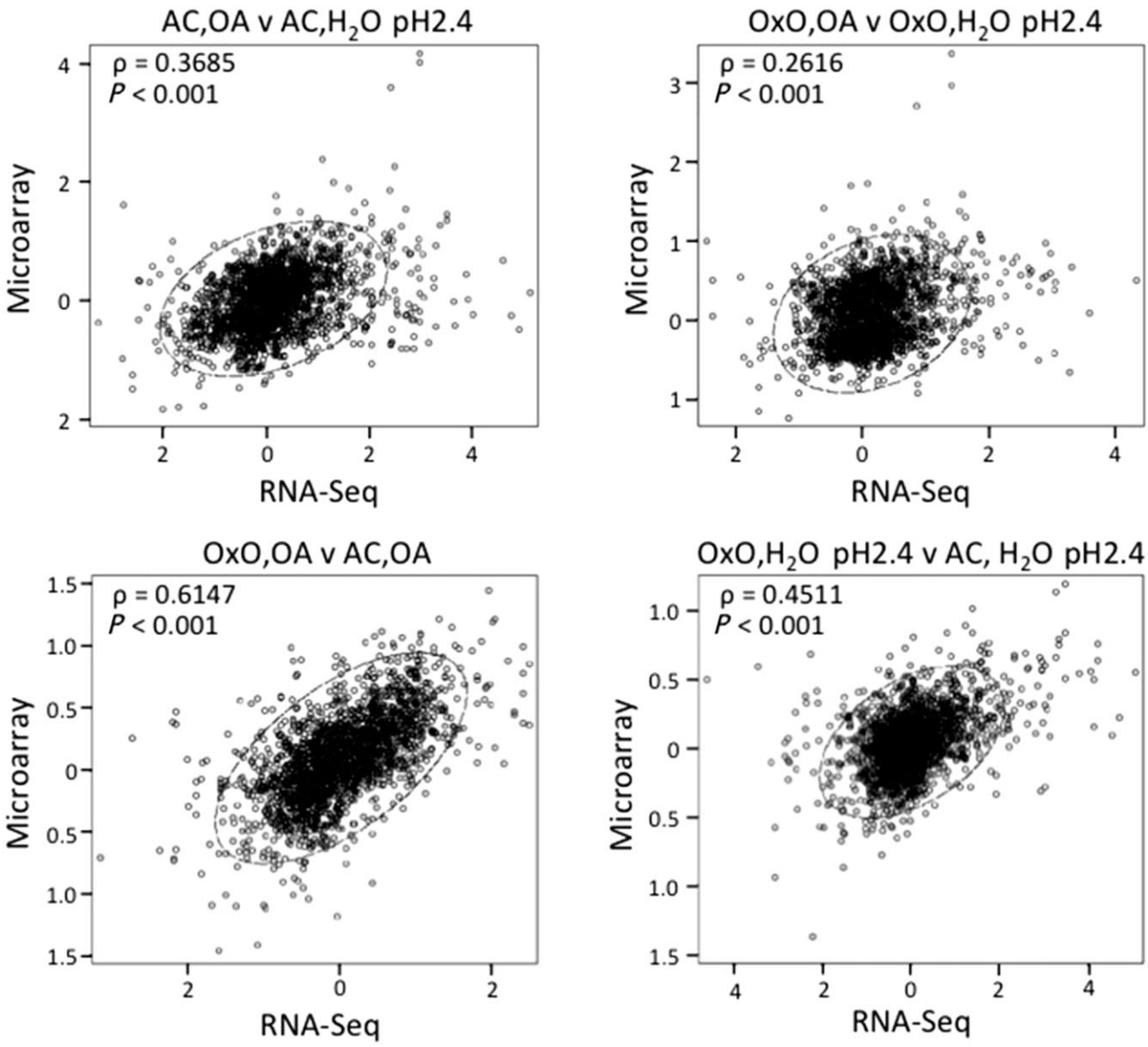

Fig. 2 Correlation between microarray and RNA-Seq data. *y* axes, microarray data as ratio of treatment over control; *x* axes, RNA-seq data as ratio of treatment over control. The Pearson correlation coefficient (ρ) and its *P* value are given within each graph. AC, AC Colibri parental soybean line; OA, oxalic acid; OxO, transgenic soybean line expressing oxalate oxidase.

whether the RNA-seq method was introducing biases caused by transcript length in the set of differentially regulated genes identified in the microarrays, the differential regulation of the 2158 coding sequences was plotted as a function of their lengths. For this experiment, the RNA-seq method tended to detect higher modulation than the microarrays for most of the differentially regulated transcripts, which fall in the range of 200–3000 bases in length. In turn, expression values for longer transcripts of 4000 or more bases showed little or no differences between the two platforms (Fig. S1, see Supporting Information).

The 31 spots showing the strongest differential expression in the microarray experiment, selected as having a $\log_2$ fold change greater than 2 or lower than −2 (Table 6), were compared side by side with their best Glyma model match in the RNA-seq data. Three of the microarray spots did not match with a Glyma model from the RNA-seq data and, of the 28 remaining sequences, 21 showed the same direction of expression (Fig. 3). In addition, although the differential expression values of the microarray were lower in all cases, they were proportional in value to their RNA-seq counterparts. Discrepancies between RNA-seq and microarrays were found in seven sequences. For one of these sequences, the difference was consistent across the four treatments. For this case, the oligo sequence probably represented a different gene than that as the best match in the Glyma models. The other six discrep-

Table 6 Top 31 genes modulated in expression in OxO (the transgenic soybean expressing oxalate oxidase) and its parent AC (AC Colibri) after infiltration with oxalic acid, water at pH 5.5 or water at pH 2.4, based on the microarray results. These data were used in the comparison with the RNA-seq results in Fig. 3.

Oligo array ID	Best match to Glyma models	Short annotation	DR	Comparison
GM18681P049K09	Glyma18g43650.1	Ferritin	UP	OA vs. H_2O at pH 2.4 in OxO
GM18751P049N07	Glyma18g43650.1	Ferritin	UP	OA vs. H_2O at pH 2.4
GM14987P040A11	Glyma03g06420.2	Ferritin 2	UP	OA vs. H_2O at pH 2.4 in OxO
GM05373P014P21	Glyma11g35610.1	Ferritin 3	UP	OA vs. H_2O at pH 2.4
GM12232P032N16	Glyma01g31300.1	Ferritin 2	UP	OA vs. H_2O at pH 2.4
GM18577P049G01	Glyma14g06160.1	Ferritin 4	UP	OA vs. H_2O at pH 2.4 in OxO
GM37303P098C07	Glyma01g36190.2	Auxin related	UP	OA vs. H_2O at pH 5.5 in AC
GM32514P085K18	Glyma01g02580.1	CAD	UP	OA vs. H_2O at pH 5.5 in AC
GM35484P093G12	Glyma02g43990.2	Glucosidase	UP	OA vs. H_2O at pH 5.5 in AC
GM37068P097I12	Glyma08g18220.1	No hits	UP	OA vs. H_2O at pH 5.5 in AC
GM03538P010D10	Glyma13g36110.1	No hits	UP	OA vs. H_2O at pH 5.5 in AC
GM18756P049N12	Glyma11g01350.2	CHS	UP	OA vs. H_2O at pH 5.5 in AC
GM00967P003I07	Glyma01g43880.1	CHS	UP	OA vs. H_2O at pH 5.5
GM09502P025L22	Glyma06g12890.3	PR protein	UP	OA vs. H_2O at pH 5.5 in AC
GM36533P096C05	Glyma09g03100.1	Electron carrier	UP	OA vs. H_2O at pH 5.5
GM37533P098L21	Glyma18g44310.1	Cationic peroxidase	UP	OA vs. H_2O at pH 5.5 in AC
GM34930P091P10	Glyma09g41450.1	Peroxidase	UP	OA vs. H_2O at pH 5.5 in AC
GM36297P095I09	Glyma03g37390.1	Pectinesterase	UP	OA vs. H_2O at pH 5.5
GM18021P047O21	Glyma06g26610.1	Blue copper-binding protein	UP	OA vs. H_2O at pH 5.5
GM32575P085N07	Glyma08g02160.1	No hits	UP	OA vs. H_2O at pH 5.5
GM12095P032H23	Glyma19g45030.1	No hits	DOWN	OA vs. H_2O at pH 5.5 in AC
GM34828P091L04	Glyma08g28370.1	CONSTANS-like	DOWN	OA vs. H_2O at pH 5.5 in AC
GM18471P049B15	Glyma15g06270.1	Unknown	DOWN	OA vs. H_2O at pH 5.5 in AC
GM08939P024E11	Glyma10g12370.1	No hits	DOWN	OA vs. H_2O at pH 5.5 in AC
GM22661P060A05	Glyma07g08150.1	Unknown	DOWN	OA vs. H_2O at pH 5.5 in AC
GM18501P049C21	Glyma16g10880.3	Terpenoids	DOWN	OA vs. H_2O at pH 5.5 in AC
GM17072P045H08	Glyma08g41460.2	Aminotransferase	DOWN	OA vs. H_2O at pH 5.5 in AC
GM24040P063J16	Glyma17g04650.1	No hits	DOWN	OA vs. H_2O at pH 5.5 in AC
GM07660P020P04	Glyma14g03580.1	No hits	DOWN	OA vs. H_2O at pH 5.5 in AC
GM11550P031B06	Glyma02g45170.1	No hits	DOWN	OA vs. H_2O at pH 5.5 in AC
GM23696P062L08	Glyma11g21400.1	No hits	DOWN	H_2O at pH 2.4 vs. H_2O at pH 5.5

CAD, cinnamyl-alcohol dehydrogenase; CHS, chalcone synthase; DR, direction of differential expression; OA, oxalic acid; PR, pathogenesis related. Italic oligo ID did not have a match in the RNA-seq experiment.

ancies were non-consistent across the treatments, and had extremely low differential expression values in the treatments disagreeing in the direction of expression (less than 0.5 up or down in either the microarray or RNA-seq), perhaps making them more susceptible to analysis artefacts, such as microarray data normalization. In general, a good agreement was shown between microarray and RNA-seq (especially for the stronger differentially expressed genes), and the data obtained by RNA-seq served to validate and expand the microarray results.

Ferritin and iron homeostasis changes induced by OA as detected by RNA-seq

The RNA-seq data provided an increased coverage of the transcriptome, enabling further investigation of changes in iron homeostasis. Genes related to iron metabolism were selected from the RNA-seq data using keyword searches, and a total of 103 sequences was found. Sequences with less than 10 counts in all the libraries were removed, leaving a total of 82 genes (Table S5, see Supporting Information). These iron-related genes were classified according to their annotation in Phytozome v8.0. Nine groups were identified: vacuolar iron transporters (VIT), stabilizers of iron transporter (SufD), iron-dependent oxygenases, iron–sulphur cluster proteins, iron-regulated transporters, ferritins, iron reduction-oxidases, iron superoxide dismutases and cytochrome b561. In addition to the ferritin genes that showed the highest expression changes, a cytochrome b561 showed very high induction in the OA-infiltrated samples. Other iron-related genes that were highly induced were AtNRAMP1 in AC treated with water and OxO treated with OA. Genes coding for iron–sulphur cluster-containing proteins showed a general induction with some degree of difference between the four samples. Both OxO samples consistently showed more induction of ferric reductases/oxidases compared with the AC samples. An iron superoxide dismutase (FeSOD) was highly induced when acidic water was infiltrated into both genotypes.

Effect of pretreatment with iron on the infection ability of the OA-deficient mutant

The gene expression results suggest that iron homeostasis is disturbed in the leaf by infiltration with OA. To test whether one possible benefit to *S. sclerotiorum* from freeing iron from the leaf would be to make this essential element available to itself, we

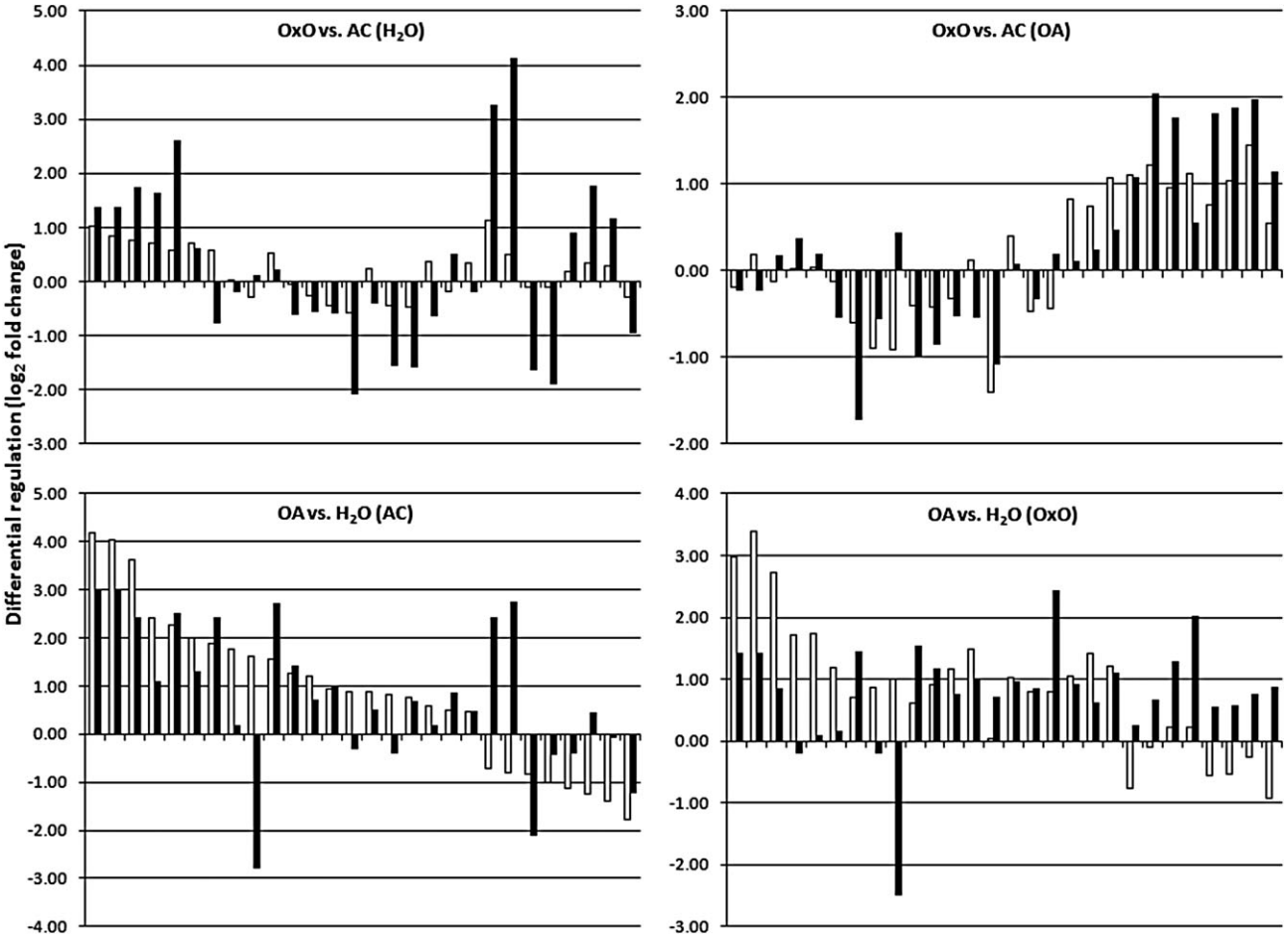

Fig. 3 Comparison between values of differential expression detected by microarrays (open bars) and RNA-seq (filled bars) for the top 28 differentially regulated oligo-spots detected in the microarray experiment, maintained in the same order along the *x* axis across the four images. AC, AC Colibri parental soybean line; OA, oxalic acid; OxO, transgenic soybean line expressing oxalate oxidase.

infiltrated *Arabidopsis thaliana* leaves with solutions of either $FeSO_4$ or $FeCl_3$ (ferrous and ferric iron, respectively), and inoculated the leaves with an OA-deficient *S. sclerotiorum*, an Oah^- knockout mutant. All leaves infiltrated with 1% and 0.1% $FeCl_3$ and/or with 1% $FeSO_4$ turned necrotic and died within 3 h. Leaves infiltrated with 0.01% $FeSO_4$ did not show any visible response. Leaves infiltrated with 0.01% $FeCl_3$ or 0.1% $FeSO_4$ showed similar responses: Oah^--inoculated leaves showed agar plug attachment after 24 h, indicating tissue penetration by mycelia. The inoculum did not show any plug attachment on leaves without iron pre-treatment (including on leaves infiltrated with water), indicating no penetration by mycelia. Curiously, for treatments that showed plug attachment (Oah^- inoculation on 0.01% $FeCl_3$- or 0.1% $FeSO_4$-infiltrated leaves), Oah^--induced disease did not progress normally. Rather, a general necrosis spread through the leaf and, after 20 days, the leaves were dead. No further growth of mycelia was observed in the necrotized tissue, indicating that, although iron infiltration might have initially helped the pathogen to penetrate and infect the host, the host tissue managed to defend the plant by limiting infection (Fig. S2, see Supporting Information). Non-inoculated control plants infiltrated with 0.01% $FeCl_3$ or 0.1% $FeSO_4$ solution only showed slight variations in chlorosis and no necrosis.

DISCUSSION

Soybean oligo microarrays and RNA-seq were utilized to analyse soybean leaf response to OA, the main virulence factor of *S. sclerotiorum*, a serious pathogen of many crops world-wide. The microarrays surveyed the expression of over 38 000 genes, and identified 2390 spots as being significant at *F*-test cut-offs of 0.001. To focus only on the genes that were statistically the most robust, this gene list was narrowed to 1054 genes that also showed an FDR-corrected *P* value of <0.001 in a treatment pairwise *t*-test. The RNA-seq analysis identified 936 genes that changed at least eight-fold, and recovered many of the same

genes as being differentially expressed, showing that the expression data are sound. However, some discrepancies were identified between the two methods used to study RNA levels. Unknown cross-hybridization can be expected to contribute to error in the microarray analysis, but RNA-seq procedures and data analysis are also not free of introduced biases. It has been suggested that the length of the transcripts may be a source of bias in RNA-seq experiments, as the density of reads would not be uniform along the transcript; small sequencing errors would have more of an effect in shorter transcripts than in long ones, affecting multigene families (Fu *et al*., 2009; Marioni *et al*., 2008; Mortazavi *et al*., 2008). Oshlack and Wakefield (2009) hypothesized that the ability of RNA-seq to detect a differentially regulated transcript depends on the length of the transcript, because this method, which uses RNA fragmentation, will give rise to more reads mapping to a longer transcript than to a shorter one. Microarrays, although not perfect, do not present this type of bias. In addition to a small bias against short-length transcripts, RNA-seq did not have the benefit of replication in this study, whereas our microarray experiment (Fig. 1) consisted of three biological replications and, each with four technical replications, enhancing the recovery of highly significant data.

Physiological studies have been reported on plant responses to OA (Cessna *et al*., 2000; Kim *et al*., 2008; Williams *et al*., 2011), but there have not been any reports on gene expression studies. The gene expression data collected here complement the physiological experiments, and suggest certain underlying mechanisms to plausibly explain some of the observed effects of OA. The expression data point to a disturbance of iron homeostasis. OA is a well-known chelator of iron, and has even been implicated in robbing iron from our diet, if the food we eat is also high in OA (Bataille and Fournier, 2001).

Ferritin is a ubiquitous iron storage protein that uses multiple subunits to surround an estimated 4500 atoms of iron per ferritin shell (Harrison and Arosio, 1996). A biochemical study of the ability of various organic acids to remove iron from ferritin has been conducted (Macur *et al*., 1991), and has found that OA is very effective, being better than citric, tartaric, succinic and fumaric acids at releasing iron from purified ferritin. Considering its high affinity for iron, we hypothesize that OA from *S. sclerotiorum* causes the release of iron from cellular storage compartments, including redox proteins and enzymes, and that this benefits the pathogen by inducing cell death and making iron readily available for sequestration by this necrotrophic pathogen. That iron is being released from host storage by OA is further supported by the findings that the main inducer of ferritin transcription in plants is iron (Briat *et al*., 1999; Torti and Torti, 2002).

Iron that has been released from ferritin and/or other cellular components may exist as Fe(II) or Fe(III) ions, which are both very reactive, and thus able to generate ROS, which can damage nucleic acids, proteins and lipids. Therefore, it is essential that iron regulation is tightly controlled to minimize this toxic condition; ferritin is a key component limiting the extent and character of the oxidative stress that can occur in cells. Ferritin has enzymatic activity via ferroxidase, which converts ferrous iron [Fe(II)] to ferric iron [Fe(III)] to allow internalization and sequestration within the ferritin core. Ferritin also serves as an antioxidant by capturing ROS-inducing iron, which protects cells from damage caused by excess iron (Lawen and Lane, 2013; Torti and Torti, 2002).

It has been shown previously, and our results support this finding, that, although OA exerts most of its function in a manner independent of its pH and its ability to acidify the environment (Coumo *et al*., 2005; Kim *et al*., 2008; Williams *et al*., 2011), low acidity favours the release of iron from ferritin (Laulhere and Briat, 1993; Macur *et al*., 1991). We noted what could be a synergistic effect of an acidic milieu and OA, as many more genes were significantly differentially expressed when comparing OA with water at pH 5.5 than with water at pH 2.4. RNA-seq results showed that ferritin was induced in plants infiltrated with water at pH 2.4.

In addition to ferritin, other proteins in the cells require iron to function. Cytochromes are haem-containing proteins which mostly use iron to perform their redox chemistry and electron transfer, and include enzymes such as cytochrome P450s, as well as components of chloroplast and mitochondrial electron transfer chains (Ross and Jakubowski, 2011). Restricting available iron from cytochromes would rapidly kill cells and greatly benefit this necrotrophic fungus.

The oxidative states of tomato leaves inoculated with either wild-type *S. sclerotiorum* or an OA-deficient mutant were investigated using the ro-GFP reporter (Williams *et al*., 2011). The authors concluded that OA creates an initial reduced environment as early as 3 h after inoculation, which suppresses the oxidative burst. This result is also consistent with the theory advocated by Cessna *et al*. (2000). Therefore, according to these studies, OA initially reduces the cell redox environment and suppresses the oxidative burst and ROS production, but later OA is involved in inducing ROS production and eliciting PCD. It appears that, if OA removes and chelates iron from iron-containing cellular proteins, this would lead to a reduced cellular environment. Higher ROS accumulation later could be the result of the absorption of light energy into the photosystem centres that have lost the capacity to pass that energy through the electron transfer chain (Allen *et al*., 1999) because of the release of iron.

Interestingly, although ferritin was strongly induced by OA infiltration, no ferritin genes were significantly induced in microarray experiments involving *S. sclerotiorum*-infected plants (Calla *et al*., 2009, 2014), indicating that, if ferritin transcripts increase in abundance as a result of increased iron levels elicited by OA, in the presence of the fungus these levels are not increased within the cell to a sufficient extent to induce detectable changes in ferritin expression levels. This observation suggests that *S. sclerotiorum* is using OA to both release iron from the host and for rapid uptake

by the pathogen. The effect of removing excess iron from infected cells would also reduce their toxicity to the pathogen, as it would minimize the induction of ROS, as free iron can react with H_2O_2 and produce the highly reactive and damaging hydroxyl radical (OH^-) (Briat *et al*., 1999; Ravet *et al*., 2009). It would be very beneficial to the pathogen to use OA to induce cell death by the removal of iron from cytochromes, including the electron transfer chains, stopping/reducing electron flow through chloroplast and mitochondria, and then absorbing the OA-chelated iron for its own metabolic needs during infection of the dead/dying cells. Our gene expression studies (this article and Calla *et al*., 2009, 2014) support this scenario.

The iron infiltration experiment showed that the provision of excess iron may complement the Oah^- pathogen which lacks OA, as this mutant gains the ability to attach to the leaves, supporting the suggestion of a role for OA in assisting the acquisition of iron for the pathogen. That the fungus was unable to advance very far into the tissue further confirms that the plant cells need to be dead or dying prior to infection, and that, in the absence of OA, *S. sclerotiorum* is unable to complete this task. However, the available data are insufficient to prove this theory as the effect was minor, and plug attachment, indicative of initial tissue penetration by the pathogen, may be a result of unmeasured effects induced in the plant by iron. However, the unexpected necrosis observed on leaves that were both infiltrated and inoculated cannot be attributed to iron or pathogen alone. Leaves that were iron infiltrated but not inoculated, would also have died if iron toxicity were responsible for necrosis. However, the leaves did not die, indicating that the necrosis was in response to the pathogen, and that iron and pathogen together were needed to induce necrosis. How this occurs is a subject for future investigations.

EXPERIMENTAL PROCEDURES

Plant growth and infiltration with OA

Seeds of the OxO transgenic soybean line 80(30)-1 (Donaldson *et al*., 2001) and its parental line AC were placed in trays of damp vermiculite (Holiday, Toronto, ON, Canada) to germinate for 7 days in a growth chamber with the following conditions: 14-h photoperiod, 26/24 °C day/night temperatures and 50% humidity. Illumination was provided by white fluorescent and incandescent bulbs at a light intensity of 300–400 μmol/m^2/s and maintained about 30 cm from the top of the growing area. Seedlings were transplanted to 10.5-cm-diameter plastic pots (Kord Products, Brampton, ON, Canada) containing a blend of sterilized topsoil, sand and ProMix 3:2:1 (Ritchie Seed and Feed, Ottawa, ON, Canada) for an additional 8 days. Fertilizer (20.20.20 N.P.K., Plant Products Co. Ltd, Brampton, ON, Canada) was applied once a week after emergence of the first trifoliate. Prior to infiltration, the tops of the pots were covered with plastic wrap (Saran) and secured with elastic bands to prevent soil from dropping into the infiltration solutions. Plants with fully opened V2 trifoliates were subjected to vacuum infiltration by drawing a vacuum and then releasing after 2 min whilst the leaves were submerged in a 500-mL solution of one of the following: 5 mM OA pH 2.4, H_2O pH 5.5 or H_2O pH 2.4 (pH adjusted with 1 M HCl). All solutions contained 0.005% Silwet L-77 (Vac-in-stuff; Lehle Seeds, Round Rock, TX, USA) as a surfactant to enhance infiltration. One leaflet from each of trifoliates 1 and 2 was collected from the plants 2 h post-infiltration, flash frozen in liquid nitrogen and stored at –80 °C. Samples from 12 AC or 80(30)-1 OxO plants were bulked for each sample and RNA was isolated at a later date. The experiment was repeated three times to generate three biological/experimental replications.

RNA extraction, purification and quantification

Total RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). Tubes containing Phase Lock Gel™ (Brinkmann Instruments, Inc., Westbury, NY, USA) were used to improve the separation of the organic and inorganic phases. After extraction, RNA concentrations were estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For microarray experiments, RNA was further purified through Qiagen RNeasy columns (Qiagen, Valencia, CA, USA), and final quality was determined by a combination of spectrophotometry (NanoDrop ND-1000) and gel electrophoresis (BioAnalyzer 2100, Agilent Technologies, Palo Alto, CA, USA).

Microarrays

Microarray labelling and hybridization procedures followed closely those described by Zou *et al*. (2005). RNA from three independent experiments was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen) in the presence of aminoallyl-dUTP. Labelled cDNA was purified (Qiagen) and quantified (NanoDrop ND-1000) to assess the adequate incorporation of the dye necessary for microarray analysis. Samples were mixed in pairs according to an interconnecting loop design (Fig. 1), suspended in hybridization buffer and applied onto the corresponding microarray slides. The oligo (70-mer) slides representing approximately 38 000 soybean transcripts (Gonzalez and Vodkin, 2007) were incubated within a sealed hybridization chamber submerged in a water bath in the dark at 42 °C for 48 h. After incubation, a series of washes was performed on the slides to remove non-binding probe (Zou *et al*., 2005), and median intensity values were determined by GenePix 5.0 (Axon, Milpitis, CA, USA) and normalized using maanova/R. Analysis of variance (ANOVA) was run on the normalized intensity values to assess the significance of the calculated differential expression, and the statistical model included the array as a random effect, and genotype and treatment as fixed effects (Calla *et al*., 2009).

RNA-seq

Total RNA from the same samples as used in the OA infiltration microarray study was digested with DNAse I (Ambion, Austin, TX, USA) and purified with Qiagen RNeasy columns as described above. RNA samples were quantified and assessed for quality using a BioAnalyzer 2100 (Agilent Technologies). One microgram of each sample (i.e. OA pH 2.4 infiltration of OxO, OA pH 2.4 infiltration of AC, H_2O pH 2.4 infiltration of OxO and H_2O

pH 2.4 infiltration of AC) was deep sequenced, using single end reads with the 'sequencing-by-synthesis' (SBS) technology at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign, IL, USA. At the sequencing facility, cDNA libraries from each of the samples were constructed, quantified by quantitative polymerase chain reaction (qPCR), pooled in equimolar concentrations in pools of two samples, sequenced on eight lanes (two samples per lane) for 100 cycles on a HiSeq2000 using a TruSeq SBS sequencing kit version 2, and analysed with the Illumina Pipeline version 1.8 (Illumina, San Diego, CA, USA). An error rate using the phiX DNA was calculated as ~1%. Raw reads of 100 bases were then processed by trimming off the adapters and aligning to the soybean genome using commercial services provided by Data2Bio (http://www.Data2Bio.com). In this pipeline, nucleotides of each raw read were scanned for low-quality bases with a PHRED quality value of <15 (out of 40) and then aligned in the soybean genome (Schmutz *et al*., 2010) release 1.0 available in Phytozome v 7.0 (http://www.phytozome.net. Reads were normalized by calculating RPKM, which accounts for gene and library size. In addition, $\log_2$ ratios between reads on different libraries were calculated for the relative assessment of changes in abundance. Furthermore, the data were filtered for coding sequences having at least four counts in at least one of the four samples sequenced. Counts of value zero were changed to unity to allow for the calculation of approximate ratios.

Arabidopsis iron infiltration

Four-week-old *Arabidopsis thaliana* ecotype Columbia plants, grown in Sunshine Mix LC1 (SunGro, Vancouver, BC, Canada) with a 13-h photoperiod and at 22/20 °C day/night temperatures, were hand infiltrated with 1%, 0.1% or 0.01% (w/v) solution of $FeSO_4$ or $FeCl_3$ on two individual leaves using a 1-mL syringe without a needle. One of the infiltrated leaves was immediately inoculated with a 5-mm-diameter potato dextrose agar (PDA) plug containing an overnight, actively growing culture of the *S. sclerotiorum* Oah$^-$ mutant (kindly provided by J. Rollins). One non-infiltrated leaf was also inoculated with the same mutant. In this manner, three treatments were applied to the same plant: infiltrated, infiltrated and inoculated, and inoculated only (without infiltration). Each of the six different iron treatments was repeated twice, for a total of 12 plants in the whole experiment. Immediately after inoculation, the plants were returned to the growth chamber where the humidity was raised to 100%.

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DISCLAIMER

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

REFERENCES

Allen, L.J., MacGregor, K.B., Koop, R.S., Bruce, D.H., Karner, J. and Brown, A.W. (1999) The relationship between photosynthesis and a mastoparan-induced hypersensitive response in isolated mesophyll cells. *Plant Physiol*. **119**, 1233–1241.

Bataille, P. and Fournier, A. (2001) Calcium supply in calcium lithiasis. *Med. Nutr.* **37**, 9–12.

Briat, J.F., Lobréaux, S., Grignon, N. and Vansuyt, G. (1999) Regulation of plant ferritin synthesis: how and why. *Cell. Mol. Life Sci*. **56**, 155–166.

Calla, B., Vuong, T., Radwan, O., Hartman, G.L. and Clough, S.J. (2009) Gene expression profiling soybean stem tissue early response to *Sclerotinia sclerotiorum* and *in silico* mapping in relation to resistance markers. *Plant Genome*, **2**, 149–166.

Calla, B., Blahut-Beatty, L., Koziol, L., Zhang, Y., Neece, D.J., Carbajulca, D., Garcia, A., Simmonds, D.H. and Clough, S.J. (2014) Genomic evaluation of oxalate-degrading transgenic soybean in response to *Sclerotinia sclerotiorum* infection. *Mol. Plant Pathol*. in press. Available at 10.1111/mpp.12115.

Cessna, S.G., Sears, V.E., Dickman, M.B. and Low, P.S. (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *Plant Cell*, **12**, 2191–2200.

Coumo, C., Dickman, M.B., Kohn, L. and Rolling, J. (2005) *Sclerotinia Sclerotiorum Sequencing Project*. Cambridge, MA: Broad Institute of Harvard and MIT.

Donaldson, P.A., Anderson, T., Lane, B.G., Davidson, A.L. and Simmonds, D.H. (2001) Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum*. *Physiol. Mol. Plant Pathol.* **59**, 297–307.

Favaron, F., Sella, L. and D'Ovidio, R. (2004) Relationships among endo-polygalacturonase, oxalate, pH, and plant polygalacturonase-inhibiting protein (PGIP), in the interaction between *Sclerotinia sclerotiorum* and soybean. *Mol. Plant–Microbe Interact*. **17**, 1402–1409.

Fu, X., Fu, N., Guo, S., Yan, Z., Xu, Y., Hu, H., Menzel, C., Chen, W., Li, Y., Zeng, R. and Khaitovic, P. (2009) Estimating accuracy of RNA-Seq and microarrays with proteomics. *BMC Genomics*, **10**, 161.

Godoy, G., Steadman, J.R., Dickman, M.B. and Dam, R. (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiol. Mol. Plant Pathol*. **37**, 179–191.

Gonzalez, D. and Vodkin, L. (2007) Specific elements of the glyoxylate pathway play a significant role in the functional transition of the soybean cotyledon during seedling development. *BMC Genomics*, **8**, 468.

Guimaraes, R.L. and Stotz, H.U. (2004) Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiol*. **136**, 3703–3711.

Harrison, P.M. and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta*, **1275**, 161–203.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.

Kim, K.S., Min, J.-Y. and Dickman, M.B. (2008) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol. Plant–Microbe Interact.* **21**, 605–612.

Laulhere, J.P. and Briat, J.F. (1993) Iron release and uptake by plant ferritin: effects of pH, reduction and chelation. *Biochem. J.* **290**, 693–699.

Lawen, A. and Lane, D.J.R. (2013) Mammalian iron homeostasis in health and disease: uptake, storage, transport, and molecular mechanisms of action. *Antioxid. Redox Signal*. **18**, 2473–2507.

Macur, R.E., Olsen, R.A. and Inskeep, W.P. (1991) Photochemical mobilization of ferritin iron. *Plant Soil*, **130**, 69–74.

Marciano, P., Di Lenna, P. and Magro, P. (1983) Oxalic acid, cell wall-degrading enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. *Physiol. Plant Pathol*. **22**, 339–345.

Marioni, J.C., Mason, C.E., Mane, S.M., Stephens, M. and Gilad, Y. (2008) RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. *Genome Res*. **18**, 1509–1517.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. and Wold, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods*, **5**, 621–628.

Oshlack, A. and Wakefield, M. (2009) Transcript length bias in RNA-seq data confounds systems biology. *Biol. Direct*, **4**, 14.

Ravet, K., Touraine, B., Boucherez, J., Briat, J.-F., Gaymard, F. and Cellier, F. (2009) Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis. *Plant J.* **57**, 400–412.

Riou, C., Freyssinet, G. and Fevre, M. (1991) Production of cell wall-degrading enzymes by the phytopathogenic fungus *Sclerotinia sclerotiorum*. *Appl. Environ. Microbiol*. **57**, 1478–1484.

Ross, P.H. and Jakubowski, N. (2011) Cytochromes—fascinating molecular machines. *Metallomics*, **3**, 316–318.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W. *et al*. (2010) Genome sequence of the palaeopolyploid soybean. *Nature*, **463**, 178–183.

Torti, F.M. and Torti, S.V. (2002) Regulation of ferritin genes and protein. *Blood*, **99**, 3505–3516.

Williams, B., Kabbage, M., Kim, H.-J., Britt, R. and Dickman, M.B. (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathog.* **7**, e1002107.

Zou, J., Rodriguez-Zas, S., Aldea, M., Li, M., Zhu, J., Gonzalez, D. *et al*. (2005) Expression profiling soybean response to *Pseudomonas syringae* reveals new defense-related genes and rapid HR-specific downregulation of photosynthesis. *Mol. Plant–Microbe Interact.* **18**, 1161–1174.

SUPPORTING INFORMATION

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

Fig. S1 Effects of transcript length on the estimated differential expression by RNA-seq compared with microarray. Longer transcripts showed higher agreement between the two platforms. AC, AC Colibri parental soybean line; OA, oxalic acid; OxO, transgenic soybean line expressing oxalate oxidase.

Fig. S2 Response of *Arabidopsis thaliana* to infiltration with 0.1% $FeSO_4$ and/or inoculation with actively growing mycelia of *Sclerotinia sclerotiorum* Oah^- mutant. Photographed 25 days after treatment immediately after leaves were detached. Three independent replications (plants) are shown.

Table S1 List of 2390 sequences significantly changing in abundance between the transgenic and parent plants, or between treatments, at overall *F*-test *P* values of <0.001 for genotype, treatment or genotype-by-treatment.

Table S2 List of 1054 significantly differentially expressed genes as determined by overall *F*-test *P* values of <0.001, and that also had at least one specific pairwise *t*-test false discovery rate (FDR)-corrected *P* value of <0.001

Table S3 List of 1045 genes that showed a change in expression as a result of a combination of oxalic acid (OA) and its low pH, as seen when comparing leaves infiltrated with OA pH 2.4 versus water at pH 5.5.

Table S4 List of the 936 predicted genes selected by RNA-seq analysis as having the greatest changes in abundance between plant lines or between treatments by comparisons of fold change of reads per kilobase per million (RPKM). Data were filtered by selecting those reads having differences greater or equal to eight-fold, or smaller or equal to 1/8-fold (log_2 of 3 or −3); reads having less than 10 raw counts in all four libraries were removed from the analysis.

Table S5 List of 103 genes related to iron metabolism selected from the RNA-seq data using keyword searches.