Annals of Ecology and Environmental Science
Volume 1, Issue 1, 2017, PP 16-26

Mitochondrial Complex 1is Important for Plant Tolerance to Fungal Biotic Stress

Alex Williams[1], Jingfang Hao[2], Moaed Al Meselmani[3], Rosine De Paepe[2], Bertrand Gakière[2], Pierre Pétriacq[1,4],

[1]Department of Animal and Plant Sciences, Univ. of Sheffield, S10 2TN Sheffield, United Kingdom

[2]Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Univ. Paris-Sud, Univ. Evry, Univ. Paris-Diderot, Univ. Paris-Saclay, Bâtiment 630, Rue Noetzlin, Gif-sur-Yvette cedex, France.

[3]Department of Molecular Biology and Biotechnology, Univ. of Sheffield, United Kingdom

[4]biOMICS Facility, Department of Animal and Plant Sciences, Univ. of Sheffield, S10 2TN Sheffield, United Kingdom

***Corresponding Author**: Dr Pierre Pétriacq, biOMICS Facility, Department of Animal and Plant Sciences, Univ. of Sheffield, S10 2TN Sheffield, United Kingdom*

ABSTRACT

Environmental constraints, such as biotic stress, are detrimental for plant productivity, survival and reproduction. Although plants have evolved metabolic mechanisms to tolerate environmental challenges, our knowledge on the importance of mitochondrial metabolism in biotic stress responses is still fragmentary. This study examined the effects of mutations in mitochondrial complex I (CI) and determined major stress-responsive metabolites associated with decreased tolerance to fungal infection. Using the pathosystem Arabidopsis thaliana-Plectosphaerella cucumerina, we demonstrated that the loss of CI function dramatically increased susceptibility to the necrotrophic pathogen. During infection, metabolomics analysis revealed that CI dysfunction caused a profound reorchestration of plant metabolism, including defence pathways. This metabolomics study demonstrates a clear role for mitochondrial CI function in tolerance to environmental biotic stress.

Keywords:*Environmental stress, mitochondria, Arabidopsis thaliana, metabolomics, Plectosphaerella cucumerina, plant immunity*

INTRODUCTION

In their environment, plants constantly face stress inducing adverse conditions, such as biotic infections (*e.g.* fungi, bacteria, pests) or abiotic constraints (*e.g.* drought, flood, heat, cold), which consequently reduce plant growth and yields. However, plant metabolism is particularly plastic and reactive, permitting acclimation to environmental fluctuations, and requires a new state of biochemical homeostasis, attained by a fine tuning of cellular processes and metabolic pools [1]. In this context, mitochondria not only house the important biochemical reactions involved in energy production in plants, but also participate in reduction/oxidation (redox) processes, which are pivotal for cellular signalling [2]. The main mitochondrial electron transport chain (mtETC) comprises four complexes (CI-CIV) and couples electron transfer to proton translocation through the inner mitochondrial membrane [3]. In addition, plants possess alternative respiratory routes, alternative NADP(H) dehydrogenases and alternative oxidase (AOX), which are not involved in energy production but allow viability of mutants of enzymes of the main pathway [4].

CI, CII and CIII are important for production of mitochondrial ROS in plants, redox homeostasis and stress responses [5–7]. Mutations affecting CII have been implicated in the reduction of transcripts associated with the defence signalling hormone salicylic acid (SA), and impart a greater susceptibility to both (hemi) biotrophic and necrotrophic pathogens[8]. In addition, mitochondrial ROS production via NAD signalling (*i.e.* a redox and stress signal [9]) leads to resistance against various pathogens [10],

which further suggests an important role for mitochondrial ROS in biotic stress responses. CI, (*i.e.* NADH: ubiquinone oxidoreductase; EC 1.6.5.3) comprises more than 40 subunits [11], and has received considerable attention regarding its role in plant response to biotic and abiotic stresses [12–16]. However, the impact of CI dysfunction on necrotrophic fungal stress is unknown.

Plectosphaerella (*P.*) *cucumerina* is a pathogenic ascomycete, which can strive on dead/decomposing plant tissues, saprophytically survive in soil and infect *Arabidopsis thaliana* (Arabidopsis) and several crops. When *P. cucumerina* is droplet-inoculated onto Arabidopsis leaves, the infection develops typical necrotrophic symptoms [17]. To resist *P. cucumerina*, Arabidopsis deploys an arsenalof chemical responses ranging from phytohormones, glucosinolates and other large-scale metabolic alterations [17–20]. In this study, we demonstrate that the disease phenotypes by *P. cucumerina* infection drastically intensify when CI function is impaired in Arabidopsis. Based on mass spectrometry analysis of metabolic markers, we provide evidence that the fungal infection causes large perturbations in Arabidopsis metabolome in response to loss of CI function. Our study has unveiled a new role for mitochondrial CI in adaptation to necrotrophic fungal stress.

Experimental Procedures

Plant Cultivation and Growth Conditions

TheArabidopsis wild-type accession Col-0 (WT) was used along with Col-0 double mutant line *ndufs8.1 ndufs8.2* (referred to as *23*) and single mutant *ndufs4* (provided by E.H. Meyer and referred to as *18*)[14,16]. Both mutants are disrupted in genes encoding subunits located in the peripheral arm of CI and display a reduced growth rate in short-day (SD) conditions [16]. Plants were grown in controlled SD conditions (8.5:15.5 h light:dark, 20:18 °C light:dark; 70% relative humidity, and 120 µmol photon m^2 s^{-1}). To analyse WT and mutant plants of similar developmental and physiological stage, seeds of *18* mutant were sown 1 week before *23* mutant, which was delayed by 1 week with WT Col-0 seeds [16]. Hence, plants of *18*, *23*, and WT were grown for 6, 5 and 4 weeks, respectively.

Pathogenicity Assay

Inoculation of *P. cucumerina* was performed by droplet (10^6 spores mL^{-1}), and disease progression was determined as lesion diameter together with microscopic observation of hyphal colonization by Trypan Blue staining [17]. Statistical differences in lesion diameter (n = 6, +/- SEM) were assessed using Student's *t*-test ($P < 0.05$ or 0.01) in Microsoft Excel.

Metabolomics

All chemicals were of analytical grade (Sigma-Aldrich, UK). Untargeted metabolic profiling by Ultra Pressure Liquid Chromatography-Quadrupole-Time Of Flight-Mass Spectrometry (UPLC-Q-TOF-MS) were performed as detailed previously [10]. Briefly, each individual sample (n = 4) consisted of 4 pooled leaves from different plants of WT, *23* or *18* genotype, which were mock-inoculated (water) or *P. cucumerina* infected (10^6 spores mL^{-1}). Samples were collected at 13 DPI, flash-frozen in liquid nitrogen, freeze-dried and stored at -80 ˚C until methanol extractions were undertaken [10]. Multivariate analysis of metabolomics data (37,522 *m/z*) was conducted in MetaboAnalyst v.3 (www.metaboanalyst.ca) using interquartile range filtering, median normalisation, cube-root transformation and Pareto scaling, after which PCA and HCA were constructed. Univariate analysis was performed using MarVis v.1 (marvis.gobics.de) to filter by ANOVA (P< 0.01) with a Benjamini-Hochberg correction for false discovery rate (FDR), yielding 1,405 significant metabolic markers. Using this filtered selection, binary comparisons between mock-inoculated or *P. cucumerina*-infected tissues were conducted in MeV v.4 (mev.tm4.org), using Student's *t*-tests (P< 0.01). Venn diagrams were constructed online (bioinformatics.psb.ugent. be/webtools/Venn). The resulting common markers for *P. cucumerina*- infected *23* and *18* mutants were identified putatively from their accurately detected *m/z* using METLIN (metlin.scripps.edu) and PubChem (pubchem. ncbi.nlm.nih.gov) online chemical databases.

Results

Loss of Mitochondrial CI in Arabidopsis Drastically Decreases Tolerance to the Fungal Pathogen P. cucumerina

Arabidopsis wild-type Col-0 plants (WT), and two independent CI mutant lines, the double mutant *ndufs8.1 ndufs8.2* (*23* [16]), and the single mutant *ndufs4* (*18* [14]), of similar developmental stage were challenged by droplet-inoculation of *P. cucumerina*. Markedly, disease lesions by 8

days of *P. cucumerina* infection were more severe in the *23* and *18* mutant lines as compared to WT (Fig. 1A). This correlated with increased staining of fungal hyphae and associated cell death at 8 DPI (Fig. 1B). Measured at 8 and 13 DPI, lesion diameters confirmed quantitative differences in disease severity between genotypes with (WT) or without a functional CI (*23* and *18*; Fig.1C), and suggested a bigger impact of *ndufs4* genotype over *ndufs8.1 ndufs8.2* genotype. Hence, our results indicate that CI dysfunction in Arabidopsis favours fungal colonization by *P. cucumerina*.

Impacts of CI Dysfunction on the Metabolic Pools during Fungal Infection

To get further insight into the metabolic mechanisms underlying the hyper-susceptibility of CI mutants to *P. cucumerina*, we conducted an untargeted metabolic profiling by UPLC-Q-TOF-MS (see Experimental procedures) from leaf tissues (n = 4) sampled at 13 days after mock inoculation (Mock) or *P. cucumerina* infection (Plecto). This metabolomics method allows the detection of changes in metabolites involved in plant-pathogen interactions [10]. Chemical signals were acquired in negative electrospray ionisation (ESI^-), yielding 37,522 detected ions (m/zratios). Global impacts of the genotypes/inoculations on the metabolic pools were displayed by Principal Component Analysis (PCA) showing the maximal variance across the first two components (PC1 and PC2; Fig. 2A). PCA indicated separation between mock-inoculated and *P. cucumerina*-infected samples (Fig. 2A). This infection effect was drastic for both CI mutants, as exemplified by a distant separation of *23*-Plecto and *18*-Plecto conditions on the plots. Furthermore, both infected mutants showed partial overlap. This was also confirmed by a clustering analysis (Pearson's correlation, average clustering) showing relationships between samples (Fig. 2B). While WT-Mock, WT-Plecto and *18*-Mock clearly clustered alone, *23*-Plecto and *18*-Plecto showed overlap, as for WT-Mock and *23*-Mock. This suggests that *23* mutant produces an intermediate phenotype between WT and *18*, which is coherent with the disease severity observed (Fig. 1C). Hence, multivariate analysis of metabolomics signatures indicate a metabolic reprogramming caused by the loss of CI function during *P. cucumerina* infection.

Next, a univariate statistical approach was adopted to identify putative markers that might explain the metabolic trends displayed in Fig. 2. The entire dataset was filtered using ANOVA (P <0.01) followed by a false discovery rate correction (FDR) to remove false positives. The resulting subset of 1,405 metabolic markers was used for binary comparisons between mock-inoculation and *P. cucumerina*-infection for each genotype (Student's t-test, P< 0.01). The three lists obtained for WT, *23* and *18* plants were analysed using a Venn diagram, thereby displaying quantitative differences for the specific and overlapping stress-responsive metabolic markers (Fig. 3A). While infection by *P. cucumerina* significantly caused quantitative changes in metabolic pools, *23* and *18* mutants showed a stronger response (190 and 303 markers, respectively) as compared to WT (121 markers). There was little overlap for WT *vs23* and WT *vs18* (27 and 29 markers, respectively). Remarkably, a larger overlap of 105 markers was observed between *23* and *18*, thus indicating common stress-responsive markers that were affected in two independent CI mutant genotypes. These 105 markers were further visualised for their intensities by a bi-dimensional clustering (48 up-regulated and 57 down-regulated markers; Fig. 3B), and putatively identified based on their accurately detected *m/z* using METLIN and PubChem chemical database [10,17,21–23]. Pie-charts in Fig. 3C show predicted pathways for up- and down-regulated markers (details are given in Table 1). Up-regulated compounds included secondary metabolites such as flavonoids (13%), terpenoids (8%), polyphenols (4%) and alkaloids (2%), and primary metabolites such as lipids (23%), amino acids (4%), organic acids (4%) and nucleotides (2%). Similar classes of metabolites were observed among down-regulated compounds including lipids (25%), flavonoids (23%), terpenoids (9%), nucleotides (5%), amino acids 5%), organic acids (4%) and alkaloids (4%). Hence, this suggests a re-adjustment of metabolites from central metabolism (lipids, nucleotides, amino and organic acids), and compounds important for stress signalling (flavonoids, polyphenols, terpenoids and alkaloids). Altogether, our results indicate that decreased tolerance to fungal biotic

stress in response to loss of CI function is underpinned by large-scale metabolic alterations.

Table1. *Putative identification of stress- responsive metabolic markers.*

Regulation[1]	*P* value[2]	RT (min)[3]	Detected *m/z*[3]	*Predicted adducts*[4]	Predicted mass[4]	*Error (ppm)*[4]	Putative Compound[4]	Predicted Formula[4]	Putative Pathway[5]
UP	1.5E-07	3.2	347.135	*[M-H]-*	348.147	*14*	Serpentine	C21H20N2O3	Alkaloids
	6.5E-07	3.1	250.063	*[M-H]-*	251.079	*36*	N-Carboxyacetyl-D-phenylalanine	C12H13NO5	Amino acids
	2.9E-05	1.3	152.010	*[M-H]-*	153.010	*50*	3-Sulfino alanine	C3H7NO4S	Amino acids
	3.7E-04	1.9	693.193	*[M-H]-*	694.175	*37*	Pelargonidin 3-(6"-succinylglucoside)-5-glucoside	C31H34O18	Flavonoids
	2.8E-07	0.6	1105.086	*[M-H]-*	1106.108	*13*	Syzyginin A	C48H34O31	Flavonoids
	1.2E-07	1.3	287.066	*[M-H]-*	288.063	*34*	Eriodictyol	C15H12O6	Flavonoids
	5.3E-08	3.5	459.108	*[M-H]-*	460.101	*32*	Apigenin 7-(6"-methylglucuronide)	C22H20O11	Flavonoids
	7.0E-05	1.4	345.135	*[M-H]-*	346.142	*1*	Catechin tetramethylether	C19H22O6	Flavonoids
	2.4E-06	6.8	492.110	*[M-H]-*	493.135	*35*	Malvidin 3-galactoside	C23H25O12	Flavonoids
	3.4E-05	2.5	463.465	*[M-H]-*	464.459	*27*	Pentadecyl oleate	C31H60O2	Lipids
	2.4E-03	0.6	1067.832	*[M+Cl]-*	1032.909	*42*	Triacylglycerol	C68H120O6	Lipids
	2.8E-07	5.6	1011.546	*[M-H]-*	1012.529	*24*	Phosphoinositide phosphate	C48H86O18P2	Lipids
	7.3E-08	5.7	820.457	*[M+Na-2H]-*	799.515	*40*	Phosphocholine	C46H74NO8P	Lipids
	1.1E-07	5.7	836.444	*[M+Cl]-*	801.458	*19*	Phosphoserine	C44H68NO10P	Lipids
	2.8E-08	8.1	597.436	*[M-H]-*	598.460	*27*	Diacylglycerol	C38H62O5	Lipids
	1.5E-05	7.6	621.311	*[M-H]-*	622.312	*10*	Phosphoinositol	C29H51O12P	Lipids
	8.0E-06	8.0	383.350	*[M-H]-*	384.339	*47*	Vitamin D3	C27H44O	Lipids
	1.3E-05	8.1	656.407	*[M-H]-*	657.437	*34*	Phosphoethanolamine	C35H64NO8P	Lipids
	7.3E-06	9.0	870.471	*[M+Cl]-*	835.515	*15*	Phosphoethanolamine	C49H74NO8P	Lipids
	1.2E-04	8.1	532.365	*[M-H]-*	533.348	*45*	Phosphoethanolamine	C27H52NO7P	Lipids
	2.5E-06	2.4	814.290	*[M-H2O-H]-*	833.275	*41*	UDP-3-O-(3-hydroxytetradecanoyl)-N-acetylglucosamine	C31H53N3O19P2	Nucleotides
	2.6E-07	2.0	209.045	*[M-H]-*	210.053	*2*	5-Hydroxyferulate	C10H10O5	Organic acids
	1.6E-08	3.5	115.002	*[M-H]-*	116.011	*14*	Fumaric acid	C4H4O4	Organic acids
	7.3E-06	0.6	1061.118	*[M+Cl]-*	1026.134	*13*	Camelliatannin F	C48H34O26	Polyphenols
	7.5E-05	8.3	667.035	*[M+Cl]-*	632.065	*0*	Vescalin	C27H20O18	Polyphenols
	2.8E-06	5.5	1153.610	*[M+FA-H]-*	1108.603	*7*	Ginsenoside Rb1	C54H92O23	Terpenoids
	8.6E-09	5.9	476.254	*[M-H]-*	477.252	*20*	1'-O-Acetylpaxilline	C29H35NO5	Terpenoids
	9.8E-06	8.4	565.449	*[M-H]-*	566.449	*13*	Anhydrorhodovibrin	C41H58O	Terpenoids
	3.1E-06	2.1	433.198	*[M-H]-*	434.194	*25*	Melledonol	C23H30O8	Terpenoids
	8.8E-04	1.9	778.131						Unknown
	4.6E-07	5.7	558.745						Unknown
	2.8E-07	0.6	303.950						Unknown
	6.4E-06	1.9	1048.094						Unknown
	5.8E-08	1.3	266.931						Unknown
	9.8E-08	5.7	819.454						Unknown
	8.6E-09	9.0	121.896						Unknown
	1.1E-07	2.5	1162.165						Unknown
	2.5E-06	9.7	987.310						Unknown
	5.9E-06	3.5	500.986						Unknown
	1.6E-05	9.0	119.900						Unknown
	5.0E-07	7.6	687.004						Unknown
	2.7E-07	2.3	896.231						Unknown
	1.0E-04	2.5	1073.207						Unknown
	1.1E-05	4.5	172.216						Unknown
	2.6E-04	9.0	132.905						Unknown
	4.0E-03	0.6	754.953						Unknown
	5.6E-03	2.5	467.663						Unknown
	7.4E-03	5.5	1100.489						Unknown
DOWN	1.3E-04	10.0	431.220	*[M-H]-*	432.231	*9*	Usambarensine	C29H28N4	Alkaloids
	5.8E-03	2.3	312.172	*[M-H]-*	313.189	*30*	Heliotrine	C16H27NO5	Alkaloids
	2.2E-07	8.4	736.137	*[M+Na-2H]-*	715.173	*14*	N2-(ADP-D-Ribosyl)-L-arginine	C21H35N9O15P2	Amino acids
	2.1E-04	2.2	407.049	*[M-H2O-H]-*	426.088	*50*	Cysteine glutathione disulfide	C13H22N4O8S2	Amino acids
	1.2E-03	5.9	435.163	*[M-H]-*	436.163	*15*	Nap-Leu-OH	C24H24N2O6	Amino acids
	6.5E-07	7.9	269.294	*[M-H]-*	270.292	*33*	3S,7S-dimethyl-hexadecan-2-ol	C18H38O	Flavonoids
	1.1E-05	1.6	809.209	*[M-H]-*	10.210	*7*	Catechin pentabenzoate	C50H34O11	Flavonoids
	7.9E-05	1.7	671.187	*[M+Cl]-*	636.205	*18*	Linoside B	C30H36O15	Flavonoids
	1.0E-04	2.3	649.169	*[M-H]-*	650.185	*12*	Hesperetin 3',7-O-diglucuronide	C30H34O16	Flavonoids
	1.1E-04	2.5	539.218	*[M-H]-*	540.215	*19*	Leucadenone A	C33H32O7	Flavonoids
	4.6E-04	10.0	829.192	*[M+Cl]-*	794.263	*49*	Epimedoside D	C37H46O19	Flavonoids
	4.7E-04	1.6	553.057	*[M+Na-2H]-*	532.085	*5*	Delphinidin-3-O-glucoside pyruvic acid	C24H20O14	Flavonoids
	7.1E-04	6.8	767.368	*[M+CH3COO]-*	708.336	*24*	Scillipheosidin 3-[glucosyl-(1->2)-rhamnoside]	C36H52O14	Flavonoids
	8.3E-04	7.9	1179.335	*[M-H]-*	1180.291	*43*	Cyanidin 3-(6"-p-coumaryl-2"'-sinapylsambubioside)-5-(6-malonylglucoside)	C55H56O29	Flavonoids
	1.7E-03	8.1	253.092	*[M-H2O-H]-*	272.105	*21*	7,3'-Dihydroxy-4'-methoxyflavan	C16H16O4	Flavonoids

	2.3E-03	6.0	627.177	*[M-2H]2-*	1256.343	*20*	Cyanidin 3-O-[2"-O-(2'"-O-(sinapoyl) xylosyl) 6"-O-(p-O-(glucosyl) p-coumaroyl) glucoside] 5-O-glucoside	C58H64O31	Flavonoids
	6.0E-03	6.5	1195.333	*[M-H]-*	1196.286	*45*	Cyanidin 3-(6"-caffeyl-2'"-sinapylsambubioside)-5-(6-malonylglucoside)	C55H56O30	Flavonoids
	6.6E-03	9.0	677.153	*[M-H]-*	678.180	*28*	Kaempferol 3-(4",6"-diacetylglucoside)-7-rhamnoside	C31H34O17	Flavonoids
	1.4E-04	5.5	566.235	*[M+Na-2H]-*	545.266	*9*	N-[(3a,5b,7a,12a)-3,12-dihydroxy-24-oxo-7-(sulfooxy)cholan-24-yl]-Glycine	C26H43NO9S	Lipids
	7.6E-06	7.2	621.310	*[M-H]-*	622.312	*8*	Phosphoinositol	C29H51O12P	Lipids
	1.0E-05	8.1	474.286	*[M-H]-*	475.270	*49*	Lyso phosphoethanolamine	C23H42NO7P	Lipids
	4.9E-05	5.4	811.428	*[M+Na-2H]-*	790.479	*30*	Phosphoglycerol	C44H71O10P	Lipids
	1.3E-04	4.6	505.256	*[M-H]-*	506.262	*2*	Didehydrovitamin D3	C27H36F6O2	Lipids
	3.1E-04	1.6	808.487	*[M-H]-*	809.500	*6*	Phosphoethanolamine	C47H72NO8P	Lipids
	7.4E-04	4.2	314.253	*[M-H]-*	630.522	*2*	Diacylglycerol	C40H70O5	Lipids
	1.0E-03	1.7	473.283	*[M-H2O-H]-*	492.322	*42*	Phosphatidic acid	C25H49O7P	Lipids
	1.0E-03	8.0	429.455	*[M-H]-*	430.454	*19*	9Z,15Z,22Z-hentriacontatriene	C31H58	Lipids
	1.1E-03	1.6	507.295	*[M-H]-*	508.280	*43*	Phosphoglycerol	C24H45O9P	Lipids
	2.0E-03	9.0	482.282	*[M-H]-*	483.296	*14*	Phosphoserine	C22H46NO8P	Lipids
	2.8E-03	3.1	848.526	*[M-H]-*	849.552	*22*	Phosphoserine	C47H80NO10P	Lipids
	5.8E-03	5.8	499.363	*[M-2H]2-*	1000.752	*11*	Triacylglycerol	C67H100O6	Lipids
	7.6E-03	4.8	232.825	*[M-3H]3-*	701.500	*3*	Phosphocholine	C38H72NO8P	Lipids
	5.2E-05	7.7	668.063	*[M-H2O-H]-*	687.038	*64*	UDP-N-acetyl-D-galactosamine 4-sulfate	C17H27N3O20P2S	Nucleotides
	1.7E-03	3.7	543.961	*[M+Na-2H]-*	522.991	*7*	Guanosine triphosphate	C10H16N5O14P3	Nucleotides
	5.4E-03	1.7	622.079	*[M+Na-2H]-*	601.082	*35*	O-acetyl-ADP-ribose	C17H25N5O15P2	Nucleotides
	1.7E-04	2.2	977.189	*[M-H2O-H]-*	996.223	*16*	1-O-Galloylfructose	C39H48O30	Organic acids
	2.6E-04	2.1	536.040	*[M+Na-2H]-*	515.046	*37*	3-(ADP)-glycerate	C13H19N5O13P2	Organic acids
	1.5E-06	4.7	393.228	*[M-H2O-H]-*	412.246	*0*	Grayanotoxin	C22H36O7	Terpenoids
	5.3E-04	2.3	549.270	*[M-H]-*	550.278	*0*	Aspecioside	C29H42O10	Terpenoids
	1.4E-03	1.7	299.201	*[M-H]-*	300.209	*2*	Retinoic acid	C20H28O2	Terpenoids
	1.5E-03	1.6	475.306	*[M-H]-*	476.314	*1*	(-)-Asbestinine 2	C28H44O6	Terpenoids
	6.9E-03	1.7	713.368	*[M-H]-*	714.373	*3*	Delavaine A	C38H54N2O11	Terpenoids
	5.9E-06	8.5	678.978						Unknown
	8.7E-06	4.8	722.369						Unknown
	1.2E-04	6.0	163.851						Unknown
	1.2E-04	10.0	161.850						Unknown
	1.7E-04	6.0	419.012						Unknown
	3.6E-04	6.3	127.870						Unknown
	7.1E-04	6.0	97.865						Unknown
	1.0E-03	7.8	316.804						Unknown
	1.2E-03	2.3	353.468						Unknown
	1.4E-03	8.9	929.162						Unknown
	2.8E-03	5.7	863.820						Unknown
	3.3E-03	9.0	889.147						Unknown
	4.5E-03	1.6	914.145						Unknown
	5.1E-03	1.7	588.004						Unknown
	6.3E-03	1.6	877.823						Unknown

[1]*Markers showing an up- or down-regulation in P. cucumerina-infected 23 and 18 mutants.*

[2]*P values are derived from ANOVA followed by false discovery rate correction (Benjamini-Hochberg).*

[3]*Retention times (RT) and accurate m/z values, detected by UPLC-Q-TOF-MS in negative ion mode.*

[4]*Predicted parameters from the METLIN chemical database using the accurately detected m/z.*

[5]*Putative metabolites and their corresponding pathways were validated by information from the PubMed chemical database.*

DISCUSSION

Mitochondria sustain energy by generating cellular ATP through oxidative phosphorylation, and house a major site of ROS produced by the mtETC [6,24]. Accordingly, several studies have suggested a role for plant mitochondria in response to biotic stress by modulating redox signalling and energy demand, including CII, alternative oxidases and other mitochondrial components [7,8,10,25,26]. The case of CI is slightly different as alternative NAD(P) dehydrogenases could compensate the loss of CI function in plants [27]. Nonetheless, CI dysfunction causes altered redox perturbations that affect responses to plant stresses [12–15]. In the present study, we have used two Arabidopsis mutant lines impaired in CI assembly/activity (*ndufs8.1 ndufs8.2* (*23*) and *ndufs4* (*18*) [14,16]). We

observed greater disease severity to the necrotrophic pathogen *P. cucumerina* in both mutants, compared to the WT (Fig. 1). Interestingly, *18* mutant, which had a more dramatic CI mutant phenotype than *23* in SD condition [16], *i)* was also more susceptible to *P. cucumerina* than *23*, and *ii)* showed a greater metabolic impact during infection than *23* (190 *vs* 303 markers, Fig. 3A). This demonstrates that increased disease severity to the fungal pathogen positively correlates with the severity of the CI mutant phenotype. This concurs with previous results showing higher susceptibility to fungal pathogen for CII mutant impaired in mitochondrial respiration [8].

For decades now, plant metabolomics studies have been used reliably to assess the physiological status of plant cells under particular stress conditions [28]. Typically, plants produce a battery of primary metabolites, crucial for central metabolism (*e.g.* amino and organic acids, sugars, lipids) and necessary to sustain normal growth and development. In addition, more chemically complex secondary metabolites, such as flavonoids, phytohormones (*e.g.* polyphenols, terpenoids) or alkaloids, are usually stress-responsive compounds. Production of secondary metabolites is effective at managing pathogenic microbes but also has a high energy demand [29]. Given the importance of mitochondrial respiration in providing energy to the cell, impairment in mtETC could plausibly lead to altered metabolic pools, as previously reported [12,14–16,30]. Here, multivariate statistical analysis of metabolomics data features revealed a greater impact of the infection on both CI mutants, with *18* being more affected than *23* (Fig. 2). This lead to the investigation of quantitative differences in metabolic markers that were common for both mutants (Fig. 3A). This approach aims to unveil the underlying metabolic state that is triggered by both *P. cucumerina* infection and dysfunction of CI. Interestingly, a similar proportion of metabolic markers were up- and down-regulated (48 and 57, respectively), indicating that loss of CI reshuffled metabolic pools under these stress conditions (Fig. 3B). Among these markers, both an increase and decrease in similar classes of compounds were observed (Fig. 3C and Table 1). Putative identification of compounds denoted a re-adjustment of central metabolites such as lipids, amino and organic acids and nucleotides, including compounds important for mitochondrial pathway (fumarate, glycerate derivative), stress metabolism (phenylalanine derivative) or redox signalling (cysteine glutathione disulphide; Table 1). Remarkably, several nucleotides involved in energy and/or stress signalling function were also altered under these conditions, in particular O-acetyl-ADP-ribose. This metabolite is intricately tied to NAD regulation upon stress responses [31], which supports the idea of a regulatory link between NAD and mitochondrial functions under stress conditions [10,16]. Hence, loss of CI function after *P. cucumerina* challenge triggers a derailment of primary metabolism. This disrupted metabolic homeostasis was further associated with changes in stress-related secondary metabolites, such as flavonoids, polyphenols, terpenoids and alkaloids (Fig. 3C and Table 1). These classes of compounds are known for their importance in plant immunity [32,33]. Under stress conditions, an alteration of their cellular homeostasis due to unbalanced mitochondrial processes might affect their efficacy in thwarting pathogen attacks, as observed in the CI mutants that proved hyper-susceptible to fungal infection (Fig. 1). Interestingly, recent data suggest a link between mitochondrial respiration and the anti-fungal effect of the polyphenol p-coumaric acid against the necrotrophic fungus *Botrytis cinerea*[34]. Furthermore. glucosinolates are known for their role against *P. cucumerina* [17]. Here, however, loss of CI in Arabidopsis is not associated with increased pools of glucosinolates after *P. cucumerina* infection, which suggests that the fungal pathogen is not appropriately resisted in these conditions.

In summary, our study broadens our understanding of mitochondrial CI function in response to environmental fluctuations. We have demonstrated that CI plays a role in Arabidopsis tolerance to the fungal pathogen *P. cucumerina*. While CI dysfunction clearly influences pools of central and defence metabolites, further experiments are required to ascertain fully how these metabolic perturbations link to plant immunity.

ACKNOWLEDGMENTS

We greatly acknowledge research support by the Plant Production and Protection (P3) centre of the University of Sheffield. The authors would

like to thank Jack Holcombe for comments on earlier version of the manuscript.

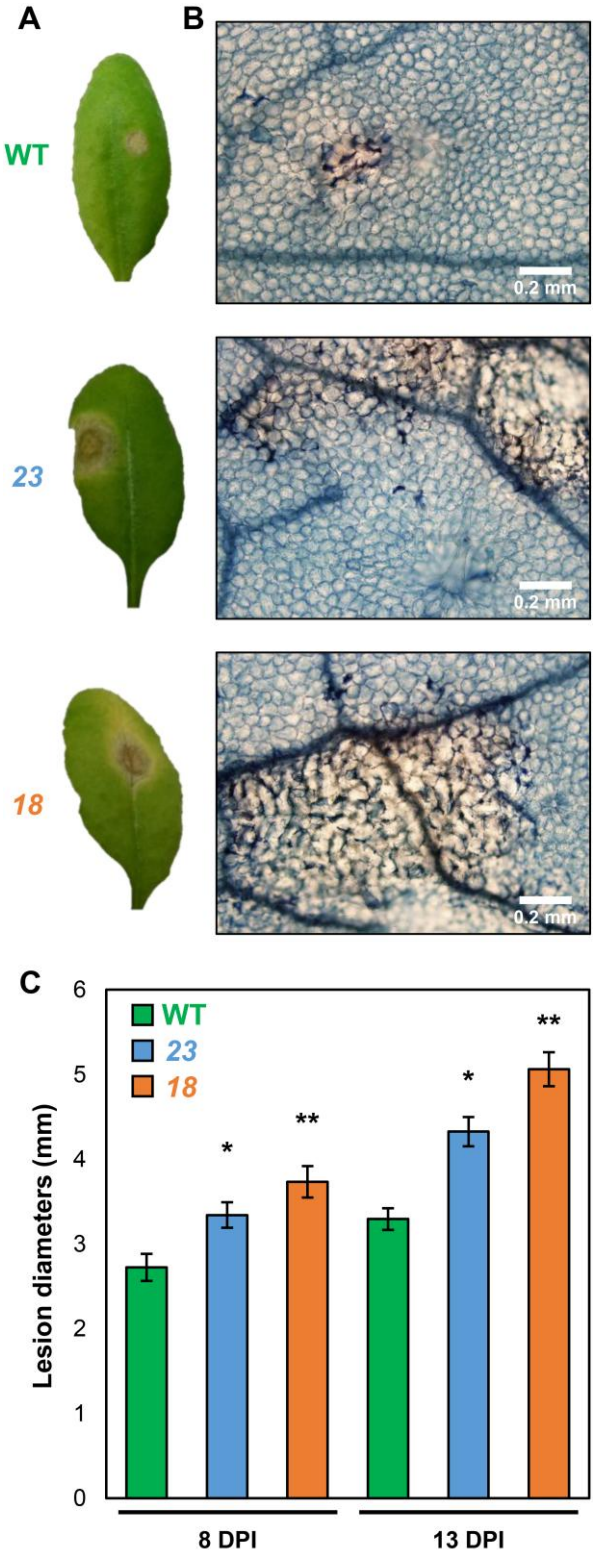

Figure1. *Disease phenotypes of Arabidopsis CI mutants against necrotrophic fungus P. cucumerina.*

Col-0 (WT) and the CI mutant plants ndufs8.1 ndufs8.2 (23) and ndufs4 (18) were droplet-infected with P. cucumerina (10^6 spores mL^{-1}), and resulting symptoms were scored at 8 and 13 days post-inoculation (DPI). ***A****, Photographs showing disease symptoms (8 DPI).* ***B****, Hyphen colonization and cell death in Trypan Blue-stained leaves (8 DPI).* ***C****, Quantitative measurements of lesion diameters. Shown are mean values from 6 leaves of different plants (n = 6, +/- SEM). Asterisks indicate statistically significant differences with the WT for each time point: *, P < 0.05; **, P< 0.01 (Student's t-test).*

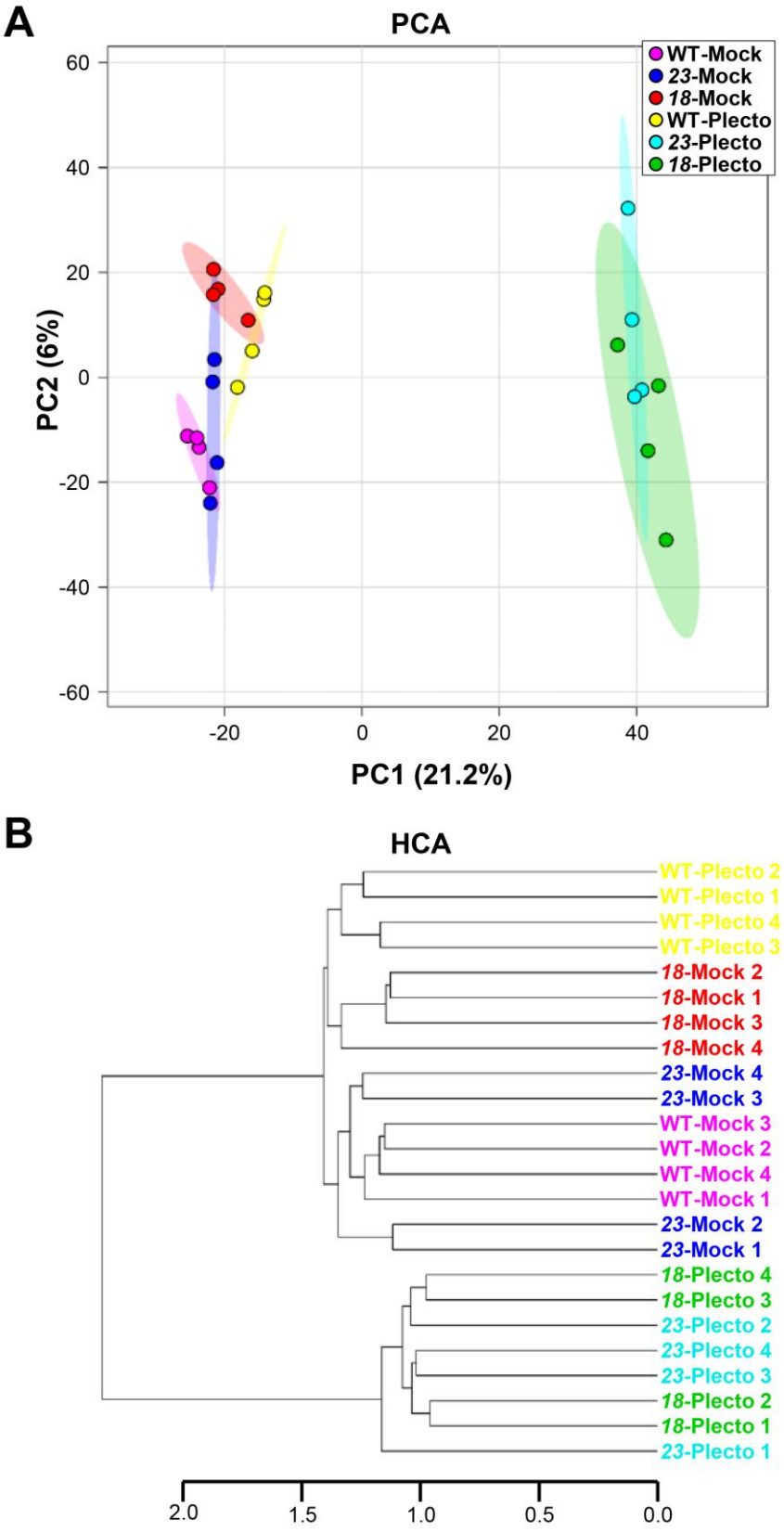

Figure2. *Multivariate analysis of metabolomics data.*

Col-0 (WT) and the CI mutant plants ndufs8.1 ndufs8.2 (23) and ndufs4 (18) were mock-inoculated (Mock) or droplet-infected with P. cucumerina (Plecto), then leaf tissues were sampled at 13 DPI. Metabolomics data were acquired by UPLC-Q-TOF-MS in negative ion mode (37,522 detected m/z) and analysed using MetaboAnalyst (interquartile range filtering, median normalisation, cube-root transformation and Pareto scaling). ***A****, Principal Component Analysis (PCA) showing global metabolic trends. Maximal variance*

explained for each PC is shown into brackets. ***B****, Clustering analysis (HCA) based on Pearson's correlation and average clustering.*

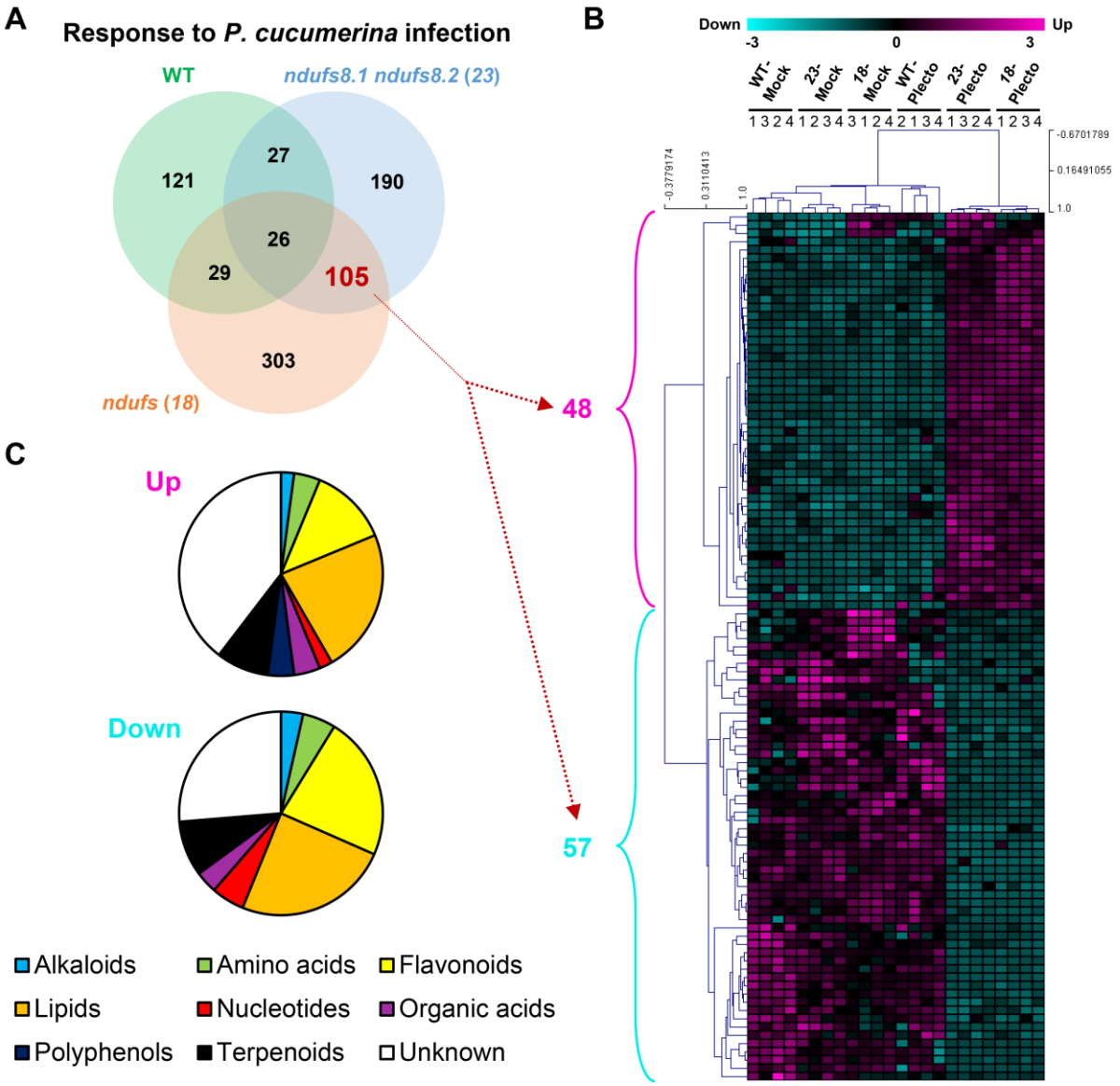

Figure3. *Quantitative and qualitative differences of stress-responsive metabolic markers.*

A*, Venn diagrams comparing significantly altered stress-responsive metabolic markers between Col-0 wild-type (WT) and CI mutant lines ndufs8.1 ndufs8.2 (23) and ndufs4 (18) after infection with P. cucumerina. The entire metabolomics dataset (37,522 m/z) was filtered by an ANOVA (P < 0.01 + FDR) and the resulting selection (1,405 m/z) was subsequently used for binary comparisons between mock inoculation (Mock) and P. cucumerina infection (Plecto) for each genotype (Student's t-test, P< 0.01). Numbers on the diagrams refer to statistically significant specific and overlapping markers.* ***B****, Bi-dimensional clustering analysis (Pearson's correlation, average clustering) of 105 markers (48 up- and 57 down-regulated) that showed common response to P. cucumerina for both CI mutants.* ***C****, Putative metabolic identification of the 48 up- and 57 down-regulated markers based on accurately detected m/z values (see Table 1 for details).*

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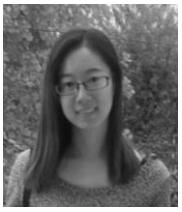
AUTHORS' BIOGRAPHY

Alex Williams, I completed my undergraduate master's degree at Sheffield on plant immune ecology in 2009. My PhD work is concerned with plant immune function and below-ground rhizosphere signalling and how alterations in atmospheric CO_2 impact these processes. I use metabolic and molecular approaches to investigate the underlying mechanisms that govern plant-biotic interactions, and focus on changes in phytohormonal signalling, peroxisomal ROS signalling and redox status of the plant.



Jingfang Hao, I obtained my bachelor degree in Biology from Henan Agricultural University in 2010, master degree in Plant Biology from Nanjing Agricultural University in 2013, and started my PhD study at the Institute of Plant Sciences Paris-Saclay in 2013. During my PhD, I am investigating the relationship between NAD levels and plant growth by using RNA-seq and Chip-seq analyses.

Moaed Al Meselmani,I completed my PhD from Indian agricultural research Institute (India) in 2006 and worked as a researcher in the General Commission for Scientific Agricultural Research in Syria studying the effect of abiotic stress in crop plants. Currently, I am a visiting researcher in the Department of Molecular Biology and Biotechnology/ The University of Sheffield (UK). I am involved in different research projects. One of the main projects is investigating the effects of reducing stomatal density on the physiology and biochemistry of wheat when grown under drought conditions.

Rosine De Paepe, After completing my PhD in 1977, I followed a "Thèse d'État" and was recruited at Centre National de la Recherche Scientifique (CNRS) in 1983. I became Directeur de Recherche (Research Director) in 1996. I have led the group "Mitochondria and Metabolism" from 1996. My main research aims at obtaining and characterising mutant plants affected in respiratory Complex I.

Bertrand Gakière, I completed a master degree in Biology and Agronomy at ENSA of Rennes (FR) in 1995, and obtained my PhD in 1999 from University of Grenoble I (FR) under Dominique Job's supervision. I investigated methionine biosynthesis in higher plants in the CNRS/Rhône-Poulenc Agrochimie "Labo Mixte"of Lyon (FR) led by Roland Douce. I moved to the Weizmann Institute of Science in Rehovot (IL) where I worked with Gad Galili on plant lysine catabolism (1999), then I moved to the Max-Planck Institute of Molecular Plant Physiology (Golm, D) working on sulfur deficiency with Rainer Hoefgen (2002). Since October 2005, I am Associate Professor at University Paris-Sud (Orsay, FR) working on NAD metabolic signalling in plant growth and immunity.

Pierre Pétriacq, After completing my master degree in Physiology & Integrative Biology in 2008 at University Pierre & Marie Curie in Paris (FR), I obtained my PhD in 2011 from University Paris-Sud (Orsay, FR) under Bertrand Gakière's supervision. Our pioneer work identified NAD as a powerful signal molecule involved in plant immunity. After a teaching position (2012), I moved to Sheffield (UK) where I worked on Plant Induced Resistance with Jurriaan Ton (2013). Since 2016, I am a group leader at the Plant Production and Protection (P3) centre of the University of Sheffield. I use mass spectrometry techniques to investigate metabolic regulations under environmental fluctuations. In October 2017, I am appointed Associate Professor at University of Bordeaux to work with Yves Gibon (INRA, FR) on metabolic fluxes of fruit.