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The cyanobacterium, *Nostoc punctiforme* can protect against programmed cell death and induce defence genes in *Arabidopsis thaliana*

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ABSTRACT

Cyanobacteria can form nitrogen-fixing symbioses with a broad range of plant species. Unlike other plant-bacteria symbioses, little is understood about the immunological responses induced by plant cyanobionts (symbiotic cyanobacteria). Here, we demonstrated that the model plant-symbiotic cyanobacteria, *Nostoc punctiforme* is capable of protecting against programmed cell death (PCD) when induced in *Arabidopsis thaliana* suspension cell cultures. We also profiled the early transcriptomic changes that were induced in response to conditioned medium (CM) from *N. punctiforme* cell cultures. Interestingly, the reduction in PCD was preceded by the induction of genes associated with defence and immunity, the most striking of which were a number of WRKY-family transcription factors. Down-regulated genes included those involved in the regulation of cell growth and differentiation. This work is the first to show that a cyanobiont can affect plant PCD and provides a useful transcriptome resource for studying early plant cell responses to symbiotic cyanobacteria.

ARTICLE HISTORY

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KEYWORDS

Nostoc punctiforme; cell suspension cultures; conditioned medium; programmed cell death (PCD); transcriptome; *Arabidopsis thaliana*

Introduction

A wide diversity of terrestrial plant taxa are capable of associating with nitrogen-fixing cyanobacteria, including bryophytes, ferns, cycads, basal eudicots of the genus *Gunnera*, and even staple food crop species such as *Oryza sativa* (rice) (Whitton 2012). However, unlike legumes and actinorhizal plants, which are colonised by nitrogen-fixing *Rhizobium* spp. and *Frankia* spp., respectively, the molecular dialogue which takes place between plants and cyanobacteria remains comparatively enigmatic (Geurts et al. 2016; Mus et al. 2016; Cissoko et al. 2018).



At present, the most characterised species of plant-symbiotic cyanobacteria is *Nostoc punctiforme* PCC 73102 (ATCC 29133). *N. punctiforme* is a heterocystous species that can colonise and support the growth of a broad range of plant hosts, including the hornwort *Anthoceros punctatus* (Ekman et al. 2013), herbaceous angiosperms of the genus *Gunnera* (Chiu et al. 2005), and more recently, rice (Álvarez et al. 2020). In addition, this species displays a positive chemotaxis toward the non-hosts species *Trifolium repens* and *Arabidopsis thaliana* (Nilsson et al. 2006), which suggests that plant-cyanobacteria interactions are evolutionarily dynamic.


To date, those works which have attempted to molecularly characterise plant-cyanobacteria interactions have primarily focused on identifying genes in the cyanobacterial symbiont (cyanobiont) rather than in the plant hosts (Duggan et al. 2007; Ekman et al. 2013; Cozy and Callahan 2014). This has mainly been due to a lack of host genome sequences, although a number have now recently been reported (Li et al. 2018; Pederson et al. 2019).

Symbiosis formation between Rhizobia and legumes is characterised by a transient activation of defence-associated genes (Gully et al. 2018; Kang et al. 2020), which is then followed by a suppression of host defence signalling (Liang et al. 2013; Wang et al. 2018). For instance, *Rhizobium* spp. produce lipochitooligosaccharides (LCOs) termed Nod factors which are capable of suppressing defence responses induced by microbe-associated molecular patterns (MAMPs) (Liang et al. 2013; Mus et al. 2016).

By contrast, the immunological dynamics of plant-cyanobacteria symbioses have gone largely unstudied. *Nostoc* spp. possess lipopolysaccharides (LPOs) in their outer membranes and peptidoglycans in their cell walls, both of which are typical MAMPs (Erbs and Newman 2012). However, they do not appear to possess any LCO biosynthetic pathway, but like Rhizobia and *Frankia* spp. are able to form intimate and, in some cases, intracellular associations without triggering an incompatible response (Geurts et al. 2016).

It is not entirely clear whether *Nostoc* LPOs are simply unrecognisable as MAMPs by plant receptors, or whether symbiotic *Nostoc* spp. can actively suppress plant immune responses, such as programmed cell death (PCD), thereby enabling colonisation. Here, *A. thaliana* suspension cell cultures were used as a system to investigate whether *N. punctiforme* can affect plant PCD. To better understand the effects of compounds secreted by *N. punctiforme* on early plant cell responses, the transcriptome of *A. thaliana* cells treated with *N. punctiforme*-conditioned medium (CM) was sequenced and analysed. Our data provide novel insights into the early immunological and developmental signalling events elicited by a plant cyanobiont.

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Materials and methods

Growth of *N. punctiforme* cells and preparation of conditioned medium

N. punctiforme strain ATCC 29133 was grown in liquid BG-11 (NH₄) medium at 21°C with shaking at 120 rpm for a photoperiod of 16 hrs light (ca. 165 μmol m⁻² sec⁻¹ of cool white fluorescent light) and 8 hrs darkness (Campbell et al. 2007; Christman et al. 2011). To harvest CM, a volume of cells equivalent to 60 μg of chlorophyll *a* was added to 100 ml of fresh growth medium. After 21 days of growth (stationary phase), cells were harvested by centrifugation at room temperature for 20 min at 3000 g in glass Corex™ round-bottomed tubes. The supernatant was then passed through two pieces of Whatman™ grade I filter paper and collected in chromic acid-washed glass vials before storing at -20°C for subsequent use. Cleaning with activated charcoal (AC) was achieved by adding AC at a concentration of 50 mg ml⁻¹, mixing for 8 hr using a magnetic stirrer and filtering as before. Finally, pH was readjusted to 7.4 using NaOH (standard BG-11 pH), as AC has been shown to reduce the pH of targeted medium (Pan and Jv 1999).

Maintenance of *A. thaliana* suspension cell cultures

A. thaliana var. Ler-0 suspension cell cultures were maintained under the same growth conditions as above but in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% sucrose, 0.5 mg L⁻¹ naphthaleneacetic acid (NAA) and 0.05 mg L⁻¹ kinetin (pH adjusted to 5.8 using KOH). Cells were sub-cultured every 7 days by transferring 10 ml of mature cells into 90 ml of fresh growth medium.

Monitoring the effects of *N. punctiforme*-CM on PCD in *A. thaliana* cells

To study the effects of *N. punctiforme*-CM on PCD in *A. thaliana* cells, 2.5 ml of CM was added to 10 ml of a 7-day-old *A. thaliana* suspension cell culture (cell density = ca. 5 × 10⁴ cells ml⁻¹) and left to pre-incubate for 1 hr under standard culturing conditions. Next, cells were either incubated at room temperature (~21°C) or placed in a water bath set to 51°C for 10 min with constant agitation at 120 rpm (Kacprzyk et al. 2017). After returning to standard culturing conditions for 24 hr, the frequency of viable, PCD-committed and necrotic cells was measured. This was done by mixing cells in a 1:1 volume of 0.004% fluorescein diacetate (FDA; w/v in 100% acetone) before quantification using epifluorescence microscopy (excitation wavelength = 490 nm, emission wavelength = 515 nm) (Alden et al. 2011). Green fluorescence as a result of FDA hydrolysis was indicative of cell viability. A retracted protoplasm, which we have demonstrated before to be a robust marker of PCD in this system (Kacprzyk et al. 2017), coupled with a loss of green fluorescence indicated PCD, whereas a lack of fluorescence and no retracted protoplasm was indicative of cell necrosis (Reape et al. 2008). To determine statistical significance, arcsine-transformed proportions were subjected to a One-way ANOVA combined with Tukey's HSD.

RNA isolation and quality control

RNA was isolated from flash-frozen 1 ml aliquots of *A. thaliana* cells immediately prior to PCD induction in cells pre-treated with either *N. punctiforme*-CM or fresh growth medium (control). Frozen cells were disrupted by bead beating at 30 h/z for 1 min before purifying total RNA using an RNeasy® plant mini kit (Qiagen™; ref. 74904) according to manufacturer's instructions. RNA was quantified using a NanoDrop® 1000 spectrophotometer and quality determined on a 1% TBE agarose gel. A Nano 6000 Assay Kit was used with a 2100 BioAnalyzer® system (Agilent®) revealed that all sample RIN scores were over 9.0.

cDNA library preparation

Library preparation and RNA-seq was performed by Novogene (Beijing, China). A total of 1 μg RNA was used as input material for library generation using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB USA), at which point index codes were added to each sample. mRNA was purified using magnetic beads harbouring poly-dT oligos. Fragmentation was performed using divalent cations in heated NEBNext First Strand Synthesis Reaction Buffer. First strand cDNA was synthesised using random hexamers and M-MuLV MuLV Reverse Transcriptase. Second strand cDNA was synthesised using DNA Polymerase I (+RNase H). Blunt ends were generated via exonuclease/polymerase activities. Next, 3' DNA ends were adenylated before ligating an NEBNext hairpin loop adaptor for hybridization. Fragments of ca. 200 bp were preferentially selected for using an AMPure XP system (Beckman Coulter, Beverly, USA). Purified, adaptor-ligated fragments were treated with 3 μl of USER® Enzyme (NEB, USA) before selective PCR enrichment using Phusion® High-Fidelity DNA polymerase using Universal and index primers. Products were purified again using the AMPure XP system and library quality assessed using the 2100 BioAnalyzer® system.

Sequencing and transcriptome assembly

Index-coded samples were clustered on a cBot Cluster Generation system using a PE Cluster Kit cBot-HS (Illumina) followed by paired-end sequencing on an Illumina NovaSeq 6000 platform. FASTAQ raw reads were filtered to remove adaptors, poly-N sequences and low-quality reads. Q20 and Q30 values were ~98.6% and ~95.3%, respectively, whereas GC content ranged between 44.7% and 45.1%. Using HISAT2 software, cleaned-up reads were mapped to the *A. thaliana* genome (TAIR10) before expressing read quantification as FPKM.

Differential expression analysis

Differential expression of genes between treatments (*n* = 3 biological replicates) was determined using the DESeq2 R package. The false discovery rate (FDR) was determined using the Benjamini and Hochberg procedure, after which genes with an adjusted *P* value (*P*_{adj}) of 0.05 were considered to be differentially expressed. Hierarchical clustering of the log₁₀FPKM values of differentially expressed genes was performed using the gplot R package.

Enrichment analysis

Gene ontology (GO) and pathway enrichment analyses were performed on the fourfold differentially regulated gene set. This was done using the ClueGO plugin (V2.5.7) (Bindea et al. 2009) for Cytoscape (V3.7.2) (Shannon et al. 2003), with the following settings: Kappa Score threshold = 0.5, Min GO level = 3, Max GO level = 8, Min genes per cluster = 3, Min % genes = 4, GO Term Fusion = True. The cut-off for enriched terms was set to a familywise error rate of $P_{adj} \leq 0.05$.

TF enrichment analysis

Peptide sequences belonging to the 962 differentially expressed genes were acquired in FASTA format using BioMart (Smedley et al. 2015) (version 0.7) through the EnsemblPlants genome resource portal (<https://plants.ensembl.org/biomart/martview/5b18fb7d060dfa269fe3dadd41bfb2a>). The TF composition of this data set was ascertained using the TF prediction tool at the plant TF database PlantTFDB (<http://planttfdb.cbi.pku.edu.cn/prediction.php>) (Jin et al. 2017). The promoter regions (region spanning 600 bp upstream of the 5'-UTR) of the 962 differentially expressed genes were also extracted using BioMart. TF names for which *cis*-binding motifs were enriched in these promoter sequences were acquired using the regulation prediction tool available through the PlantRegMap portal (http://plantregmap.cbi.pku.edu.cn/regulation_prediction.php) (Tian et al. 2020). The FDR was controlled using a Benjamini-Hochberg correction ($P_{adj} \leq 0.05$).

STRING network analysis

The STRING protein-protein interaction network was generated based on the fourfold differentially regulated gene set using the stringApp plugin (V1.5.1) (Doncheva et al. 2019) for Cytoscape. All possible evidence channels were selected and a confidence score of 0.7 (high strength) was selected ($P_{adj} \leq 0.05$).

Results

N. punctiforme can protect against plant programmed cell death (PCD)

To investigate whether *N. punctiforme* can regulate PCD, we used a well-established method involving a 10-min, 51°C temperature stress to induce PCD in *A. thaliana* suspension cell cultures. This treatment has previously been demonstrated to induce an interruptible form of 'apoptotic-like' PCD which is marked by a distinct protoplasmic retraction that is associated with DNA fragmentation (Doyle et al. 2010; Alden et al. 2011; Kacprzyk et al. 2017), proving that protoplasmic retraction is a robust and reliable marker for PCD. To test whether *N. punctiforme* could affect this process, *A. thaliana* cells were pre-incubated for 1 hr with conditioned medium (CM; 20% v/v) from mature *N. punctiforme* liquid cultures prior to inducing PCD. As a control treatment, cells were pre-incubated with fresh *N. punctiforme* growth medium (20% v/v). This workflow is depicted in Figure 1(a). Twenty-four hr after applying the temperature stress, cell viability in cultures pre-incubated with fresh growth medium was reduced to ~68%, with ~30% of cells committing to PCD (FDA negative, protoplasmic retraction), and <0.25% of cells dying by necrosis

(FDA negative, no protoplasmic retraction) (Figure 1(b)). However, when cells were pre-incubated with *N. punctiforme*-CM, viability was maintained at a significantly higher level (~85%) whereas PCD levels were significantly attenuated (~14%) compared to control cells ($P < 0.001$; Figure 1(b)). Importantly, necrosis levels did not change (Figure 1(b)), which suggested that *N. punctiforme*-CM could specifically protect against PCD activation. To demonstrate that the protective effects imparted by *N. punctiforme* could be reversed, the CM was washed with 50 mg ml⁻¹ of activated charcoal (AC). AC has been well proven to remove growth regulatory compounds from plant cell cultures but will not remove highly soluble nutrients such as sugars and major/trace elements (Yu et al. 1993; Thomas 2008). Indeed, when pre-incubated with AC-treated CM, PCD levels did not differ from control-treated cells, which suggests that bioactive compounds secreted by *N. punctiforme* were effectively stripped by the AC.

N. punctiforme-CM elicits major transcriptomic changes

As a 1-hr pre-incubation with *N. punctiforme*-CM sufficed to protect against PCD, we hypothesised that a differential induction of stress-associated genes would be discernible at this time-point. To investigate this, RNA was extracted and a cDNA library generated ahead of paired-end RNA-sequencing. After applying a 0.3 FPKM cut-off, a total of 19,064 genes were determined to be expressed, 396 of which were unique to *N. punctiforme*-CM treated cells (Figure 2(a)). We observed that between control and CM-treated cells, 7,032 genes were differentially expressed after controlling for false discovery ($P_{adj} \leq 0.05$). Hierarchical clustering analysis revealed that large numbers of these genes grouped into similar patterns of expression between treatments (Figure 2(b)). After applying a threshold of a fourfold change in expression, we deemed 483 and 479 genes to be significantly up- and down-regulated, respectively in response to *N. punctiforme*-CM (Figure 2(c)). A list of the differentially regulated genes can be found at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155980>.

Functional categorisation of genes regulated by *N. punctiforme*-CM

To functionally interpret these transcriptomic changes, we performed gene ontology (GO) and pathway enrichment analyses to systematically evaluate the biological significance of the genes which were fourfold differentially expressed (Figure 3). Up-regulated genes were mainly associated with defence responses, chitinase activity, indole-containing metabolic processes, and responses to chitin. Notable up-regulated pathways included phenylpropanoid biosynthesis, photosynthesis and glutathione metabolism. The most enriched processes associated with the down-regulated expression gene set were primary metabolic processes, such as transcription and the synthesis of macromolecules, responses to hormones, and meristem and shoot system development (Figure 3). Photosynthesis was also suggested to be a down-regulated pathway, however, the expression of only 3 photosynthesis-associated genes were down-regulated, whereas the expression of 22 photosynthesis-associated genes were up-regulated,

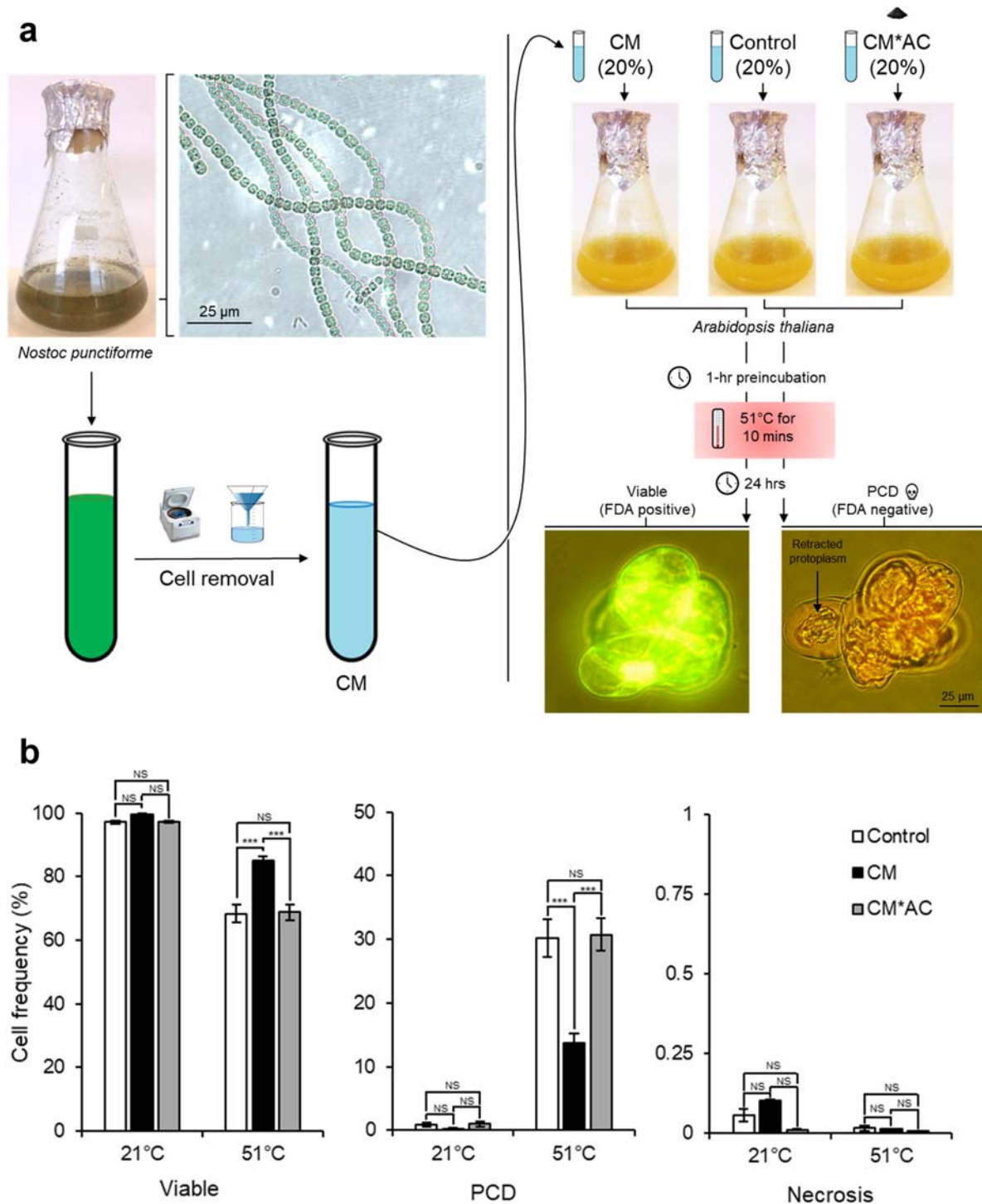


Figure 1. *Nostoc punctiforme*-conditioned medium (CM) can regulate plant programmed cell death (PCD). (a) Workflow used to examine the effects of *N. punctiforme*-CM on PCD in *A. thaliana* suspension cell cultures. Cells were removed from a mature (21 d-old) *N. punctiforme* liquid culture. A 7-day-old *A. thaliana* suspension cell culture (following sub-culture) was split into two flasks. *N. punctiforme*-conditioned medium (CM) was added at a 20% concentration (v/v) to one of the flasks, while a 20% (v/v) of fresh medium was added as a control treatment to the other flask. As an additional treatment, CM pre-treated with activated charcoal (CM*AC) was added (after removal of AC). After a 1-hr preincubation, cells were heat-shocked for 10 min at 51°C and returned to 22°C for 24 hr, before viable, PCD-committed and necrotic (not shown) cells were quantified. (b) Frequency of viable, PCD-committed and necrotic *A. thaliana* cells after a 1-hr pre-incubation with either fresh *N. punctiforme* growth medium (Control), *N. punctiforme*-CM, and CM pre-treated with AC (CM*AC) 24 hr after a 10-min heat stress treatment (51°C). *** $P \leq 0.001$. Error bars are S.E., $n = 5$, ca. 750–1050 scored cells per replicate.

suggesting an overall up-regulation of photosynthesis. In general, the expression of hormone signalling-associated genes was down-regulated (Figure 3).

Transcription factor (TF) enrichment analysis

To better understand the significance of the CM-induced transcriptional reprogramming, we analysed the TF composition of the differentially expressed gene set. Of the

962 differentially regulated genes, 121 were predicted to encode TFs (Supplementary data file 1). Expression of 24 TF genes belonging to 8 different families were up-regulated, with the most represented encoding WRKY family TFs. Conversely, a total of 97 TF genes spread across 27 families were significantly down-regulated, with genes encoding DOF and MYB family TFs being the most enriched. To investigate this further, we screened the promoters of the differentially expressed genes for TF-binding

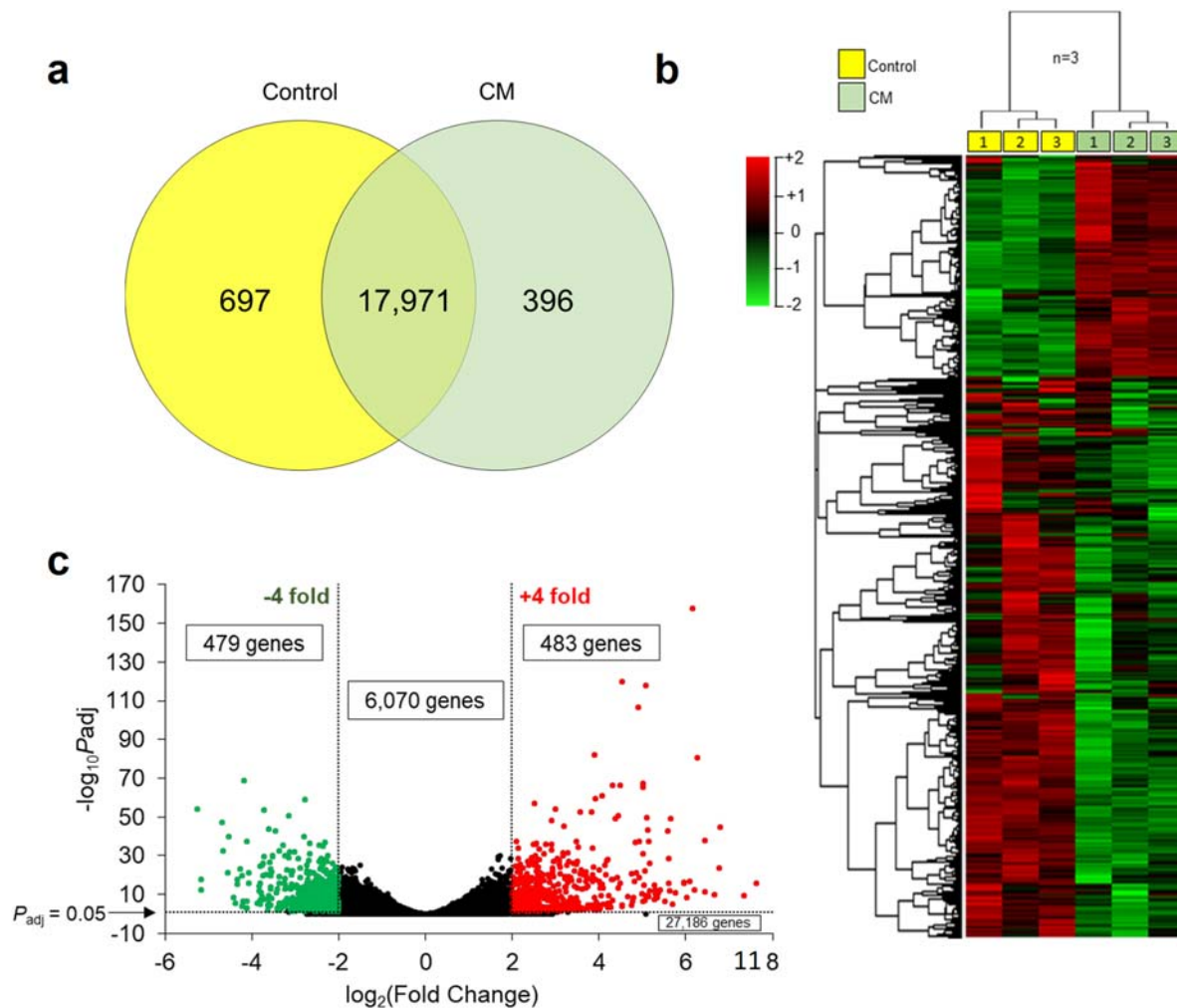


Figure 2. Transcriptomic changes in *A. thaliana* suspension cell cultures induced by *N. punctiforme*-CM. Changes in gene expression following a 1-hr incubation with either fresh *N. punctiforme* growth medium (Control) or *N. punctiforme*-CM (20% v/v) are shown. (a) Venn diagram depicting co-expressed and uniquely expressed genes between control and CM treatments. (b) Hierarchical clustering of 7032 genes differentially expressed between control and CM treatments. (c) Volcano plot depicting the distribution of all 34,218 annotated reads, where a fourfold change cut-off in expression is delimited.

motifs and observed a significant enrichment of *cis*-motifs corresponding to known binding sites of 43 different TFs in the up-regulated gene set (462 genes) (Figure 4). Of these, almost all (41/43) were W-box recognition elements for WRKY TFs, with WRKY50 motifs being associated with 56 different genes. Only 9 TFs were associated with the down-regulated gene set, most of which belonged to the TCP class, a family of plant-specific developmental regulators (Li 2015) (Figure 4). A list of all the TF binding motifs can be found in Supplementary data file 2.

Analysis of potential protein–protein interaction networks

We generated a STRING protein–protein interaction network using the 962 significantly regulated genes to render the transcriptomic changes into an interaction network (Figure 5). Of these, the STRING algorithm computed the protein products of 170 as being highly likely to interact with the product of at least one other differentially regulated gene. An additional 72 proteins were also linked in this network from the STRING database. The entire network comprised 1,752 edges (interactions), 103 hubs (proteins with 3 or more edges) and 40 components, the largest of which comprised 77 proteins (nodes), 45 of which were

up-regulated. Most of these are implicated in defence responses to biotic and abiotic stresses, with WRKY33 appearing as a major hub linked with 20 different proteins. Also included were a range of proteins (i.e. IGMT1-3, BGLU26, CYP81F2 and CYP71A12) which are implicated in the metabolism of defence metabolites, such as glucosinolates and cyanogenic glycosides (Clay et al. 2009; Pfalz et al. 2016) (Figure 5).

The next two largest components (58 and 12 nodes) mainly consisted of proteins which are associated with photosynthesis and the transport of nitrate and nitrite, respectively. The remaining 37 components (each comprising 8 nodes or less) appeared to be relevant in cell division and differentiation (15 components), stress/defence responses (12 components), or have functions that could not be easily curated (8 components) (Figure 5). Included in the cell division and differentiation components are enzymes involved in cytokinin and brassinosteroid metabolism, as well as a range of proteins implicated in the regulation of cell fate. One such component comprises *WOX4*, *CLV3* and *PXY*. *WOX4* and *CLV3*, which negatively regulate shoot stem cell identity (Wahl et al. 2010; Somssich et al. 2016), were strongly down-regulated (36- and 10-fold, respectively), suggesting that *N. punctiforme* may also negatively regulate cell differentiation.

Plant Interactions

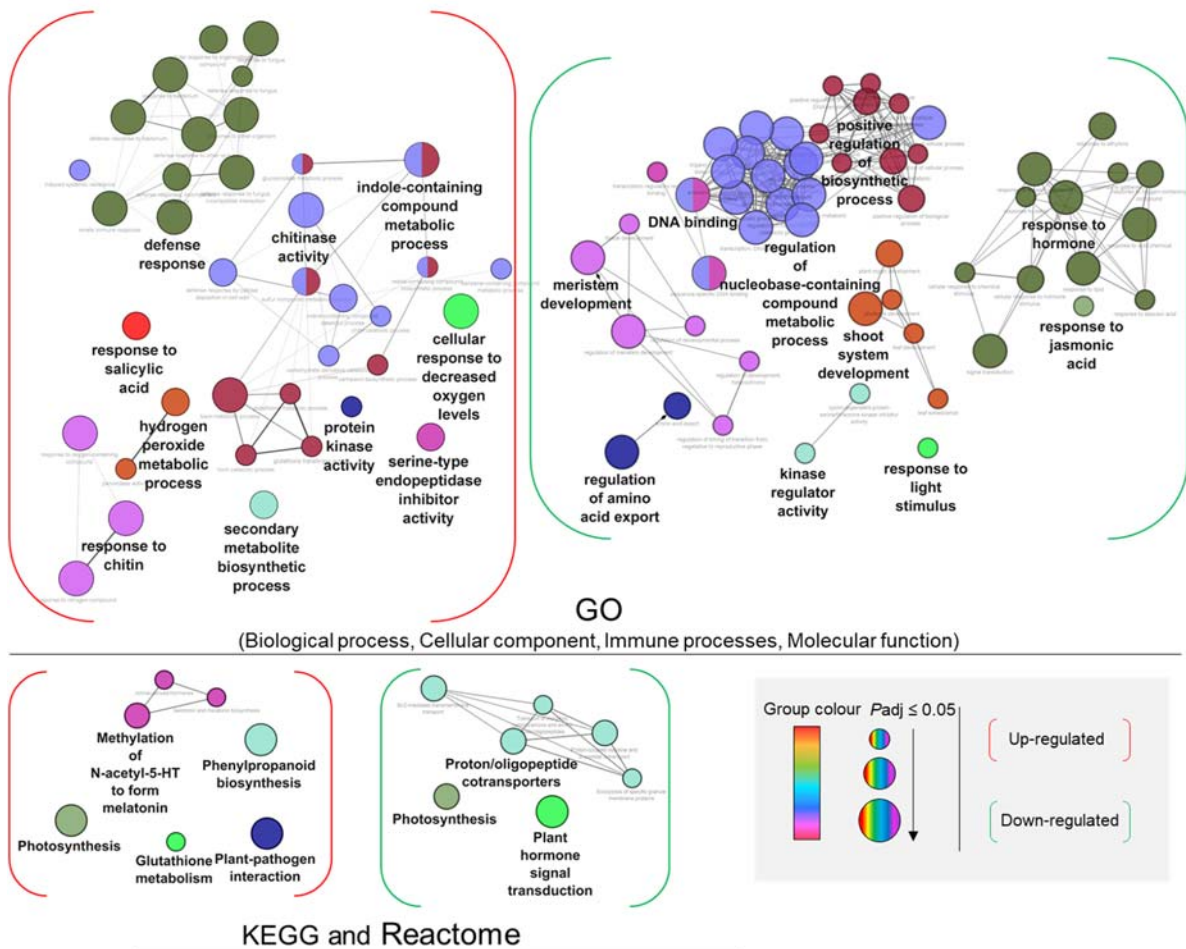


Figure 3. Functional categorisation of genes that were fourfold differentially regulated in response to *N. punctiforme*-CM. GO terms and pathways (nodes) are connected by common genes. Terms in bold are the most statistically significant with respect to other terms in a group. As indicated in the legend, node colour corresponds to GO/KEGG and Reactome group, whereas node size denotes statistical significance. The number of genes for each term are provided in Supplementary data file 1.

Discussion

Is the regulation of host PCD by cyanobionts important in plant-cyanobacteria associations?

We demonstrated that CM from *N. punctiforme*, a well-characterised plant cyanobiont, could protect *A. thaliana* cells against PCD (Figure 1(b)). To demonstrate this, we used a well-established model system involving a heat-shock (HS) treatment to induce PCD in *A. thaliana* suspension cell cultures (Doyle et al. 2010; Alden et al. 2011; Kacprzyk et al. 2017). The PCD which occurs in plant cell cultures as a result of HS treatment shares the hallmarks of PCD (e.g. protoplasmic retraction and DNA fragmentation (Doyle et al. 2010; Kacprzyk et al. 2017)) induced by H₂O₂ (Houot et al. 2001), cell culture dilution (McCabe et al. 1997), salicylic acid (Poór et al. 2013) and that which occurs during suspensor cell death (Havel and Durzan 1996). However, we can expect some of the upstream events to be distinct and that the ability of *N. punctiforme* to suppress a HS-induced PCD might be reflected in the differentially expressed gene set.

HS-induced PCD has been hypothesised to occur due to heat-induced protein misfolding, as evidenced by the observation that heat shock proteins (HSP) can protect against HS-induced PCD (Qi et al. 2011). In *A. thaliana* suspension cell cultures, synthesis of the small HSP,

HSP17.6 is induced at 37°C and is suspected to play an anti-PCD role at 50°C (Rikhvanov et al. 2007). Interestingly, *Hsp17.6a* was up-regulated 7.3-fold by *N. punctiforme*-CM. Protein misfolding, which likely results from the disruption of redox homeostasis that occurs during HS-induced PCD (Vacca et al. 2004), can be buffered against by S-glutathionylation (Dalle-Donne et al. 2009). It may therefore be noteworthy that 6 genes (Supplementary data file 3) predicted to encode glutathione S-transferase (GST) enzymes were significantly up-regulated by *N. punctiforme*-CM, with *GSTF7* expression being induced 43-fold. In addition, 8 genes (Supplementary data file 3) predicted to encode peroxidase enzymes were also up-regulated. This included *PER4*, which was elevated by 50-fold. Whether a capacity of *N. punctiforme* to protect against PCD is significant in the context of its ability to colonise various plant species deserves to be discussed.

In a diverse range of bacteria-eukaryote symbioses, bacterial symbionts are known to produce effectors which suppress host immune responses to enable symbiosis establishment and maintenance (Pannebakker et al. 2007; An et al. 2014; Kitchen and Weis 2017). However, unlike many of these symbioses, including the ones formed between Rhizobia and legumes or *Frankia* spp. and actinorhizal plants, most of the associations formed between

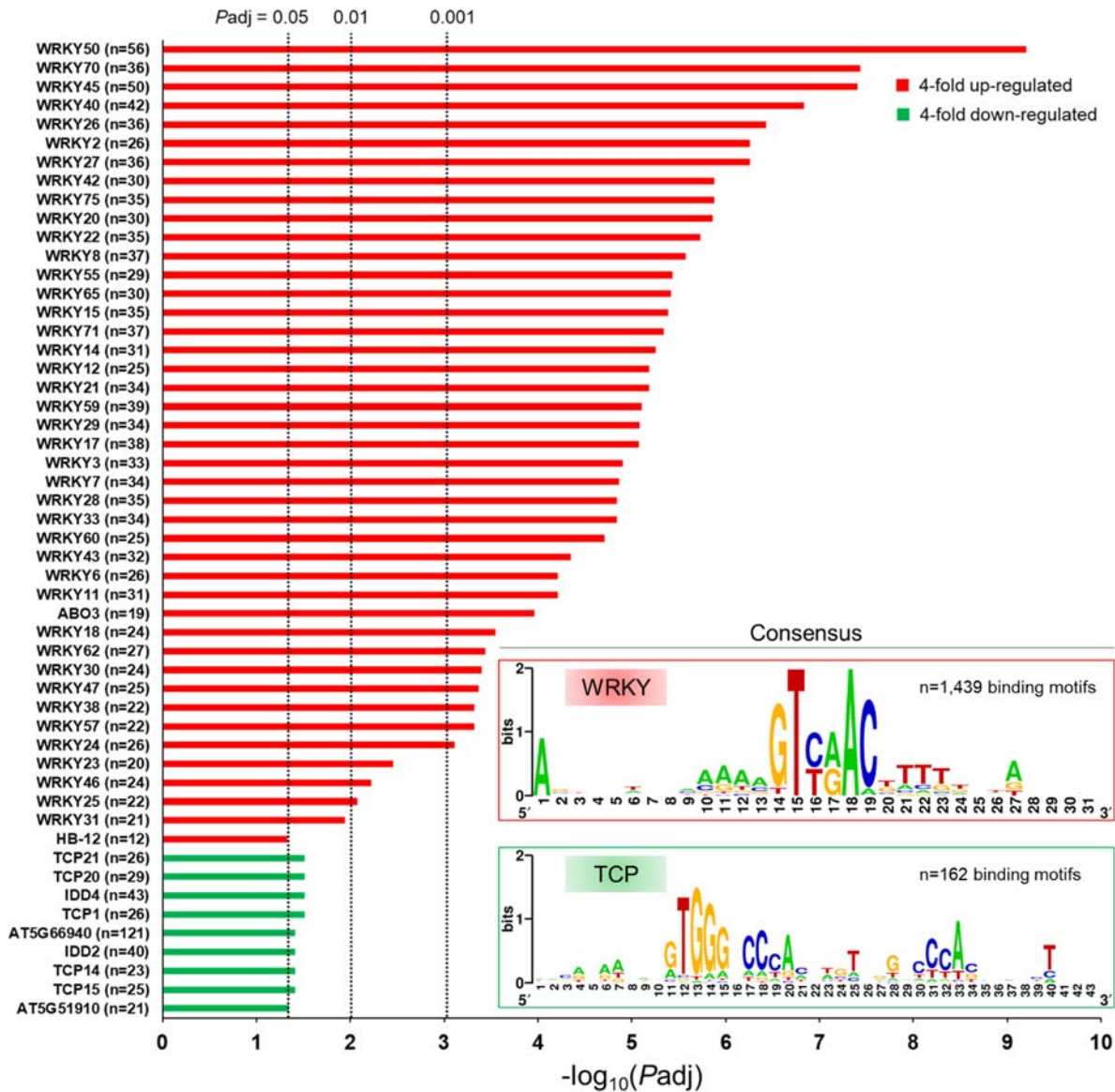


Figure 4. cis-Motif analysis of differentially expressed genes. Transcription factors (TFs) for which binding motifs were found in the promoter regions of the 962 differentially expressed genes (\geq fourfold) in response to *N. punctiforme*-CM. For each TF, the number of genes for which canonical binding motifs were found is given. Also depicted are the enriched consensus sequences for WRKY and TCP family transcription factors.

cyanobacteria and plants do not involve intracellular colonisation (Geurts et al. 2016). With the exception of *Gunnera* spp., which are intracellularly colonised by *Nostoc* spp. (Johansson and Bergman 1992), most plant-symbiotic cyanobacteria either grow epiphytically (Whitton 2012) or, more loosely, in the rhizosphere (Prasanna et al. 2009).

Whilst *N. punctiforme* PCC 73102 – the strain used in this work – can colonise *Gunnera manicata* (Chiu et al. 2005), it was originally isolated from the coralloid roots of a cycad (*Macrozamia* spp.) (Rippka et al. 1979), which extracellularly harbours the cyanobiont between its outer and inner cortical layers (Lindblad and Costa 2002). This would suggest that the ability of cyanobacteria such as *N. punctiforme* to reduce PCD may not necessarily be a trait that is selected exclusively by hosts which harbour their cyanobionts intracellularly. Rather, an ability to buffer cells against PCD could be the result of a more general selection for bacteria which prime plant immunity and tolerance to stress, as cyanobacteria are known to promote plant growth by improving biotic and abiotic stress tolerance (Singh 2014).

Although one gene (*SBT1.9*) encoding a putative subtilisin-like protease was listed in the down-regulated gene set

(fourfold), no genes, such as the core developmental PCD marker genes outlined by Olvera-Carrillo et al. (Olvera-Carrillo et al. 2015) involved directly in the plant PCD pathway were differentially regulated, suggesting that the PCD reduction was not due to specific components of the plant PCD pathway being regulatory targets. Rather, the observed reduction in PCD was more likely the result of an improved cellular redox homeostasis that functioned to increase the threshold above which PCD would have been activated.

Whilst the transcriptomic data can glean some insights about the genetic responses of the plant, the nature of the *N. punctiforme*-derived compounds responsible for eliciting such responses is still unclear. Here, pre-treatment of the *N. punctiforme*-CM with AC appeared to abrogate its capacity to reduce PCD. AC is used in plant cell cultures to remove growth regulatory substances and has an adsorptive preference for polar rather than apolar compounds, although it will not remove highly water-soluble compounds such as sucrose or major and trace elements (Yu et al. 1993; Thomas 2008). The few studies which have investigated the composition of the exo-proteome and exo-metabolome of symbiotic *Nostoc* spp. have revealed an enrichment of small peptides,

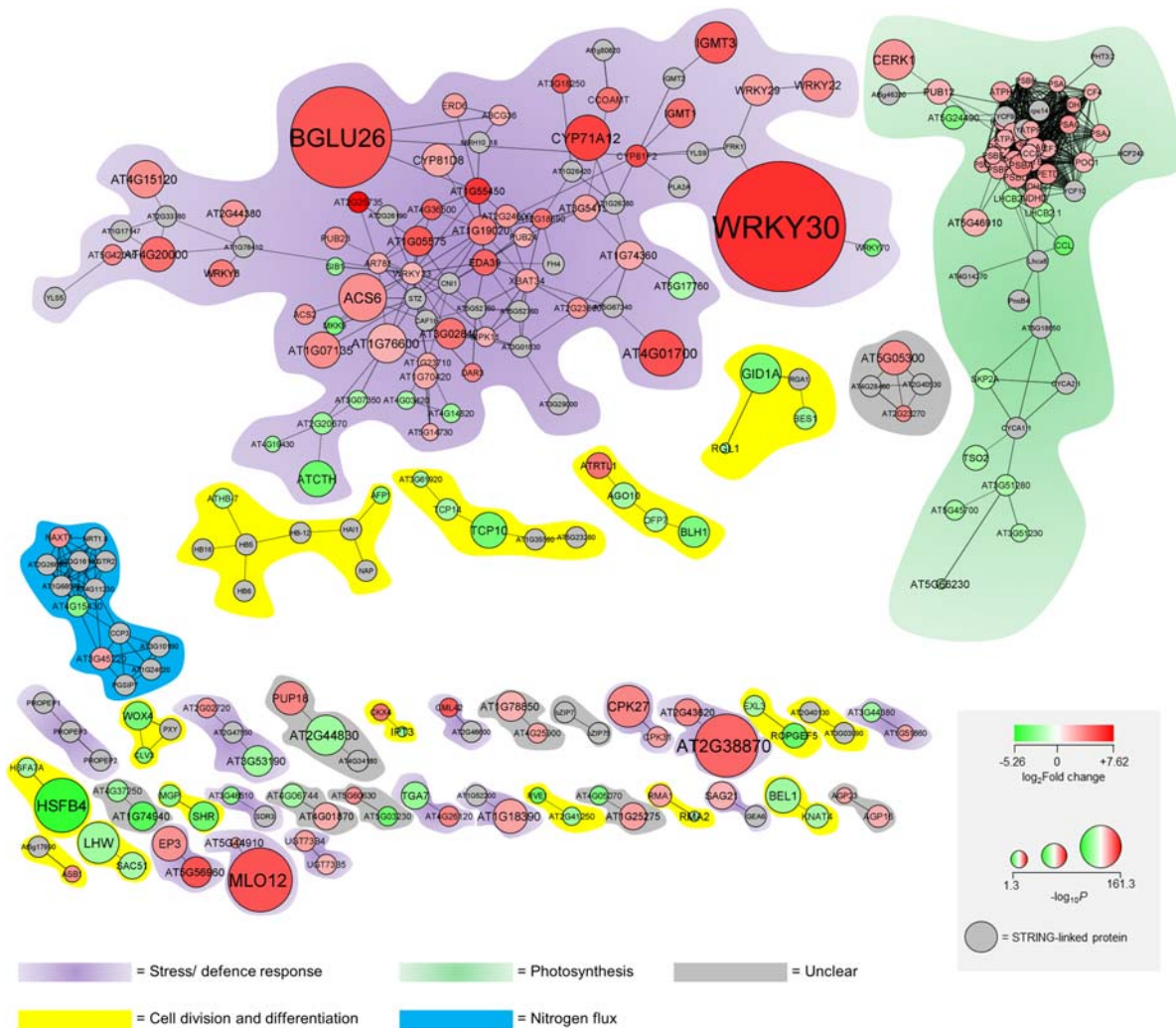


Figure 5. STRING protein-protein interaction network generated using genes that were at least fourfold differentially regulated by *N. punctiforme*-CM. The network comprises 40 separate components which have been grouped into four different biological processes based on the predicted function of the majority of nodes (proteins) within each. As indicated in the legend, node colour and colour intensity correspond to change in gene expression level, node size denotes statistical significance of differential expression and grey nodes are associated through the STRING database. Network topology attributes can be found in Supplementary data file 3.

polyketides and peptidoglycan-derived tetrasaccharides (Vilhauer et al. 2014; Liaimer et al. 2015). At present, other than a group of polyketides which were demonstrated to be important in suppressing the motile growth phase of *N. punctiforme* during symbiotic association (Liaimer et al. 2015), none of these compounds have been functionally characterised.

It was recently demonstrated that *Nostoc* cyanobionts possessed 32 additional gene families compared to a free-living strain, and that intracellular plant-symbiotic Nostocales spp. likely evolved from a single common ancestor, whereas extracellular plant-symbiotic species likely evolved convergently (Warshan et al. 2017, 2018). Future works should test whether this genetic diversity impacts capacity for plant PCD reduction and determine whether PCD can be attenuated if induced using different methods, including biotic and developmental cues.

N. punctiforme can modulate the expression of plant genes involved in immunity and cell growth

Unsurprisingly, the biological processes most associated with the up-regulated gene set were genes linked with plant defence and immunity. This was mirrored in the STRING network, in which the largest component primarily

comprised of up-regulated genes encoding proteins involved in stress and defence responses (Figure 5). The induction of defence genes would presumably have been preceded by the recognition of compounds from *N. punctiforme*. In total, 17 receptor kinases and 3 mitogen-activated protein kinase (MAPKs) were significantly up-regulated (Supplementary data file 3), some of which could be key components in transducing the signals into the observed transcriptional changes. Represented in these transcriptional changes were a strikingly wide range of WRKY TFs. WRKYs, which are master regulators of biotic stress responses (Phukan et al. 2016), were amongst the most strongly induced genes (8 in total) and their *W*-box binding motifs were highly represented in the promoter regions of the up-regulated gene set (Figure 5). In addition, STRING prediction using the differentially regulated gene set revealed that *WRKY33* was a core component in the up-regulated set of defence genes.

Although few plant genes which are regulated by cyanobionts have been characterised, a recent study showed that the symbiotic association between the water fern, *Azolla filiculoides* and its cyanobiont, *Nostoc azollae* resulted in the positive and negative regulation of 88 and 72 genes, respectively (Eily et al. 2019). These were termed ‘putative symbiosis genes’ and were selected on the basis of their

differential expression in the *A. filiculoides* host in the presence of *N. azollae* but in the absence of a source of combined nitrogen, which was reasoned to be the ‘optimal symbiosis growth condition.’ The *Nostoc-Azolla* system (Eily et al. 2019) obviously differed radically to the one used here, in which nitrogen-supplemented, un-differentiated *A. thaliana* cells were exposed to CM derived from vegetative *N. punctiforme* cell cultures (non-diazotrophic). Nonetheless however, both approaches identified a range of *WRKY* genes as being differentially regulated by *Nostoc* cyanobionts, including *WRKY72*, which was strongly up-regulated in both systems.

It is worth mentioning that the strong induction of immunity-associated genes was not followed by an incompatible response (i.e. PCD). Rather, cells were buffered against the activation of heat-induced PCD. That this occurred in the non-host *A. thaliana* may not be that surprising given the observation that in both *A. thaliana* and host legume spp., Rhizobia initially activate immune responses (ROS accumulation, cytosolic Ca²⁺ burst, MAPK signalling) only for Nod factors to quickly suppress them (Liang et al. 2013). The up-regulated defence gene set may then, in part, represent an initial triggering of immune signalling that is subsequently suppressed, or it represents the beginning of a more protracted priming effect against biotic and abiotic stresses. Additional timepoints would certainly aid in clarifying this.

Whilst a distinctive defence transcriptome was strongly induced, the expression of genes which were down-regulated by *N. punctiforme* appeared to primarily be important in regulating cell division and differentiation. At least 5 genes involved in auxin biosynthesis were significantly down-regulated (Supplementary data file 3), whereas *LOG1*, which encodes a cytokinin riboside 5'-monophosphate phosphoribohydrolase that converts cytokinin into its active form (Kuroha et al. 2009), was strongly up-regulated (5.7-fold). Notable genes included *WOX4*, *CLV3* and *FAF4*, which were down-regulated 36-, 10- and 9-fold, respectively. These genes play key roles in promoting cellular differentiation in meristematic regions, thereby exerting a negative control on cell division (Wahl et al. 2010; Etchells et al. 2013; Somssich et al. 2016). Another gene, *MYB59*, which is a negative regulator of cell proliferation (Mu et al. 2009), was down-regulated 21-fold. MYBs were the second-most represented TFs in the down-regulated gene set, with DOF family TFs being the most abundant. DOF and MYB TFs are involved in a range of developmental processes (Ambawat et al. 2013; Le Hir and Bellini 2013), which suggests that broadly, defence signalling was up-regulated at the expense of developmental signalling. This is worth noting as nodule formation in legumes is preceded by the induction of de-differentiation and cell division in root cortical cells, and the induction by *N. punctiforme* of multiple rounds of cell division within the glands of *Gunnera* spp. is expected to be critical for symbiosis establishment (Geurts et al. 2016).

In summary, our results demonstrate for the first time that a cyanobacteria is capable of protecting plant cells against PCD. This may be an important preceding step to successful colonisation, as initial immune responses may require suppression. What, in our opinion, is more likely however is that the observed PCD reduction was the result of bioactive compounds which confer a more general cellular tolerance to stress, something which could be attributable to a selective pressure for bacteria which promote plant fitness.

This is distinct from an active suppression of the PCD process, as genes typically involved in the PCD pathway were not differentially regulated. Rather, genes associated with protecting cells against oxidative stress (*GST*- and *PER*-family genes) were induced. In relation to this, future works should investigate whether cellular oxidative stress tolerance in plants can indeed be improved by cyanobacteria. Other significant transcriptomic changes included the up-regulation of a strikingly high number of *WRKY* genes and the down-regulation of a wide range of genes which function in the regulation of cell division and differentiation. Our study brings light to the early defence responses of plant cells to symbiotic cyanobacteria and is the first to show that a cyanobacteria can affect plant PCD. In addition, a valuable transcriptome is provided which could serve as a molecular blueprint for future endeavours to better understand the relatively enigmatic plant-cyanobacteria symbioses.

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Author contributions

S.B., P.F.M, and C.K.Y.N conceived of the study and designed the experiments. S.B. performed all the experiments and analysed the data. S.B. prepared the draft of the manuscript with the help of P.F.M and C.K.Y.N. All authors read, edited, and approved the manuscript.

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Appendix

Statement on data availability

Full transcriptome data – including raw data (.FASTQ files), processed data (annotated read counts, FPKM values and differentially expressed genes) and a metadata sheet – have been deposited at the Gene Expression Omnibus (GEO) at NCBI. The data (series record: GSE155980) will be made publicly available on Nov 10, 2020 but can be accessed early using an access token (see below).

Access token: yzatqokqrdexnaj

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155980>