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ORIGINAL ARTICLE

Cell division and turgor mediate enhanced plant growth in *Arabidopsis* plants treated with the bacterial signalling molecule lumichrome

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Abstract

Main conclusion **Transcriptomic analysis indicates that the bacterial signalling molecule lumichrome enhances plant growth through a combination of enhanced cell division and cell enlargement, and possibly enhances photosynthesis.**

Lumichrome (7,8 dimethylalloxazine), a novel multitrophic signal molecule produced by *Sinorhizobium meliloti* bacteria, has previously been shown to elicit growth promotion in different plant species (Phillips et al. in Proc Natl Acad Sci USA 96:12275–12280, https://doi.org/10.1073/pnas.96.22.12275, 1999). However, the molecular mechanisms that underlie this plant growth promotion remain obscure. Global transcript profiling using RNA-seq suggests that lumichrome enhances growth by inducing genes impacting on turgor driven growth and mitotic cell cycle that ensures the integration of cell division and expansion of developing leaves. The abundance of *XTH9* and *XPA4* transcripts was attributed to improved mediation of cell-wall loosening to allow turgor-driven cell enlargement. Mitotic *CYCD3.3, CYCA1.1, SP1L3, RSW7* and *PDF1* transcripts were increased in lumichrome-treated *Arabidopsis thaliana* plants, suggesting enhanced growth was underpinned by increased cell differentiation and expansion with a consequential increase in biomass. Synergistic ethylene–auxin cross-talk was also observed through reciprocal over-expression of *ACO1* and *SAUR54,* in which ethylene activates the auxin signalling pathway and regulates *Arabidopsis* growth by both stimulating auxin biosynthesis and modulating the auxin transport machinery to the leaves. Decreased transcription of jasmonate biosynthesis and responsive-related transcripts (*LOX2; LOX3; LOX6; JAL34; JR1*) might contribute towards suppression of the negative effects of methyl jasmonate (MeJa) such as chlorophyll loss and decreases in RuBisCO and photosynthesis. This work contributes towards a deeper understanding of how lumichrome enhances plant growth and development.

Keywords Cell wall · Cyclins · Defence response · Expansins · Mitotic cell cycle · Stress response · Transcript profiling

Abbreviations

CK	Cytokinin
GA	Gibberellic acid
JA	Jasmonic acid
PGPR	Plant growth-promoting rhizobacteria

Introduction

Plant growth progresses through different developmental phases and the transitions are controlled by distinct genetic cues that integrate endogenous and environmental signals. A variety of bacterial genera are present in soils, some of which produce substances that enhance plant growth. Such plant growth-promoting rhizobacteria (PGPR) stimulate plant growth through mobilizing nutrients in soils, producing

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numerous plant growth regulators, protecting plants by controlling or inhibiting phytopathogens, improving soil structure and bioremediating polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (Bhattacharyya and Jha 2012). Diverse symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and non-symbiotic (*Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*, *Azomonas*) rhizobacteria are used worldwide as bio-inoculants to promote plant growth and development, including under various stresses. Other than nitrogen fixing symbioses, many PGPR release phytohormones that promote cell growth and massively increase root hair production (Yanni et al. 2001). PGPR also facilitate plant growth directly by assisting in nutrient resource acquisition. Root-nodule bacteria synthesize signal molecules in soil that indirectly promote plant growth via an increase in nutrient availability and uptake through enhanced root absorptive capacity in the transpiration stream (Zhang and Smith 2002; Matiru and Dakora 2004, 2005a).

The discovery of lumichrome (7,8 dimethylalloxazine) as a novel plant growth promoting multitrophic signal molecule produced by the bacterium *Sinorhizobium meliloti* (Phillips et al. 1999), has driven research to elucidate the molecular mechanisms through which this compound induces plant growth. Previous studies reported that its application leads to enhanced root respiration which resulted in increasing concentrations of rhizospheric CO_2 needed for growth of N_2-fixing rhizobia and mycorrhizal fungi (Phillips et al. 1999). Lumichrome's effect on improving plant growth and development has been attributed to enhanced leaf stomatal conductance, transpiration and enhanced photosynthetic rates in soybean and corn (Zhang and Smith 2002; Dakora 2003; Matiru and Dakora 2005b). Conversely, its addition led to significantly decreased root respiration in lupin, while in cowpea it decreased stomatal conductance, which subsequently affected CO_2 intake and reduction by RuBisCo (Matiru and Dakora 2005b).

At the molecular level, a microarray gene expression study indicated changes in carbon and ethylene metabolism in roots of both *Lotus japonicus* and tomato treated with lumichrome. Enhanced starch accumulation was attributed to an increase in plastidial *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) transcripts and NAD-dependent enzyme activity (Gouws et al. 2012), which has previously been shown to generate this phenotype (Muñoz-Bertomeu et al. 2009). Additionally, lumichrome treatment resulted in a reduction of transcript levels of genes involved in ethylene metabolism, including ethylene response factor/elements, *ACC OXIDASE 1* (*ACO1*) and transcriptional factors *AP2/EREBP* and a *C2H2 ZINC FINGER PROTEIN* (Gouws et al. 2012). It was speculated that the interaction of lumichrome with ethylene metabolism was the result of a transient redox mimicry and independent from the biomass accumulation. Despite these physiological experimental advances in exploring the growth stimulatory mechanisms of lumichrome, a comprehensive molecular analysis remains elusive. Here, we use next generation sequencing (RNA-seq) in the model plant *Arabidopsis thaliana* to examine this. Although we previously worked on *Lotus japonicus* and tomato, the enormous variety of genetic resources offered by *A. thaliana* make it an obvious choice for further investigations. We, therefore, aimed to identify specific changes in gene expression relating to the enhanced growth observed in *A. thaliana* plants treated with lumichrome, utilising an RNA-seq approach.

Materials and methods

Plant material, treatment and growth

Arabidopsis thaliana (ecotype Columbia-0) seeds were stratified (4 °C, 48 h) prior to seed sowing. The experiment was conducted in a controlled environment growth chamber (16/8 h day/night, 22 ± 2 °C, 75% relative humidity). Pots were arranged in a factorial randomised complete block design consisting of 6 replications and blocks. Lumichrome stock solutions (100 µM) were freshly prepared for each treatment in methanol/1 M HCl (49:1, v/v) with constant stirring. Intact plants were treated with 5 nM lumichrome by a combination of root drenching and foliar application (50 ml) at intervals of 2 days throughout the entire growth period (Phillips et al. 1999). Control plants were treated using the same dilution of 49:1 methanol/HCl, without added lumichrome, in dH_2O. Above-ground plant material (rosette leaves) was harvested from 5-week-old plants for fresh biomass determination. The same plant materials were then oven dried at 70 °C for 3 d and subjected to dry biomass determination.

RNA extraction, library preparation, and Illumina sequencing

Total RNA was isolated from *Arabidopsis* rosette leaf tissue material (250 mg) according to a CTAB protocol (Hu et al. 2002). RNA was further purified using the Qiagen RNase-free DNase kit (Cat #79254), and eluted in RNase-free water according to the manufacturer's instructions. Library preparation and sequencing were performed at the Agricultural Research Council Biotechnology Platform (South Africa). For RNA-seq, total RNA was subjected to removal of ribosomal RNA using the Plant Leaf Ribo-Zero™ Magnetic Kit (Illumina), according to the manufacturer's instructions. Ribosomal RNA-depleted RNA samples were fragmented, and first-strand cDNA synthesis performed using random hexamers and reverse transcriptase. The cDNA

was converted to double-stranded cDNA, subjected to end repair and 3′ adenylation and ligated to Illumina TrueSeq's paired-end index adaptor before the DNA fragments were PCR enriched (15 cycles). The purity and size of resulting libraries were verified on an Agilent Technologies 2100 Bioanalyzer with an expected fragment of approximately 260 bp. The prepared libraries were sequenced on the Illumina Platform, generating paired-end reads of length 125 nt.

Differential gene expression analysis

Adaptor sequences were removed from the raw sequencing reads and low-quality bases at read ends were trimmed (minimum quality 20 Phred score over a 3 nt window, minimum read length 20 nt) using Trimmomatic v. 0.33 (Bolger et al. 2014). The Tuxedo software suite v.2.2 (Bowtie, TopHat, Cufflinks, Cuffdiff; Trapnell et al. 2012) was used to compare samples and calculate differential expression. Trimmed sequencing reads were aligned against the wild type *Arabidopsis* (Columbia-0; TAIR10) genome and gene expression was quantified as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Differential expression was calculated based on Cuffdiff statistical tests of three replicates of treated relative to untreated samples, using a statistical significance of q (adjusted P value) < 0.05.

Validation of RNA sequencing data by reverse transcription quantitative PCR (RT-qPCR)

The same DNase-treated RNA pool as was used for library construction was used for validation of the RNA-seq data. The complementary DNA (cDNA) template was obtained via reverse transcription of 1 µg of total RNA, using an oligo (dT_{18}) primer and M-MLV (H-) reverse transcriptase (Promega) following the manufacturer's instruction. The integrity of cDNA template was checked with RT-qPCR using *MONENSIN SENSITIVITY1* (*At2g28390; MON1*) as a constitutively expressed gene (Supplementary Fig. S1). To validate the reliability of the RNA-seq, we performed RT-qPCR using SYBR-Green dye in reactions containing KAPA SYBR® FAST qPCR Kit Master Mix (2X) ROX Low (KAPA Biosystems, Cape Town, South Africa), forward and reverse primers (200 nM each) and a 5X diluted cDNA template (10 ng) according to the manufacturer's instructions. The primer pairs (Supplementary Table S1) were designed with Quant Primer tool (Arvidsson et al. 2008), and PCR reactions were run in a 7500 Real-Time PCR System (Applied Biosystems) under conditions that included initial denaturing/enzyme activation at 95 °C for 3 min and 40 cycles at 95 and 60 °C for 3 and 30 s, respectively. Data for at least three technical replicates were analysed using the Applied Biosystems SDS software (version 1.4), while the cycle threshold (Ct) was used to determine the relative expression level of a given gene using the $E^{-\Delta\Delta}$Ct method [PCR efficiency (E) was calculated using LinRegPCR (version 2014.5)] (Ramakers et al. 2003). *MON1*, a member of the SAND family, shown by RNA-seq to be invariantly expressed in the samples, was used as a housekeeping reference control gene (Czechowski et al. 2005).

Results

Growth response of *Arabidopsis* upon lumichrome treatment

To test for the effect of lumichrome, *Arabidopsis thaliana* Columbia-0 plants were watered with a combination of root drenching and foliar application every 2 days with 5 nM lumichrome. Fresh and dry weights of 5-week-old rosette leaves showed significantly increased biomass production in treated plants (Fig. 1a, b). Rosettes from treated plants were visually distinguishable as larger than those of untreated plants after 5 weeks of growth (Fig. 1c).

Functional classification and gene enrichment of differentially expressed responsive genes

A microarray study on *Lotus japonicus* and tomato (Gouws et al. 2012) has previously assisted in our understanding the complexity of the transcriptional regulation and its impact on phenotype after application of lumichrome. However, next-generation sequencing is a more sensitive methodology to examine differential gene expression because of its high throughput and accuracy (Szittya et al. 2008). The current study, therefore, adopted a high-throughput paired-end sequencing of ribosomal-depleted RNA on the Illumina HiSeq 2500 platform, and used the Tuxedo analysis protocol to further investigate lumichrome-associated transcriptional modulation. Statistics of clean reads in RNA sequencing are shown in Supplementary Table S1. Out of the total 32 845 different transcripts analysed, only 198 showed significant differential expression, with 66 and 132 transcripts being significantly up or down-regulated, respectively.

To gain insight into which transcripts were most highly over-represented, transcriptome data were loaded into PageMan and a Wilcoxon test was applied to each category. Based on this statistical overview, enrichment analysis of differentially expressed genes that were up-regulated in lumichrome-treated *Arabidopsis* rosette leaves relative to their untreated control were associated with lipid metabolism, RNA regulation of transcription was over-represented. In contrast, down-regulated genes resulted in a shift of the gene enrichment functional categories associated to secondary metabolism, hormone metabolism (jasmonates), biotic stress (pathogenesis-related and plant defensins),

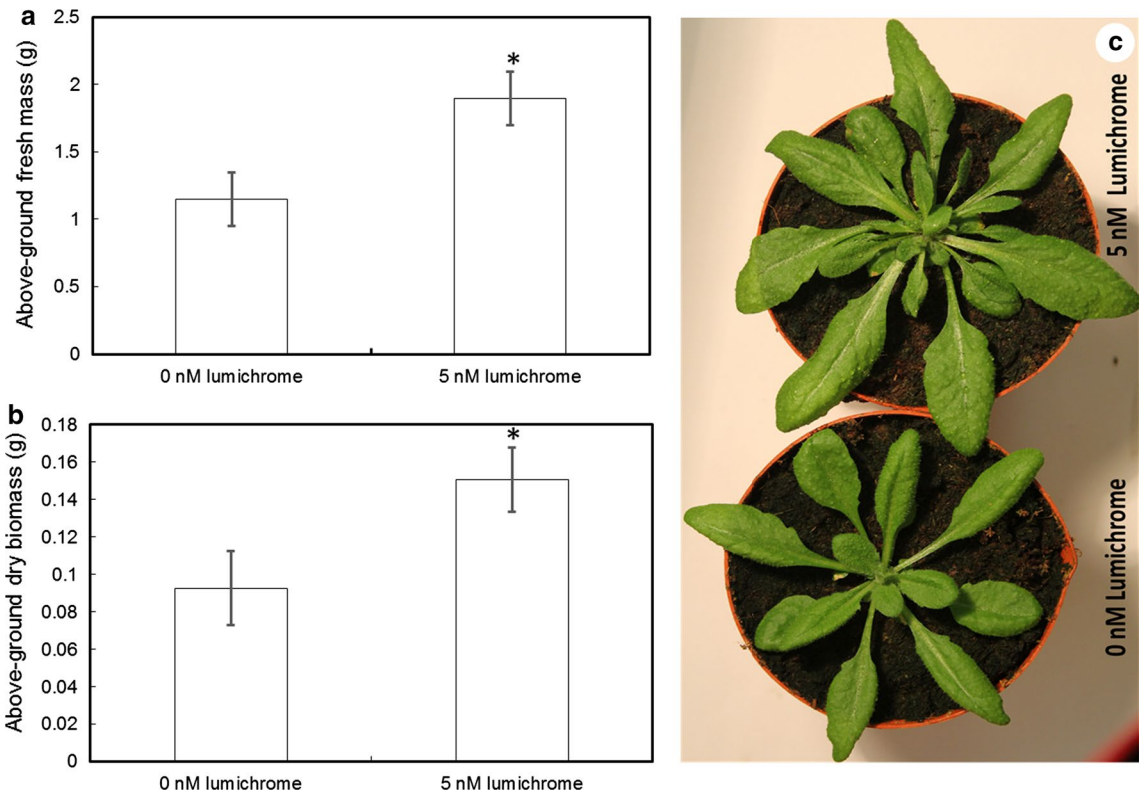

Fig. 1 Biomass production analysis of lumichrome-treated *Arabidopsis thaliana* plants. Lumichrome treatment (5 nM) enhanced fresh (**a**) and dry biomass production (**b**) in *Arabidopsis* compared to the untreated control. Treated plants had a visibly larger rosette than untreated plants after 5 weeks of treatment (**c**). Error bars represent standard error (mean ± SE, $n = 6$) of 6 individual plants, while asterisks represent significant differences between the treatments ($P \leq 0.05$)

miscellaneous myrosinases (jacalin-lectin) and development storage proteins. Besides the over-represented biological functional categories revealed by Wilcoxon, several transcripts unique to the lumichrome treatment were associated with phytohormone-related pathways including auxin, gibberellin, ethylene, and jasmonate. In addition, a significant up-regulation of mitotic transcripts was also observed in the category cell cycle, division and development (Table 1).

Cell cycle, division and development related metabolism

Lumichrome treatment of *Arabidopsis* prompted an up-regulated expression of cell wall and cell cycle transcripts such as *RADIALLY SWOLLEN 7* (*RSW7, At2g28620*) and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9* (*XTH9; At4g03210*) and *EXPANSIN A4* (*EXPA4*; *At2g39700*). Lumichrome treatment additionally resulted in an increase in transcription of cell mitosis *CYCLIN A1;1* (*CYCA1;1; At1g44110*), and *CYCLIN D3;3* (*CYCD3;3; At3g50070*). Similarly plant development-associated transcripts, namely, *PROTODERMAL FACTOR 1* (*PDF1; At2g42840*), *SPIRAL 1-LIKE3* (*SP1L3; At3g02180*) and *UNIVERSAL STRESS PROTEIN* (*USP*) *FAMILY PROTEIN/EARLY NODULIN ENOD18 family protein* (*At3g03270*) were also induced upon lumichrome treatment (Table 1).

Hormone metabolism and signalling

Transcripts for ethylene biosynthesis (Table 1) were altered through increased expression of the *1-AMINOCYCLOPROPANE-1-CARBOXLATE OXIDASE* (*ACO1; At2g19590*) gene. This was accompanied by expression of several transcription factors belonging to proteins predicted to contain an ethylene-responsive element, including an *AP2/EREBP/RAP2.4* transcription factor (*At1g22190*). Interestingly, transcripts for proteins from other hormone signalling pathways that have been implicated in cross-talk with ethylene signalling during *Arabidopsis* growth, such as the auxin responsive protein *SMALL AUXIN RNA51* (*SAUR54; At1g19830*), and the gibberellin-induced transcript, *GAST1 PROTEIN HOMOLOG 1* (*GASA1; At1g75750*), were also induced. A distinct suppression of jasmonate metabolism-related transcripts was demonstrated through the down-regulated expression of *Arabidopsis* lipoxygenases *LOX2* (*At3g45140*), *LOX3* (*At1g17420*) and *LOX6* (*At1g67560*). In addition, we

Table 1 Categories of differentially expressed genes of 5-week-old *Arabidopsis* rosette leaves following lumichrome treatment

AGI	Gene name	Log_2 fold change ratio	*Q* value
Cell cycle, division and development			
At2g39700	*ARABIDOPSIS THALIANA EXPANSIN A4* (*EXPA4*)	0.982	0.015
At4g03210	*XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9* (*XTH9*)	1.996	0.006
At2g28620	*RADIALLY SWOLLEN 7,* (*RSW7*)	1.251	0.015
At1g44110	*CYCLIN A1;1* (*CYCA1;1*)	1.309	0.044
At3g50070	*CYCLIN D3;3* (*CYCD3;3*)	1.354	0.030
At3g02180	*SPIRAL 1-LIKE3* (*SP1L3* | *SP1L3*)	0.838	0.042
At2g42840	*PROTODERMAL FACTOR 1* (*PDF1*)	2.616	0.027
At3g03270	*UNIVERSAL STRESS PROTEIN* (*USP*) *FAMILY PROTEIN/EARLY NODULIN ENOD18 FAMILY PROTEIN*	1.713	0.037
Hormone metabolism and signalling			
At1g19830	*SMALL AUXIN UPREGULATED RNA 54* (*SAUR54*)	3.327	0.006
At2g19590	*1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE* (*ACC OXIDASE 1* (*ACO1*)	1.696	0.018
At1g75750	*GAST1 PROTEIN HOMOLOG 1* (*GASA1*)	1.301	0.006
At4g00970	*CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASE 41* (*CRK41*)	1.188	0.006
At1g03430	*HISTIDINE-CONTAINING PHOSPHOTRANSFER FACTOR 5* (*AHP5*)	0.905	0.042
At1g17420	*LIPOXYGENASE 3* (*LOX3*)	−1.110	0.030
At1g72520	*LIPOXYGENASE 6* (*LOX6*)	−1.228	0.027
At3g45140	*LIPOXYGENASE 2* (*LOX2*)	−1.555	0.042
At3g16460	*JACALIN-RELATED LECTIN 34* (*JAL34*)	−1.786	0.040
At3g16470	*JASMONATE RESPONSIVE 1* (*JR1*)	−1.922	0.006
At3g16390	*NITRILE SPECIFIER PROTEIN 3* (*NSP3*)	−1.765	0.006
Regulation of transcription, translation and post-translational modification			
At5g26210	*AL4* (*ALFIN-LIKE 4*	1.023	0.021
At1g22190	*RELATED TO AP2.4* (*RAP 2.4*)	0.864	0.018
At2g19810	*ARABIDOPSIS OXIDATION-RELATED ZINC FINGER 1* (*ATOZF1*)	1.003	0.006
At1g11870	*SERYL-tRNA SYNTHETASE*	1.155	0.006
At3g56910	*PLASTID-SPECIFIC 50S RIBOSOMAL PROTEIN 5* (*PSRP5*)	0.818	0.034
At5g67380	*CKA1* (*CASEIN KINASE ALPHA 1); KINASE*	1.161	0.018
At5g43060	*RESPONSIVE TO DEHYDRATION 21B* (*RD21B*)	1.048	0.006
At5g27660	*ARABIDOPSIS PARK13 HOMOLOG* (*AtPARK13*)	1.327	0.006
At4g28270	*RING MEMBRANE-ANCHOR 2* (*RMA2*)	0.960	0.006
At1g47570	*RING/U-BOX SUPER FAMILY PROTEIN*	1.003	0.011
At2g24860	*CHAPERONE PROTEIN DNAJ-RELATED*	1.172	0.044
Stress and defence response			
At3g44480	*RECOGNITION OF PERONOSPORA PARASITICA 1* (*RPP1*)	−1.412	0.027
At2g38870	*PROTEASE INHIBITOR, PUTATIVE*	−1.091	0.048
At1g19670	*ARABIDOPSIS THALIANA CORONATINE-INDUCED PROTEIN 1* (*ATCLH1*)	−1.233	0.044
At2g43590	*CHITINASE, PUTATIVE*	−1.251	0.025
At1g66100	*THIONIN, PUTATIVE*	−2.096	0.006
At2g43620	*CHITINASE, PUTATIVE*	−2.061	0.006
At2g14610	*PATHOGENESIS-RELATED GENE 1* (*PR1*)	−2.142	0.027
At3g04720	*PATHOGENESIS-RELATED 4* (*PR4*)	−2.538	0.006
At2g34930	*DISEASE RESISTANCE FAMILY PROTEIN*	−1.678	0.015
At2g43530	*DEFENSIN-LIKE PROTEIN 194*	−1.419	0.018
At1g13609	*DEFENSIN-LIKE PROTEIN 287.*	−1.433	0.006
At5g44430	*PLANT DEFENSIN 1.2C* (*PDF1.2C*)	−4.011	0.006
At5g44420	*PLANT DEFENSIN 1.2* (*PDF1.2A*)	−4.011	0.006
At2g43510	*DEFENSIN-LIKE*)	−1.770	0.006

Table 1 (continued)

AGI	Gene name	Log_2 fold change ratio	*Q* value
At2g26010	*PLANT DEFENSIN 1.3* (*PDF1.3*)	−3.596	0.006
At2g26020	*PLANT DEFENSIN 1.2B* (*PDF1.2B*)	−3.596	0.006
Transport			
At4g35100	*PLASMA MEMBRANE INTRINSIC PROTEIN 3* (*PIP3A*)	0.920	0.018
At1g01620	*PLASMA MEMBRANE INTRINSIC PROTEIN 1C* (*PIP1C*)	0.827	0.030
At2g25060	*PLASTOCYANIN-LIKE DOMAIN-CONTAINING PROTEIN*	1.909	0.037
At5g48485	*DEFECTIVE IN INDUCED RESISTANCE 1* (*DIR1*)	2.396	0.006

Values are represented as log_2 fold changes of gene expression of treated leaves normalized to the untreated control leaves of three independent replications and the q value (adj. *P* value < 0.05). Gene annotations are based on the *Arabidopsis* TAIR10 annotation and represented by the *Arabidopsis* genome initiative (AGI) code. MapMan annotation was used to assign genes to 35 functional categories

observed down-regulated transcripts for jasmonate responsive transcripts, including *JACALIN-RELATED LECTIN 34* (*JAL34; At3g16460*) and *JASMONATE RESPONSIVE 1* (*JR1; At3g16470*) in lumichrome-treated *Arabidopsis* plants.

Stress and defence response

Treating *Arabidopsis* rosette leaves with lumichrome resulted in a distinct decrease in transcript levels for genes involved in systemic acquired resistance against pathogens (Table 1). This includes ten plant immunity-related transcripts such as *PATHOGENESIS-RELATED GENE 1* (*PR1; At2g14610*), *PATHOGENESIS-RELATED 4* (*PR4; At3g04720*), *DISEASE RESISTANCE FAMILY PROTEIN* (*At2g34930*), *DEFENSIN-LIKE PROTEIN 194* (*At2g43530*), *DEFENSIN-LIKE PROTEIN 287* (*At1g13609*), *PLANT DEFENSIN 1.2C* (*PDF1.2C; At5g44430*), *PLANT DEFENSIN 1.2* (*PDF1.2A; At5g44420*), *DEFENSIN-LIKE* (*At2g43510*), *PLANT DEFENSIN 1.3* (*PDF1.3; At2g26010*) and *PLANT DEFENSIN 1.2B* (*PDF1.2B; At2g26020*). Other than reduced plant immunity-related transcripts, lumichrome-treated plants showed increased levels of transcripts encoding *PLASMA MEMBRANE INTRINSIC PROTEIN 3* (*PIP3A*) and *PLASMA MEMBRANE INTRINSIC PROTEIN 1C* (*PIP1C*), both aquaporins involved in the salt stress response, and *DEFECTIVE IN INDUCED RESISTANCE1* (*DIR1*), which is a lipid transfer protein involved in systemic acquired resistance.

Validation of RNA-seq gene expression by RT-qPCR

To validate RNA-seq results, a number of differentially expressed transcripts were randomly selected for expression analysis by RT-qPCR (Supplementary Table S2). The up-regulated transcripts (*XTH9*, *RSW7*, *CYCA1;1*, *GASA1*, *ACO1*) and down-regulated transcripts (*LOX3*, *JAL34*) were analysed by RT-qPCR using *MON1* (AT2G28390) as reference gene. *MON1,* a member of the SAND family, has previously been identified as a useful reference gene in *Arabidopsis* expression studies (Czechowski et al. 2005) and was also invariant in the RNA-seq expression data. Comparisons between the RT-qPCR and RNA-seq analysis showed a positive correlation between the two approaches, indicating that the RNA-seq expression analysis performed is highly reliable (Fig. 2).

Discussion

There is ongoing rigorous research worldwide exploring a range of plant growth promoting compounds for use in improving crop production. Lumichrome is one such plant growth promoting rhizobacterial signal molecule that stimulates growth in a variety of plant taxa (Phillips et al. 1999; Zhang and Smith 2002; Dakora 2003; Matiru and Dakora 2005a, b; Khan et al. 2008; Gouws et al. 2012). Our results confirm its growth promoting effect, as demonstrated by significantly increased rosette leaf fresh and dry biomass. This prompted RNA-seq and RT-qPCR validation analysis to elucidate the molecular mechanisms allied to growth promotion on the gene transcription level.

Lumichrome induces genes involved in plant growth reconfiguration through the control of turgor-driven cell elongation and mitotic cell cycle

In plants, the balance between growth and cell cycle progression requires coordinated regulation of four different processes: macromolecular synthesis (cytoplasmic growth), turgor-driven cell wall extension, mitotic cycle expansion and endocycle. The up-regulated expression of *XTH9* and *EXPA4* transcripts following lumichrome treatment suggested the disruption of elaborate microtubule arrays, cellulose deposition and cell-wall thickening, thereby allowing cell wall loosening and turgor-driven cell enlargement (Wolf et al.

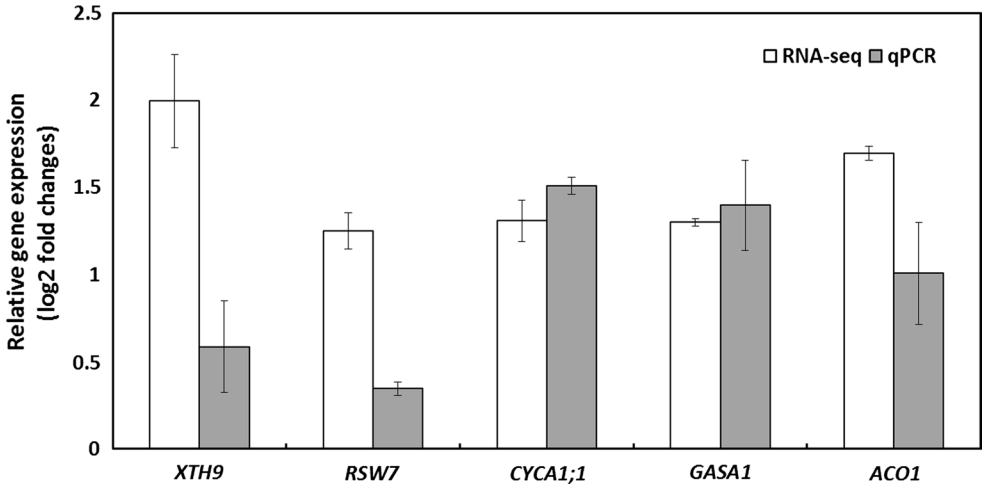

Fig. 2 RT-qPCR validation of differentially expressed genes from lumichrome-treated *Arabidopsis* RNA-seq data. Histograms represent relative transcript expression levels of RNAseq and RT-qPCR of lumichrome-treated against untreated rosette leaves. Log2 fold change of transcript levels was determined from replicates ($n = 3$) of each sample while for qPCR, the Ct values were averaged and normalized to *MON1* according to $E^{-\Delta\Delta}$Ct method (Ramakers et al. 2003). Error bars represent standard error (mean ± SE, $n = 3$). Relative expression of all transcripts was significantly different at $P \leq 0.05$

2012). In *Arabidopsis*, *XTH*s tend to be expressed strongly in rapidly dividing and expanding tissues (Hyodo et al. 2003; Jan et al. 2004), whilst expression of *EXPANSINs* improved cell wall loosening and promoted above-ground biomass in transgenic rice (Choi et al. 2003). *EXPANSINs* initiate the development of the leaf primordium, which later recapitulates the entire process of leaf formation (Pien et al. 2001), enhancing leaf cell size and results in larger leaves (Kuluev et al. 2012).

Besides this turgor-driven growth, increased expression of the core mitotic *CYCD3.3* and *CYCA1.1* transcripts suggested concurrent cell proliferation and cell differentiation during leaf development (Braybrook and Kuhlemeier 2010), which in turn possibly contributed to an increase in biomass. Cell cycle control and plant development are mainly integrated at the cell cycle checkpoints (G1/S and G2/M) and the molecular machineries involved (De Veylder et al. 2003; Inzé 2005). Expression of *CYCD3* is promoted by cytokinin (CK), hence loss of *CYCD3;1-3* activity reduces the capacity for exogenous CK to initiate shoot formation (Riou-Khamlichi 1999; Dewitte et al. 2007). Conversely, over-expression of *CYCD3;1* is sufficient to confer CK-independent shoot formation from callus tissue (Riou-Khamlichi 1999). In that regard, a positive correlation of increased biomass and increased expression of *CYCD3.3* following lumichrome treatment in our study could be due to a CK-independent activity that directly enhanced G2/M phase transition (Sorrell et al. 1999). In addition, the promoted G1/S transition phase was suggested through an increase in mitotic *CYCA1;1* transcripts. Increased core mitotic cycle transcripts were coupled with an increased *CASEIN KINASE ALPHA 1* (*CKA1*) expression, suggesting their reciprocal function in regulating mitotic cell cycle in plants (Reichheld et al. 1996). This is in agreement with reports that tissues with high mitotic activity, such as meristems, show a higher level of *CK2* transcripts, indicating a role for *CK2* in cell proliferation in these tissues. This has previously been shown in dominant negative mutant of *CK2* in *Arabidopsis*, which demonstrated an up-regulated expression of the core cell cycle-related genes at the G2/M transition (Moreno-Romero et al. 2011). Therefore, an increase in another kinase such as *CKA1* in lumichrome-treated plants suggested signalling of increased mitotic cell cycle transcripts, which further suggests increased cell division and expansion with a consequential increase in biomass.

Other than the increased core mitotic cyclins, transcripts that are involved in spindle assembly and cell cycle progression from G2 phase to metaphase, namely *SPIRAL1-LIKE3* (*SP1L3; At3g02180*) and *RADIALLY SWOLLEN 7* (*RSW7; At2g28620*) were also increased. *SP1L3* is required for cortical microtubule directional control of rapidly expanding *Arabidopsis* cells through directional deposition of cellulose microfibrils (Nakajima et al. 2006; Foteinopoulos and Mulder 2014). Defects in *SP1L3* phosphorylation impair events that participate in the spatiotemporal regulation of acentrosomal spindles, leading to mitotic defects which in turn result in enhanced ploidy, development arrest of apical meristems, ectopic meristem formation and defects in tissue patterning (Petrovská et al. 2012). *RSW7* plays a role in the formation of unique cellular structures such as the phragmoplast and the cell plate, both of which are required to divide the cell after nuclear division (Gillmor et al. 2016).

Mutations in *RSW7* retard growth by disrupting the normal pattern of wall placement (Wiedemeier et al. 2002).

Still on the theme of cell expansion and differentiation, our results demonstrated an induced expression of *PROTODERMAL FACTOR 1* (*PDF1*), which encodes a proline-rich cell-wall protein that is expressed exclusively in the protodermal tunica layer (L1) of shoot meristems (Abe et al. 2001). Together with *Arabidopsis thaliana MERISTEM LAYER1* (*AtML1*) and *PROTODERMAL FACTOR2* (*PDF2*), *PDF1* encodes an L1 box-binding homeodomain protein with high homeobox sequence similarity and shows expression exclusively in the L1 of vegetative meristem and epidermis of the *Arabidopsis* shoot apical meristem (SAM) and throughout the shoot development (Lu et al. 1996; Abe et al. 2003). Failure to differentiate epidermal cells in a *PDF2-1* and *AtML1-1* double mutant explains the role of these genes in the differentiation of epidermal cells from the L1 of shoot meristems (Abe et al. 2003). In that respect, due to their high homeobox sequence similarities, an increase in *PDF1* expression in the current study suggested its role in the differentiation of epidermal cells from the L1 of shoot meristems giving rise to cell division and differentiation of the SAM, resulting into an increase in above-ground biomass (Fletcher and Meyerowitz 2000).

Reciprocal hormonal cross-talk and signalling may play a role in enhanced *Arabidopsis* growth

Plant hormones play essential roles in coordinating external and internal signals to elicit the appropriate growth and developmental responses to precisely regulate responses to both temporal and spatial stimuli. A pronounced reduction in transcripts of genes involved in ethylene metabolism, such as *ACO1*, *AP2/EREBP* and a *C2H2 zinc finger protein,* was previously reported in *Lotus japonicus* and tomato (Gouws et al. 2012). We observed, however, increased expression of the ethylene biosynthesis-related *ACO1* transcript and the *AP2.4/EREBP* transcription factor (*At1g22190*), hence suggesting that the response might be species-specific. In addition, we observed increased transcript levels of a primary auxin response transcript, *SAUR54*, which positively regulates cell expansion to promote hypocotyl growth and leaf cell expansion (Spartz et al. 2012; Stamm and Kumar 2013). This response also suggested an auxin and ethylene cross-talk mechanism. Ethylene is produced from methionine (Met) via *S*-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylate (ACC) in which ACC synthase and ACC oxidase (ACO1), respectively, catalyze the last two steps in this biosynthetic pathway (Kende and Zeevaart 1997). Auxin is known to greatly stimulate ethylene production in vegetative tissues by inducing the synthesis or activation of *ACC SYNTHASE*, *CS1* and *ACO1* (Abel et al. 1995). As such, the increased *SAUR54* expression could have resulted in activation of *ACO1* and *AP2.4/EREBP* transcription in lumichrome-treated plants. Consistent with a previous study (Li et al. 2015), an increase in *SAUR54* expression might confer reduced sensitivity to ethylene, resulting in enhanced rosette growth in lumichrome-treated plants. Simultaneous increases in expression of *SAUR54* and endoplasmic reticulum (ER) localized RING *MEMBRANE-ANCHOR 2* (*At4g28270; RMA2*) transcripts might have positively regulated auxin transport across the ER membrane, thereby regulating cellular auxin homoeostasis and hormonal control of *Arabidopsis* vegetative growth (Peret et al. 2012; Bou-Torrent et al. 2014). A reciprocal increase in *SAUR54* and *GASA1* expression may be linked to increases in auxin and gibberellin signalling, respectively, which may in turn enhance growth via synergistic hormonal cross-talk between these two hormones. Classically, gibberellins are a growth-promoting class of phytohormones, regulating a wide range of growth and developmental processes throughout the life cycle of a plant, including leaf expansion, induction of flowering, as well as flower and seed development (Davière and Achard 2013). Auxin has previously been shown to regulate the expression of a number of gibberellic acid (GA) metabolic genes involved in the synthesis of active GAs in pea stem and *Arabidopsis* seedlings (Frigerio et al. 2006; Chapman et al. 2012). Our results suggest that an increase in *SAUR54* transcripts may have increased levels of auxin, which in turn up-regulated the expression of *GASA1* for GA metabolism. The transcriptional regulation between auxins and GA hormone pathways and their signalling role is, therefore, likely to be important for the synergistic cross-talk mediated cell division and cell expansion (Ross et al. 2000).

Jasmonates are oxylipin signalling molecules, including 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), and derivatives such as the methyl ester and amino acid conjugates of JA. JA is synthesized from linolenic acid, which is first oxygenated by lipoxygenase, to yield 13(*S*)-hydroperoxy linolenic acid (13-HPOT) (Vick and Zimmerman 1984). The *Arabidopsis* genome contains six lipoxygenase genes, of which *LOX2* (*At3g45140*), *LOX3* (*At1g17420*), and *LOX6* (*At1g72520*) contain chloroplast signalling peptides and show 13*S*-lipoxygenase activity, both features that are required for JA biosynthesis upon wounding and during senescence in leaves (Bell et al. 1995; He et al. 2002; Chung et al. 2008; Seltmann et al. 2010). The leaf senescence-promoting effect of methyl jasmonate (MeJa) is accompanied by chlorophyll loss and decreases in RuBisCO and photosynthesis (Weidhase et al. 1987), which is detrimental for plant growth and productivity. However, *LOX2*, *LOX3*, *LOX6, JAL34* and *JR1* were all down-regulated in response to lumichrome treatment. These genes are essential for JA biosynthesis in wounded or senescing leaves (Bell et al. 1995; Seltmann et al. 2010) except for *LOX3,* which is

transcribed in the roots and the transcript is transported to leaves and activated during leaf senescence (He et al. 2002; Chung et al. 2008). In that regard, decreased levels of these transcripts in the current study suggested a substantive role of lumichrome in delayed leaf senescence through the suppression of the effects of MeJa, namely chlorophyll loss and decreases in RuBisCO and photosynthesis. In addition, this response suggested a decrease in plant immunity against pathogen-induced injury through the expression of *LOX6,* which is essential for stress-induced jasmonate accumulation in *Arabidopsis* leaves (Chauvin et al. 2013). Reduced transcripts encoding *PATHOGENESIS-RELATED* (*PR1*; *PR4*), *PLANT DEFENSINS,* (*PDF1.2C; PDF1.2A; PDF1.3; PDF1.2B*) and *PLANT DEFENSIN-LIKE* (*At2g43530, At2g43530* At1g13609) also suggested reduced plant immunity against pathogens. *Arabidopsis thaliana* mutants that are impaired in JA production or perception exhibit enhanced susceptibility to a variety of pathogens, including fungal pathogens (Thomma et al. 1998; Norman-Setterblad et al. 2000). Similarly, growth promotion effects in lumichrome-treated plants were accompanied by down-regulation of a large suite of plant defence genes, including the jasmonic acid biosynthetic pathway, *PLANT PATHOGENESIS* and *DEFENSINS*. However, this study did not expose *Arabidopsis* to pathogenic attack or fungal infection, highlighting the need to challenge lumichrome-treated *Arabidopsis* with pests and pathogens to test the validity of the idea that their defence responses may be impaired.

Despite the possibility of reduced plant immunity against pathogens including fungi, lumichrome-treated plants exhibited increased expression of transcripts encoding *CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASE 41* (*CRK41*, also designated *DUF26 26* [Wrzaczek et al. 2010]; Table 1). The extracellular domain of CRKs encompasses two copies of the DUF26 (Domain of Unknown Function 26) domain, which contains three cysteine residues in a conserved configuration (C-X_8-C-X_2-C). The presence and spacing of the conserved cysteines in the DUF26 domain suggest that CRKs might be connected to ROS and redox signalling (Chen et al. 2004; Wrzaczek et al. 2010, 2013). Elevated transcript levels of several *CRK* genes trigger intracellular signalling cascades, allowing cells to respond and adapt to internal and external stimuli. For instance, CRKs play an important role as mediators of signalling specificity during regulation of stomatal aperture (Bourdais et al. 2015). Therefore, increased levels of transcripts encoding *CRK41,* coupled with up-regulated *PLASMA MEMBRANE INTRINSIC PROTEIN 3* (*PIP3A*) and *PLASMA MEMBRANE INTRINSIC PROTEIN 1C* (*PIP1C*), suggest a mutual functional relationship of redox modifications and plasma membrane permeabilities for better water and nutrient uptake and movement of sugars for metabolism in the plant (Viger et al. 2014). These responses are also known to improve photosynthetic efficiency and to increase biomass production (Farquhar and Sharkey 1982; Foyer and Shigeoka 2011).

Conclusion

The addition of 5 nM lumichrome elicited a growth promotion effect in *Arabidopsis* (Fig. 1). Based on our RNAseq data, we propose a model for growth enhancement via hormonal cross talk, intracellular signalling cascades and mitotic cell differentiation and expansion in lumichrome-treated plants. Firstly, increased abundance of *XTH9* and *AtEXP4* transcripts suggested loosening and rearrangement of the cell wall and subsequent cell expansion. Likewise, an increase in specific mitotic cell cycle genes (*CYCA1;1; CYCD3;3; SP1L3*; *RSW7*) suggested that the integration of cell division and expansion to developing leaves was promoted through the regulation of mitotic cell cycle phase and microtubule cellular organization and proliferation. Secondly, the reciprocal over-expression of *ACO1* and *SAUR54* suggested the synergistic ethylene–auxin cross-talk effect in which ethylene activated the auxin-signalling pathway and regulated *Arabidopsis* growth by both stimulating the auxin biosynthesis and by modulating the auxin transport machinery to the leaves (*SAUR54*; *RMA2*). Thirdly, simultaneous reduced expression levels of lipoxygenases (*LOX2*; *LOX3*; *LOX6*) and jasmonate-related transcripts (*JAL34; JR1*) suggested delayed jasmonate-associated leaf senescence, which might further contribute to improved chlorophyll biosynthesis and photosynthetic productivity. Although our study did not experimentally expose plants to stress conditions, we also observed an upregulated expression of stress induced genes associated to an ABA-independent dehydration and salinity stress signalling. While these findings gave us new insights and enhanced our knowledge of how lumichrome induces growth promotion in *Arabidopsis*, reverse genetic analysis with mutants and over-expressor lines, using some of these genes, will help show that these genes are actually involved.

Author contribution statement MP, JK, JL and PH designed the research. The research presented in this manuscript forms a part of the PhD dissertation for MP at Stellenbosch University and which may be accessed online at http://scholar.sun.ac.za/handle/10019.1/101430. MP conducted all the experiments and analysed the data. BC and HM conducted the RNA-seq bioinformatics analysis and PY the RT-qPCR experiments. MP, JK, JL and PH prepared the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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