

**Molecular analysis of plant growth promoted with low molecular weight compounds in relation to genetically altered photosynthetic carbohydrate partitioning in higher plants**

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*Dissertation submitted in fulfillment of the academic requirements for the degree of  
Doctor of Philosophy in Plant Biotechnology at Stellenbosch University*



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March 2017

## **Declaration**

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This dissertation includes 3 original papers which will be submitted to the peer-reviewed journal *Frontiers in Plant Physiology*. The development and writing of these unpublished papers was the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors. The findings of this work were presented at the 43<sup>rd</sup> Annual Congress of the South African Association of Botanists (8-11 January 2017, Cape Town, South Africa).

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## Abstract

Despite the impressive advances that have been made over the years in improving both plant growth and yields, there is little reason to become complacent about these developments, especially with regard to food supply for the world's increasing population. Other than conventional and marker assisted breeding, the use of crude extracts from plant growth-promoting rhizobacteria (PGPR) bacteria that possess biostimulatory properties has been considered as an alternative practice for enhancing plant development and crop productivity. One such substance is lumichrome (*7,8 dimethylalatoxazine*), a novel plant growth promoting multitrophic signal molecule produced by the bacterium *Sinorhizobium meliloti*. Lumichrome has been shown to elicit growth promotion and trigger a compensatory increase in whole-plant net carbon assimilation through enhanced starch accumulation and altered ethylene metabolism. Despite physiological experimental advances in exploring the growth stimulatory mechanisms of lumichrome, a comprehensive molecular analysis in various plant species still remains elusive.

This study focused on understanding the genetic, molecular and biochemical regulatory networks as key determinants for crop growth and productivity in relation to lumichrome in *Arabidopsis thaliana*. Our specific objectives focused on unravelling i) global changes in gene expression, ii) protein expression and iii) key metabolites or biochemical pathways which were seemingly affected by lumichrome treatments in *Arabidopsis thaliana*. The study further made use of *Arabidopsis* mutant lines deficient in the primary gene encoding the *ADP-GLUCOSE PYROPHOSPHORYLASE* small subunit (*APSI*) to study the regulatory mechanisms of carbon metabolism. Subsequently, the findings were used to infer functional interactions among genes, proteins and metabolites in order to speculate on the possible control mechanism(s) involved.

Application of biologically-active levels of lumichrome has been demonstrated to enhance plant development through changes in photosynthetic rates, leaf stomatal conductance and transpiration in several plant species. Enhanced growth is reported to be attributed to xylem transport and *in situ* accumulation of lumichrome in leaves, which subsequently triggers events that promote cell division and leaf expansion in both monocots and dicots. However, previous studies have also demonstrated mixed physiological responses between plants

species to lumichrome, hence molecular processes responsible for growth promotion in other species remain somewhat elusive. Consistent with previous studies, our study demonstrated that the addition of 5 nM lumichrome to *Arabidopsis thaliana* plants elicited a growth promoting effect to increase overall plant size and biomass. The increased overall plant size was attributed to enhanced photosynthesis and the use of higher levels of photoassimilates for cell division and cell enlargement. This was further supported by Next Generation Sequencing (Transcriptomic profiling-True-Seq) which revealed that the growth stimulatory effect was effected predominantly through genes associated to cell wall modification, cell division and expansion. Our proteomics study results further suggested that lumichrome treatment enhances and stabilizes photosynthesis, providing increased photoassimilates for growth in wild type *Arabidopsis*. Although starch levels were increased in lumichrome-treated wild type plants, levels of APS1 were unexpectedly decreased.

To demonstrate that silencing *APS1* enhances *Arabidopsis* growth, we profiled both proteins and metabolites in *Arabidopsis* T-DNA knockout lines which were deficient in APS1. The reverse genetics approach revealed that enhanced growth of *aps1* mutant plants relative to the wild type can be ascribed to enhanced photosynthetic efficiency, which ensured the provision of energy and carbon supply for *Arabidopsis* growth. The results further indicated that similar levels of enhanced growth and photosynthesis following lumichrome-treatment of wild type plants could be achieved in the knockout plants even in the absence of lumichrome. There was no further effect on the growth of these mutant lines following lumichrome, strongly suggesting that APS1 is responsible for mediating the lumichrome-associated growth response in *Arabidopsis*. Unlike wild type plants, starch levels in the *aps1* lines were extremely low and were not affected by lumichrome treatment. We therefore conclude that lumichrome enhances growth in *Arabidopsis* plants via enhanced photosynthesis in a process mediated via APS1, and that the enhanced levels of starch seen in lumichrome treated wild type plants are merely an artifact of this enhanced photosynthesis. It will be interesting to investigate further the means by which APS1 regulates this mechanism. In summary, proteomic and metabolomic analyses all suggest that down-regulation of APS in lumichrome-treated plants enhanced photosynthesis, leading to increased availability of C for enhanced plant growth.

## Opsomming

Ten spyte van die indrukwekkende vooruitgang wat gemaak is oor die jare in die verbetering van beide plantgroei en -opbrengste, is daar min rede om selfvoldaan oor hierdie ontwikkelings te raak, veral ten opsigte van voedselvoorsiening vir die wêreld se groeiende bevolking. Anders as konvensionele en merkerbemiddelde teling, word die gebruik van ru-uittreksels uit plantgroei-bevorderende-rhizobakterieë (PGPR) wat biostimulerende eienskappe besit, beskou as 'n alternatiewe praktyk vir die verbetering van plantontwikkeling en gewas-produktiwiteit. Een sulke stof is lumichrome (*7,8 dimethylalatoxazine*), 'n nuwe plantgroei-bevorderende multitrofiese seinmolekule, wat deur die bakterie *Sinorhizobium meliloti* afgeskei word. Lumichrome is bewys om groei-bevordering te ontlok, gepaard met 'n kompenserende toename in geheel-plant netto-koolstof-assimilasie deur verbeterde stysel-opeenhoping en veranderde etileen-metabolisme. Ten spyte van vooruitgang in die fisiologiese verkenning van die groei-stimulerende meganismes van lumichrome, is 'n omvattende molekulêre analise in verskillende plantspesies steeds ontwykend.

Hierdie studie fokus op die begrip van die genetiese, molekulêre en biochemiese regulatoriese netwerke as sleutelfaktore vir gewasgroei en produktiwiteit met betrekking tot lumichrome in *Arabidopsis thaliana*. Ons spesifieke doelwitte het gefokus daarop om die volgende te ontrafel: i) globale veranderinge in geenuitdrukking, ii) proteïen uitdrukking en iii) die belangrikste metaboliete of biochemiese-paaie in *Arabidopsis thaliana* wat skynbaar beïnvloed word deur behandeling met lumichrome. Om die regulatoriese meganismes van koolstofmetabolisme te bestudeer, het hierdie studie verder gebruik gemaak van gemuteerde *Arabidopsis*-lyne met 'n tekort in die primêre geen wat die *ADP-GLUKOSE PYROPHOSPHORYLASE* klein subeenheid (*APSI*) encodeer. Daarna is die bevindinge gebruik om funksionele interaksies tussen gene, proteïene en metaboliete af te lei om sodoende te spekuleer oor die moontlike beheermeganisme(s) wat betrokke is.

Die aanwending van biologies-aktiewe vlakke van lumichrome is getoon om plantontwikkeling te verbeter in verskeie plantspesies deur veranderinge aan te bring in die tempo van fotosintese, blaarhuidmondjie geleiding en transpirasie. Verbeterde groei word na bewering toegeskryf aan die vervoer van lumichrome deur die xileem en *in situ* versameling in die blare, waar dit gevolglik gebeur veroorsaak wat seldeling en blaar-uitbreiding bevorder (in beide monokotiele en dikotiele). Tog het vorige studies ook gemengde fisiologiese reaksies tussen plantspesies getoon ten opsigte van lumichrome-behandeling.

Daarom blyk molekulêre prosesse wat verantwoordelik is vir groei bevordering in ander spesies ietwat ontwykend. In ooreenstemming met vorige studies, het ons studie getoon dat die toevoeging van 5 nM lumichrome tot *Arabidopsis thaliana* plante groei-bevordering ontlok wat 'n algehele verhoging in plantgrootte en biomassa tot gevolg het. Die verhoging in plantgrootte word toegeskryf aan verbeterde fotosintese en die gebruik van hoër vlakke van fotosintese-produkte vir seldeling en selvergroting. Dit word verder ondersteun deur "Next Generation Sequencing" (Transcriptomic profiling-True-Seq), wat aan die lig gebring het dat die groei-stimulerende effek oorwegend bewerkstellig word deur gene wat verband hou met selwandverandering, seldeling en uitbreiding. Die resultate van ons proteomika studie het verder voorgestel dat lumichrome-behandeling fotosintese verhoog en stabiliseer, en sodoende verhoogde fotosintese-produkte verskaf vir groei in wilde-tipe *Arabidopsis*. Hoewel styselvlakke verhoog was in wilde tipe plante wat met lumichrome behandel is, het die vlakke van APS1 onverwags afgeneem.

Om te demonstreer dat die afskakeling van *APS1* groei in *Arabidopsis* verhoog, het ons beide proteïene en metaboliete bestudeer in *Arabidopsis* T-DNA 'knockout' lyne met 'n tekort aan APS1. Die tru-genetika benadering het getoon dat verbeterde groei van *aps1* gemuteerde plante, in vergelyking met die wilde-tipe plante, toegeskryf kan word aan verbeterde fotosintetiese-doeltreffendheid wat die energie- en koolstoftoevoer verskaf vir groei in *Arabidopsis*. Die resultate het verder aangedui dat soortgelyke vlakke van verbeterde groei en fotosintese as gevolg van lumichrome-behandeling van wilde tipe plante bereik kan word in die 'knockout' gemuteerde plante, selfs in die afwesigheid van lumichrome. Die aanwending van lumichrome het geen verdere effek op die groei van hierdie 'knockout' plante gehad nie, wat daarop dui dat APS1 verantwoordelik is vir die bemiddeling van die lumichrome-geassosieerde groei reaksie in *Arabidopsis*. In teenstelling met wilde-tipe plante, was die styselvlakke in die *aps1* gemuteerde plante bitter laag en ongeaffekteer deur behandeling met lumichrome. Ons kan dus aflei dat lumichrome groei in *Arabidopsis* plante verhoog deur verbeterde fotosintese in 'n proses bemiddel deur APS1, en dat die verhoogde styselvlakke wat waargeneem is in lumichrome-behandelde wilde-tipe plante slegs 'n byproduk is van hierdie verbeterde fotosintese. Dit sal interessant wees om die manier waarop APS1 hierdie meganisme reguleer verder te ondersoek. Om op te som, dui proteomiese en metabolomiese analises daarop dat die vermindering van APS in lumichrome-behandelde plante fotosintese verbeter, wat gevolglik lei tot die verhoogde beskikbaarheid van koolstof vir beter plantgroei.

## Acknowledgements

I would like to acknowledge my supervisors, Professor Jens Kossmann and Dr Paul Hills for academic guidance and tremendous financial supports towards my studies. To my co-supervisor, Dr James Lloyd, your valuable technical and scientific research oriented inputs and professional ideas are really appreciated. My sincere gratitude to Dr Christell van der Vyver, for always procuring chemicals in time and also providing other laboratory facilities during the tenure of my studies. Thank you to Ingrid Jacobs for assistance in translating the abstract into Afrikaans.

Gratitude is expressed to the following valuable collaborations:

Waltraud X. Schulze (University of Hohenheim-Germany), Alisdair Fernie, Saleh Alseekh and Stephanie Arrivault (Max-Planck Institute for Molecular Plant Physiology, Potsdam-Golm-Germany) for their respective proteomics and metabolomics analytical and bioinformatics services. Many thanks to Hans J. Maree and Beatrix Coetzee (Department of Genetics (Stellenbosch University) as well Phillip Young (Institute of Wine Biotechnology-University of Stellenbosch) and Alexander Valentine (Department of Botany and Zoology-University) for RNA-seq data analyses, real time PCR and IRGA data collection respectively.

My Fiancée, Laurie, thank you for your amazing patience, perseverance and support during hard times and good times.

Above all, to God be the glory, this consecration and anointing that always breaks every chain. Thank you for the grace and mercy upon my life and I can confidently say *“I can do all things through Christ who strengthen me.....Little children you have overcome, because greater is He that is in you than the one in the world” .....Mountains shall depart...but my kindness shall not depart from you, neither shall the covenant of my peace be removed, says the Lord that has mercy on you”, Therefore in everything, I give thanks and praise to God.*

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## CHAPTER 1

### General introduction

#### Understanding the functional cellular processes that underpin growth

The plant leaf, by providing the basis for energy capture, is at the core of plant growth and ultimately an important part of human economic activities. Throughout their development, *Arabidopsis* leaves undergo different growth stages to mature into planar, photosynthetic organs organized in a flat rosette with little overlap (Rodriguez et al., 2014). During the vegetative developmental phase of *Arabidopsis thaliana*, leaves arise from the shoot apical meristem (SAM) as rod-shaped structures in a spiral pattern with very short internodes, which initially grow exclusively by cell proliferation (Rodriguez et al., 2014). The SAM is the source of all cells that ultimately form the shoot, including the subset that ends up building the leaves (Traas and Monéger, 2010). Primary morphogenesis follows when leaf growth is sustained by successive cell divisions and specific structures, such as trichomes, vasculature, and stomata, begin to form. Lastly, during secondary morphogenesis, the cells cease proliferating and begin to expand, mainly by cell wall loosening that continues to fuel further leaf growth (Cosgrove, 2005). The growth of organs and whole plants depends on both cell growth (Braybrook and Kuhlemeier, 2010; de Reuille et al., 2006) and cell-cycle progression regulation (Beemster et al., 2005b; Boudolf et al., 2009; Donnelly et al., 1999). The balance between growth and cell-cycle progression requires coordinated regulation of four different processes, namely cytoplasmic growth, turgor-driven cell-wall extension, the mitotic cycle, and the endocycle.

#### *Cytoplasmic and turgor-driven cell wall expansion growth*

Growth ultimately results from the net accumulation of macromolecules and cellular components (cytoplasmic growth) while cell expansion refers to increased cell volume caused by enlargement of the vacuole. The volume of individual cells increases through cytoplasmic growth and turgor-driven cell-wall extension. Cytoplasmic growth and the cell cycle provide the building blocks required for plant growth, but the space that can be occupied by these building blocks is constrained by the cell walls. Cell enlargement requires that the walls yield to the cell's turgor pressure and cell-wall relaxation appears to be the major control point in this process. Controlled cell-wall relaxation is made possible by the action of different proteins on the cell wall matrix which weakens the cell wall, allowing it to

yield to turgor and maintaining cell wall plasticity (Wolf et al., 2012). Cytoplasmic and turgor-driven cell wall expansion growth is regulated by numerous cell wall proteins such as expansins (EXPs) and xyloglucan endotransglucosylase/hydrolases (XTHs) (Cosgrove, 2000a; Rose *et al.*, 2002; Sampedro and Cosgrove, 2005; Van Sandt *et al.*, 2007; Eklöf and Brumer, 2010).

Expansins are believed to bind to the surface of cellulose microfibrils, thereby disrupting the hydrogen bonds formed with xyloglucan and allowing the wall to extend (Cosgrove, 2000a; Darley et al., 2001; Li et al., 2003). Ectopic expression of *EXPA1* in cucumber initiated development of the leaf primordium, which later developed into a normal leaf (Pien *et al.*, 2001). These authors also suggested that local expression of expansins within the meristem induces a developmental program that recapitulates the entire process of leaf formation. Furthermore, Cho and Cosgrove (2000), demonstrated considerably greater *EXP10* expression in young growing petioles and leaf blades than in older non-growing leaves in *Arabidopsis*. Also in recent studies, overexpression of *EXPA10* from *Arabidopsis* and *EXPA1* from poplar were reported to significantly affect tobacco leaf cell sizes, resulting in larger leaves (Kuluev et al., 2012). Interestingly, overexpression of guard cell wall expressed expansins such as *EXPA1* and *Vicia faba EXPA1* in *Arabidopsis* and tobacco respectively, has been reported to increase the rate of light-induced stomatal opening, while inhibition reduced the sensitivity of stomata to the same stimuli. An increase in light-induced stomatal opening in *Arabidopsis EXPA1* and *Vicia faba EXPA1* overexpressing plants was accompanied by an increase in transpiration and photosynthetic rate, which clearly indicates that expansins participate in the regulation of stomatal movement by modifying the cell walls of guard cells (Wei et al., 2011a, 2011b). In contrast, suppression of *EXPB2* in rice resulted in significant physiological changes, including a significant reduction in the width of leaf blades (Zhou et al., 2015).

In addition to expansins, a class of enzymes known as xyloglucan endotransglucosylases/hydrolases (XTHs), catalyzes the endo-cleavage of xyloglucan polymers and the subsequent transfer of the newly generated reducing ends to other polymeric or oligomeric xyloglucan molecules ( Rose *et al.*, 2002; Yokoyama and Nishitani,

2001). XTHs have figured prominently in plant cell wall models, due to the potential of these enzymes to cause transient matrix cleavage without hydrolysis, thus providing a potential molecular mechanism for controlled, turgor-driven wall expansion (Rose et al., 2002). In *Arabidopsis*, it has been reported that *XTH9* tended to be expressed strongly in rapidly dividing and expanding tissues, suggesting that it functions in the development and morphogenesis of tissues close to shoot apices (Hyodo et al., 2003). Similarly, *in situ* hybridization and *XTH8* promoter GUS fusion analysis in rice revealed that *XTH8* was highly expressed in vascular bundles of leaf sheath and young nodal roots where the cells are actively undergoing elongation and differentiation (Jan et al., 2004). Taken together, the above-mentioned reports highlight the significance of expansin and XTH genes in leaf development and growth through the disruption of elaborate microtubule arrays, cellulose deposition and cell-wall thickening thereby resulting in cell division and expansion.

#### ***Function of mitotic cell-cycle as a growth regulator***

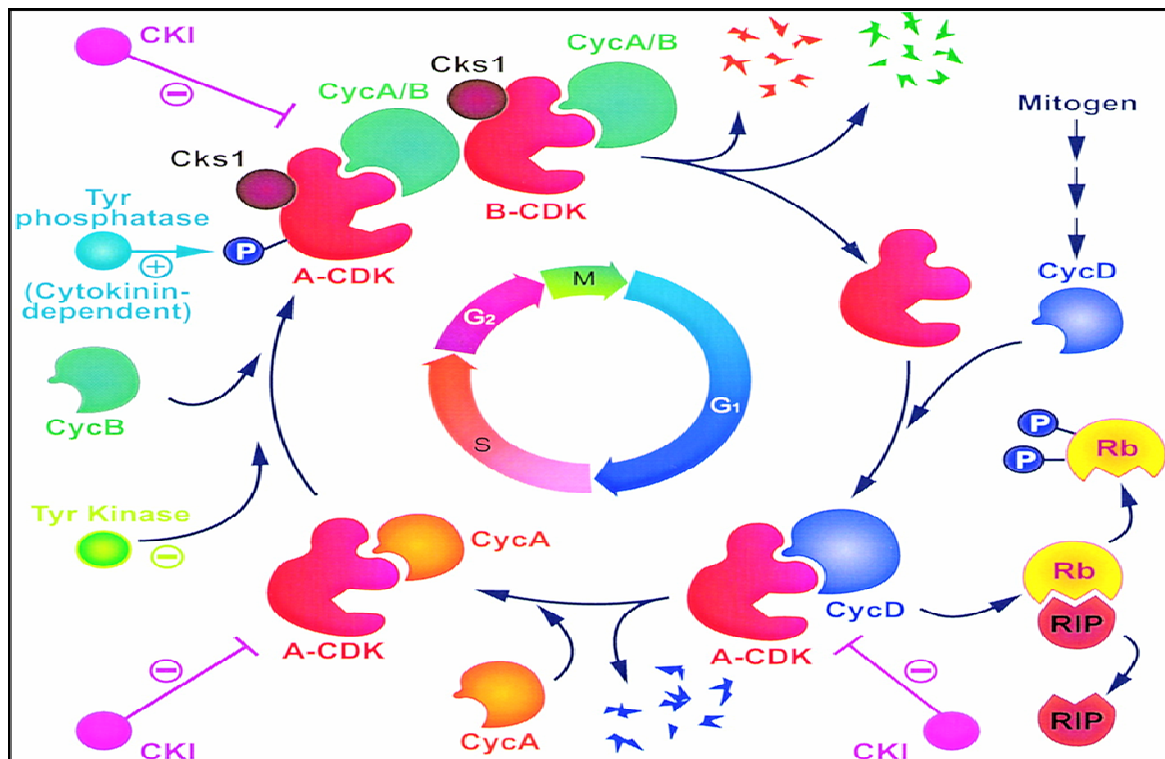
In plants, post-embryonic developmental processes are localized to meristems, organized regions of cell proliferation that have the property of self-perpetuation, such as the shoot apical meristem, and to meristematic regions where cell proliferation occurs. Some regulatory proteins, such as cyclins and inhibitors of cyclin-dependent kinases, are particularly numerous in plants, possibly reflecting the remarkable ability of plants to modulate their post-embryonic development. Cell proliferation and cell expansion are tightly controlled in order to generate organs with a determinate final size such as leaves. As cell division ceases, the cell continues expanding and this transition from division to expansion is manifested as a cell cycle arrest front which remains at a fixed position for a particular time period and then moves rapidly toward the base of the leaf blade (Donnelly et al., 1999; Andriankaja et al., 2012). After a few days, this cell cycle arrest front abruptly disappears at the base of the leaf and further leaf growth is driven by cell expansion (Kazama et al., 2010; Andriankaja et al., 2012) and the asymmetric division of meristemoids (Geisler, 2000; Kazama et al., 2010; Gonzalez et al., 2012). As such, cell cycle regulation is of pivotal importance for plant growth and development including cell proliferation within tissues and cell differentiation (Donnelly et al., 1999).

Cell cycle-regulated gene expression plays an important role in the activity of cell cycle regulators and it is therefore of pivotal importance for correct leaf growth and to achieve final organ size. Cell cycle-regulated gene expression plays major roles in both cell cycle control and progression of cells through the cycle. The four phases of the mitotic cell cycle are conserved in all eukaryotes and consist of four distinct phases, including an undifferentiated DNA pre-synthetic phase with a 2C nuclear DNA content (G1 phase), DNA synthesis phase with a nuclear DNA content intermediate between 2C and 4C (S phase), a second undifferentiated phase (DNA post-synthetic phase) with a 4C nuclear DNA content (G2 phase) and the ultimate mitosis (M phase). Progression through these different phases needs to be tightly controlled at the molecular level in order to allow correct transmission of the genetic information (Harashima et al., 2013; De Veylder et al., 2003).

Upon mitogenic stimulation (Fig.1), D-type cyclins (CYCD) are produced and associate with the A-type Cyclin-dependent kinases (CDKA). The resulting CDKA/CYCD complexes phosphorylate a retinoblastoma-like protein (pRb), resulting in the release of pRB-interacting proteins (RIP) that in turn trigger the onset of S phase, hence CDKA/CYCD association controls the Gap1 phase/Synthesis phase (G1/S) transition (Dewitte and Murray, 2003; Menges et al., 2006; De Veylder et al., 2003). Although expression of *CYCD3* genes is promoted by cytokinins (CK), overexpression of *CYCD3;1* is sufficient to confer CK-independent shoot formation from calli, while loss of *CYCD3;1-3* activity reduces the capacity for exogenous CK to regulate shoot formation (Dewitte et al., 2007; Riou-Khamlichi, 1999). Further studies on the role of *CYCD* showed that overexpressing *Arabidopsis CYCD2.1* with the 35S promoter in tobacco stimulated growth, hence substantiating that Cyclin D gene is capable of improving plant growth rates and accelerating plant development (Beemster et al., 2005a; Cockcroft et al., 2000). On the contrary, overexpression of *CYCD2.1* in *Arabidopsis* did not accelerate growth of either root or leaf, but resulted in abnormal morphology in *Arabidopsis* (Sanz et al., 2011; Zhou et al., 2003). Similarly, overexpression of *CYCD3.1* in *Arabidopsis* resulted in abnormal organs, including curled leaves due to an increased number and smaller size of epidermal cells. In addition, it was difficult for the transgenic plants to develop distinct spongy and palisade mesophyll layers (Dewitte et al., 2003). These previous studies proposed that *CYCD3s* can either promote mitotic cell division or inhibit endoreduplication and cellular differentiation

processes that are generally incompatible with the indeterminate, mitotic nature of meristematic cells (Dewitte et al., 2003).

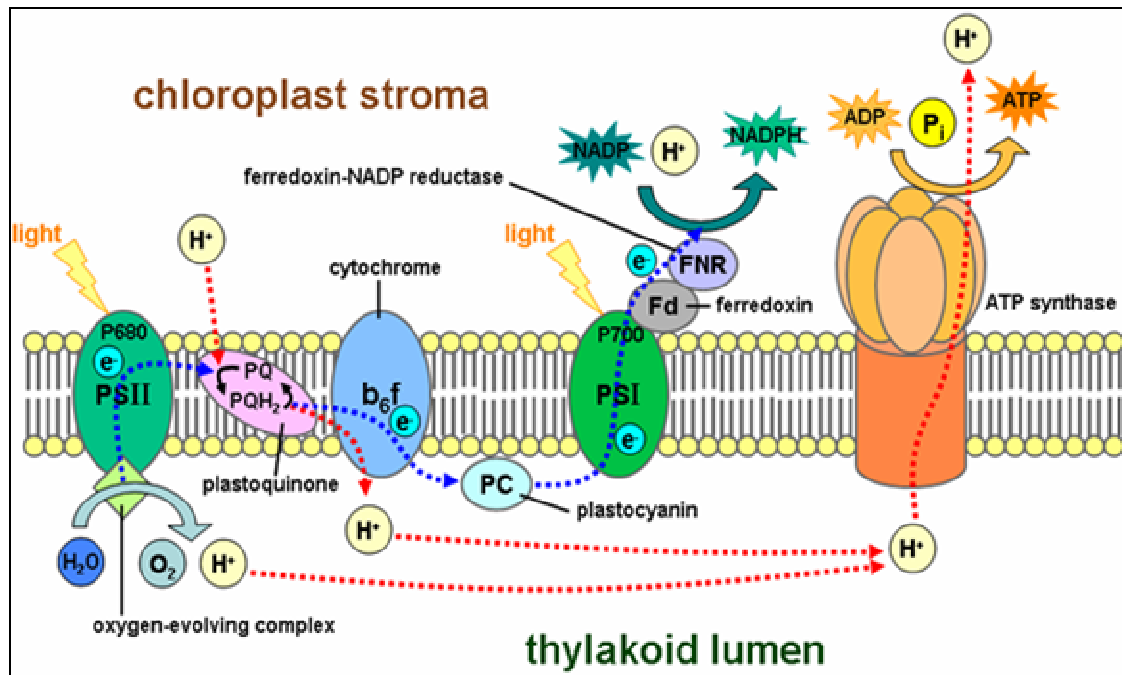
During the S phase, A-type cyclins (CYCA) are synthesized to activate A-CDKs, and CDK activity is inhibited by tyrosine phosphorylation as cells reach the end of the S phase. During the Gap 2 (G2) phase, B-type cyclins (CYCB) appear and associate with CDKA and CDKB, both with kinase activity, at the Gap2/ Mitotic (G2/M) transition point. The correct functioning and activation of the mitotic CDKs requires association with CKS1 docking factors (Cks1) and the cytokinin-dependent removal of the inhibitory phosphate group. Cyclin A1, in complexes with a succession of various CDK partners, may control microtubule dynamics, as suggested by its association with all appropriate structures throughout the cell cycle (Fig. 1) (Mironov et al., 1999).



**Figure 1.** Proposed model for mitosis cell cycle regulation in plants (Mironov et al., 1999). The four phases of the mitotic cell cycle are conserved in all eukaryotes and consist of four distinct phases; G1 phase, S phase, G2 phase and the ultimate M phase.

**Photosynthesis: capturing solar energy and the conversion of this energy into vegetative tissues that can accumulate plant biomass**

Through its photosynthetic capacity, the leaf provides the basis for growth of the whole plant. Cells are both the basic building blocks of the leaf and the regulatory units that integrate genetic and environmental information into the developmental program. The plant leaf, by providing the basis for energy capture, is at the core of plant growth and ultimately an important part of human economic activities. Plants, unlike animals, are auxotrophic multicellular organisms that utilize solar energy, carbon dioxide and water to make sugars and other organic compounds and thereby biomass (Demura and Ye, 2010). Therefore, the production of plant biomass depends on many factors, including the ability to capture solar energy and the conversion of this energy into vegetative tissues that can accumulate plant biomass. Accumulation of biomass in the vegetative stage of plant growth can be considered as the ultimate expression of its metabolic activities (Meyer et al., 2007). In that regard, oxygenic photosynthesis is a highly-regulated, chloroplast-defined, energy-transducing, multi-step process which involves a series of electron transfer reactions resulting from water oxidation at the Photosystem II (PSII) lumen side, to  $\text{NADP}^+$  on the stromal side of Photosystem I (PSI) (Fig. 2). The energy conversion involves the absorption of photons by antenna pigments in the light harvesting complexes (LHCs), which then transfer the excitation energy to the reaction centers of PSII and PSI, driving the primary photochemical reactions and creating charge separation (Baker et al., 2007). The resultant light-driven charge separation is responsible for the linear electron flow between PSII and PSI through plastoquinol (PQ), cytochrome-b6f complex (Cyt-b6f) and plastocyanin (PC) (Baker et al., 2007). Coupled to this linear electron flux is the release of protons during water oxidation at the lumen side of PSII, and cyclic electron flow into the thylakoid lumen due to the Q-cycle at cyt-b6f, generating a pH gradient across thylakoid membrane ( $\Delta\text{pH}$ ) that contributes to ATP synthesis from ADP and Pi (Merchant and Sawaya, 2005; Nelson and Ben-Shem, 2004) and triggers the protective process of nonphotochemical quenching (NPQ) under stress conditions (Baker et al., 2007; Heber, 2002; Johnson, 2011; Joliot and Johnson, 2011). The products from this photosynthetic electron transport chain (ETC), such as chemical energy (ATP) and reducing power (NADPH), are subsequently consumed by metabolic reactions like the Calvin cycle, resulting in incorporation of  $\text{CO}_2$  from the atmosphere into carbohydrates which are transported to all other plant organs to meet their energy demands.



**Figure 2.** Overview of the light dependent photosynthetic electron and proton transport in the thylakoid membrane and associated reactions (Meyer et al., 2009). In the chloroplast's thylakoid membranes, antenna pigments in the light harvesting complexes (LHCs) absorb photons and transfer the excitation energy to the reaction centres of PSII and PSI. Light-driven charge separation is responsible for the linear electron flow between PSII and PSI through plastoquinol (PQ), cytochrome-b6f complex (Cyt-b6f) and plastocyanin (PC) to ferredoxin (Fdx). Reduced Fdx reduces either thioredoxins (Trx) via ferredoxin:thioredoxin reductase (FTR) or NADP via ferredoxin:NADP reductase (FNR), while protons released into the thylakoid lumen in the oxidation of H<sub>2</sub>O and by PQ are used for the synthesis of ATP.

***Chloroplast functioning as an integral control mechanism for photosynthetic electron transport chain (PETC) redox homeostasis***

The chloroplast is an important apparatus for photosynthetic efficiency. Photosynthetically-active chloroplasts are central organelles that develop during the expansion phase, providing the plant with energy (Sakamoto et al., 2008). Chloroplast biogenesis and development needs to be coordinated with seedling growth to ensure optimal rates of photosynthesis without oxidative damage upon seedling emergence. In higher plants, chloroplasts develop from

proplastids, small organelles that are present primarily in meristematic cells and in young post-mitotic cells (Vothknecht and Westhoff, 2001). As meristematic cells begin to differentiate into mesophyll and palisade cells, proplastids coordinately differentiate into chloroplasts. This differentiation process is also modulated by environmental cues such as light, and thus certain *Arabidopsis* mutants with an altered light signal transduction pathway are able to develop leaves even in the absence of chloroplast development (Li et al., 1994). The conversion of proplastids into functional chloroplasts is accompanied by high transcription levels of plastid- and nuclear-encoded genes involved in the transcription/translation apparatus (Baumgartner et al., 1989), because nucleus and chloroplasts, have to exchange information to regulate photosynthesis. However, expression of these genes decreases once the mature chloroplast is established, suggesting that one central regulatory point during chloroplast differentiation is the activation of chloroplast transcription (Kusumi et al., 2010; Mullet, 1993)

Among others, chaperones have crucial roles in chloroplast biogenesis and functioning. For instance, plastid CPN60A null or deficient mutations negatively affect photosynthesis due to abolished greening of plastids, resulting in an albino phenotype with no developed chloroplasts and impaired plastid division and reduced chlorophyll levels respectively. This further results in severely impaired plant development (Peng et al., 2011; Suzuki et al., 2009). In addition, chaperones are essential for chloroplast protein import and processing through the outer and inner envelope (membranes) of the chloroplasts which is in turn essential in chloroplast biogenesis and maturation. The majority of chloroplast-targeted proteins enter the plastids via the translocon on the outer envelope of chloroplast (TOC) and translocon on the inner envelope of chloroplast (TIC), while others are targeted via the endoplasmic reticulum (Kessler and Schnell, 2009). Loss of function of proteins such as stromal chloroplastic heat shock protein 70 (cpHSC70; cpHSC70-1 or cpHSC70-2), which are involved in chloroplast import and processing, leads to a white seedling phenotype and seedling lethality (Cline and Dabney-Smith, 2008; Shipman-Roston et al., 2010), with a consequential negative effect on photosynthetic efficiency.

Photosystem I (PSI) and photosystem II (PSII), as previously discussed, are the two photosystems in the thylakoid membranes that function as charge-separation devices in the process of photochemical energy transduction. An imbalance of energy distribution between



the two photosystems tends to occur in natural environments, where light quality and quantity fluctuate with time (Bellafiore et al., 2005). Therefore, there is a need to constantly balance their excitation levels to ensure optimal efficiency of electron flow. Thus highly flexible regulation of photosynthetic light reactions in plant chloroplasts is a prerequisite to provide sufficient energy flow to downstream metabolism and plant growth, to protect light reactions against photodamage, and to ensure controlled cellular signaling from the chloroplast to the nucleus. In that respect, chloroplast retrograde signaling and the subsequent response in the chloroplast is integral to the photosynthetic electron transport chain (PETC) redox homeostasis. Such chloroplast retrograde signaling occurs via the control of excitation energy transfer between the two photosystems (PSII and PSI), electrochemical gradient across the thylakoid membrane ( $\Delta pH$ ), and of electron transfer from PSII to PSI electron acceptors (Munekage et al., 2002; Tikkanen and Aro, 2014). The chloroplast not only coordinates the expression of nuclear and chloroplast genes for chloroplast biogenesis, photosynthesis efficiency, photoprotection, and the maintenance of chloroplast functioning at optimal levels in response to fluxes in metabolites, but also influences other aspects of plant development and adaptation (Chi et al., 2015). The following review will not exhaust the entire retrograde signaling mechanisms during state transitions, rather, a particular emphasis will be on chlorophyll tetrapyrrole synthesis, the oxygen evolving complex (OEC),  $F_0F_1$ -ATPase synthase and the role of photosynthetic metabolites controls.

### ***Chlorophyll is essential for photosynthetic efficiency***

Chlorophyll is one of the most abundant biological molecules on earth and plays an essential role in global carbon cycling through plant solar energy capture and photosynthetic carbon fixation. Thus it plays an essential role in photosynthesis by harvesting light energy and driving electron transfer (Nelson and Yocum, 2006). Precursor molecules of chlorophylls, which are a major component of photosynthesis, act as a chloroplast-derived signal (Kobayashi et al., 2009). Thus as light-harvesting antennae, they act as photon funnels, absorbing light and channeling its energy to reaction centers (RCs) (Hohmann-Marriott and Blankenship, 2011; Kusaba et al., 2009), where chlorophylls perform their functions including photochemical charge separation to create strong oxidants and reductants and sending low-potential electrons through the electron transport chain (ETC) (Hohmann-Marriott and Blankenship, 2011). The chlorophyll precursors, tetrapyrroles, have been

implicated in the coordination of chloroplast biogenesis (Zhang et al., 2006), as well as the ratio of different carotenoids in photosynthetic tissues that is regulated by nuclear gene transcription and a histone methyl transferase (Cazzonelli et al., 2009). The photosynthetic complexes are formed during thylakoid formation, which requires a coordinated import of pigment-binding proteins such as chlorophyll *a/b*-binding proteins (CAB/light-harvesting chlorophyll-binding) and the biosynthesis of the pigments. Thus, carotenoid and chlorophyll biosynthesis is tightly regulated during chloroplast development as they are essential for the assembly of photosynthetic complexes and photoprotection (Cazzonelli and Pogson, 2010). Impairment of chlorophyll synthesis leads to either a lack or a reduced amount of LHC proteins in thylakoid membranes (Terao et al., 1985), implying an impaired thylakoid structure flexibility, PSII supercomplex structure during state transition and thermal dissipation for PSII protection (Havaux et al., 2007; Jansson, 1999; Pietrzykowska et al., 2014; Ramel et al., 2013). Molecular analysis using LHCB6 knock-out mutants of *A. thaliana* demonstrated a negative effect on the packing of PSII in the membrane and thus the efficiency of photosynthesis and photoprotection (de Bianchi et al., 2008; Kovács et al., 2006). In addition, Holt *et al.* (2005) and Avenson *et al.* (2008) proposed that LHCB6, together with the other minor antenna complexes, is the site of formation of a zeaxanthin radical cation, and thus is directly involved in non-photochemical quenching (NPQ) that protects plants from photodamage under high light conditions (Nott et al., 2006; Staneloni et al., 2008). Overproduction of singlet oxygen in mutant plants lacking of chlorophyll *b* has been attributed to a lack of thermal dissipation for PSII protection due to the absence of most chlorophyll *b*-binding proteins, which is indeed likely to cause major disruptions to fluent energy transfer (Havaux et al., 2007; Ramel et al., 2013). Moreover, chlorophylls are essential for the proper folding of some photosynthetic proteins (Paulsen et al., 1993). Carotenoids have several roles in photosynthesis, which include structural stabilisation and assembly of protein complexes in the thylakoid membrane (Paulsen et al., 1993), light absorption and excited state energy transfer to the chlorophylls (Gradinaru et al., 2000; Mimuro and Katoh, 1991). Furthermore, carotenoids provide protection against photo-oxidative damages by acting as important antioxidants in the thylakoid membrane and are involved in dissipation of excited states of chlorophylls in cases of over-excitation (Havaux and Niyogi, 1999).

***The oxygen-evolving complex (OEC) of photosystem II (PSII)***

Photosystem II (PSII) is a light-driven water–plastoquinone oxidoreductase, in which the oxygen-evolving complex (OEC) catalyzes the water-splitting reaction (Debus, 1992; Hillier and Messinger, 2005; McEvoy and Brudvig, 2006). It consists of both membrane-intrinsic and membrane-extrinsic subunits (Renger and Renger, 2008). On the thylakoid lumenal side of PSII, the  $\text{Mn}_4\text{CaO}_5$  cluster catalyzes water oxidation, leading to the production of molecular oxygen, essential for most living organisms on earth (Vinyard et al., 2013). The membrane-intrinsic subunits of PSII are involved in pigment and/or cofactor binding for photochemical reactions, whereas the membrane-extrinsic subunits on the lumenal side play crucial roles in stabilizing the  $\text{Mn}_4\text{CaO}_5$  cluster and in retaining  $\text{Ca}^{2+}$  and  $\text{Cl}^-$ , required cofactors for water oxidation (Bricker et al., 2012). Green eukaryotes, including higher plants and green algae, have a set of three extrinsic proteins, namely PSBO (33 kDa), PSBP (23 kDa), and PSBQ (17 kDa), that bind to the lumenal surface of PSII. PSBP and PSBQ proteins are thought to have evolved from their cyanobacterial homologs; cyanoP and cyanoQ (Ifuku et al., 2011; Shen and Inoue, 1993). The main purpose of PSBP and PSBQ is to optimize the availability of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  cofactors for efficient water oxidation by PSII. However, the N-terminal region of PSBP specifically interacts with PSII to induce conformational changes around the  $\text{Mn}_4\text{CaO}_5$  cluster, required for  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  binding (Tomita et al., 2009). In that regard, PSBQ supports this PSBP function as it stabilizes PSBP binding, thereby contributing to the maintenance of the catalytic Mn cluster of the water oxidation machinery in higher plant PSII (Ifuku et al., 2011; Kakiuchi et al., 2012). Investigations of the molecular functions of PSBP and PSBQ *in vivo* using RNAi-knockdown plants has shown that suppression of PSBP results in a slow growth rate and various defects in PSII function, such as lower oxygen-evolving activity, lower quantum yield, and a slower electron transfer rate at the donor side of PSII (Chaiwongsar et al., 2012). On the other hand, PSBQ-knockdown plants cannot survive under prolonged (~ 3 weeks) low-light stress (Yi et al., 2006). These results confirm that the oxygen-evolving complex (OEC) associated with PSII of the chloroplast thylakoid membrane is a vital component of the photosynthetic machinery. Moreover, both PSBP and PSBQ can be structurally-linked in stabilizing the association of a minor antenna protein, LHCB5 (CP26), to the inner membrane-facing domains of PSBC (CP43), which in turn provides relevant significance of these extrinsic subunits as structural basis for the overall stabilization of the LHCII-PSII supercomplex in higher plants.

***Photosynthetic efficiency: The role of the chloroplast ATP synthase***

Chloroplast ATPase is a protein complex, located within the chloroplast thylakoid membranes, which synthesizes ATP from ADP and inorganic phosphate (Pi) through a proton-motive force (PMF) formed by the photosynthetic electron transport (Hisabori et al., 2002). CF<sub>0</sub>F<sub>1</sub> ATP synthase contains multiple subunits arranged in a hydrophilic structure (F<sub>1</sub>) interacting with nucleotides and coupling ATP synthesis to electron transport, and a hydrophobic structure (F<sub>0</sub>) forming a channel which transports H<sup>+</sup> across the membrane. This enzyme belongs to a large family of F<sub>0</sub>F<sub>1</sub> type ATPases and shares a common structure with the ATPase from mitochondria and bacteria (Evron et al., 2000). F<sub>0</sub>F<sub>1</sub>-ATP synthase (F<sub>0</sub>F<sub>1</sub>) is a critical enzyme for energy conversion in the cell and consists of a membrane-peripheral component F<sub>1</sub>, which in turn is formed of five different subunits with a stoichiometry of  $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$  (Vik, 2007), and a membrane-embedded part F<sub>0</sub>, the proton translocation device, is composed of three different subunits, a, b, and c with a stoichiometry of  $a_1b_2c_{10-15}$  (Mitome et al., 2004; Pogoryelov et al., 2005; Stock et al., 1999). In synthesis mode, the F<sub>0</sub> motor converts the electrochemical gradient of protons into torque, to force the F<sub>1</sub> motor to act as an ATP generator, while in the hydrolysis mode, F<sub>1</sub> converts the chemical energy of ATP hydrolysis into a torque, causing the membrane-embedded F<sub>0</sub> motor to act as anion pump (Dimroth et al., 2006). In the light, the ATPase complex is activated in order to achieve a high capacity of ATP synthesis, whereas in the dark, it is converted to an inactive state, thereby preventing the dissipative cleavage of stromal ATP (Wu et al., 2007). To optimize photosynthetic productivity, particularly when light availability is limiting, there must be a tight regulation between efficient energy absorption and energy conversion, to avoid over-reduction and damage to the cell (Oelze et al., 2008). This tight regulation of the chloroplast ATPase complex includes, among others, nucleotide binding, thiol modulation; proton transmembrane electrochemical potential ( $\Delta\mu\text{H}^+$ ) and formation across the thylakoid membrane and phosphorylation followed by 14-3-3 protein binding. PMF is a proton gradient responsible for release of inhibitory, tightly bound MgADP and also for the reduction of the disulfide bridge in the subunit (Evron et al., 2000). Thus in the presence of light, the generation of  $\Delta\mu\text{H}^+$  induces conformational changes within the ATPase complex, causing activation of the enzyme. These conformational changes and ADP release are reversed, inactivating the enzyme and avoiding energy losses as  $\Delta\mu\text{H}^+$  dissipates in the dark (Kleefeld et al, 1990; Strotmann et al, 1998; Wu et al, 2007).

The role of the chloroplast ATPase includes the control of light driven induced-electron pump and inhibition of plastoquinol reoxidation at the Cyt-bf due to acidification of the thylakoid lumen ,which in-turn leads to the activation of non-photochemical quenching (NPQ) (Witt 1979; Cruz *et al.*, 2001; Cruz *et al.*, 2005). The importance of CF<sub>0</sub>F<sub>1</sub> ATP synthase was previously evident in tobacco and *Arabidopsis* leaves, in which, at a given proton flux, decreasing pH will increase trans-thylakoid proton motive force (pmf), thus lowering lumen pH and contributing to the activation of NPQ (Kanazawa and Kramer, 2002). An impaired ATPase synthase complex has been reported to impair starch accumulation. In leaves the flux of carbon into the starch-biosynthetic pathway is thought to be controlled by modulation of AGPase activity and is very sensitive to ATP and free Pi availability. Inhibition of AGPase by ATPB has been shown to result in reduced starch levels (Zeeman *et al.*, 2007). Therefore, any disturbance of ATPase can be harmful to the cell and hence must be tightly regulated in order to prevent cell death (Johnson, 2008).

#### ***Intercellular signaling mediated by photosynthetic metabolites***

Other than its integral photosynthetic role, the chloroplast is essential for the production of hormones and metabolites. Sugar control of metabolism, growth and development, stress, and gene expression has long been thought to be a metabolic effect (Rolland *et al.* 2006). Sugars can act as signals in retrograde and other signaling pathways, integrating environmental and developmental changes during plant growth. Chloroplasts are central players in sugar-induced leaf growth (Van Dingenen *et al.*, 2016), and as such chloroplasts need to differentiate to start photosynthesis, producing sugars and other retrograde signals, which could trigger the transition to cell expansion. Young proliferating leaves first depend on the supply of sugars, produced by photosynthetically active source leaves, to grow, while the reduced photosynthetic activity of source leaves or reduced sugar availability triggers young, proliferating leaves to produce their own sugars and energy for further growth (Li *et al.*, 2006). This regulation is controlled at numerous levels in plants, for example by allosteric regulation of metabolic enzymes, and tissue-specific or temporal-specific expression of genes (Rook and Bevan, 2003). For example, sugars can modulate nuclear gene expression, especially the repression of nucleus-encoded photosynthesis genes, such as *CHLOROPHYLL A/B BINDING PROTEIN* (CAB) and the small subunit of *RUBISCO*, to control feedback regulation of photosynthesis.

### **Carbon metabolism: Starch and sucrose have a central regulatory function in steering plant growth**

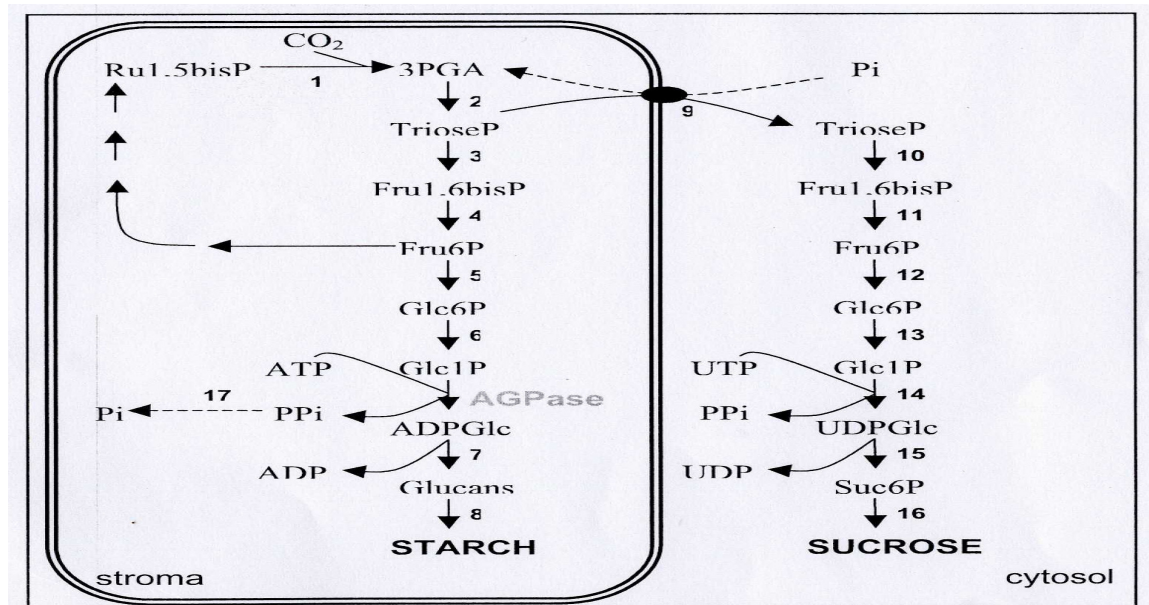
Subsequent to photosynthesis is the conversion of physical and chemical sources of energy into carbohydrates (Habibi and Hajiboland, 2012; Lodish et al., 2000). Carbon fixation through the reductive pentose phosphate pathway (Calvin cycle or C<sub>3</sub> cycle) is the most biologically abundant and economically-relevant pathway that can be targeted for enhancing photosynthetic efficiencies. The C<sub>3</sub> cycle is the primary pathway of carbon assimilation in the majority of photosynthetic organisms and it is the single largest flux of organic carbon and assimilates. The C<sub>3</sub> cycle utilizes the products of the light reactions of photosynthesis, ATP and NADPH, to fix atmospheric CO<sub>2</sub> into carbon skeletons that are used to fuel the rest of plant metabolism (Stitt et al., 2010). The C<sub>3</sub> cycle is initiated by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) that catalyzes the carboxylation of the CO<sub>2</sub> acceptor molecule ribulose-1,5-bisP (RuBP) resulting in the formation of the three-carbon compound 3-phosphoglycerate (3-PGA). 3-PGA formed by this reaction is used to form the triose phosphates glyceraldehyde phosphate (G-3-P) and dihydroxyacetone phosphate via two reactions that consume ATP and NADPH. The regenerative phase of the cycle involves a series of reactions that convert triose phosphates into the CO<sub>2</sub> acceptor molecule RuBP (Fig. 3). Carbon compounds produced in this cycle are essential for growth and development of the plant (Raines and Paul, 2006; Smith and Stitt, 2007a)

The primary end-products of photosynthesis are triose phosphates, which can be the immediate source for starch synthesis within the chloroplast (Slewinski and Braun, 2010; Stitt et al., 1984). In that regard, the pathway of starch synthesis in *Arabidopsis* leaves during the day involves the conversion of Calvin cycle intermediates into ADP-glucose (ADP-Gluc), the substrate for starch synthases in the chloroplast. Chloroplastic isoforms of phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM) catalyse the conversion of fructose 6-phosphate into glucose 1-phosphate and ATP (Ghosh and Preiss, 1966; Sweetlove *et al.*, 1996; Tsai and Nelson, 1966). It is widely accepted that ADP-glucose pyrophosphorylase (AGPase) is the sole enzyme catalysing the production of ADP-glucose used in this pathway (Fig 3). This substantiation has been obtained with the use of maize, spinach, potato and *Arabidopsis* (Ghosh and Preiss, 1966; Müller-Röber *et al.*, 1992; Sweetlove *et al.*, 1996; Tsai and Nelson, 1966). AGPase-deficient transgenic potatoes

produced more tubers per stolon but, with reduced size relative to the wild type plants due to competition for a limited supply of photoassimilates. In addition, those tubers stopped growing at an earlier stage of development which further contributed to poor tuber yields (Müller-Röber et al., 1992). In starch biosynthesis metabolism, AGPase is exquisitely sensitive to allosteric regulation, being activated by 3-phosphoglyceric acid (3PGA) and inhibited by inorganic orthophosphate (Pi; (Sowokinos and Preiss, 1982). The supply and demand for photoassimilates influences changes in 3PGA/Pi ratio. Production of photoassimilates leads to a high 3PGA/Pi ratio in the chloroplast stroma, resulting in activation of AGPase which increases flux into the pathway of starch biosynthesis (Stitt and Quick, 1989; Zeeman et al., 2007). This allows the rate of starch synthesis to be adjusted in response to changes in the balance between sucrose synthesis in leaves and to changes in the balance between sucrose breakdown and respiration in tubers (Geigenberger et al., 1998; Stitt et al., 1987). The sugar-dependent redox regulation of AGPase through trehalose-6-phosphate and sucrose has been elaborated on previously.

Alternatively, triose phosphates can be exported to the cytosol for sucrose metabolism via the triose phosphate translocator (Fig. 3). Sucrose, the major product of photosynthesis, can be utilized directly by glycolysis or translocated within the plant as a soluble carbohydrate via the phloem. It constitutes the most abundant form of soluble storage carbohydrate and also serves as a signalling molecule that triggers essential metabolic events (Koch, 1996b). In the source leaves, sucrose, metabolized to its hexose products (glucose and fructose), can be stored in the vacuole, or it can be transported through the phloem to the sink tissues and used for the maintenance of cellular metabolism, cell wall biosynthesis, respiration, or converted into starch for later use (Kutschera and Heiderich, 2002; Lemoine et al., 2013; Sturm et al., 1999). The synthesis of sucrose in plants involves a two-step process catalyzed by sucrose-6-phosphate synthase (SPS) and sucrose-6-phosphate phosphatase (SPP). The first important regulatory step in controlling sucrose synthesis involves the synthesis of sucrose-6-phosphate (Suc-6-P) from fructose-6-phosphate (Fru-6-P) and UDP-glucose, the reaction catalyzed by SPS (Stitt et al., 1988). In the final step, SPP catalyzes the final step in the pathway of sucrose biosynthesis, where Suc-6-P produced by SPS is irreversibly hydrolyzed to sucrose (Lunn and Ap Rees, 1990). Lately, studies have demonstrated that SPS interacts with SPP and that this interaction impacts soluble carbohydrate pools and affects carbon

partitioning to starch, which in turn promote plant growth and biomass accumulation. The action of the elevated SPS and SPP activity manifests in the synthesis of sucrose that is rapidly broken down to glucose and fructose by either invertase or sucrose synthase counterparts, and the overall level of sucrose maintains a homeostatic level. Increased growth and yield of plants with higher SPS activity may be the result of decreased energy costs associated with direct export of sucrose during the day rather than the synthesis of starch and its conversion back to sucrose for export at night (Worrell et al., 1991; Coleman et al., 2010; Laporte et al., 1997; Micallef et al., 1995; Park et al., 2008). In that regard, altering sucrose metabolism may be key to improving the biomass quantity and quality for bioenergy applications (Maloney et al., 2015).



**Figure 3.** Regulation of the photosynthetic carbohydrate partitioning between starch and sucrose in the chloroplast and cytosol (Kolbe, 2005). RuBisCo (1), 3PGA kinase and NADP GAPDH (2), Fru1.6bisP aldolase (3 and 10), FBPase (4 and 11), PGI (5 and 12), PGM (6 and 13), GBSS (7), SBE, debranching enzymes (8), TPT (9), 14 – UGPase (14), SPS(15), SPP (16), pyrophosphatase (17).

The partitioning of newly fixed carbon is one important resource allocation mechanism influencing plant growth and yield (Müller-Röber et al., 1992; Tiessen and Padilla-Chacon,



2013). Therefore, sucrose and starch are the major quantitative end-products of CO<sub>2</sub> assimilation in most plants and they provide the organic carbon source for the synthesis of other cellular constituents (Iddai and Scott, 2011; Schlosser et al., 2014; Sulpice et al., 2009). Plant growth and productivity is dependent upon photoassimilate production and the efficient allocation to sinks such as developing leaves or storage organs (Iddai and Scott, 2011; Schlosser et al., 2014; Sulpice et al., 2009). Therefore, photosynthetic carbohydrate partitioning has become an important target to breed for enhanced plant growth and yield to address the rising demand for both food and bioenergy (Sulpice et al., 2009).

Normal growth of plants depends on the coordinated regulation of sink and source metabolism. The growth of new sinks, such as fruits or new leaves, and the demand of non-photosynthetic tissues, such as roots, must be balanced with the source acquisition of nutrients, such as carbon assimilation during photosynthesis in fully-expanded leaves (Van Camp, 2005; Müller-Röber et al., 1992; Smith and Stitt, 2007b; Tiessen and Padilla-Chacon, 2013). Source photosynthesis and sink utilization occur in a coordinated fashion with fine control of enzyme activity by metabolic intermediates and coarse control by changes in photosynthetic gene expression (Sonnewald, 2001). Photosynthetically-active leaves (source leaves) produce their own energy and carbon sources for growth and development, while non-photosynthetic organs such as roots, flowers and young growing leaves depend on these source leaves for carbon supply to grow (Turgeon, 1989).

Starch is the major non-structural carbohydrate in plants. It serves as an important store of carbon that fuels plant metabolism and growth when they are unable to photosynthesize. In *Arabidopsis*, as in most vascular plants, starch plays an important role in the day-to-day carbohydrate metabolism of the leaf. Growth is a highly energy-demanding process, hence in *Arabidopsis*, starch synthesis and degradation are diurnally regulated such that an optimal carbohydrate balance is maintained during both day and night, and energy stress can be avoided (Gibon et al., 2009; Smith and Stitt, 2007b; Stitt and Zeeman, 2012; Zeeman and Rees, 1999). Severe slow-growth phenotypes of mutants unable to make starch, or unable to efficiently degrade it, are a clear indication that starch is integral to growth. Growth of such mutants is further compromised due to premature exhaustion of carbohydrates and the

subsequent triggered starvation response in which valuable cellular components are degraded to support cellular housekeeping activities and keep the plant alive (Buchanan-Wollaston et al., 2005; Koch, 1996a; Streb and Zeeman, 2012). Incomplete mobilization of starch reserves would also compromise growth as it would mean that assimilated carbon, which could be used for the production of new photosynthetic biomass, languishes as an unproductive storage compound (Graf et al., 2010; Pantin et al., 2011). These starch excess or deficient phenotypes advocate for efficient control of starch metabolism for plant growth.

### ***Thioredoxins and thiol modulation of photosynthesis and carbon metabolism***

Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. In plants, ROS are always formed by high-energy exposure or the inevitable leakage of electrons onto O<sub>2</sub> from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments resulting into progressive oxidative damage and ultimately cell death (Maheshwari and Dubey, 2009; Meriga et al., 2004; Mishra et al., 2011; Shah et al., 2001). Because of the direction of information flow, photosynthetic redox signals can be defined as a distinct class of retrograde signals in addition to signals from organelle gene expression or pigment biosynthesis. Therefore, the subsequent paragraphs will introduce plants mechanism that restrain the chloroplast from under and/or over-excitation under fluctuating light conditions and rapidly restore redox homeostasis before generation of harmful side products. Furthermore, our review will shed more light of the redox control of carbon metabolism with more particular emphasis on starch biosynthesis.

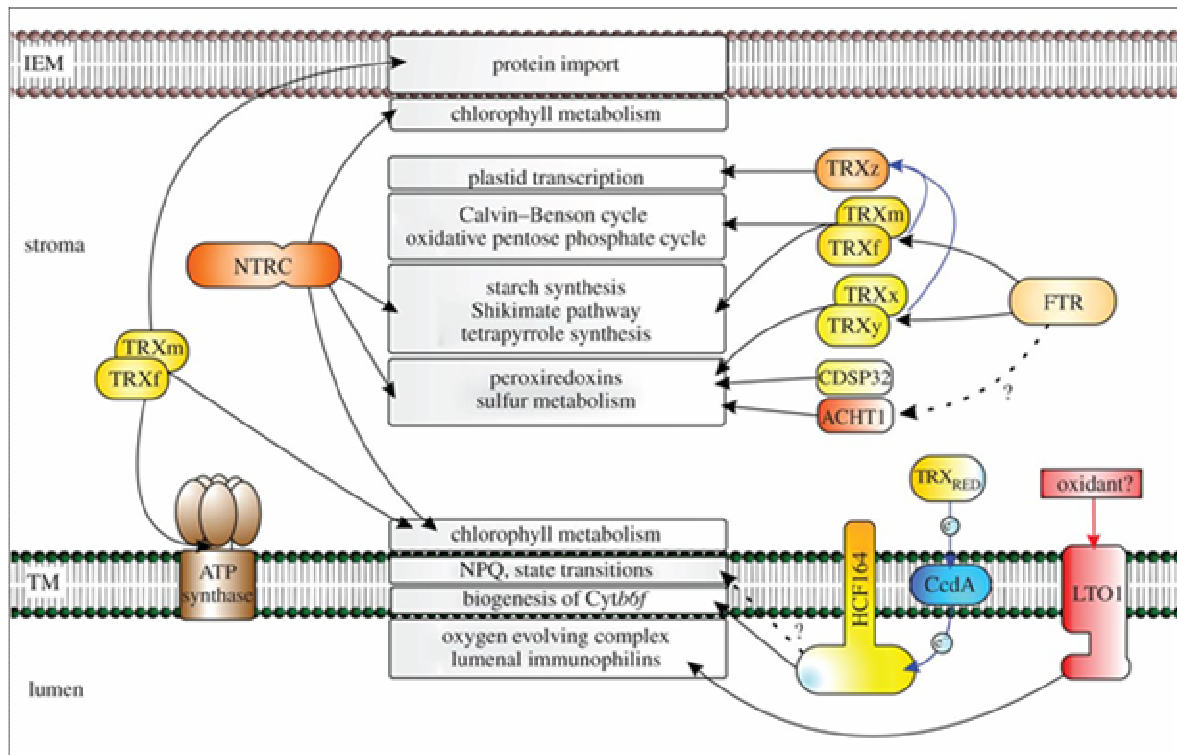
Chloroplast thioredoxin systems are crucial components of this redox network, mediating environmental signals to chloroplast proteins. Thioredoxins (TRXs) are protein oxidoreductases that have a redox active dithiol/disulfide motif in their active site. In the reduced state, they induce reductive cleavage of a disulfide bond in target proteins via a bimolecular nucleophilic substitution reaction. Thioredoxins (TRXs) mediate light-dependent activation of primary photosynthetic reactions in plant chloroplasts by reducing disulfide bridges in redox-regulated enzymes. Plants are distinguished from other organisms by having highly versatile TRX systems, including two TRXs dependent on ferredoxin (FTR) and

NADPH (NTR) as reducing power, respectively, and multiple types of TRXs including h, o, f, m, x, y, z, and several TRX-like proteins (Buchanan and Balmer, 2005; Meyer et al., 2008). In *Arabidopsis*, five types of classical low-molecular weight TRXs (f, m, y, x, z) are localized to plant chloroplasts, of which f, m and y exist in *Arabidopsis* as two, four and two isoforms respectively, emphasizing the impact of TRX-dependent regulation of proteins in chloroplasts (Arsova et al., 2010; Buchanan and Balmer, 2005; Schürmann and Buchanan, 2008).

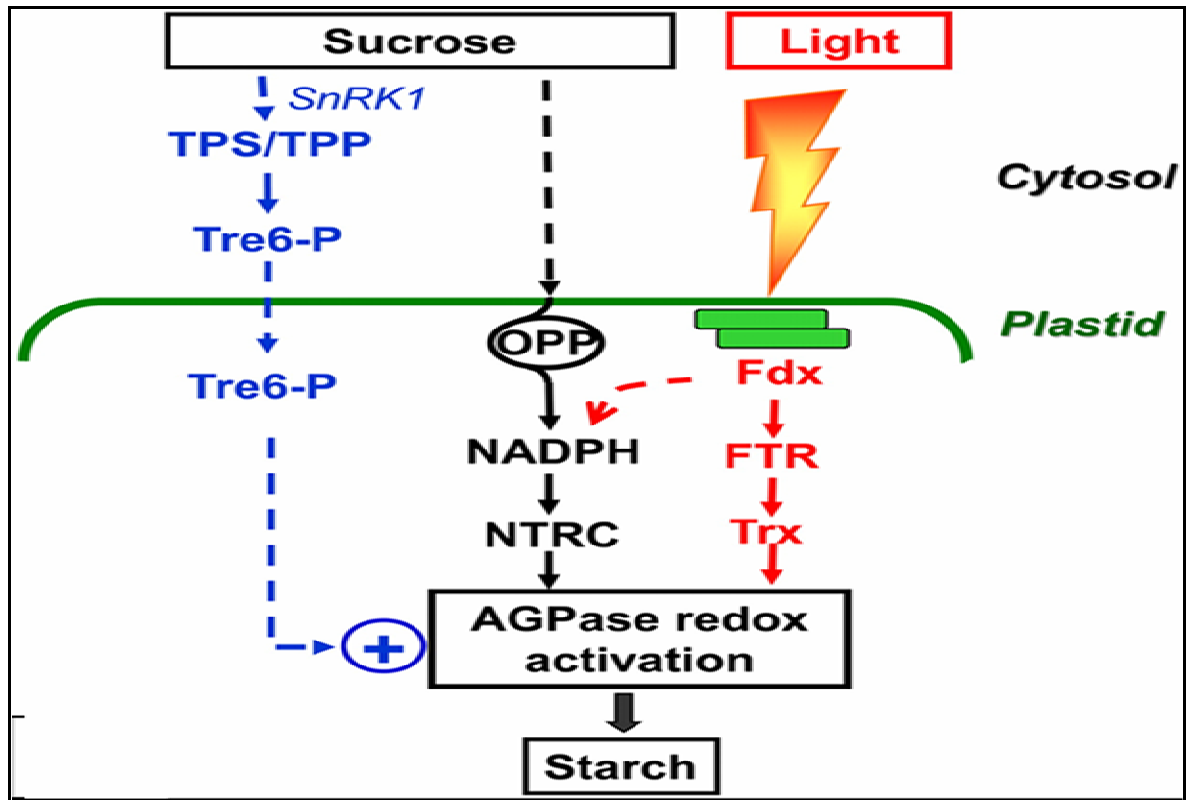
Light regulation of photosynthetic enzymes is a process that links photosynthetic electron transport and the activity of specific chloroplast enzymes via of the ferredoxin-thioredoxin system (Mikkelsen et al., 2005). Photosynthetic electron transport leads to reduction of ferredoxin (Fdx), and reducing equivalents are transferred by ferredoxin reductase (FTR) to thioredoxin (Trx) f or m, which activate target enzymes by the reduction of regulatory disulfides (Thormählen et al., 2013). As ferredoxin is mainly reduced during the light reactions of photosynthesis, the ferredoxin-dependent TRX system is responsible for light-induced activation of primary photosynthetic reactions (Fig. 4), namely, the Calvin–Benson cycle (Geigenberger and Fernie, 2014; Michelet et al., 2013), ATP synthesis (Hisabori et al., 2013), the malate-oxaloacetate shuttle (Miginiac-Maslow et al., 2000) and starch metabolism (Thormählen et al., 2013).

The chloroplast-type thioredoxin (Trx) is a critical reducing equivalent mediator responsible for the reduction of the cysteine residues located within the enzymes in the Calvin-Benson cycle, namely glyceraldehyde 3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and phosphoribulokinase. Hence the Calvin-Benson cycle is indirectly controlled by photosynthetic electron transport via the Trx system in the cell or organelle (Ballicora et al., 2000; Buchanan, 1991). Regarding starch biosynthesis modulation, NADPH-dependent thioredoxin reductase C (NTRC), containing both an NADP-Trx reductase and a Trx in a single polypeptide, serves as an alternate system for transferring reducing equivalents from NADPH to AGPase, thereby enhancing storage starch synthesis (Michalska et al., 2009). In addition, redox activation of AGPase by both light and metabolites in *Arabidopsis* has been previously reported (Tiessen et al., 2002; Hendriks et al., 2003). Under dark conditions, NTRC is primarily linked to sugar oxidation via the initial

reactions of the oxidative pentose phosphate pathway (OPP) and, in this way, regulates AGPase independently of the Fdx/Trx system. It has been shown that NADP-thioredoxin reductase C (NTRC) mediates the reduction of the APS1 subunits of AGPase by NADPH *in vitro*. NTRC is an unusual plastid-localized bi-functional protein that contains NADP-thioredoxin reductase (NTR) and a thioredoxin domain in a single polypeptide. The use of *ntrc* deletion mutants provided evidence that NTRC performs this function *in vivo*. Monomerization of APS1 in the light was decreased, and the sucrose-dependent monomerization in the dark was completely suppressed in *ntrc* mutants compared to the wild type (Michalska et al., 2009). Moreover, trehalose-6-phosphate (Tre-6-P) acts an intracellular signal, linking sucrose in the cytosol with AGPase in the plastid (Kolbe, 2005). An increase in sucrose in the cytosol leads to an increase in the level of Tre-6-P by modulating Tre-6-P synthase (TPS) and/or Tre-6-P phosphatase (TPP). *Arabidopsis* plants with altered levels of Tre-6-P provided genetic evidence that Tre-6-P, rather than trehalose, leads to AGPase redox-activation (Kolbe, 2005). In addition, Tre-6-P is taken up into the plastid and promotes NTRC and/or FTR/Trx-dependent redox activation of AGPase by a yet unresolved mechanism (Geigenberger et al., 2005; Li et al., 2012) (Fig. 5).



**Figure 4.** Chloroplast processes controlled by Thioredoxin (TRX) systems in the stroma, thylakoid membranes (TM), lumen and in inner envelope membrane (IEM). (Nikkanen and Rintamäki, 2014).



**Figure 5.** Post-translational redox regulation of starch metabolism (Li et al., 2012). Light and metabolites modulation inputs plays an important role in the redox regulation of starch metabolism.

### Manipulation of plant growth using phytohormones and/or plant growth regulators

Recent molecular and genetic studies have identified a number of factors that regulate processes such as vegetative meristem activities, cell elongation, photosynthetic efficiency, and secondary wall biosynthesis, all of which are crucial for plant biomass production (Demura and Ye, 2010). The regulators of these processes could potentially be applied to improve the yield of biomass crops. This include among others several growth regulators known as plant hormones which includes both hormonal substances of natural occurrence (phytohormones) as well their synthetic analogues referred to plant growth regulators (Gaspar et al., 1996; Lone et al., 2010; Spartz and Gray, 2008). PGRs are known to influence a wide array of physiological parameters (Marimuthu and Surendran, 2015), including alteration of plant architecture, uptake of nutrients and metabolism, promotion of photosynthesis, partitioning and mobilization of assimilates to defined sinks as well as yield (Marimuthu and

Surendran, 2015; Sharma et al., 2013). These growth regulators, when applied as a foliar spray at specific growth stages in optimum concentrations, could play a significant role in increasing crop yield and quality of produce (Nagasubramaniam et al., 2007). Plant hormones act at minute concentrations to induce developmental control by regulating cell division, differentiation, elongation and death (Spartz and Gray, 2008). Among others, hormones such as auxins, ethylene, cytokinins and gibberellins have been shown to regulate intercellular signals that pattern tissues and can also participate in growth control. Jasmonate is one such hormone that negatively affects chlorophyll biosynthesis and photosynthesis efficiency. 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 that originate from  $\alpha$ -linolenic acid ( $\alpha$ -LeA) of chloroplast membranes. Upon oxygenation of  $\alpha$ -linolenic acid ( $\alpha$ -LeA) by 13-lipoxygenase (13-LOX), an unstable allene oxide is formed by a 13-allene oxide synthase (13-AOS) and subsequently cyclized by an allene oxide cyclase (AOC) to *cis*-(+)-OPDA (Howe et al., 2000; Howe and Schilmiller, 2002; Schaller and Stintzi, 2008). An essential step in the biosynthesis of jasmonic acid (JA) is catalysed by allene oxide cyclase (AOC) which establishes the naturally occurring enantiomeric structure of jasmonates (Stenzel et al., 2012). Reduction of the cyclopentenone ring by OPDA reductase3 (OPR3) and three-times  $\beta$ -oxidative degradation of the carboxylic acid side chain result in the formation of (+)-7-*iso*-JA (Staswick and Tiryaki, 2004). All the steps by AOS, AOC, and OPR3 are truly committed to the JA biosynthetic pathway and their features are required for JA biosynthesis upon wounding and during senescence in leaves (Bell et al. 1995; He et al. 2002; Chung et al. 2008; Selmann et al. 2010). An increase in JA biosynthesis leads to a progressive leaf senescence and is accompanied by chlorophyll loss and decreases in RuBisCO and photosynthesis (Weidhase *et al.* 1987), which is detrimental for plant growth and productivity. This was demonstrated in a quick-leaf-senescence inbred maize line, Huangzao-4(HZ4), in which light absorption, energy transformation and electron transfer capacity were decreased, leading to a faster decreases of the density of active PSII reaction centres, the O<sub>2</sub> evolution rate and the PSI photo-activity (Papuga et al., 2010).

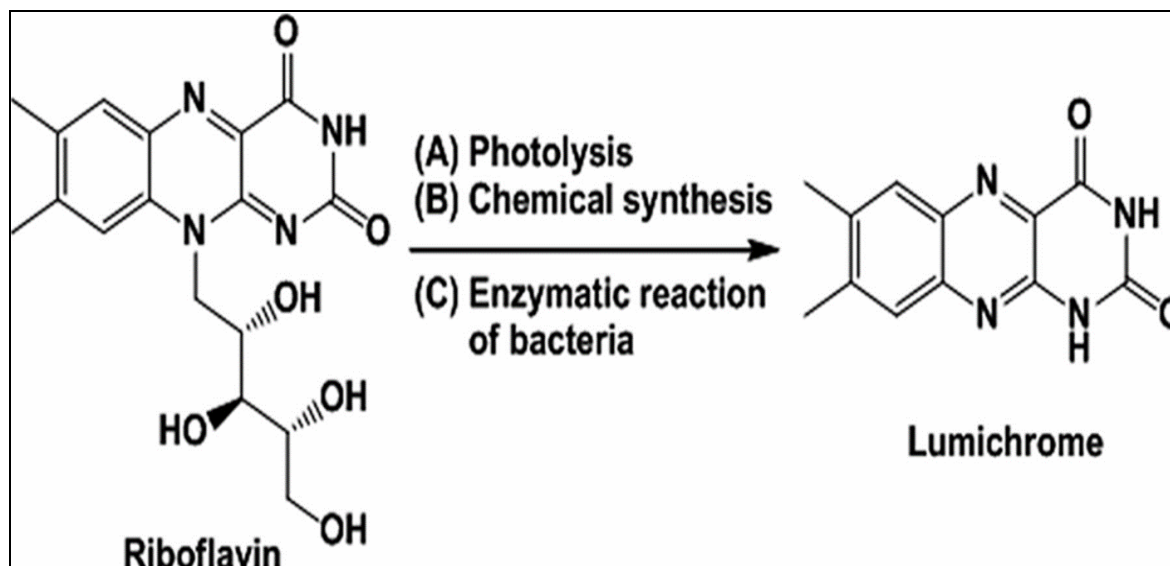
Other than the well-known classified plant hormones, plant growth promoting rhizobacteria (PGPR) have been shown to regulate fundamental processes in plant development. These free-living soil bacteria have the ability to form nitrogen-fixing symbioses with legumes and

facilitate plant growth either directly, by providing nutrients (nitrogen, phosphorus and essential minerals), modulating plant hormones and development, or indirectly, by suppressing inhibitory effects of various plant pathogens, improving soil structure and bioremediating polluted soils (Glick, 2012; Howieson et al., 2000; Lugtenberg and Kamilova, 2009). Apart from their well-known role in nitrogen fixation in legumes, symbiotic nitrogen-fixing bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium* and *Mesorhizobium* affect fundamental processes in plant development through the release of powerful molecules such as the phytohormones cytokinin (Phillips and Torrey, 1972) and the auxin indoleacetic acid (IAA) (Hirsch et al., 1997; Law and Strijdom, 1989; Vessey, 2003).

***Lumichrome – A novel signal molecule from rhizobial exudates that stimulate plant growth***

Over a decade ago, lumichrome (*7,8-dimethylalloxazine*) was identified from filtrates of *Sinorhizobium meliloti* cultures (Phillips et al., 1999) as a signalling molecule between the growth promoting rhizobacterium and its host plants (Gouws et al., 2012; Khan et al., 2008; Matiru and Dakora, 2005a, 2005b). Lumichrome is a known degradation product of the vitamin riboflavin (Fig. 6), being generated either through photochemical or enzymatic cleavage of the ribityl group under neutral or acidic conditions in the presence of sunlight (Khan et al., 2008; Phillips et al., 1999; Yanagita and Foster, 1956). As such, the role of lumichrome has often been linked with that of riboflavin (Yanagita and Foster, 1956).





**Figure 6.** Structures of riboflavin and lumichrome molecules functioning as plant growth promoters.

Lumichrome and riboflavin are known to act as signal molecules stimulating root respiration and plant growth, which is closely related to a triggered compensatory increase in whole-plant net carbon assimilation (Phillips et al., 1999) which is needed for growth of  $N_2$ -fixing rhizobia (Lowe and Evans, 1962) and mycorrhizal fungi in rhizosphere soils (Becard et al., 1992). Application of biologically-active levels of lumichrome has been demonstrated to enhance plant development through changes in photosynthetic rates, leaf stomatal conductance and transpiration in several plant species (Matiru and Dakora, 2005b). Consistent with this, foliar application of lumichrome on soybean and maize also enhanced photosynthetic rates and plant growth (Khan et al., 2008). Enhanced growth is reported to be attributed to xylem transport and *in situ* accumulation of lumichrome in leaves which subsequently triggers events that promote cell division and leaf expansion in both monocots and dicots (Dakora, 2003; Matiru and Dakora, 2005a).

Recently, the impact of lumichrome on the root of tomato and *Lotus japonicus* was assessed using a variety of '-omics' techniques which resulted in a comprehensive profile of possible lumichrome-dependent modes of action associated with enhanced plant growth (Gouws et al., 2012). A microarray-based gene expression study described carbon and ethylene metabolism in roots of both *Lotus japonicus* and tomato treated with lumichrome. An increase in starch

metabolism 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 lead to enhanced starch accumulation (Muñoz-Bertomeu *et al.* 2009). An increase in starch accumulation was further explained by a significant reduction in the cytosolic non-phosphorylating GAPDH isoforms which led to a significant shift in carbohydrate metabolism in lotus upon lumichrome treatment. Therefore, the significant enhanced starch accumulation in the absence of microorganisms strongly suggested that lumichrome act as a modulator of carbon fluxes within the plant cell. In respect to an altered ethylene metabolism, lumichrome treatment resulted in a reduction of transcripts of genes such 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. Photosynthetic carbohydrate partitioning between starch and sucrose has become an important target to breed for enhanced plant growth and yield to address the rising demand for both food and bioenergy (Sulpice et al., 2009). Despite these physiological experimental advances in exploring the growth stimulatory mechanisms of lumichrome, a comprehensive molecular explanation still remains elusive, hence further studies are required to examine the underlying roles of lumichrome-mediated stimulation of plant growth in various plant species.

### **Aims and objectives**

This project employed high-throughput analytical tools coupled with molecular genetic techniques to test correlations between the signal molecule lumichrome and carbohydrate partitioning, in order to better understand the control loops that integrate metabolism. The aim of the study was to further our understanding on the genetic, molecular and biochemical regulatory networks as key determinants for crop growth and productivity in relation to lumichrome, a low molecular weight signalling molecule. Therefore the specific objectives were to further elucidate the i) physiological biomass and photosynthetic response, ii) global changes in gene transcription, iii) differential expression of proteins and their functionality and iv) key metabolites or biochemical pathways which are seemingly affected by lumichrome treatments in *Arabidopsis*. These data would be used to infer functional interactions among genes, proteins and metabolites in order to speculate on the possible

control mechanism(s) involved, finally testing any derived hypotheses via a reverse genetic approach utilising knockout mutant plant lines.

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## CHAPTER 2

**Analysis of the mode of action of lumichrome treatment in *Arabidopsis* (L): transcriptome analysis suggests that cell division and turgor is mediating enhanced plant growth**

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**Abstract**

Lumichrome (*7,8 dimethylalatoxazine*), a novel multitrophic signal molecule produced by *Sinorhizobium meliloti* bacteria, has previously been shown to elicit growth promotion in different plant species. However, the molecular mechanisms that underlie this plant growth promotion remain obscure. Global transcript profiling using RNA-seq in the current study, 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*, *SPIL3*, *RSW7* and *PDF1* transcripts were increased in lumichrome-treated *Arabidopsis*, 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 effect of



methyl jasmonate (MeJa) such as chlorophyll loss and decreases in RuBisCO and photosynthesis.

**Keywords:** Lumichrome, *Arabidopsis*, transcript profiling, cell wall, mitotic cell cycle ethylene-auxin cross-talk, 13-lipoxygenases

## Introduction

Plant development progresses through different developmental phases and the transitions are controlled by distinct genetic cues that integrate endogenous and environmental signals (Luo and Nobel, 1992; Schlosser et al., 2014; Tugizimana et al., 2013). A variety of bacterial genera are vital components of soils and the potential of utilizing plant growth-promoting rhizobacteria (PGPR) in agriculture is steadily increasing due to their ability to produce growth promoting substances. PGPR stimulate plant growth through mobilizing nutrients in soils, producing 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 (Hynes et al., 2008; Russo et al., 2008; Braud et al., 2009; Rajkumar et al., 2010; Ahemad and Khan, 2012; Bhattacharyya and Jha, 2012; Ahemad, 2015). Hence, diverse symbiotic (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) and non-symbiotic (*Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*, *Azomonas*) rhizobacteria are now being used worldwide as bio-inoculants to promote plant growth and development under various stresses. Rhizobacteria-mediated plant growth promotion has been described in detail in various studies (Mayak et al., 2004; Wani and Khan, 2010; Ma et al., 2011; Ahemad and Khan, 2011, 2012). Other than nitrogen fixing symbioses, many PGPR release phytohormones (Phillips and Torrey, 1972) that promote bacterial cell growth and massively increase plant 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 (Simillion *et al.* 2002; Zhang and Smith, 2002; Matiru and Dakora, 2004; Matiru and Dakora, 2005).

The discovery of lumichrome (*7,8 dimethylalatoxazine*), 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<sub>2</sub> (Phillips et al., 1999) needed for growth of N<sub>2</sub>-fixing rhizobia (Lowe and Evans 1962) and mycorrhizal fungi (Becard et al., 1992). Its 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 2005). Conversely, lumichrome addition led to significantly decreased root respiration in lupin, while in cowpea it decreased stomatal conductance, which subsequently affected CO<sub>2</sub> intake and reduction by RuBisCo (Matiru and Dakora 2005).

At the molecular level, a microarray gene expression study described carbon and ethylene metabolism in roots of both *Lotus japonicus* and tomato treated with lumichrome. An increase in starch metabolism 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 lead to enhanced starch accumulation (Muñoz-Bertomeu *et al.* 2009). Additionally, lumichrome treatment resulted in a reduction of transcripts 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 still remains elusive. Here, we use next generation sequencing (RNA-seq) in the model plant *Arabidopsis thaliana* to examine this. We propose that the coordinated role of lumichrome in growth promotion of *Arabidopsis* is through inducing genes associated with the cell cycle that involves cell wall plasticity, the mitotic cell cycle and hormonal cross-talk signalling.

## **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,  $22\pm 2^{\circ}\text{C}$ , 75% relative humidity). Pots were arranged in a factorial randomised completely block design (RCBD) consisting of 6 replications and blocks respectively. Lumichrome stock solutions (5 nM) were freshly prepared for each treatment in methanol/1 M HCl (49:1) with constant stirring. Plants were treated with 5 nM lumichrome by a combination of root drenching and foliar application (50 ml) of intact plants at intervals of 2 days throughout the entire growth period (Phillips *et al.* 1999). The control plants were treated in the same way, using the same dilution of 49:1 methanol/HCl (without added lumichrome) in  $\text{dH}_2\text{O}$  as was used in the treated plants in order to account for the methanol/HCl in the treatments. 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  $70^{\circ}\text{C}$  for three consecutive days and subjected to dry biomass determination.

#### ***cDNA library preparation and Sequencing-Illumina-TrueSeq***

Total RNA was isolated from *Arabidopsis* rosette leaf tissue material (250 mg) according to a CTAB protocol (Hu *et al.* 2002). cDNA library preparation and sequencing were performed at the ARC Biotechnology Platform-Agricultural Research Council (South Africa). In brief, 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. For RNA-seq, total RNA was subjected to removal of ribosomal RNA using the Plant Leaf Ribo-Zero<sup>TM</sup> Magnetic Kit (Plant Leaf), 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 band of approximately 260 bp.

#### ***Differential gene expression analysis***

Raw FASTQ data was adapter and quality trimmed using Trimmomatic Illuminaclip with minimum Phred score of 20 and read length of 20 nt to control for reads quality (<http://www.usadellab.org/cms/?page=trimmomatic>). Tuxedo software suite v.2.2 (Bowtie,

TopHat, Cufflinks, Cuffdiff; Trapnell *et al.* 2012) with an integrated R statistical computing environment was used to compare samples and calculate differential expression. Trimmed sequencing reads were aligned against the wild type *Arabidopsis* (Col-0; TAIR10) genome and gene expression was quantified using Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Significantly differentially expressed transcripts were calculated based on Cuffdiff statistical tests of 3 replicates of treated relative to untreated samples using adjusted values ( $p < 0.005$  and  $q < 0.005$ ), a false discovery rate (FDR; 0.1) and absolute log<sub>2</sub>-fold change.

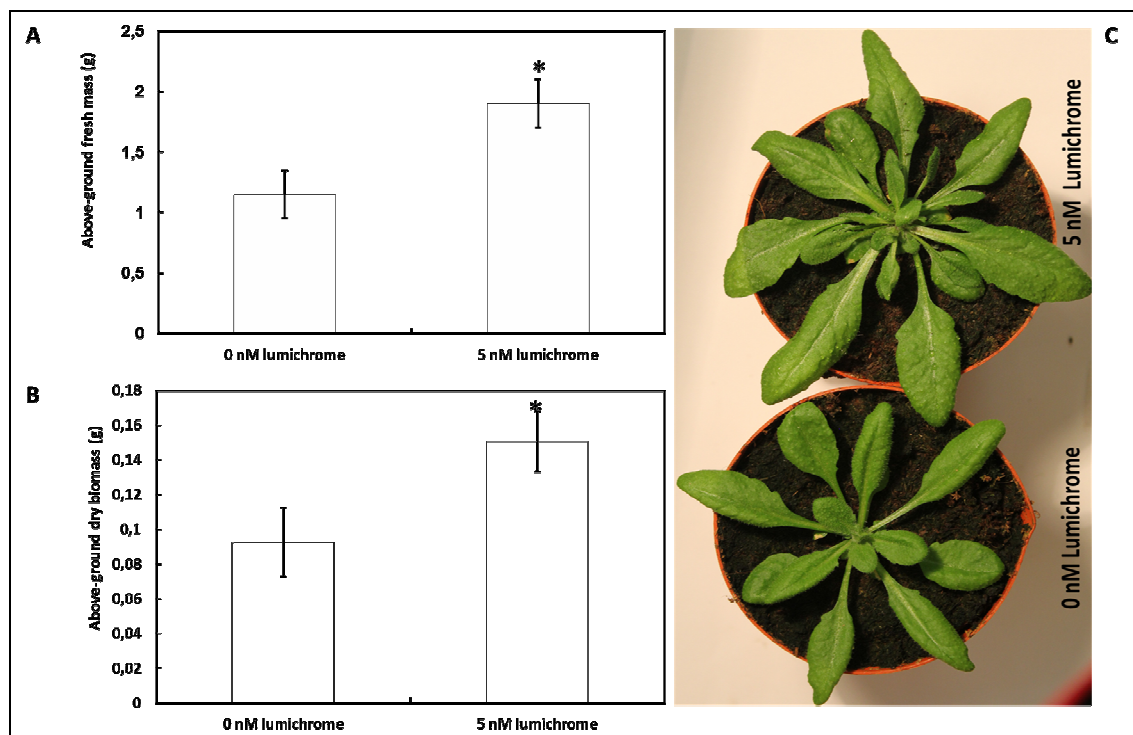
#### ***Validation of RNA sequencing data by quantitative real-time PCR (qRT-PCR)***

The complementary DNA (cDNA) template was obtained via reverse transcription of 1 µg of total RNA, using an oligo (dT<sub>18</sub>) primer and M-MLV (H-) reverse transcriptase (Promega, Anatech, South Africa) following the manufacturer's instruction. The integrity of cDNA template was checked on an RT-PCR using *MONENSIN SENSITIVITY1* (*At2g28390*; *MON1*) gene as a constitutively expressed gene (Supplementary Fig. S1). To validate the reliability of the RNA-seq, we performed qRT-PCR using SYBR-Green dye in reactions containing KAPA SYBR<sup>®</sup> 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/activation enzyme activation at 95°C for 3 minutes and 40 cycles at 95°C and 60°C for 3 and 30 seconds 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  $2^{-\Delta\Delta Ct}$  method (Pfaffl, 2001) using PCR efficiency calculated by LinRegPCR (version 2014.5). *MON1*, 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 two days with 5 nM lumichrome. Fresh and dry weights of five week old rosette leaves showed significantly increased biomass production in treated plants (Fig. 1A and 1B). Rosettes from treated plants were visually distinguishable from those of untreated plants after 5 weeks of growth (Fig. 1C).



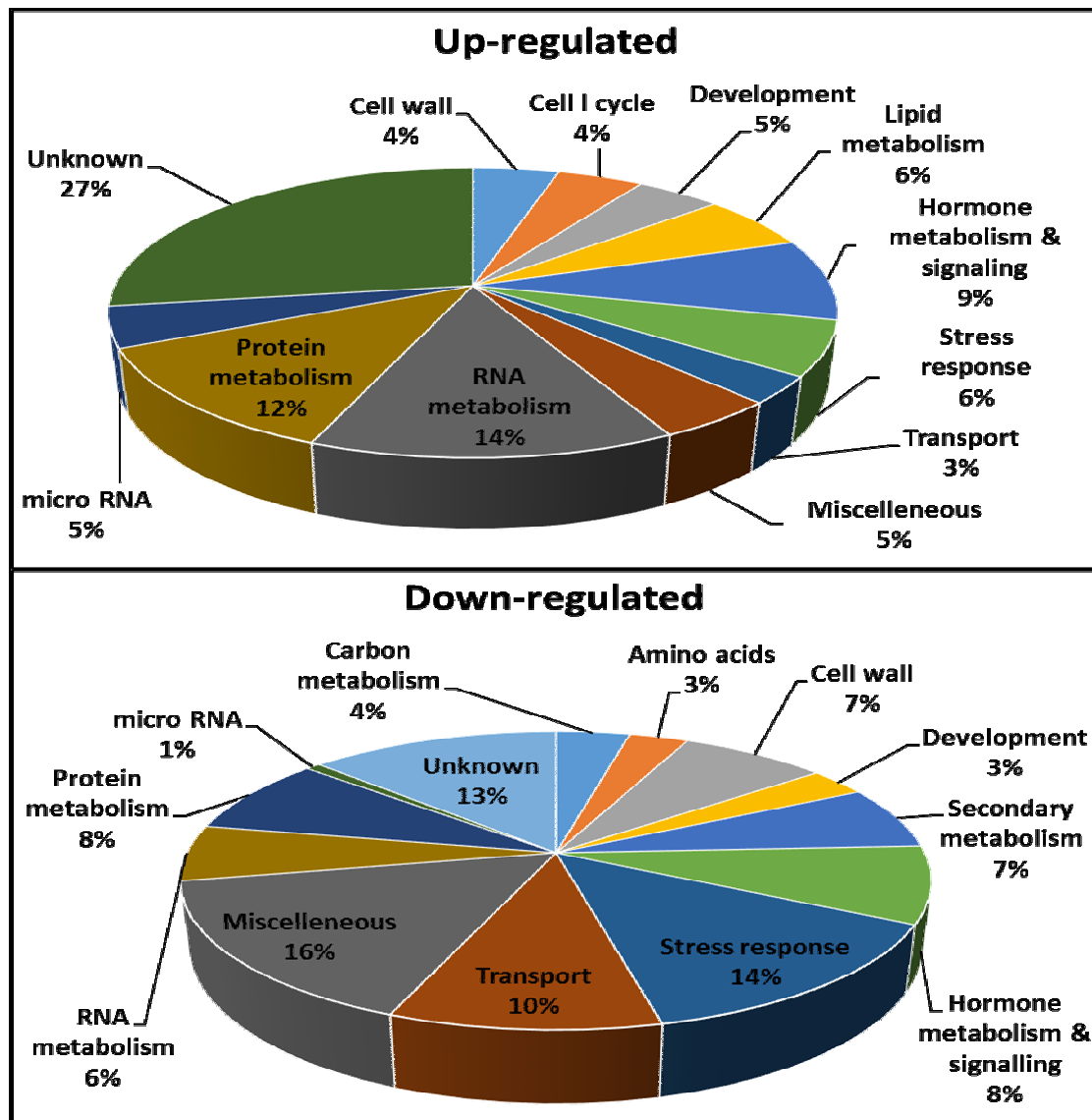
**Figure 1.** Biomass production analysis of lumichrome-treated *Arabidopsis thaliana* plants. Lumichrome treatment (5nM) enhanced fresh (A) and dry biomass production (B) in *Arabidopsis* compared to the untreated control. Treated plants had a visibly larger rosette than untreated plants after 5 weeks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

***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 (Lu et al., 2006; Moxon et al., 2008; Szittyá et al., 2008). The current study, therefore, adopted a high-throughput paired-end mRNA sequencing (Illumina HiSeq2500 platform) platform using the tuxedo analysis protocol to further investigate lumichrome-associated transcriptional modulation. Statistics of clean reads in RNA sequencing are shown in Supplementary Table S2. Out of the total 32 845 different transcripts analyzed, only 198 showed significant differential expression, with 66 and 132 transcripts being significantly up or down-regulated respectively (Fig. 2). We then adopted Mapman annotation for the *Arabidopsis* transcriptome using the mapping file Ath\_AGI\_TAIR9\_Jan2010 to assign genes to 35 functional categories (Fig. 2). Other than 27% (18) which were unassigned, (category 35), the greatest number of up-regulated genes were related to RNA metabolism (9; 14%), protein metabolism (8; 12%), stress response (4; 6%), lipid metabolism (6%), hormone metabolism and signalling (6; 9%). Transcripts from the bins representing miscellaneous (21; 16%), biotic stress response (18; 14%), transport (13; 10%), protein metabolism (11; 8%), hormone and signalling metabolism (11; 8%), secondary metabolism (9; 7%), and RNA transcriptional regulation (9; 6%) were significantly down-regulated.

To gain insight into which genes were most highly over-represented, transcriptome data were loaded into PageMan and a Wilcoxon test (Wilcoxon, 1945) 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 transcripton and Not assigned categories were over-represented. In constrast, 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), miscellaneous myrosinases (jaclin-lectin) and development storage proteins (Supplementary Fig. S2). Besides the over-represented biological functional categories revealed by Wilcoxon,

several genes unique to the lumichrome treatment were associated with phytohormone-related pathways including auxin, gibberellin, ethylene and jasmonate. In addition, an up-regulated transcription of mitotic transcripts were also observed (Table 1). Changes in transcripts expression following lumichrome treatment and the proposed role of lumichrome in regulating *Arabidopsis* growth are illustrated in Fig. 3 and 4 respectively.



**Figure 2.** Functional biological classification of significantly differentially expressed genes of *Arabidopsis* plants treated with 5 nM lumichrome. Differentially expressed genes were assigned by MapMan into different classes. The differentially-expressed transcripts are indicated percentiles.

***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; Fig. 4).

***Lumichrome-regulated transcripts related to hormone and signaling metabolism***

Transcripts for ethylene biosynthesis (Table 1) were altered through increased expression of the *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE (ACO1; At2g19590)*. 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*). Intriguingly, transcripts that putatively have intricacies of synergistic cross-talk effect with ethylene in *Arabidopsis* growth such as an auxin responsive protein, designated *SMALL AUXIN RNA51 (SAUR54; At1g19830)*, and gibberellin-induced regulated responsive 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* lipoxygenase (*LOX*) such as *LOX2 (At3g45140)*, *LOX3 (At1g17420)* and *LOX6 (At1g67560)*. In addition, we observed down-regulated transcripts for jasmonate biosynthesis, including *JACALIN-RELATED LECTIN 34 (JAL34; At3g16460)* and a jasmonate responsive transcripts such as *JASMONATE RESPONSIVE 1 (JRI; At3g16470)* in lumichrome-treated *Arabidopsis* plants.

***Stress and defense response***

Treating *Arabidopsis* rosette leaves with lumichrome, resulted in a distinctive decrease in transcripts levels for systemic acquired resistance against pathogens (Table 1). This includes ten plant immunity-related transcripts such as *PATHOGENESIS-RELATED GENE 1 (PRI;*



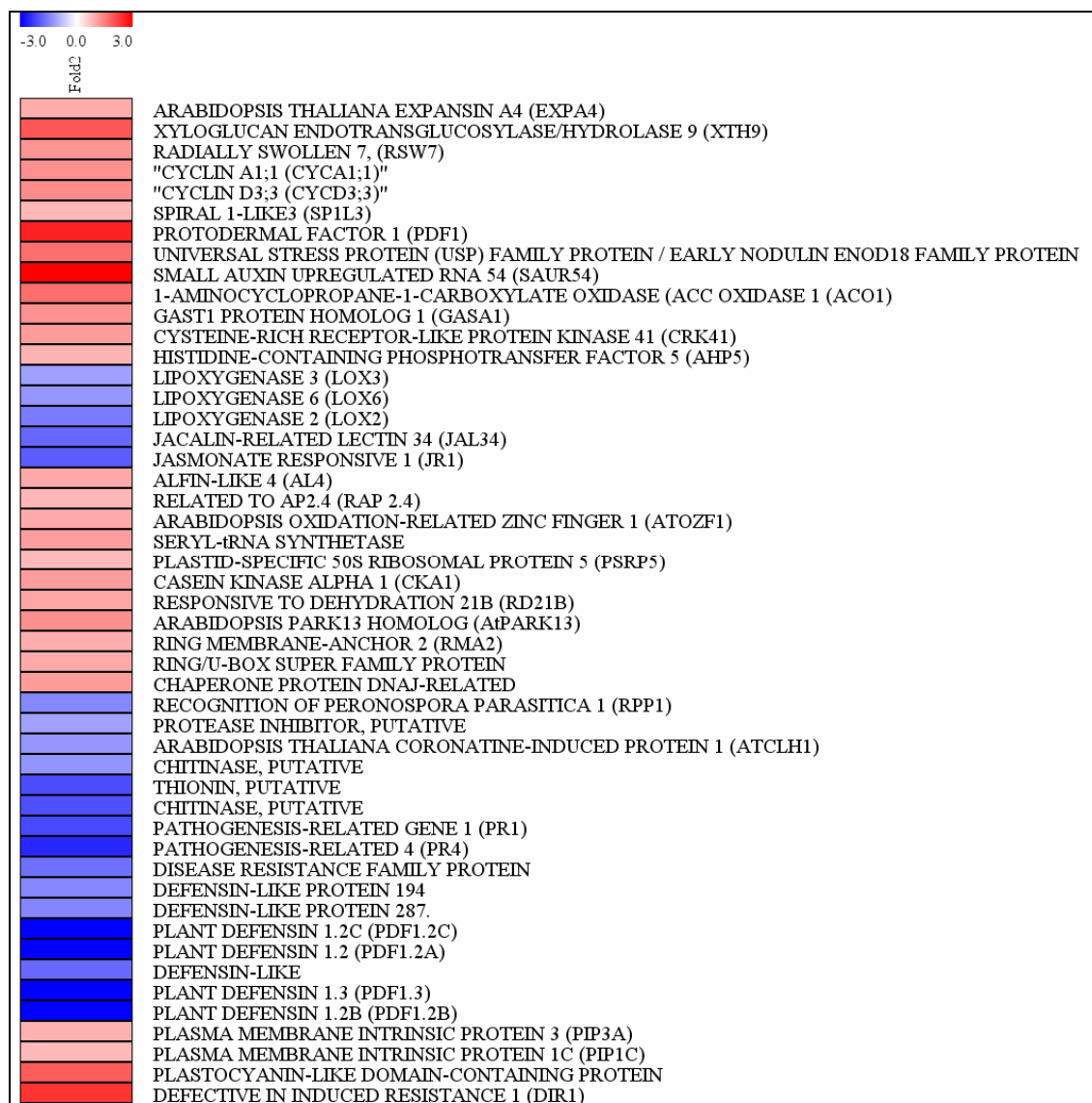
*At2g14610*), *PATHOGENESIS-RELATED 4 (PR4; At3g04720)*, *DISEASE RESISTANCE FAMILY PROTEIN (At2g34930)*, *DEFENSIN-LIKE PROTEIN 194 (At2g43530)*, *DEFENSIN-LIKE PROTEIN 287 (At1g13609)*, *PLANT DEFENSIN 1.2C (PDF1.2C; At5g44430)*, *PLANT DEFENSIN 1.2 (PDF1.2A; At5g44420)*, *DEFENSIN-LIKE (At2g43510)*, *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)*.

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

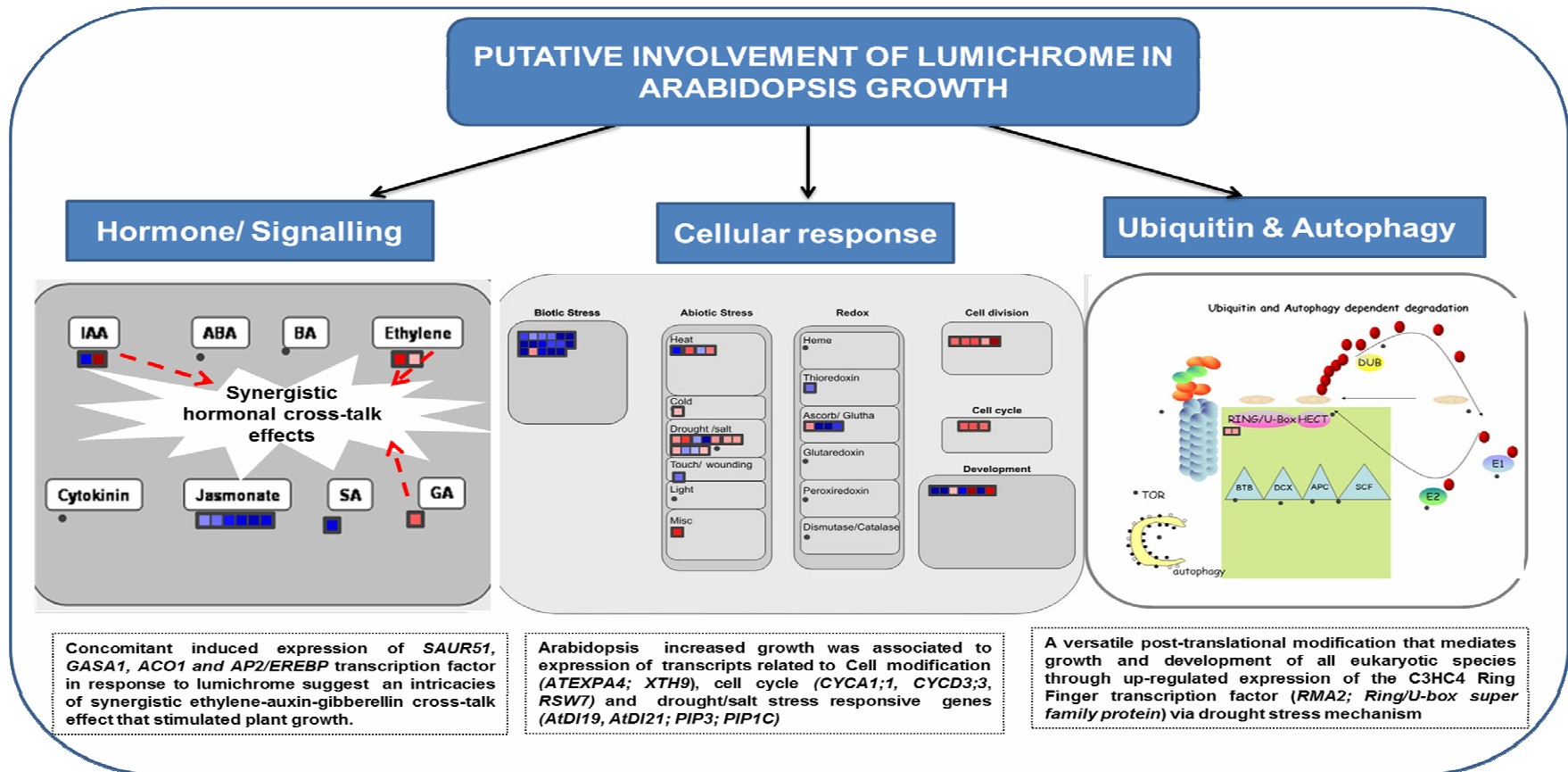
AGI	Gene name	Fold2 change ratio	<i>q</i> -value (adj. <i>p</i> ≤ 0.05)
<i>Cell cycle, division and development</i>			
<i>At2g39700</i>	<i>ARABIDOPSIS THALIANA EXPANSIN A4 (EXPA4)</i>	0.982	0.015
<i>At4g03210</i>	<i>XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9 (XTH9)</i>	1.996	0.006
<i>At2g28620</i>	<i>RADIALLY SWOLLEN 7, (RSW7)</i>	1.251	0.015
<i>At1g44110</i>	<i>CYCLIN A1;1 (CYCA1;1)</i>	1.309	0.044
<i>At3g50070</i>	<i>CYCLIN D3;3 (CYCD3;3)</i>	1.354	0.030
<i>At3g02180</i>	<i>SPIRAL 1-LIKE3 (SPIL3   SPIL3)</i>	0.838	0.042
<i>At2g42840</i>	<i>PROTODERMAL FACTOR 1 (PDF1)</i>	2.616	0.027
<i>At3g03270</i>	<i>UNIVERSAL STRESS PROTEIN (USP) FAMILY PROTEIN / EARLY NODULIN ENOD18 FAMILY PROTEIN</i>	1.713	0.037
<i>Hormone metabolism and Signaling</i>			
<i>At1g19830</i>	<i>SMALL AUXIN UPREGULATED RNA 54 (SAUR54)</i>	3.327	0.006
<i>At2g19590</i>	<i>1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE (ACC OXIDASE 1 (ACO1)</i>	1.696	0.018
<i>At1g75750</i>	<i>GAST1 PROTEIN HOMOLOG 1 (GASA1)</i>	1.301	0.006
<i>At4g00970</i>	<i>CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASE 41 (CRK41)</i>	1.188	0.006
<i>At1g03430</i>	<i>HISTIDINE-CONTAINING PHOSPHOTRANSFER FACTOR 5 (AHP5)</i>	0.905	0.042
<i>At1g17420</i>	<i>LIPOXYGENASE 3 (LOX3)</i>	-1.110	0.030
<i>At1g72520</i>	<i>LIPOXYGENASE 6 (LOX6)</i>	-1.228	0.027
<i>At3g45140</i>	<i>LIPOXYGENASE 2 (LOX2)</i>	-1.555	0.042
<i>At3g16460</i>	<i>JACALIN-RELATED LECTIN 34 (JAL34)</i>	-1.786	0.040
<i>At3g16470</i>	<i>JASMONATE RESPONSIVE 1 (JR1)</i>	-1.922	0.006
<i>At3g16390</i>	<i>NITRILE SPECIFIER PROTEIN 3 (NSP3)</i>	-1.765	0.006

<b>Regulation of transcription, translation and post translational modification</b>			
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
<b>Stress and defense response</b>			
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
At2g26010	PLANT DEFENSIN 1.3 (PDF1.3)	-3.596	0.006
At2g26020	PLANT DEFENSIN 1.2B (PDF1.2B)	-3.596	0.006
<b>Transport</b>			
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<sub>2</sub> fold changes of gene expression of treated leaves normalized to the untreated control leaves of three independent replications and the P value adjusted (adj. P value). Gene annotations were based on probe oligosequence similarity to the *Arabidopsis* genome (Ath\_AGI\_TAIR9\_Jan2010), and represented by the *Arabidopsis* genome initiative (AGI) code. MapMan annotation was used assign genes to 35 functional categories.



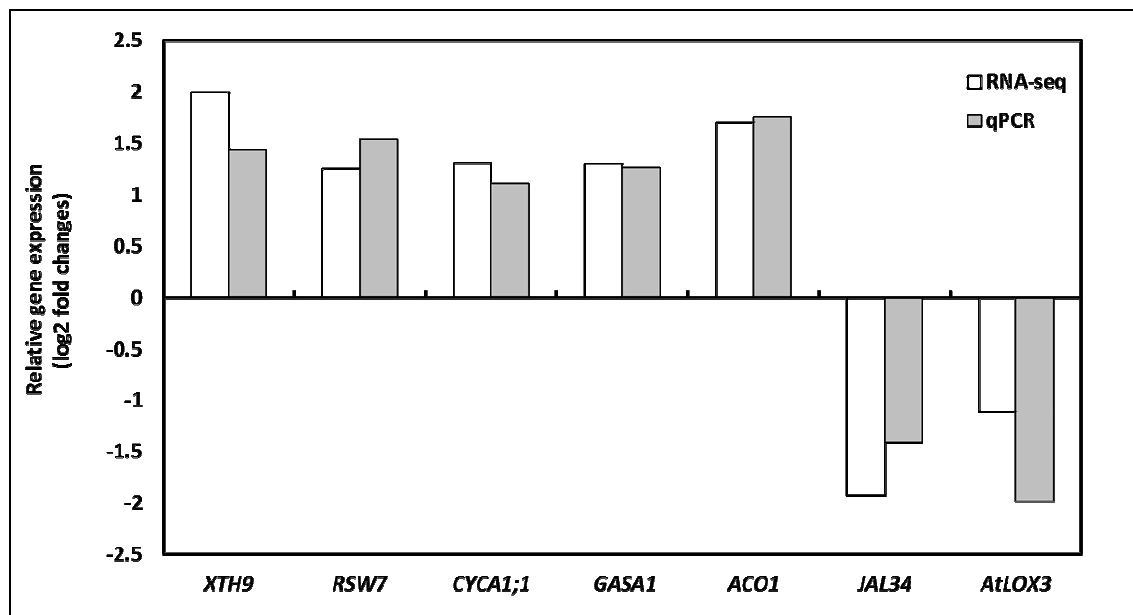
**Figure 3.** Overlay Heat Map of differentially expressed genes. The heat map values represent fold2 change ratio and  $P = (<0.05)$  of wild type *Arabidopsis* plants following lumichrome treatment. The magnitude of change is indicated in the color scale represented in the figure (between +3 and -3), and red represents transcripts that are increased while blue represents those that are decreased.



**Figure 4.** Proposed involvement of lumichrome in *Arabidopsis* growth. *Arabidopsis* growth is promoted via a synergistic ethylene-auxin-gibberellin cross-talk mechanisms, cell expansion and drought responsive gene expression.

### Validation of RNA-seq gene expression by qRT-PCR

To validate RNA-seq results, we randomly selected several differentially-expressed transcripts for expression analysis by qRT-PCR (Supplementary Table S1). The up-regulated transcripts (*XTH9*, *RSW7*, *CYCA1;1*, *GASA1*, *ACO1*) and down-regulated transcripts (*LOX3*, *JAL34*) were analysed by qRT-PCR using *MON1*, which was invariant in the RNA-seq experiment, as reference. Comparisons between the qRT-PCR and RNA-seq analysis showed a positive correlation between the two approaches, indicating that the RNA-seq expression analysis performed is highly reliable (Fig. 5).



**Figure 5.** qRT-PCR validation of differentially up-regulated and down-regulated expressed genes from lumichrome-treated *Arabidopsis* RNA-seq data. Histograms represent relative transcript expression levels of RNAseq and qRT-PCR of lumichrome-treated against untreated rosette leaves. Log<sub>2</sub> 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  $2^{-\Delta\Delta Ct}$  method (Pfaffl, 2001). All the relative transcript expression were significantly different at  $p \leq 0.05$ .

## 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 (Dakora, 2003; Gouws et al., 2012; Khan et al., 2008; Matiru and Dakora, 2005a, 2005b; Phillips et al., 1999; Zhang and Smith, 2002). Our results confirm its growth promoting effect, as demonstrated by significantly increased rosette leaf fresh and dry biomass. This prompted RNA-seq and qRT-PCR validation analysis to elucidate the molecular mechanisms allied to growth promotion on the gene transcription level.

### *Lumichrome confers mechanisms 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 (Cosgrove, 2000; Rose et al., 2002; Sampedro and Cosgrove, 2005; Van Sandt et al., 2007; Choi et al., 2003; Wolf et al., 2012). In *Arabidopsis*, *XTHs* tend to be expressed strongly in rapidly dividing and expanding tissues (Hyodo et al., 2003; Jan et al., 2004), while expression of *EXPANSINS* improved mediation of 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), which in turn enhances 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 the concurrent cell proliferation and cell differentiation during leaf development (Braybrook and Kuhlemeier, 2010; Donnelly et al., 1999; Sugimoto-Shirasu and Roberts, 2003; Veit, 2004; De Veylder et al., 2001), 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 (Inzé, 2005; De Veylder et al., 2003). Expression of *CYCD3* is promoted by cytokinin (CK), hence loss of *CYCD3;1-3* activity reduces the capacity for exogenous CK to instruct shoot formation (Dewitte et al., 2007; Riou-Khamlichi, 1999). Conversely, over-expression of *CYCD3;1* is sufficient to confer CK-independent shoot formation from calli (Riou-Khamlichi et al., 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 (SPIL3)* and *RADIALLY SWOLLEN 7 (RSW7; At2g28620)* were also increased. *SPIL3* is required for cortical microtubule directional control of rapidly expanding *Arabidopsis* cells through directional deposition of cellulose microfibrils (Nakajima et al. 2004; Wasteneys 2004; Nakajima et al. 2006; Foteinopoulos and Mulder 2014). Defects in *SPIL3* 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 (Abe et al., 2003; Lu et al., 1996). 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 stimulate greatly ethylene production in vegetative tissues by inducing the synthesis or activation of *ACC SYNTHASE*, *CS1* and *ACO1* (Abel et al., 1995; Böttcher et al., 2013; Yu and Yang, 1979). As such, the increased *SAUR54* expression could have resulted in activation of *ACO1* and *AP2.4/EREBP* transcripts 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 homeostasis and hormonal control of *Arabidopsis* vegetative growth (Bou-Torrent et al., 2014; Golisz et al., 2008; Peret et al., 2012). 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 auxins and gibberellins. Classically, gibberellins are another 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; Hedden, 2001; Sun and Gubler, 2004). 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 (Chapman et al., 2012; Frigerio et al., 2006). Our results propose that an increase in *SAUR54* transcripts suggested 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 signaling 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 13S-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; Seltsmann 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 *JRI* were all down-regulated in response to lumichrome treatment. These genes are essential for JA biosynthesis upon wounding and during senescence in leaves (Bell et al. 1995; Seltsmann 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 (Norman-Setterblad et al., 2000; Staswick et al., 1998; Stintzi et al., 2001; Thomma et al., 1998). 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 defense responses are 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* (Chen et al., 2004; Wrzaczek et al., 2010); Table 1]. The extracellular domain of CRKs encompasses two copies of the DUF26 domain (stress-antifungal domain), which contains

three cysteine residues in a conserved configuration (C-X<sub>8</sub>-C-X<sub>2</sub>-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 CRKs 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*; *SPIL3*; *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.

### **Supplementary data**

The following supplemental materials are available

Supplementary Figure S1: RNA integrity denatured in 1 % agarose gel and cDNA (E) transcribed from RNA for real time qPCR.

Supplementary Figure S2. PAGEMAN visualisation of comparison of significantly over-represented functional gene categories of lumichrome-treated *Arabidopsis*.

Supplementary Table S1. Primer pairs for the selected genes for qRT-PCR validation

Supplementary Table S2. Statistics of clean reads in RNA sequencing.

### **Authors and contributors**

MP, JK, PH and JL designed the research. MP conducted all the experiments and analysed the data. BC and HM conducted the RNA-seq bioinformatics analysis and PY the qRT-PCR experiments. MP, JK, PH and JL prepared the manuscript.

### **Acknowledgments**

We are very grateful to Professor Martin Kidd for aid in the statistical analysis.

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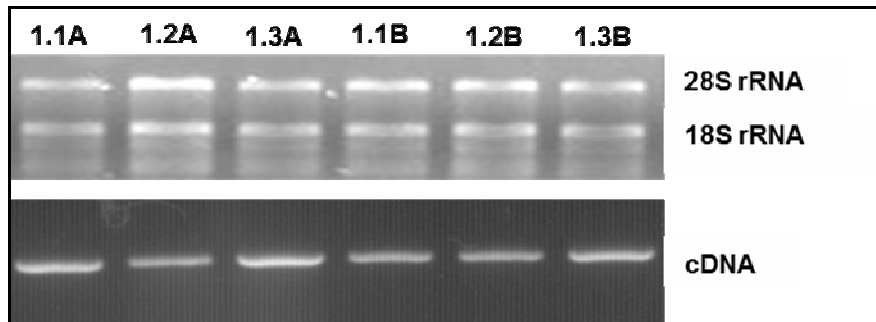
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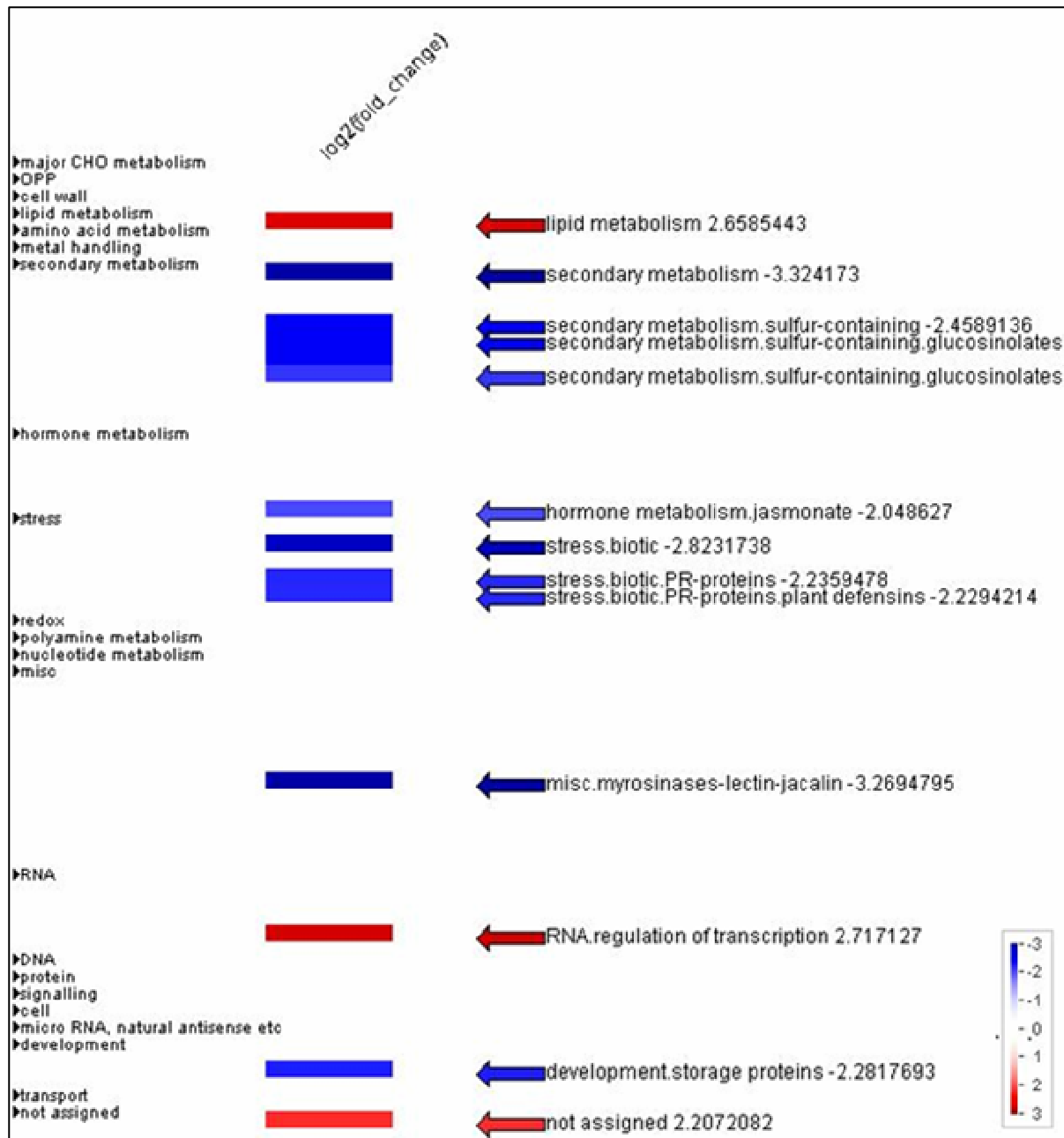
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## Supplementary data



**Figure S1.** RNA integrity denatured in 1 % agarose gel and cDNA transcribed from RNA for real time qPCR. Letters A denotes the untreated plants (0 nM lumichrome), while B represents the treated plants (5 nM lumichrome). cDNA integrity was checked in RT-PCR using *MONENSIN SENSITIVITY1* (*At2g28390*; *MON1*) as a constitutively expressed control.



**Figure S2:** PAGEMAN visualisation of comparison of significantly over-expressed functional gene categories of lumichrome-treated *Arabidopsis* plants. Values are representative of the log<sub>2</sub> transformed values of three statistically analysed untreated and treated replicates respectively. Data was then subjected to Wilcoxon rank sum test ( $P < 0.05$ ) (Usadel et al., 2006). Red indicates an increase whereas blue indicates a decrease.

**Table S1. Primer pairs for the selected genes for RT-qPCR validation**

Gene ID	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
At2g28390	<i>MONENSIN SENSITIVITY1 (MON1)</i>	CAAGGCAGGAAATCACCAGGTTG	CTGTACAGCTGATGCAGACCAG
At4g03210	<i>XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE (XTH9)</i>	GTGTCAACGAAGGCGAAGTCAC	TCTTGATTCAAACCCAGCTCCAG
At2g28620	<i>RADIALLY SWOLLEN 7 (RSW7)</i>	TGTCTTGGTCACGAGTACAAGGTG	ACTCTCTCTTCCTCGGTGTTGAGC
At1g44110	<i>CYCLIN A1;1 (CYCA1;1)</i>	ACCCAACAAGACGACCATGGAAC	AGCCGTGAGCAGTACTACACAAT CATCATC
AT1G75750	<i>GAST1 PROTEIN HOMOLOG 1 (GASA1)</i>	TTCTCCAACCTCGTCCAGGCTGATG	TACACACGCACTCCCACAATCATC ATCG
AT2G19590	<i>ACC OXIDASE 1 (ACO1)</i>	ACCAGTCAGAGATGGTCAAGGC	TCATCCATCGTCTTGCTGAGTCC

**Table S2. Statistics of clean reads in RNA sequencing.**

Replication	Read pairs before trim	Read pairs after trim	% of read pairs retained
1.1A	11,678,990	9,678,201	82.87
1.2A	9,167,958	7,608,804	82.99
1.3A	9,542,620	7,810,013	81.84
1.1B	8,285,442	6,771,778	81.73
1.2B	9,746,348	7,791,433	79.94
1.3B	25,760,361	21,407,442	83.1

Letters A and B under replicates column represent untreated control and lumichrome-treated *Arabidopsis* plants respectively

## CHAPTER 3

### **Analysis of the mode of action lumichrome treatment in *Arabidopsis* (II.): Proteome analysis suggests that chloroplast biogenesis and carbon metabolism are mediating enhanced photosynthesis and plant growth**

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#### **Abstract**

The influence of lumichrome on *Arabidopsis thaliana* rosette leaves was assessed by physiological measurements combined with global expression profiling (proteomics and metabolomics). Physiological analyses revealed an increase in photosynthetic rate, stomatal conductivity, transpiration rate and chlorophyll levels. Enhanced photosynthesis rate was linked to a reciprocal increase in DIFFERENTIATION AND GREENING-LIKE 1 (DAL1) and chaperone (RUBISCO INTERACTING CHAPERONIN-60A; CPN60A, CHLOROPLAST HEAT SHOCK PROTEIN 70-1; cpHSC70-1) proteins, which are involved in early chloroplast development, plastid differentiation and maturation. In addition, an increase in chaperone proteins (cpHSC70-1, COLD-REGULATED 15A; COR15A) involved in facilitating protein translocation into the stroma and stabilization of chloroplast membranes respectively might have contributed to enhanced photosynthesis rate. Efficient chloroplast functioning was possible through concomitant increased chlorophyll levels and LIGHT HARVESTING COMPLEX PHOTOSYSTEM II SUBUNIT 1 and 6 proteins (LHCB1; LHCB6), with an important functional role in the formation or aggregation of the discs in the chloroplast, which in turn

might have contributed to an optimal thylakoid structure flexibility, maintenance of Photosystem II (PSII) supercomplex structure during state transition and thermal dissipation for PSII protection. Furthermore, an improved attainable photosynthesis rate, possibly through a highly flexible regulation of photosynthetic light reactions in plant chloroplasts, comprising i) the oxygen evolving complex (OEC) of PSII, ii) the electron transport pump via CF<sub>0</sub>F<sub>1</sub> ATP synthase, iii) thiol-based redox state control, and iv) non-photochemical quenching (NPQ), was suggested. The present study also suggested that synergy between increased stomatal conductivity, RUBISCO ACTIVASE and CPN60A might have contributed to an increased rate of CO<sub>2</sub> fixation, while the partitioning of the fixed carbon into cytosolic glycolysis via FRUCTOSE-BISPHOSPHATE ALDOLASE (FBA8) and PHOSPHORYLATING NAD GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2 (GAPC2) might have implied increased growth due to concomitant increases in sucrose and reducing sugars. The intimate molecular connections between increased soluble sugars and redox homeostasis, as well as between sugar accumulation and carbon fixation, were demonstrated.

**Key words:** *Arabidopsis*, chloroplast development, photosynthesis efficiency, retrograde signalling, proteomics, metabolomics.

## Introduction

Photosynthetic active plants utilise the pigment chlorophyll to provide chemical energy (ATP) and reducing power (NADPH), to drive the so-called dark reactions of photosynthesis (Kramer and Evans, 2011). The photosynthetic process is divided into four stages, each occurring in a defined area of the chloroplast. These stages include absorption of light, electron transport leading to the reduction of NADP<sup>+</sup> to NADPH, generation of ATP, and conversion of CO<sub>2</sub> into carbohydrates (carbon fixation). Reactions for the first three are catalyzed by proteins in the thylakoid membrane. Conversion of CO<sub>2</sub> into chemical intermediates and starch occurs in the chloroplast stroma, while formation of sucrose from three-carbon intermediates takes place in the cytosol (Lodish *et al.*, 2000).

Photosynthetic competence is dependent on proper chloroplast biogenesis and its efficient functions. Chloroplast biogenesis is a multistage process leading to fully differentiated and functionally mature plastids. Chloroplasts develop from proplastids, small organelles in leaf primordia, which possess small vesicles containing no photosynthetic complexes. Upon illumination, during natural photomorphogenesis, proplastids develop the thylakoid network and photosynthetic capacity (Pogson and Albrecht, 2011). Higher plant chloroplasts possess a thylakoid membrane network, which hosts the protein complexes that carry out the light reactions of oxygenic photosynthesis and provide a medium for energy transduction (Sakamoto et al., 2008). This includes four multi-subunit protein complexes (Photosystem I [PSI], Photosystem II [PSII], ATP synthase, and cytochrome b6f complexes), each with multiple cofactors (Rast et al., 2015; Wollman et al., 1999). PSI and PSII are the two photosystems in the thylakoid membranes that function as charge-separation devices in the process of photochemical energy transduction. Therefore, a highly flexible regulation of photosynthetic light reactions in plant chloroplasts is a prerequisite to provide sufficient energy flow to downstream metabolism and plant growth. Regulation activities occur via the control of OEC, excitation energy transfer between PSII and PSI, electrochemical gradient across the thylakoid membrane ( $\Delta\text{pH}$ ), and chloroplast related homeostasis (Munekage et al., 2002; Tikkanen and Aro, 2014).

Central to plant growth processes are several growth regulators known as plant hormones and their synthetic analogues referred to plant growth regulators (PGRs) (Gaspar et al., 1996; Spartz and Gray, 2008), which are extremely important agent in the integration of developmental activities (Lone *et al.*, 2010). Other than the well-known classified plant hormones, plant growth promoting rhizobacteria (PGPR) have been shown to regulate the fundamental processes in plant development under normal and stress conditions. Apart from their well-known role for nitrogen fixation in legumes, symbiotic nitrogen-fixing bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium* and *Mesorhizobium* affect fundamental processes in plant development through the release of plant hormones such as cytokinin (Phillips and Torrey, 1972) and auxins like indoleacetic acid (IAA) (Hirsch et al., 1997; Law and Strijdom, 1989; Vessey, 2003). Another PGPR-produced growth promoting substance, lumichrome, has been isolated from *Sinorhizobium meliloti* (Phillips et al., 1999).

Lumichrome has been shown to act as a signal molecule stimulating root respiration and overall growth in the bacterium's symbiotic plant partner (Khan et al., 2008; Phillips et al., 1999; Volpin and Phillips, 1998; Yanagita and Foster, 1956). Lumichrome increased photosynthesis, stomatal conductance and leaf transpiration and triggered a compensatory increase in whole-plant net carbon assimilation. (Phillips *et al.*, 1999; Matiru & Dakora 2005; Matiru & Dakora 2005; Khan *et al.*, 2008). Enhanced growth was also attributed to transport of lumichrome in the xylem and its accumulation in leaves, which subsequently triggered events that promote cell division and leaf expansion (Matiru & Dakora 2005). At the molecular level, protein profiling of lumichrome-treated *Lotus japonicus* and tomato (*Solanum lycopersicum*) roots suggested induction of proteins implicated in protecting plants against stress by re-establishing normal protein conformation and thus cellular homeostasis (Gouws, 2009). Moreover, enhanced growth was attributed to an increase in enzymes involved in glycolysis and heat stress signalling chaperones (Gouws *et al.*, 2012). However, previous studies have demonstrated mixed physiological responses between plants species to lumichrome (Matiru and Dakora, 2005a, 2005b; Phillips et al., 1999). Proteomic data are only available for lumichrome-treated tomato and *Lotus japonicus* plants (Gouws, 2009). Due to mixed physiological responses among different plant species, molecular processes responsible for growth promotion in other species remain somewhat elusive. In the current study, proteomics, metabolomics and biochemical photosynthesis-related approaches were followed to investigate the lumichrome-associated growth stimulatory mechanisms in *Arabidopsis thaliana*.

## **Materials and methods**

### ***Plant material, growth condition and photosynthesis related measurements***

*Arabidopsis thaliana* (ecotype Columbia-0) was grown in a controlled growth cabinet with a 16/8 h day/night photoperiod at 22±2°C and 75% relative humidity. Pots were arranged in a factorial randomised completely block design (RCBD) consisting of 6 replications and blocks respectively. Plants were treated with 5 nM lumichrome by a combination of root drenching and foliar application (50 ml) of intact plants at intervals of 2 days throughout the entire growth period. Lumichrome stock solutions (5 nM) were freshly prepared in methanol/1 M HCl (49:1) for each treatment (Phillips *et al.* 1999). Control plants were similarly treated with dH<sub>2</sub>O

containing the same amounts of MeOH/HCl (49:1) as the lumichrome treated plants. All data was collected from 5-week-old *Arabidopsis* rosette leaves. Photosynthetic parameters were concurrently measured with an open-system photosynthesis meter (Li-Cor 6400, Li-Cor Inc., Lincoln, NE, USA) equipped with the standard leaf chamber (encloses 6 cm<sup>2</sup> of leaf area) and CO<sub>2</sub> injection system (model 6400-01, Li-Cor Inc., Lincoln, NE, USA) for control of CO<sub>2</sub>. The light intensity for all measurements was 400 μmol m<sup>-2</sup>.s<sup>-1</sup>, provided by a red–blue light source (model 6400-02, Li-Cor Inc., Lincoln, NE, USA). Photosynthetic readings were taken at 12 irradiance intervals (PAR 0– 2000 μmol m<sup>-2</sup>.s<sup>-1</sup>). All photosynthetic values were adjusted and expressed on a leaf dry mass basis. The light-saturated rate of photosynthesis (P<sub>max</sub>), quantum yield (φ), light compensation point (LCP) and dark respiration were derived from the light–response curves, whereas photosynthetic water-use efficiency (PWUE) was calculated from measurements of P<sub>max</sub> and transpiration rate. Pigments associated with photosynthesis were extracted from frozen plant material and assayed in 80% (v/v) acetone and determined and calculated as previous described (Arnon, 1949). Physiological data was analyzed using the STATISTICA Version 12 (StatSoft Inc.), following two-way analysis of variance (ANOVA) and a Games-Howell *post-hoc* test to infer differences between treatments at 95% confidence level.

### ***Protein preparation and LC-MS/MS analysis of peptides***

*Arabidopsis* leaf tissue (100 mg) was ground to a fine powder in liquid nitrogen using a mortar and pestle. The ground tissue was solubilised in 6M Urea/50 mM Tris-HCl (pH 8.0). Protein disulfide bridges were reduced in 5 mM dithiothreitol (DTT) for 30 min at 37°C. Subsequently, cysteine residues were alkylated in 15 mM iodoacetamide (IAA) in the dark for 30 min. The total soluble protein was determined using Bio-Rad Protein Assay Kit I5000001 according to the manufacturer's instructions by measuring absorbency of the dilution spectrophotometrically at 595 nm, using bovine gamma globulin as a standard (Bradford, 1976). Subsequently, 50 μg total protein for each sample was digested for 4 h at 37°C with endoproteinase (Trypsin/Lys-C Mix, Mass Spectrometry grade, Promega). After 6-fold dilution with 50 mM Tris-HCl (pH 8.0), samples were digested further overnight at 37°C. These were then acidified by addition of trifluoroacetic acid (TFA; 1% [v/v]) to terminate the reaction. Samples were centrifuged at 13



000xg for 10 min in order to remove particulate materials and immediately desalted using Millipore ZipTip18 micropipette tips (Rappsilber *et al.*, 2003).

Tryptic peptide mixtures were analyzed by LC/MS/MS using a Nanoflow Easy-nLC1000 (Thermo Scientific) as an HPLC-system and a Quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive Plus, Thermo Scientific) as a mass analyzer. Peptides were eluted from a 75  $\mu\text{m}$  x 50 cm analytical column (Thermo Scientific) on a linear gradient running from 4 to 64% (v/v) acetonitrile over 240 min and sprayed directly into the Q-Exactive mass spectrometer. Proteins were identified by MS/MS using information-dependent acquisition of fragmentation spectra of multiple charged peptides. Up to twelve data-dependent MS/MS spectra were acquired for each full-scan spectrum acquired at 60,000 full-width half-maximum resolution. Overall cycle time was approximately one second.

Protein identification and ion intensity quantitation was carried out by MaxQuant version 1.4.0.1 (Cox and Mann, 2008). Spectra were matched against the *Arabidopsis* proteome (TAIR10, 35386 entries) using Andromeda (Cox and Mann, 2011). Carbamidomethylation of cysteine was set as a fixed modification; oxidations of methionine were set as variable modifications. Mass tolerance for the database search was set to 20 ppm on full scans and 0.5 Da for fragment ions and multiplicity was set to 1. For label-free quantitation, retention time matching between runs was chosen within a time window of 2 min. Peptide false discovery rate (FDR) and protein FDR were set to 0.01, while site FDR was set to 0.05. Hits to contaminants (e.g. keratins) and reverse hits identified by MaxQuant were excluded from further analysis (Benjamini and Hochberg, 1995).

Reported ion intensity values were used for quantitative data analysis. cRacker (Zauber and Schulze, 2012) was used for label-free data analysis of phosphopeptide ion intensities based on the MaxQuant output (evidence.txt). All proteotypic peptides were used for quantitation. Within each sample, ion intensities of each peptide ion species (each m/z) were normalized against the total ion intensities in that sample (peptide ion intensity/total sum of ion intensities).

Subsequently, each peptide ion species (i.e. each  $m/z$  value) was scaled against the average normalized intensities of that ion across all treatments. For each peptide, values from three biological replicates then were averaged after normalization and scaling. Comparisons between lumichrome treatment and the control were evaluated using pairwise t-tests and multiple testing corrections.

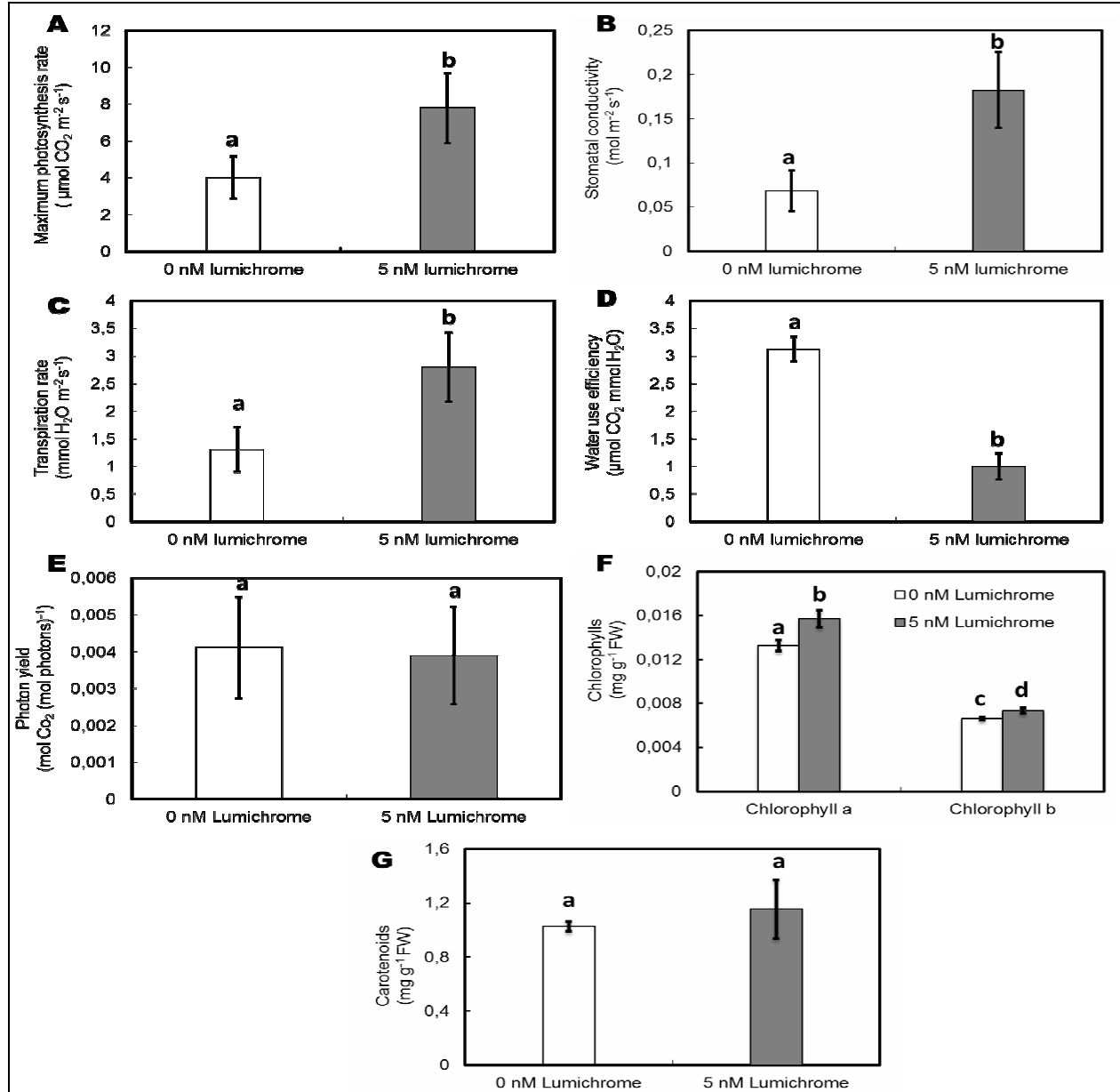
### ***Metabolite extraction and derivatization for GC-TOF-MS analysis***

Metabolites were extracted and derivatized for gas chromatography–time of flight-mass spectrometry (GC-TOF-MS) profiling, according to established procedures (Lisec et al., 2006b). Normalization of raw data followed the procedure outlined in Roessner *et al.* (2001), while peaks were identified using the Tagfinder software (Luedemann et al., 2012) coupled with mass tags deposited in the Golm Metabolome Database (Kopka et al., 2005).

## **Results**

### ***Lumichrome enhances various facets of photosynthesis in Arabidopsis***

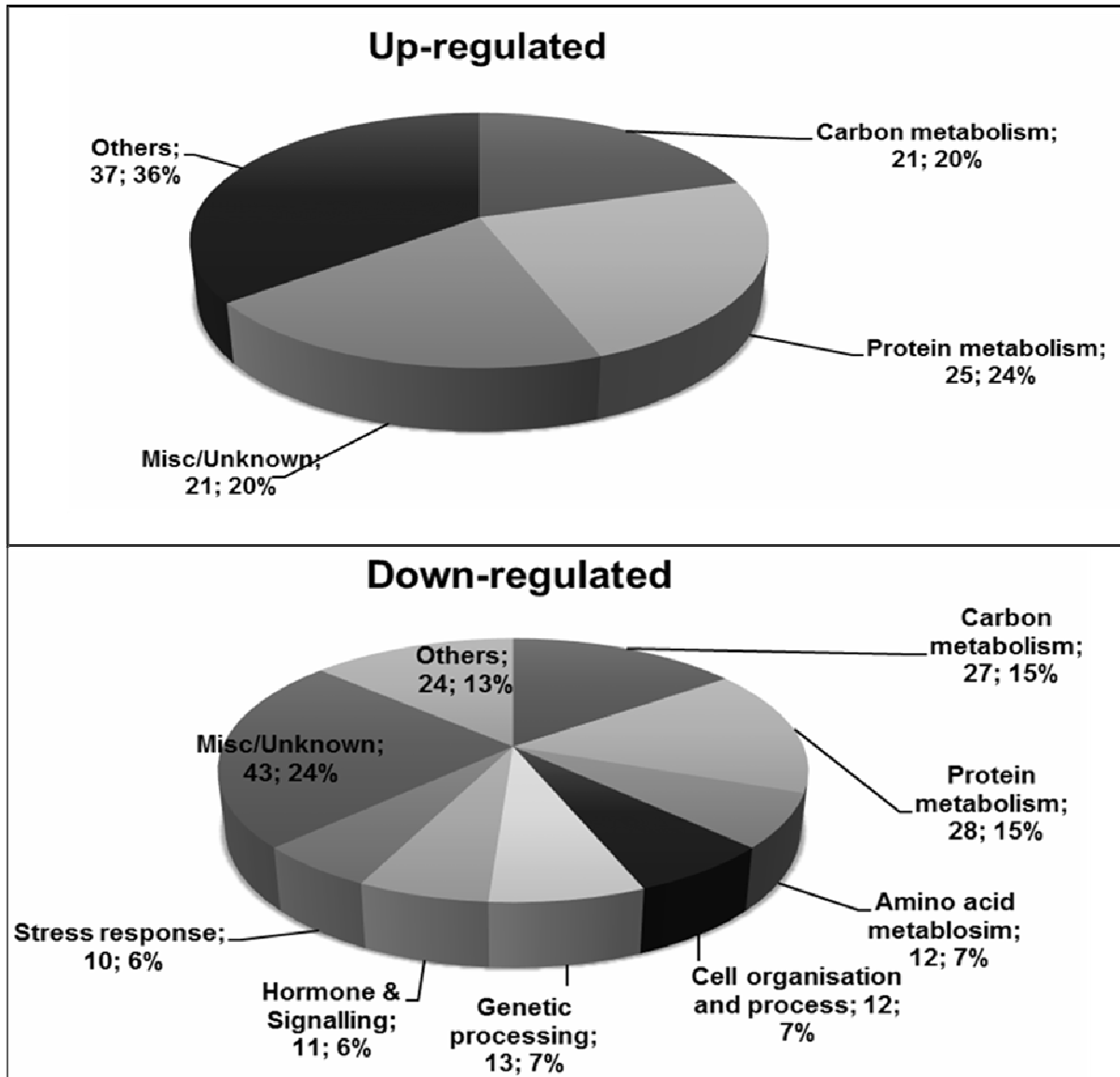
Analysis of the 5 week old *Arabidopsis* rosette leaves showed that lumichrome-treated plants exhibited an increase in maximum photosynthetic rate (Fig. 1A), stomatal conductance (Fig 1B) and transpiration rate (Fig. 1C) and a decrease in water use efficiency (Fig. 1D) compared to corresponding untreated plants. There was a significant increase in chlorophyll *a* and chlorophyll *b* concentrations (Fig 1F), whereas carotenoid levels were unaltered (Fig.1G).



**Figure 1.** Photosynthetic parameters and pigments in *Arabidopsis* in response to 5 nM lumichrome treatment. Photosynthetic  $\text{CO}_2$  assimilation rates (A), stomatal conductance to water vapour (B), transpiration rates (C), intrinsic WUE (D), photon yield (E), chlorophyll pigments (F) and carotenoids (G) of 5-week-old *Arabidopsis* leaves. Error bars denotes standard error of the means ( $n=6$ ). Within graphs, treatments marked with the same letters were not significantly different from one another ( $P < 0.05$ ; Games-Howell *post-hoc* test).

***Differential protein profiles in response to lumichrome***

A previous proteomic study to examine the effect of lumichrome on tomato and *Lotus japonicus* revealed that it alters proteins involved in glycolysis and protein folding (Gouws, 2009). Lumichrome treatment in the present study resulted in a total of 284 significantly altered proteins, with 88 and 135 being up-regulated and down-regulated respectively. According to functional analysis (Fig. 2), protein and carbon associated metabolism were the two most widely represented categories, constituting 23% and 21% of up-regulated proteins respectively. A similar response trend was demonstrated for the down-regulated proteins, with the largest number of proteins also being associated with protein and carbon metabolism (15% each). Other functional categories containing proteins that were down-regulated were cell division, cell cycle, stress response, hormone, signalling and genetic processing, which includes proteins related to transcription, translation and post-translational modifications.



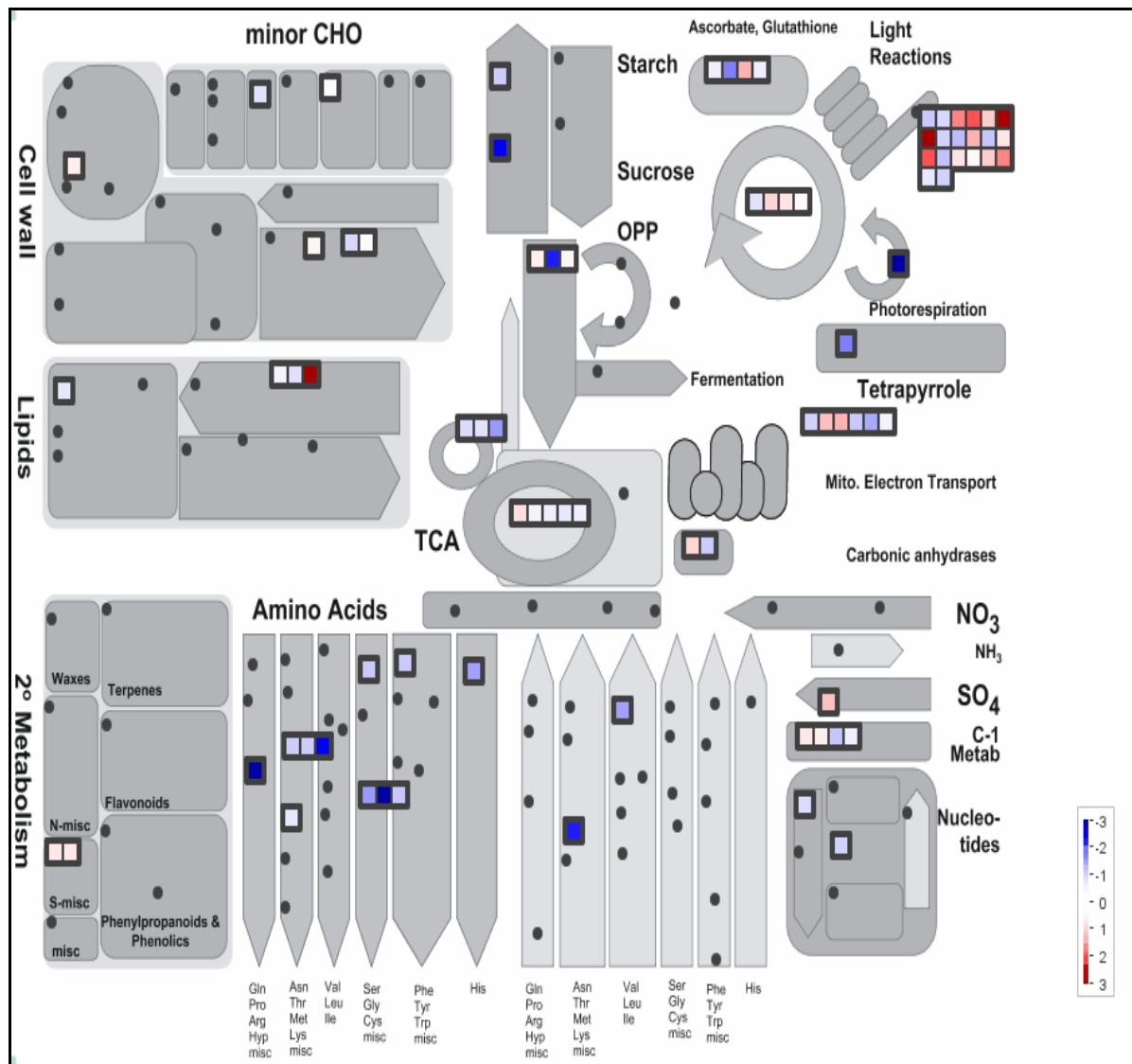
**Figure 2.** Functional differential protein distribution of differential protein profiles in *Arabidopsis* ( $P \leq 0.05$ ). The percentage of each functional group was obtained based on the calculated absolute number of proteins under each of the functional groups subtracted from the total number of significantly differentially-expressed proteins.

***Carbohydrate metabolism related protein change response***

According to the Mapman metabolism overview (Fig. 3), the most affected proteins were related to photosynthesis. These included an increase in OEC such as PSII SUBUNIT P-1 (PSBP-1, At1g06680), PSII SUBUNIT TN (PSBTN, At3g21055), and the oxygen evolving protein 2 (OEE2), namely PSBQ-1 and PSBQ-2 (At4g21280, At4g05180). In addition, our result revealed significantly increased chloroplast ATPase synthase proteins, which form the catalytic entity in the chloroplast ATP synthase electron transport chain. These include ATP SYNTHASE SUBUNIT ALPHA (ATPA, Atcg00120), ATP SYNTHASE SUBUNIT BETA (ATPB, Atcg00480), ATP SYNTHASE DELTA-SUBUNIT (ATPD, At4g09650) and ATP SYNTHASE EPSILON CHAIN (ATPE, Atcg00470). However, LIGHT HARVESTING COMPLEX PHOTOSYSTEM II SUBUNIT 6 (At1g15820, LHCB6) and LIGHT-HARVESTING CHLOROPHYLL-PROTEIN COMPLEX II SUBUNIT B1B2 (At2g34430, LHCB1B2) were significantly decreased following lumichrome treatment. With respect to PSI, the light antenna protein, ONE-HELIX PROTEIN 2 (OHP2, At1g34000), was present at reduced levels following lumichrome treatment. Despite decreased *PSAE-1* (At4g28750), the PSI reaction centre was promoted through an increase in PSI SUBUNIT E-2 (PSAE-2, At2g20260) and PSI-N (PSAN, At5g64040) (Table 1; Supplementary Fig. 1). In addition, lumichrome treatment resulted in increased levels of the chloroplast DIFFERENTIATION AND GREENING-LIKE 1 (DAL1, At2g33430) protein (Table 1).

Furthermore, treating *Arabidopsis* with lumichrome affected proteins levels for carbon metabolism. Regarding CO<sub>2</sub> assimilation, several Calvin cycle proteins were significantly increased, plastidial glycolytic PHOSPHOGLYCERATE KINASE FAMILY (At1g56190), RUBISCO INTERACTING CHAPERONIN-60A (CPN60A, At2g28000) and RUBISCO ACTIVASE (RCA, At2g39730). Partitioning of carbohydrates between starch and sucrose was also significantly altered (Table 1). This was revealed through a suppressed plastid PHOSPHOGLUCOMUTASE 1 (PGM1, At5g51820) and ADP-GLUCOSE PYROPHOSPHORYLASE (AGPase, At5g19220), while the cytosolic proteins including PHOSPHORYLATING NAD GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

C2 (GAPC-2, At1g13440) and FRUCTOSE-BISPHOSPHATE ALDOLASE (FBA8, At3g52930).



**Figure 3.** MapMan general overview of a pairwise comparison of lumichrome-treated *Arabidopsis* rosette leaves relative to the untreated control. Proteins that were shown to be differentially expressed using  $p < 0.05$  as a cut-off value were imported. Red represents proteins that were up-regulated while blue indicates those that were down-regulated upon 5 nM lumichrome treatment. The magnitude of protein profiles is indicated by the colour intensity.

***Stress, redox homeostasis and hormone metabolism-associated proteins***

Lumichrome significantly induced several proteins, which are essential for scavenging reactive oxygen species in the light regulation of photosynthetic metabolism (Table 1). This includes high abundance reactive oxygen species (ROS) redox signalling-related proteins such as ARABIDOPSIS THIOREDOXIN M-TYPE 1 (AtHM1, At1g03680), THIOREDOXIN M-TYPE 2 (TRX-M2, At4g03520) and THIOREDOXIN M-TYPE 4 (TRX-M4, At3g15360) (Supplementary Fig. S2). Furthermore, theoredoxin-related proteins including PDI-LIKE 1-1 (ATPDIL1-1, At1g21750) and THIOREDOXIN Y2 (TRX-Y2, At1g43560) were significantly increased. *Arabidopsis* also accumulated increased amounts of CHLOROPLAST HEAT SHOCK PROTEIN 70-1 (cpHSC70-1, At4g24280) and BIP1-HEAT SHOCK PROTEIN 70 (BIP1-Hsp70, At5g28540) family proteins. This was further accompanied by an abundance of chloroplast stromal COLD-REGULATED 15A (COR15A, At2g42540). Conversely, proteins related to jasmonate biosynthesis, such as ALLENE OXIDE CYCLASE 2 (AOC2, At3g25770), ALLENE OXIDE SYNTHASE (AOS, At5g42650) and LIPOXYGENASE 2 (AtLOX2, At3g45140), were down-regulated (Table 1).

**Table 1. Protein profiling of *Arabidopsis* rosette leaves in response to 5 nM lumichrome treatment**

AGI	Protein name	Biological pathway	log2 fold change ratio	t-test p-value
<b>Photosynthesis</b>				
At2g33430	DAL1  differentiation and greening-like 1	development	1.71	0.00
At1g06680	Photosystem II subunit P-1 (PSBP-1)	PSII light reaction	1.03	0.00
At1g51400	Photosystem II 5 kD protein	PSII light reaction	3.31	0.01
At1g15820	Light harvesting complex photosystem II subunit 6 (LHCB6)	PS II LHC	-0.93	0.05



At2g34430	Photosystem II light harvesting complex gene B1B2 (LHB1B2)	PSII LHC	-1.10	0.03
At3g21055	Photosystem II subunit TN (PSBTN) I	PSII light reaction	3.37	0.02
At4g05180	Photosystem II subunit Q-2 (PSBQ-2)	PSII light reaction	2.02	0.00
At4g21280	Photosystem II subunit Q-1 (PSBQ-1)	PSII light reaction	1.68	0.00
At5g64040	Photosystem I reaction center subunit PSI-N (PSAN)	PSI light reaction	1.34	0.00
Atcg00120	ATP synthase subunit alpha (ATPA)	PS light reaction-ATP synthase	0.82	0.00
Atcg00470	ATP synthase epsilon chain (ATPE)	PS light reaction-ATP synthase	1.09	0.03
Atcg00480	ATP synthase subunit beta (ATPB)	PS light reaction-ATP synthase	0.41	0.03
At4g09650	ATP synthase delta-subunit gene (ATPD)	PS light reaction-ATP synthase	1.69	0.00
Atcg00730	Photosynthetic electron transfer D (PETD)	PS light reaction-cytochrome b6/f	-1.19	0.03
Atcg01060	PSAC	PSI light reaction	1.97	0.02
At4g28750	Photosystem I reaction centre subunit IV / PSAE protein ( <i>PSAE-1</i> )	PSI light reaction	-1.24	0.00
Atcg00350	Photosystem I, PsaA/PsaB protein (PSAA)	PSI light reaction	-1.13	0.00
At2g20260	Photosystem I subunit E-2 (PSAE-2)	PSI light reaction	0.74	0.00
At1g34000	One-helix protein 2 (OHP2)	PSI LHC I	-1.03	0.02
At1g20020	Ferredoxin-NADP(+)-oxidoreductase 2 (ATLFNR2)	PS light reaction-(ox/red) ferredoxin reductase	-0.73	0.02
At3g15840	Post-illumination chlorophyll fluorescence increase (PIFI)	PS light reaction-cyclic electron flow-chlororespiration	-1.03	0.00
At1g56190	Phosphoglycerate kinase family protein	PS-Calvin cycle	0.97	0.05

At2g28000	RuBisCo interacting chaperonin-60alpha (CPN60A)	PS calvin cycle	0.38	0.02
At2g39730	RuBisCo activase (RCA)	PS calvin cycle RuBisCo interacting	0.77	0.01
At2g47400	CP12 domain-containing protein 1 (CP12-1)	PS calvin cycle	-0.81	0.00
<b>Carbon metabolism</b>				
At1g13440	Glyceraldehyde-3-phosphate dehydrogenase C2 (GAPC-2)	Cytosolic glycolysis	0.61	0.01
At3g52930	Dually targeted aldolase	Cytosolic glycolysis.	0.49	0.00
At5g51820	Phosphosphoglucomutase 1 (PGM1)	Plastid glycolysis	-2.28	0.00
At5g48300	ADP glucose pyrophosphorylase 1 (AGPase)	major CHO metabolism-starch synthesis	-1.08	0.05
<b>Hormone metabolism</b>				
At3g25770	Allene oxide cyclase 2 (AOC2)	Jasmonate hormone metabolism	-0.68	0.00
At3g45140	Lipoxygenase 2 (ATLOX2)	Jasmonate hormone metabolism	-0.56	0.01
At5g42650	Allene oxide synthase (AOS)	Jasmonate hormone metabolism	-0.61	0.04
<b>Redox homeostasis</b>				
At1g03680	Thioredoxin M-type 1 (TRX-M1)	Redox-thioredoxin	0.73	0.00
At1g07890	ascorbate peroxidase 1 (ATAPX01)	Redox-ascorbate and glutathione	1.31	0.00
At1g21750	Protein disulfide isomerase-like 1-1 (PDIL1-1)	Redox-thioredoxin	0.27	0.01
At1g43560	Thioredoxin Y2 (TRX Y2)	Redox-thioredoxin	0.52	0.01
At2g20270	Thioredoxin superfamily protein	Redox-glutaredoxins	1.09	0.00
At3g15360	Thioredoxin M-type 4 (TRX-M4)	Redox-thioredoxin	0.74	0.00

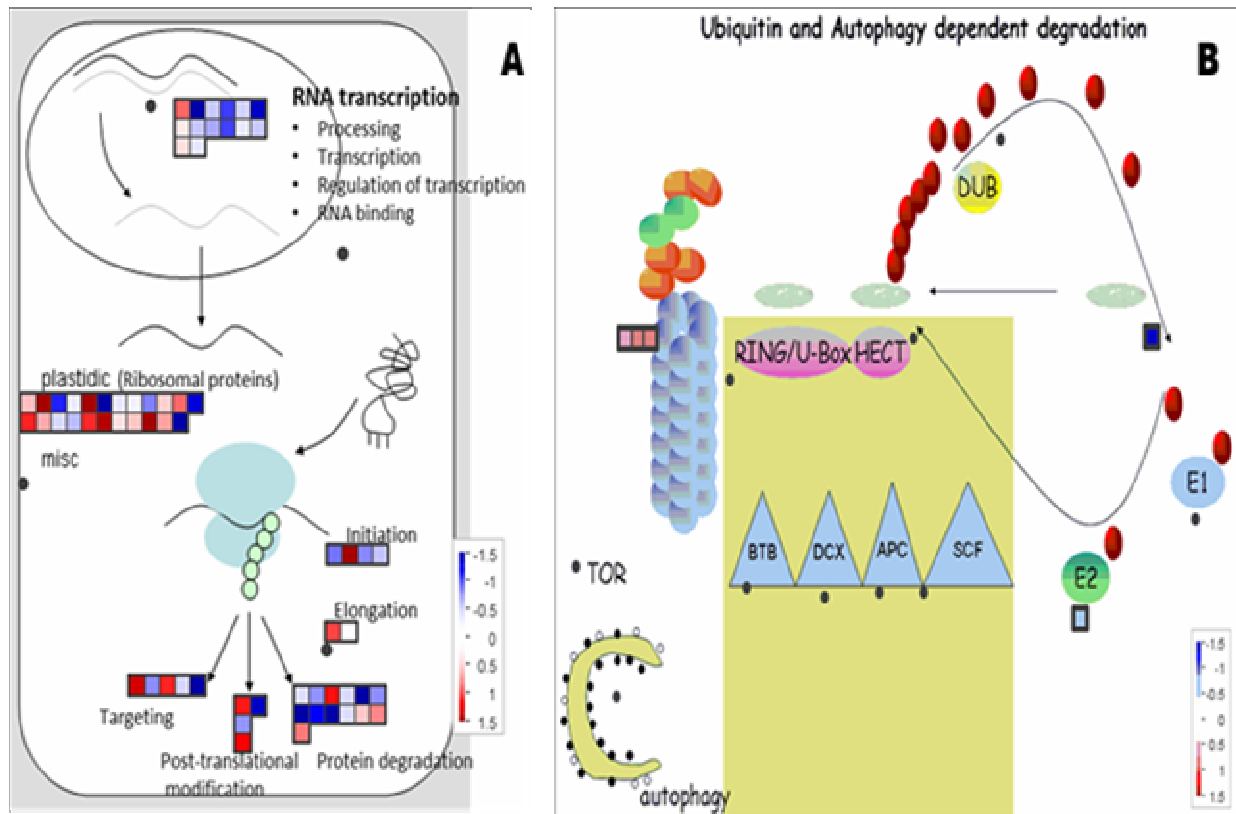
At3g52960	Peroxiredoxin-II-E, (PRXIIIE)	Redox-peroxiredoxin	1.00	0.00
At4g03520	Thioredoxin superfamily protein (TRX-M2)	Redox-thioredoxin	0.86	0.00
<b>Stress response</b>				
At2g42540	COR15A  cold-regulated 15a	Stress-abiotic/cold	0.78	0.04
At4g24280	cpHSC70-1  chloroplast heat shock protein 70-1	stress-abiotic/heat	0.54	0.00
At5g28540	BIP1  heat shock protein 70 (Hsp 70) family protein	stress-abiotic/heat	0.32	0.05
At1g12270	Hop1  stress-inducible protein, putative	Stress	-0.41	0.01
At1g20450	ERD10  Dehydrin family protein	stress-abiotic/unspecified	-0.52	0.03
At1g76180	ERD14  Dehydrin family protein	stress-abiotic/unspecified	-0.70	0.00
At1g80030	Molecular chaperone Hsp40/DnaJ family protein	stress-abiotic/heat	-1.36	0.03
At3g44110	ATJ3  DNAJ homologue 3	stress-abiotic/heat	-0.89	0.02
At4g16660	heat shock protein 70 (Hsp 70) family protein	stress-abiotic/heat	-0.57	0.03
At4g36020	CSDP1  cold shock domain protein 1	stress-abiotic/cold	-0.49	0.03
At4g37910	mtHsc70-1  mitochondrial heat shock protein 70-1	stress-abiotic/heat	-0.60	0.01

Values are represented as log<sub>2</sub> fold changes of protein profiles of lumichrome-treated compared to the untreated control and the *p* value adjusted (adj. value) to correct for the probability of false positives using the FDR correction (Benjamini and Hochberg, 1995). *p* value <0.05 was deemed significant.

### ***Ubiquitin and autophagy dependent protein degradation in lumichrome-treated Arabidopsis plants***

Lumichrome treatment in *Arabidopsis* increased proteasome-dependent degradation (Fig. 4) through high abundance of CYSTEINE-TYPE ENDOPEPTIDASE INHIBITOR (CYS1, At5g12140). In addition, our results showed an increase in ubiquitin proteinases such as REGULATORY PARTICLE TRIPLE-A1 (RPT1A, At1g53750), N-TERMINAL

NUCLEOPHILE AMINO HYDROLASES (Ntn HYDROLASES, At1g79210) and PROTEASOME REGULATOR1 (PTRE1, At3G53970). However, the four serine proteases including CLP PROTEASE PROTEOLYTIC SUBUNIT 3 (CLPP3, AT1G66670), PEPTIDASE S41 FAMILY PROTEIN (At4g17740), TRYPSIN FAMILY PROTEIN WITH PDZ DOMAIN (DEG8, At5g39830) were significantly reduced.



**Figure 4.** MapMan RNA-protein synthesis and ubiquitin-proteasome dependent degradation overview. RNA-protein synthesis overview maps (A) and ubiquitin and autophagy dependent protein degradation (B) of differentially expressed proteins calculated from three independent replicates. Red and blue histogram denotes up-regulated and down-regulated proteins respectively.

***Metabolite profile changes in response to lumichrome***

GC-TOF-MS metabolite profile analysis was performed. This identified a total of 160 metabolites. To test for the significance of changes in these upon lumichrome treatment, we performed a Student *t*-test ( $p \leq 0.05$ ), which resulted in only 15 upregulated metabolites (Table 2) that were significantly increased upon lumichrome treatment. This included accumulation of soluble sugars and alcohols including fructose, glucose, maltose, sucrose, trehalose, alpha- alpha and rhamnose. Among these, the most highly induced were fructose and glucose.

**Table 2. Changes in metabolite abundance of *Arabidopsis* rosette leaves in response to 5 nM lumichrome treatment**

Compound	Fold2 change ratio	<i>t</i> -test ( $p \leq 0.05$ )	Response
Sugars and sugar alcohols			
Fructose	1,15	0,006	up-regulated
Glucose	1,13	0,036	up-regulated
Maltose	0,71	0,033	up-regulated
Sucrose	0,23	0,019	up-regulated
Trehalose, alpha-alpha	0,60	0,010	up-regulated
Rhamnose	0,30	0,016	up-regulated

GC-MS analyses of *Arabidopsis* rosette leaves metabolites in response to lumichrome. Compound values are expressed as fold change ratio value of the treated against the untreated control. Response ratio = intensity of the mass of the specific metabolite normalized to fresh weight and the internal standard ribitol. Significant changes were evaluated using *t*-test ( $p \leq 0.05$ ).

## Discussion

Phytohormones have been known to stimulate growth of plant organs such as leaves, roots and stems via cell division and expansion (Letham *et al.*, 1978; Ross *et al.*, 2002; Campanoni *et al.*, 2003; van der Graaff *et al.*, 2003). Recent studies have, however, discovered new bacterial metabolites such as lumichrome that also affect seed germination and alter seedling development when applied to different plant taxa (Zhang & Smith, 2001; Smith *et al.*, 2002; Dakora, 2003). Consistent with previous studies (Matiru & Dakora 2005; Khan *et al.*, 2008), our study revealed that lumichrome treatment of *Arabidopsis* plants increased photosynthetic rate, stomatal conductivity and chlorophyll levels. Increased photosynthetic rates may be ascribed to stomatal opening, allowing CO<sub>2</sub> entry into leaves and/or more efficient CO<sub>2</sub> utilization, pointing to well-functioning mesophyll metabolism. To link phenotype with the underlying mechanisms determining it, proteomic analysis was performed on lumichrome-treated and untreated plants to investigate the effects of lumichrome on plant metabolism.

### ***Chloroplast biogenesis and efficient functioning possibly contributed to an increased photosynthesis rate***

Chloroplast biogenesis is a multistage process leading to fully differentiated and functionally mature plastids. Its biogenesis and competent functioning needs to be coordinated with seedling growth to ensure optimal rates of photosynthesis without oxidative damage (Sun, 1963). In that respect, an increase in DIFFERENTIATION AND GREENING-LIKE 1 (DAL1 also designated as MORF2) and RUBISCO INTERACTING CHAPERONIN-60A (CPN60A) proteins, suggested improved early chloroplast development, differentiation and maturation. Arrested plastid development in DAL mutants at the early steps of thylakoid assembly explains the importance of DAL1 in the early chloroplast differentiation (Bisanz *et al.*, 2003). Altered pigmentation is an easily scored and sensitive monitor of plastid function, hence green leaves contain normal chloroplasts whereas the white ones have small plastids that resemble proplastids (Chatterjee *et al.*, 1996). This has been previously demonstrated in *Antirrhinum majus* and *Arabidopsis*, in which a null mutation of a gene required for chloroplast differentiation and palisade development showed white leaves due to deficiencies in plastid RNA editing (Bisanz *et al.*, 2003; Chatterjee *et al.*, 1996; Takenaka *et al.*, 2012). Moreover, plastid CPN60A null or

deficient mutation negatively affect photosynthesis due to abolished greening of plastids, resulting in an albino phenotype with either no developed chloroplasts or impaired plastid division and reduced chlorophyll levels, with a consequential severely impaired plant development (Peng et al., 2011; Suzuki et al., 2009).

In addition, chloroplast protein import and processing through the outer and inner envelope (membranes) of the chloroplasts, are essential in chloroplast biogenesis and maturation. The majority of chloroplast-targeted proteins enter the plastids via the translocon on the outer envelope of chloroplast (TOC) and translocon on the inner envelope of chloroplast (TIC), while others are targeted via the endoplasmic reticulum (Kessler and Schnell, 2009). We observed an increase in Stromal CHLOROPLAST HEAT SHOCK PROTEIN 70 (cpHSC70), which is a stable part of the translocon (Su and Li, 2010). *Physcomitrella patens* and *Arabidopsis* impaired in stromal cpHSC70-1 or cpHSC70-2 have shown to be defective in protein import into chloroplasts (Shi and Theg, 2010b), while loss of chloroplast import and processing lead to a white seedling phenotype and seedling lethality (Cline and Dabney-Smith, 2008; Shipman-Roston et al., 2010). Therefore our results suggested that the enhanced photosynthesis rate could be ascribed to protein translocation at inner envelope of chloroplast (TIC) possibly via an enhanced cpHSC70 (Bisanz et al., 2003; Chatterjee et al., 1996; Rolland et al., 2011; Romani et al., 2012; Schultes et al., 2000).

The synthesis of chlorophyll, plays a role not only in the formation or aggregation of the discs in the chloroplast, but also in determining their final structure (Gibbs, 1962). During thylakoid formation the photosynthetic complexes are formed, which requires a coordinated import of pigment-binding proteins such as chlorophylla/b-binding proteins (CAB/light-harvesting chlorophyll-binding) and the biosynthesis of the pigments (Cazzonelli and Pogson, 2010). Impairment of chlorophyll synthesis in rice leads to either a lack or a reduced amount of LHC proteins in thylakoid membranes (Terao et al., 1985). The present study revealed a simultaneous increase in chlorophyll levels and LHCB proteins (LHCB6 and LHCB1), implying an optimal thylakoid structure flexibility, maintenance of PSII supercomplex structure during state transition

and thermal dissipation for PSII protection (Havaux et al., 2007; Jansson, 1999; Pietrzykowska et al., 2014; Ramel et al., 2013). Molecular analysis using LHCB6 knock-out mutants of *A. thaliana* demonstrated that lack of this protein has a negative effect on the packing of PSII in the membrane and thus the efficiency of photosynthesis and photoprotection (de Bianchi et al., 2008; Kovács et al., 2006). In addition, Holt *et al.* (2005) and Avenson *et al.* (2008) proposed that LHCB6, together with the other minor antenna complexes, is the site of formation of a zeaxanthin radical cation, and thus it is directly involved in non-photochemical quenching (NPQ) that protects plants from photodamage in high light conditions (Nott et al., 2006; Staneloni et al., 2008). In addition, increased chlorophyll levels were accompanied by reduced Jasmonate acid (JA) biosynthesis-associated proteins such LIPOXYGENASE 2 (LOX2), ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE 2 (AOC2). Reduced JA biosynthesis leads to delayed leaf senescence. This was demonstrated in a quick-leaf-senescence inbred maize line, Huangzao-4(HZ4), in which light absorption, energy transformation and electron transfer capacity were decreased, leading to a faster decreases of the density of active PSII reaction centres, the O<sub>2</sub> evolution rate and the PSI photo-activity (Papuga et al., 2010).

Increased RUBISCO ACTIVASE (RCA) and THIOREDOXIN -M TYPE1, 2 and 4 (TRX-M1; TRX-M2; TRX-M4) in response to lumichrome might have contributed to increased CO<sub>2</sub> fixation. The enzyme RuBisCo incorporates CO<sub>2</sub> into plants during photosynthesis and it is widely accepted as the ultimate rate-limiting step in photosynthetic carbon fixation. Reduction of RCA in higher plants reduces the maximum attainable rates of photosynthesis (Eckardt and Snyder, 1997; Mate et al., 1993; Pollock et al., 2003). However, both RuBisCo and RCA contain numerous cysteines, highlighting the need for reduction of disulfide bonds to enhance the enzymes' catalytic activities (Wang *et al.*, 2013). It was previously reported that RCA regulates the activity of RuBisCo in response to light-induced changes in both the ADP/ATP ratio and the redox potential via thioredoxin-f (Motohashi et al., 2001). However, an increase in CO<sub>2</sub> assimilation in lumichrome-treated *Arabidopsis* in the current study may be linked to a preferential TRX-M (TRX- M1; TRX-M2; TRX-M4) targeted redox activation of chloroplastic RCA. This is in agreement with a previous report in which deficiency of TRX-M impaired plant growth and decreased the CO<sub>2</sub> assimilation rate (Okegawa and Motohashi, 2015).



Activation of RuBisCo by RCA serves an important regulatory function in linking the rate of CO<sub>2</sub> fixation to the rate of electron transport activity via the production of ATP. ATPase activity is an intrinsic property of RCA (Robinson and Portis, 1989). Increased RCA was accompanied by upregulated chloroplast F<sub>0</sub>F<sub>1</sub> complex (CF<sub>0</sub>F<sub>1</sub> ATP synthase) proteins, such as ATPA, ATPB, ATPD and ATPE in the current study. CF<sub>0</sub>F<sub>1</sub> ATP synthase contains multiple subunits arranged in a hydrophilic structure (F<sub>1</sub>) interacting with nucleotides and coupling ATP synthesis to electron transport, and a hydrophobic structure (F<sub>0</sub>) forming a channel which transports H<sup>+</sup> across the membrane. Likewise, as previously reported in tobacco and *Arabidopsis* leaves (Witt 1979; Cruz *et al.*, 2001; Cruz *et al.*, 2005), an increase in CF<sub>0</sub>F<sub>1</sub> ATP synthase might have stimulated the light driven induced-electron pump, which further implied a triggered acidification of the thylakoid lumen, leading to the activation of non-photochemical quenching (NPQ) (Kanazawa and Kramer, 2002).

Other than CF<sub>0</sub>F<sub>1</sub> ATP synthase, electron transport was possibly improved via an increase in optimal OEC. Green eukaryotes, including higher plants and green algae, have a set of three extrinsic proteins, PSBO, PSBP, and PSBQ, which bind to the lumenal surface of PSII. Generally, PSBO is most strongly bound to PSII and stabilizes the manganese (Mn) cluster (Kuwabara *et al.*, 1985). PSBP is involved in Ca<sup>2+</sup> and Cl<sup>-</sup> retention, while PSBQ participates primarily in Cl<sup>-</sup> retention (Ghanotakis *et al.*, 1984; Miyao and Murata, 1985; Yi *et al.*, 2008). PSBP and PSBQ collectively protects the Mn cluster from reductants in the bulk solution (Ghanotakis *et al.*, 1984). Significant defects in PSII function, such as lower oxygen-evolving activity, lower quantum yield, slower electron transfer rate at the donor side of PSII and reduced photoautotrophic growth have been previously reported on cyanobacterial mutants lacking PSBP and PSBQ (Chaiwongsar *et al.*, 2012; Thornton *et al.*, 2004). Likewise, increased levels of OEE1 and OEE2 proteins, including PSBP, PSBQ1, PSBQ2 and the 5 kD protein (At1g51400), suggests an improved stabilization of PSBP binding by PSBQ, thereby contributing to the maintenance of the catalytic Mn cluster of the water oxidation machinery in *Arabidopsis* PSII (Ifuku *et al.*, 2011; Kakiuchi *et al.*, 2012) following lumichrome treatment. This in-turn suggests

that increased growth in the *Arabidopsis* rosette could be attributed to optimum quantum yield, electron transfer rate at the donor side of PSII (Chaiwongsar et al., 2012; Thornton et al., 2004).

***Lumichrome might confer a signal functional role that alters carbon metabolism***

Proteomic studies on tomato roots previously reported repression of starch biosynthesis as a result of reduced levels of plastid PHOSPHORYLATING NAD-SPECIFIC GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH), also designated GAPC1 (Gouws, 2009). GAPC1 reversibly converts the glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by coupling with the reduction of NAD<sup>+</sup> to NADH (Backhausen et al., 1998; Meyer-Gauen et al., 1994). Plastid GAPC, along with the PHOSPHOGLYCERATE KINASE, is involved in starch breakdown for the production of ATP needed for starch metabolism in green and non-green plastids (Backhausen et al., 1998; Plaxton, 1996). We however, observed a decrease in plastid PHOSPHOGLUCOMUTASE 1 (PGM1) and the ADP-GLUCOSE PHOSPHORYLASE (AGPase, also designated APS1) proteins. PGM1 is essential in the second enzymatic step of starch biosynthesis that converts glucose-6-phosphate into glucose-1-phosphate, while AGPase converts glucose-1-phosphate and ATP into ADP-glucose and inorganic pyrophosphate (PPi), thus constituting the first step in starch biosynthesis. *Arabidopsis* mutants in which the activities of PGM1 or AGPase are reduced or abolished have greatly reduced levels of leaf starch or a starchless phenotype (Caspar *et al.*, 1985; Lin *et al.*, 1988). Growth of the starchless mutant is known to be restricted relative to wild type, due to its inability to synthesize starch in both source and sink tissues for use at night (Huber and Hanson, 1992).

High abundance of the cytosolic glycolysis-related FRUCTOSE-1,6-BISPHOSPHATE 8 (FBA8) and cytosolic phosphorylating, NAD-specific GAPC2 protein in lumichrome-treated plants, suggested that lumichrome confers a signal functional role in sucrose biosynthesis. Cytosolic FBA8 and cytosolic NAD-specific GAPC2 play an important role in glycolysis by providing the cell with ATP and NADH (Valverde et al., 2005; Zaffagnini et al., 2013). GAPC2 and AtFB8 have highly reactive catalytic cysteine structures, that can undergo multiple redox-

induced post-translational modifications in response to reactive oxygen species and reactive nitrogen species (Zaffagnini et al., 2013). Therefore, an upregulated cytosolic PROTEIN DISULFIDE ISOMERASE–LIKE 1-1 (PDIL1-1) might have played an important role in reducing disulfide bonds to enhance GAPC2 and FBA8's catalytic activities (Balmer et al., 2001; Noiva, 1999), resulting in improved glycolytic activities of these proteins. This might in-turn lead to accumulation of water soluble carbohydrates and synthesis of ATP (Lu et al., 2012). Our results further suggested that lumichrome modifies sugar signaling pathways, in which FBA8 and GAPC2 underpinned increased levels of sucrose, glucose and fructose. Sucrose provides both an energy source and the building blocks for the production and maintenance of biomass, illustrating the dependence of biomass production on carbon fixation in lumichrome-treated plant (Koch, 2004).

#### ***Ubiquitin-proteasome dependent degradation in response to lumichrome treatment***

Much of plant physiology, growth, and development is controlled by the selective removal of short-lived regulatory proteins through the small protein ubiquitin and the 26S proteasome proteolytic pathway. Our study suggested that lumichrome regulates the 26S proteasome, a multi-subunit ATP-dependent protease responsible for degrading most short-lived intracellular proteins targeted for breakdown by ubiquitin conjugation (Chung and Tasaka, 2011). Lumichrome appears to induce a high abundance of PROTEASOME REGULATOR1 (PTRE1), CYSTEINE-TYPE ENDOPEPTIDASE INHIBITOR (CYS1), N-TERMINAL NUCLEOPHILE AMINO HYDROLASES (Ntn HYDROLASES) and REGULATORY PARTICLE TRIPLE-A1 (RPT1A). PTRE1 is a positive regulator of the 26S proteasome through fine-tuning the homeostasis of Aux/IAA repressor proteins, thus modifying auxin activity. PTRE1 deficient plants have a dwarf phenotype with small and curved leaves, and impaired shoot apical dominance (Yang et al., 2016). Overexpression of *CYS1* plays a crucial role in inhibiting cell death resulting from nitro-oxidative stress (Belenghi et al., 2003). Therefore, an increase in CYS1 protein in lumichrome-treated *Arabidopsis* plants suggested suppression of cell death from virulent pathogens, oxidative and nitrosative stresses.

Plants with a functional circadian clock have an enhanced performance and fitness because they correctly phase their daily life to the light/dark cycle in the environment. It is now well established that *Arabidopsis* clock genes and their protein products operate through auto-regulatory feedback loops that promote rhythmic oscillations in cellular, metabolic and physiological activities (Más, 2008; Staiger et al., 2003). Treating *Arabidopsis* with lumichrome in the current study altered RNA binding proteins, which are involved in the post-transcriptional control in the circadian system through low abundance of GLYCINE-RICH RNA-BINDING PROTEIN 7 and 8 (GRP7, GRP8). GRP7 negatively auto-regulates oscillations of its own mRNA by alternative splicing coupled to nonsense-mediated mRNA decay (NMD) mechanism, while GRP8 encoding a related RRM-protein is an GRP7 target that also negatively auto-regulates and reciprocally cross-regulates GRP7 via a post-transcriptional mechanism (Heintzen et al., 1997; Staiger et al., 2003). When GRP7 protein levels are high, an alternatively spliced transcript is produced at the expense of the fully spliced mRNA, resulting in mRNA variants with premature termination codons that may give rise to non-functional proteins upon translation (Heintzen et al., 1997). These non-functional proteins are rapidly degraded via the NMD mechanism (Heintzen et al., 1997; McGlincy and Smith, 2008; Staiger et al., 2003). This mechanism is essential in the prevention of accumulation of potentially harmful truncated proteins (McGlincy and Smith 2008). Therefore, reduction in GRP7 and GRP8 in lumichrome-treated plants might have prevented the production of alternatively spliced transcripts that cannot be translated into functional proteins at the expense of the fully spliced mRNAs (Heintzen et al., 1997; McGlincy and Smith, 2008; Staiger et al., 2003).

## Conclusions

Treating *Arabidopsis* with lumichrome enhanced photosynthesis related processes, including maximum photosynthesis rate and CO<sub>2</sub> fixation. Molecular analysis using proteomics revealed that the enhanced photosynthesis rate could be linked to enhanced levels of proteins involved in early chloroplast development, differentiation and maturation (DAL1; CPN60A; cpHSC70). Effective chloroplast functioning was linked to an increase in chlorophyll levels and light reactions proteins (LHCB6 and LHCB1), implying an optimal thylakoid structure flexibility, maintenance of PSII supercomplex structure during state transition and thermal dissipation for

PSII protection. Furthermore, an increase in photosynthesis rate was linked to a possible provision of sufficient energy flow to downstream metabolism and plant growth via an increase in OEC proteins of the PSII [PSBP; PSBQ1; PSBQ2; 5 kD protein (At1g51400)]. This was further supported by an increase in CF<sub>0</sub>F<sub>1</sub> ATP synthase (ATPA; ATPB; ATPD; ATPE) for a possible stimulated light driven induced-electron pump, which further implied a triggered acidification of the thylakoid lumen, leading to the activation of NPQ. Efficient chloroplast functioning was also linked to high abundance of CO<sub>2</sub> assimilation, which was possibly underpinned by preferential TRX-M (TRX-M1; TRX-M2; TRX-M4) targeted redox activation of chloroplastic RCA. TRX-M type also suggested anti-oxidative defense system while an improved facilitation of protein translocation into the stroma and stabilization of chloroplast membranes was possibly via increased chaperones proteins (CPN60A). In addition to effects on the light reaction systems, stomatal conductivity and CO<sub>2</sub> fixation, possibly via up-regulated CPN60A and RCA proteins, might have contributed to enhanced photosynthesis rate. Other than the suggested chloroplast biogenesis and efficient functioning, enhanced growth could be linked to proteins relating to carbon metabolism. This was revealed by increased cytosolic proteins (FBA8; GAPC2) in lumichrome-treated plants, implying enhanced sucrose biosynthesis, which in-turn provided the cell with ATP and NADH. Taken together, we found that the enhancement of photosynthesis and chlorophyll levels associated with lumichrome treatment was correlated with changes in photosynthesis related protein levels, while increased growth may be due to changes in carbon metabolism, particularly relating to the plastidial pPGM-AGPase starch biosynthesis pathway in lumichrome plants, with concomitant increases in sucrose and reducing sugars. Future research work that involves the use of either mutants or transgenic plants to reduce or overexpress these photosynthetic and carbon metabolism related candidate genes will assist in confirming these proposed lumichrome-associated mode of action.

### **Author contributions**

MP, JK, JL and PH designed the experiments. Experimental work was conducted by MP, JL, SA, WS and AF. Data analysis was performed by MP, WS, AF, SA and AV. The manuscript was prepared by MP, PH, JK, and JL.

## Acknowledgements

We are very grateful for Professor Martin Kidd for assistance in the statistical analysis. We also acknowledge Malira Masoabi for assisting with the management of *Arabidopsis* plants.

## Supplementary material

**Fig. S1.** Photosynthesis light reaction and Calvin cycle overview in *Arabidopsis* following lumichrome application.

**Fig. S3.** Redox homeostasis and jasmonate metabolism in *Arabidopsis* in response to lumichrome treatment.

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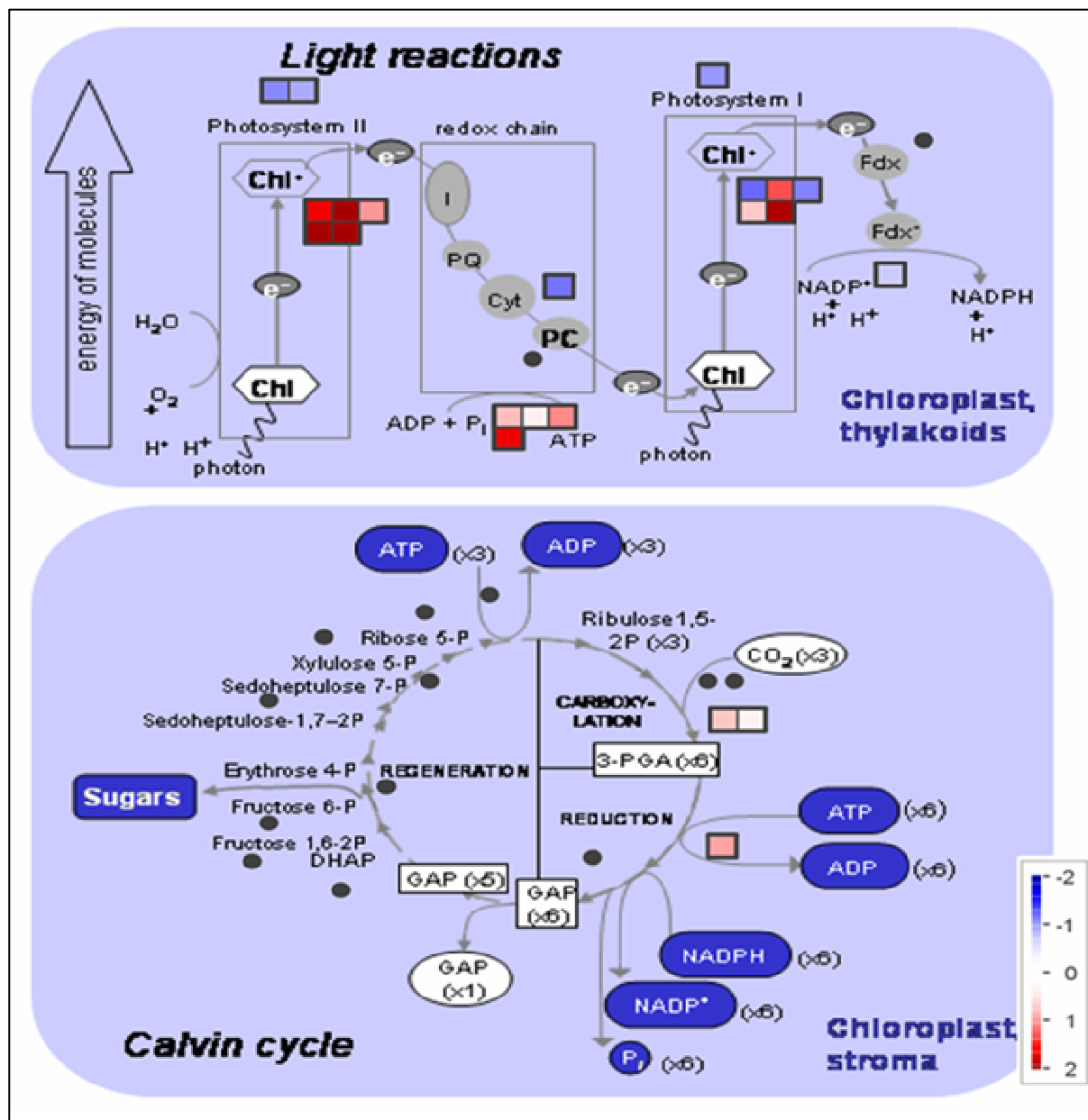
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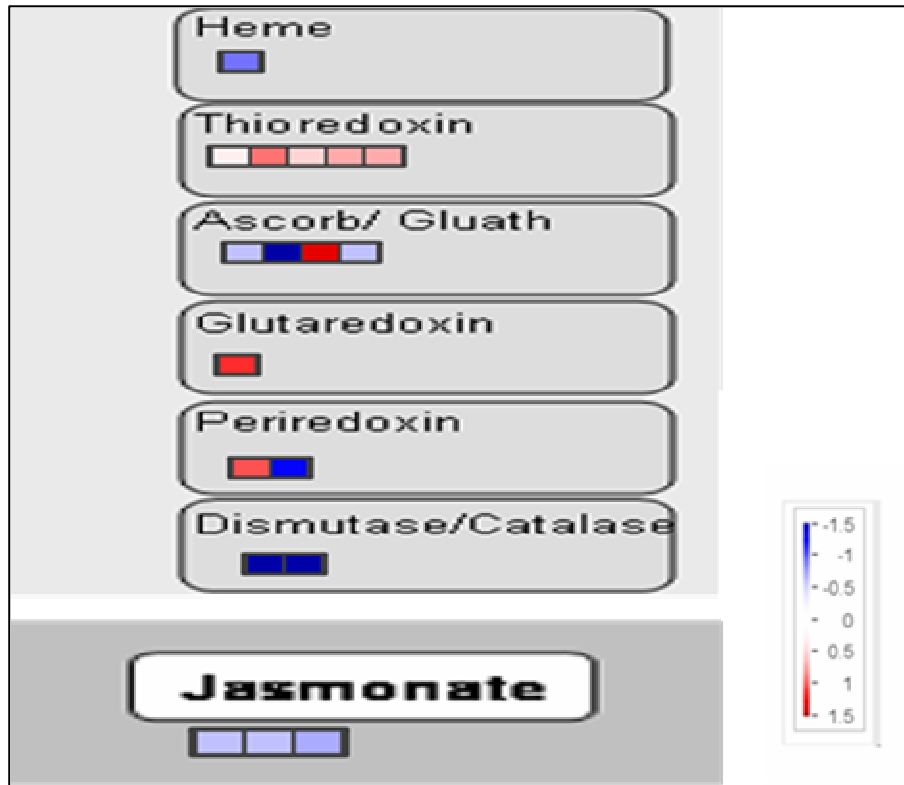


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## Supplementary data



**Figure S1.** Photosynthesis light reaction and Calvin cycle overview in *Arabidopsis* following application of 5 nM lumichrome. Red denotes proteins that were up-regulated while blue indicates those that were down-regulated upon 5 nM lumichrome treatment. The magnitude of protein expression is indicated by the colour intensity



**Figure S2.** Redox homeostasis and jasmonate metabolism in *Arabidopsis* in response to lumichrome treatment. Red denotes proteins that were up-regulated while blue indicates those that were down-regulated upon 5 nM lumichrome treatment.

## CHAPTER 4

### **Analysis of the mode of action of lumichrome treatment in *Arabidopsis* (III.): Proteome analysis suggests that either reduction or deficiency in ADP-Glucose Pyrophosphorylase small subunit (APS1) enhanced photosynthetic energy provision, carbon supply and utilization for plant growth**

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#### **Abstract**

Previously we found that the enhancement of photosynthesis associated with lumichrome (7,8-*dimethylalatoxazine*) treatment in *Arabidopsis* was correlated with changes in photosynthesis-related proteins, particularly those involved in effective chloroplast biogenesis and functioning. In addition, previous studies on tomato and *Lotus japonicus* proposed that the lumichrome-dependent growth increase may be due to altered starch metabolism, while we suggested that increased growth in lumichrome-treated plants could be linked to changes in carbon metabolism, particularly relating to reduced starch and concomitant increased levels of sucrose and reducing sugars. In this study, proteomic analyses were performed in *Arabidopsis* plants deficient in ADP-GLUCOSE PYROPHOSPHORYLASE small subunit (APS), a primary protein for the first committed step of starch biosynthesis, to test for the effect of the mutation and further elucidate lumichrome-dependent changes in carbon metabolism. Enhanced photosynthesis in *aps* mutants compared to wild type was linked to gravitropism-related proteins whose role is to ensure proper positioning of the leaves for efficient photosynthesis and gas exchange. This was accompanied

by an increase in PSII light harvesting complexes, photosystems reaction centre, and  $cF_0F_1$  ATPase proteins, implying effective photosynthetic efficiency. We have demonstrated through a reverse genetic approach that silencing *APS1* (*At5g48300*) enhanced photosynthetic efficiency and biomass in *Arabidopsis*. An increase in the growth of mutant plants compared to wild type plants was linked to an increase in carbon supply and utilization in the plastid. Additionally, lumichrome application did not further increase biomass accumulation in the mutants, as it did in the wild type plants. Rather, the size and biomass of both lumichrome-treated wild type *Arabidopsis* and untreated *aps* mutant plants were similar. Despite an increase in biomass, *aps1* mutants produced miniscule starch levels compared to the wild type, indicating that starch was not the main integrator of enhanced plant growth in *aps1* mutant lines. Rather, the growth of lumichrome *aps1* mutant lines was sustained by an enhanced photosynthesis efficiency which ensured energy generation and carbon supply for *Arabidopsis* growth.

**Keywords:** *Arabidopsis*, APS1, lumichrome, proteomics, photosynthesis, carbon metabolism

## Introduction

Starch is the predominant storage carbohydrate in the plant kingdom that fuels plant metabolism and growth when the plant is unable to photosynthesize (Streb and Zeeman, 2012). Starch has been identified as a major integrator in the regulation of plant growth, allowing the plant to cope with the daily alternation between a positive carbon balance in the light and a negative carbon balance in the dark (Schurr et al., 2006; Sulpice et al., 2009). The partitioning of photoassimilates is important for normal growth in a diurnal cycle and is finely controlled to suit the growth conditions (Gibon et al., 2009), the means by which this is done varies from species to species (Paul and Foyer, 2001). In *Arabidopsis*, a large fraction of photoassimilates stored in the chloroplast during the day as starch are remobilized during the subsequent night (Zeeman and Rees, 1999). Disrupting leaf-starch metabolism affects the way that *Arabidopsis* plants develop as well as the rate of growth. Starchless and reduced-starch lines grown under day/night conditions flower at a later developmental stage than the wild type (Corbesier et al., 1998). Disruption of starch metabolism leads to changes in the pools of key metabolites and such changes are perceived by signal transduction pathways involved in controlling the overall growth

and development of the plant (Schulze et al., 1994). Moreover, primary and secondary metabolites can in-turn influence gene expression and protein stability (Tugizimana et al., 2013).

The importance of storing carbohydrates as starch is reflected in the growth of plants that are unable to synthesize or to fully degrade their transitory starch. Mutations affecting starch metabolism have been instrumental in defining the biosynthetic and degradative pathways, as well as the mechanisms that control them. Such studies date from the first genetic screens for *Arabidopsis* leaves with altered starch levels in the mid-1980s (Caspar et al., 1985b). Subsequent studies include functional genomics approaches in confirming the starch synthesis pathway (Hanson and McHale, 1988; Harrison et al., 2000; Lin et al., 1988a; Yu et al., 2000), elucidating the pathway of starch breakdown (Baunsgaard et al., 2005; Caspar et al., 1991; Kötting et al., 2005; Yu et al., 2001), and in studying the factors that determine starch structure and properties (Zeeman et al., 2007). Both genetic and biochemical studies have demonstrated the primary role of ADP glucose pyrophosphorylase (AGPase) in starch biosynthesis. AGPase exclusively catalyses the synthesis of ADP glucose (ADPG) and pyrophosphate (PPi), and acts as the major limiting step of the gluconeogenic process (Caspar et al., 1985b; Müller-Röber et al., 1992). A decreased shoot-to-root ratio has been reported in starchless mutants in respect to the wild type (Schulze et al., 1991)

Besides its importance in plant growth, starch represents a large proportion of the daily caloric intake for food consumers (Burrell, 2003) and is also used for non-food purposes such as feedstock and for a host of industrial applications, including bioethanol production (Smith, 2008). Given the importance of starch, it is important to improve carbohydrate metabolism and ultimately plant growth to help offset the pressure on agriculture resulting from the increased use of starch crops for non-food purposes. Furthermore, there is need for novel crop varieties with tailored starches specifically suited to particular industrial needs (Burrell, 2003; Santelia and Zeeman, 2011; Zeeman et al., 2010). In an effort to identify innovative technologies to improve plant growth, bacterially-synthesised plant hormones and a number of biologically-active novel molecules have been purified from rhizobial exudates that stimulate cell growth and nodule

organogenesis (Dakora, 2003; Dyachok et al., 2000; De Jong et al., 1993). Lumichrome (7,8-dimethylaloxazine), a morphogen secreted by symbiotic rhizobia (Phillips et al., 1999), elicits developmental changes that consequently result in growth promotion and greater plant biomass in both monocotyledonous and dicotyledonous plants (Gouws et al., 2012; Khan et al., 2008; Matiru and Dakora, 2005a, 2005b; Phillips et al., 1999).

Molecular analysis on tomato and *Lotus japonicus* revealed that the underlying growth promotion effect was related to genes involved in plant-bacterial symbiosis. In addition, significantly enhanced starch accumulation strongly suggested that lumichrome acts as a modulator of carbon fluxes within the plant cell (Gouws et al., 2012). The authors suggested that an increase in starch could have been attributed to up-regulated plastidial GAPDH transcription and enhanced plastidial NAD-dependent enzyme activity. However, proteomics studies on wild type *Arabidopsis* (Columbia 0) revealed that the enhanced photosynthetic rate could be linked to enhanced levels of proteins involved in early chloroplast development, differentiation and maturation. We further suggested that an increase in biomass in lumichrome-treated wild type *Arabidopsis* may be linked with decreased levels of ADP-GLUCOSE PYROPHOSPHORYLASE small subunit (APS) with a concomitant increase in carbon metabolism-related proteins, particularly those relating to increases in sucrose and reducing sugars which are then further invested in growth (Chapter 3). These different findings suggest that the effect of lumichrome treatment varies slightly depending on the plant species. Despite these findings on altered carbon metabolism in lumichrome-treated plants, loss-of-function mutants of potential genes have not previously been analysed. In this study, we have used proteomics and metabolomics tools to dissect the link between the physiological and the underlying molecular mechanisms associated with growth in two *Arabidopsis* knockout lines deficient in APS (also designated *APSI*) treated with lumichrome.

## Materials and methods

### *Plant material and growth conditions*

Seeds for the wild type *Arabidopsis thaliana* ecotype Columbia (Col-0) and two independent AGPase (At5g48300) homozygous T-DNA insertion lines (Supplementary Table S1) from the SALK collection T-DNA knock-out mutant lines (*Salk\_059083* [*aps1-1*] and *Salk\_040155* [*aps1-2*]), both in a Columbia-O background, were surface decontaminated for 90 s in 70% ethanol, followed by 15 min in 1.75% sodium hypochlorite containing 0.02% (v/v) Tween-20, and then rinsed six times with sterile dH<sub>2</sub>O. Thereafter, the surface decontaminated seeds were stratified for 48 hrs at 4°C in the dark and sown in pots containing sterilized planting mix (Jiffy Co. Hydroponics, South Africa). All experiments were conducted in a controlled environment growth chamber with a 16 h light / 8 h dark photoperiod at 22 ±2°C, 75% relative humidity in a factorial randomised completely block design (RCBD) consisting of 6 replications and blocks respectively. Lumichrome stock solutions (1M) were freshly prepared for each treatment in methanol/1 M HCl (49:1) and further diluted to a final concentration of 5 nM in dH<sub>2</sub>O. Plants were supplied with 5 nM lumichrome by a combination of root drenching and foliar application (50 ml) to intact plants at intervals of 2 days throughout the entire growth period (Phillips et al., 1999). Control plants were similarly treated with water containing the same amount of methanol/HCl as the lumichrome-treated plants. 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 72 h for dry biomass determination.

### *Photosynthesis biochemical measurements*

An open-system photosynthesis meter (Li-Cor 6400, Li-Cor Inc., Lincoln, NE, USA) equipped with the standard leaf chamber (encloses 6 cm<sup>2</sup> of leaf area) and CO<sub>2</sub> injection system (model 6400-01, Li-Cor Inc., Lincoln, NE, USA) for control of CO<sub>2</sub> was used to perform photosynthesis physiological parameters. The red–blue light source (model 6400-02, Li-Cor Inc., Lincoln, NE, USA) for light intensity was set at 400 μmol photons m<sup>-2</sup>s<sup>-1</sup>.



### ***Starch measurement***

Starch was isolated from 50 mg frozen leaf material of 5 week old rosette leaves in 80% (v/v) ethanol at 80°C, according to Lin et al. (1988b) . The pellet was washed twice with 80% (v/v) ethanol and incubated at 95°C for 4 hours. After cooling sample was neutralized by addition of 0.2 M potassium hydroxide, followed by addition of 50 mM Sodium acetate (NaAc) buffer pH 4.5 containing 10% (v/v) amyloglucosidase (*Hormoconis Resinae*; Megazyme) at 37°C. Glucose in the supernatant was assayed in buffer (250 µl) consisting of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM ATP, 1 mM NAD and 1 U mL<sup>-1</sup> hexokinase, followed by addition of 1 U mL<sup>-1</sup> Gluc-6-PDH (*Leuconostoc mesenteroides*, Megazyme).

### ***Statistical analysis***

Data collected on components of plant growth, photosynthesis and starch parameters were analyzed using STATISTICA Version 12 (StatSoft Inc.), using two-way analysis of variance (ANOVA) and a Games-Howell *post-hoc* test to infer differences between treatments at the 95% confidence level.

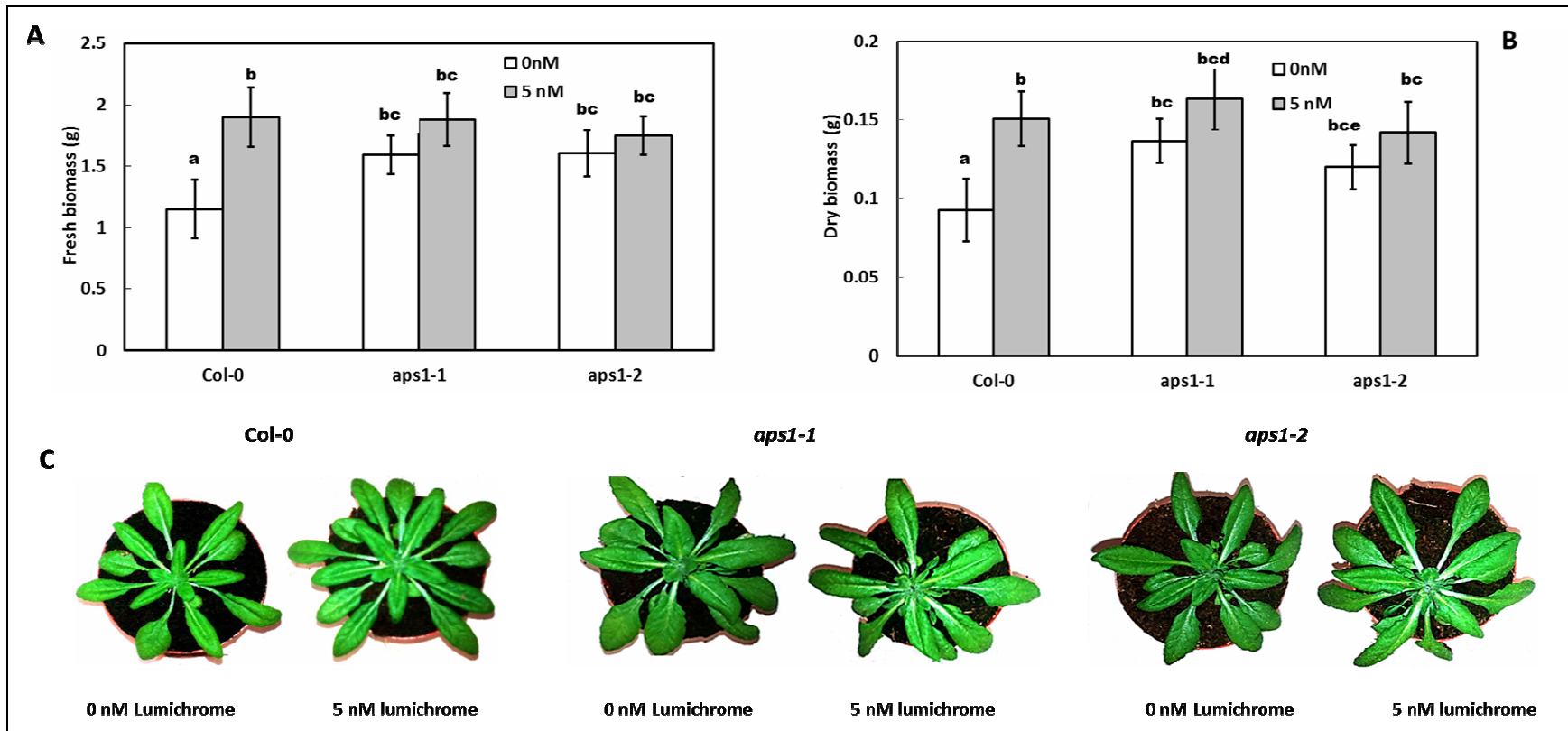
### ***Determination of metabolite and protein expression profiles***

Primary metabolites (50 mg) and photosynthesis phosphorylated intermediates (15 mg) were extracted as described by (Heise et al., 2014). Gas chromatography coupled to electron impaction/time-of-flight mass spectrometry (GC-EI-TOF-MS) was performed for metabolites using an Agilent 6890N gas chromatograph and splitless injection connected to a Pegasus III time-of-flight mass spectrometer (LECO), and analysis according to corresponding LECO software (Lisec et al., 2006a). For protein profiling, samples were each homogenized in 8 M urea and 50 mM Tris-HCl (pH 8). Approximately, 50 µg of protein was reduced, alkylated and digested using Trypsin/Lys-C (Trypsin/Lys-C Mix, Mass Spectrometry grade, Promega) according to the manufacturer's manual. Liquid chromatography-mass spectrometry (LC/MS/MS) based metabolite measurement was conducted as previously described (Chapter 3).

## Results

### *Physiological responses elicited by lumichrome treatment*

*Arabidopsis thaliana* Columbio-0 wild type plants displayed a significant increase in biomass upon lumichrome treatment compared to untreated control plants. We also observed a remarkable increase in biomass and photosynthetic rate in both untreated *aps1-1* and *aps1-2* mutants relative to untreated wild type plants (Fig. 1, Table 1). However, when treated with lumichrome, mutant plants showed neither any further increase in biomass accumulation nor photosynthesis rate and were similar in size to untreated plants from the same line, as well as to the lumichrome-treated wild type plants. In addition, photosynthesis components such as leaf respiration, water use efficiency, stomatal conductivity and transpiration rate were not significantly affected following lumichrome application in *aps1-1* and *aps1-2* mutants compared to their relative untreated controls (Table 1).



**Figure 1.** Physiological response of 5-week-old wild type *Arabidopsis* and APS1-deficient mutant plants following lumichrome application. Fresh biomass (A), dry biomass (B) and phenotypic response (C) of lumichrome-treated wild type *Arabidopsis* (far left) and APS1-deficient mutant plants (*aps1-1* and *aps1-2* [middle centre and far right]) compared to the untreated control plants. Error bars show the standard error of the means (mean  $\pm$ SE, n=6), while treatments differing significantly, ( $P \leq 0.05$ ) are indicated with different letters (ANOVA with *post hoc* LSD tests).

**Table 1. Physiological response of *Arabidopsis* Columbia-0 wild type and APS1-deficient mutant plants following lumichrome application**

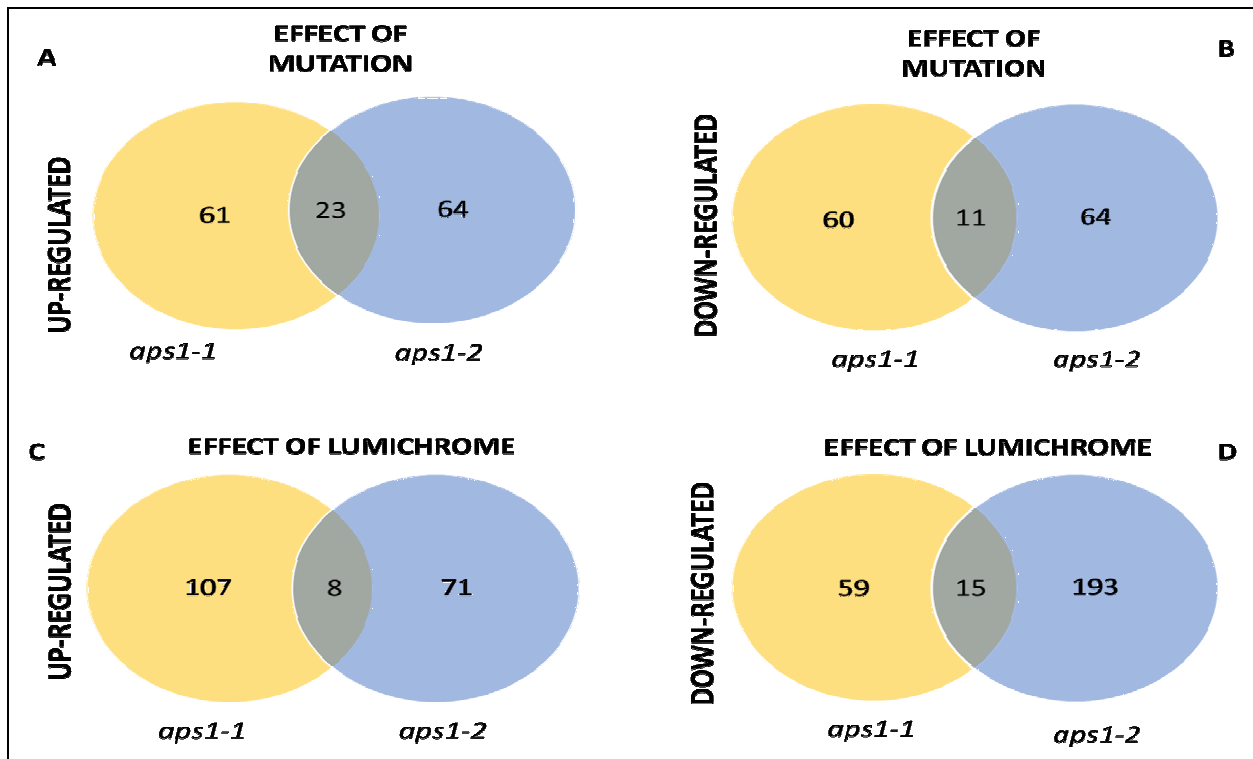
Parameters	Treatment	Col-0	<i>aps1-1</i>	<i>aps1-2</i>
	<b>Photosynthesis</b> ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	0 nM	3,29 $\pm$ 0,12 c	5,65 $\pm$ 0,49 a
	5 nM	5,13 $\pm$ 0,22 ab	5,98 $\pm$ 0,50 a	5,39 $\pm$ 0,36 ab
<b>Leaf respiration</b> ( $\mu\text{mol CO}_2 \text{ release g}^{-1} \text{ DW}$ )	0 nM	2,00 $\pm$ 0,21 a	3,22 $\pm$ 0,13 b	2,58 $\pm$ 0,17 ab
	5 nM	1,76 $\pm$ 0,56 ab	2,07 $\pm$ 1,26 ab	2,48 $\pm$ 0,11 a
<b>Stomatal conductance (gs)</b> ( $\text{mol m}^{-2} \text{ s}^{-1}$ )	0 nM	0.83 $\pm$ 0.18 ab	1.00 $\pm$ 0.28 a	0.46 $\pm$ 0.04 cb
	5 nM	0.49 $\pm$ 0.04 cb	0.47 $\pm$ 0.03 cb	0.41 $\pm$ 0.03 c
<b>Intercellular CO<sub>2</sub> (Ci)</b> ( $\mu\text{mol CO}_2/\text{mol air}$ )	0 nM	379.44 $\pm$ 1.82 a	370.00 $\pm$ 3.46 b	366.89 $\pm$ 2.38 bc
	5 nM	367.67 $\pm$ 1.05 bc	361.44 $\pm$ 3.22 c	361.00 $\pm$ 3.06 c
<b>Water Use Efficiency (A/gS)</b> ( $\mu\text{mol CO}_2 \text{ mmol H}_2\text{O}$ )	0 nM	1.42 $\pm$ 0.02 a	1.46 $\pm$ 0.18 ab	1.40 $\pm$ 0.12 a
	5 nM	0.81 $\pm$ 0.06 b	1.85 $\pm$ 0.16 a	1.75 $\pm$ 0.15 a
<b>Transpiration rate (E)</b> ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ )	0 nM	4.15 $\pm$ 0.20 a	4.14 $\pm$ 0.45 b	3.41 $\pm$ 0.15 ab
	5 nM	3.60 $\pm$ 0.14 ab	3.26 $\pm$ 0.14 ab	3.12 $\pm$ 0.07 a
<b>Maximum quantum yield(<math>\square</math>)</b> (mol CO <sub>2</sub> fixed mol <sup>-1</sup> absorbed photons)	0 nM	0.0040 $\pm$ 0.0004 b	0.0052 $\pm$ 0.001ab	0.0035 $\pm$ 0.0003b
	5 nM	0.0036 $\pm$ 0.0004b	0.0068 $\pm$ 0.010a	0.0049 $\pm$ 0.010ab

Mean  $\pm$  SE (n = 9), values marked with the same letter within a row are not significantly different from one another (ANOVA with *post hoc* LSD tests)

### ***Protein profile changes of AGPase-deficient Arabidopsis mutants following lumichrome application under long photoperiod growth regimes***

Statistical pairwise ANOVA revealed a total of 155 and 162 significant protein changes for untreated *aps1-1* and *aps1-2* plants respectively (Fig. 2) compared to untreated *Arabidopsis* control plants. From these proteins, only 23 (Fig 2A) up-regulated and 11 down-regulated (Fig. 2B) proteins were commonly shared between the two independent *aps1* lines. These were

largely related to photosynthesis, carbon metabolism, lipid metabolism, amino acid metabolism, RNA metabolism, protein metabolism and signalling (Table 2). To test for the effect of lumichrome on *aps* mutant plants, the same statistical pairwise ANOVA analysis was performed in lumichrome-treated *aps* knockout plants relative to their untreated controls. A pairwise analysis approach revealed a total of 189 and 287 significantly expressed proteins in *aps1-1* and *aps1-2* respectively upon lumichrome treatment (Fig. 2C & 2D). Further analysis to identify proteins that were common to both lumichrome-treated *aps* lines showed only 8 (Fig. 2C) and 15 (Fig. 2D) up- and down-regulated proteins respectively.



**Figure 2.** Venn diagram showing overlapping and non-overlapping changes in protein profiles. Differentially altered proteins in untreated *aps* mutants relative to the untreated wild type *Arabidopsis* (Fig 2A & 2B) and the lumichrome-treated *aps* mutants relative to the untreated mutant plants (Fig 2C & 2D). A two-fold change and  $p \leq 0.05$  in protein levels between untreated *aps1* mutant and untreated wild type *Arabidopsis* control plants were used to profile variation in protein levels.

### ***Photosynthesis-related proteins***

Our results revealed increased LIGHT HARVESTING COMPLEX PHOTOSYSTEM II SUBUNITS (LHCB) such as LHCB4.2, LHB1B1, LHCB5, LHCB6 as well as the PHOTOSYSTEM II REACTION CENTER PROTEIN A and B (PSBA and PSBB) for both untreated *aps1-1* and *aps1-2* compared to *Arabidopsis* untreated-wild type. In addition, photosystem I (PSI)-related proteins such as LHCA1 and the core PHOTOSYSTEM I P700 CHLOROPHYLL APOPROTEIN A1 and A2 (PSAA; PSAB, Table 4) were significantly up-regulated. Despite an increase in stromal PHOTOSYSTEM I SUBUNIT D-1 (PSAD-1), untreated *aps1-1* and *aps1-2* mutants revealed a significant decrease in LIGHT HARVESTING COMPLEX PHOTOSYSTEM II SUBUNIT 6 (LHCB6). Other than the light harvesting reaction related proteins, comparing untreated *aps1-1* and *aps1-2* mutants to untreated wild type *Arabidopsis* also showed an increase in photosynthesis-related proteins such as SHOOT GRAVITROPISM 4 (SGR4) and ATPase ALPHA SUBUNIT (ATPA), while ATPase/MAGNESIUM CHELATASE (CHLI1) and DEG PROTEASE 8 (DEG8) were significantly decreased (Table 3). On the other hand, lumichrome-treated *aps* mutants had up-regulated chaperone-related proteins such as stromal Hsp70s, chloroplast cpHsc70-1 and cpHsc70-2 compared to the corresponding untreated *aps* mutants (Table 4).

### ***Redox homeostasis and antioxidant signalling***

Generally, the photosynthetic redox regulation of untreated *aps1-1* and *aps1-2* compared to untreated wild type *Arabidopsis* was decreased via low abundance of GLUTATHIONE PEROXIDASE 2 (GPX2; Table 2). However, following lumichrome application, *aps* mutants showed an increase in THIOREDOXIN M-TYPE 2 and 4 (TRX-M2; TRX-M4), FERREDOXIN-THIOREDOXIN REDUCTASE CATALYTIC BETA CHAIN FAMILY PROTEIN (FTR). Additionally, lumichrome-treated mutants showed an abundance of stress-related proteins such as up-regulated CHLOROPLAST HEAT SHOCK PROTEIN 70-2 (CPHSC70-2) and CHAPERONIN 20 (CPN20; Table 3).

***Changes in carbon metabolism-related proteins***

Our results identified up-regulated conserved transketolase paralogues TKL1 and TKL2 and ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT (APL1) in *aps1-1* and *aps1-2* mutants compared to the untreated wild type *Arabidopsis* control plants (Table 2). There were no carbohydrate metabolism-related proteins that were altered in both and *aps* mutant lines compared to their relative untreated controls following lumichrome application (Table 3). We therefore analyzed the effect of lumichrome treatment in *aps1-1* and *aps1-2* relative to lumichrome-treated wild type *Arabidopsis* plants (Table 4). Our results demonstrated a consistent increase in cytosolic proteins such as FRUCTOSE-1,6-BISPHOSPHATASE (FBPase) and PHOSPHOGLUCOMUTASE 2 (PGM2), while levels of the plastidial DISPROPORTIONATING ENZYME 2 (DPE2) for starch degradation were significantly decreased.

**Table 2. Proteins that were differentially expressed in both independent untreated *Arabidopsis aps1* T-DNA knock-out mutant lines compared to the untreated wild type *Arabidopsis***

AGI	Biological Function	Protein name	Fold change ratio	
			<i>aps1-1</i>	<i>aps1-2</i>
<b>Photosynthesis</b>				
At3g08940	Photosystem II.LHC-II	LHCB4.2	1.176	1.529
At2g34430	Photosystem II.LHC-II	LHB1B1	1.251	1.893
At4g10340	Photosystem II.LHC-II	LHCB5	1.174	1.168
At1g15820	Photosystem II.LHC-II	LHCB6	1.24	1.685
Atcg00680	Photosystem II.PSII polypeptide subunits	PSBB	1.68	1.787
Atcg00020	Photosystem II.PSII polypeptide subunits	PSBA	1.288	2.05
At3g54890	Photosystem I.LHC-I	LHCA1	1.223	1.26
Atcg00340	Photosystem I. polypeptide subunits	PSAB	1.12	1.574

Atcg00350	Photosystem I. polypeptide subunits	PSAA	0.985	1.451
Atcg00120	ATP synthase.alpha subunit	ATPA	1.09	0.728
<b>Tetrapyrrole biosynthesis</b>				
At4g18480	Tetrapyrrole synthesis	CHL11; ATPase/ magnesium chelataase	-0.77	-0.704
<b>Cell vesicle</b>				
At5g39510	Cell vesicle transport	SHOOT GRAVITROPSIM 4 (SGR4)	0.741	0.884
<b>Carbon metabolism</b>				
At3g60750	Calvin cyle.	TRANSKETOLASE 1 (ATKL1)	0.15	0.526
At3g60750	OPP-non-reductive	TRANSKETOLASE (ATKL2)	0.15	0.526
At5g19220	Starch synthesis	ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1(APL1)	1.265	0.952
<b>Amino acid metabolism</b>				
At1g23310	Central amino acid metabolism.	GGT1	0.614	0.665
<b>Protein metabolism</b>				
At5g20290	Protein.synthesis-40S subunit.S8	RPS8A	0.5	0.807
At1g04270	Proteinsynthesis-.40S subunit.S15	RPS15	0.945	1.659
At5g39830	Protein degradation.	DEG8	-1.389	-0.991
<b>Unknown/Miscellaneous</b>				
At2g14880	Not assigned.	SWIB COMPLEX BAF60B DOMAIN-CONTAINING PROTEIN	0.662	0.941
At5g53490	Not assigned	THYLAKOID LUMENAL 17.4 KDA PROTEIN	0.606	0.615
At4g38710	Not assigned	GLYCINE-RICH PROTEIN	-0.477	-0.262

Negative log<sub>2</sub> fold change ratios represent down-regulated genes and while positive log<sub>2</sub> fold ratios indicate up-regulated genes



**Table 3. Proteins that were differentially expressed in both lumichrome-treated *aps1* mutants compared to their respective untreated control lines**

AGI	Biological function	Protein name	Fold change ratio	
			<i>aps1-1</i>	<i>aps1-2</i>
<b><i>Photosynthesis related proteins</i></b>				
At1g15820	PS II light reaction light harvesting complex	LHCB6	-0.879	-1.339
Atcg00560	PSII light reaction polypeptide subunits	PSBL	-1.292	-1.979
Atcg00340	PSI light reaction polypeptide subunits	PSAB	-1.04	-1.55
Atcg00350	PSI light reaction polypeptide subunits	PSAA	-1.02	-1.699
At4g02770	PSI light reaction polypeptide subunits	PSAD-1	0.267	0.061
<b><i>Stress response related proteins</i></b>				
At5g20720	Protein folding	CHAPERONIN 20 (CPN20)	0.431	0.455
At5g49910	Heat stress	CHLOROPLAST HEAT SHOCK PROTEIN 70-2 (CPHSC70-2)	0.529	0.368
At5g52310	Cold stress	COLD REGULATED 78 (COR78)	-0.306	-0.771
<b><i>Redox Homeostasis and Antioxidant Signalling</i></b>				
At4g03520	Redox-thioredoxin	THIOREDOXIN M-TYPE (TRX-M2)	0.592	0.367
At3g15360	Redox-thioredoxin	THIOREDOXIN M-TYPE (TRX-M4)	0.529	0.431
At2g04700	Redox-thioredoxin	FERREDOXIN-THIOREDOXIN REDUCTASE CATALYTIC BETA CHAIN FAMILY PROTEIN (FTR)	0.31	0.298

Pairwise comparison of significant proteins is represented in log<sub>2</sub> fold change ratio at a significance level of  $p \leq 0.05$ .

**Table 4. Commonly altered proteins in both lumichrome-treated *Arabidopsis aps1*-deficient mutant lines in respect to lumichrome-treated wild type *Arabidopsis* plants**

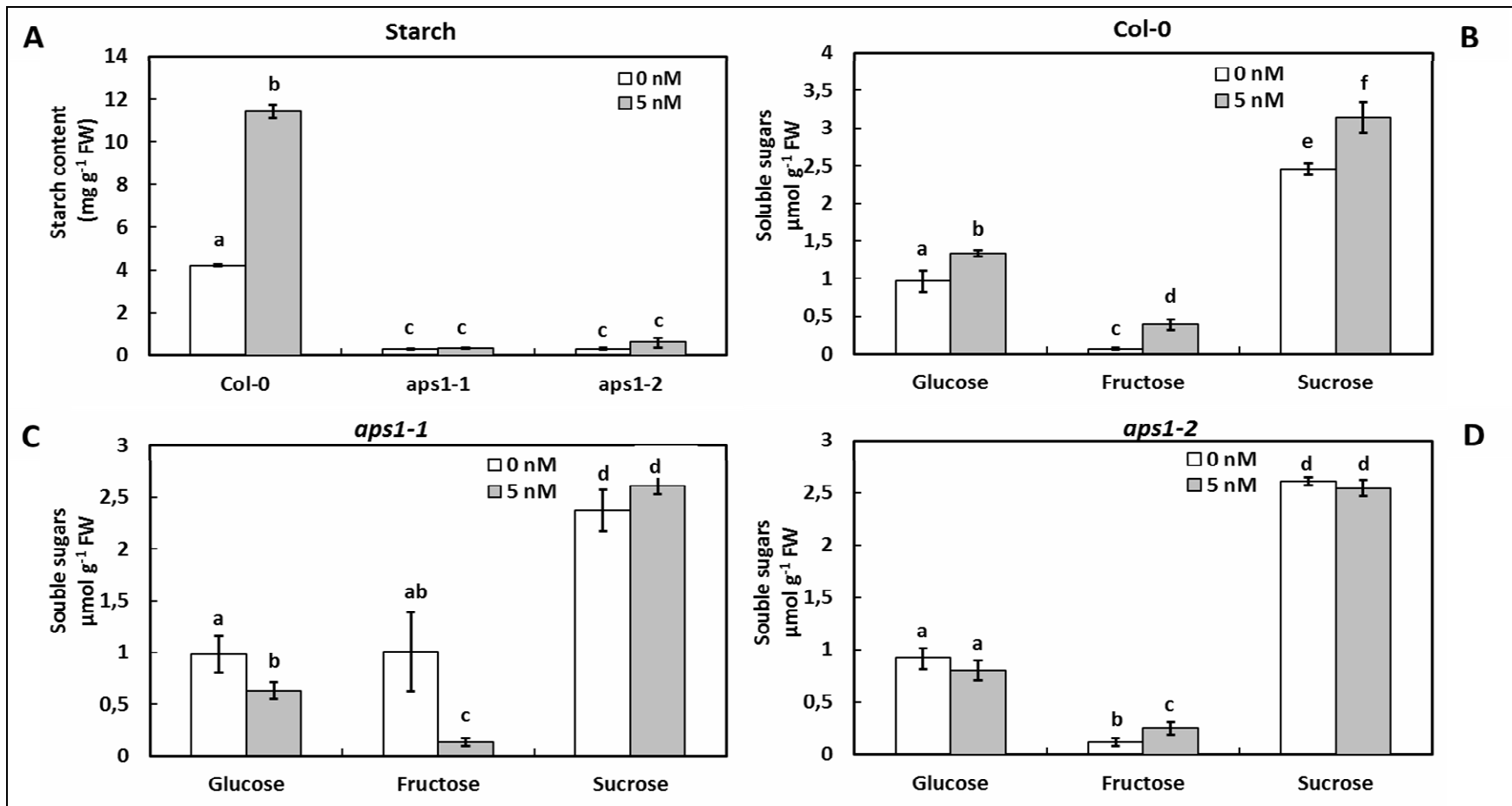
AGI	Biological Function	Protein name	Fold2 change ratio	
			<i>aps1-1</i>	<i>aps1-2</i>
At1g43670	Sucrose synthesis	FRUCTOSE-1,6-BISPHOSPHATASE (FBPase)	1.528	1.758
At1g70730	Cytosolic glycolysis	PHOSPHOGLUCOMUTASE2 (PGM2)	0.856	0.902
At2g40840	Starch degradation	DISPROPORTIONATING ENZYME 2) (DPE2)	-0.791	-0.954

***Metabolomics profiling to evaluate biochemical changes in aps1-deficient mutant following lumichrome treatment***

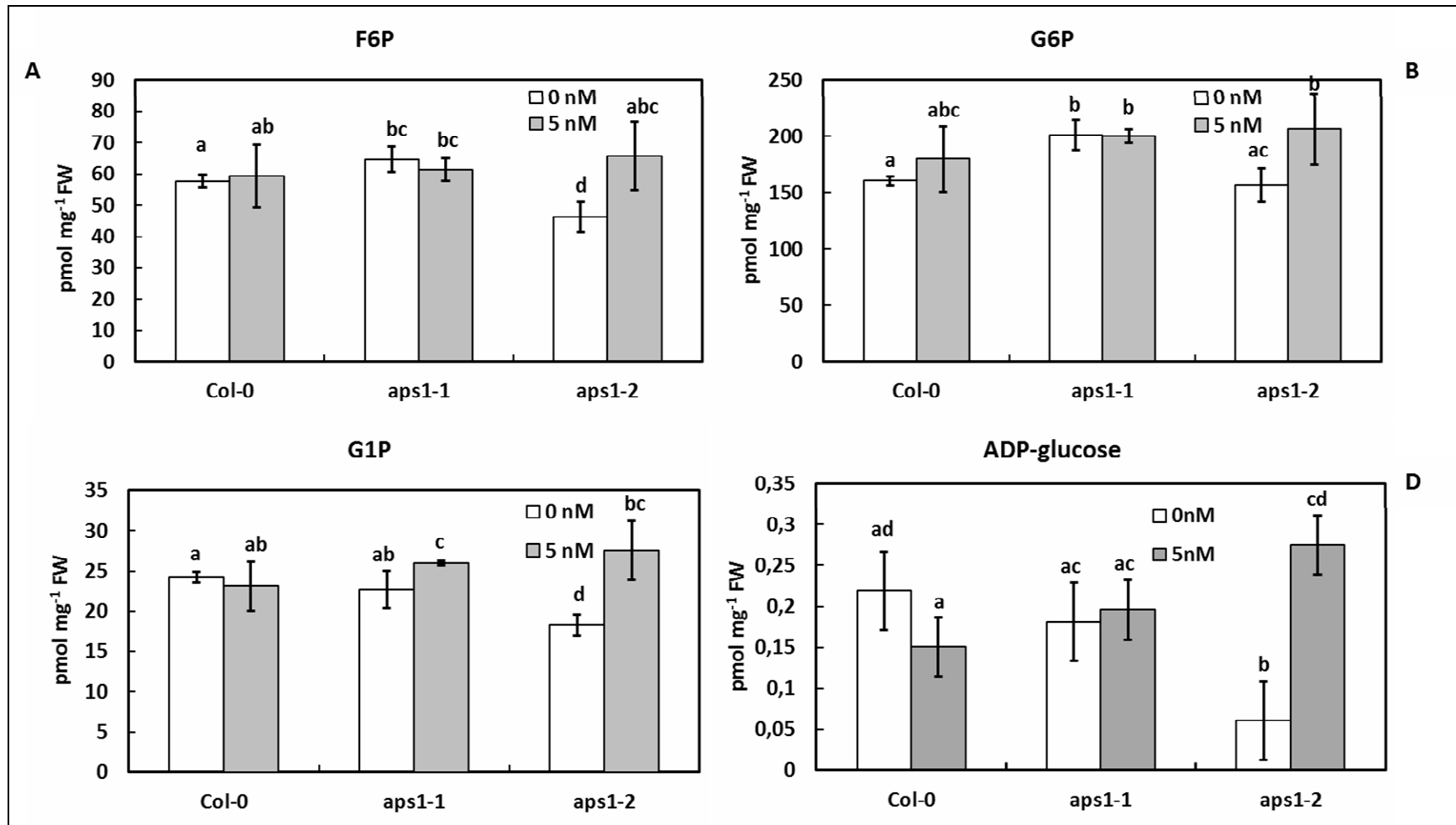
Profiling of central pools of metabolites and phosphorylated intermediates was performed to test for the effects of lumichrome on *Arabidopsis* metabolism (Fig. 3). Starch content was significantly higher in wild type *Arabidopsis* plants compared to *aps1-1* and *aps1-2* respectively. Our results showed a remarkable increase in starch content in lumichrome-treated wild type *Arabidopsis* compared to the relative control (Fig. 3A). Lumichrome application did not affect starch levels in *aps* mutant lines. In addition, we observed that the starch levels in *aps* mutant lines, whether treated with lumichrome or not, were significantly decreased to miniscule levels compared to the wild type (Fig. 3A). Total soluble sugars such as glucose, fructose and sucrose were significantly increased in wild type *Arabidopsis* following lumichrome treatment (Fig. 3B). However, sucrose content did not change in lumichrome-treated *aps1-1* and *aps1-2* relative to their corresponding untreated controls (Fig. 3C and 3D). In addition, LC-MS photosynthetic phosphorylated substrate analysis (Fig. 4) revealed a significant increase in glucose-1-phosphate (G1P; Fig. 4C) in both lumichrome-treated *aps* mutant lines compared to their untreated controls.

Similar ADP-glucose levels were observed in wild type and *aps1-1* plants, and these levels did not change following lumichrome treatment. Untreated *aps1-2* plants had significantly lower levels of ADP-glucose than the other two lines, however, following lumichrome treatment the levels of ADP-glucose in these plants rose to similar levels to those observed in the other lines (Fig. 4D).

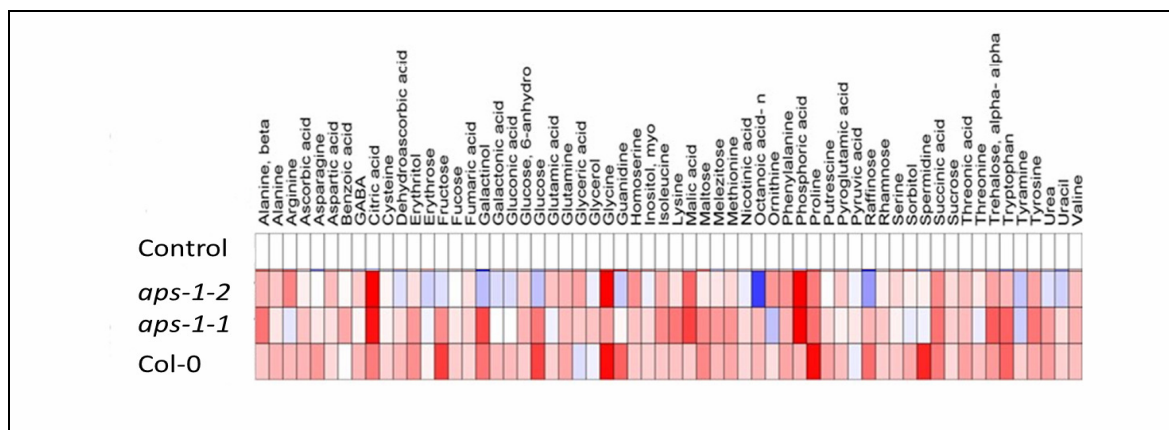
Profiling of central pools of metabolites using GC-TOF-MS identified a total of 60 metabolites. According to heatmap visualization of the 60 altered metabolites, lumichrome treatment resulted in a general increase in levels of the metabolites examined (Fig. 5). However, only 5 metabolites were commonly expressed between wild type *Arabidopsis*, *aps1-1* and *aps1-2*. These included the sugar  $\alpha$ - $\alpha$  trehalose and amino acids  $\beta$ -alanine, phenylalanine, tyrosine and valine (Table 5).



**Figure 3.** Metabolite changes in wild type *Arabidopsis* and *APS1*-deficient mutant plants in response to lumichrome treatment. Starch content for wild type *Arabidopsis* (A) and mutant lines *aps1-1* (B) and *aps1-2*(C). Total soluble sugars for wild type *Arabidopsis* (D), *aps1-1* (E) and *aps1-2* (F) following lumichrome treatment. Error bars denotes the means with standard error bars (mean  $\pm$ SE, n=6) while different letters represent significant differences among the treatments ( $p \leq 0.05$ ).



**Figure 4.** Changes in photosynthesis intermediates levels of lumichrome-treated *Arabidopsis* rosette leaf compared to the respective untreated control. Error bars denotes the means with standard error bars (mean  $\pm$ SE, n=6), while different letters denote significant differences among the treatments, ( $p \leq 0.05$ ).



**Figure 5.** Overlay Heat Map of 60 metabolite profiles from lumichrome-treated wild type *Arabidopsis* and *aps1* mutant plants. The heat map values represent Fold2 ratio and  $p = (<0.05)$  of *Arabidopsis aps1*-deficient mutant following lumichrome treatment. The magnitude of change is indicated in the color scale represented in the figure (between +2 and -2), and red represents metabolites that are increased while blue represents those that are decreased.

**Table 5. Metabolites profiles that were common in both lumichrome-treated *Arabidopsis aps1* mutant lines compared to their corresponding untreated mutant lines.**

Compound	Col-0		<i>aps1-1</i>		<i>aps1-2</i>	
	Fold2 change	<i>p</i> -value	Fold2 change	<i>p</i> -value	Fold2 change	<i>p</i> -value
$\alpha$ - $\alpha$ -Trehalose	0,60	0,010	0,29	0,010	0,03	0,027
B-Alanine,	NS.	NS.	0,28	0,001	0,42	0,036
Phenylalanine	0,53	0,026	0,08	0,033	0,27	0,009
Tyrosine	0,49	0,034	0,25	0,021	0,27	0,025
Valine	0,38	0,037	0,19	0,021	0,32	0,008

Response ratio = intensity of the mass of the specific metabolite normalized to fresh weight and internal standard ribitol. Significant changes were evaluated using log2 fold change and t-test ( $p \leq 0.05$ ).

## Discussion

Studies on tomato and *Lotus japonicus* proposed that lumichrome-dependent growth enhancement may be due to an increase in starch accumulation (Gouws et al., 2012). We, however, reported (Chapter 3) that lumichrome enhanced growth in *Arabidopsis* by reducing plastidial starch biosynthesis proteins such as pPGM1 and ADP-glucose pyrophosphorylase1 small subunit (APS1). To conclusively demonstrate that silencing APS1 enhances *Arabidopsis* growth, we profiled both proteins and metabolites in T-DNA knockout lines which are deficient in APS1 compared to wild type *Arabidopsis*. Further analyses were performed in lumichrome-treated *aps1* mutant lines relative to their corresponding untreated mutant.

### ***Enhanced photosynthesis efficiency and biomass in Arabidopsis-APS1 deficient mutant compared to wild type Arabidopsis may be attributed to changes in photosynthesis and carbon metabolism-related proteins***

Both *aps1* mutant lines exhibited a remarkable increase in photosynthetic rate compared to wild type *Arabidopsis* plants. This could be associated with an increase in SHOOT GRAVITROPISM 4 (SGR4) protein, suggesting a promoted upward shoot growth to ensure a proper positioning of the leaves for efficient photosynthesis (Chen et al., 1999; Yamauchi et al., 1997). However, the ATPase/ MAGNESIUM CHELATASE (CHLI1) protein, which is involved in the first committed step in chlorophyll biosynthesis (Mochizuki et al., 2001), was decreased in the mutant plants. Although *CHLI1* and *CHLI2* transcripts accumulate to similar levels, at the protein level CHLI2 is undetectable in both the wild type and the CHLI1 mutant, suggesting unusual posttranslational protein instability of CHLI2 (Rissler *et al.*, 2002). Although we did not observe any changes in CHLI2 protein levels, we assumed that *aps* mutant lines could make chlorophylls, probably using this second isoform *CHLI2*.

In our previous studies on lumichrome-treated wild type *Arabidopsis* plants, we proposed that the enhanced photosynthetic rate may be associated with changes in photosynthesis-related protein levels, particularly those involved in the effective chloroplast functioning via an increase in optimal thylakoid structure flexibility, maintenance of PSII supercomplex structure during state transition and thermal dissipation for PSII protection (Chapter 3). In the current study, we observed that the biomass of *aps1* mutant lines were comparable to that of lumichrome-treated wildtype plants. Likewise, comparing *aps1* mutant lines to the wild type

suggested an effective chloroplast biogenesis and functioning through a remarkable property of the antenna system's ability to actively regulate PSII quantum efficiency and photosynthetic electron transport to avoid the damaging effects of excess light. An increase in PSII LHCB4.2, LHB1B1, LHCB5 and LHCB6 proteins in untreated *aps* mutants relative to untreated wild type *Arabidopsis* may be linked to a high functional degree of redundancy among LHCB subunits (de Bianchi et al., 2008, 2011; Ruban et al., 2003) in ensuring stable PSII supramolecular architecture and maintains its stoichiometry with the reaction center. This in-turn may have enhanced photo-protection by inhibiting radical chain reactions of peroxy-lipids occur during photo-oxidative stress (Ballottari et al., 2007; Dall'Osto et al., 2010). In addition, an increase in PSII (PSBA; PSBB) reaction centre proteins might have attributed to stabilization of PSII function and an increase in *de novo* synthesis and replacement of photodamaged proteins at high light intensities (Fromm et al., 1985; Mattoo et al., 1981). Furthermore an effective photosynthetic functioning may be linked with an increase in both PSI core complex (PSAA and PSAB) and cF0F1 ATPase, suggesting the promotion of electron transport and the presence of protective non-photochemical mechanisms that quench singlet-excited chlorophylls and harmlessly dissipate excess excitation energy as heat (Witt 1979; Cruz et al., 2001; Cruz et al., 2005). We therefore speculate that these may collectively have resulted in an enhanced photosynthetic efficiency with a concomitant sustained non-photochemical quenching. Collectively, we observed that the effect of *aps1* deficiency and lumichrome treatment were similar at protein level.

The increase in biomass production in *aps1* mutants relative to the wild type *Arabidopsis* may be linked to an increase in two highly conserved paralogues of TRANSKETOLASES TKL1 and TKL2. In most plants, transketolase activity in the Calvin cycle is mostly located in the plastid membrane where photosynthesis takes place (Teige et al., 1998). Plastid transketolase (TK) has been shown to have a significant effect on photosynthesis and growth in antisense tobacco plants. Small reductions (20 to 40%) in the activity of TK in tobacco inhibited photosynthesis and significantly decreased the levels of aromatic amino acids and phenylpropanoids (Henkes, 2001). This effect of TK on carbon allocation may be due to its central location in the C3 cycle where it is involved in the reductive Calvin cycle and non-oxidative part of the pentose phosphate pathway, hence playing a critical role in connecting the pentose phosphate pathway to glycolytic intermediates (Jung et al., 2004; Schenk et al.,



1998). Therefore, increased growth in untreated *aps1* mutants compared to the untreated *Arabidopsis* control plants could have been at least partially attributed to the participation of TKL1 and TKL2 in generating energy for the Calvin cycle as well as improved carbon flow to the glycolysis pathway.

Our results also revealed an upregulated ADP-GLUCOSE PYROPHOSPHORYLASE large unit (APL1) and in *aps1* mutants compared to the wild type. It is widely accepted that ADP-GLUCOSE PYROPHOSPHORYLASE (AGPase) is the sole source of ADP-glucose linked to plant starch biosynthesis (Müller-Röber et al., 1992), and starch is regarded as a major integrator of plant growth (Iddai and Scott, 2011; Schlosser et al., 2014; Sulpice et al., 2009). In *Arabidopsis*, six genes encode proteins with homology to AGPase, two of these genes encode for distinct small subunits (*APS1* and *APS2*) and four encode modulatory large subunits (*APL1*–*APL4*) (Crevillén et al., 2003). The different large subunits are encoded by related genes that evolved by divergence, specialization, and sub-functionalization from a common catalytic ancestor (Ballicora, 2005; Crevillén et al., 2003; Simillion et al., 2002). *APS1*, *APL1*, and *APL2* have catalytic activity, thus indicating the evolution from a common ancestor for both types of subunits (Ballicora, 2005; Ventriglia et al., 2008). Despite an increase in APL1 in *aps1* mutant lines compared to the wildtype, there was still a miniscule amount of starch in the *aps1* mutant lines, indicating that the increase in APL1 could not compensate for loss of the catalytic role of APS1 in starch biosynthesis. In contrast to reports that plants which are unable to make starch and show severe slow-growth phenotypes due to competition for a limited supply of photoassimilates (Ghosh and Preiss, 1966; Müller-Röber et al., 1992; Sweetlove et al., 1996; Tsai and Nelson, 1966), growth of *aps1* mutant plants was significantly enhanced compared to the wildtype. This collectively provided very strong evidence for the hypothesis that enhanced biomass production in *aps1* mutant compared wild type was not dependent on starch. Rather it is directly linked to an increased carbon supply resulting from an enhanced photosynthesis.

***Treating Arabidopsis aps1 mutant plants with lumichrome enhances photosynthetic efficiency***

Treating *aps1* mutant plants with lumichrome revealed an increase in PHOTOSYSTEM I SUBUNIT D-1 (PSAD-1) relative to their untreated corresponding mutants. Down-regulation (5–60%) of PSI-D de-stabilizes PSI, resulting in increased photosensitivity and altered thiol disulfide redox state of the stroma (Haldrup et al., 2003), while complete lack of the D subunit leads to seedling lethality under photoautotrophic conditions (Ihnatowicz et al., 2004). Therefore, an increase in PSAD-1 might have contributed to enhanced binding and stabilization of the Ferredoxin-PSI complex, specifically by guiding ferredoxin to the binding site on PSI, thus resulting improved PSI affinity and stability (Busch and Hippler, 2011; Fromme et al., 2001; Sétif et al., 2002; Zanetti and Merati, 1987; Zilber and Malkin, 1988). An increase in PSI-D1 was accompanied by high abundance of chaperones such as CHAPERONIN (CPN20) and CHLOROPLAST HEAT SHOCK PROTEIN 70-2 (CPHSC70-2). CPN20 is a co-chaperonin that aids chaperonins in their protein-folding function in an ATP-dependent manner and facilitates the folding of the bound polypeptide substrate to its native form (Hartl and Hayer-Hartl, 2009; Horwich et al., 2007). Independently of its known function in the chaperonin system, CPN20 also plays a role in the activation of iron superoxide dismutase (FeSOD) for oxidative stress protection and chloroplast development. Previous studies in which CPN20 expression was silenced in tobacco and tomato resulted in decreased FeSOD activity *in vivo*, a pale-green phenotype and poorly developed mesophyll tissues with a reduced number of chloroplasts due to damage to the chloroplast nucleoids by superoxide anions, which suggests a role for CPN20 in chloroplast development (Hanania et al., 2007; Myouga et al., 2008). On the other hand, CPHSC70-2 is essential for chloroplast protein import and processing (Bisanz et al., 2003; Chatterjee et al., 1996; Rolland et al., 2011; Romani et al., 2012; Schultes et al., 2000; Su and Li, 2010). *Arabidopsis* impaired in cpHSC70-2 are defective in protein import into chloroplasts (Shi and Theg, 2010) with white seedling and seedling lethality (Cline and Dabney-Smith, 2008; Shipman-Roston et al., 2010). Therefore, a collective increase in PSAD-1, CPN20 and cpHSC70 may be linked to enhanced chloroplast biogenesis and maturation through improved regulation of the assembly state of proteins important for thylakoid biogenesis and maintenance (Yalovsky *et al.*, 1992; Liu *et al.*, 2007; Shi and Theg, 2010). These may have in-turn improved PSI stability, protection and repair as well enhancing protein-folding of *de novo*-synthesized polypeptides (Martin and Hartl, 1994).

Intriguingly, lumichrome-treated *aps1* mutant plants showed an increase in THIOREDOXIN M-TYPE (TRX-M2; TRX-M4) and FERREDOXIN THIOREDOXIN REDUCTASE CATALYTIC B-CHAIN FAMILY PROTEIN (FTR) relative to their untreated control plants. This is suggestive of an interaction between FTR TRX isoforms in which the unique 4Fe–4S cluster enzyme ferredoxin:thioredoxin reductase (FTR) uses photosynthetically-reduced ferredoxin as an electron donor to reduce the disulfide bridge of different TRX isoforms (Dai et al., 2004), particularly TRX-M2 and TRX-M4 in the current study.

In respect to carbon metabolism, we observed similar levels of ADP-glucose in both untreated and treated *aps1-1* plants and wild type plants. However, there was a significant decrease and increase in ADP-glucose content in treated *aps1-1* and *aps1-2* plants respectively. Despite this inconsistent response in the treated *aps1* mutant lines, the levels of starch in the mutant plants, whether treated with lumichrome or not, were extremely low in the mutant lines in comparison to the wild type plants. These observations are not consistent with reports that in *Arabidopsis* leaves, most of ADP-glucose linked to starch biosynthesis occurs outside the chloroplast in source leaves, hence the plastid phosphoglucoisomerase (PGI)-plastidial phosphoglucomutase (pPGM)-AGPase pathway and pPGM and AGPase are not major determinants of intracellular ADP-glucose content (Bahaji et al., 2014, 2015; Baroja-Fernández et al., 2004; Li et al., 2013; Muñoz et al., 2005). Interestingly, our results revealed a consistent increase in cytosolic fructose-1,6-bisphosphatase (FBPase) protein. FBPase catalyzes the first irreversible reaction during conversion of triose phosphate to sucrose in the cytosol and plays an important role in regulating photosynthetic carbon partitioning between sucrose and starch (Daie, 1993; Sharkey et al., 1992; Zrenner et al., 1996). However, an increase in FBPase and sucrose in lumichrome-treated *aps1* mutant lines did not possibly attributed to an alternative ADP-glucose production, in which sucrose might have been hydrolysed to ADP-glucose and subsequently transported to the chloroplast for to starch production (Zhou and Cheng, 2004). Therefore, an increase in biomass despite miniscule levels of starch in *aps1* mutant lines compared to the wild type, indicated that starch was not the main integrator of growth in the *aps1* mutant plants; rather growth was directly linked to enhanced photosynthesis with increased carbon supply for plant growth.

## Conclusions

In summary, our study showed unambiguously that silencing APS1 (At5g48300) enhanced photosynthesis efficiency and biomass in *Arabidopsis*. Enhanced photosynthesis was attributed to an increase in proteins involved in a promoted upward shoot growth to ensure a proper positioning of the leaves for efficient photosynthesis efficiency. Furthermore, improved photosynthetic efficiency in *aps1* mutant plants may be linked to an increase in proteins involved in ensuring PSII macrostructure and stability, promoted electron transport and protective non-photochemical mechanisms that quench singlet-excited chlorophylls and harmlessly dissipate excess excitation energy as heat may have contributed to an improved photosynthetic efficiency. In respect to plant growth, an increase in biomass in *aps1* mutants compared to the wild type could be allied to an increase in photosynthesis capacity through the participation of transketolases TKL1 and TKL2 in energy generation for the Calvin cycle as well as improved carbon flow to the glycolysis pathway.

We have also demonstrated that no further growth effect was observed following lumichrome treatment in *aps1* mutant lines. Additionally, the starch levels in lumichrome-treated *aps1* mutant lines compared to their corresponding untreated mutant plants did not change, once again indicating that starch was not the main integrator of plant growth in *aps1* mutant lines. Our results therefore suggested that the enhanced growth of *aps1* mutant plants can be ascribed to enhanced photosynthetic efficiency, which ensured the provision of energy and carbon supply for *Arabidopsis* growth. In summary, transcriptomic, proteomic and metabolomic analyses all suggest that down-regulation of APS in lumichrome-treated plants enhanced photosynthesis, leading to increased availability of C for growth. Consistent with this hypothesis, a reverse genetics approach using *aps1* knockout mutants indicated that similar level of enhanced growth and photosynthesis could be achieved even in the absence of lumichrome. It will be interesting to investigate further the means by which APS1 regulates this mechanism.

## Acknowledgements

We are very grateful to Martin Kidd for statistical analysis. Our gratitude is due to Shaun Peters for sourcing *Arabidopsis* AGPase deficient *Salk-lines* and assisting in genotyping the lines for T-DNA insertions.

## Author contributions

MP, JK, JL and PH designed the experiments. Experimental work was conducted by MP, JL, SA, WS and AF. Data analysis was performed by MP, WS, AF, SA and AV. The manuscript was prepared by MP, PH, JK, and JL.

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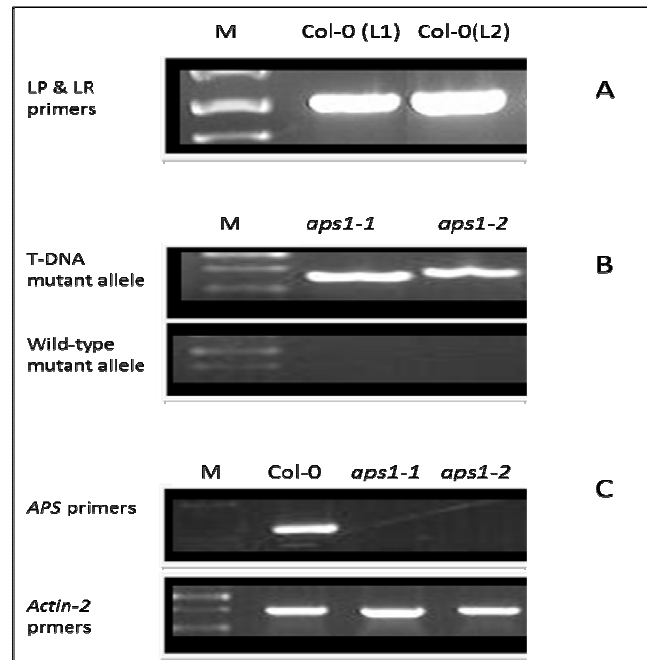
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**Supplementary data****Table S1. Primer list for *aps1* *Arabidopsis* T-DNA knockout Salk-lines for homozygosity screening**

<b>AGPase-At5g48300</b>	<b>Primer Sequence (5'-3')</b>
Salk_059083 Fwd	TAGGGGAACCCGTTTACAATC
Salk_059083 Rev	TACAACCTCCAATGGACTTGGC
Salk_040155 Fwd	AACATTTCTCTAATGTGTGTTATTCATG
Salk_040155 Rev	ACACACAGCCGCGTTATTTAC
LBb1.3	ATTTTGCCGATTCGGAAC



**Figure S1:** Molecular characterisation of *apsI* T-DNA knockout *Arabidopsis* Salk-lines. Homozygous plants from two of APS-independent lines (At5g48300: *Salk\_059083* and *Salk\_040155*) were genotyped using a PCR approach from genomic DNA (Edwards et al., 1991). T-DNA insertions were confirmed by using the flanking primers for the specific gene and primers from the T-DNA right border as generated by Signal T-DNA verification primer design. Both left (LP) and right (RP) flanking primers for the specific gene were used to confirm wild type allele (Fig S1A) while the T-DNA right border (LBb1.3) primer paired with RP primer were used to confirm homozygous showing absence of the wild type background (Fig. S1B). Total RNA was extracted from leaves using the RNeasy mini kit (Qiagen), following the manufacturer's instructions. The cDNA template for semi-quantitative PCR (sqPCR) were obtained by reverse transcription of 1  $\mu$ g total RNA with an oligo (dT<sub>18</sub>) primer and the RevertAid reverse transcriptase (M-MuLV RT, Fermentas) following the manufacturer's instructions. The sqRT-PCR was carried out in 50  $\mu$ l containing 5  $\mu$ l cDNA, 0.5 mM of each dNTP, and 0.5  $\mu$ mol of each primer, 1X PCR buffer and 1.25 U GoTaq DNA polymerase (Promega). The sqRT-PCR was performed at a primer annealing temperature of 60°C for 25 cycles, which occurs in the linear range of the constitutively expressed *ACTIN2* gene (*ACT2*, At3g18780). All *Salk* lines showed clear absence of AGPase small subunit transcripts in their leaves (Fig S1C).

## CHAPTER 5

### General conclusion

Despite the impressive advances that have been made over the years in improving both plant growth and yields, there is little reason to become complacent about these developments, especially with regard to food supply for the increasing world population (Cassman, 2001). This therefore calls for crop improvement technologies, in order to supply the increasing demand for food while still protecting natural resources for future generations. Other than conventional and marker assisted breeding, the use of crude extracts from plant growth-promoting rhizobacteria (PGPR) bacteria that possess biostimulatory properties has been considered as an alternative practice for enhancing plant development and crop productivity (Velázquez-Becerra et al., 2011; Zou et al., 2010). Such bacterial products have been applied to several agricultural crops for the purposes of growth and yield enhancement and disease control (Kloepper and Schroth, 1981; Zablutowicz *et al.*, 1991). One such substance is lumichrome (*7,8 dimethylalatoxazine*), a novel plant growth promoting, multitrophic signaling molecule produced by the bacterium *Sinorhizobium meliloti* (Phillips et al., 1999). Lumichrome has been shown to enhance photosynthesis and growth of legumes and tomato (Gouws et al., 2012; Khan et al., 2008; Matiru and Dakora, 2005; Matiru and Dakora, 2005), although lumichrome treatments did not affect any measured growth variable of corn (Khan et al., 2008). Combined transcriptional and proteomics analyses on tomato (*Solanum lycopersicon*) and *Lotus japonicus* suggested that the growth promotion effect may be associated with enhanced starch accumulation and altered ethylene metabolism (Gouws et al., 2012). However, due to mixed responses amongst plant species (Khan et al., 2008; Matiru and Dakora, 2005a), the underlying physiological growth promotion mechanism elicited by lumichrome is still unclear. To further contribute towards understanding the mode of action underlying lumichrome-associated plant growth promotion, this study utilised transcriptomic, proteomic and metabolomic analyses on *Arabidopsis thaliana* rosette leaves.

***Lumichrome treatment elicited plant growth promotion in wild type Arabidopsis rosette leaves***

Our current study proceeded from the postulate that lumichrome elicits plant growth and development promotion (Gouws et al., 2012; Khan et al., 2008; Matiru and Dakora, 2005a, 2005b; Phillips et al., 1999). Physiological and gene expression analysis was performed in lumichrome-treated *Arabidopsis thaliana* (ecotype Columbia-0) plants compared to untreated control plants (Chapter 2). As observed in previous studies, lumichrome demonstrated the ability to increase the biomass of *Arabidopsis* plants. Gene expression profiling, suggested that lumichrome-dependent growth enhancement in *Arabidopsis* plants was attributable to an increase in mitosis-related transcripts, whose role is to ensure cell wall loosening to allow turgor-driven cell enlargement (*XTH9*, *AtXPA4*; *RSW7*) and integration of cell division and expansion to developing leaves (*CYCA1;1*, *CYCD3;3*, *PDF1*). In addition, an increase in *CKA1* transcripts in lumichrome-treated plants suggested enhanced signaling of mitotic cell cycle-associated transcripts, which further suggests increased cell division and expansion with a consequential increase in biomass.

Other than an increase in expression of core mitosis cycle transcripts, changes in expression of either hormone biosynthesis or signaling also suggested the role of lumichrome in mediating cell division and cell expansion. Over-expression of *SAUR54* may be linked to signaling of an increase in auxin, while an increase in *RMA2* transcripts might have increased auxin transport across the ER membrane, thereby improving cellular auxin homeostasis and hormonal control of *Arabidopsis* vegetative growth. The possible resultant increase in auxin could have greatly activated the expression of *ACO1* and *AP2.4/EREBP* transcripts, hence stimulating ethylene production in vegetative tissues. Similar observations were reported in various plant species in which an increase in ethylene production following exogenous auxins was due to auxin-induced transcription of *ACS* and *ACO* genes for ethylene biosynthesis (Abel et al., 1995; Böttcher et al., 2013; Kondo et al., 2006; Yoshii and Imaseki, 1982; Yu and Yang, 1979). We also observed gibberellic acid (GA) responsive transcripts such as such as *GASAI*, signaling increased levels of GA hormone (Aubert et al., 1998; Roxrud et al., 2007; Shi and Olszewski, 1998). Therefore, the suggested increased levels of auxin and GA further implied auxin/gibberellic acid synergistic cross-talk with a functional role in mediating cell division and cell expansion.

Comparative proteomic analysis, however demonstrated an increase in proteins related to both photosynthesis rate and biomass production (Chapter 3). First, an increase in photosynthesis rate in lumichrome-treated wild type *Arabidopsis*, could be linked to proteins whose roles are to enhance early chloroplast development, differentiation and maturation. These includes an increase in CPN60A, cpHSC70, cpHSC70-1 and COR15, which implied an improved early steps of thylakoid assembly, plastid division, protein translocation into the stroma and stabilization of chloroplast membrane. This may have in turn attributed to efficient functioning of the chloroplast, as demonstrated by an increase in chlorophyll *a* and *b* levels. These increased levels of chlorophylls may be positively linked to an increase in expression of *LOX3*, *LOX4* and *LOX6* transcripts (Chapter 2), and *LOX2*, *AOS* and *AOC2* proteins (Chapter 3), which implied a reduced jasmonate biosynthesis in lumichrome-treated wild type *Arabidopsis* plants. Jasmonates are cyclopentanone derivatives that originate biosynthetically from linolenic acid (LA) via an inducible octadecanoid pathway consisting of several enzymatic steps. The early steps convert LA to 12-oxo-phytodienoic acid (OPDA) via lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) in the chloroplast, hence these methyl jasmonate-related enzymes increase upon wounding and during senescence in leaves (Bell *et al.* 1995; He *et al.* 2002; Chung *et al.* 2008; Seltsmann *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. Indeed, the leaf senescence-promoting effect of methyl jasmonate has been reported to decrease absorption, energy transformation and electron transfer capacity, leading to decreases in the density of active PSII reaction centres, the O<sub>2</sub> evolution rate and the PSI photo-activity (Papuga *et al.*, 2010). Therefore, reduction of JA biosynthesis-related transcripts and proteins in lumichrome-treated wild-type could be linked with an increase in chlorophyll biosynthesis and delayed leaf senescence, as well as an increase in photosynthetic efficiency.

The biosynthesis and signal transduction of ethylene was previously evaluated on roots of lumichrome-treated tomato and *Lotus japonicus*. That study revealed enhanced ACC synthase levels in the roots of both species. However, ethylene evolution rates were significantly enhanced only in *Lotus japonicus* roots, while those of tomato remained invariable from the control. Further analysis revealed that the differences in ethylene levels

were due to post-transcriptional regulation of ethylene biosynthetic enzymes, which was confirmed by the maximal catalytic activities of the respective biosynthetic proteins. Thus, the enhanced ACC synthase catalytic activity significantly increased ACC synthase levels and ethylene in *Lotus japonicus* (Gouws et al., 2012). In this light, phytohormone profiling of candidate hormones, particularly, ethylene, auxin and gibberellic acid, would have been useful for understanding any regulation in hormone homeostasis and possible hormonal cross-talk in lumichrome-treated plants. Although we did not perform hormone analysis due to time restraints, this would be a valuable area of investigation for future research.

An increase in chlorophyll *a* and *b* levels, further suggested an improved sensing of the level of excitation energy via the thylakoid proton gradient, which subsequently will have allowed excess energy to be dissipated as heat by formation of a non-photochemical quencher (Horton et al., 1996). This could be explained by an high abundance of PSII light harvesting complex (LHCB) proteins, which are involved in optimal thylakoid structure flexibility, maintenance of PSII supercomplex structure during state transition, and thermal dissipation for PSII protection (Havaux et al., 2007; Jansson, 1999; Pietrzykowska et al., 2014; Ramel et al., 2013). Other than the increased light harvesting chlorophyll-binding proteins, the enhanced photosynthetic rate in lumichrome-treated wild type *Arabidopsis*, was possibly attributed to an improved oxygen evolving complex (OEC) and electron transport. An increase in PSBP and PSBQ implied an improved stabilization of PSBP binding by PSBQ, thereby contributing to the maintenance of the catalytic Mn cluster of the water oxidation machinery in *Arabidopsis* PSII (Ifuku et al., 2011; Kakiuchi et al., 2012), found that these proteins, once again indicating that lumichrome enhances chloroplast functionality. Furthermore, an increase in membrane-extrinsic proteins coupled with increased CF<sub>0</sub>F<sub>1</sub> ATP synthase proteins (ATPA, ATPB, ATPD and ATPE) collectively implied an efficient cyclic electron flow into the thylakoid lumen, generating a pH gradient across thylakoid membrane ( $\Delta$ pH) that contributes to ATP synthesis from ADP and Pi (Cruz *et al.*, 2001; Cruz *et al.*, 2005) (Merchant and Sawaya, 2005; Nelson and Ben-Shem, 2004). In addition, it implied a triggering of the photoprotective process of nonphotochemical quenching (NPQ) that harmlessly dissipate excess excitation energy as heat (Baker et al., 2007; Heber, 2002; Johnson, 2011; Joliot and Johnson, 2011).

Consistent with the previous study on tomato and *Lotus japonicus* roots (Gouws et al., 2012), lumichrome-dependent growth increase in wild type *Arabidopsis* plants may be attributed to an enhanced starch accumulation (Chapter 4). Our study surprisingly revealed that lumichrome-treatment resulted in decreases in starch biosynthesis proteins, such as, plastidial pPGM1 and AGPase small subunit (APS1) proteins. Chloroplastic isoforms of PGM catalyze the conversion of fructose 6-phosphate into glucose 1-phosphate while AGPase uses glucose-1-phosphate and ATP to generate ADP-Glc and PP<sub>i</sub>. Genetic and biochemical evidence shows that mutations affecting these enzymes decrease or abolish starch biosynthesis in leaves (Caspar et al., 1985b; Hanson and McHale, 1988; Lytovchenko et al., 2002; Müller-Röber et al., 1992). We however, observed that an increase in biomass be ascribed to stomatal opening allowing CO<sub>2</sub> entry into leaves and/or more efficient CO<sub>2</sub> utilization, pointing to well-functioning mesophyll metabolism (Pagter et al., 2005). This further positively correlated with up-regulated levels of TRX-M1, 2, 4, CPN60A and RCA protein, implying a preferential TRX-M targeted redox activation of chloroplastic CPN60A and RCA protein during CO<sub>2</sub> fixation. An implied increase in CO<sub>2</sub> fixation, might have turn provided carbon supply for plant growth (Raines and Paul, 2006; Smith and Stitt, 2007). In addition, the results revealed an increases in cytosolic proteins, such as FBA8 and GAPC2, implying enhanced sucrose biosynthesis and levels of reducing sugars, once again indicating an increase in carbon supply and energy from photosynthesis as building blocks to produce biomass.

***Silencing AGPase small subunit (APS1) enhances Arabidopsis growth, while lumichrome treatment on APS1-deficient mutants result in no further increase in growth***

Since we had observed a significant decrease in levels of APS1 in lumichrome-treated *Arabidopsis* plants, which was in contrast to the observed increase in starch levels in lumichrome-treated plants, as well as previous reports in which lumichrome-associated plant growth was linked to starch, we tested the effects of lumichrome on *Arabidopsis* plants deficient in APS1 (Chapter 4). Both proteins and metabolites were profiled in 2 independent *aps1* T-DNA knockout lines. Plants from both knockout lines showed enhanced growth compared with wild type plants. The *aps1* mutant plants were similar in size to lumichrome-treated wild type plants, and furthermore, lumichrome treatment had no additional effect on their growth, suggesting that APS1 is indeed an important mediator of lumichrome-associated

growth enhancement. Interestingly, although wild type plants treated with lumichrome had significantly increased starch levels, consistent with the previous studies, the *aps* mutant lines by comparison had only miniscule amounts of starch which did not change in response to lumichrome application. This strongly suggests that starch *per se* is not important in the growth enhancement mechanism, but rather that enhanced starch reserves in lumichrome-treated wild type plants may instead be an artifact of improved photosynthesis and photoassimilate production.

Our proteomic data revealed that *aps1* mutant lines exhibited an increase in SHOOT GRAVITROPISM 4 (SGR4) protein compared with wild type plants, suggesting better positioning of the leaves for efficient photosynthesis. Our study showed unambiguously that silencing *APSI* (At5g48300) enhanced photosynthetic efficiency and biomass in *Arabidopsis*. The increased biomass of *aps1* mutants compared to wild type plants could be allied to an increase in two conserved transketolase paralogues (ATKL1, ATKL2), implying an increase generation of NADPH energy and the interconversion of phosphorylated sugars for carbon supply and utilization for growth through the Calvin cycle.

In addition to being of similar sizes to lumichrome-treated wild type plants, untreated *aps1* plants also had similar increases in PSII light-harvesting chlorophyll a/b binding proteins (LHCB4.2; LHB1B1; LHCB5; LHCB6) and photosystems reaction centre-related proteins (PSDI, PSAA and PSAB) as the treated wild type plants. This may imply an improved absorption of photons and/or the transfer of the excitation energy to the reaction centers of PSII and PSI, and thus improved photosynthesis efficiency. Furthermore, treated wild type plants and untreated mutant plants demonstrated a similar increase in CF<sub>0</sub>F<sub>1</sub> ATPase, suggesting an improved PSII stability, and an enhanced light driven induced-electron pump. This in turn might have triggered inhibition of plastoquinol re-oxidation at the Cytochrome b6f due to acidification of the thylakoid lumen as well as activation of NPQ, once again indicating an enhanced photosynthesis efficiency.

Our results also demonstrated that no further growth effect was observed following lumichrome treatment in *aps1* mutant lines. Additionally, lumichrome treatment of the *aps1* mutant lines had no effect on starch levels in these plants, once again indicating that starch is not the main integrator of plant growth in lumichrome-treated *Arabidopsis* plants. We



however, observed increase in PSAD-1, CPN20, cpHSC70, ferredoxin:thioredoxin reductase (FTR) proteins which are involved in ensuring PSI affinity, stability and photo-protection. This further demonstrated that growth was dependent on enhanced photosynthesis capacity.

### **Closing remarks**

Consistent with previous studies, the addition of 5 nM lumichrome to *Arabidopsis thaliana* plants elicited a growth promoting effect to increase overall plant size and biomass. Transcriptomic analyses linked the increased growth to changes in genes relating to cell division and cell expansion. Proteomic results suggested lumichrome treatment enhances and stabilizes photosynthesis, providing increased photoassimilates for growth. Although both transcriptomic and proteomic changes reflected enhanced growth processes, the proteomic data gave a clearer indication of the possible mechanism(s) associated with this growth. We attribute this to the fact that the plant material used in these transcriptomic and proteomic studies was harvested at the end of the experimental growth period. Hence, initial transcriptomic changes relating to the observed changes in photosynthesis may have occurred earlier in the experiment and thus been missed, with the observed transcriptomic changes rather reflecting the end-result of the enhanced photosynthesis, namely the use of higher levels of photoassimilates for cell division and cell enlargement. Therefore, different sampling points would have been useful for understanding any transcriptional regulation, and this would be a valuable area of investigation for future research.

Despite an increase in starch levels in lumichrome-treated wild type plants, levels of APS1 were paradoxically decreased. The use of *aps1* knockout lines revealed that untreated *aps1* plants were of similar size to lumichrome-treated wild type plants. Photosynthetic levels in these *aps1* plants were also enhanced, and similar changes in photosynthesis-related proteins were observed in these plants as were seen in lumichrome-treated wild type plants. Additionally, lumichrome treatment had no further effect on the growth of these mutant lines, strongly suggesting that APS1 is responsible for mediating the lumichrome-associated growth response in *Arabidopsis*. Unlike wild type plants, starch levels in the *aps1* lines were extremely low and were not affected by lumichrome treatment. Consequently, we conclude that lumichrome enhances growth in *Arabidopsis* plants via enhanced photosynthesis in a process mediated via APS1, and that the enhanced levels of starch seen in lumichrome treated

wild type plants are merely an artifact of this enhanced photosynthesis. Although, we postulate that APS1 is a critical modulator of lumichrome response in *Arabidopsis*, a valuable area of investigation will be to further unravel the mechanisms by which APS1 regulates growth enhancement, particularly considering transcriptional responses at different sampling points, following lumichrome inoculation.

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