Analysis of the molecular and physiological effects following treatment with BC204 in *Arabidopsis thaliana* and *Solanum lycopersicum*

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Declaration

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- 43rd Annual Congress of the South African Association of Botanists (8-11 January 2017, Cape Town, South Africa)
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Summary

Plant biostimulants have been earmarked as one of the pivotal role players in the next much-needed agricultural revolution. Plant biostimulants are mostly from natural sources and they do not directly provide the plant with any nutrients. To date, many different biostimulants have been produced and tested on several different plant species. Although several reports indicate that they elicit an increase in overall plant growth, induce resistance to both abiotic and biotic stresses, increase crop yield and improve fruit/vegetable quality, the molecular data to back up these claims has generally been missing. One such plant biostimulant, BC204, is a citrus-based plant extract used on a variety of crop species in South Africa, China and Australia. There are internal reports from tests conducted by the producers of BC204 which show that it elicits physiological responses such as an increase in crop yield and fruit quality. One postgraduate research study reported that Croplife, a product closely related to BC204, has the potential to improve water utilisation efficiency in table grape cultivars (Van Zyl, 2007). However, no molecular data is available to explain the specific mechanisms associated with the increase in plant growth and tolerance to environmental stresses. Environmental stress is predicted to worsen due to climate change, but also due to irrigation practices on arable land areas, which can result in soil salinity. Although some progress has been made towards understanding plant mechanisms towards salt tolerance in efforts to combat the negative effects of salinity, these mechanisms are still a long way from being fully understood. BC204, like other plant biostimulants, could be a short-term alternative whilst salt tolerance and other abiotic stress mechanisms in plants are further unravelled. Such biostimulants can also be used to study salt tolerance, as the first part of this study provides preliminary evidence that BC204 significantly alleviates salt stress in *Arabidopsis thaliana*. BC204 treatment increased chlorophyll content, fresh and dry weights, whilst reducing proline, anthocyanin and malondialdehyde content in the presence of 10ds·m^{-1} EC salt stress. Stomatal conductivity was also reduced by BC204 in source leaves. In addition, BC204 had a significant effect on the expression of salinity-related genes, stimulating the expression of salinity-related genes *RD29A* and *SOS1* independently of NaCl-stress, whilst suppressing the expression of *SOT1* and *P5CS1*. In the second part of study, an RNA-seq approach was adopted to elucidate the effects of BC204 at the molecular level in the model plant species, *Arabidopsis thaliana*. BC204, applied via a soil drench at a low concentration of 0.01% (v/v), stimulated above-ground biomass production whilst eliciting a large change in gene expression levels across several biochemical pathways in *Arabidopsis thaliana*. Of the entire transcriptomic profile examined, a total of 8.212% of genes were significantly differentially expressed between the treated and control groups, of which 5.136% were upregulated and 3.076% downregulated. Most notably, genes involved photosynthesis, several aspects of cell wall metabolism, carbohydrate metabolism, signalling, stress and secondary metabolism were upregulated, which could explain the increase in plant growth. Genes related to transcription and RNA regulation were both strongly up- and downregulated, which suggests that BC204 plays a role in inducing and suppressing several pathways. In the third part of this study, the same RNA-seq approach was adopted to elucidate the effect of BC204 in *Solanum lycopersicum*, an important model crop species, at the molecular level under unstressed conditions. BC204, applied via foliar spray at a concentration of 0.05% (v/v), stimulated tomato root and shoot biomass production, root and shoot length and stem width compared to the untreated control plants. Of the 33308 transcripts analysed, a total of 18.059% genes were significantly differentially expressed between the control and treated groups, of which 8.776% were upregulated and

9.283% downregulated. Most notably, genes involved in signalling, stress and protein metabolism were upregulated, which could explain the increased growth that was observed. In both plant species, BC204 seemed to induce pathways involved in several environmental stresses. Together, the results of this study provide evidence that BC204 elicits a major change in a variety of metabolic processes which forms part of a complex network activating a broad priming response. These priming responses seem to start with enhanced photosynthesis, allowing additional energy to be channelled towards complex metabolic changes through RNA regulation and signalling. Very few metabolic plant processes seem to be unaffected by BC204 treatment.

Opsomming

Plantbiostimulante is geoormerk as een van die belangrikste rolspelers in die volgende broodnodige landbou-rewolusie. Plantbiostimulante word meestal onttrek uit natuurlike bronne en voorsien nie direk die plant van voedingstowwe nie. Tot op hede is baie soorte plantbiostimulante geproduseer en getoets op verskillende plantsoorte en alhoewel verskeie verslae aandui dat dit 'n toename in algehele plantgroei tot gevolg het, weerstand teen abiotiese sowel as biotiese spanning veroorsaak het, die oesopbrengs verhoog is en die vrugte / groente-kwaliteit verbeter het, ontbreek die molekulêre gegewens om hierdie aansprake te ondersteun. BC204 is 'n sitrus-gebaseerde plantuittreksel wat as 'n plantbiostimulant gebruik word vir 'n verskeidenheid plantsoorte in Suid-Afrika, China en Australië. Alhoewel daar verslae is dat dit fisiologiese reaksies ontlok, soos 'n toename in gewasopbrengs en vrugkwaliteit, is daar geen molekulêre gegewens beskikbaar om die spesifieke meganismes te verduidelik wat verband hou met die toename in plantgroei en verdraagsaamheid teenoor omgewings-stressors nie. Een nagraadse navorsingsstudie het verslag gelewer dat Croplife, ‘n produk nabyverwant aan BC204, die potensiaal het om waterverbruik effektiwiteit in tafeldruiwe kultivars te verbeter (Van Zyl, 2007). Hierdie tipe stressors sal na verwagting vererger as gevolg van klimaatsverandering, maar ook as gevolg van besproeiingspraktyke in bewerkbare gebiede. Alhoewel daar 'n mate van vordering gemaak is met betrekking tot die begrip van plantmeganismes ten opsigte van souttoleransie in die pogings om die negatiewe gevolge van soutgehalte te bekamp, is hierdie meganismes grootliks onbekend. BC204, soos ander plantbiostimulante, kan 'n korttermyn alternatief wees terwyl souttoleransie en ander abiotiese stresmeganismes in plante verder ontrafel word. Sulke biostimulante kan ook gebruik word om soutverdraagsaamheid te bestudeer, aangesien die eerste deel van hierdie studie voorlopige bewys lewer dat BC204 soutstres in *Arabidopsis thaliana* aansienlik verlig. Met die behandeling van BC204 het die chlorofil-inhoud, vars en droë gewigte verhoog, terwyl die inhoud van prolien, antosianien en malondialdehied in die teenwoordigheid van 10ds · m-1 EC soutstres verlaag is. Blaarhuidmondjie geleiding is ook verminder deur BC204 en NaCl in die bronblare. Verder het BC204 'n beduidende invloed op die uitdrukking van soutreaktiewe-gene gehad, wat die uitdrukking van soutgereaktiewe-gene *RD29A* en *SOS1* onafhanklik van NaCl-spanning stimuleer, terwyl die uitdrukking van *SOT1* en *P5CS1* onderdruk is. In die tweede deel van die studie is 'n RNS-seq-benadering aangewend om die gevolge van BC204 op molekulêre vlak by die modelle plantspesies, *Arabidopsis thaliana*, toe te lig. BC204, toegedien via 'n gronddeurdrenking met 'n lae konsentrasie van 0.01% (v / v), stimuleer bogrondse biomassa-produksie, terwyl dit 'n groot verandering in geen-uitdrukkingsvlakke oor verskeie biochemiese padweë in *Arabidopsis thaliana* ontlok. Van die hele transkriptomiese profiel wat ondersoek is, is 'n totaal van 8.212% van die gene beduidend onderskeibaar tussen die behandelde groepe en die kontrolegroepe, waarvan 5.366% geherreguleer en 3.076% afgereguleer is. Die belangrikste veranderinge is die toename in uitdrukking van gene wat fotosintese, verskeie aspekte rondom selwand metabolisme, koolhidraatmetabolisme, seine, spanning en sekondêre metabolisme insluit, wat die toename in plantgroei kan verklaar. Die uitdrukking van gene wat met transkripsie en RNS-regulering verband hou, het beide sterk toegeneem ena afgeneem, wat daarop dui dat BC204 'n rol speel in die induksie en onderdrukking van verskeie padweë. In die derde deel van hierdie studie is dieselfde RNS-seq-benadering gebruik om die effek van BC204 in *Solanum lycopersicum*, 'n belangrike plantmodelspesie, op molekulêre vlak onder onbedrukte toestande toe te lig. BC204, toegedien via blaarbespuiting met 'n lae konsentrasie van 0.05%, stimuleer wortel- en

skietbiomassa-produksie, wortel- en lootlengte en stamwydte in vergelyking met die onbehandelde kontrole groep. Uit die 33308 transkripsies wat geanaliseer is, is 'n totaal van 18.059% gene betekenisvol onderskei tussen die kontrole- en behandelde groepe, waarvan 8.776% geherreguleer is en 9.283% afgereguleer is. Die belangrikste is dat die uitdrukking van gene wat betrokke is by seinttransduksie, stres en proteïenmetabolisme toegeneem het, wat die toename in waargenome groei kan verklaar. Gesamentlik lewer die resultate van hierdie studie bewyse dat BC204 'n groot verandering opwek in 'n verskeidenheid metaboliese prosesse wat deel uitmaak van 'n komplekse netwerk wat 'n breë basisrespons aktiveer. Dit lyk asof hierdie reaksies begin met verbeterde fotosintese, waardeur addisionele energie gekanaliseer kan word na ingewikkelde metaboliese veranderinge deur middel van RNS-regulering en seintransduksie. Baie min metaboliese plantprosesse blykbaar nie deur BC204-behandeling beïnvloed te word nie.

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List of abbreviations and non-SI units

Abbreviation/Symbol	Description
%	Percentage
°C	Degrees Celsius
ABA	Abscisic acid
AGP	Arabinogalactan protein
AMF	Arbuscular mychorrhizal fungi
At	*Arabidopsis thaliana*
BC204	Code-name for biostimulant used in this study
BR	Brassinosteroids
Ca^{2+}	Calcium ion
cDNA	Complementary deoxyribonucleic acid
cGMP	3'5'-cyclic guanosine monophosphate
CHO	Minor carbohydrates
Cl^-	Chloride ion
Col-0	Columbia 0
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle threshold
DAG	Days after germination
DAVID	The Database for Annotation, Visualization and Integrated Discovery
ddH_2O	De-ionised distilled water
DEG	Differentially expressed gene
dH_20	Distilled water
$ds{\cdot}m^{-1}$	DeciSiemens per metre
EC	Electrical Conductivity
ER	Endoplasmic reticulum
ET	Ethylene
FAO	Food and Agriculture Organization
FC	Fold change
FDR	False discovery rate
Fig	Figure
Fv/Fm	Variable fluorescence divided by maximum fluorescence
GA	Gibberellic acid
g·FW	Gram fresh weight
GM	Genetic modification
GO	Gene ontology
HS	Humic substances
inf	Infinite
JA	Jasmonic acid
K	Potassium
K^+	Potassium ion
L	Litres
LRR	Leucine-rich repeats
LSD	Least Significant Difference
MDA	Malondialdehyde
Mg	Magnesium

Mg^{2+}	Magnesium ion
mL	Millilitre
mM	Millimolar
mRNA	Messenger RNA
MS	Murashige and Skoog
n	Number of replicates
N	Nitrogen
n/a	Not applicable
Na	Sodium
Na^{+}	Sodium ion
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
OPP	Oxidative pentose phosphate pathway
P	Phosphorus
P5CS	Δ1-1-pyrroline-5-carboxylate synthetase
PANTHER	Protein Analysis THrough Evolutionary Relationships
PB	Plant biostimulant
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
PGPS	Plant growth promoting substances
PR	Pathogenesis-related (genes or proteins)
PS	Photosystem
RD29A	Responsive-to-Dehydration 29A
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse Transcriptase-Quantitative PCR
S	Sulfur
SA	Salicylic acid
SEA	Singular Enrichment Analysis
SI	Sustainable intensification
SOD	Superoxide dismutase
SOS	Salt Overly Sensitive
SOT12	Sulfotransferase 12
TCA	Tricarboxylic acid cycle
T-DNA	Transfer DNA
v/v	Volume / volume
µg	Microgram
µL	Microlitre
w/v	Weight per volume

CHAPTER 1: General introduction and literature review

1.1 Feeding an insatiable world

Since the start of human civilization approximately 10 000 years ago (Hallauer, 2011), plant growth has been pivotal to the success, survival and advancement of humankind, whether directly or indirectly (Guo et al., 2010). Humans rely on plants for oxygen, clothing, shelter, medicine, food, feed, biofuel and other industrial products. Currently, and for the foreseeable future, plant growth and health will be a determining factor in the survival of humankind.

1.1.1 Booming population and an increase in life expectancy

Overall improvements in life quality, a steady increase in life expectancy over the past 200 years (Oeppen and Vaupel, 2002) and improvements in medical technology in recent decades are leading to an increase in the world population which is set to continue (Bongaarts, 2009). Asian and African countries have displayed the largest increase in population, which is projected to continue increasing at the same rate for the next 30 to 40 years (Bavel, 2013). These are also the countries where issues of food security are most pressing (Satterthwaite et al., 2010). The direct effect of an increasing world population means that our food, feed, energy and plant biomass production for industry-related products needs to be increased. Between 2015 and 2017, world hunger increased and it isestimated that 821 million people were undernourished in 2018 (http://www.fao.org/state-of-food-security-nutrition/en/).

Agricultural production alone will need to increase by at least 60% (FAO. World Livestock 2011, Popp et al., 2014), but possibly by as much as 110% (Ray et al., 2013; Tilman et al., 2011). Imperative agricultural challenges include the rapid human population growth, increasing water scarcity, climatic changes and declining soil fertility (Kremser and Schnug, 2002).

1.1.2 Environmental constraints

Plant communities are limited by resource and recruitment limitations, predators and pathogens, and disturbances such as fires, climate and temporal variation (Tilman and Lehman, 2002). Humans are negatively contributing to the deterioration of all three components of environmental quality (Bünemann et al., 2018), namely soil, water and air quality (Andrews et al., 2002; Bünemann et al., 2018). Environmentally damaging agriculture practices lead to deteriorating soil quality and polluted waterbodies, while industrial practices lead to air, water and soil pollution (Moss, 2008). It is well-documented that pesticides, phosphorus and nitrate are the main agricultural water pollutants (Ertani et al., 2012). While agriculture is supposed to contribute to human health positively (Bhat, 2008), current practices are achieving the opposite, harming the environment and human health.

1.1.2.1 Deteriorating soil

Agriculture is the largest consumer of freshwater as it diverts the water away from natural habitats, which has severe effects on biodiversity and ecosystems (Godfray and Garnett, 2014). These anthropogenic actions (Anand et al., 2016) are also possibly the cause of environmental changes leading to plants experiencing an increase in abiotic stress caused by extreme temperatures, drought, waterlogging and

salinity stress (Raza et al., 2019). These effects can be seen in deteriorating soil across the globe, of which soil salinity is a major problem.

1.1.2.2 Salinity stress

Soil salinity is a global problem, affecting more than 100 countries (Rengasamy, 2006; Suter and Widmer, 2013) and approximately 20% of the world's irrigated cropland (Machado and Serralheiro, 2017; Yamaguchi and Blumwald, 2005). It is estimated that more than 50% of global arable land will be affected by 2050 (Wang et al., 2003). Primary salinisation occurs naturally, resulting from rock erosion over long periods of time via precipitation and through groundwater (Parihar et al., 2015), while secondary or human-induced salinisation is caused by a combination of poor drainage and irrigation (Zhu, 2007). Both combine to change the soil for the worse, leading to the accumulation of ions such as Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-. Although all these ions can cause soil salinisation, high concentrations of Na^+ as a result of excess NaCl are the most prevalent and the subject of most salt-stress related research (Tavakkoli et al., 2010).

Like drought and extreme temperature, the ion build-up contributing to soil salinity described above causes osmotic stress in plant tissue which affects water potential and hampers the plant's ability to take up water through the roots (Babu et al., 2011; Park et al., 2016). High salinity influences all aspects of plant growth and growth stages, leading to ion toxicity, osmotic stress, nutrient deficiency and oxidative stress (Shrivastava and Kumar, 2015). Most plant/crop species that are used to feed the global population are highly sensitive to high salinity (Yang and Guo, 2018; Zhang and Shi, 2013) and are known as glycophytes. Glycophytes can loosely be defined as plants able to grow healthily in soil with a low content of sodium salts, whilst a higher content of these salts results in stunted growth (Cheeseman, 2015). Halophytes, in turn, can grow and reproduce in high salinity environments due to their ability to control uptake and compartmentalize Na^+, K^+ and Cl^- ions (Flowers and Colmer, 2008). Salt stress has been increasingly studied in efforts to unravel the mechanisms by which plants deal with such stress. Saline soils in general affect plants by reducing the plant's ability to take up water or by ion-excess within the plant leading to cell damage within transpiring leaves (Parihar et al., 2015), also known as the salt-specific effect of salinity (Greenway and Munns, 2018). The effect of salt stress in plants has recently been extensively reviewed (Isayenkov and Maathuis, 2019).

The build-up of Na^+ is the major culprit in the detrimental effects seen on plant growth, but it remains unclear how this is sensed by the plant (Wu, 2018). The earliest components in NaCl-sensing and signalling include a rapid elevation of Ca^{2+} in the cytosol, an increase in reactive oxygen species (ROS) production and synthesis of cyclic nucleotides such as 3'5'-cyclic guanosine monophosphate (cGMP) (Isayenkov and Maathuis, 2019). Salty soil initially induces osmotic stress which results in nutrient imbalances, interruption of membranes and disrupts the plant's ability to detoxify ROS, which damage the plant at a molecular level (Gupta and Huang, 2015). The exact mechanisms by which plants mitigate salt stress have not been fully elucidated, but several role-players have been implicated.

In response to environmental stimuli such as salt stress, cellular abscisic acid (ABA) levels are increased (Yang and Guo, 2018), which triggers the expression of numerous stress-responsive genes (Shinozaki and Yamaguchi-Shinozaki, 2007). This regulation occurs via transcription factors which are

elevated in response to salt stress and through histone H3 acetylation and methylation, which further regulates stress-inducible gene expression (Fernando and Schroeder, 2016). One example is the induction of *RESPONSIVE TO DESICCATION 29A* (*RD29A*) expression (Lee et al., 2016), which is commonly used in research as a marker for salinity stress. The expression of *RD29A* is also induced by cold and drought stress and although its induction and overexpression increases a plant's resistance to abiotic stress, it was concluded that the RD29A protein is unlikely to serve directly as a protective molecule (Msanne et al., 2011).

The increase in ABA levels also stimulates an increase in the production of ROS species, which is used in research to monitor intracellular levels of oxidative stress in plants (Choudhury et al., 2017; You and Chan, 2015). The increase in production of ROS species, previously thought to only be a toxic by-product of stress, also serves as a source of signalling molecules leading to the production of antioxidants and antioxidative enzymes forming part of a concerted plant defence reaction (Mhamdi and Van Breusegem, 2018; Xia et al., 2015). If not sufficiently detoxified and scavenged, ROS species can cause oxidative damage to proteins, lipids and DNA (Tripathy and Oelmüller, 2012). Following prolonged oxidative stress caused by the Na^+ build-up and subsequent ROS species production, ion-toxicity is the inevitable next stage unless the plant sufficiently deals with the excess Na^+, which is not the case in most plants. The build-up of Na^+, generally not an essential element for plants, in the cytosol causes K^+ deficiency (Wu, 2018), which disrupts enzymatic processes since K^+ activates more than 50 key metabolic enzymes which cannot be substituted with Na^+ (Tester and Davenport, 2003). The enzymes activated by K^+ belong to the classes ligases (synthetases), transferases, oxidoreductases and pyruvate kinases (Bhandal and Malik, 1988).

Accumulation of proline (Gharsallah et al., 2016), malondialdehyde (MDA) (AbdElgawad et al., 2016) and anthocyanin (Eryılmaz, 2006) are commonly used in research as indicators of salt stress. Proline, a low molecular weight non-enzymatic amino acid and antioxidant (Singh et al., 2014) is an osmolyte biosynthesized through the glutamate and ornithine pathways. It plays an important protectory role in plant cells experiencing salt stress (Huang et al., 2013) by alleviating the negative impact of salt by decreasing osmotic stress to maintain membrane integrity and function (Singh et al., 2014). Under saline conditions, histone demethylase irreversibly removes the methylation of the *Δ^1-1-pyrroline-5-carboxylate synthetase (P5CS)* coding sequence, leading to the overexpression of *P5CS*. The *P5CS* gene encodes the P5C5 enzyme which catalyses proline synthesis from glutamate, a rate limiting step in proline synthesis (Signorelli and Monza, 2017). The induction of the expression of *P5CS* therefore leads to an accumulation of proline (Banerjee and Roychoudhury, 2017; Roychoudhury et al., 2015). As an extended effect of the presence of ROS species, cell membranes are damaged via the oxidation of acids in the lipid bilayer, a process also known as lipid peroxidation (Carrasco-Ríos and Pinto, 2014). Lipid peroxidation causes an increase in levels of malondialdehyde (MDA) (AbdElgawad et al., 2016), which is the first product formed during free radical-induced damage and the decomposition of polyunsaturated fatty acids in membranes (Chutipaijit et al., 2011). The production of anthocyanins, which also act as antioxidants, increases in the presence of salt stress as these play a similar protectory role to proline, while serving as signal molecules activating downstream stress-responsive pathways (Chunthaburee et al., 2016; Wahid and Ghazanfar, 2006). Anthocyanins are also suggested to play roles in quenching ROS, photoprotection and xenohormesis (Kovinich et al., 2015). Elevated levels of anthocyanins in *A. thaliana* have been shown to enhance salinity tolerance (Oh et al., 2011).

The model organism *A. thaliana* can only tolerate moderate concentrations of up to 50 mM NaCl (Sanders, 2000), but has been pivotal in unravelling the Salt Overly Sensitive (SOS) signalling pathway which is involved in salt stress, and which was also the first abiotic stress-signalling pathway elucidated in plants (Ji et al., 2013; Zhu, 2000, 2016). Three membrane transporters, AtSOS1, AtHKT1 and AtNHX1 are critical Na^+ carriers which reduce salt toxicity in plants (Zhang and Shi, 2013). AtSOS1 and AtHKT1 are suggested to mediate Na^+ transport, control ion uptake and spatial distribution of Na^+ and K^+ by regulating the expression levels of relevant Na^+ and K^+ transporter genes (Wang et al., 2019). These membrane transporters remove Na^+ from the cytoplasm by transporting it into the vacuole or out of the cell (Qiu et al., 2002). The signalling pathway involves a salt-elicited Ca^{2+} signal in the cytosol, where a myristoylated calcium binding protein, SOS3, which by itself has no enzymatic activity, activates a serine/threonine protein kinase, SOS2 (Gong et al., 2004). The SOS3/SOS2 complex subsequently phosphorylates and activates SOS1 (Zhu, 2002, 2016), a Na^+/H^+ antiporter located at the plasma membrane, which exports Na^+ out of the cytosol into the apoplastic space (Blumwald et al., 2000; Liu et al., 2007; Qiu et al., 2002; Shi et al., 2002). AtSOS1 transcripts can be stabilised by plasma membrane-localized NAPDH oxidase-generated ROS. This occurs via a positive feedback loop in which SOS1 is required for maintenance and activation of NAPDH oxidase activity. The ROS generated by NAPDH oxidase activity then stabilise *SOS1* mRNA, which increases SOS1 activity as well as NADPH activity (Chung et al., 2008). This pathway is highly conserved in plants, as previously reviewed (Isayenkov and Maathuis, 2019; Ji et al., 2013). Another study also suggested the involvement of an endoplasmic reticulum (ER) membrane-associated transcription factor AtbZIP17 targeted by a subtilisin-like serine protease, AtS1P. AtbZIP17 functions as a stress transducer (Liu et al., 2007). In conjunction with another transcription factor, AtbZIP28, AtZIP17 up-regulates expression of several different sets of genes suggested to mitigate stress (Liu et al., 2008).

Independently of ABA signalling, salt stress upregulates the expression of the *AtWRKY8* transcription factor (Hu et al., 2013) which directly binds to the *AtRD29A* promoter, leading to the gene's upregulation (Rao et al., 2016). NaCl-stress also strongly induces *AtSOT12*, which codes for a sulfotransferase that is also implicated in pathogen resistance via salicylic acid signalling (Baek et al., 2010). During the initial phase of salt shock in *Oryza sativa* (rice), approximately 10% of the transcripts were significantly differentially expressed (Kawasaki et al., 2007). In *A. thaliana* root tissue, a microarray study revealed that 150 mM salt stress affected the expression of more than 20% of the genome, which included transcription factors, effectors of homeostasis, kinases/phosphatases and hormone-related genes. (Jiang and Deyholos, 2006). In a proteomic study by the same authors, 86 proteins were differentially synthesised in the roots of *A. thaliana* under saline conditions. These results correlated poorly to the previous microarray study, suggesting that post-transcriptional regulation plays a pivotal role in stress-responsive gene expression (Jiang et al., 2007). An RNA-seq study of citrus roots revealed that 1831 genes were differentially expressed under salt stress conditions, which included a multitude of transcription factors and genes involved in hormone metabolism and signalling (Xie et al., 2018).

1.2 Sustainability

Plants rarely experience a single environmental stress (Ahanger et al., 2017), emphasizing the fact that a single approach to overcoming an environmental stressor is not often a viable solution. Scientists are constantly looking for novel, environmentally sustainable methods to improve and accelerate plant growth in

order the meet the growing demands of a hungry and insatiate world. The philosophies of ‘’producing more at all costs’’ or reducing the input while maintaining production levels, loosely termed as ‘’agricultural intensification’’ (Struik and Kuyper, 2017), will have to make way for more sustainable agriculture if we are to keep up with the demand while protecting and preserving the global environment.

The five pillars of sustainable agriculture, as outlined in a review paper by Khwidzhili and Worth (2016), are biological productivity, economic viability, protection of natural resources, reduced levels of risk and social acceptance. This concept is also known as sustainable intensification (SI) (Pretty, 1997), which is needed to increase agricultural produce while simultaneously conserving and protecting the environment and planet (Pretty and Bharucha, 2014). There are numerous traditional and modern methods that can be employed to stimulate and improve plant growth and health in efforts to increase stress tolerance, overall yield, end-product quality and shelf/storage-life. These include an increase in use of agrochemicals, fertilizers and biotechnological techniques such as selective breeding and genetic modification (Borlaug, 2002; Fedoroff, 2010).

Agriculture has improved and developed, but not in line with the rapid increase in population and life expectancy as a result of improved medical technology, increases in birth-rates globally and greater life expectancy (Bongaarts, 2009). Massive strides have been made, for example, since 1992, the number of undernourished people in the world has declined by 21.4%, even whilst the average amount of cropland per capita decreased from 0.5 ha to 0.23 ha (Kanianska, 2016). This is mainly due to the Green Revolution that started in the 1960s (Borlaug, 1971; Pingali, 2012).

1.2.1 The Green Revolution

Traditional plant growth promoting strategies involve using chemical plant protectors commonly known as herbicides and pesticides, as well as soil manipulation and conditioning, either by the addition of compost, biochar (Gurwick et al., 2013) or fertilizers. Supplementing the soil with nutrients can improve plant growth and subsequent yield as it enriches the rhizosphere of the plants directly adjacent to the roots. There is a positive correlation between the availability of macronutrients such as nitrogen (N), phosphorus (P) and potassium (K) and the growth rate and yield of plants (White and Brown, 2010).

Preceding the work of Darwin and Mendel (Fairbanks and Abbott, 2016; Gayon, 2016; Walker and Blanc, 1906), plant and animal breeding has been around for at least 10 000 years, in parallel with the start of human civilizations (Hallauer, 2011). The findings of these two iconic scientists, however, represents the starting point for modifying and enhancing the genetic potential of crops for the future (Miflin, 2000) and the unprecedented improvement in crop yields observable over the last few centuries (Bennett, 2010).

The scientist who eventually integrated the principles of Darwin and Mendel’s work was Norman Borlaug (Borlaug, 1971). His work, especially in third-world countries, led to a period known as the Green Revolution (Pingali, 2012) which markedly increased the production of, but not limited to, wheat, maize and rice in developing countries on the Southern American and Asian continents and, most recently, Africa (Figure 1.1). Developing countries on the Asian, South American, and African continents benefitted the most from the Green revolution, a period which started in the 1960s (Evenson and Gollin, 2003).

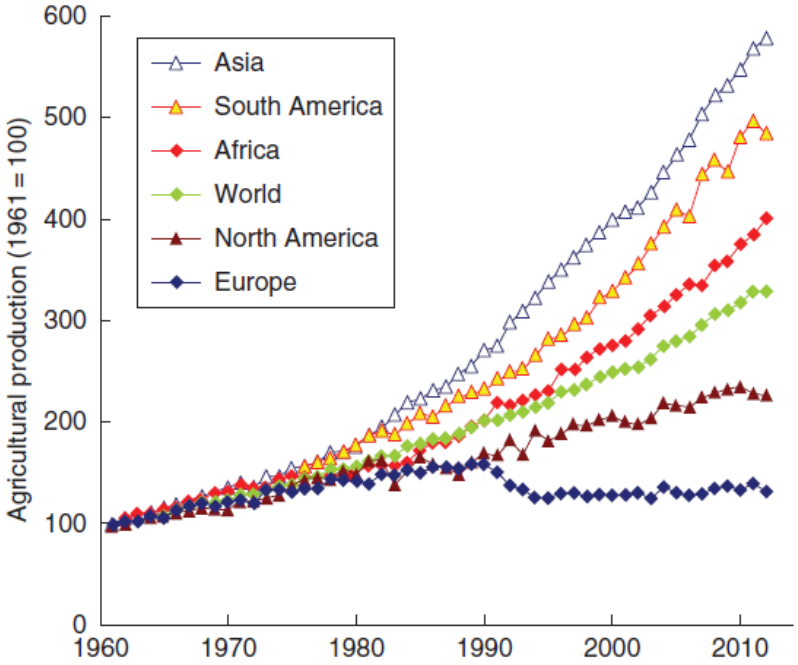

Figure 1.1 Relative changes in net tonnes of food produced between 1961 and 2012 across the world (Pretty and Bharucha, 2014).

The Green Revolution happened as a result of both increased agrochemical usage and by applying genetic knowledge to create improved varieties, via selective breeding, that are able to channel the nutrients towards greater seed production instead of other biomass (Pinstrup-Andersen and Hazell, 1985). ‘’Dwarfing traits’’ were introduced into the plants, which affected the production and action of gibberellin (GA) plant hormones. This decrease in GA and GA signalling resulted in plants with reduced height as nutrients were channelled towards yield rather than plant height, which also reduced the lodging of plants that resulted in yield loss (Hedden, 2003). This led to reduced photoperiodicity and shorter growing periods (Pinstrup-Andersen and Hazell, 1985) which ultimately increased food production. A trademark of the Green Revolution was that it was able to increase the food supply without increasing the amount of farmland required (Taiz, 2013).

Plant breeders have capitalised on the advances made in genetics and entire genome sequencing (Joosen et al., 2009; Varshney et al., 2009). Genome sequences have provided researchers access to the genetic code of the sought-after phenotypic traits humans have been trying to bring out in crops for centuries. By 2017, the genomes of 236 angiosperm species had been fully sequenced and made publicly available (Chen et al., 2018), with several others in the process of sequencing and assembly. This has led to the creation of large-scale DNA marker-trait associations (Collard and Mackill, 2008) which have the potential to accelerate breeding in order to create more sought-after crop varieties. However, the source for better traits within a certain species is limited since the genomic potential has been pushed to the brink. In conjunction with our lack of knowledge and limited diversity, we have also destroyed thousands of plant species, as there is a negative correlation between human presence and plant species richness (Pautasso, 2007). Therefore, possible sources of genome potential have already been destroyed.

1.2.2 Plant transgenic technology: Genetic Engineering and Modification

Although selective breeding and artificial selection can technically be regarded as genetic modification (GM), GM is generally defined as the modification of the genetic material of an organism by inserting genes (DNA) from another source (organism) which do not occur naturally within the genome of the organism. The first GM plants were antibiotic resistant tobacco and petunia, created in 1983 (reviewed by Zhang, Wohlhueter and Zhang, 2016). Since then, plant transgenic technology has been demonstrated to have great potential for crop improvement (Jhansi Rani and Usha, 2013; Kumar et al., 2011) and became commercially available several years later (Christou, 1996). Between 1996 and 2016, commercially planted transgenic crops increased from 1.7 to 178 million ha (Brookes and Barfoot, 2018). The most sought-after commercially available GM traits are herbicide tolerance and insect resistance, but other available GM varieties include drought tolerance, altered growth, modified product quality and pollination control (Brookes and Barfoot, 2018) (http://www.isaaa.org/gmapprovaldatabase/commercialtraitlist/default.asp).

Transgenic technology, despite having great potential for crop improvement, is expensive, laborious and subjected to several regulations (Sareen et al., 2014). Plant genomes are complex and stable T-DNA insertions events are not always guaranteed (Jupe et al., 2019). A considerable hurdle for transgenic technology is public perception and ignorance, as consumers are leaning towards organic/natural produce because they fear the term ‘GMO’ (Buiatti et al., 2013). With the addition of CRISPR-Cas9 technology to the biotechnology and genome engineering toolbox, the possibilities have now been drastically increased, but this system also carries its own set of limitations (Wilson et al., 2018). Since there is no transfer of genes from other organisms, CRISPR-modified organisms are not classified as GM (Shew et al., 2018) which would circumvent the public acceptance hurdle. However, just like transgenic technology, CRISPR also has the potential to be used as a tool to improve plant growth and production and is not a stand-alone solution for the growing need to increase agricultural output. It holds great potential to be used in conjunction with other tools and methods as described in this review.

1.2.3 We may have exhausted the genome potential of important crops

The multifaceted agricultural challenges we are facing need to be addressed through multifaceted solutions. Ideally, combinations of plant growth promoting strategies, both traditional and modern, can be implemented together for an overall positive effect on plant growth and, ultimately, total yield. A combination of conventional technology and biotechnology is needed for optimal output (Borlaug, 2002).

As mentioned, the two most successful methods to increase plant growth are an increase in use of agrochemicals and selective breeding. This ultimately led to the Green Revolution (Pingali, 2012) which resulted in a massive increase in crop production and yield. However, commercial soil is being oversaturated with nutrients and pesticides, leading to over-fertilisation and toxic soils. Over-fertilising with nitrogen adversely affects soil microbial life (Geisseler and Scow, 2014; Singh, 2018). This changes the soil pH, resulting in the non-availability of micronutrients to the plant (Kashem and Singh, 2002). Excess nutrients or poor irrigation practices leads to nutrients leaching into groundwater, which ends up in fresh-water rivers and washes out to the ocean. In the rivers, these nutrients cause havoc as they stimulate water-plants and algae to grow. This, in turn, suffocates the water of oxygen after the plants die and rot, leading to dead fish, and, ultimately, rotten water. This phenomenon is known as eutrophication (Yang et al., 2008).

Selective breeding is an established and successful way of increasing crop yield, but it can be time-consuming, expensive, skill-demanding and still relatively limited in terms of output, even with recent advances (Witcombe et al., 2013). After 10 000 years of artificial selection followed by a few decades of more targeted breeding, we are currently probably in possession of the best varieties possible, based on the available genetic resources.

1.2.4 Sustainable intensification has a time constraint

In a rapidly growing world population with limited resources, time is running out and knowledge gained in agrisciences is not keeping up with medical technology and the exponentially growing world population. Another factor to keep in mind is that environmental conditions are deteriorating for the worse, with nutrient-poor land, increasing temperatures and unpredictable climate changes and fluctuations putting a lot of pressure on agriculture (Drake and Griffen, 2010). Since expanding farmland is not a viable option (Taiz, 2013), current agricultural productivity needs to be improved with minimal impact on the environment. The need for SI is necessary for global food security while protecting the environment (Pretty and Bharucha, 2014). Since it is a rather new concept, it is still relatively undefined, and experts are divided as to what exactly it entails.. While to some SI means to only make marginal changes to the existing system in order to increase food production, others think it is to alter an agricultural system causing environmental damage which would continue to leave close to a billion people malnourished (Petersen and Snapp, 2015). Others are of the opinion that SI is also just a component of what is needed and is not synonymous with food security (Garnett et al., 2013). This SI approach can possibly be aided by the usage of plant biostimulants (PBs), since PBs are inexpensive to produce, are of natural/organic origin and do not adversely affect the environment like chemically-based agrochemicals do.

1.2.5 Plant Biostimulants (PBs) as another tool in the toolkit

The previously described methods of plant growth promotion have been responsible for the relatively high crop yields to date, most notably the Green Revolution (Pingali, 2012). However, consistent increased pressure on the currently available resources means that we are in dire need of another green revolution or something similar. A relatively novel method of improving plant growth is through the application of a PB, either via a foliar spray, directly to the soil/rhizosphere, or by treating seeds prior to germination (Yakhin et al., 2017).

1.3 Plant Biostimulants - Turning on a metabolic switch

A PB can be defined as a substance applied at low concentrations that does not provide the plant with nutrients or direct protection against external stress, but rather stimulates the plant's endogenous metabolism to modify its physiological processes (du Jardin, 2015, 2012). The earliest mention of this concept, known as a 'biogenic stimulant', was discussed in 1993 (reviewed by Yakhin *et al.*, 2017). PBs promote plant growth in minute quantities (Zhang and Schmidt 1997). Although it is generally accepted that the term 'biostimulant' was originally coined and defined in 1997 by a web journal (http://grounds-mag.com, reviewed by du Jardin, 2015) and only appeared in a peer-reviewed paper describing the alleviating effects of a PB on perennial ryegrass (*Lolium perenne* L.) in 2007 (Kauffman et al., 2007), the term ''plant

biostimulant'' was actually used twice before this, in papers on *Zea Mays* L. (Ohlrogge, 1977) and *S. lycopersicum* (Castro et al., 1988).

There is still no consensus on the definition of a PB, but the US FDA published the following: *"[A] substance or micro-organism that, when applied to seeds, plants, or the rhizosphere, stimulates natural processes to enhance or benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, or crop quality and yield."*. Since this definition could still include general fertilizers and crop protectors, the European Biostimulants Industry Council (EBIC) added two further parts to the definition (www.biostimulants.eu/):

1. *Regardless of nutrient presence, PBs operate through different mechanisms than fertilizers.*
2. *They do not act directly against pests or disease, but rather affect the plant's vigour, therefore differing from crop protection products.*

The most recent definition of a PB is the following:
''*A material which contains substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and/or crop quality, independently of its nutrient content.*'' (Ricci et al., 2019).

In a recent review, the challenge in defining PBs was highlighted and the following definition was proposed: ''*A formulated product of biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds*'' (Yakhin et al., 2017). However, this definition excludes synthetic PBs. PBs have also been referred to as bioeffectors (Van Oosten et al., 2017), metabolic enhancers, phytostimulators (Yakhin et al., 2017) or elicitors (Le Mire et al., 2016). An elicitor is a PB with a biotic origin (Alvarez-Arquieta et al., 2017; du Jardin, 2015). The term plant biostimulants (PBs) will be used for the purpose of this review, regardless of its origin or mode of action.

PBs have a broad effect on plants, including stimulating plant growth by increasing plant metabolism, enhancing photosynthesis, stimulating germination and increasing the absorption of nutrients from the soil (Yakhin et al., 2017). PBs may also mitigate stress in plants, with the alleviation of abiotic stress by PBs being frequently cited (Blaszczak et al., 2016; Bulgari et al., 2019; Guinan et al., 2013; Sharma et al., 2014; Pushp S. Shukla et al., 2018; Trevisan et al., 2019; Van Oosten et al., 2017; Yamauchi, 2018).

1.3.1 The characterisation of PBs

PBs can be categorised into several individual groups based on their source. This includes humic and fulvic acids, beneficial bacteria and fungi, protein hydrolysates, seaweed extracts and botanicals, biopolymers such as chitosan, and inorganic compounds (du Jardin, 2012). These diverse PBs each stimulate different aspects of plant growth, but a vast overlap of effects has been reported. These effects include increased defence against abiotic and biotic stress (Trevisan et al., 2019) and increased uptake of nutrients (Desoky et al., 2018; Halpern et al., 2015). Nutrient use efficiency in terms of nutrient mobilization, uptake from the soil, transport, storage and assimilation can also be improved by PBs (du Jardin, 2015). Furthermore, PBs improved crop quality and yield and exhibited phytohormone-like activity (Colla et al., 2014). The stimulatory effect is usually holistic, improving several aspects of plant growth and health

simultaneously. PBs, or rather the elucidation of their effects on plants, are a rather novel field of research and are still poorly understood. Due to their holistic effects on the plant, it is difficult and expensive to analyse the effects of PB treatments. Because of this lack of fundamental research into their active ingredients and modes of action, it is also challenging to classify the products (Fleming et al., 2019). However, the number of publications focussing on PBs is growing exponentially, with interest on the rise, particularly over the last decade (Rouphael and Colla, 2018). For this review, PBs will be characterized into six groups, namely microorganisms, humic and fulvic acids, seaweed-derived, plant- and animal-derived PBs and inorganic compounds. As evident from the tables in the below sections, biostimulants often elicit a very broad effect on plant growth. Even those that are well characterised and tested on several plant species have shown to affect multiple metabolic pathways. Therefore, it is likely that not just one or even two mechanisms are involved in their mode of action.

1.3.1.1 Microorganisms, humic and fulvic acids as the first known PBs

Two of the first known PBs, although not defined as such at the time, were arbuscular mycorrhizal fungi (AMF) (Rouphael et al., 2015) and plant growth-promoting bacteria (PGPB) (Hayat et al., 2010; Ruzzi and Aroca, 2015), collectively known as plant growth-promoting microbes (PGPM). Supplementing the soil with these beneficial fungi and bacteria is a method routinely used by farmers (de Souza et al., 2015). The AMF-plant relationships aid the plants by affecting soil structure, improving nutrient uptake, increasing resistance to abiotic and biotic stresses, and increasing overall plant health (Maksimov et al., 2011). The PGPB, in turn, aid the plant with phosphate solubilization, nitrogen fixation, nutrient uptake and alleviation from stress (reviewed by de Souza, Ambrosini and Passaglia, 2015). Other effects reported also include pathogen exclusion or suppression (Köhl et al., 2019). *Trichoderma* species are present in more than 60% of all biofungicides (Mukherjee et al., 2012), which illustrated an increase in bioactivity which kills and suppresses non-beneficial fungi (López-Bucio et al., 2015; Verma et al., 2007). It is well-documented that AMF-relationships can induce systemic resistance to disease and pest attack in crops, also known as mycorrhiza-induced resistance' (MIR) (Cameron et al., 2013; Woo and Pepe, 2018).Beneficial effects on growth of a number of plant species have been reported for a variety of different microorganism-based PBs (Table 1.1).

Humic and fulvic acids are the principal components of soil organic matter (Klucáková 2018). They are closely related, but fulvic acids differ from humic acids with regards to the degree of polymerization and their carbon and oxygen content. Both have been reported to elicit a wide variety of responses in plants (Table 1.1). Known effects include a general increase in plant growth and health, as well as improved responses to environmental stress (Canellas et al., 2015).

1.3.1.2 Seaweed-derived PBs

Seaweeds, or derivatives thereof, are well-studied in term of their effects on different plant species in comparison to other PBs and have been used in agriculture for centuries (reviewed by Stirk, 2006). Seaweed-derived PBs are economically viable because cultivating them requires no fresh water, pesticides, fertiliser or energy inputs (https://www.futurefarming.com/Smart-farmers/Articles/2019/12/Seaweed-based-biostimulants-to-enhance-productivity-511049E/). Due to the well-studied nature of seaweeds, broad effects

have been reported including basic stimulation of plant growth, increased resistance to abiotic stress and pathogenic resistance (Shukla et al., 2019). Some of the available products and their effects in plants have been extensively reviewed recently (Sharma et al., 2014), however, a brief list of examples is presented in Table 1.2. *Ascophyllum nodosum* is the main species that is exploited and is present in most of the commercially available products.

1.3.1.3 Plant-derived PBs

Similar to seaweed extracts, plant-derived PBs are also well-studied and similarly broad effects on plant growth and resistance to environmental stresses have been reported in a variety of model and non-model plant species (Table 1.3). Plant-derived PBs, often containing high concentrations of amino acids and micronutrients, have phytohormone activity and have been shown to specifically target plant growth promotion to increase sustainable food production in the agricultural sector (Zulfiqar et al., 2019). More specifically, these PBs can protect plants against abiotic stress (freezing, salinity, drought, osmotic) while accelerating plant growth and improve nutrient use efficiency (Posmyk and Szafrańska, 2016). Furthermore, certain plant-derived PBs mitigate pathogenic responses in plants (Bargiacchi et al., 2012; Gavelienė et al., 2018). In a recent study, the potential lack of consistency in the formulation of biostimulants, particularly those containing plant extracts, was highlighted and an enzyme-assisted extraction method was proposed (EL Boukhari et al., 2020). However, even if the extraction method is consistent to maintain quality and consistency and the exact ratio of each ingredient (Povero et al., 2016), the environment from which the plant material is obtained will never be, which could significantly affect the type and quantity of metabolites extracted from that plant material. It is therefore important that each new batch of products should be analysed in terms of active compound content such as amino acids and phytohormones. Several analytic techniques are available which should be used to compare the content of different batches of biostimulants (Sharma et al., 2016). This applies to seaweed-based biostimulants as well.

1.3.1.4 Animal-derived PBs

Animal-derived PBs are used to a lesser extent in agriculture due to some studies reporting adverse effects. The application of some of these PBs resulted in chlorosis, growth depression and some phytotoxic effects, most likely due to an imbalance in amino acid composition and high salinity content (reviewed by Colla *et al.*, 2015). There are also some concerns regarding the safety and ethical considerations of this group of PBs (Madende and Hayes, 2020). Regardless of these concerns, certain animal-derived PBs have been shown to increase tomato fruit yield, contribute to an overall increase in plant growth and also increase resistance to drought and pathogens in other plant species (Table 1.4). The contents of animal-derived PBs are somewhat similar to those of plant-derived PBs in that they also contain amino acids or peptides, vitamins, carbohydrates and other biologically active elements (reviewed by Baroccio et al., 2017).

1.3.1.5 Inorganic compounds, synthetics, uncategorised and undefined

The classification of PBs is challenging and they can be categorised either by their source or by mode of action. But since many have not been tested with regards to all physiological effects and/or stress defence, categorising by source is mostly used. Inorganic and synthetic PBs do not have a biological source and therefore fall under a different category. They also display similar PB effects as described above, with

some of these PBs being combined with organic matter, proteins, amino acids and other biologically-derived components. The reported effects include increased resistance to freezing, a protectory effect against Cd^{2+}-stress, enhanced water efficiency and inducing the production of metabolites providing defence against possible pathogens (Table 1.5).

As previously mentioned, broad increases in growth and beneficial affects observed in plants treated with PBs are often reported. Additionally, possible discrepancies in the consistency of the composition of specific PBs also makes it challenging to attribute specific mechanisms to their mode of action.

Table 1.1 Humic acids, fulvic acids and microorganisms and their biostimulatory effects on several plant species.

Plant Biostimulant Description and/or name	Effect	Plant species tested on	Alleviating effect towards abiotic or biotic stress?		References and further information
			Yes/No	Type	
Organic matter, polysaccharides, peptides and amino acids, vitamin complex and chelated zinc (Radifarm®)	Used to aid transplanting	*Begonia semperflorens*	n/a	n/a	Koleška et al., 2017
Humic acid	Alleviated salt and temperature stress and stimulate seed germination	parsley, celery, leek, tomato, lettuce, basil, radish and garden seed	Yes	Salinity stress, heat stress	Yildirim et al., 2002
Organic matter, polysaccharides, peptides and amino acids, vitamin complex and humic acids	Decreased SOD and POD activity in the leaves when nitrogen, phosphorus and potassium (NPK) nutrition was reduced by 40%	Tomato (*S. lycopersicum*)	Yes	Nutrient stress	Koleška et al., 2017
A suspension–solution containing humic and fulvic acids, obtained from compost of worm (Radicon®)	Effectively reduced the infestation of the root parasite *Phelipanche ramose* while increasing the yield	Tomato (*S. lycopersicum*)	Yes	Biotic; root parasite	Disciglio et al., 2016
Arbuscular mychorrhizal fungi (AMF)	AMF-plant relationships aid the plants by affecting soil structure, improving nutrient uptake, increasing resistance to abiotic and biotic stresses and increasing overall plant health AMF can also induce systemic resistance to disease and pest attack in crops, also known as mycorrhiza-induced resistance' (MIR)	Tomato (*S. lycopersicum*) and several others as reviewed by Maksimov et al., 2011	Yes	Abiotic; Biotic (MIR)	Rouphael et al., 2015; Maksimov et al., 2011; Cameron et al., 2013; Woo and Pepe, 2018

Tricooderma	Increase in bioactivity which kills and suppresses non-beneficial fungi	Several including maize and tomato	Yes	Biotic, antifungal	López-Bucio et al., 2015 Verma et al., 2007 Woo and Pepe, 2018
Plant growth-promoting bacteria (PGPB)	Aid the plant with phosphate solubilization, nitrogen fixation, nutrient uptake and alleviation from stress Enhance crop tolerance to drought and salinity by reducing soil levels of 1-amino cyclopropane-1-carboxylate deaminase (ACC) and pollutants such as heavy metal detoxification, pesticides and herbicides	Several (including wheat)	Yes	Drought, salinity	reviewed by de Souza, Ambrosini and Passaglia, 2015 Nguyen et al., 2017 Le Mire et al., 2016 Upadhyay and Singh, 2015
Chitosan; salicylic acid-chitosan nanoparticle	Induces pathogen resistance, abiotic stress resistance while also stimulating plant growth as a whole Induced antifungal and growth promoting activities	*Thymus daenensis* Coffee Basil	Yes	Drought, Biotic	Bistgani et al., 2017 Pichyangkura and Chadchawan, 2015Pirbalouti et al., 2017 Kumaraswamy et al., 2019
Proprietary fermentation metabolites (CYT31)	Triggered the upregulation of genes relating to the ROS scavenging system. It also activated transcription factors (TF) involved in drought resilience	*A. thaliana*	Yes	Drought stress	Blaszczak et al., 2016

Table 1.2 Seaweeds -extracts, microalgae and other bioactive marine-substances as a major group of plant biostimulants and their positive effects on the growth and stress responses in different plant species.

Plant Biostimulant Description and/or name	Effect	Plant species tested on	Alleviating effect towards abiotic or biotic stress? Yes/No	 Type	References and further information
(Fylloton)	Increased photosynthesis in. Stimulate growth and yield in soybean	*Dracocephalum moldavica* L Soybean	n/a	n/a	Kocira 2015 Kocira 2019
Ascophyllum nodosum-derived extract (BiozymeTM)	Increase overall growth Enhance freezing tolerance by reducing expression of chlorophyllase genes while modulating the expression of cold responsive genes such as *COR15A*, *RD29A* and *CBF3* involved in protecting membrane integrity Improve plant growth in NaCl-stressed plants	*A. thaliana* Soybean	Yes	Freezing stress, Salt stress, Drought	Tandon and Dubey, 2015 Rayirath et al., 2009 Jithesh et al., 2018 Fleming et al., 2019
Stimplex® (alkaline extract of A. nodosum)	Alleviate drought stress	*Citrus sinensis* L.	Yes	Drought	Spann and Little, 2010
Seaweed-sap derived from *Kappaphycus* and *Gracilaria*	Increased grain yield by more than 10%, carbohydrate content by 17.4% and protein content by 4.8%	*Zea mays* L	n/a	n/a	Layek et al., 2015
Contains polyuronic components from sea algae (Bio-algeen S 90)	Increased leaf number, plant height, total yield and vitamin C content	Lettuce (*Lactuca sativa*)	n/a	n/a	Dudaš et al., 2016
Formulation of PGPB and fresh water algae	Increased fresh weight by up to 20% while increasing the antioxidant capacity and total carotenoid content	Lettuce (*Lactuca sativa*)	n/a	n/a	Kopta et al., 2018
Ecklonia maxima-derived (Kelpak®)	Increase leaf and shoot fresh weight	Swiss chard	n/a	n/a	Arthur et al., 2013

Seaweed and black peat (AZAL5 and HA7)	increasing biomass and chloroplast numbers and increased the concentrations of Na, Mn, Cu and Mg while increasing expression of a Cu transporter (*COPT2*) and S transporters (*SULTR1.1* and *SULTR1.2*)	Winter oilseed rape	n/a	n/a	Billard et al., 2014
Algreen [(fresh seaweed (*Sargassum* sp., *Ascophyllum nodosum*, *Laminaria* sp.) extract containing vitamins, alginates, free amino acids and hormones]	Enhanced growth, fruit quality and yield	Strawberry (cv. Sweet Charlie)	n/a	n/a	El-Miniawy et al., 2014
Algal polysaccharides	Activate several plant defence mechanisms	Several including tomato, green bean, wheat and rice	Yes	Abiotic, Biotic	reviewed by Stadnik and de Freitas, 2014
Algae extract laminarin, and benzo-(1, 2, 3)-thiadiazole- 7-carbothioic acid S-methyl ester (BION®)	Induced the expression of defence genes against fungal pathogens and increased their resistance to infections	*A. thaliana* and several others	Yes	Biotic, antifungal	Vergnes et al., 2014 Le Mire et al., 2016)
Combination of two marine bioactive substances NA9158 and EXT1116	Increase in root biomass and ammonium absorption	Grapevine	n/a	n/a	Mugnai et al., 2008
Dunaliella salina exopolysaccharides	Alleviated salt stress in tomato plants	Tomato (*S. lycopersicum*)	Yes	Salt stress	Arroussi et al., 2018

Table 1.3 The source, description and general bio stimulatory effects of plant extracts, raw plants extracts and protein-based protein hydrolysates from plant material on plants.

Plant Biostimulant Description and/or name	Effect	Plant species tested on	Alleviating effect towards abiotic or biotic stress? Yes/No	 Type	References and further information
Soy protein-based protein hydrolysate	Increased fresh weight, dry weight, leaf area, plant height, chlorophyll and nitrogen	Broccoli	n/a	n/a	Amirkhani et al., 2016
Boraga officinalis raw extracts	Increased photosynthetic pigments in the leaves, photosynthetic activity, fresh weight, total flavonoids and phenols and protein levels	Lettuce (*Lactuca sativa*)	n/a	n/a	Bulgari et al., 2017
Aqueous garlic extract	Improved root growth and development and photosynthetic pigments	Eggplant and pepper	n/a	n/a	Hayat et al., 2018
Sunflower seed-based protein hydrolysate	Displayed auxin-like activity	Garden cress (*Lepidium sativum* L) and lettuce (*Lactuca sativa*)	n/a	n/a	Ugolini et al., 2015
Oak extract	Enhanced grape composition and produced less acidic wines with more stable colour and intensity as well as a higher content of polyphenols imported in wine quality	*Vitis vinifera*	n/a	n/a	Pardo-García et al., 2014
Alfalfa protein hydrolysate	Effect on the tricarboxylic acid (TCA) cycle by enhancing plant growth and sugar accumulation, while simultaneously inducing the expression of asparagine (AS) in the roots	*Zea mays* L	n/a	n/a	Schiavon et al., 2008
Protein hydrolysate (APR®)	Increased root dry mass while inducing the expression of	*Zea mays* L	n/a	n/a	Trevisan et al., 2017

	genes coding for enzymes involved in cell wall biosynthesis and remodelling suppressed the expression of genes involved in actin and microtubule polymerization or depolymerisation				
Glycine betaine, acetyl-thioproline, folic acid, seaweed and other plant extracts (FOLICIST®)	Improved seed germination and radicle extension	Wheat and *Zea mays* L	n/a	n/a	Ziosi et al., 2012
Free amino acids-based (Ruter AA, Terra Sorb, Razormin)	Increased the freezing tolerance of seedlings Bioprotective effect against pathogenic microcytes (Terra Sorb)	Winter rapeseed Wheat and chickpea	Yes	Freezing tolerance, Pathogenic resistance	Gavelienė et al., 2018
Moringa oleifera leaf extracts	Alleviated drought stress	Squash (*Cucurbita pepo* L.)	Yes	Drought tolerance	Abd El-Mageed et al., 2017
Auxins, cytokinins, gibberellic acid, seaweed extract, hydrolysed proteins and trace elements (spic cytozyme)	Reduced fruit cracking and increased fruit length, diameter, weight and volume	Pomegranate (*Punica granatum* L. cv. Kandhari kabuli)	Yes	Water stress	Abubakar et al., 2013
Beetroot and seaweed extract with added urea, glucose, amino acids, sodium hydroxide and citric acid (SUNRED)	Stimulated the production of anthocyanin in grape skins	*Vitis vinifera*	n/a	n/a	Deng et al., 2019
Raw plant extracts (VAL-P01 and VAL-P02)	Induced genes related to ABA and osmotic stress treatment; significantly altered the transcription of senescence genes	*A. thaliana*	Yes	Osmotic stress	Santaniello et al., 2012

Mineral elements, amino acids, vitamins and phytohormone-like substances (EXPANDO®)	Induced the expression of ROS enzyme (SOD, POX and CAT) gene expression	Tomato (*S. lycopersicum*)	Yes	Osmotic stress	Contartese et al., 2016
Complex of vitamins, aminoacids, proteins and betaines (Megafol®)	Increased biomass and improved chlorophyll fluorescence in plants subjected to drought-stress	*Solanum lycopersicum*	Yes	Drought	Petrozza et al., 2014
Sweet chestnut raw extracts	Positively affected plant growth while enhancing plant resistance to pathogenic nematodes in tobacco plants	Tobacco (*Nicotiana tabacum*	Yes	Biotic, pathogen	Bargiacchi et al., 2013
Quick-link (vegetal-based biopolymer containing amino acids and peptides)	Triggered the accumulation of metabolites involved in defence mechanisms such as carotenoids, flavonoids and glucosinolates in fruits Enhances adventitious rooting via brassinosteroid-mediated processes	Basil, tomato, *Chrysanthemum*, *Cucumis melo* L.	Yes	Abiotic and biotic	Kim et al., 2019 Rouphael et al., 2018a
Cycoflow (plant and yeast extracts containing amino acids and boro, zinc and manganese)	Increased ascorbic acid content and overall plant growth	*Solanum lycopersicum*	Yes	Heat stress	Francesca et al., 2020
Amino acids from plant extracts (AminoPrim, AminoHort)	Improved yield and grain quality	*Triticum aestivum* L. (winter wheat)	n/a	n/a	Popko et al., 2018

Table 1.4 Animal-derived and animal protein hydrolysates as a lesser explored and defined category of plant biostimulants.

Plant Biostimulant Description and/or name	Effect	Plant species tested on	Alleviating effect towards abiotic or biotic stress?		References and further information
			Yes /No	Type	
Animal protein hydrolysate	Enhanced plant shoot number, ground dry weight, root morphology, N-content in leaf and shoots and gas exchange	Snapdragon (*Antirrhinum majus* L.)	n/a	n/a	Christiano et al., 2018
Shrimp extract	Altered the soil's functional microbial community while also increasing its nutrient contents	*Chrysanthenum*	n/a	n/a	Ji et al., 2017
Chitosan; Salicylic acid-chitosan nanoparticle	Induced pathogen resistance, abiotic stress resistance while also stimulating plant growth as a whole; induced antifungal and growth promoting activities	*Thymus daenensis* Coffee Basil	Yes	Drought, pathogen resistance	Bistgani et al., 2017 Dzung et al., 2011 Pichyangkura and Chadchawan, 2015 Pirbalouti et al., 2017 Kumaraswamy et al., 2019
Pepton	Increased yield	Gold cherry tomatoes	n/a	n/a	Polo and Mata, 2018
Meat hydrolysate	Enhanced plant growth and microelement concentrations while decreasing NO^{-3}, PO^{3-4} and $SO2^{-4}$ concentrations	*Zea mays* L	n/a	n/a	Ertani et al., 2013
Fish protein hydrolysates	Increase plant's resistance to insects and other environmental stimuli	Several fruit and vegetable species	Yes	Insects, heat stress, drought stress	Reviewed by Madende and Hayes, 2020

Table 1.5 Plant biostimulants not derived from a biotic/natural source but from inorganic compounds, synthetics, uncategorised and undefined sources.

Plant Biostimulant Description and/or name	Effect	Plant species tested on	Alleviating effect towards abiotic or biotic stress?		References and further information
			Yes /No	Type	
Inorganic nitrogen and polysaccharides (Erger®)	Break the bud dormancy in warmer geographical areas	*Actinidia deliciosa*	n/a	n/a	Hoeberichts et al., 2017
Combination of sodium para-nitrophenolate PNP, sodium orthonitrophenolate and sodium 5-nitroguaiacolate (Asahi SL)	A foliar spray accelerated adaptation to chilling by decreasing electrolyte leakage from leaf tissues	Coriander (*Coriandrum sativum*) *A. thaliana*	Yes	Freezing/chilling; Protectory effect against Cd^{2+}-stress	Pokluda 2016 Przybysz et al., 2016
Low doses of sodium selenite, glycine betaine and a spray adjuvant	Enhanced water efficiency	Cabbage and cauliflower	Yes	Drought stress	Seciu et al., 2016
Lateral root promoting peptides and other biopolymers like lignosulphonates and micronutrients	Triggered the accumulation of metabolites involved in defence mechanisms such as carotenoids, flavonoids and glucosinolates	Tomato (*Solanum lycopersicum*)	Yes	Biotic	Rouphael et al., 2018a
Radifarm® (Combination of organic matter, polysaccharides, peptides, amino acids, vitamin complex and chelated zinc)	Used to aid in transplanting explants; higher fresh and dry weights of root and above-ground plant tissue	*Begonia semperflorens*	n/a	n/a	(Koleška et al., 2017)

Viva® (folic acid, polysaccharides, humic acids, proteins, pepetides, amino acids and vitamins)	Decreased SOD and POD activity in the leaves when nitrogen, phosphorus and potassium (NPK) nutrition was reduced by 40%	*S. lycopersicum*	Yes	Oxidative stress	(Koleška et al., 2017)

1.3.1.6 The search for new PBs

As discussed, PBs have diverse modes of action, reported in a variety of crop and model species. Although the effects differ, PBs increase yield and confer some sort of resistance to abiotic, but also biotic, stresses in some instances. The increase in biomass is usually due to an increase in cell wall biosynthesis which acts as an extra layer of defence. The latter is a result of increased nutrient uptake and, sometimes, increased photosynthetic rates. PBs are reportedly eco- and cost friendly, organic and have the potential to be used as alternatives to chemical fertilizers, pesticides and fungicides (Ronga et al., 2019).

Recently, nanoparticles and nanomaterials have also been reported to have potential PB effects. These nanoparticles of metals and nanomaterials of carbon have been shown to increase plant growth at specific, low concentrations (Benavides-Mendoza et al., 2019). A salicylic acid-functionalized chitosan nanoparticle promoted plant growth and defense in maize plants (Kumaraswamy et al., 2018). In a study on how PBs affect plant microbiota, it was reported that certain PBs can enhance the growth of PGPB (Ruzzi et al., 2019). PB research has increased substantially over the last few years, with certain scientists dedicating entire studies to the search for novel PBs (Povero et al., 2016).

1.3.2 PBs almost universally play a role in relieving stress in plants

As described in a previous section, PBs stimulate/induce the plant's own metabolism to elicit a response. They are therefore also known as elicitors and are able to induce plant resistance (Le Mire et al., 2016). PBs have been shown to display priming characteristics. Priming involves the activation of plant defence signalling by a substance to prepare the plant for an environmental stress, both abiotic and biotic. Some PBs can enhance plant tolerance to both abiotic and biotic stress (Guinan et al., 2013), as illustrated above (Tables 1.1-1.5).

1.3.3 Understanding the modes of PB action

As discussed, PBs have mostly been assessed based on their effects on basic parameters of plant growth as well as their ability to mitigate environmental stress conditions. Most of these parameters have already been discussed in this review. These include fresh and dry biomass, root growth and development, photosynthesis, metabolite and mineral content in leaves, sugar and starch analysis and germination. In fruits, overall fruit quality is examined before and after storage. Fruit content is also analysed. Most studies have made use of general growth parameters to indicate an improvement in plant growth. Despite the progress made in characterising the effect of PBs on plant growth, major questions remain largely unanswered, particularly at the biochemical and molecular level. Many published studies are available, but only a few have attempted to unravel the molecular mechanisms responsible for the observed physiological mechanisms. Only a limited number of studies have focussed on determining the mechanistic aspects of PBs, especially at the molecular level.

With PBs having such a broad effect on plant growth and development, methodologies using a more holistic approach will be more effective to elucidate the mechanistic aspects of PB action. Genomics, transcriptomics, proteomics (Suwabe and Yano, 2008) and metabolomics (Hong et al., 2016) have the potential to unravel the holistic effects of PBs on plant growth and development. These approaches,

however, have been underutilised to date. The rich bed of knowledge generated by studies conducted on *Arabidopsis thaliana* has made this species a popular choice for transcriptomic analyses of PB action. *A. thaliana* is a classic genetic model because its relatively small genome has been fully sequenced and a wide variety of mutants are available, which aids any future research and verification of data obtained by transcriptomic analyses (Koornneef and Meinke, 2010). It has been the major plant model system for the past three decades (Chang et al., 2016). *A. thaliana* is valuable as a model plant for PB research because of the extensive molecular data available for this species. Although several plant species have been utilized in PB research, using model species like *A. thaliana* and *S. lycopersicum* is important because of the extensive research that has been conducted on their genomes, allowing these to be well annotated and described. It is therefore possible to gain a good understanding of exactly which genes and proteins are affected by any particular treatment. Consequently, *A. thaliana* has been relatively widely used in a number of PB studies. Humic substances (HS), one of the more well-studied PBs, reportedly altered the expression of genes involved in primary metabolism, growth and development in *Arabidopsis*. Further analysis, however, suggested the involvement of both IAA-dependent and independent signalling pathways (Trevisan et al., 2011). The application of HS derived from earthworm faeces influenced the metabolite profile of *Arabidopsis* plants (Conselvan et al., 2018). Humic substances also induced the expression of *IAA19I* and formation of lateral roots (Trevisan et al., 2010). Humic and fulvic acids have been shown to stimulate root and shoot structural changes and induce shifts in metabolism relating to abiotic stress tolerance (Canellas et al., 2015). A transcriptomic study where *A. thaliana* plants were subjected to NaCl stress and treated with *Ascophyllum nodosum* extracts revealed that this PB modulated the expression of genes involved in stress response, carbohydrate metabolism and phenylpropanoid metabolism (Jithesh et al., 2018). Alternatively, low concentrations of green seaweed extracts stimulated root growth while higher concentrations inhibited germination and root growth (Ghaderiardakani et al., 2018). A further study on *Ascophyllum nodosum* extracts revealed that it modulated the expression of three miRNA, ath-miR399, ath-miR827, ath-R2111b and also their target genes, suggesting that these extracts play a role in phosphate homeostasis in *A. thaliana* (Pushp Sheel Shukla et al., 2018). Stimplex®, an *A. nodosum* concentrate, stimulated cytokinin-like activity (Khan et al., 2011). Another study reported an increase in total cytokinins and abscisic acid (ABA) whereas auxin levels where reduced (Wally et al., 2013). An unusual agricultural compound, the major mammalian female sex hormone 17 β-estradiol, displayed PB properties by increasing growth, yield and primary metabolism at low concentrations while regulating phenylpropanoid pathway genes, negatively affecting the phenylpropanoid and flavonoid biosynthetic pathways in *Arabidopsis* (Upadhyay and Maier, 2016).

Using *Solanum lycopersicum* as a model plant species, in conjunction with *A. thaliana,* is important in this study due to the major differences between the two different plant models. *S. lycopersicum* is a model crop for fruit-bearing plants (Kimura and Sinha, 2008), and is also an economically important crop plant in comparison to *A. thaliana,* which is only used for research purposes. Although both are dicotyledonous plants, *A. thaliana* is a non-mycotrophic plant, while tomato plants are mycotrophic and known for their close beneficial relationships with various species of arbuscular mychorrhizal fungi (Chitarra et al., 2016) and beneficial bacteria (Harman and Uphoff, 2019). *S. lycopersicum* has been used with great success in transcriptomic studies (Chang et al., 2016), and has also been suggested as a model plant species to be used for the discovery of new PBs (Povero et al., 2016).

The effect of PBs has been widely studied on several tomato cultivars. PBs have been shown to increase overall plant growth (Ali et al., 2019; Bulgari et al., 2019; Drobek et al., 2019; Hernández-Herrera et al., 2014; Ibrahim, K.H. Ghoniem, 1970; Kavipriya and Boominathan, 2018), tomato fruit yield (Saraswathi and Praneetha, 2013; Zodape et al., 2011) and fruit quality (Castro et al., 1988; Chehade et al., 2018; Grabowska et al., 2012), stimulate root growth (Kim et al., 2019; Polo and Mata, 2018) and alter flowering patterns. PBs have also been shown to aid and possibly prime plants (Hayat et al., 2018) to mitigate certain environmental stresses such as salt stress (Arroussi et al., 2018), drought stress (Goñi et al., 2018; Paul et al., 2019; Petrozza et al., 2014), nutrient stress (Sestili et al., 2018) and biotic stress cause by several pathogens (Agarwal et al., 2016; Disciglio et al., 2016). Protein hydrolysates elicited hormone-like activity and increased nitrogen-uptake in tomato (Colla et al., 2014).

Since molecular characterisation of the effects of PBs on plants is important, several studies have adopted a transcriptomic approach. In a microarray study, a PB known as EXPANDO® was shown to alter the expression of genes involved in transcription, signal transduction, stress responses, carbohydrate metabolism, protein metabolism, transport and secondary metabolism (Contartese et al., 2016). The determination that *Ascophyllum nodosum* extracts applied as PBs increased drought tolerance in tomato plants has been revealed by using RT-qPCR analysis in conjunction with several other physiological measurements (Goñi et al., 2018). In a microarray transcriptomic study, it was shown that *Alfalfa*-protein hydrolysates upregulated genes involved in stress-related responses (Ertani et al., 2017). Transcriptomic analysis revealed a total of 620 differentially expressed genes (DEGs), which included transcription factors, transporter genes and S-transferases (Wilson et al., 2015). In the second study, a PB identified as APR® elicited a total of 1006 DEGs in the lateral roots of maize seedlings (Trevisan et al., 2017). TEA, a vermicompost-based PB in the category 'humic substances', enhanced proton extrusion in *S. lycopersicum* Micro-Tom plants, which promoted root growth by exerting an auxin-like activity (Zandonadi et al., 2016). A legume-derived protein hydrolysate (PH) induced the expression of nitrate reductase (*NR*), nitrite reductase (*NiR*) and ferredoxin-dependent glutamate synthase (*GLT*) as well as the expression of amino acid and other ammonium transporters (Sestili et al., 2018). It simultaneously increased plant dry weight leaf nitrogen content and SPAD index (Sestili et al., 2018). By-products from fennel processing residues enhanced both plant productivity and fruit quality (Chehade et al., 2018). Lycopene content increased in tomato fruits after treatment with Asahi SL, Biozyme™ and Goëmar BM 86 (Grabowska et al., 2015). Food processing by-products improved tomato yield, increased fruit mineral content, enhanced titratable acidity, increased vitamin C and phenol content (Chehade et al., 2018). An increase in fruit yield due to the application of a PB known as *panchakavya* was also reported (Saraswathi and Praneetha, 2013). Pepton 85/16®, containing more than 16% free amino acids, and a seaweed extract (Acadian) both increased yield and all other vegetative parameters in gold cherry tomatoes (Polo and Mata, 2018). Transcriptomic analysis revealed the induction of a large number of genes in the roots of maize seedlings (Trevisan et al., 2017). Also in maize seedlings, protein hydrolysates induced genes involved in cell wall organization, hormone metabolism, transport processes and stress responses (Santi et al., 2017), while another protein hydrolysate regulated the expression of genes involved in ROS metabolism and nitrate transport (Trevisan et al., 2019). An alfalfa protein hydrolysate induced the expression of root genes involved in nitrogen use efficiency (Schiavon et al., 2008). Non-model organisms have also been used as subjects in transcriptomic studies, although to a lesser extent. This methodology was also applied in kiwifruit in an effort to characterize the breaking of bud

dormancy (Hoeberichts et al., 2017). In cucumber plants, gelatin capsule seed treatment differentially enhanced the expression of 620 genes (Wilson et al., 2015). Metabolic analysis of PBs on plants have been utilized to a lesser extent. A plant-derived protein hydrolysate elicited large changes in phytohormones and lipids while improved tolerance to ROS-mediated oxidative imbalance (Paul et al., 2019).

1.3.4 BC204 as a PB

BC204 is a product from BioRevolution Pty.Ltd (South Africa). The product is a mixture of bioflavonoids from *Citrus aurantium* extractions, as well as added organic acids and other plant extracts. The exact formula is patented and only known to the company. The product is currently not registered as an organic PB due to the lack of peer-reviewed research on it. Data obtained from BC204 trials is unpublished and represents company intellectual property.

No molecular data is available that could explain the observations, such as an increase in overall yield, increased fruit set and retention, increased firmness and nutrient value of fruits and enhanced fruit colour. Other changes reported by the company include enhanced root development and a positive effect on soil compaction due to an increase in root exudates. The treatment of plants with BC204 resulted in increased resistance to pathogens and predatory insects as well as an increase in beneficial rhizosphere micro-organisms and saprophytic nematodes. BC204 is also claimed to increase secondary metabolite levels in plants (specifically flavonoids); its organic acid content assists with anti-microbial action, and increases activity of beneficial soil organisms, improves colonisation of soil fungi on roots, and improves availability and uptake of nutrients. These effects have been reported in a very broad range of fruits, vegetables and other economically important crop species. This makes it very difficult to pin-point specific possible mechanisms elicited by BC204, but they serve as clues that BC204 probably does not function via a single mechanism but rather stimulates a broad range of effects.

The direct advantages to the plants include: improved quality related to nutrient levels in fruit; a healthier root zone; enhanced colour development; improved flowering and fruit set; improved marketable yield and size distribution of fruit/tubers/bulbs and suppressed insect and disease attacks through improved overall plant health. To date, positive results have been reported for the following crops: lettuce; maize; grapes; bananas; tobacco; sugar cane; macadamias; potatoes; broccoli; cauliflower; avocados; olives; soybean; tomatoes; plums; pears; apples; citrus; cereals; lupines and canola (N Hanekom, unpublished results).

A. thaliana is a valuable plant model organism because of its small size, generation time and genetic resources (The Arabidopsis Genome Initiative, 2000), and *S. lycopersicum* because of its ability to form symbiotic relationships with arbuscular mycorrhizal fungi, its status as a model organism for fruit-bearing plants and the recent decoding of its genome (Consortium, 2012). Genetic resources for both organisms are also readily available, which enables further analysis of the data which is obtained through such transcriptomic studies.

There is considerable debate regarding the sustainability and usage of PBs and PGPS. A decade and even less ago, PBs were viewed in a similar way to ‘’snake oils’’ (Basak, 2008) and even homeopathic

medication. Due to the lack of peer-reviewed studies and absence of physiological, biochemical and molecular data, the claims made by PB-producing companies were often rejected by the agricultural and scientific community. However, as evident form the section above, the beneficial effects of PBs are now being reported on and publications on PBs modes of mechanisms are publicly available in the form of scientific journals and not just as pictures and testimonies. The topic for debate is whether PBs and PGPS merely ''speed up'' plant metabolism and growth, which merely depletes the water and nutrients in the soil at a faster rate. The use of some PBs may lead to exactly that outcome, and this issue is then amended by increasing the nutrient content of the soil through fertilisation and more water. This would make their increased usage irrelevant towards sustainability. However, improved nutrient use efficiency has been reported for several PBs (De Pascale et al., 2017; Halpern et al., 2015), consequently, nutrient use efficiency should be a standard test to be included when testing the effects of specific PBs. Another two factors that makes PBs a sustainable solution is the fact that they are obtained/extracted from natural/environmental-friendly sources and act at extremely low concentrations. Furthermore, they also minimize the need for more traditional fertilisers, pesticides, insecticides and other PGPS (Calvo et al., 2014; Yakhin et al., 2017). PBs are therefore ear-marked as a main role player in finding sustainable solutions in agriculture (Du Jardin, 2015).

1.4 Concluding remarks – looking forward

Even with the limited knowledge available, consumers and farmers need to be properly informed about the effects and potential benefits of PBs. Due to the holistic effect of PBs on overall plant growth, it is challenging to elucidate the specific mechanisms by which these PBs exert their effects on plants. For this reason, a holistic approach towards a mechanistic understanding, such as a transcriptomic analysis, is an ideal solution. As discussed in this review, it is also clear that in most studies the commercial PBs were tested on non-model plant species. The mostly likely reasons for this are that the specific company was only interested in the effect of its PB on that specific plant species because their target market lies within that specific agricultural sector.

Characterizing the molecular effects of PBs in plants, specifically model plants, can be used as a starting point to improve the formulations of currently available PBs. This is also important to establish a more science-based PB industry and develop more reliable and fairer regulations regarding the marketing, sales and use of these products (Yakhin et al., 2017). Understanding the effects of PBs to a greater extent could also result in them being used more efficiently as a tool in order to get closer to the goal of sustainable intensification.

1.4.1 Benefits of PBs in South Africa and the African continent

Sub-Saharan Africa is particularly vulnerable to excess pressure on the agricultural sector and food security for various reasons including high exposure to climate extremes (http://www.fao.org/state-of-food-security-nutrition/en/). Crop productivity for major crops such as wheat, maize, sorghum and millet are lower in Africa and South Asia in comparison to global averages (Knox et al., 2012). For a deeper review refer to Chauvin et al. (2012). The major challenge is mostly access to affordable PGPS. Providing peer-reviewed scientific data that explains the beneficial effects of locally produced PBs like BC204 would also instil confidence in these products, which could drive their distribution and widespread usage. Although more

established PBs and PGPS, which are mostly imported, can improve local production, yield and food security, importing these products is expensive due to unfavourable exchange rates and import taxes. Using locally-produced products will also stimulate small business development and inspire other local agriculture businesses to follow suit.

South Africa is an agricultural powerhouse, exporting products globally. The latter is vital to its economy, reportedly worth R273 344 million in 2016/2017, amounting to 2% of the total GDP (Economic Review of the South African Agriculture, 2016/2017). With widespread drought and increasing pressure on the agricultural sector to deliver, the use of BC204 could benefit not only current farmers, but also emerging ones. PB development and characterisation is important for agriculture as well as the economy (Rouphael et al., 2018). It is estimated that global PB markets will reach US$2.91 billion by 2021, reaching approximately 24.9 million hectares globally (Fleming et al., 2019). This would also drive local and international investments towards the African continent.

1.5 Aims and Objectives

The commercially available plant biostimulant BC204 is widely used across a number of crop species in South Africa, and has been shown to have positive effects on a variety of different aspects of crop productivity and quality. Nonetheless, very little is known about how this specific PB achieves these effects *in planta*. The overall aim of this study was to elucidate the molecular mechanisms responsible for the enhancements in plant growth and physiology observed following treatment with BC204 in the model species *Arabidopsis thaliana* and *Solanum lycopersicum,* through a combination of basic plant physiological measurements, biochemical characterisation and transcriptomic analyses.

Specific objectives were:

i) To determine the physiological responses of *A. thaliana* plants to BC204 treatment under optimal conditions;
ii) To determine whether BC204 was able to enhance tolerance of *A. thaliana* plants to salinity (NaCl) stress, and to determine the associated physiological responses to both the stress and the presence of the PB during this stress;
iii) To determine global gene expression changes in *A. thaliana* following BC204 treatment *via* an RNA-seq approach;
iv) To determine the physiological responses of hydroponically-grown *Solanum lycopersicum* plants to BC204 treatment; and
v) To analyse the transcriptomic response of *S. lycopersicum* seedlings to treatment with BC204, again using an RNA-seq approach.

The data obtained from these analyses was then used in combination with a bioinformatics approach to explain how BC204 enhances plant growth and development in these two model species, providing a clearer understanding of how this plant biostimulant could lead to enhanced agricultural yield and productivity.

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The Application of a Commercially Available Citrus-Based Extract Mitigates Moderate NaCl-Stress in *Arabidopsis thaliana* Plants

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Abstract: Aims: The aim of this study was to assess the effect of BC204 as a plant biostimulant on *Arabidopsis thaliana* plants under normal and NaCl-stressed conditions. Methods: For this study, ex vitro and in vitro growth experiments were conducted to assess the effect of both NaCl and BC204 on basic physiological parameters such as biomass, chlorophyll, proline, malondialdehyde, stomatal conductivity, Fv/Fm and the expression of four NaCl-responsive genes. Results: This study provides preliminary evidence that BC204 mitigates salt stress in *Arabidopsis thaliana*. BC204 treatment increased chlorophyll content, fresh and dry weights, whilst reducing proline, anthocyanin and malondialdehyde content in the presence of 10 $dS \cdot m^{-1}$ electroconductivity (EC) salt stress. Stomatal conductivity was also reduced by BC204 and NaCl in source leaves. In addition, BC204 had a significant effect on the expression of salinity-related genes, stimulating the expression of salinity-related genes *RD29A* and *SOS1* independently of NaCl-stress. Conclusions: BC204 stimulated plant growth under normal growth conditions by increasing above-ground shoot tissue and root and shoot growth in vitro. BC204 also increased chlorophyll content while reducing stomatal conductivity. BC204 furthermore mitigated moderate to severe salt stress (10–20 $dS \cdot m^{-1}$) in *A. thaliana*. Under salt stress conditions, BC204 reduced the levels of proline, anthocyanin and malondialdehyde. The exact mechanism by which this occurs is unknown, but the results in this study suggest that BC204 may act as a priming agent, stimulating the expression of genes such as *SOS1* and *RD29A*.

Keywords: Anthocyanin; Arabidopsis; BC204; biostimulants; malondialdehyde; proline; salinity; *SOS1*

1. Introduction

Soil salinity is a global problem, affecting more than 100 countries [1,2] and approximately 20% of the world's irrigated cropland [3,4]. It is estimated that more than 50% of global arable land will be affected by 2050 [5]. Primary salinisation occurs naturally via precipitation and through groundwater, resulting from rock erosion over long periods of time [6], while secondary or human-induced salinisation is caused by a combination of poor drainage and irrigation [7]. Both combine to change the soil for the worse, leading to the accumulation of ions such as Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-. Although all of these ions can cause soil salinisation, high concentrations of Na^+ as a result of excess NaCl is the most prevalent and the subject of most salt-stress related research [8]. This build-up of Na^+ is the major culprit in the detrimental effects seen on plant growth, but it remains unclear how this is sensed by the plant [9].

Saline conditions drastically reduce the yield of major crops, due to the toxic effects of Na^+ accumulating in plant tissue leading to stunted growth and eventually cell death. High salinity influences all aspects of plant growth and growth stages, leading to ion toxicity, osmotic stress, nutrient deficiency and oxidative stress [10]. Most plant/crop species that are used to feed the global population are highly sensitive to high salinity [11,12].

Salty soil initially induces osmotic stress which results in nutrient imbalances, interruption of membranes and disrupts the plant's ability to detoxify reactive oxygen species (ROS) which damage the plant at a molecular level [13]. The exact mechanisms by which plants mitigate salt stress have not been fully elucidated, but several role-players have been implicated.

In response to environmental stimuli such as salt stress, cellular abscisic acid (ABA) levels are increased [11], which triggers the expression of numerous stress-responsive genes [14]. This regulation occurs via transcription factors which are elevated in response to salt stress and also through histone H3 acetylation and methylation which further regulates stress-inducible gene expression [15]. One example is the induction of *RD29A* expression [16]. The increase in ABA levels also stimulates an increase in the production of ROS, which is used in research to monitor intracellular levels of oxidative stress in plants [17,18]. The increase in production of ROS, previously thought to only be a toxic by-product of stress, also serves to provide signaling molecules leading to the production of antioxidants and antioxidative enzymes, forming part of a concerted plant defense reaction [19,20]. If not sufficiently detoxified and scavenged, ROS can cause oxidative damage to proteins, lipids and DNA [21]. The role of ROS in the mitigation of oxidative stress has recently been extensively reviewed [22]. Following prolonged oxidative stress caused by the Na^+ build-up and subsequent ROS production, ion-toxicity is the inevitable next stage, unless the plant sufficiently deals with the excess Na^+, which is not the case in most plants. The build-up of Na^+, which is generally not essential for plants, in the cytosol causes K^+ deficiency, which disrupts enzymatic processes since K^+ activates more than 50 key enzymes [9].

The accumulation of proline [23], malondialdehyde [24] and anthocyanin [25] are commonly used in research as indicators of salt stress. Proline, a low molecular weight non-enzymatic antioxidant [26], is an osmolyte biosynthesized through the glutamate and ornithine pathways. It plays an important protectory role in plant cells experiencing salt stress [27], alleviating the negative impact of salt by decreasing osmotic stress to maintain membrane integrity and function [26]. Under saline conditions, histone demethylase irreversibly removes the methylation of the *Δ^1-1-pyrroline-5-carboxylate synthetase (P5CS)* coding sequence, leading to the overexpression of *P5CS* and thus an accumulation of proline [28,29]. As an extended effect of the presence of ROS, cell membranes are damaged via the oxidation of acids in the bilayer, a process also known as lipid peroxidation [30]. Lipid peroxidation causes an increase in malondialdehyde levels [2,4], which is the first product formed during free radical-induced damage and the decomposition of polyunsaturated fatty acids in membranes [31]. The production of anthocyanins, also antioxidants, increases in the presence of salt stress as these play a similar protectory role to proline while serving as a signal molecule activating downstream stress-responsive pathways [32,33]. Anthocyanins are also suggested to play roles in quenching ROS, photoprotection and xenohormesis [34]. Elevated levels of anthocyanins in *A. thaliana* have been shown to increase salinity tolerance [35].

Salinity stress induces the expression of a large number of genes and pathways as outlined in two extensive review papers [36,37]. The expression of these genes is often used as indicators of salt stress. The model organism *A. thaliana* has been pivotal in unravelling the Salt Overly Sensitive (SOS) signaling pathway which is involved in salt stress, which was also the first abiotic stress-signaling pathway elucidated in plants [38,39,40]. Independently of ABA signaling, salt stress upregulates the expression of the *AtWRKY8* transcription factor [41] which directly binds to the *RD29A* promoter, leading to upregulation of the gene [37]. The expression of *RD29A* is also induced by cold and drought stress and although its induction and overexpression increase a plant's resistance to abiotic stress, it was concluded that the RD29A protein is unlikely to serve directly as a protective molecule. The exact function of RD29A is still unknown but it likely serves as a warning signal for abiotic stress responses [42]. NaCl-stress also strongly induces *AtSOT12*, which codes for a sulfotransferase that is also implicated in pathogen resistance via salicylic acid signaling [43].

Three membrane transporters, AtSOS1, AtHKT1 and AtNHX1 are critical Na+ carriers which reduce salt toxicity in plants [12]. AtSOS1 and AtHKT1 are suggested to mediate Na^+ transport, control ion uptake and spatial distribution of Na^+ and K^+ by regulating the expression levels of relevant Na^+ and K^+ transporter genes [44]. These membrane transporters remove Na^+ from the cytoplasm by transporting it into the vacuole or out of the cell [45]. The signaling pathway involves a salt-elicited Ca^{2+} signal in the cytosol, where a myristoylated calcium binding protein, SOS3, activates a serine/threonine protein kinase, SOS2 [46]. The SOS3/SOS2 complex subsequently phosphorylates and activates SOS1 [40,47] which codes for a Na^+/H^+ antiporter, expressed and located at the plasma membrane, which exports Na^+ out of the cytosol into the apoplastic space [48,49,45,50]. *AtSOS1* transcripts can be stabilized by plasma membrane-localized

NAPDH oxidase generated ROS [51]. This pathway is highly conserved in plants and its main role seems to be to maintain ion homeostasis [38,52].

Some strategies implemented thus far to address salt stress in agriculture have been to elucidate salt tolerance mechanisms and signaling [53,13,54,55,56,11] and use the information for two genetic approaches to create more salt-tolerant crops. The first approach is creating more salt tolerant varieties through breeding programs, while the second approach is to genetically modify crop plant to be more salt tolerant [57,4,58]. In addition to the usual fertilizers and crop protection agrochemicals used in efforts to improve the plant's immediate environment, plant biostimulants (PBs) are a novel collection of agrochemicals recently introduced in agriculture. PBs, described as materials that can promote plant growth in minute quantities regardless of nutrient composition [59], have been shown to alleviate the effects of abiotic stress, which includes salt stress, in crop plants. There are a wide variety of commercially available PBs routinely used in agriculture. Examples of commercially patented PBs are extensively reviewed elsewhere [60]. PBs improve plant growth, yield, fruit quality and tolerance to abiotic stress [61,62,63,64,60,65]. Some researchers are developing and finetuning efforts to discover and characterize new PBs suitable for agricultural use [66,67,68]. The priming effect of PBs can be described as preparing the plant for abiotic stress by activating plant defense mechanisms against stress [69,70,71,72].

Ascophyllum nodosum extracts (seaweed-extracts), the most well-characterized group of PBs, have been shown to induce salinity tolerance in *A. thaliana* by regulating the expression of stress responsive genes [73] and by modulating miRNAs involved in nutrient acquisition and stress tolerance [74]. There are also other reports where exogenous application of PBs has alleviated or mitigated the effects of NaCl-stress. In sweet pepper, exogenous application of citric acid, humic acid, putrescine and seaweed extracts to unstressed and NaCl-stressed plants increased sugar and potassium (K^+) content while decreasing Na^+ and proline content, which was exactly the opposite to the NaCl-stressed plants [75]. In salt -stressed *Solanum lycopersicum* plants, an exopolysaccharide-type PB reduced the levels of proline, Na^+, phenolic compounds and antioxidant enzyme activity [76]. The effects of PB applications on crop plants to alleviate abiotic stresses such as salinity and improve plant growth have been recently reviewed [61]. BC204 is a citrus-based PB currently used in South Africa, Australia, China and USA as an agrochemical to improve crop growth. There is no published literature which describes the effects of BC204 on plant growth and abiotic stress tolerance. Here, we examined the effects of BC204 on the growth of the model organism *A. thaliana*, and whether BC204 was able to mitigate salinity stress in this species.

2. Results

BC204 Increases A. thaliana Above-Ground Fresh and Dry Weight While Increasing Leaf Number under Both Normal and Salinity Stress Conditions

BC204 treatment resulted in enhanced shoot growth of *A. thaliana* Columbia-O plants, even in the presence of 50 mM and 100 mM salinity stress. Plants were germinated and grown on peat discs for three weeks before being treated with 0.01% (*v/v*) BC204 every six days and/or NaCl every three days. BC204-treated plants (Figure 1D) were visibly larger and healthier than the untreated control plants (Figure 1A). Salinity stress had a significant impact on plant growth (Figure 1B,C), salt-stressed plants were visibly smaller than their non-stressed counterparts. BC204 treatment resulted in an obvious enhancement of plant growth under saline conditions (Figure 1 E,F), such that BC204-treated stressed plants had similar growth characteristics to the untreated unstressed plants. Fresh (Figure 2A) and dry (Figure 2B) mass measurements reflected these observations, with BC204-treated plants being significantly heavier than their control counterparts under both non-stressed and salinity-stressed conditions. BC204 treatment was able to return biomass levels in both 50 mM and 100 mM salt-treated plants to at least those of the untreated control plants. BC204 treatment also increased the leaf number (Figure 2C). The average electroconductivity (EC) of the peat discs for control (water only) and BC204-treated plants were similar, ranging between 0 and 1 $dS \cdot m^{-1}$, while 50 mM NaCl-treated peat discs had an average EC of 10 $dS \cdot m^{-1}$ and 100 mM NaCl-treated disks recorded an average EC of 20 $dS \cdot m^{-1}$.

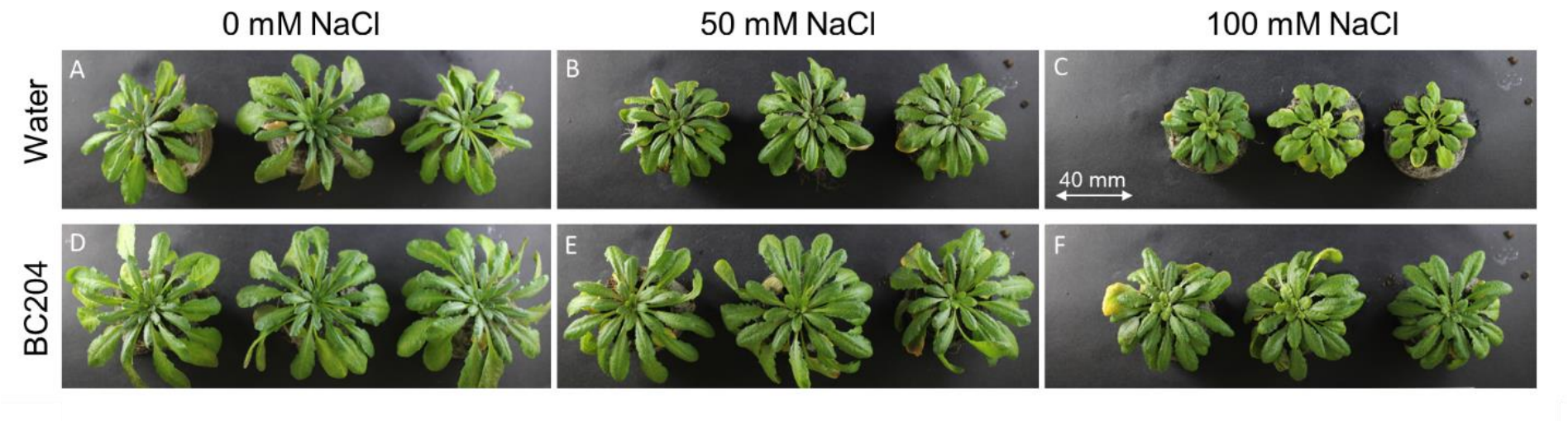

Figure 1. Rosette growth of *Arabidopsis thaliana* plants in response to salt (NaCl) and BC204 treatments. Plants were treated with water (**A**), 50 mM NaCl (**B**), 100 mM NaCl (**C**), 0.01% (*v/v*) BC204 (**D**) and combinations of BC204 and 50 mM (**E**) and 100 mM (**F**) NaCl.

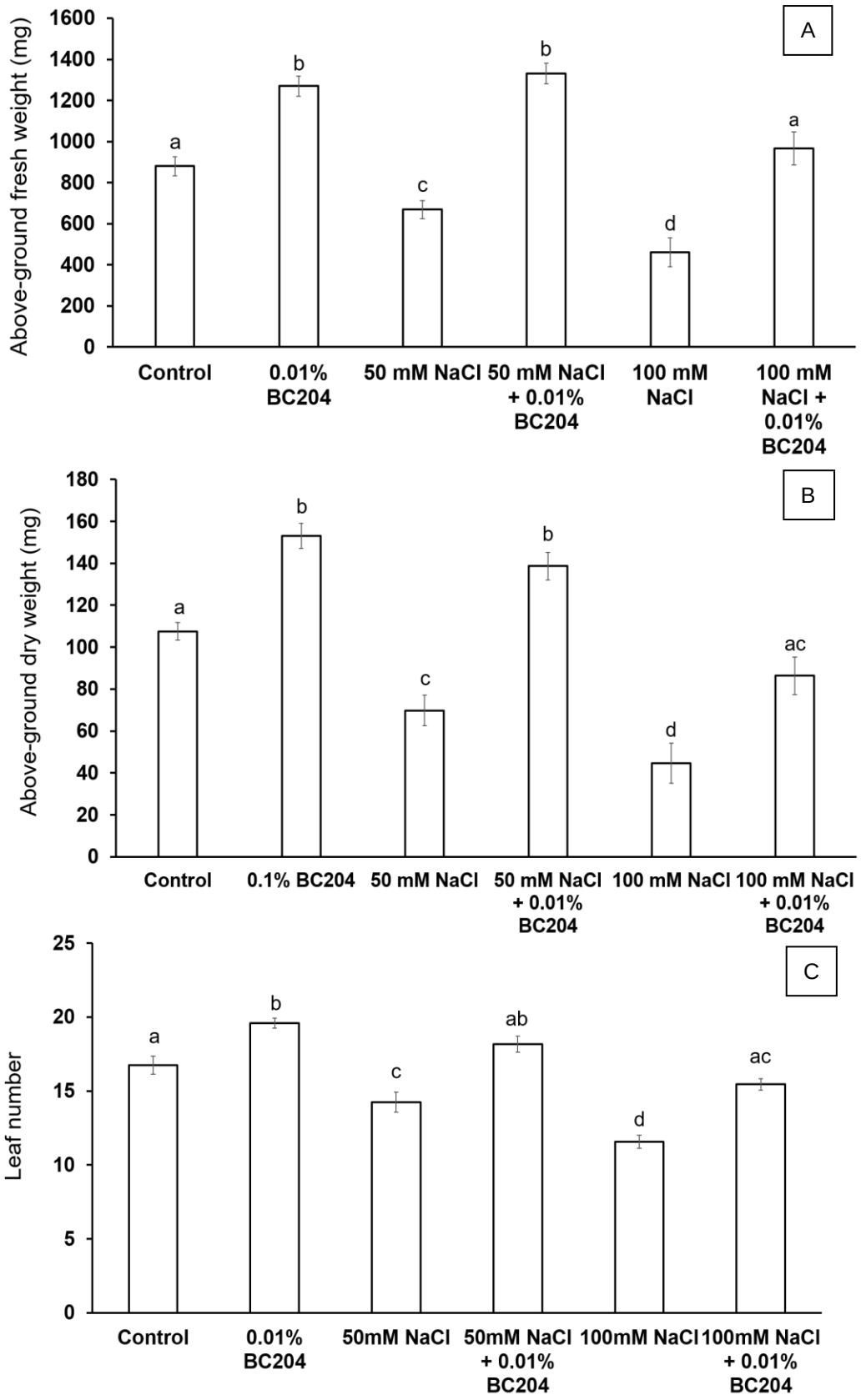

Figure 2. Above-ground fresh (**A**) and dry (**B**) biomass production and total leaf number (**C**) of *Arabidopsis thaliana* plants treated with BC204 and two different concentrations of NaCl. Bars represent the mean of 20 replicates (n = 20) ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD post-hoc test to be significantly different (p< 0.05) from the control.

BC204 Increases A. thaliana Root and Shoot Growth in the Presence and Absence of NaCl-Stress in In Vitro *Growth Conditions*

To test for the effect of BC204 in vitro, *A. thaliana* seedlings were grown on media supplemented with BC204, NaCl and a combination of both. After germination on media containing no BC204 or NaCl, 4-day old *A. thaliana* Columbia-0 seedlings of identical sizes were transferred to ½ MS media supplemented with either 0.001% (*v/v*) BC204, 50 mM NaCl, 50 mM NaCl + 0.001% (*v/v*) BC204, 100 mM NaCl or 100 mM NaCl + 0.001% (*v/v*) BC204. Roots of BC204-treated seedlings (Figure 3D–F) were visibly larger in comparison to the control (Figure 3A) and NaCl-stressed seedlings (Figure 3B,C). Shoots of BC204-treated seedlings were visibly larger than the untreated control plants on media containing 0 mM and 100 mM NaCl, although no differences were observed between the shoots of 50 mM NaCl treated seedlings and their BC204-treated counterparts. Fresh (Figure 4A,B) and dry masses (Figure 4C,D) of shoots and roots were significantly increased in the presence of 0.001% BC204 after 10 days. BC204 treatment improved biomass accumulation for both shoots and roots under both 50 mM and 100 mM salt to levels comparable to those of the untreated control plants. Primary root length was also increased by BC204, both in the absence and presence of NaCl-stress (Figure 4E).

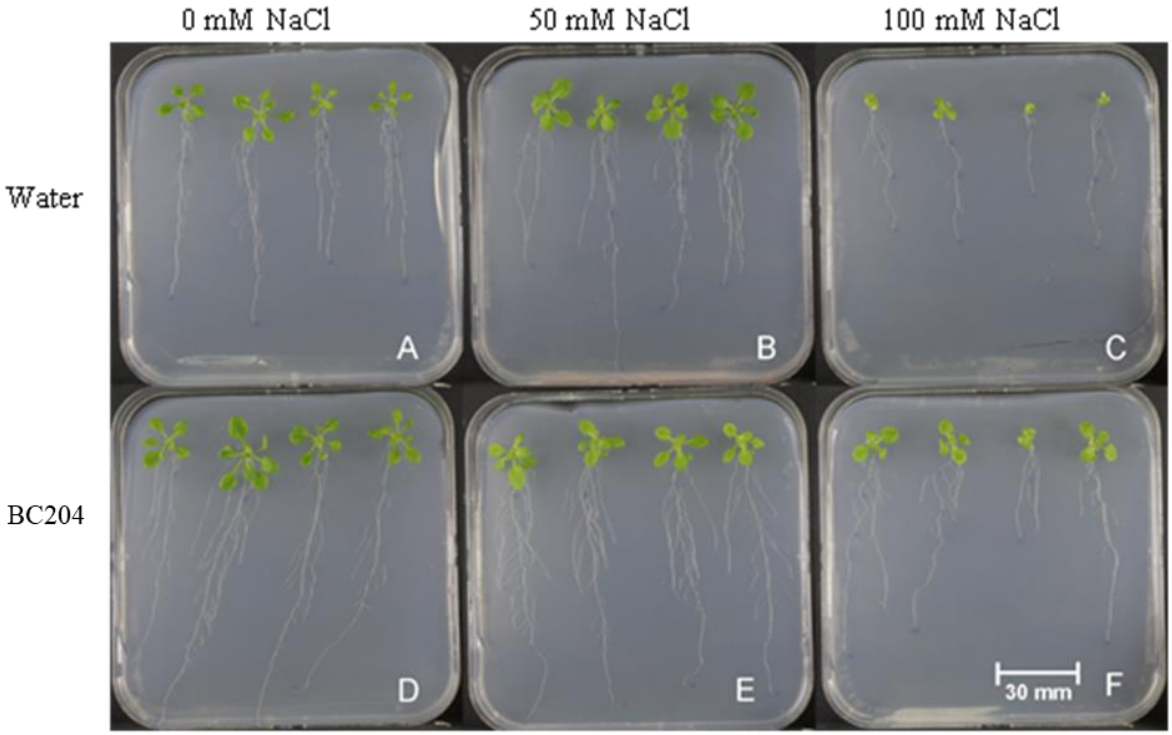

Figure 3. Growth of *Arabidopsis thaliana* seedlings in vitro in response to salt (NaCl) and BC204 treatments. Seedlings were grown on half-strength Murashige and Skoog (MS) media (**A**), supplemented with 50 mM NaCl (**B**), 100 mM NaCl (**C**), 0.001% (*v/v*) BC204 (**D**), 50 mM NaCl and BC204 (**E**), and 100 mM NaCl and BC204 (**F**).

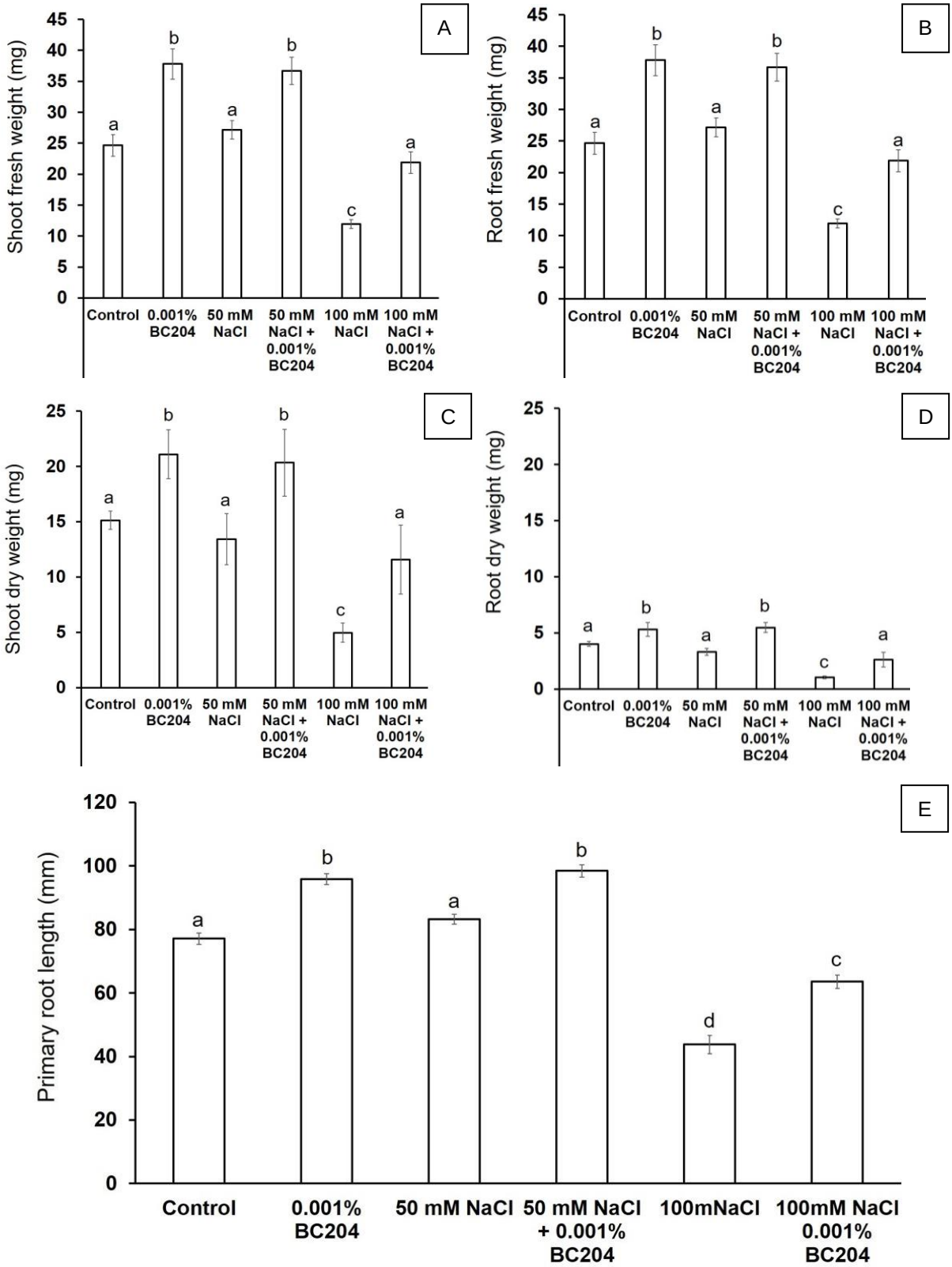

Figure 4. Shoot (**A**) and root (**B**) fresh mass, shoot (**C**) and root (**D**) dry mass and primary root length (E) of in vitro grown *Arabidopsis thaliana* seedlings treated with BC204 and NaCl. Bars for tissue fresh weight and primary root length represent the mean of 20 replicates (n = 20) ± standard error. Bars for tissue dry weight represent the mean of three pooled samples of 10 replicates (n = 3). Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD post-hoc test to be significantly different (p < 0.05) from the control.

BC204 Increased Chlorophyll Content, Lowered Stomatal Conductance and Increased Fv/FM in Unstressed and NaCl-Stressed Conditions

To test for the effects of BC204, NaCl, and a combination of both, on Fv/Fm measurements, the same plants from the initial growth experiment were used. The 100 mM NaCl-stressed plants were excluded from these analyses in order to investigate a more moderate salt stress rather than a severe stress [77,78,79]. All measurements were taken and tissues harvested 90 min after the final BC204 treatment. Chlorophyll content increased in BC204-treated plants in comparison to the control. While 50 mM NaCl reduced the amount of chlorophyll in the shoot tissue (Figure 5A), plants treated with BC204 in conjunction with 50 mM NaCl had a chlorophyll content that was similar to that observed in the control group. Fv/Fm values were slightly enhanced by 0.01% BC204 and 50 mM NaCl as individual treatments compared to the untreated control, and a combination of both resulted in the highest Fv/Fm value (Figure 5B). There were no significant differences in stomatal conductance rates in the sink leaves between any of the treatments. However, in the source leaves, 0.01% BC204, 50 mM NaCl and the combination of both all resulted in similarly reduced stomatal conductance levels compared to control plants (Figure 5C).

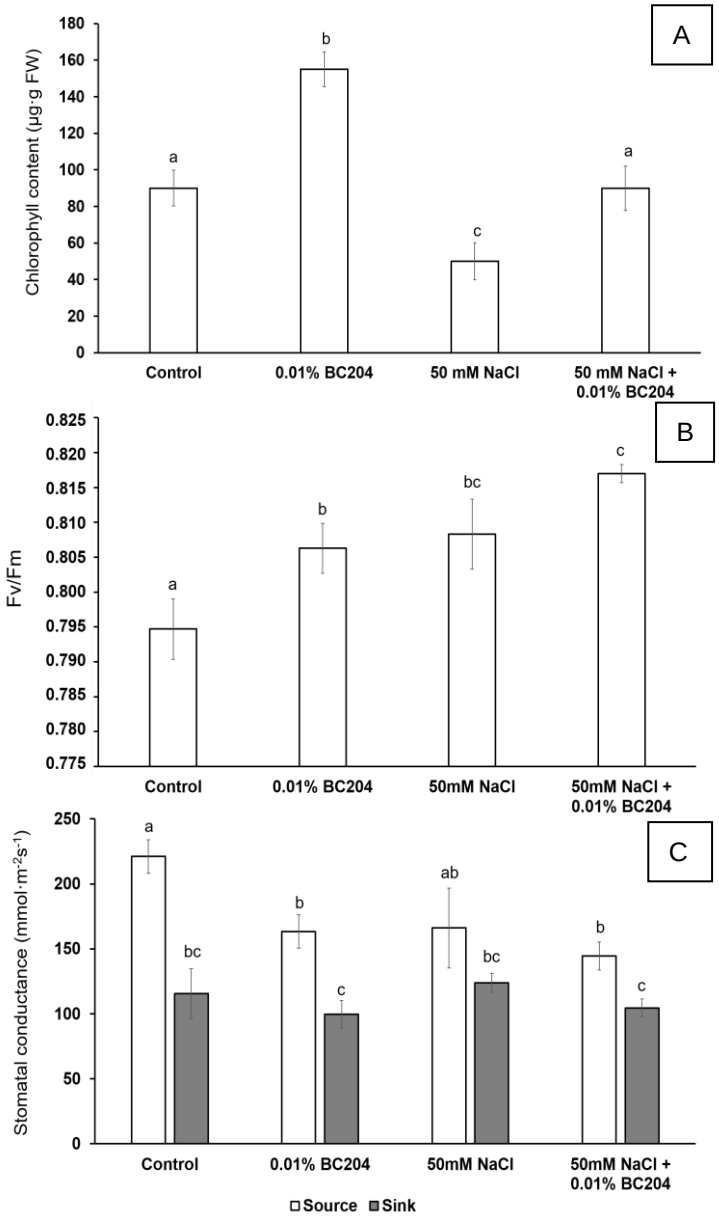

Figure 5. Total chlorophyll content (A), Fv/Fm (B) and stomatal conductance (C) in *Arabidopsis thaliana* plants subjected to NaCl-stress, BC204 treatment and a combination of BC204 and NaCl. Bars represent the mean of 9 replicates (n=9) ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different (P<0.05) from the control.

BC204 Attenuates the High Levels of Anthocyanin, Proline and Malondialdehyde Content Elicited by NaCl-Stress. To test for the effect of BC204 and NaCl on anthocyanin, proline and malondialdehyde content, the same plants from the initial growth experiment were used, again excluding the 100 mM NaCl-stressed plants since the aim of the study was to investigate a moderate rather than a severe stress [77,78,79]. Anthocyanin (Figure 6A), proline (Figure 6B) and malondialdehyde (Figure 6C) contents were significantly higher in NaCl-treated plants than in the control plants. BC204 on its own had no direct effect on anthocyanin and malondialdehyde levels, but significantly reduced proline levels even under unstressed conditions. The addition of BC204 to the NaCl-treated plants reduced the levels of anthocyanins and malondialdehyde to those of the control plants. BC204-treatment also significantly reduced proline levels under saline conditions, although these were still elevated compared with control plants. None of the treatments affected SOD activity (Figure 6D).

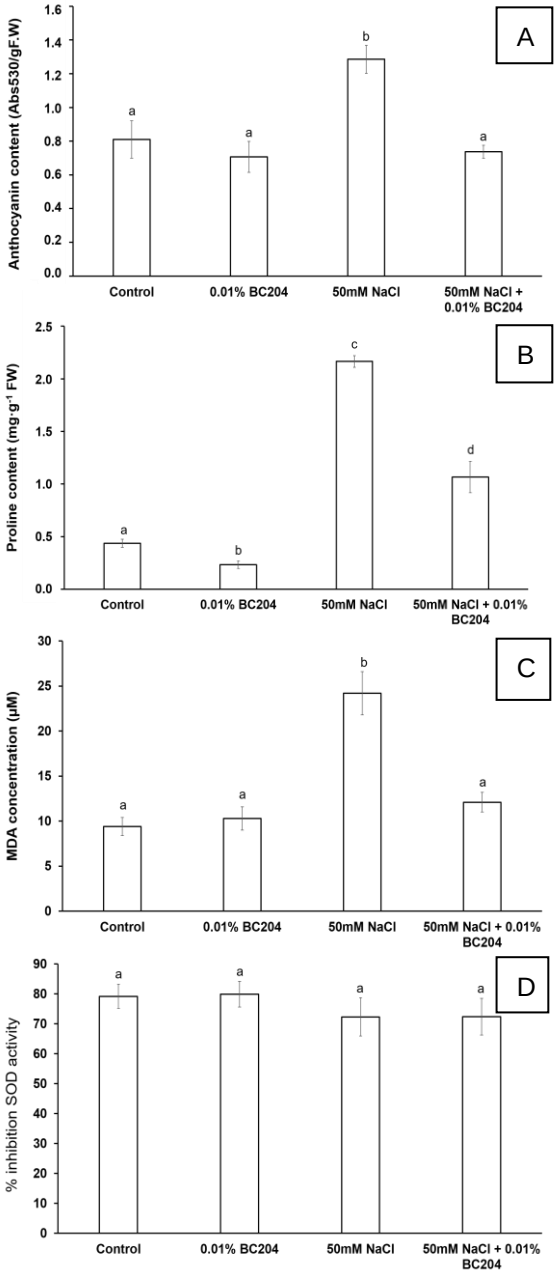

Figure 6. Anthocyanin content (A), proline content (B), malondialdehyde concentration (C) and % SOD inhibition (D) in *Arabidopsis thaliana* shoot tissue following BC204 and NaCl treatment. Bars represent the mean of 9 replicates (n=9) ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different ($P<0.05$) from the control.

BC204 Stimulates the Expression of Two NaCl-Responsive Genes

Expression of salt-responsive genes *P5CS1, SOT1* and *SOS1* was examined through RT-qPCR to determine the effects of salinity and BC204 treatments. BC204 significantly increased the expression of *RD29A* and *SOS1,* while decreasing the expression of *P5CS1* and *SOT1* (Figure 7). NaCl induced the expression of *SOT1* and *SOS1,* while having no significant effect on the expression of *P5CS1* and *RD29A* (Figure 7). A combination of both BC204 and NaCl resulted in a similar expression profile to NaCl treatment, except for a significant increase in *SOS1* expression levels (Figure 7), similar to that observed following the individual BC204 treatment (Figure 7).

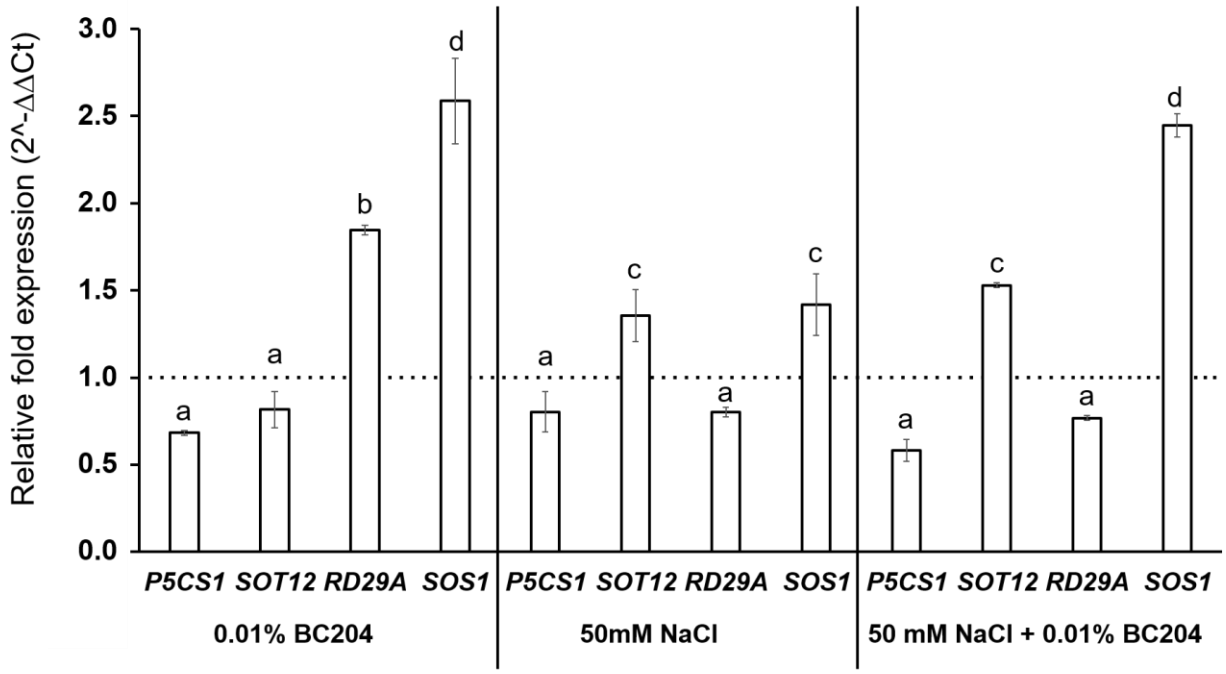

Figure 7. Relative fold expression of NaCl-responsive genes in *A. thaliana* shoot tissue under BC204 and NaCl-stress and a combination of both. Histograms represent relative transcript expression levels of Reverse Transcriptase Quantitative PCR (RT-qPCR) in the shoot tissue of plants treated with BC204 (0.01%), NaCl-stressed (50mM) and BC204 (0.01%) combined with NaCl-stress (50 mM). Ct values were averaged and normalized to *Eflα* (At1g18070) according to the $2^{-\Delta\Delta Ct}$ method [80]. The expression values for all genes for the untreated control plants were set as 1. Bars represent the mean of three replicates (n = 3) ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD post-hoc test to be significantly different (p< 0.05) from the control.

3. Discussion

Soil salinity is one of the largest constraints in agriculture, leading to a decrease in plant productivity and agriculture, resulting in approximately 20% of all yield loss [1,2,81]. There is a time constraint on this issue as the world population is growing extensively and a 50% increase in food production will be needed by as early as 2030 [82], while it is estimated that 50% of all arable soil will be salinized by 2050 [36]. However, unravelling these mechanisms and implementing the knowledge gained is a slow process and a large number of questions still remain unanswered [52], a common theme in plant research due to the complexity of the regulatory networks and crosstalk between signaling pathways and plant metabolism as a whole. Our results confirm a mitigating and possible priming effect of BC204 as a PB towards salt-stress, as it enhances tissue fresh and dry mass accumulation, primary root length, and certain stress markers including the accumulation of stress-responsive metabolites and alters the expression patterns of genes involved in NaCl-stress responses.

BC204 Improved Plant Growth in A. thaliana Grown under Moderate and High Saline Conditions

Plant size, fresh and dry mass and leaf number are the most basic indicators of plant health and are used as initial markers of a positive response to plant growth promotion. The majority of PBs display an increase in these basic indicators of plant growth promotion [83,84,85,86] while plants experiencing abiotic stress are generally smaller with reduced biomass [87,88]. A protein hydrolysate PB from *Medicago sativa L*. has been shown to alleviate salt-stress effects in maize plants by increasing biomass and stimulating plant nitrogen metabolism and antioxidant systems [89]. In tomato plants, *Dunaliella salina* exopolysaccharides alleviated salt stress by increasing tissue fresh and dry weight, root and shoot length while decreasing proline levels, phenolic compounds, Na^+ levels and antioxidant enzyme activity [76]. In tall fescue turf-grass, foliar application of glycine betaine increased fresh weight clippings under salt stress conditions [90]. Under saline stress conditions, sorghum tissue fresh and dry weight were also increased by humic substances and *Moringa oleifera* leaf extract [91]. In cowpea plants, selenium, glycine betaine and seaweed extract increased biomass under salt stress conditions [92].

In this study, BC204 increased plant biomass under normal growth conditions in both *ex vitro* (Figure 1) and in vitro (Figure 3) experiments. Furthermore, BC204 visibly increased above-ground rosette growth of *A. thaliana* under both 50 Mm and 100 Mm saline conditions (Figure 1), while an increase in root growth was also observed in vitro at the same NaCl concentrations (Figure 3). BC204 significantly increased rosette fresh (Figure 2A) and dry biomass (Figure 2B) of these plants in unstressed and NaCl-stressed conditions, while an increase in leaf number was also observed (Figure 2C). Tissue (root and shoot) fresh and dry biomass and primary root length were also significantly increased in vitro (Figure 3,4). The ex vitro experiment simulated salinity conditions which would closely correlate to how NaCl build-up occurs in nature. The addition of 10 mL of either 50 Mm or 100 Mm NaCl to each plant every three days for 21 days resulted in EC values of the peat disks of approximately 10 $dS \cdot m^{-1}$ and 20 $dS \cdot m^{-1}$ respectively at the end of the experiment. Moderately saline water (primary drainage water and groundwater) has an EC of 2 to 10 $dS \cdot m^{-1}$ while highly saline water (secondary drainage water and groundwater) falls between 10 and 25 $dS \cdot m^{-1}$. Irrigation water usually has a measurement of 0.7 to 2 $dS \cdot m^{-1}$ [79]. Most studies on salt responses focus more on short term responses (6 to 48 hours) and single salt treatments, which could often simulate salt shock rather than salt stress [93]. Also, most studies focus on the response in the roots rather than the shoot tissue, since roots represent the site where the plants directly experience the stress [94,95].

Studies investigating PB action in vitro are rather scarce, with only a few reporting that certain PBs improve root growth of plants in vitro [96] or an increase in biomass production under saline conditions [97]. For the in vitro experiment in this study, 100 Mm NaCl would be regarded as high salt stress, but *A. thaliana* can grow and survive in concentrations of up to 150 Mm NaCl, which is regarded as salt shock [93,98]. *A. thaliana* grows relatively normally in 50 Mm NaCl [77], as shown in our results (Figure 3). BC204 stimulated root growth under saline conditions (Figure 4). A reduction in primary root length is one of the most common indicators of salt stress in plants [99,100]. Primary root length was significantly reduced in vitro on media containing 100 mM NaCl, however, a significant increase in primary root length was observed in these plants where BC204 was added to the medium in addition to the salt (Figure 4E). To narrow the scope of the study,

further analysis was conducted only on peat-grown plants treated with 50 mM NaCl, in order to investigate plant response to moderate salinity stress (~10 $dS \cdot m^{-1}$).

BC204 increased total chlorophyll content while reducing stomatal conductivity in unstressed and NaCl-stress conditions

It is well documented that abiotic stresses such as salinity stress inhibit photosynthesis, including via stomatal closure. The exact mechanism by which salinity affects photosynthesis, however, remains largely unclear [87]. Since photosynthesis is the main driver of energy production and storage in plants, it is tightly regulated and highly sensitive to environmental stimuli [101]. Chlorophyll, a photosynthetic pigment, is one of the most important components of photosynthesis [102]. An increase in chlorophyll content usually correlates with an increase in photosynthesis and subsequent increase in plant biomass [103]. It is well-documented that under saline stress conditions, chlorophyll content decreases [64,104,105,106,107]. In several reports, PB treatment led to a significant increase in total chlorophyll [108,109,110]. PBs have also been shown to preserve chlorophyll in plants under abiotic stress conditions [67,111]. In this study, BC204 mitigated the reduced total chlorophyll levels observed in 50 mM NaCl-stressed plants, returning these to the same levels as in the untreated control plants (Figure 5A). A significant increase in chlorophyll was also recorded in unstressed plants treated with BC204 (Figure 5A), which may partly explain the enhanced biomass accumulation observed in these plants via its contribution to possible enhanced photosynthesis.

Chlorophyll fluorescence can also be an indicator of the level of stress the plant experiences. Salt stress has been reported to decrease Fv/Fm values [112]. Slight decreases in Fv/Fm have also been reported in *A. thaliana* under salinity stress [113]. In another study, exposure to either 100 Mm or 150 Mm NaCl resulted in Fv/Fm values of approximately 0.78 and 0.75 respectively, compared to the control of 0.8 [114]. Atonik, a PB, did not affect Fv/Fm measurements [115]. BC204, 50 Mm NaCl and a combination of both significantly increased Fv/Fm values compared to the control (Figure 5B).

Plants regulate stomatal conductance to optimize carbon uptake with respect to water [116]. Stomatal conductance is also used in plant research as an indicator of the level of stress the plant experiences [13] and is mediated through ABA signaling [54]. Increased stomatal conductivity is therefore an indication that plants are experiencing no to low levels of environmental stress. Under saline conditions, plants experience a variety of stresses including water stress. In order to prevent water loss, plants close their stomata which lead to a decrease in carbon dioxide in chloroplasts [93]. Under saline conditions, foliar application of glycine betaine as a PB significantly increased stomatal conductance of tomato leaves [117]. In the source leaves in *Arabidopsis*, BC204, 50 Mm NaCl, and a combination of both all reduced stomatal conductance compared to the control (Figure 5C). This was a surprising result as PBs have been reported to increase stomatal conductivity [115,118,119]. Plants close their stomata when they experience abiotic stress in order to prevent water-loss [120]. The lowered stomatal conductance could have been a short-term priming response to the BC204 treatments, as only one measurement was taken at the end of the experiment, 90 min after the final BC204 treatment. Alternatively, increased chlorophyll levels and enhanced Fv/Fm by BC204 allowed the plants to photosynthesize more efficiently without the need to open their stomata further. This remains highly speculative since Fv/Fm as an indicator of stress was measured, and photosynthesis was not measured directly. The lack of effect of any of the treatments on stomatal conductance in the sink leaves is most likely explained by the fact that these leaves are still growing and developing and are not yet fully photosynthetic.

BC204 attenuated increased anthocyanin, proline and malondialdehyde levels under NaCl-stress

Anthocyanin and proline accumulation under stress-conditions, such as NaCl-stress, provides important protection in plant leaves [31] possibly mediated by salicylic acid [121]. BC204 reduced the levels of both anthocyanin (Figure 6A) and proline (Figure 6B) in shoot tissues of NaCl-stressed plants. Proline is involved in membrane stability while malondialdehyde is a breakdown product of unsaturated fatty acids [122]. Flavonoids, one of the major ingredients in BC204, are known to be synthesized under stress conditions in the plant. If BC204 provides the plants with anthocyanin-like metabolites or precursors, the plant does not have to produce these itself and can rather invest its energy into primary metabolism such as biomass production. This, however, remains highly speculative as the concentration of BC204 used in the treatments is extremely low and would be unlikely to induce such a major metabolic process. Oxidative stress is known

to increase the activity of antioxidant enzymes such as ascorbate peroxidase, phenol peroxidase, glutathione peroxidase, catalase and dismutase [17,18]. The percentage (%) SOD activity, however, remained unchanged under all experimental conditions (Figure 6D).

BC204 treatment alters the expression of genes involved in the salt response

NaCl-stress induces the expression of a large number of genes, some of which have been flagged as markers for NaCl-stress [123]. For example, NaCl-stress commonly induces the expression of *RESPONSIVE TO DESICCATION* (*RD29A*) [59], *SALT OVERLY SENSITIVE (SOS1*) [124] and *SULFOTRANSFERASE 12* (*SOT12*) [43]. *SOT12* codes for a sulfotransferase known to sulfonate salicylic acid [43]. The up-regulation of *SOT12* by BC204 suggests a possible activation of defense against biotic stress as well as abiotic stress, something not investigated in this study.

The expression of *RD29A* is induced by abiotic stresses such as cold temperatures, drought and saline conditions [124,125], while ABA has also been also been shown to up-regulate the expression of *RD29A* [126]. PBs developed from brown seaweed extracts have been shown to increase the expression of *RD29A* in *A. thaliana* [127], acting as a priming agent. *RD29A* and its homologue, *RD29B,* code for enzymes unlikely to be directly involved in a protectory role in abiotic stress although their exact functions are still unknown [42]. Transcript abundance of *RD29A* is the highest approximately 2 h after salt treatment [16]. In our results, NaCl treatment did not induce the expression of *RD29A* (Figure 7). This is strange but could be explained by the fact that plant tissue was harvested at the end of the experiment, meaning that possible elevated levels of *RD29A* returned to normal at the time of harvesting.

In the absence of *SOS1* expression, plants are highly susceptible to salt stress [128] while the overexpression of this gene results in an increase in salt tolerance [58]. SOS1 is suggested to also be involved in the control of long-distance Na^+ transport from the root to the shoot, where under mild salt stress it loads Na^+ into the xylem and under severe salt stress it retrieves Na^+ from the xylem [50]. Although the expression of *SOS1* is generally more abundant in root tissue under saline conditions, NaCl has also been shown to up-regulate its expression in shoot tissue [124]. As *SOS1* mRNA has a half-life of approximately 10 min [51], expression might have been even higher if the plant tissue was harvested earlier than 90 min after treatment.

P5CS1 codes for an enzyme involved in a rate-limiting step of proline biosynthesis [129]. The unchanged levels of expression of *P5CS1* in the presence of BC204 is in contrast to the reduction in proline content observed in this study following BC204 treatments, and particularly with the increase in proline levels observed following 50 mM salt treatment. As mentioned, the expression of this gene is a rate-limiting step in proline biosynthesis and is also subjected to feedback mechanisms. High levels of proline have been shown to suppress the expression of proline biosynthesis genes in *A. thaliana* [130] and *Enterobacteriaceae* [131]. It is possible that at the time of tissue harvesting, the *P5CS1* mRNA levels had been reduced in response to feedback inhibition, although the metabolite levels remained elevated in response to the stress.

BC204 could aid the plant in dealing with salt stress by stimulating the expression of *RD29A* and *SOS1* in the absence of salt stress. BC204 therefore possibly primes the plant by activating the SOS pathway, which is known to improve salt stress tolerance in plants [38]. Although the function of RD29A is still unknown [42], a study revealed that *RD29A* promoter functions in almost all tissues and organs in vegetative plants during water deficiency [132]. Therefore, it is hypothesized that *RD29A*-induced expression by BC204 further serves as possible priming.

Conclusion

BC204 stimulates plant growth under normal growth conditions by increasing above-ground shoot tissue and root and shoot growth *in vitro*. BC204 also increases chlorophyll content while reducing stomatal conductivity. BC204 furthermore mitigates moderate salt stress (10-20 $dS \cdot m^{-1}$) in *A. thaliana*. Under salt stress conditions, BC204 reduces the levels of proline, anthocyanin and malondialdehyde. The exact mechanism is unknown, but the results in this study indicate that BC204 acts as a priming agent in some sense, stimulating the expression of genes such as *SOS1* and *RD29A*.

This study focused on salt stress at a single growth stage, at the end of vegetative growth. However, investigating how the plant reacts to BC204 during oxidative stress/early salt stress will be valuable as it will give insight into a possible priming effect of BC204 during earlier stages of plant growth. Ion measurements (Na^+ and K^+) will also be valuable to shed light on the effect of BC204. For gene expression, several samples should be harvested at different time points within the experiment to account for the circadian control of specifically genes like *RD29A* [16].

There is an overlap in how plants perceive and respond to salt, drought and, to a lesser extent cold stress as many genes that are regulated by salt stress also respond to cold or drought stress [39]. Therefore, it is possible that BC204 could have similar alleviating effects on these other abiotic stress conditions. This, however, remains purely speculative and an interesting topic for future investigation.

4. Materials and Methods

Plant Material and Growth Conditions

For pot trials, *A. thaliana* (ecotype Columbia-0) seeds were surface decontaminated via vapour sterilization from an adapted protocol [133] by placing open microcentrifuge tubes containing the seed under a glass dome with a beaker containing 100 mL sodium hypochlorite and 2 mL hydrochloric acid (37%) for 4 h. After vapor sterilization, seeds were sown onto peat disks (Jiffy™ no.9, Johannesburg, South Africa), subjected to seed stratification (48 h, 4°C) before being placed in controlled conditions (10 h light, , 22±1°C, 14 h dark, 18±1°C, 75% relative humidity). As the seeds germinated, excess seedlings were removed with forceps until only one seedling remained on each peat disc. Care was taken to ensure that all remaining seedlings were of the same size. Plants were maintained for three weeks and received no fertilizer. Plants were arranged randomly within the growth chamber and harvested in a random order. Plants were treated with 10 mL 0.01% BC204 weekly, starting 21 days after germination (DAG). Also starting 21 DAG, NaCl-treated plants were treated with 10 mL NaCl solution (either 50 mM or 100 mM) every three days via a soil drench. BC204 and NaCl treatment continued for 3 weeks and all tissues were harvested 3 days after the final treatment. Although *A. thaliana* can tolerate and grow normally at 50 mM NaCl [78], the salt concentration in the peat discs was gradually increased to simulate a moderate salt stress rather than imposing an immediate salt shock [78]. Control plants were watered with 10 mL dH_2O and all plants were similarly watered on non-treatment days to prevent the peat discs from drying out. Electroconductivity was measured by at room temperature by combining the wet peat from three plants and gently compressing it before inserting the electrodes from a Procheck soil conductivity sensor (Decagon Devices, Pullman, WA, USA). This was repeated three times for each treatment group.

For in vitro trials, *A. thaliana* seeds were surface decontaminated via vapor sterilization as described above. Surface decontaminated seed was sown onto petri dishes containing half-strength Murashige and Skoog (MS) media (Sigma-Aldrich, St Loius, MO, USA) solidified with 0.9% (*w/v*) Phytoagar (DUCHEFA Biochemie, Haarlem, The Netherlands) with the pH adjusted to 5.8 using potassium hydroxide (KOH). Growth media were sterilized by autoclaving for 20 min at a temperature of 121 °C and pressure of 103 kPa. Five DAG, seedlings were transferred to a CELLSTAR® petri dish (120 mm × 120 mm) (Greiner Bio-One, Frickenhausen, Germany) with media supplemented with 50 mM NaCl, 100 mM NaCl, 0.001% BC204 and combinations of these. The plates were placed almost vertically under cool white fluorescent tubes (Osram L 58V/740, 50 µmol photons.m^{-2}·s^{-1}) in controlled conditions (16 h light, 23 ± 2 °C, 8 h dark, 18 ± 1 °C, 75% relative humidity). After 10 d further growth, the plates were opened and photographed with a Canon camera (model E0S 550D). Primary root length was determined using ImageJ (version 1.49) software [134].

Stomatal Conductance, Fv/Fm Measurements

Ninety minutes after the final BC204 treatment, 12 plants from each treatment group (Control, 0.01% [*v/v*] BC204, 50 mM NaCl, 50 mM NaCl+ 0.01% [*v/v*] BC204) were selected for measurement of stomatal conductance, using a SC-1 leaf porometer (ICT International, Armidale, Australia), and Fv/Fm, using an 0S30p+ chlorophyll fluorometer (Optiscience, Hudson, NH, USA).

Total Chlorophyll Extraction and Quantification

Chlorophyll was extracted from plants grown on peat disks using an adapted protocol [135]. Nine plants were ground to a fine powder using liquid nitrogen in a pre-chilled mortar and pestle. Thereafter, 1 mL dimethylsulfoxide (DMSO) was added to approximately 50 mg ground tissue in a microcentrifuge tube. The extract was vortexed and centrifuged at 10,000 x*g* for 2 min. The supernatant was removed to a fresh tube and retained. The pellet was re-extracted twice in further 1 mL aliquots of DMSO and the supernatant liquids combined in a microcentrifuge tube. Absorbance of the samples was measured in triplicate at 645 nm and 663 nm against a DMSO blank. Total chlorophyll was calculated using the formula: (0.0202 x A_{663}) + (0.00802 x A_{645}).

Anthocyanin, Proline, Malondialdehyde and Superoxide Dismutase (SOD)

Anthocyanins were extracted from plants grown on Jiffy peat disks (Jiffy™ no.9, South Africa) using an adapted protocol [136]. Nine plants were randomly selected per treatment, flash frozen in liquid nitrogen and ground to a fine powder in a pre-chilled mortar and pestle. Total anthocyanins were extracted using a methanol/acetic acid/H_2O (9:1:10) buffer. Absorbance was measured at 530 nm and 637 nm, then anthocyanin content was calculated as $A_{530}-(0.25-A_{637})$ and expressed as Abs530/g·FW.

Proline content was determined using an adapted protocol [137]. Nine plants were selected at random, pooled, flash frozen and ground in liquid nitrogen using a mortar and pestle and proline extracted using approximately 50 mg tissue and 5 µL/mg FW 3% (*w/v*) sulfosalicylic acid (SAS). Samples were vortexed and centrifuged at 10,000 ×*g* for 5 min. A 100 µL aliquot of the supernatant was added to 500 µL of a reaction mixture of 3% (*w/v*) SAS/glacial acetic acid/acidic ninhydrin (1:2:2). This mixture was heated at 95 °C for 60 min before being centrifuged at 10,000 ×*g* for 5 min. Absorbance was measured at 520 nm.

Malondialdehyde was extracted and quantified according to Rao and Sresty [136]. Leaf tissue was homogenized with 0.1% (*w/v*) trichloroacetic acid (TCA), centrifuged at 10,000 ×*g* for 5 min and the supernatant mixed with 20% (*w/v*) TCA and 0.5% (*w/v*) thiobarbituric acid (TBA). The mixture was boiled for 15 min before being transferred to ice and centrifuged at 10,000 ×*g* for 2 min. The absorbance of the supernatant was measured at 532 nm. SOD activity was determined according to manufacturer's protocol using the Invitrogen™ K335-100 Superoxide Dismutase (SOD) Activity Colorimetric Assay kit (ThermoFisher Scientific, Waltham, MA, USA).

RNA Extractions and Reverse Transcriptase Quantitative PCR (RT-qPCR) Analysis

Nine plants from each treatment were randomly selected and pooled into three representative samples (3 plants per sample), flash frozen, ground in liquid nitrogen using a mortar and pestle and total RNA extracted in a Maxwell® 16 AS2000 Instrument with the Maxwell® 16 Total RNA Purification Kit, as per the manufacturer's protocol (Promega, Madison, WI, USA). One microgram of total RNA was used to obtain complementary DNA (cDNA) via reverse transcription using an oligo(dT)18 primer and RevertAid reverse transcriptase (Thermo Scientific™, Waltham, MA, USA), according to manufacturer's protocol. The PowerUp™ SYBR™ Green Master Mix kit and the QuantStudio 3 Real-Time PCR System was used for reverse transcriptase quantitative PCR (RT-qPCR) analysis and the relative expression calculated using the $2^{-\Delta\Delta CT}$ method [80]. Expression of each gene for the untreated control plants was set as 1. *Ef1α* (At1g18070) was used for as an internal control as it has previously been shown to be a suitable reference gene for *A. thaliana* under abiotic stress conditions, including salt stress [138]. Primer sequences for RT-qPCR analyses are given in Supplementary Table S1.

Data and Statistical Analysis

All experiments were independently replicated at least three times to ensure reproducibility. Statistical significance between control, treated and NaCl-stressed groups was determined by the one-way ANOVA function in Excel followed by the Fischer's least significant difference (LSD) test at the 0.05 probability level.

Abbreviations

ABA	abscisic acid
DAG	days after germination
EC	electroconductivity
g·FW	gram fresh weight
LSD	least significant difference
MS	Murashige and Skoog
NaCl	sodium chloride
P5CS	Δ^1-1-pyrroline-5-carboxylate synthetase
PB	plant biostimulant
PCR	polymerase chain reaction
RD29A	responsive to desiccation
ROS	reactive oxygen species
RT-qPCR	reverse transcriptase quantitative PCR
SOD	superoxide dismutase
SOS	Salt Overly Sensitive
SOT12	sulfotransferase 12

Author Contributions: J.L. and P.H. designed the research. J.L. conducted all the experimental work and analysed the data. The research presented in this manuscript will form a part of the PhD dissertation for J.L. at Stellenbosch University. J.L. prepared and P.H. edited and revised the manuscript.

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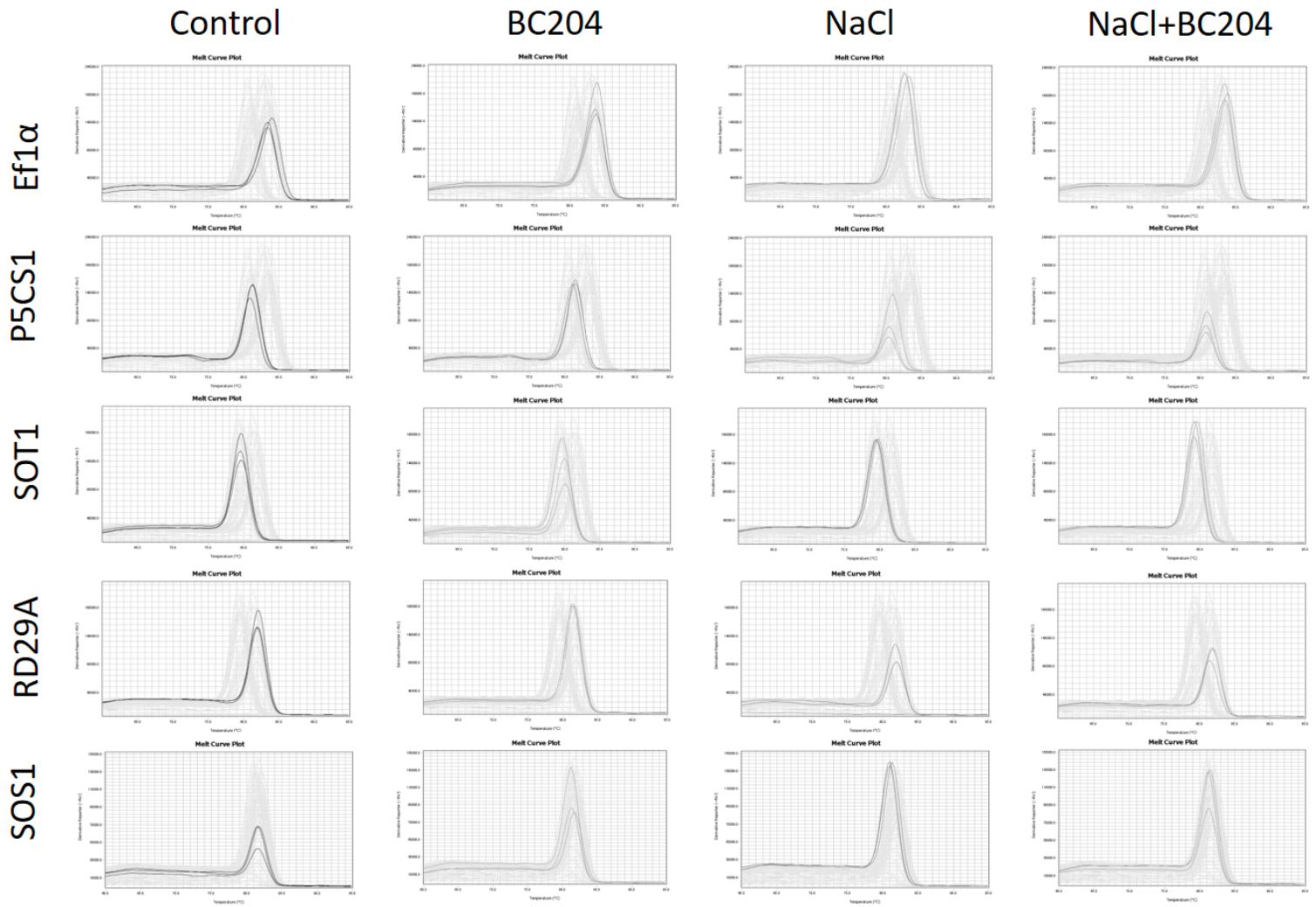
Supplementary Material

Table S2.1 Primer pairs sequences for RT-qPCR analysis in *Arabidopsis thaliana*

Gene ID	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size	Annealing temperature
At2g39800	*P5CS1*	AGGTCATGCTGATGGAATCTGT	GGCTGCTGGATAGTCCAACTT	103 bp	60°C
At5g52319	*RD29A*	GAGCTCCGTTGGGAGGAAAT	GGTTCTCCGTCAAATCCCGT	103 bp	60°C
At2g03760	*SOT12*	CGAAAAAGCGGTTGAAGCGT	GATTCTCGCGGCTTGCATAC	95 bp	60°C
At2g01980	*SOS1*	CCCAGCTCAAGGTCTCGTTT	TTCAGAGGAAGCTGACACGC	96 bp	60°C
At1g18070	*EF1α*	CGAAAACCCTAGACACCTCGT	TCTGAAAGGAGTCTTGCGGC	105 bp	60°C



Suppl Fig 2.1 Melt curves of all the genes used for RT-qPCR analysis

CHAPTER 3: A citrus-based plant extract stimulates plant growth by inducing cell wall biogenesis, photosynthesis, and stress-related gene expression

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3.1 Abstract

BC204 is a citrus-based plant extract used as a plant biostimulant on a variety of plant species in South Africa, China and Australia. Although there are reports that it elicits physiological responses such as an increase in crop yield and fruit quality, no molecular data is available to explain the specific mechanisms associated with the increase in plant growth and tolerance to environmental stresses. In this study, an RNA-seq approach was adopted to elucidate the effects of BC204 at the molecular level in *Arabidopsis thaliana*. BC204, applied via a soil drench at low concentrations of 0.01% (v/v), stimulated above-ground biomass production whilst eliciting a large change in gene expression levels across several biochemical pathways. Of the entire transcriptomic profile examined, a total of 8.212% of genes were significantly differentially expressed between the treated and control groups, of which 5.136% were upregulated and 3.076% downregulated. Most notably, genes involved in photosynthesis, several aspects of cell wall biogenesis, remodelling and restructuring, carbohydrate metabolism, signalling, stress and secondary metabolism were upregulated, which could explain the increase in plant growth. Genes related to transcription and RNA regulation were both strongly up- and downregulated, which suggests that BC204 plays a role in inducing and suppressing several pathways. This novel study provides valuable information to be used as starting point for targeted future research.

Keywords: BC204, *Arabidopsis thaliana*, RNA-seq, plant biostimulant, gene expression, RT-qPCR, transcription, RNA regulation, secondary metabolism, signalling, hormone metabolism

3.2 Introduction

The need for more sustainable agriculture in recent decades has led to the search for novel plant growth promoting substances (PGPS) to increase agricultural output to feed a growing world population while simultaneously minimizing the impact on the environment. This is known as agricultural intensification (Struik and Kuyper, 2017). Plant biostimulants (PB) have been identified as a group of natural extracts or compounds set to revolutionize agriculture because of their broad range of effects on plant growth, as extensively reviewed previously (Bulgari et al., 2015). PBs stimulate plant growth by, but not limited to, enhancing tolerance to abiotic (Bulgari et al., 2019; Van Oosten et al., 2017) and biotic stress (Bargiacchi et al., 2012; Guinan et al., 2013;), photosynthesis (Bulgari et al., 2019; Drobek et al., 2019; Wilson et al., 2015), auxin, ethylene, jasmonic acid and salicylic acid signalling (Agarwal et al., 2016; Eshraghi et al., 2011; Ghaderiardakani et al., 2018; Trevisan et al., 2019), primary and secondary metabolism (Jithesh et al., 2018; Khan et al., 2011; Santaniello et al., 2012), yield and nutraceutical potential (Kocira et al., 2017) and nutrient uptake (Drobek et al., 2019; Halpern et al., 2015; Xu and Geelen, 2018). The broad range of physiological and biochemical processes stimulated by PBs from different origins has been extensively reviewed (Yakhin et al., 2017).

Despite the progress made in characterising the observed effects of PBs on general plant growth and physiology, major questions remain unanswered regarding the specific mechanisms involved. Although numerous published studies on PB are available, only a few have attempted to unravel the molecular mechanisms responsible for the observed physiological effects. The result is that marketing of these products is being held back because the companies do not have the necessary peer-reviewed evidence to back up their claims (Caradonia et al., 2018), which limits the sales and distribution of their products. Furthermore, the studies that have been conducted are mostly only relevant to the specific product they are testing on the non-model plant species of interest (reviewed in Chapter 1 of this dissertation).

BC204 has been used to great success as a PB in several countries for crop improvement, with unpublished reports that it increases crop yield, fruit quality, shelf life, overall plant growth and development and tolerance to stress conditions in a variety of plant species including, but not limited to, tomatoes, tropical fruits, citrus, grapes, bananas and several nut tree species. However, there are no biochemical or molecular data available to explain and validate these results seen in practice.

Methodologies implemented to study the effect of PBs on plant growth are high-throughput plant phenotyping (Rouphael et al., 2018b; Ugena et al., 2018), high-throughput physiological functional phenotyping (Dalal et al., 2019) and limited biochemical analysis (Parađiković et al., 2018). Molecular characterization has also been used to a much smaller extent in the past, with the majority of studies using quantitative Reverse Transcriptase-PCR (RT-qPCR) analysis (Ali et al., 2019; Fleming et al., 2019; Upadhyay and Maier, 2016), which enables researchers to investigate the expression of a small set of genes in order to hypothesize the effects that PBs have on certain genetic pathways.

Due to the broad range of physiological effects reported (Bulgari et al., 2019, 2015; Calvo et al., 2014; Colla et al., 2015; Yakhin et al., 2017), it would be fair to assume that PB would have some overlap in gene expression. Recently, some researchers have begun using more holistic approaches such as

transcriptomics to study the effects of PBs on plants (Povero et al., 2016), since the effects of PBs are themselves wide ranging, including effects on transcription, carbohydrate metabolism protein metabolism, secondary metabolism, transport, signalling and genes involved in plant defence (Contartese et al., 2016; Ertani et al., 2013; Jannin et al., 2013; Sestili et al., 2018; Shukla et al., 2018; Weeda et al., 2014).

Microarray studies have been used to analyse the effects of PBs at the molecular level, mostly in *Arabidopsis* (Fleming et al., 2019; Trevisan et al., 2010; Wally et al., 2013). In *A. thaliana*, chitosan was shown to induce defence against biotic stress (*Botrytis cinerea*) by upregulating genes involved in camalexin biosynthesis through an CERK1-independent pathway (Povero et al., 2011). In another study, *A. thaliana* was treated with raw plant-derived protein extracts and gene expression mimicked plant responses to osmotic stress and ABA treatment, while inducing the transcription of senescence-related *DIN* genes, which are typically expressed in the absence of light and during sugar starvation. *DIN* gene expression is also induced during natural leaf senescence (Santaniello et al., 2012). Two other studies in *Arabidopsis* revealed that *Ascophyllum nodosum* seaweed extracts induced salt tolerance by regulating the expression of abiotic and redox stress-responsive genes (Goñi et al., 2016; Jithesh et al., 2018). Another PB known as CYT31 induced the expression of genes involved in ROS scavenging in *Arabidopsis* plants exposed to drought stress (Blaszczak et al., 2016). In a study where maize seedlings were treated with protein hydrolysates, microarray analysis revealed the role of genes related to cell wall organization, stress response, transport processes and hormone metabolism (Santi et al., 2017). Although microarrays were successfully used in these studies, transcriptomic analysis in the form of next-generation sequencing, specifically RNA sequencing (RNA-seq), has been underutilized in PB research.

RNA-seq enables scientists to follow the expression of novel genes that have not previously been annotated (Brunskill and Steven Potter, 2012). This is advantageous in a study where no or limited prior molecular or biochemical data is available on the effect of the specific PB, as is the case with BC204. An RNA-seq study on cucumber seedlings treated with a gelatin seed capsule revealed that genes coding for amino acid and nitrogen source transporters were upregulated. Furthermore, two transcriptional factors regulating xenobiotic detoxification could also explain the enhanced growth and increased abiotic stress tolerance (Wilson et al., 2015). Only three studies so far have adopted an RNA-seq approach for *A. thaliana* plants treated with PBs. RNA-seq analysis combined with metabolite profiling revealed that *Ascophyllum nodosum* extracts mediated freezing tolerance by increasing the accumulation of osmoprotectants and altering cellular fatty acid composition through a large shift in gene expression towards proline biosynthesis (Nair et al., 2012). In another study, RNA-seq analysis of miRNAs revealed that *A. nodosum* extracts mitigated salinity stress by modulating the expression of miRNAs involved in nutrient acquisition and salinity stress tolerance (Shukla et al., 2018). Melatonin, only recently classified as a PB (Arnao and Hernández-Ruiz, 2019), induced the expression of genes involved in plant defence and also genes involved in abscisic acid (ABA), ethylene, salicylic acid and jasmonic acid pathways at several levels, including biosynthesis and signalling. At the same time, genes pertaining to auxin signalling and responses, peroxidases and those associated with cell wall modifications and synthesis were downregulated (Weeda et al., 2014).

Choosing to use a model species with a fully sequenced and thoroughly annotated genome is important as it will provide more information than using a non-model species. *A. thaliana* is a classical

genetic model because it has a small genome which is fully sequenced and annotated, and a wide variety of mutants are available which aid any future research and verification of data obtained by transcriptomic analysis (Koornneef and Meinke, 2010). It has been the major plant model system for the past three decades (Chang et al., 2016) and any information gained by RNA-seq analysis would be valuable, both academically and agriculturally.

Due to the absence of any peer-reviewed data on BC204, this study aimed to determine the effects of BC204 treatment on *A. thaliana* at the molecular level, using an RNA-seq approach to investigate the effects of BC204 on gene expression in *A. thaliana* shoot tissue after three weeks of treatment with 0.01% (v/v) BC204. BC204 induced a large shift in gene expression towards increased photosynthesis, cell wall biosynthesis, signalling and major changes in transcription, regulation of transcription and large gene families. The results of this study provide a solid platform that can be used to further investigate specific biochemical pathways in order to confirm the effects seen at the molecular level. Simultaneously, the results obtained here corroborate and explain some of the effects observed at the phenotypic and biochemical level (see Chapter 2**)**. This study highlights the importance of using next-generation sequencing as an important analytical tool to characterise the effect of PBs on plant growth.

3.3 Materials and Methods

3.3.1 Plant Material and growth conditions

Arabidopsis thaliana (ecotype Columbia-0) seeds were surface decontaminated via vapour sterilisation from an adapted protocol (Lindsey et al., 2017) by placing open microcentrifuge tubes containing the seed under a glass dome with a beaker containing 100 mL sodium hypochlorite and 2 mL hydrochloric acid (37% [w/w]) for 4 h. After vapour decontamination, seeds were sown onto peat disks (Jiffy™ no.9, South Africa), and subjected to seed stratification for 48 h at 4°C before being grown under controlled conditions in a Snijders Economic Deluxe controlled environment growth chamber. Growth conditions were 120 µMol photons/s/m^2 under cool white fluorescent tubes (Osram L 58V/740) in a 10 h:14 h light:dark photoperiod with day:night temperatures of 22:18±1°C at 75% relative humidity. As the seeds germinated, excess seedlings were removed using forceps until only one seedling remained on each peat disc. Care was taken to ensure that all remaining seedlings were of the same size. Plants were maintained for three weeks and received no fertiliser. Plants from the different treatments were arranged randomly within the growth chamber and harvested in a random order. Plants were treated with 10 mL 0.01% (v/v) BC204 weekly, starting 21 days after germination (DAG). All experiments were conducted using the same batch of BC204 extract. Control plants were watered with 10 mL dH_2O and all plants were similarly watered on non-treatment days to prevent the peat discs from drying out. After 21 d further growth, the plants were photographed with a Canon E0S 550D camera. The rosette tissue was harvested for fresh biomass determination before being oven dried for 48h at 70°C for dry biomass determination.

3.3.2 Data and statistical analysis

All growth experiments were independently replicated at least three times to ensure reproducibility. Statistical significance between control and treated groups was determined by the one-way ANOVA function in Microsoft Excel, followed by the Fischer's least significant difference (LSD) test at the 0.05 probability level.

3.3.3 RNA extractions for RNA-seq and quantitative RT-PCR

Arabidopsis rosette tissue was harvested after four weeks of BC204 treatment, 90 min after the final treatment. A total of 9 plants per treatment were selected at random, harvested and divided into three pooled samples, each pool containing the rosette tissue of three plants. Total RNA was then extracted from each of the three pooled samples for each treatment (n=9 plants per treatment). The plant tissue (~100 mg) was ground in liquid nitrogen using a pre-chilled mortar and pestle and total RNA extracted in a Maxwell® 16 AS2000 Instrument with the Maxwell® 16 Total RNA Purification Kit, as per the manufacturer's protocol. RNA integrity was determined using a Agilent 2100 Bioanalyzer (Agilent Technologies, USA) at the Central Analytical Facilities (CAF) at Stellenbosch University, with RNA samples with RIN scores of 9 or above used for library construction.

3.3.4 Library preparation and Illumina sequencing

Library preparation and sequencing from the purified RNA were performed at the Agricultural Research Council Biotechnology Platform (South Africa). Sample preparation was conducted using 1 µg of RNA, quantified using an Invitrogen Qubit fluorometer. Library preparation utilised the Illumina TruSeq

Stranded mRNA library Kit which preferentially amplifies polyA RNA, according to the manufacturer's instructions. The quality of the constructed libraries was confirmed using a PerkinElmer LabChip® GX system. The libraries were then sequenced on the Illumina HiSeq 2500 Platform using the version 4 sequencing chemistry, which resulted in the generation of 10 million paired end reads of 125 nucleotides (nt) in length.

3.3.5 Differential gene expression analysis

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

3.3.6 Gene Ontology (GO) and gene enrichment analysis

Several online software and databases were consulted in order to provide a more complete visual representation of the differentially expressed genes. These include The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org), UniProt (https://www.uniprot.org), Mercator v.3.6 (https://plabipd.de/portal/mercator-sequence-annotation), PANTHER (www.pantherdb.org), DAVID (https://david.ncifcrf.gov) and MapMan (https://mapman.gabipd.org). PageMan analysis, coupled with a Wilcoxon test (Wilcoxon, 1945), was also used to visualize gene expression involved in specific processes in greater detail. The web-based AgriGO V2.0, specifically the Singular Enrichment Analysis (SEA) visualization feature, was also utilized to create a summary of GO ontologies associated with BC204 action (Tian et al., 2017).

3.3.7 Quantitative RT-qPCR analysis

One microgram of the same DNase-treated total RNA as was used for the RNA-seq analysis was used to obtain complementary DNA (cDNA) *via* reverse transcription using an oligo$(dT)_{18}$ primer and RevertAid reverse transcriptase (Thermo Scientific™, United States), according to manufacturer's protocol. The PowerUp™ SYBR™ Green Master Mix kit and the QuantStudio 3 Real-Time PCR System was used for quantitative RT-qPCR analysis and the relative expression calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *MON1* (At2g28390) was used for as an internal control as it has previously been shown to be a suitable reference gene for *A. thaliana* (Czechowski et al., 2005; Pholo et al., 2018), and expression levels for *MON1* were stable across all the RNAseq analyses. For each sample, 1 µL of cDNA and 0.8 µL of each primer (10µM, Supplementary Table S3.2)) was added to the PowerUp™SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) and each reaction mixture transferred to a 0.1 mL MicroAmp™, optical 96-well clear reaction plate (Applied Biosystems, USA). The reactions were performed using a Quantstudio™ 3 Real-Time PCR system (Thermo Fisher Scientific, USA). The incubations for the RT-qPCR reactions were

as follows: 95°C for 10 min to activate the Dual-Lock *Taq* DNA polymerase, followed by 40 cycles of 95°C for 15 s (Denaturation) and 60°C for 1 min (Annealing and Extension).

3.4 Results

3.4.1 Dose-dependence and mode of treatment of BC204 for Arabidopsis thaliana

In order to determine the most optimal concentration and mode of BC204 treatment, *A. thaliana* plants were treated with different concentrations (0.001%; 0.01%; and 0.05% [v/v]) of BC204 via soil drench and foliar spray methods. There were no statistically significant changes in shoot biomass production between the different concentrations or methods of treatment (data not shown). It was therefore decided to move forward with a soil drench concentration of 0.01% (v/v) in order to ensure that the same amount was applied to all plants, since a foliar spray cannot guarantee that each plant receives the same amount of BC204.

3.4.2 BC204 increased Arabidopsis thaliana *above-ground fresh and dry biomass*

To investigate the effect of BC204 on plant shoot biomass, similarly-sized 3-week old *Arabidopsis thaliana* Col-0 plants were treated with BC204 and the control group only with water via a soil drench. The control group (Figure 3.1A) received water and the BC204-treated group (Figure 3.1B) was drenched with 10 mL 0.01% (v/v) BC204 once every 6 days, and all plants were watered routinely every second day. An increase in plant shoot growth was clearly visible in the BC204-treated plants, which were visibly larger than the plants in the control group. Fresh (Figure 3.2A) and dry (Figure 3.2B) mass were also significantly higher in BC204-treated plants compared with the control plants.

Figure 3.1 Above-ground biomass production of *Arabidopsis thaliana* Columbia-0 plants treated with either water as a control group (A), or a soil drench of 0.01% (v/v) BC204 (B). BC204-treated plants (**B**) were visibly larger than their untreated counterparts (**A**).

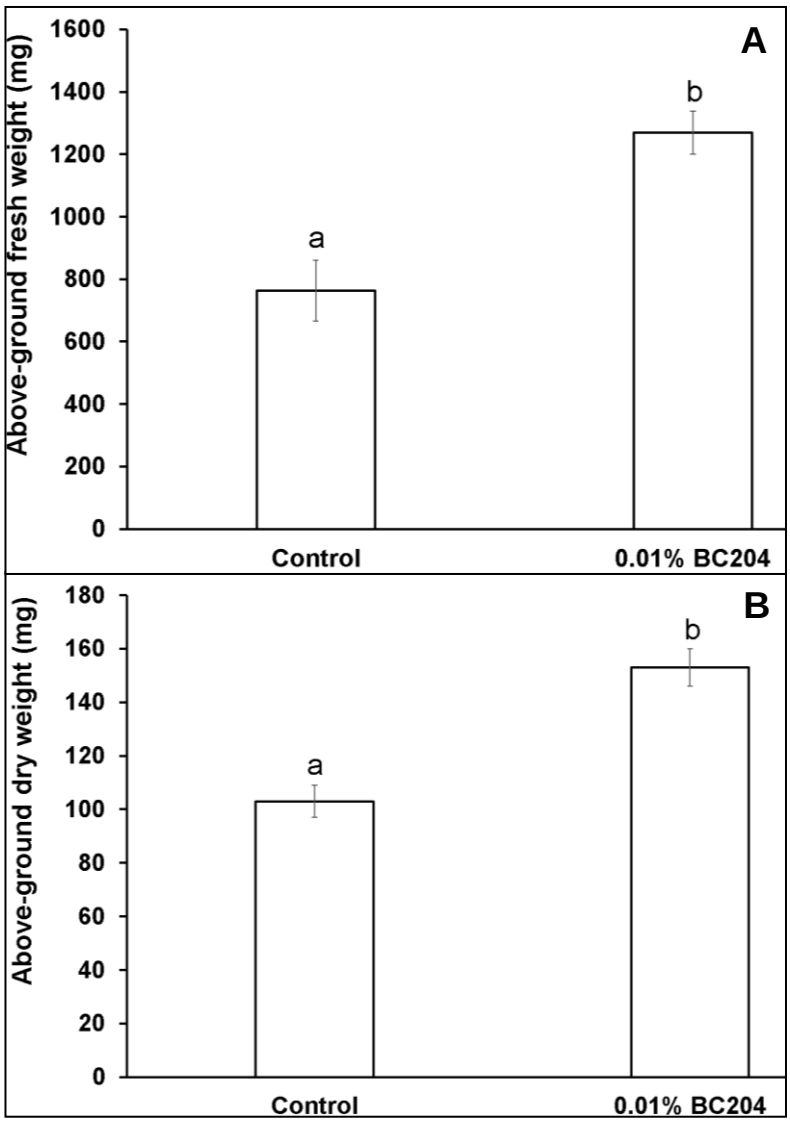


Figure 3.2 Fresh and dry weight of above-ground biomass of *Arabidopsis thaliana* plants treated with BC204. BC204 treatment (0.01% [v/v]) increased fresh (**A**) and dry (**B**) biomass production in *Arabidopsis thaliana* compared to the control plants. Bars represent the mean of 12 (n=12) replicates ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different (P<0.05) from the control.

3.4.3 Transcriptomic comparison of untreated versus BC204-treated Arabidopsis *shoot tissue samples*

Illumina RNA sequencing of the 6 samples (two different growth conditions, with three biological replicates for each) generated a total of 45.155423 million single reads, translating to a mean of 7.525903 million reads per sample. After trimming the data, a total of 41.094641 million reads remained, translating to a mean of 6.849107 million reads per sample.

Out of the 32710 transcripts analysed, a total of 2686 (8.212%) were significantly differentially expressed between the treated and control groups, of which 1680 (5.136%) were upregulated and 1006 (3.076%) were downregulated (Table 3.1). Most upregulated genes, 1399 (83.27% of the total upregulated genes), had a $\log_2$fold value ranging between 0 and 1. Most downregulated genes, 819 (81.4% of the total

downregulated genes), had a negative log_2fold value smaller than 0 but larger than -1. A total of 11 genes were upregulated with a log_2fold value of larger than 3 (Table 3.2). The ''inf'' value calculated from the transcriptomic analysis was changed to and displayed as a value of a 100 in order to ease downstream analysis and visualization of the data. However, these transcripts are unlikely to be biologically relevant and are generally left out of downstream analyses (Yendrek et al., 2012). A total of 6 genes were downregulated by a log_2fold of at least -3 (Table 3.3). ITAG2.3 descriptions were obtained from The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org; 19 October 2019) database, while The Universal Protein Resource (UniProt; https://www.uniprot.org; 23 October 2019) and EMBL-EBI Expression Atlas (https://www.ebi.ac.uk; 23 October 2019) were also consulted for further descriptions, gene information and function. A complete list of upregulated (Supplementary Table S3.3) and downregulated (Supplementary Table S3.4) genes (log_2fold change values of at least 1 or -1) can be found in the Supplementary Material section of this chapter.

Table 3.1 Differentially expressed genes (DEG) significantly altered by BC204 (0.01% [v/v]) treatment compared to the control in *Arabidopsis thaliana* shoot tissue

	Number of DEG	Number of upregulated genes	Number of downregulated genes
All	2686	1680	1006
Filtered (>1log_2fold)	468	281	187
Filtered (>2log_2fold)	90	57	33
Filtered (>3log_2fold)	17	11	6

Table 3.2 Genes significantly upregulated by a log_2fold of larger than 3 in *Arabidopsis thaliana* shoot tissue following BC204 (0.01% [v/v]) treatment

Gene ID (AGI)	ITAG2.3 descriptions	Name and accession number (UniProt/Expression Atlas)	Expression Atlas (gene information)	Log_2ratio	q-value (adj. p≤ 0.05)
AT1G21910	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family.	Accession: Q9SFE4 Name: ERF012.	Ethylene activated DNA-binding transcription factor activity in response to freezing, jasmonic acid, salicylic acid and heat.	4.23074	0.000776133
AT5G57560	Encodes a cell wall-modifying enzyme.	Accession: Q38857 Name: Xyloglucan endotransglucosylase/hydrolase protein (XTH22).	Xyloglucosyl transferase and hydrolase activity; response to auxin, mechanical stimulus, cell wall biogenesis, carbohydrate metabolic process, cold, heat.	3.97935	0.000776133
AT1G66760	MATE efflux family protein.	Accession: Q9C9M8 Name: Protein DETOXIFICATION 9 (DTX9).	Drug transmembrane transporter activity, antiporter activity, response to wounding, integral component of membrane.	3.78785	0.000776133
AT4G28040	Nodulin MtN21-like transporter family protein.	Accession: Q9SUD5 Name WAT1-related protein.	Transmembrane transporter activity, plasma membrane, integral component of membrane.	3.72004	0.00477784
AT2G20670	Sugar phosphate exchanger, putative (DUF506).	Accession: Q9SIU5/Q9SK36 Name: F23N11.1.	Protein of unknown function, PDDEXK-like, expressed in the chloroplast (Q9SIU). Microtubule binding, chromosome segregation (Q9SK36).	3.60024	0.000776133

AT5G52300	Encodes a protein that is induced in expression in response to water deprivation such as cold, high-salt, and desiccation. The response appears to be via abscisic acid.	Accession: Q04980 Name: Low-temperature-induced 65 kDa protein (LTI65).	Response to abscisic acid, salt stress, leaf senescence and water deprivation.	3.55512	0.000776133
AT4G08950	Phosphate-responsive 1 family protein.	Accession: Q9ZPE7 Name: Protein EXORDIUM (EXO).	Response to brassinosteroid, required for cell expansion in leaves, possibly involved in signalling process coordinating BR responses with environmental or developmental signals.	3.32798	0.000776133
AT1G50040	formin-like protein, putative (DUF1005).	Accession: Q9LPM5 Name: F2j10.8 protein.	Protein of unknown function DUF1005 family.	3.2814	0.000776133
AT1G49500	Transcription initiation factor TFIID subunit 1b-like protein.	Accession: Q9XIB7 Name: F13F21_6	Translation initiation factor activity.	3.20134	0.000776133
AT1G35140	EXL1, EXODIUM LIKE 1, PHI-1, PHOSPAHTE-INDUCED 1, a mutant of this gene showed diminished biomass production.	Accession: Q9C6E4 Name: Protein EXORDIUM-like 1 (EXL1)	May play a role in a brassinosteroid-dependent regulatory pathway that controls growth and development under low carbon and energy availability.	3.12155	0.000776133

Table 3.3 Genes significantly downregulated by a log_2fold of at least -3 in *Arabidopsis thaliana* shoot tissue following BC204 (0.01% [v/v]) treatment

Gene ID (AGI)	ITAG2.3 descriptions	Name and accession number UniProt/Expression Atlas	Expression Atlas (gene information)	Log_2ratio	q-value (adj. p≤ 0.05)
AT4G16640	Collagen catabolism.	Accession: O23507 Name: Metalloendoproteinase 1-MMP (1MMP)	MMPs may play a role in the degradation and remodelling of the extracellular matrix (ECM) during development or in response to stresses.	-3.80444	0.0219843
AT4G12735	Mitochondrion function.	Accession: Q8LCC1 Name: At4g12735	Possible involved in the peroxisome and plasma membrane.	-3.20858	0.0494053
AT2G17660	RPM1-interacting protein.	Accession: Q9SEY4 Name: At2g17660	Defense response signalling pathway, resistance gene-independent, located at the plasma membrane.	-3.11634	0.0062571
AT1G20180	DUF677 transmembrane protein	Accession: Q6DYE5 Name: UPF0496 protein	Integral component of membrane.	-3.03657	0.00142905
AT2G36780	UDP-Glycosyltransferase superfamily protein.	Accession: Q9ZQ96 Name: UDP-glycosyltransferase 73C3 (UGT73C3)	Transferase activity, transferring hexosyl groups, UDP-glycosoltransferase activity.	-3.02382	0.000776133

Mercator (Schwacke et al., 2019) was used to assign significantly up and downregulated genes into functionally categorized groups. Apart from the genes not assigned into a bin (25.56%), miscellaneous (8.30%), RNA metabolism (7.13%), signalling (6.36%), cell wall (4.95%), stress (4.77%), transport (4.59%) and PS (4.24%) related genes were the most highly upregulated by BC204-treatment (Figure 3.3). The downregulated processes included not assigned (27.09%), RNA (13.45%), protein (13.25%), transport (6.47%) and development (4.58%) (Figure 3.4). As an extension of this analysis, up and downregulated genes were assigned into categories by the Protein Analysis THrough Evolutionary Relationships (PANTHER) Classification System Version 14.1 (Thomas et al., 2003; www.pantherdb.org) based on cellular component, biological process, molecular function (Figure 3.5) and protein class (Figure 3.6). BC204 simultaneously induced and suppressed almost the same number of genes in every biochemical process (Figure 3.5). More than 100 genes were upregulated and downregulated in the cell and organelle (GO: cellular component), in metabolic and cellular processes (GO: biological process) and catalytic activity and binding (GO: molecular function). Only two of the 28 categories, protein-containing complex and transporter activity, had more genes that were downregulated than upregulated. More than 70 genes were upregulated in the protein class sections hydrolases, oxidoreductases and transferases (Figure 3.6). Proteins associated with transcription factors, signalling molecules and nucleic acid binding had more downregulated than upregulated genes

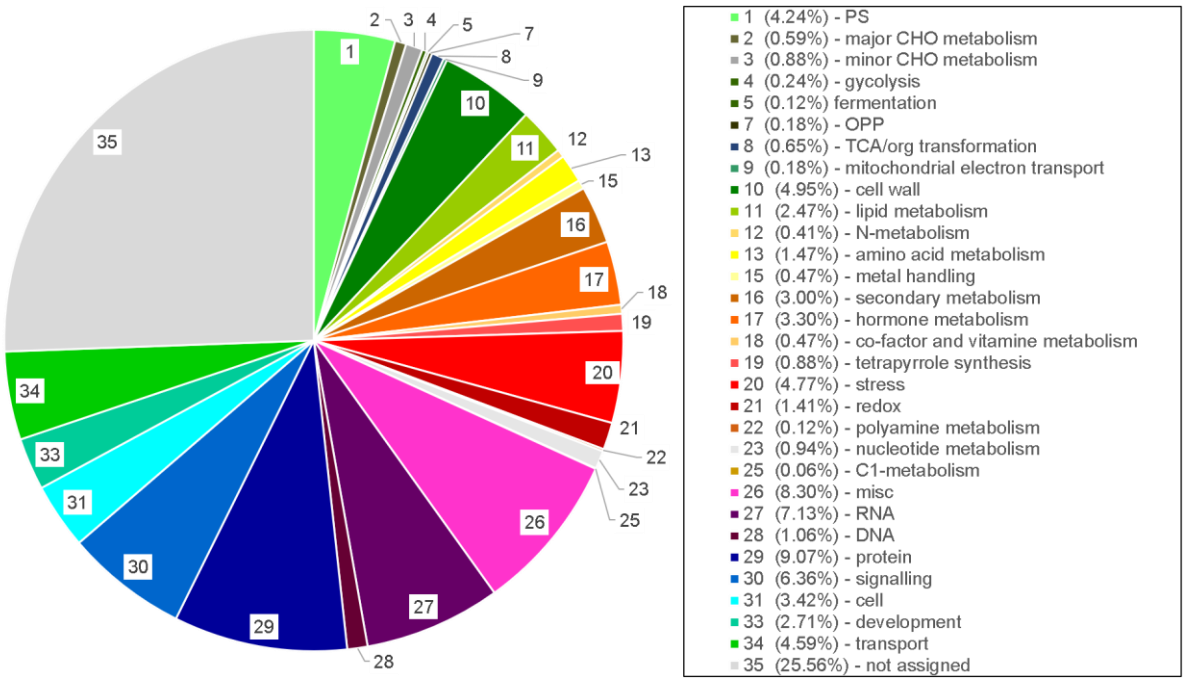


Figure 3.3 Functional biological classification by Mercator of significantly upregulated genes in *Arabidopsis thaliana* plants treated with 0.01% BC204 soil drench. Upregulated genes were assigned by the Mercator functional annotation tool into different bins. The differentially expressed transcripts are indicated by percentiles.

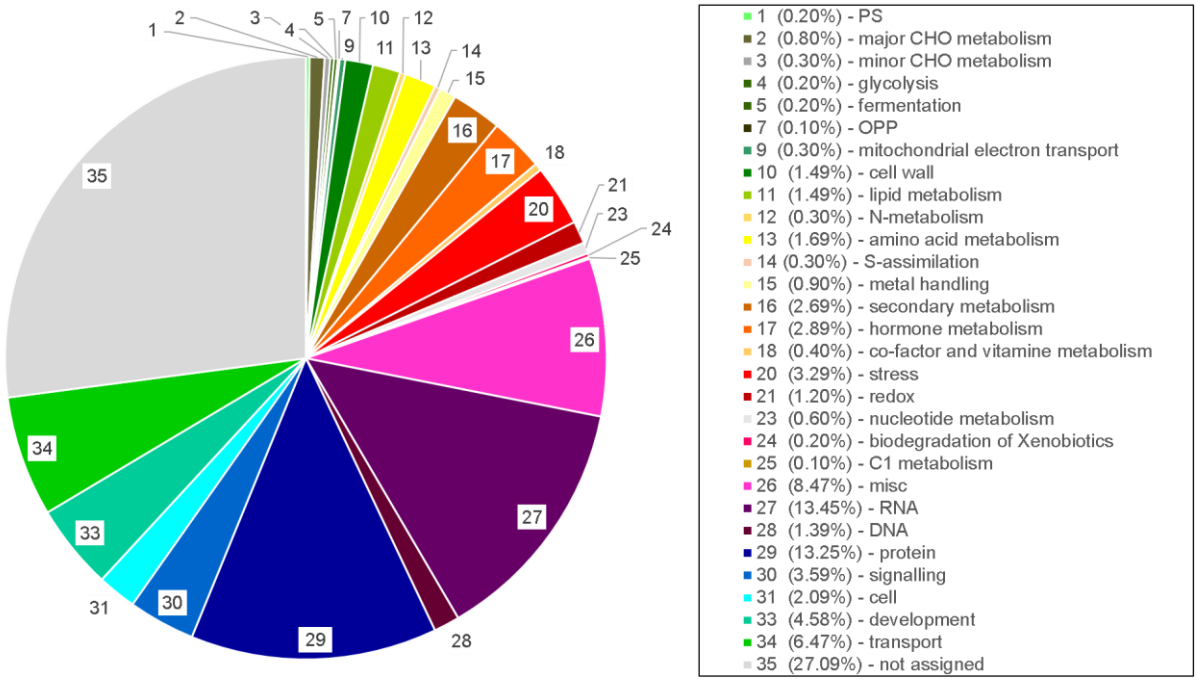


Figure 3.4 Functional biological classification by Mercator of significantly downregulated genes of *Arabidopsis thaliana* plants treated with 0.01% BC204. Downregulated genes were by the Mercator functional annotation tool into different bins. The differentially expressed transcripts are indicated by percentiles.

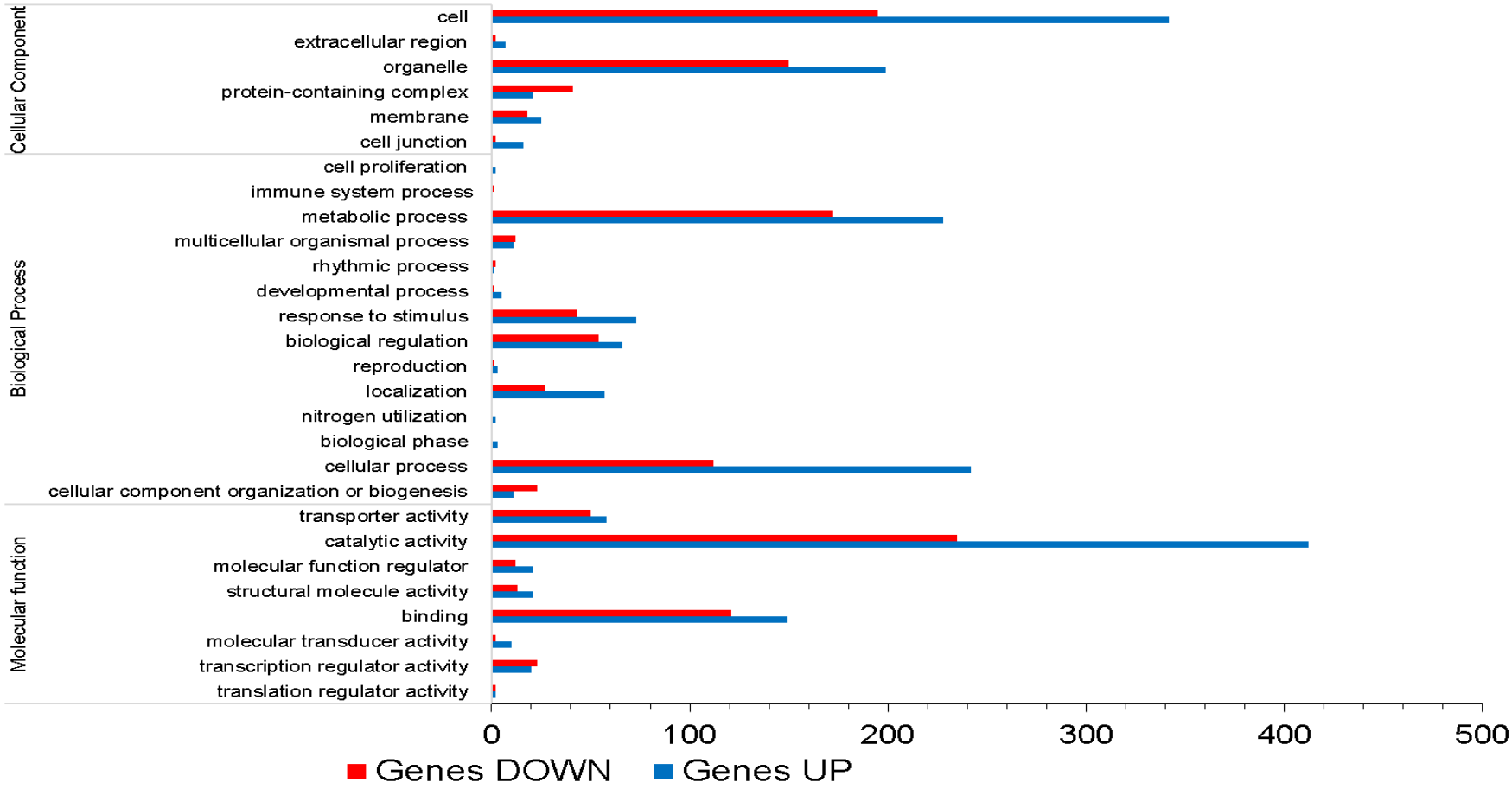


Figure 3.5 Up and downregulated genes in *Arabidopsis thaliana* shoot tissue elicited by BC204 soil drench treatment functionally categorised into bins by the PANTHER database into either cellular component, biological process or molecular function gene ontologies. Red bars represent total downregulated genes, while blue bars indicate total upregulated genes.

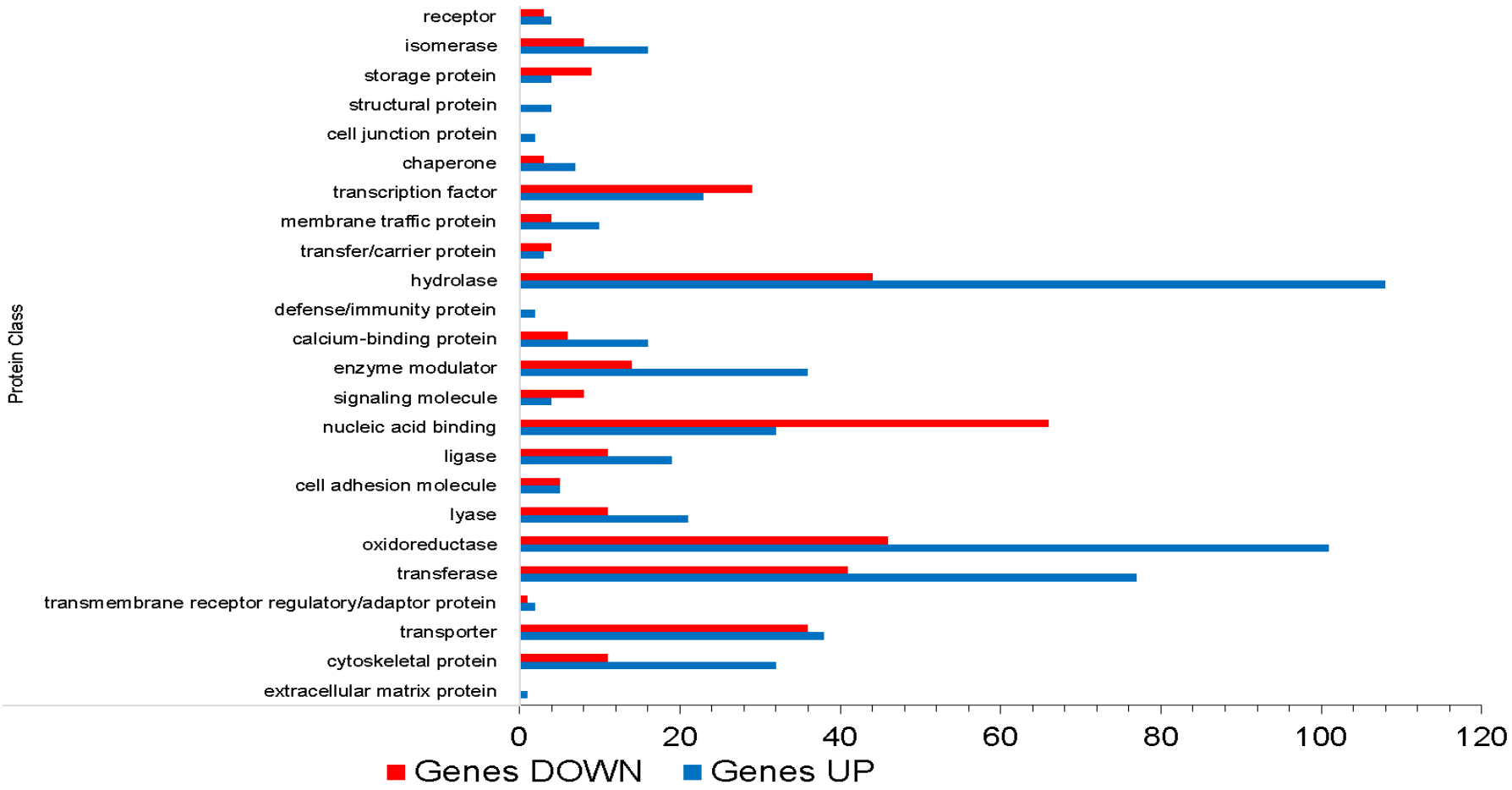


Figure 3.6 Up and downregulated genes in *Arabidopsis thaliana* shoot tissue categorised into bins by the PANTHER database into different protein classes. Red bars represent total downregulated genes while blue bars indicate total upregulated genes.

In addition to Mercator, Mapman annotation (Thimm et al., 2004) was adopted for the *A. thaliana* transcriptome using the mapping file (Ath_AGI_LOCUS_TAIR10_Aug2012.m02) to assign genes to 35 functional categories (Figure 3.7). A total of 32971 out of 32709 transcripts were mapped, with some of the data points mapped multiple times to different bins. Figure 3.7 represents 2696 data points. Most upregulated genes (blue) were involved in cell wall biogenesis, lipid metabolism, minor carbohydrates (CHO), light reactions, calvin cycle, tetrapyrrole and several aspects of secondary metabolism.

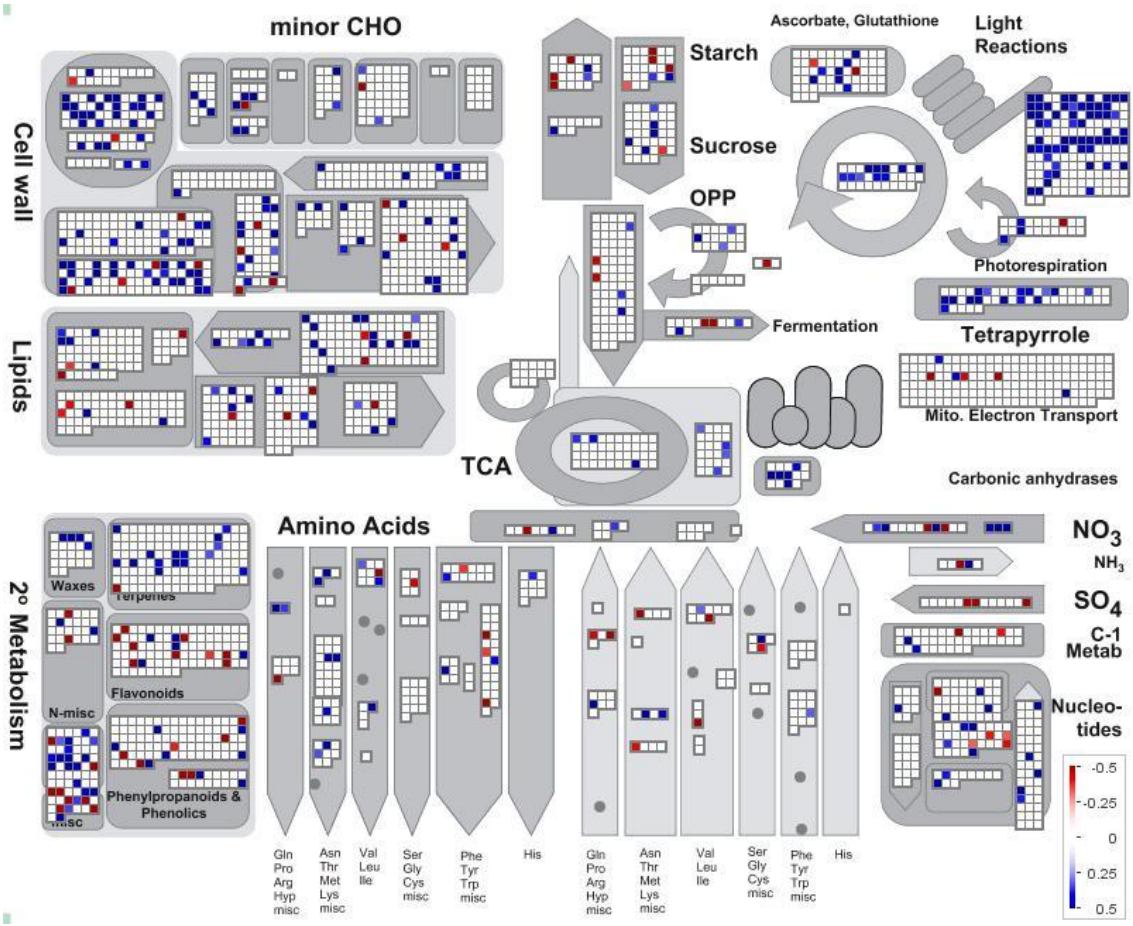


Figure 3.7 MapMan general overview of BC204-treated *Arabidopsis thaliana* DEGs in above-ground shoot tissue relative to the untreated control using the mapping file (Ath_AGI_LOCUS_TAIR10_Aug2012.m02). A total of 32971 genes out of 32709 were mapped, with some of the data points mapped multiple times to different bins. A total of 2696 data points is visible. Genes that were shown to be differentially expressed using $p < 0.05$ as a cut-off value were imported. Blue boxes represent genes that were upregulated while red indicates genes that were downregulated by BC204. Intensity of the colours are indicative of the levels of expression.

Of the 468 DEGs with a $\log_2$fold larger than 1 and smaller than -1, 85 annotated genes were characterised into the major MapMan ontology bins of Metabolism_overview (Table 3.4). These genes are mostly involved in cell wall metabolism, lipid metabolism, energy, secondary metabolism and amino acids, with a few involved in N-metabolism, S-assimilation and nucleotide metabolism.

As an extension of MapMan functional categorisation, PageMan analysis (Usadel et al., 2006) and a Wilcoxon test (Wilcoxon, 1945) were conducted to provide a more detailed visualisation of genetic changes

elicited by BC204 of gene expression altered with a log_2fold change of between -1 and 1 (Figure 3.8). An even more detailed figure, including all up and downregulated processes, may be found in the *Supplementary Material* (Supplementary Figure S4.4). As observed with the MapMan analysis output, BC204 altered a large set of processes, as categorized into different bins. The largest concentration of upregulated processes was photosystem (PS), oxidative pentose phosphate pathway (OPP), tricarboxylic acid cycle (TCA), cell wall, lipid metabolism, hormone metabolism, co-factor and vitamin metabolism, stress, DNA and signalling. The largest concentration of downregulated processes was in mitochondrial electron transport/ATP synthesis, N-metabolism, amino acid metabolism, S-assimilation, secondary metabolism, redox, nucleotide metabolism, biodegradation of xenobiotics, protein, miscellaneous and transport. RNA processing was both up- and downregulated.

Table 3.4 Genes significantly upregulated by a log_2fold of larger than 1 and smaller than -1 in *Arabidopsis thaliana* shoot tissue following BC204 (0.01% [v/v]) treatment as annotated and classified by MapMan (metabolism_overview) ontology.

Bin Code	BinName	Locus identifier	Description	Log_2fold change
Cell wall biogenesis				
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT1G03870*	Symbols: FLA9 | FLA9 (FASCICLIN-LIKE ARABINOOGALACTAN 9)	1.853
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT1G55330*	Symbols: AGP21, ATAGP21 | AGP21	1.093
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT2G04780*	Symbols: FLA7 | FLA7 (FASCICLIN-LIKE ARABINOOGALACTAN 7)	1.046
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT2G23130*	Symbols: AGP17, ATAGP17 | AGP17 (ARABINOGALACTAN PROTEIN 17) |	2.6
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT4G12730*	Symbols: FLA2 | FLA2 (FASCICLIN-LIKE ARABINOGALACTAN 2)	1.102
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT4G37450*	Symbols: AGP18, ATAGP18 | AGP18 (ARABINOGALACTAN PROTEIN 18)	1.256
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT5G10430*	Symbols: AGP4, ATAGP4 | AGP4 (ARABINOGALACTAN PROTEIN 4)	1.236
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	*AT5G44130*	Symbols: FLA13 | FLA13 (FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 13 PRECURSOR)	1.676
10.5.3	cell wall.cell wall proteins.LRR	*AT4G13340*	leucine-rich repeat family protein / extensin family protein	2.027
10.8.1	cell wall.pectin*esterases.PME	*AT3G10720*	pectinesterase, putative	1.108
10.8.1	cell wall.pectin*esterases.PME	*AT4G02330*	Symbols: ATPMEPCRB | ATPMEPCRB; pectinesterase	1.037

10.8.1	cell wall.pectin*esterases.PME	*AT5G47500*	pectinesterase family protein	1.154
10.8.2	cell wall.pectin*esterases.acetyl esterase	*AT5G45280*	pectinacetylesterase, putative	1.227
10.7	cell wall.modification	*AT1G10550*	Symbols: XTH33, XET | XTH33; hydrolase, acting on glycosyl bonds / hydrolase, hydrolyzing O-glycosyl compounds / xyloglucan:xyloglucosyl transferase	2.034
10.7	cell wall.modification	*AT1G20190*	Symbols: ATEXPA11, EXP11, ATEXP11, ATHEXP ALPHA 1.14 | ATEXPA11 (ARABIDOPSIS THALIANA EXPANSIN 11)	1.182
10.7	cell wall.modification	*AT1G32170*	Symbols: XTR4, XTH30 | XTR4 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 4); hydrolase, acting on glycosyl bonds / hydrolase, hydrolyzing O-glycosyl compounds / xyloglucan:xyloglucosyl transferase	1.577
10.7	cell wall.modification	*AT1G65310*	Symbols: ATXTH17, XTH17 | XTH17 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 17); hydrolase, acting on glycosyl bonds / hydrolase, hydrolyzing O-glycosyl compounds / xyloglucan:xyloglucosyl transferase	1.442
10.7	cell wall.modification	*AT2G01850*	Symbols: EXGT-A3, XTH27, ATXTH27 | EXGT-A3; hydrolase, acting on glycosyl bonds / xyloglucan:xyloglucosyl transferase	1.108
10.7	cell wall.modification	*AT2G14620*	xyloglucan:xyloglucosyl transferase, putative / xyloglucan endotransglycosylase, putative / endo-xyloglucan transferase, putative	-2.55
10.7	cell wall.modification	*AT2G20750*	Symbols: ATEXPB1, EXPB1, ATHEXP BETA 1.5 | ATEXPB1 (ARABIDOPSIS THALIANA EXPANSIN B1)	1.179
10.7	cell wall.modification	*AT3G45960*	Symbols: ATEXLA3, EXPL3, ATEXPL3, ATHEXP BETA 2.3 | ATEXLA3 (arabidopsis thaliana expansin-like a3)	2.149

10.7	cell wall.modification	*AT3G45970*	Symbols: ATEXLA1, EXPL1, ATEXPL1, ATHEXP BETA 2.1 | ATEXLA1 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A1)	1.826
10.7	cell wall.modification	*AT3G55500*	Symbols: ATEXPA16, EXP16, ATEXP16, ATHEXP ALPHA 1.7 | ATEXPA16 (ARABIDOPSIS THALIANA EXPANSIN A16)	1.181
10.7	cell wall.modification	*AT4G30280*	Symbols: ATXTH18, XTH18 | XTH18 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 18); hydrolase, acting on glycosyl bonds / hydrolase, hydrolyzing O-glycosyl compounds / xyloglucan:xyloglucosyl transferase	1.012
10.7	cell wall.modification	*AT4G30290*	Symbols: ATXTH19, XTH19 | XTH19 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 19); hydrolase, acting on glycosyl bonds / hydrolase, hydrolyzing O-glycosyl compounds / xyloglucan:xyloglucosyl transferase	2.834
10.7	cell wall.modification	*AT4G38400*	Symbols: ATEXLA2, EXPL2, ATEXPL2, ATHEXP BETA 2.2 | ATEXLA2 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A2)	1.634
10.7	cell wall.modification	*AT5G02260*	Symbols: ATEXPA9, EXP9, ATEXP9, ATHEXP ALPHA 1.10 | ATEXPA9 (ARABIDOPSIS THALIANA EXPANSIN A9)	-1.582
10.7	cell wall.modification	*AT5G57560*	Symbols: TCH4, XTH22 | TCH4 (Touch 4); hydrolase, acting on glycosyl bonds / xyloglucan:xyloglucosyl transferase	3.979
10.3.2	cell wall.hemicellulose synthesis.glucuronoxylan	*AT5G22940*	Symbols: F8H | F8H (FRA8 HOMOLOG); catalytic	1.192
10.2	cell wall.cellulose synthesis	*AT3G28180*	Symbols: ATCSLC04, CSLC04, ATCSLC4, CSLC4 | ATCSLC04 (CELLULOSE-SYNTHASE LIKE C4); cellulose synthase/ transferase, transferring glycosyl	1.17

			groups	
10.2.1	cell wall.cellulose synthesis.cellulose synthase	*AT4G24000*	Symbols: ATCSLG2, CSLG2 | ATCSLG2; cellulose synthase/ transferase/ transferase, transferring glycosyl groups	-1.699
10.1.6	cell wall.precursor synthesis.GAE	*AT4G30440*	Symbols: GAE1 | GAE1 (UDP-D-GLUCURONATE 4-EPIMERASE 1); UDP-glucuronate 4-epimerase/ catalytic	1.022
10.6.1	cell wall.degradation.cellulases and beta -1,4-glucanases	*AT1G19940*	Symbols: AtGH9B5 | AtGH9B5 (Arabidopsis thaliana Glycosyl Hydrolase 9B5); catalytic/ hydrolase, hydrolyzing O-glycosyl compounds	1.345
10.6.2	cell wall.degradation.mannan-xylose-arabinose-fucose	*AT5G49360*	Symbols: BXL1, ATBXL1 | BXL1 (BETA-XYLOSIDASE 1); hydrolase, hydrolyzing O-glycosyl compounds	2.202
10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	*AT1G02460*	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	-1.372
10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	*AT2G41850*	Symbols: PGAZAT, ADPG2 | PGAZAT (POLYGALACTURONASE ABSCISSION ZONE A. THALIANA); polygalacturonase	-2.845
10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	*AT3G06770*	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	1.219
10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	*AT5G06860*	Symbols: PGIP1, ATPGIP1 | PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1); protein binding	1.14
Lipid metabolism				
11.3	lipid metabolism.Phospholipid synthesis	*AT4G01950*	Symbols: ATGPAT3, GPAT3 | GPAT3 (GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 3); acyltransferase	1.61

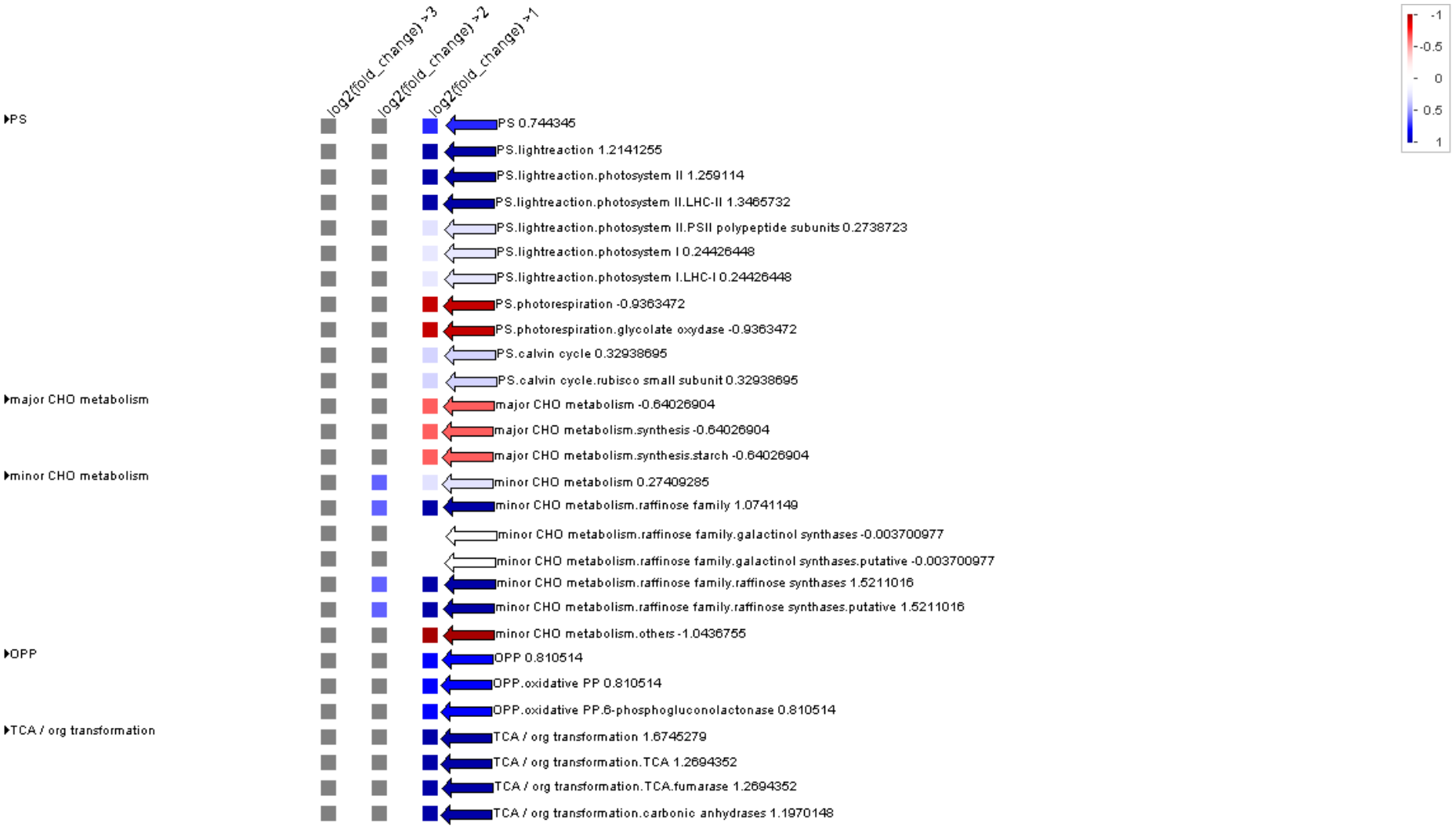
11.1.1.2.3	lipid metabolism.FA synthesis and FA elongation.Acetyl CoA Carboxylation.heteromeric Complex.Biotin Carboxyl Carrier Protein	*AT5G15530*	Symbols: BCCP2, CAC1-B | BCCP2 (BIOTIN CARBOXYL CARRIER PROTEIN 2); biotin binding	2.121
11.1.10	lipid metabolism.FA synthesis and FA elongation.beta ketoacyl CoA synthase	*AT1G01120*	Symbols: KCS1 | KCS1 (3-KETOACYL-COA SYNTHASE 1); acyltransferase/ fatty acid elongase	1.584
11.1.10	lipid metabolism.FA synthesis and FA elongation.beta ketoacyl CoA synthase	*AT2G28630*	Symbols: KCS12 | KCS12 (3-KETOACYL-COA SYNTHASE 12); acyltransferase/ catalytic/ transferase, transferring acyl groups other than amino-acyl groups	1.032
11.1.15	lipid metabolism.FA synthesis and FA elongation.ACP desaturase	*AT1G43800*	acyl-(acyl-carrier-protein) desaturase, putative / stearoyl-ACP desaturase, putative	-1.671
11.9.2.1	lipid metabolism.lipid degradation.lipases.triacylglycerol lipase	*AT1G02660*	lipase class 3 family protein	1.219
11.9.3.3	lipid metabolism.lipid degradation.lysophospholipases.glycerophosphodiester phosphodiesterase	*AT4G26690*	Symbols: SHV3, MRH5, GPDL2 | SHV3 (SHAVEN 3); glycerophosphodiester phosphodiesterase/ kinase	1.243
11.9.4.13	lipid metabolism.lipid degradation.beta-oxidation.acyl CoA reductase	*AT3G44550*	Symbols: FAR5 | FAR5 (FATTY ACID REDUCTASE 5); binding / catalytic/ oxidoreductase, acting on the CH-CH group of donors	1.953
Energy				
2.1.2	major CHO metabolism.synthesis.starch	*AT3G30720*	Symbols: QQS | QQS (QUA-QUINE STARCH)	-1.113
7.1.2	OPP.oxidative PP.6-phosphogluconolactonase	*AT1G13700*	glucosamine/galactosamine-6-phosphate isomerase family protein	1.402

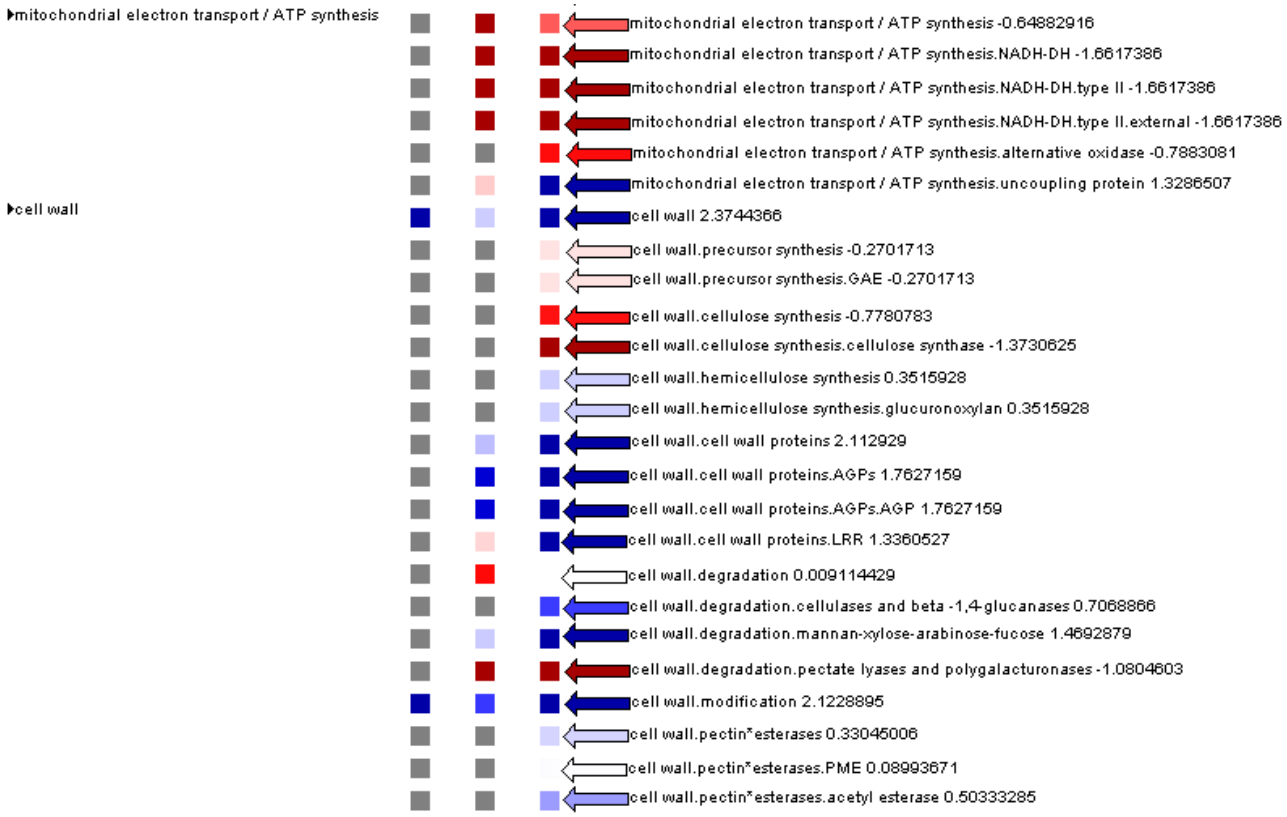
8.1.8	TCA / org transformation.TCA.fumarase	*AT5G50950*	fumarate hydratase, putative / fumarase, putative	1.949
1.3.2	PS.calvin cycle.rubisco small subunit	*AT5G38410*	ribulose bisphosphate carboxylase small chain 3B / RuBisCO small subunit 3B (RBCS-3B) (ATS3B)	1.188
8.3	TCA / org transformation.carbonic anhydrases	*AT2G28210*	Symbols: ATACA2 | ATACA2 (ALPHA CARBONIC ANHYDRASE 2); carbonate dehydratase/ zinc ion binding	1.297
8.3	TCA / org transformation.carbonic anhydrases	*AT3G01500*	Symbols: CA1 | CA1 (CARBONIC ANHYDRASE 1); carbonate dehydratase/ zinc ion binding	1.309
8.3	TCA / org transformation.carbonic anhydrases	*AT3G52720*	Symbols: ATACA1, ACA1 | ACA1 (ALPHA CARBONIC ANHYDRASE 1); carbonate dehydratase/ zinc ion binding	1.397
9.2.1.2	mitochondrial electron transport / ATP synthesis.NADH-DH.type II.external	*AT4G21490*	Symbols: NDB3 | NDB3; NADH dehydrogenase	-2.78
9.4	mitochondrial electron transport / ATP synthesis.alternative oxidase	*AT1G32350*	Symbols: AOX1D | AOX1D (alternative oxidase 1D); alternative oxidase	-1.175
9.8	mitochondrial electron transport / ATP synthesis.uncoupling protein	*AT4G24570*	Symbols: DIC2 | mitochondrial substrate carrier family protein	2.014
1.2.2	PS.photorespiration.glycolate oxydase	*AT4G18360*	(S)-2-hydroxy-acid oxidase, peroxisomal, putative / glycolate oxidase, putative / short chain alpha-hydroxy acid oxidase, putative	-1.268
1.1.1.1	PS.lightreaction.photosystem II.LHC-II	*AT2G34430*	Symbols: LHB1B1, LHCB1.4 | LHB1B1; chlorophyll binding	1.944
1.1.1.1	PS.lightreaction.photosystem II.LHC-II	*AT3G27690*	Symbols: LHCB2.4, LHCB2.3, LHCB2 | LHCB2.3; chlorophyll binding	1.306

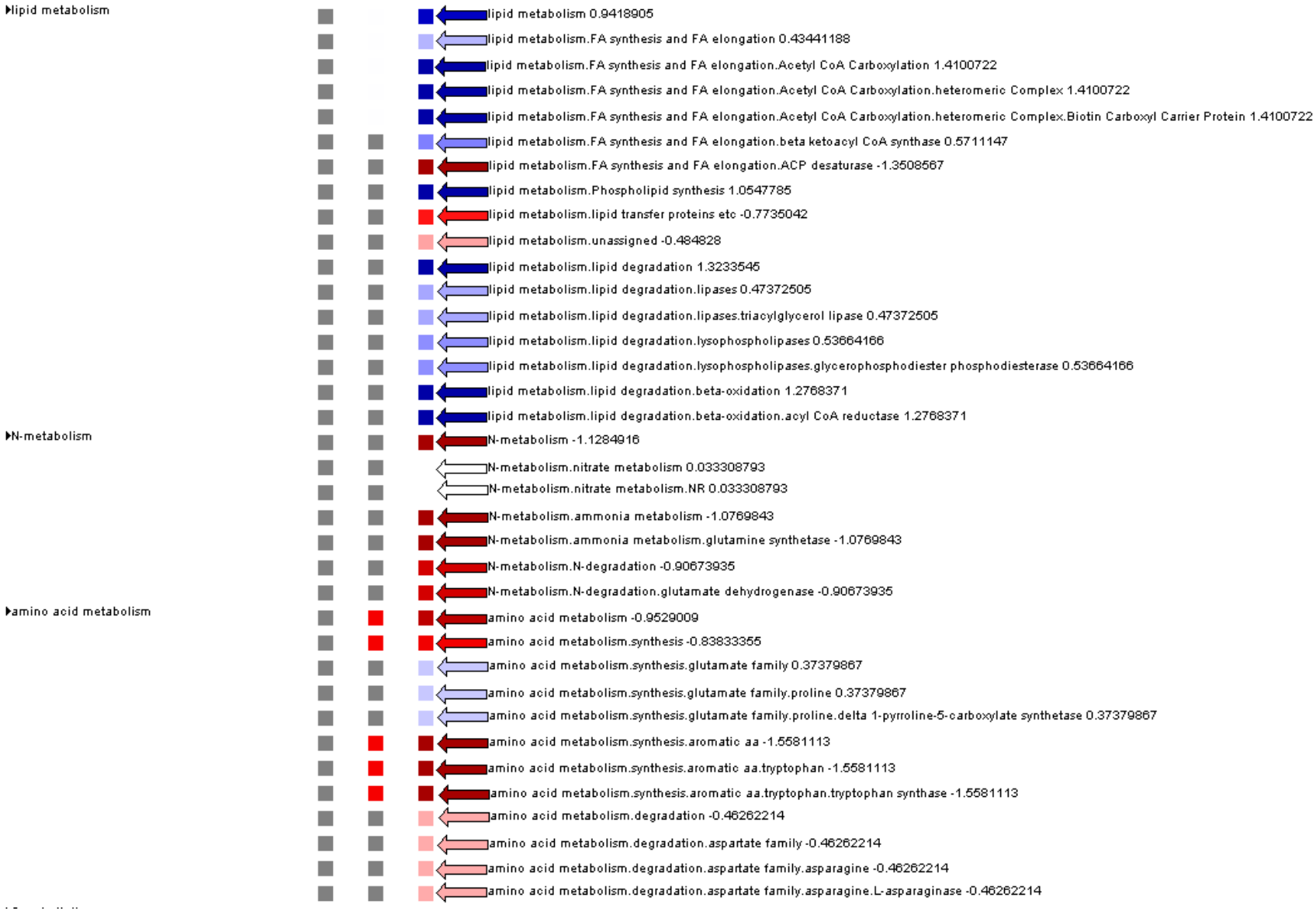
1.1.1.2	PS.lightreaction.photosystem II.PSII polypeptide subunits	*AT2G01918*	calcium ion binding	1.17
1.1.2.1	PS.lightreaction.photosystem I.LHC-I	*AT5G28450*	chlorophyll A-B binding protein, chloroplast, putative / LHCI type II CAB, putative	1.154
Secondary metabolism				
16.1	secondary metabolism.isoprenoids	*AT1G54660*	pseudogene, similar to vetispiradiene synthase, blastp match of 54% identity and 3.0e-79 P-value to GP|5360685|dbj|BAA82108.1||AB022719 vetispiradiene synthase {Solanum tuberosum}	1.51
16.1.5	secondary metabolism.isoprenoids.terpenoids	*AT1G61120*	Symbols: TPS04, GES | TPS04 (TERPENE SYNTHASE 04); (E,E)-geranyllinalool synthase	1.12
16.1.5	secondary metabolism.isoprenoids.terpenoids	*AT4G16740*	Symbols: ATTPS03 | ATTPS03; (E)-beta-ocimene synthase/ myrcene synthase	-1.385
16.4.1	secondary metabolism.N misc.alkaloid-like	*AT2G29290*	tropinone reductase, putative / tropine dehydrogenase, putative	1.061
16.8.4	secondary metabolism.flavonoids.flavonols	*AT2G36790*	Symbols: UGT73C6 | UGT73C6 (UDP-glucosyl transferase 73C6); UDP-glucosyltransferase/ UDP-glycosyltransferase/ quercetin 3-O-glucosyltransferase/ quercetin 4'-O-glucosyltransferase/ quercetin 7-O-glucosyltransferase/ transferase, transferring glycosyl groups	1.419
16.8.4	secondary metabolism.flavonoids.flavonols	*AT3G60290*	oxidoreductase/ oxidoreductase, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	1.898
16.8.4.2	secondary metabolism.flavonoids.flavonols.flavonol 3-O-glycosyltransferase	*AT4G34135*	Symbols: UGT73B2 | UGT73B2 (UDP-GLUCOSYLTRANSFERASE 73B2); UDP-glucosyltransferase/ UDP-glycosyltransferase/ flavonol 3-O-glucosyltransferase/ quercetin 7-O-glucosyltransferase	-1.012

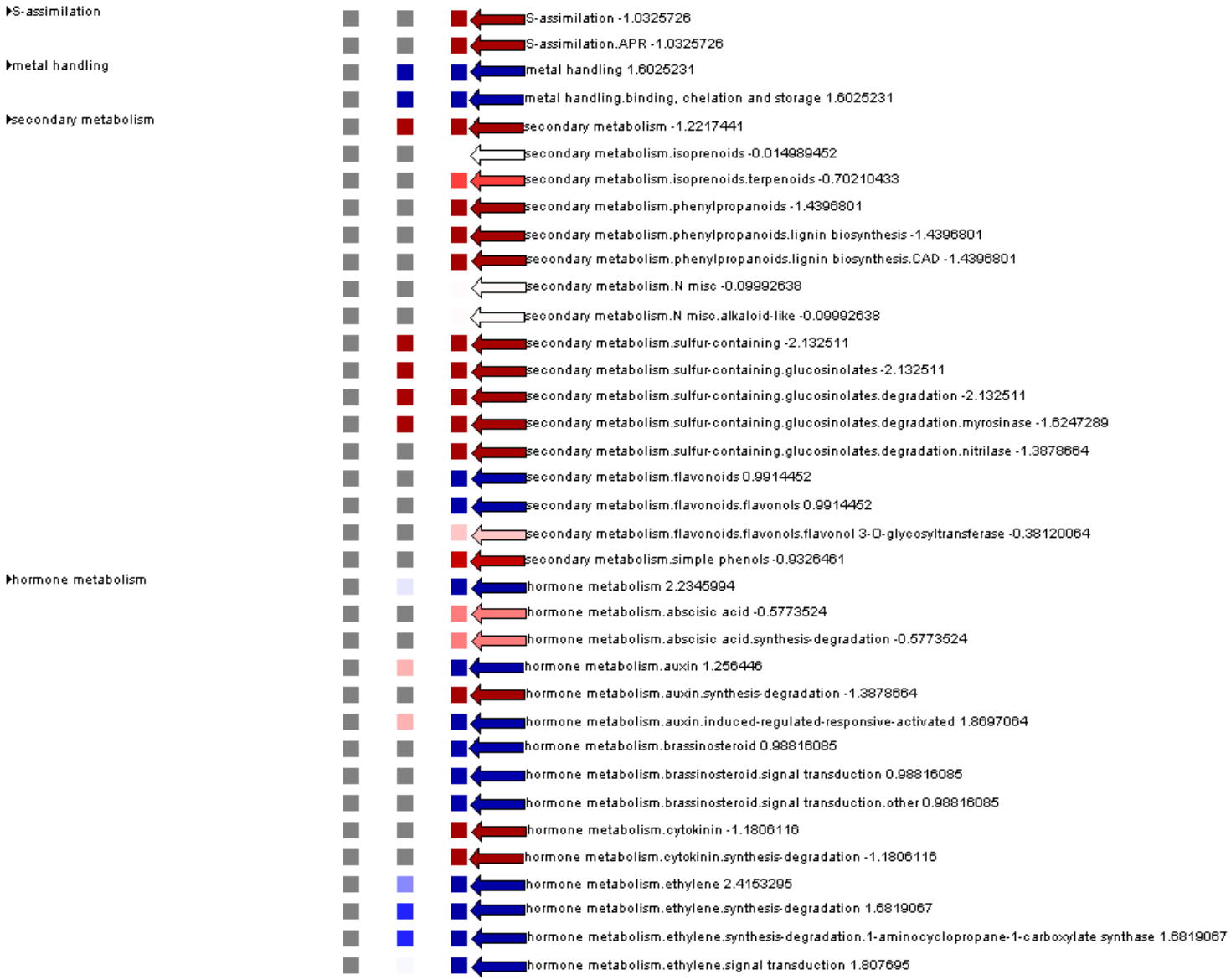
16.5.1.3.1	secondary metabolism.sulfur-containing.glucosinolates.degradation.myrosinase	*AT1G54020*	myrosinase-associated protein, putative	-2.453
16.5.1.3.3	secondary metabolism.sulfur-containing.glucosinolates.degradation.nitrilase	*AT3G44300*	Symbols: NIT2 | NIT2 (nitrilase 2); indole-3-acetonitrile nitrilase/ indole-3-acetonitrile nitrile hydratase/ nitrilase	-1.707
16.2.1.10	secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	*AT4G37990*	Symbols: ELI3-2, ELI3, ATCAD8, CAD-B2 | ELI3-2 (ELICITOR-ACTIVATED GENE 3-2); aryl-alcohol dehydrogenase/ mannitol dehydrogenase	-1.784
16.10	secondary metabolism.simple phenols	*AT1G33030*	O-methyltransferase family 2 protein	-1.326
16.10	secondary metabolism.simple phenols	*AT2G29130*	Symbols: LAC2, ATLAC2 | LAC2 (laccase 2); laccase	1.015
Amino acid metabolism				
13.1.2.2.1	amino acid metabolism.synthesis.glutamate family.proline.delta 1-pyrroline-5-carboxylate synthetase	*AT2G39800*	Symbols: P5CS1, ATP5CS | P5CS1 (DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1); delta1-pyrroline-5-carboxylate synthetase	1.196
13.1.6.5.5	amino acid metabolism.synthesis.aromatic aa.tryptophan.tryptophan synthase	*AT5G28237*	tryptophan synthase, beta subunit, putative	-2.237
13.2.3.1.1	amino acid metabolism.degradation.aspartate family.asparagine.L-asparaginase	*AT3G16150*	L-asparaginase, putative / L-asparagine amidohydrolase, putative	-1.042
Other				
12.2.2	N-metabolism.ammonia	*AT5G16570*	Symbols: GLN1;4 | GLN1;4; glutamate-ammonia ligase	-1.355

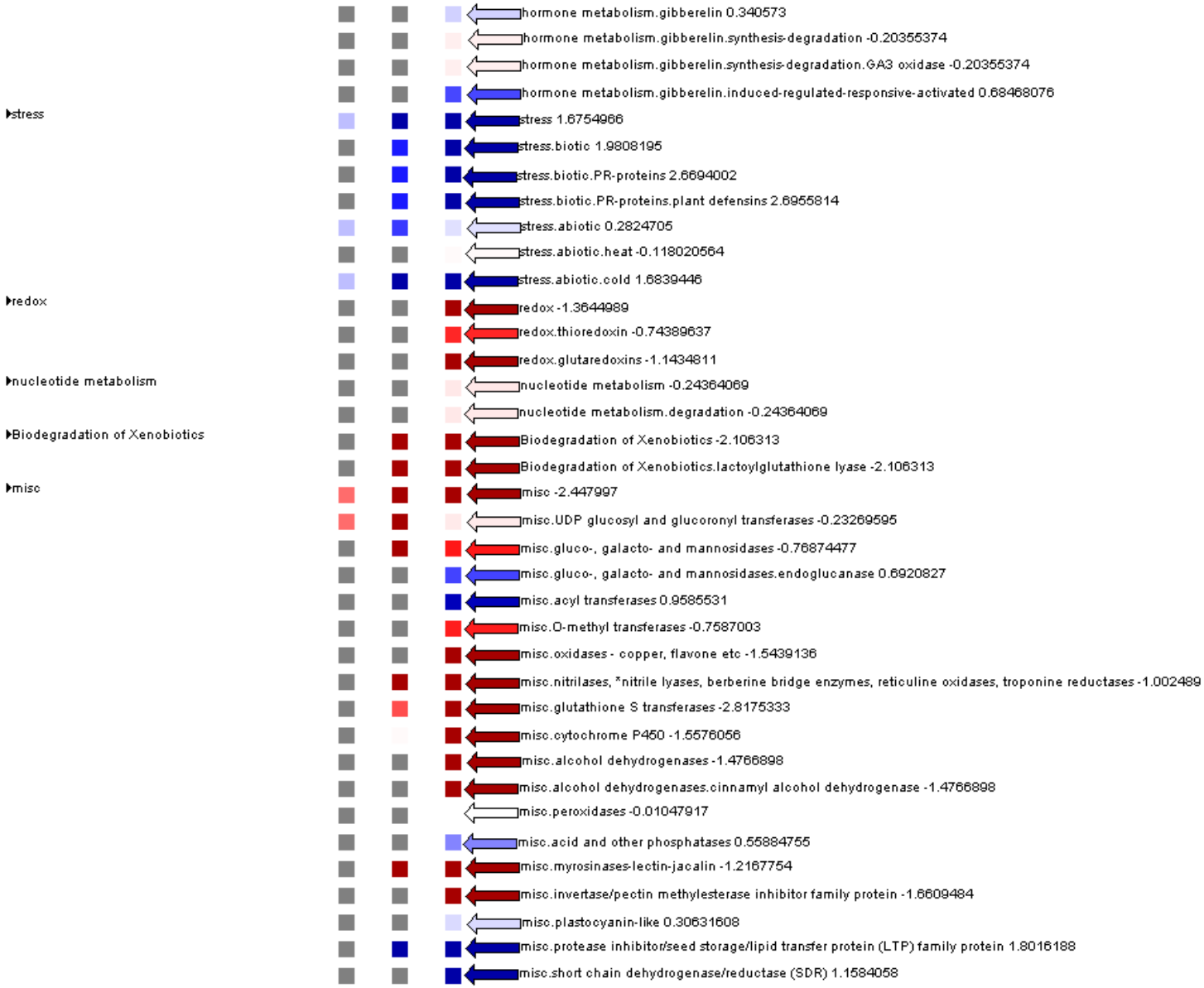
	metabolism.glutamine synthetase			
12.1.1	N-metabolism.nitrate metabolism.NR	*AT1G37130*	Symbols: NIA2, B29, NIA2-1, CHL3, NR, NR2, ATNR2 | NIA2 (NITRATE REDUCTASE 2); nitrate reductase (NADH)/ nitrate reductase	1.095
12.3.1	N-metabolism.N-degradation.glutamate dehydrogenase	*AT3G03910*	Symbols: GDH3 | GDH3 (GLUTAMATE DEHYDROGENASE 3); binding / catalytic/ oxidoreductase/ oxidoreductase, acting on the CH-NH2 group of donors, NAD or NADP as acceptor	-1.255
14.2	S-assimilation.APR	*AT4G21990*	Symbols: APR3, PRH-26, PRH26, ATAPR3 | APR3 (APS REDUCTASE 3); adenylyl-sulfate reductase	-1.339
23.2	nucleotide metabolism.degradation	*AT1G14250*	nucleoside phosphatase family protein / GDA1/CD39 family protein	1.045
23.2	nucleotide metabolism.degradation	*AT4G29610*	cytidine deaminase, putative / cytidine aminohydrolase, putative	1.036

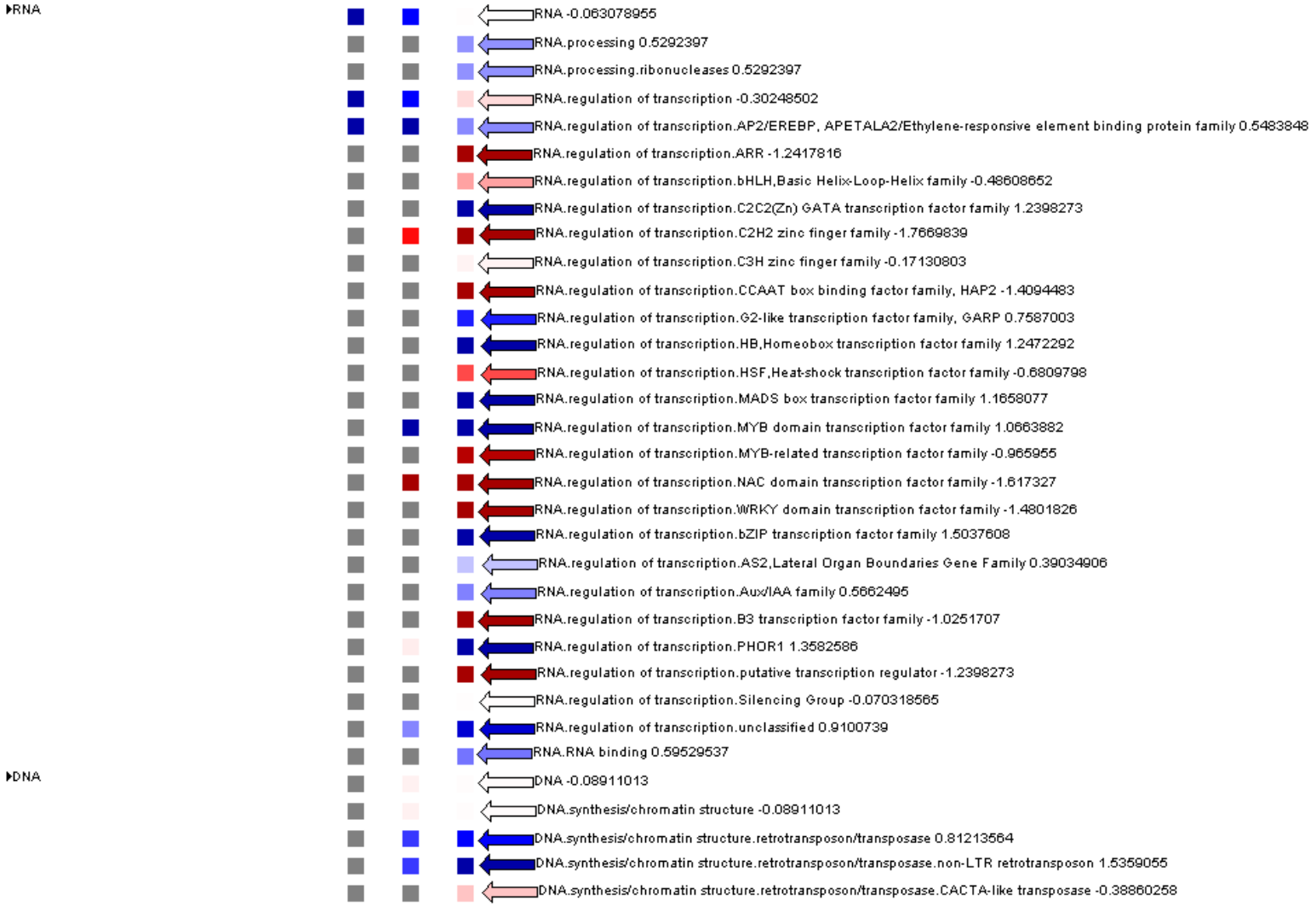


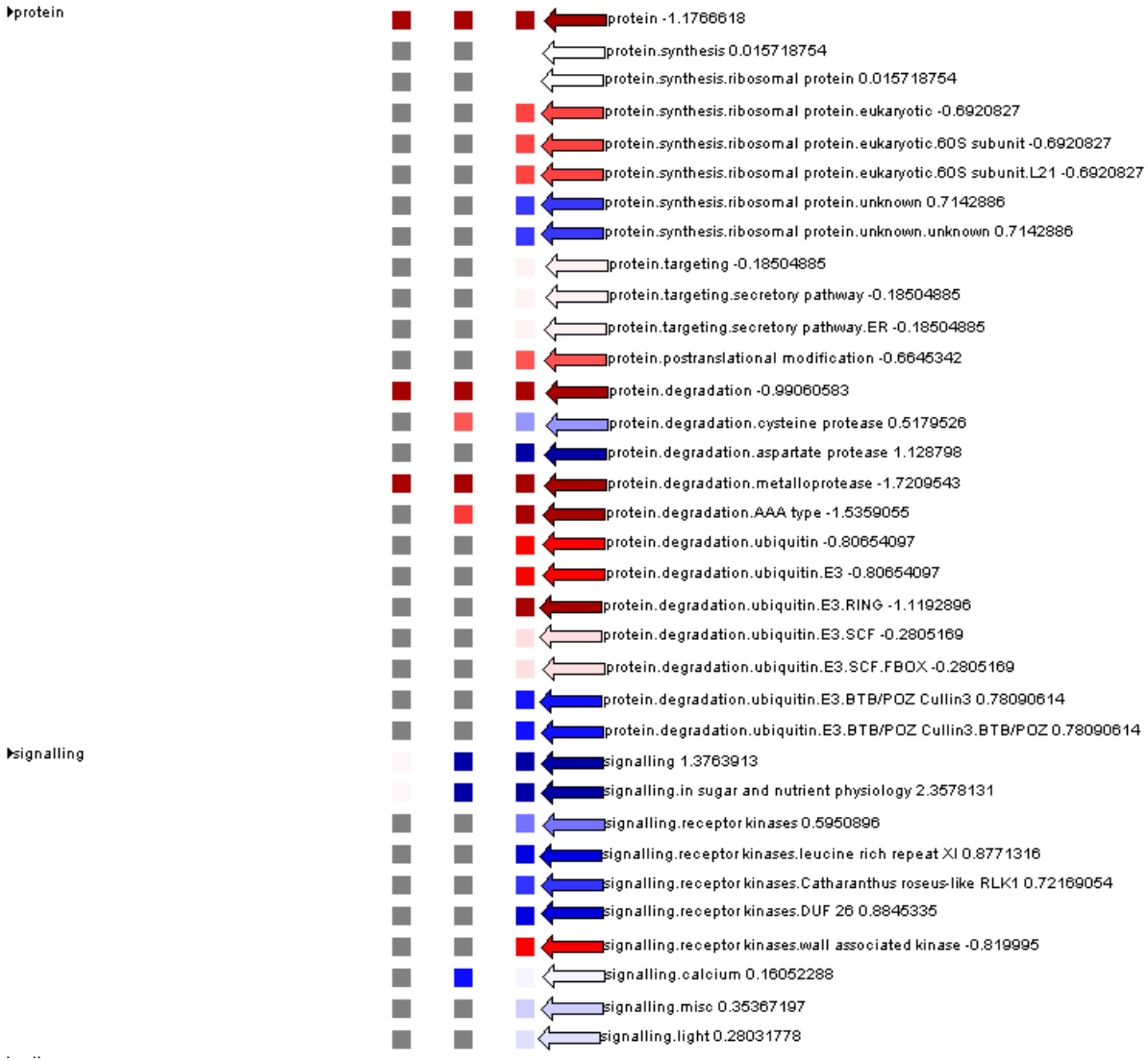












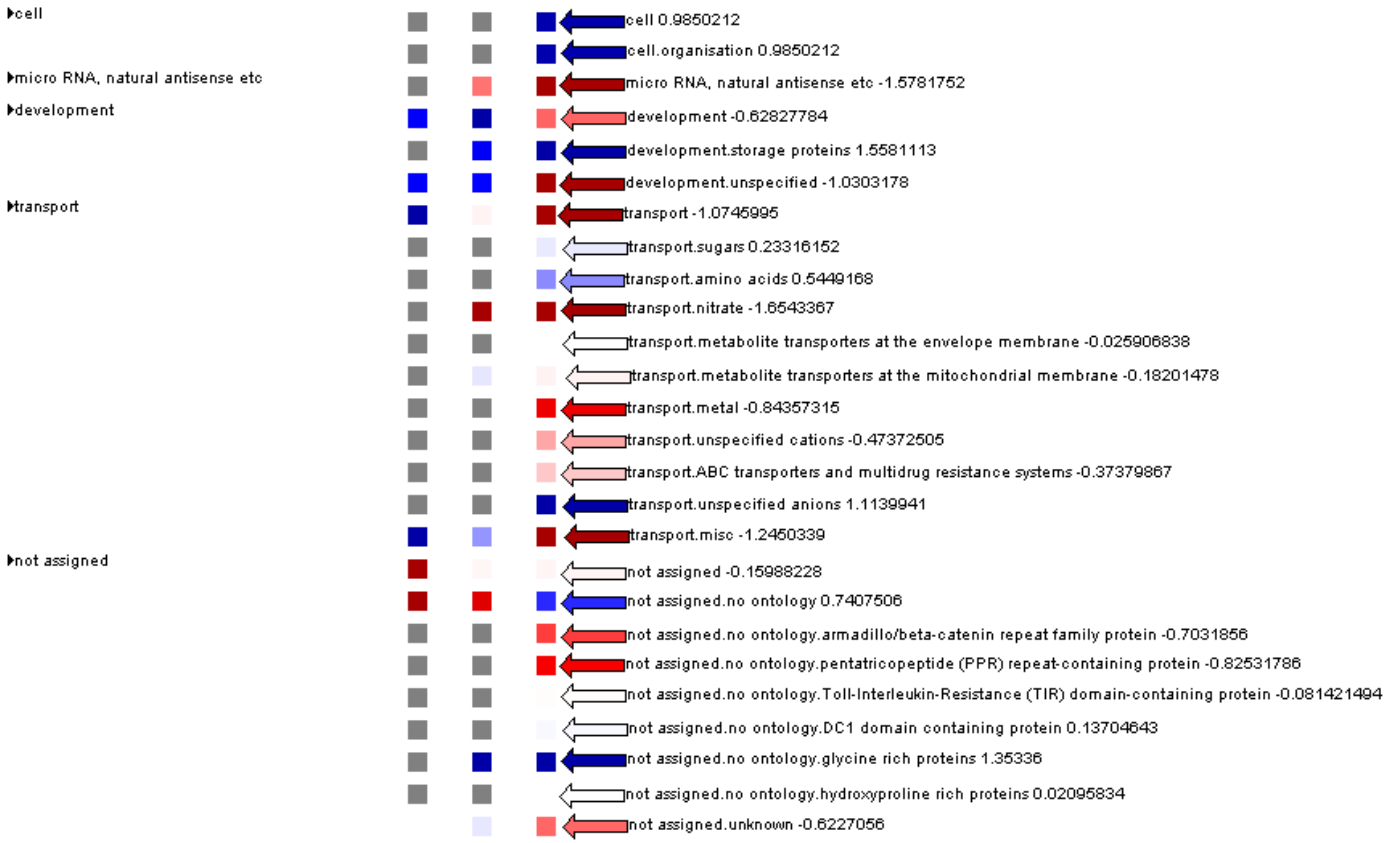


Figure 3.8 Genes upregulated by a $\log_2$fold of at least 1 and -1 in the *Arabidopsis thaliana* transcriptome were categorized into MapMan bins and displayed in PageMan, an extension of the MapMan analysis. Data was subjected to a Bin-wise Wilcoxon test, with p-values not considered for this analysis. Blue indicates upregulated processes while red/pink is indicative of downregulated processes.

Like the PANTHER analysis, the agriGO v2.0 online analysis tool was used to classify and categorise DEGs into either Biological Process, Molecular Function and Cellular component (Table 3.5). Genes with a log_2fold of larger than 1 and smaller than -1 were used for this analysis.

Table 3.5 Genes significantly upregulated by a log_2fold of larger than 1 and smaller than -1 in *Arabidopsis thaliana* shoot tissue following BC204 (0.01% [v/v]) treatment as annotated and classified by Singular Enrichment Analysis (SEA) (agriGO v2)

	GO term	Process	False Discovery Rate (FDR)		Number	
			Up	Down	Up	Down
Biological Process (GO:0008150)	GO:00223052	signalling	$1.8e^{-10}$	1	201	70
	GO:0050789	regulation of biological process	$2.2e^{-05}$	0.00071	396	241
	GO:0071840	cellular component organization or biogenesis	0.045	1	204	86
	GO:0000003	reproduction	1	0.42	91	76
	GO:0048511	rhythmic process	0.39	0.036	16	14
	GO:0065007	biological regulation	0.0014	0.00012	427	276
	GO:0044699	single-organism process	$1.9e^{-32}$	$5.4e^{-08}$	808	428
	GO:0048518	positive regulation of biological process	1	0.0019	47	56
	GO:0048519	negative regulation of biological process	0.92	0.75	62	39
	GO:0001906	cell killing	1	-	8	-
	GO:0032502	developmental process	0.36	0.015	219	149
	GO:0032501	multicellular organismal process	1	0.0066	180	144
	GO:0009987	cellular process	$1.2e^{-14}$	0.002	924	510
	GO:0022414	reproductive process	1	0.4	90	76
	GO:0008152	metabolic process	$1.3e^{-09}$	$7.8e^{-06}$	847	507
	GO:0040007	growth	0.00016	1	73	20
	GO:0051179	localization	1	0.46	161	106
	GO:0051704	multi-organism process	0.00011	0.0077	145	86
	GO:0050896	response to stimulus	$4.1e^{-38}$	$2.1e^{-13}$	618	333
	GO:0002376	immune system process	0.00039	0.018	46	27
Molecular Function (GO:0003674)	GO:0060089	molecular transducer activity	0.028	1	26	7
	GO:0005215	transporter activity	1	0.00069	91	85
	GO:0016209	antioxidant activity	0.17	1	20	5
	GO:0005198	structural molecule activity	1	1	24	19
	GO:0098772	molecular function regulator	1	1	27	16
	GO:0003824	catalytic activity	$2.5e^{-15}$	0.1	709	362
	GO:0001071	nucleic acid binding transcription factor activity	1	0.0012	100	98
	GO:0004871	signal transducer activity	0.013	1	44	13
	GO:0000988	transcription factor activity, protein binding	1	1	15	8
	GO:0009055	electron carrier activity	0.19	0.18	21	15

	GO:0005488	binding	0.038	0.1	773	467
Cellular Component (GO:0005575)	GO:0099512	supramolecular fiber	1	1	17	5
	GO:0031974	membrane-enclosed lumen	1	0.0037	20	61
	GO:0043226	organelle	0.18	0.081	1129	690
	GO:0030054	cell junction	$3.3e^{-10}$	1	109	36
	GO:0016020	membrane	$3.2e^{-35}$	1	753	297
	GO:0055044	symplast	$5.7e^{-10}$	1	108	36
	GO:0032991	macromolecular complex	1	1	149	88
	GO:0044464	cell part	$8.8e^{-14}$	$9.3e^{-06}$	1452	858
	GO:0005623	cell	$9e^{-14}$	$9.3e^{-06}$	1452	858
	GO:0005576	extracellular region	$1.1e^{-16}$	1	294	83
	GO:0044425	membrane part	$7.7e^{-18}$	1	510	203
	GO:0044421	extracellular region part	0.54	1	14	6
	GO:0044422	organelle part	$7.2e^{-15}$	1	423	181

3.4.4 BC204 affects the expression of genes involved in cell wall metabolism, photosynthesis, transcription factors and stress responses

BC204 has a holistic effect on gene expression, influencing the expression of genes across almost all characterised bins (Figure 3.3, Figure 3.4). Although difficult to pinpoint which process is mostly affected, several seem to be pivotal to the observed increase in growth. Genes coding for cell wall biogenesis and modification were upregulated (Table 3.4), as were 9 genes coding for key enzymes involved in cell wall precursor biosynthesis (Figure 3.9, Table 3.6).

Photosynthesis genes in the chloroplast involved in both PSI and PSII were upregulated (Figure 3.10, Figure 3.11). Chlorophyll synthesis, as a branch in tetrapyrrole metabolism, was also upregulated (Figure 3.12, Table 3.7). Starch synthesis was downregulated at more than one point while sucrose metabolism was upregulated (Figure 3.13, Table 3.8).

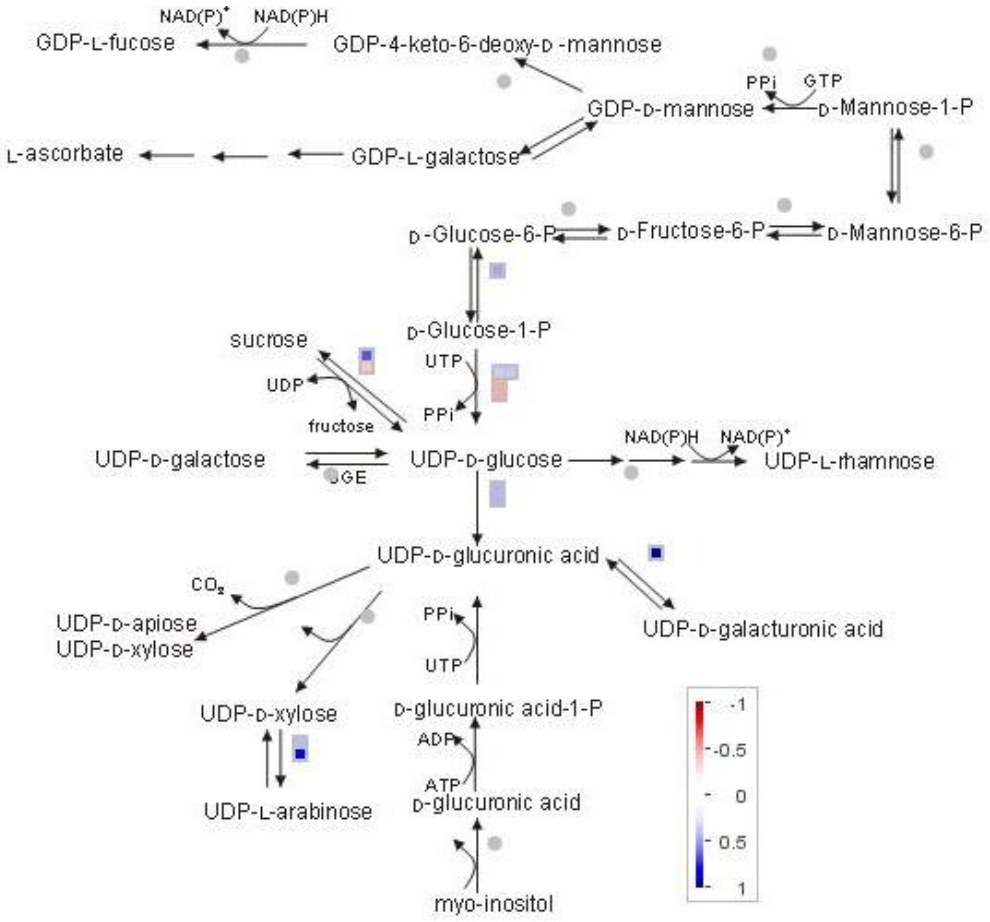


Figure 3.9 Twelve genes coding for key enzymes involved in the biosynthesis of cell wall precursors following BC204 (0.01% [v/v]) treatment as annotated and classified by MapMan. 2730 of 26868 data points were mapped, with 12 visible here.

Table 3.6 Genes involved in cell wall precursor metabolism in *Arabidopsis thaliana* shoot tissue metabolism that were significantly altered following 0.01% (v/v) BC204 treatment

BinCode	BinName	Gene ID	Description	Log_2fold change
4.2.8	glycolysis.plastid branch.glyceraldehyde 3-phosphate dehydrogenase (GAP-DH)	*AT1G79530*	Symbols: GAPCP-1 | GAPCP-1 (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF PLASTID 1); NAD or NADH binding / binding / catalytic/ glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)/ glyceraldehyde-3-phosphate dehydrogenase	0.479
4.1.5	glycolysis.cytosolic branch.pyrophosphate-fructose-6-P phosphotransferase	*AT1G12000*	pyrophosphate--fructose-6-phosphate 1-phosphotransferase beta subunit, putative / pyrophosphate-dependent 6-phosphofructose-1-kinase, putative	0.359
4.1.12	glycolysis.cytosolic branch.phosphoglycerate mutase	*AT2G17280*	phosphoglycerate/bisphosphoglycerate mutase family protein	-0.46
4.1.14	glycolysis.cytosolic branch.pyruvate kinase (PK)	*AT3G49160*	pyruvate kinase family protein	-0.472
4.1.16	glycolysis.cytosolic branch.phospho-enol-pyruvate carboxylase kinase (PPCK)	*AT1G12580*	Symbols: PEPKR1 | PEPKR1 (Phosphoenolpyruvate carboxylase-related kinase 1); ATP binding / kinase/ protein kinase/ protein serine/threonine kinase	0.373
2.2.1.5	major CHO metabolism.degradation.sucrose.Susy	*AT1G73370*	Symbols: SUS6, ATSUS6 | SUS6 (SUCROSE SYNTHASE 6); UDP-glycosyltransferase/ sucrose synthase	0.661
2.2.1.5	major CHO metabolism.degradation.sucrose.Susy	*AT4G02280*	Symbols: SUS3, ATSUS3 | SUS3 (sucrose synthase 3); UDP-glycosyltransferase/ sucrose synthase/ transferase, transferring glycosyl groups	-0.374
10.1.4	cell wall.precursor synthesis.UDP-Glc dehydrogenase (UGD)	*AT1G26570*	Symbols: UGD1, ATUGD1 | UGD1 (UDP-GLUCOSE DEHYDROGENASE 1); NAD or NADH binding / UDP-glucose 6-dehydrogenase/ binding / catalytic/ coenzyme binding / oxidoreductase/ oxidoreductase, acting on the CH-OH group of donors, NAD or NADP as	0.437

			acceptor	
10.1.4	cell wall.precursor synthesis.UDP-Glc dehydrogenase (UGD)	*AT3G29360*	UDP-glucose 6-dehydrogenase, putative	0.444
10.1.6	cell wall.precursor synthesis.GAE	*AT4G30440*	Symbols: GAE1 | GAE1 (UDP-D-GLUCURONATE 4-EPIMERASE 1); UDP-glucuronate 4-epimerase/ catalytic	1.022
10.1.9	cell wall.precursor synthesis.MUR4	*AT1G30620*	Symbols: HSR8, MUR4, UXE1 | MUR4 (MURUS 4); UDP-arabinose 4-epimerase/ catalytic	0.416
10.1.9	cell wall.precursor synthesis.MUR4	*AT2G34850*	Symbols: MEE25 | MEE25 (maternal effect embryo arrest 25); UDP-glucose 4-epimerase/ binding / catalytic/ coenzyme binding	0.858

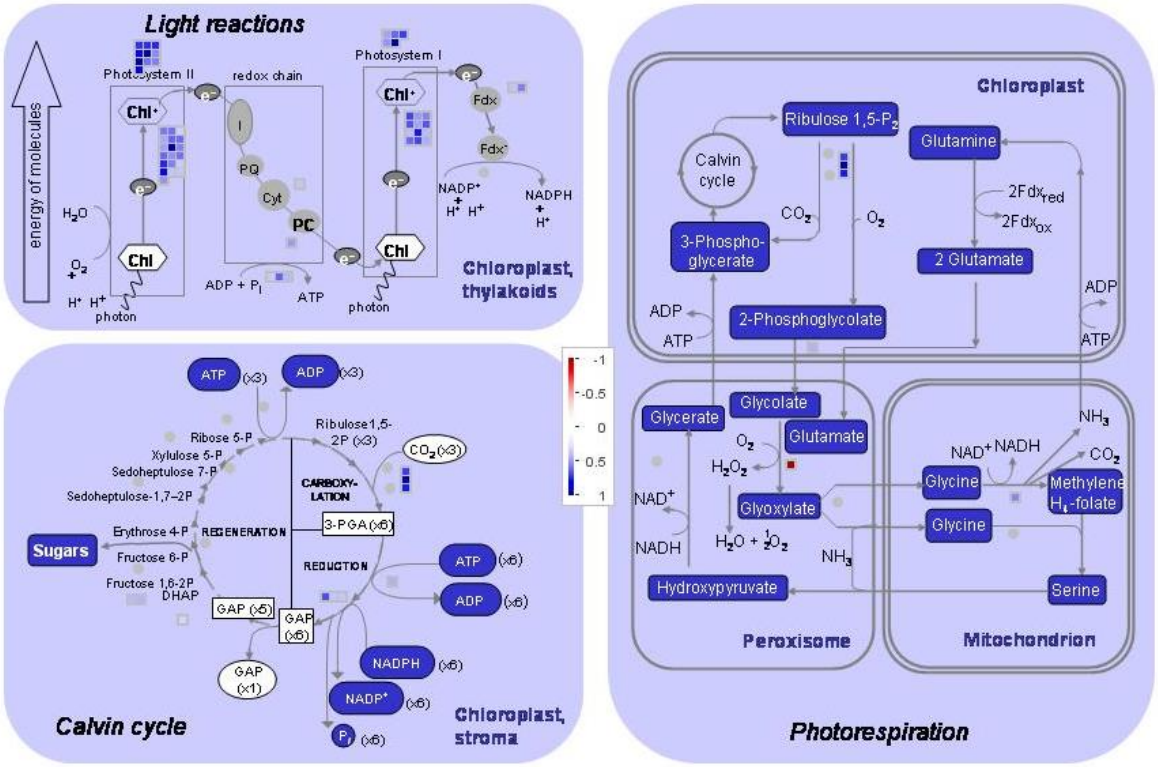


Figure 3.10 BC204 putative involvement in photosynthesis in *Arabidopsis thaliana* shoot tissue, as predicted by MapMan ontology bin-wise characterisation. 2730 of 26868 data points were mapped. In total, the expression of 69 genes involved in photosynthesis was induced by BC204 treatment.

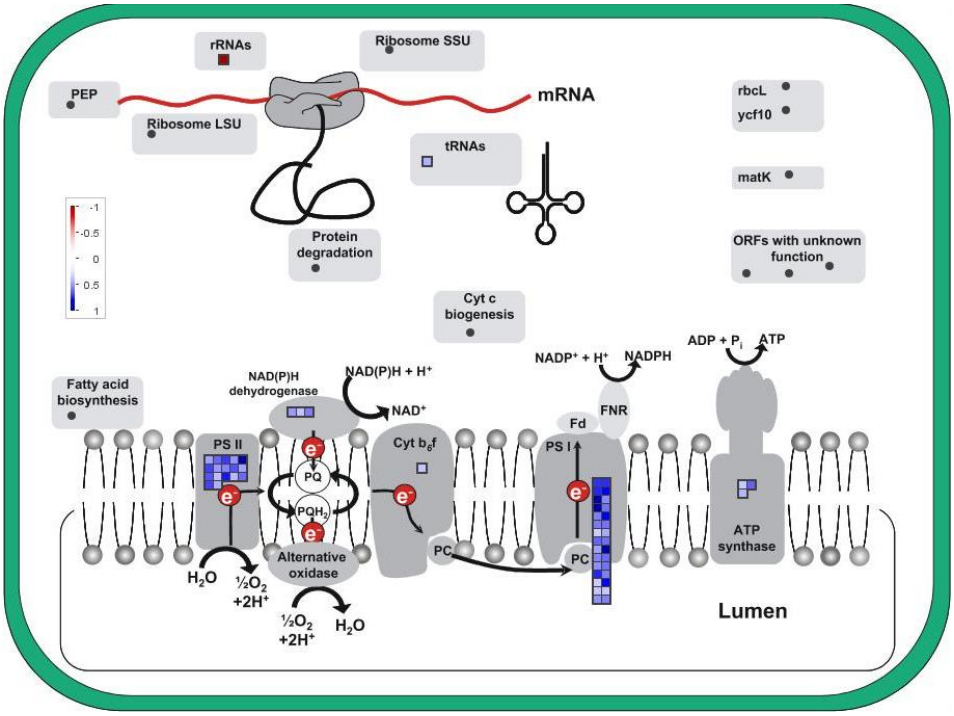


Figure 3.11 Effect of BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue on gene expression in the chloroplast. 2730 of 26868 data points were mapped, with 56 chloroplast-related genes mostly upregulated by BC204 treatment. Both PSII and PSI-related genes were strongly upregulated.

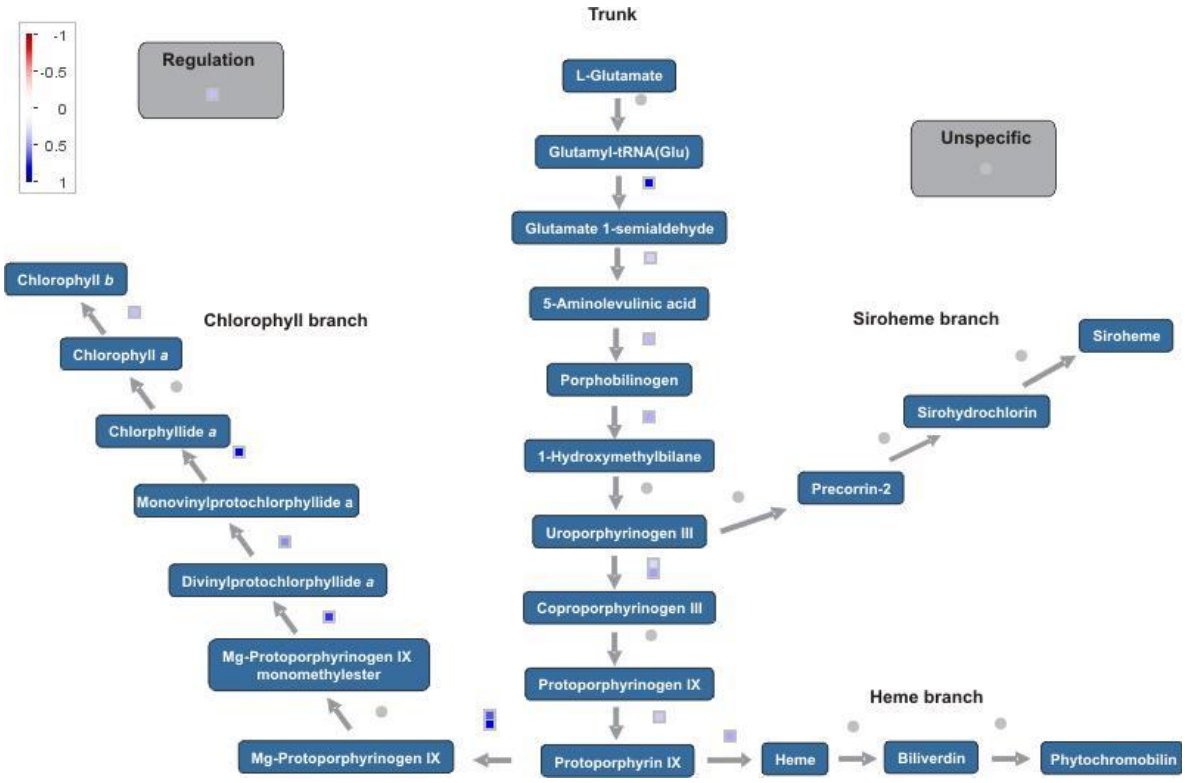


Figure 3.12 Effect of BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue on genes involved in tetrapyrrole metabolism and chlorophyll biosynthesis. 2730 of 26868 data points were mapped, with 15 genes involved in chlorophyll metabolism upregulated by BC204.

Table 3.7 Effect of BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue on genes coding for enzymes involved in tetrapyrrole metabolism

BinCode	BinName	Gene ID	Description	Log_2fold change
19.40	tetrapyrrole synthesis.regulation	*AT3G59400*	Symbols: GUN4 | GUN4; enzyme binding / tetrapyrrole binding	0.402
19.2	tetrapyrrole synthesis.glu-tRNA reductase	*AT1G58290*	Symbols: HEMA1 | HEMA1; glutamyl-tRNA reductase	0.875
19.3	tetrapyrrole synthesis.GSA	*AT3G48730*	Symbols: GSA2 | GSA2 (glutamate-1-semialdehyde 2,1-aminomutase 2); catalytic/ glutamate-1-semialdehyde 2,1-aminomutase/ pyridoxal phosphate binding / transaminase	0.335
19.4	tetrapyrrole synthesis.ALA dehydratase	*AT1G69740*	Symbols: HEMB1 | HEMB1; catalytic/ metal ion binding / porphobilinogen synthase	0.406
19.5	tetrapyrrole synthesis.porphobilinogen deaminase	*AT5G08280*	Symbols: HEMC | HEMC (HYDROXYMETHYLBILANE SYNTHASE); hydroxymethylbilane synthase	0.457
19.7	tetrapyrrole synthesis.uroporphyrinogen decarboxylase	*AT2G40490*	Symbols: HEME2 | HEME2; uroporphyrinogen decarboxylase	0.32
19.7	tetrapyrrole synthesis.uroporphyrinogen decarboxylase	*AT3G14930*	Symbols: HEME1 | HEME1; uroporphyrinogen decarboxylase	0.491
19.9	tetrapyrrole synthesis.protoporphyrin IX oxidase	*AT4G01690*	Symbols: PPOX, HEMG1, PPO1 | PPOX; protoporphyrinogen oxidase	0.363
19.20	tetrapyrrole synthesis.ferrochelatase	*AT5G26030*	Symbols: FC1, FC-I, ATFC-I | FC1 (ferrochelatase 1); ferrochelatase	0.469
19.10	tetrapyrrole synthesis.magnesium chelatase	*AT4G18480*	Symbols: CHLI1, CH42, CH-42, CHL11, CHLI-1 | CHLI1; ATPase/ magnesium	0.663

			chelatase	
19.10	tetrapyrrole synthesis.magnesium chelatase	*AT5G13630*	Symbols: GUN5, CCH, CHLH, CCH1 | GUN5 (GENOMES UNCOUPLED 5); magnesium chelatase	0.825
19.12	tetrapyrrole synthesis.magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase	*AT3G56940*	Symbols: CRD1, CHL27, ACSF | CRD1 (COPPER RESPONSE DEFECT 1); DNA binding / magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase	0.722
19.13	tetrapyrrole synthesis.divinyl chlorophyllide-a 8-vinyl-reductase	*AT5G18660*	Symbols: PCB2 | PCB2 (PALE-GREEN AND CHLOROPHYLL B REDUCED 2); 3,8-divinyl protochlorophyllide a 8-vinyl reductase	0.539
19.14	tetrapyrrole synthesis.protochlorophyllide reductase	*AT5G54190*	Symbols: PORA | PORA; oxidoreductase/ protochlorophyllide reductase	0.817
19.16	tetrapyrrole synthesis.chlorophyll b synthase	*AT1G44446*	Symbols: CH1, ATCAO, CAO | CH1 (CHLORINA 1); chlorophyllide a oxygenase	0.396

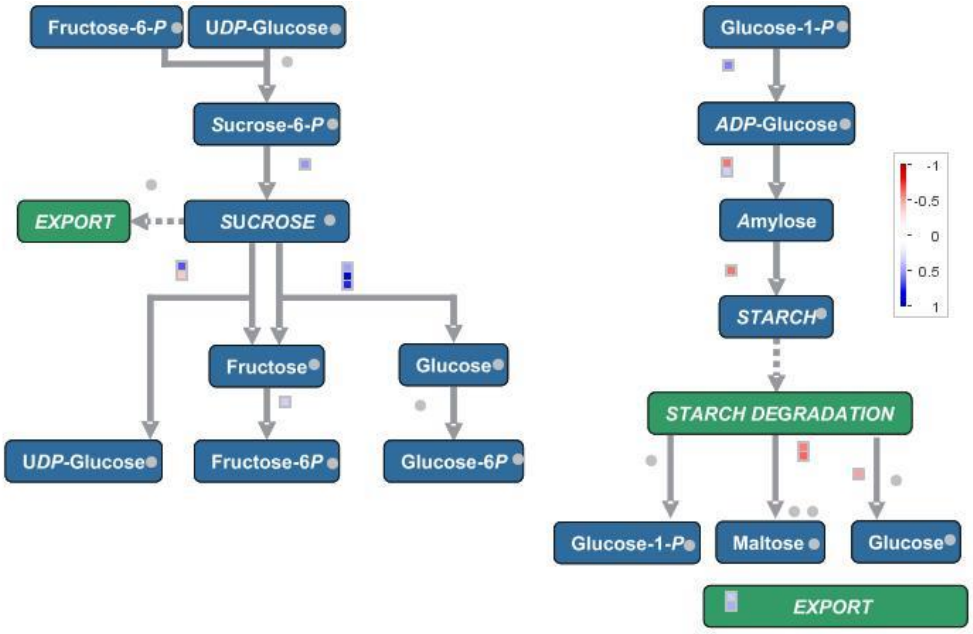


Figure 3.13 Changes in genes expression involved in sugar and starch metabolism after BC204 (0.01% [v/v]) treatment in *A. thaliana* shoot tissue BC204 treatment. 2730 of 26868 data points were mapped, with 16 visible here.

Table 3.8 Effect of BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue on genes involved in sugar and starch metabolism

BinCode	BinName	Gene ID	Description	Log_2fold change
2.1.1.2	major CHO metabolism.synthesis.sucrose.SPP	*AT3G52340*	Symbols: SPP2, ATSPP2 | SPP2 (SUCROSE-6F-PHOSPHATE PHOSPHOHYDROLASE 2); catalytic/ magnesium ion binding / phosphatase/ sucrose-phosphatase	0.52
2.2.1.5	major CHO metabolism.degradation.sucrose.Susy	*AT1G73370*	Symbols: SUS6, ATSUS6 | SUS6 (SUCROSE SYNTHASE 6); UDP-glycosyltransferase/ sucrose synthase	0.661
2.2.1.5	major CHO metabolism.degradation.sucrose.Susy	*AT4G02280*	Symbols: SUS3, ATSUS3 | SUS3 (sucrose synthase 3); UDP-glycosyltransferase/ sucrose synthase/ transferase, transferring glycosyl groups	-0.374
2.2.1.3.1	major CHO metabolism.degradation.sucrose.invertases.neutral	*AT3G06500*	beta-fructofuranosidase, putative / invertase, putative / saccharase, putative / beta-fructosidase, putative	0.484
2.2.1.3.2	major CHO metabolism.degradation.sucrose.invertases.cell wall	*AT3G13790*	Symbols: ATCWINV1, ATBFRUCT1 | ATBFRUCT1; beta-fructofuranosidase/ hydrolase, hydrolyzing O-glycosyl compounds	0.843
2.2.1.3.3	major CHO metabolism.degradation.sucrose.invertases.vacuolar	*AT1G62660*	beta-fructosidase (BFRUCT3) / beta-fructofuranosidase / invertase, vacuolar	0.75
2.2.1.1	major CHO metabolism.degradation.sucrose.fructokinase	*AT1G66430*	pfkB-type carbohydrate kinase family protein	0.345
2.1.2.1	major CHO metabolism.synthesis.starch.AGPase	*AT5G19220*	Symbols: ADG2, APL1 | APL1 (ADP GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 1); glucose-1-phosphate adenylyltransferase	0.565

2.1.2.2	major CHO metabolism.synthesis.starch.starch synthase	*AT1G32900*	starch synthase, putative	-0.604
2.1.2.2	major CHO metabolism.synthesis.starch.starch synthase	*AT5G65685*	soluble glycogen synthase-related	0.345
2.1.2.3	major CHO metabolism.synthesis.starch.starch branching	*AT3G20440*	Symbols: EMB2729, BE1 | alpha-amylase/ catalytic/ cation binding	-0.609
2.2.2.1.1	major CHO metabolism.degradation.starch.starch cleavage.alpha amylase	*AT4G25000*	Symbols: ATAMY1, AMY1 | AMY1 (ALPHA-AMYLASE-LIKE); alpha-amylase	-0.575
2.2.2.1.2	major CHO metabolism.degradation.starch.starch cleavage.beta amylase	*AT4G17090*	Symbols: CT-BMY, BAM3, BMY8 | CT-BMY (CHLOROPLAST BETA-AMYLASE); beta-amylase	-0.63
2.2.2.3	major CHO metabolism.degradation.starch.glucan water dikinase	*AT5G26570*	Symbols: PWD, OK1, ATGWD3 | ATGWD3; carbohydrate kinase/ catalytic/ phosphoglucan, water dikinase	-0.497
2.2.2.6	major CHO metabolism.degradation.starch.transporter	*AT5G16150*	Symbols: GLT1, PGLCT | PGLCT (PLASTIDIC GLC TRANSLOCATOR); carbohydrate transmembrane transporter/ sugar:hydrogen symporter	0.366
2.2.2.6	major CHO metabolism.degradation.starch.transporter	*AT5G46110*	Symbols: APE2, TPT | APE2 (ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT 2); antiporter/ triose-phosphate transmembrane transporter	0.462

At the regulation level, major shifts in gene expression were observed, with 676 of these regulatory genes influenced at different levels (Figure 3.14). A massive shift in the expression of transcription factors, as well as genes involved in protein modification and protein degradation was observed. The types of transcription factors fall into a many different categories (Table S3.1). Categories of transcription factors mostly influenced were AP2 (ethylene-response element), bHLH (basic helix-loop-helix family), several zinc finger family transcription factors, GATA transcription factor family, MYB, WRKY, bZIP and many others (Table 3.9). A large group of mainly unclassified transcription factors were also altered by BC204. The expression of these transcription factors were both up- and downregulated with no classes spefically mostly up- or downregulated. Downstream of regulation, the cellular response to BC204 included large changes in expression of genes related to biotic stress, cell division, cell cycle and development (Figure 3.15)**.** The plants in this study were not subjected to biotic stress, but 774 genes involved in biotic stress metabolism were altered at varied magnitudes by BC204 (Figure 3.16).

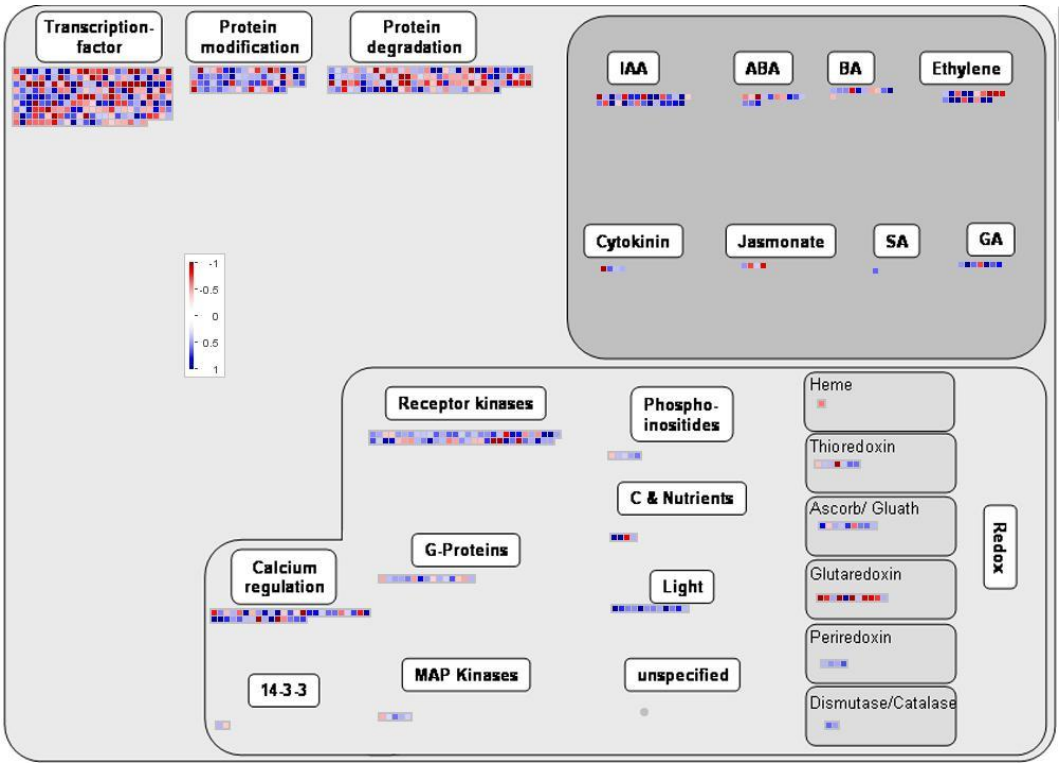


Figure 3.14 Effect of BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue on genes involved in major regulatory processes in the plant including genes involved in phytohormone metabolic regulation. 2730 of 26868 data points were mapped, with 676 genes coding for regulatory role-players induced and repressed at variable levels of predicted log_2fold change.

Table 3.9 Transcription factor categories altered by BC204 treatment in *Arabidopsis thaliana* shoot tissue

Transcription factor category (with BinCode)	Total	Induced	Repressed
RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family (27.3.3)	21	11	10
RNA.regulation of transcription.unclassified (27.3.99)	21	8	13
RNA.regulation of transcription.MYB domain transcription factor family (27.3.25)	16	10	6
RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family (27.3.6)	14	7	7
RNA.regulation of transcription.WRKY domain transcription factor family (27.3.32)	12	6	6
RNA.regulation of transcription.C2H2 zinc finger family (27.3.11)	11	5	6
RNA.regulation of transcription.HB,Homeobox transcription factor family (27.3.22)	11	6	5
RNA.regulation of transcription.bZIP transcription factor family (27.3.35)	10	5	5
RNA.regulation of transcription.putative transcription regulator (27.3.67)	9	6	3
RNA.regulation of transcription.G2-like transcription factor family, GARP (27.3.20)	7	5	2
RNA.regulation of transcription.C3H zinc finger family (27.3.12)	6	3	3
RNA.regulation of transcription.Aux/IAA family (27.3.40)	6	4	2
RNA.regulation of transcription.CCAAT box binding factor family, HAP2 (27.3.14)	5	0	5
RNA.regulation of transcription.MYB-related transcription factor family (27.3.26)	5	2	3

RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family (27.3.37)	4	3	1
RNA.regulation of transcription.GeBP like (27.3.49)	4	-	4
RNA.regulation of transcription.PHOR1 (27.3.64)	4	3	1
RNA.regulation of transcription.ARR (27.3.5)	3	-	3
RNA.regulation of transcription.C2C2(Zn) DOF zinc finger family (27.3.8)	3	2	1
RNA.regulation of transcription.C2C2(Zn) GATA transcription factor family (27.3.9)	3	1	2
RNA.regulation of transcription.NAC domain transcription factor family (27.3.27)	3	-	3
RNA.regulation of transcription.Trihelix, Triple-Helix transcription factor family (27.3.30)	3	2	1
RNA.regulation of transcription.General Transcription (27.3.50)	3	1	2
RNA.regulation of transcription.Psudo ARR transcription factor family (27.3.66)	3	-	3
RNA.regulation of transcription.ABI3/VP1-related B3-domain-containing transcription factor family (27.3.1)	2	1	1
RNA.regulation of transcription.C2C2(Zn) CO-like, Constans-like zinc finger family (27.3.7)	2	1	1
RNA.regulation of transcription.HSF,Heat-shock transcription factor family (27.3.23)	2	1	1
RNA.regulation of transcription.MADS box transcription factor family (27.3.24)	2	2	-
RNA.regulation of transcription.Orphan family (27.3.34)	2	1	1

RNA.regulation of transcription.B3 transcription factor family (27.3.41)	2	-	2
RNA.regulation of transcription.Bromodomain proteins (27.3.42)	2	-	2
RNA.regulation of transcription.Global transcription factor group (27.3.52)	2	1	1
RNA.regulation of transcription.HDA (27.3.55)	2	-	2
RNA.regulation of transcription.Nucleosome/chromatin assembly factor group (27.3.62)	2	1	1
RNA.regulation of transcription.zf-HD (27.3.80)	2	-	2
RNA.regulation of transcription.ARF, Auxin Response Factor family (27.3.4)	1	-	1
RNA.regulation of transcription.TCP transcription factor family (27.3.29)	1	1	-
RNA.regulation of transcription.Chromatin Remodeling Factors (27.3.44)	1	1	-
RNA.regulation of transcription.DNA methyltransferases (27.3.46)	1	1	-
RNA.regulation of transcription.JUMONJI family (27.3.57)	1	1	-
RNA.regulation of transcription.NIN-like bZIP-related family (27.3.60)	1	-	1
RNA.regulation of transcription.PWWP domain protein (27.3.68)	1	1	-
RNA.regulation of transcription.SET-domain transcriptional regulator family (27.3.69)	1	1	-
RNA.regulation of transcription.Silencing Group (27.3.70)	1	1	-

RNA.regulation of transcription.Transcriptional Adaptor Zinc Bundle (TAZ) domain family (27.3.72)	1	1	-
RNA.regulation of transcription.BBR/BPC (27.3.84)	1	-	1
RNA.regulation of transcription.sigma like plant (27.3.85)	1	1	-

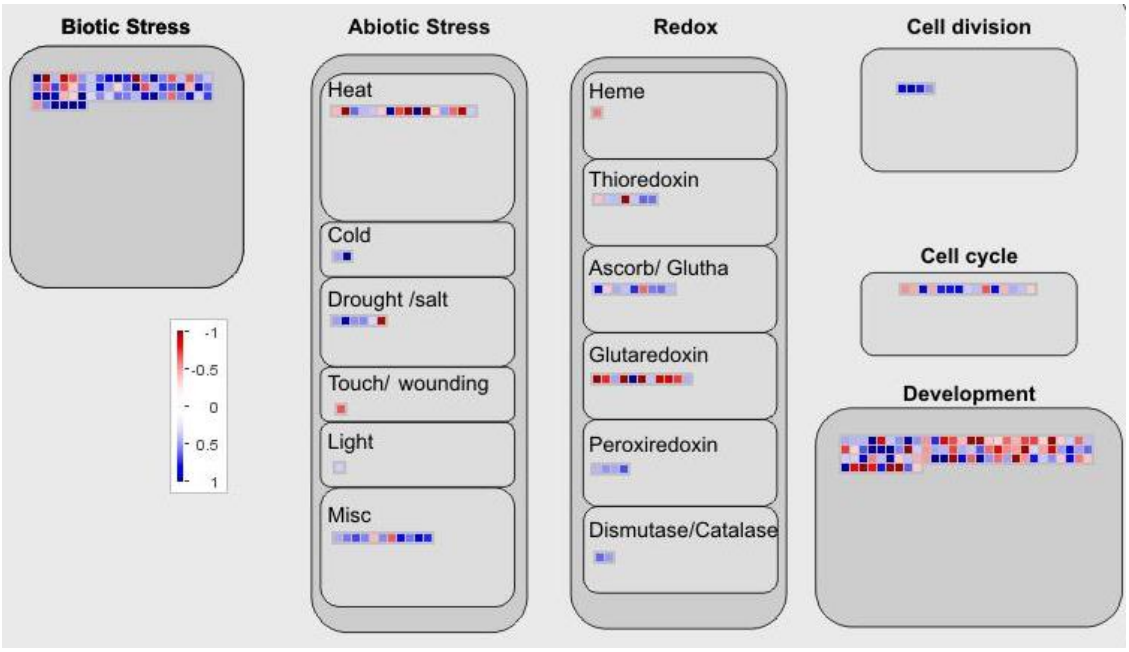


Figure 3.15 Cellular response downstream of regulation processes in response to BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue. 2730 of 26868 data points were mapped by Mapman. The expression of 249 genes involved in major cellular responses were altered in response to BC204 treatment.

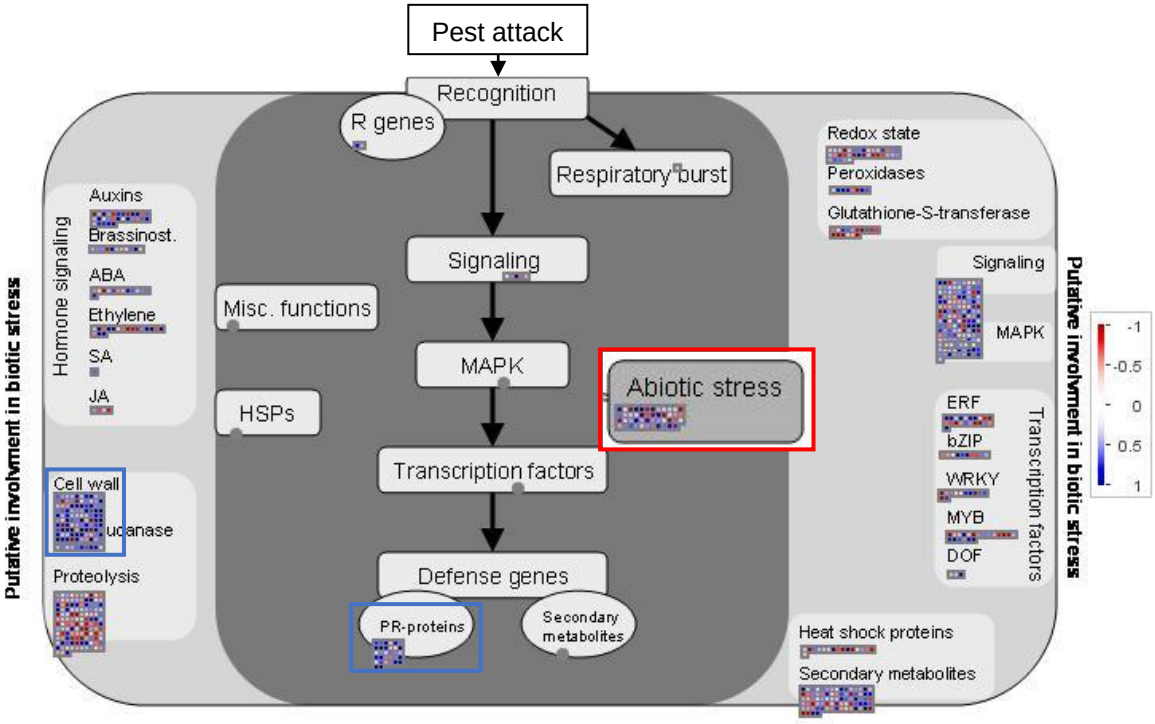


Figure 3.16 Putative involvement of BC204 in biotic stress metabolism in *Arabidopsis thaliana* as per MapMan bin-wise categorisation. 2730 of 26868 data points were mapped, with 774 visible here.

Genes involved in secondary metabolism were evenly distributed with regards to up- and downregulation (Figure 3.17). Downregulated genes were found in all major secondary metabolism processes affected by BC204 treatment. The inositol phosphate biosynthesis pathway, on the other hand, was upregulated at 12 stages (Figure 3.18, Table 3.9).

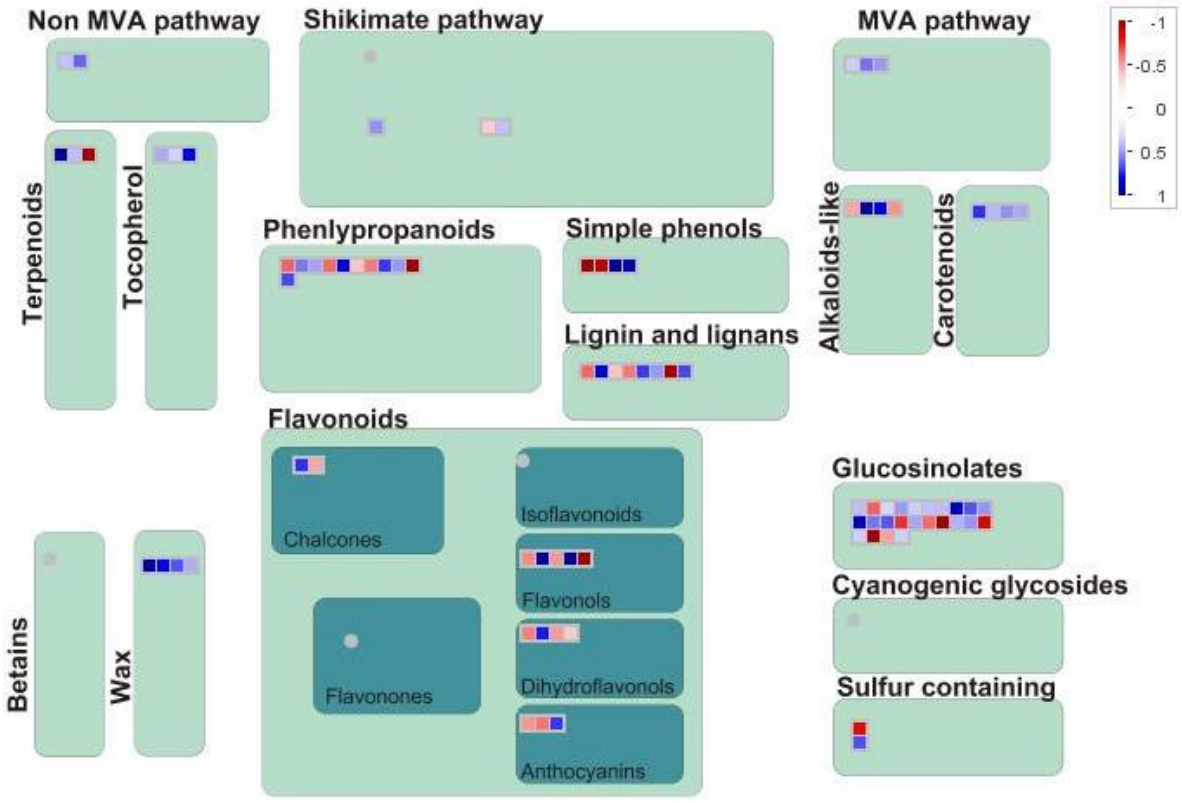


Figure 3.17 Putative effect of BC204 on 89 genes involved in secondary metabolism in *Arabidopsis thaliana* shoot tissue. 2730 of 26868 data points were mapped by MapMan.

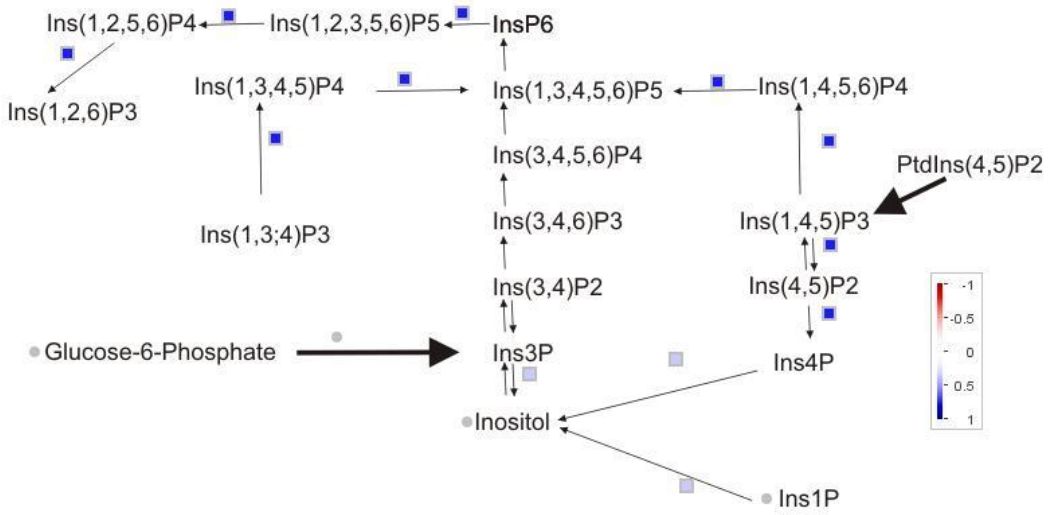


Figure 3.18 The induction of 12 genes coding for inositol phosphates synthesis by BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue.

Table 3.10 Genes involved in inositol phosphatase induced by BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue

BinCode	BinName	Gene ID	Description	Log_2fold change
3.4.5	minor CHO metabolism.myo-inositol.inositol phosphatase	*AT1G31190*	Symbols: IMPL1 | IMPL1 (MYO-INOSITOL MONOPHOSPHATASE LIKE 1); 3'(2'),5'-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase/ inositol-1(or 4)-monophosphatase	0.367
3.4.5	minor CHO metabolism.myo-inositol.inositol phosphatase	*AT1G31190*	Symbols: IMPL1 | IMPL1 (MYO-INOSITOL MONOPHOSPHATASE LIKE 1); 3'(2'),5'-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase/ inositol-1(or 4)-monophosphatase	0.367
3.4.5	minor CHO metabolism.myo-inositol.inositol phosphatase	*AT1G31190*	Symbols: IMPL1 | IMPL1 (MYO-INOSITOL MONOPHOSPHATASE LIKE 1); 3'(2'),5'-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase/ inositol-1(or 4)-monophosphatase	0.367
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761

3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761
3.4.1	minor CHO metabolism.myo-inositol.poly-phosphatases	*AT4G18010*	Symbols: IP5PII, AT5PTASE2 | AT5PTASE2 (MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2); inositol-polyphosphate 5-phosphatase	0.761

3.4.5 Quantitative RT-PCR (RT-qPCR) validation of RNA-seq data

To validate RNA-seq results, we randomly selected four genes, two of which were upregulated and two that were downregulated. The upregulated (AT1G50040*,* AT3G50060*)* and downregulated transcripts (AT1G58340*,* AT3G60140) were analysed by RT-qPCR using *MON1*, which was unchanged in the RNA-seq experiment, as a reference. Comparisons between the RNA-seq and RT-qPCR analysis showed positive correlation between the two approaches (Table 3.10). Although RT-qPCR indicated that AT1G58340 expression was up-regulated*,* the standard error was relatively large, placing the value within range of the downregulation predicted by RNA-seq analysis.

Table 3.11 RT-qPCR validation of differentially up- and downregulated genes obtained from RNA-seq data following BC204 treatment in *Arabidopsis thaliana* shoot tissue. Log_2fold change of transcript levels from the RNA-seq analysis was determined from replicates (n=3) of each sample while for quantitative RT-qPCR, the Ct values were averaged and normalized to MON1 according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the relative transcript expression was significantly different at $p \leq 0.05$. For RT-qPCR, values lower than 1 represent downregulation of the transcript.

Gene	RNA-seq (log_2fold)	RT-qPCR (foldchange ± SE)
AT1G50040	3.2814	2.927 ±1.696
AT1G58340	-2.24741	1.780 ±1.056
AT3G50060	2.68942	19.339 ±7.648
AT3G60140	-2.79986	0.560 ±0.286

3.5 Discussion

As the need for more sustainable agriculture solutions are being sought in order to reach the collective goal of sustainable intensification (Struik and Kuyper, 2017), scientists are turning towards characterising the molecular effects of PBs on plant growth in order to validate the claims made by the producers of these products (Ricci et al., 2019). BC204 has been shown, in practice and in unpublished results (Van Zyl, 2007), to elicit major physiological benefits to plant growth and development, a phenomenon also seen with the use of other PBs on a range of plant species (Drobek et al., 2019; Yakhin et al., 2017). Our results confirmed that BC204 increases *A. thaliana* above-ground rosette growth with respect to fresh and dry biomass. This prompted RNA-seq analysis to elucidate the effects of BC204 on global gene expression in this model plant species.

3.5.1 BC204 increased aboveground biomass after 4 weeks of exogenous treatment

As observed in Chapter 2, BC204 elicited an increase in *A. thaliana* aboveground biomass compared to the untreated control. This visible increase in rosette size (Figure 3.1) was also reflected in biomass measurements, where a significant increase in both fresh and dry weights of above-ground rosette tissue elicited by BC204 was observed (Figure 3.2). This confirms a growth response related to increased shoot growth by BC204. PBs from different origins are widely reported to at least increase tissue fresh weight in a variety of plant species (Bulgari et al., 2015; Dudaš et al., 2016; Fleming et al., 2019; Gavelienė et al., 2018; Petrozza et al., 2014; Przybysz et al., 2014; Rouphael et al., 2018a; Santoso et al., 2018; Trevisan et al., 2019; Zeljković et al., 2013). Considering the absence of any prior molecular or biochemical data for this PB, there were no biochemical or molecular markers which could be used as indicators of the optimal time to harvest material in order to obtain the best understanding of the molecular changes brought about by BC204 treatment. Since BC204 is usually sprayed three times per season, we assumed that a total of three treatments over three weeks and a final treatment 90 min prior to harvesting would allow for the detection of most long-term and short-term molecular responses to BC204 in *Arabidopsis*.

3.5.2 Transcriptomic analysis reveals deep sequencing covering the entire genome

Although recent advances made in next generation sequencing depth and coverage have rendered the need for many replicates unnecessary (Bass et al., 2019; Liu et al., 2014), we utilised three pooled samples per treatment (n=9) to provide sufficient coverage and variation to instil confidence in the outcomes of this transcriptomic analysis. After trimming the data, a mean of 6.849107 million reads per samples was retained, which is considered more than sufficient for further analysis (Conesa et al., 2016). Although trimming of RNA-seq data can possibly alter gene expression estimates (Williams et al., 2016), it is routinely used in order to only use high-quality data for downstream analysis.

3.5.3 Filtering low expression genes for downstream analysis

There is considerable debate around which cut-off point for $\log_2$fold changes should be used in RNA-seq studies. This normalization and significance issues have their origin in microarray studies but affect next-generation sequencing data analysis as well since cut-offs modulate the outcome of the data and subsequent analysis and discussion points (Dalman et al., 2012). Filtering of low expression genes is routinely used in studies, and although no universal optimal thresholds have been set, most opt for a cut-off

of 1.5 or 2 (Sha et al., 2015). Using a high fold-change (FC) cut-value might provide a well-defined and concise set of genes to ease downstream analysis but increases the likelihood of missing many complex regulatory processes (Zhao et al., 2018). Furthermore, a higher fold change does not necessarily equal a greater effect. Certain upstream regulators may have a much smaller foldchange in comparison to downstream genes which are most likely targeted by multiple pathways. Bias can be introduced by researchers in order to strengthen their argument/hypothesis. A balance must therefore be found between statistical significance and biological relevance. For an exploratory study like this it was decided to use a fold change value of 1/-1 as a bench mark for the majority of the analyses, while including lower DEGs at times, particularly with regards to signalling, regulation and transcription, since small changes in the expression of these genes can have large effects on plant metabolism and growth.

For the more detailed analyses (e.g PageMan analysis), genes with a smaller log_2fold value (between 1 and -1) were omitted in order to highlight the most important changes at the molecular level elicited by BC204. These genes were considered highly induced or repressed. In the *Supplementary Material*, a list of all genes with altered expression levels can be visualized in a detailed PageMan figure (Supplementary Figure S3.5). As mentioned, the effect of filtering out lower expressed genes changes the overall outcome of the processes altered by BC204, especially with the automated analysis such as PageMan which includes a Wilcoxon test (Figure 3.8; Supplementary Figure S3.5). This figure also indicates how the inclusion of lower expression genes alters the hypothetical outcome of the processes involved in the BC204 mode of action. For example, processes involved in major carbohydrate metabolism were shown to be downregulated when genes with a log_2fold smaller than -1 and greater 1 was included in the analysis (Figure 3.8), but when all the DEGs (no foldchange cut-off) were included these processes were mostly unchanged (Supplementary Figure S3.5). This can also be observed in large sections of each bin.

3.5.4 BC204 induces a large shift in gene expression across many pathways in primary and secondary metabolism

BC204 elicited changes in gene expression across many biochemical pathways involved in both primary and secondary metabolism (Figure 3.7). There have been only a limited number of studies on PBs which have used an RNA-seq approach to analyse the effects of PBs at the molecular level, and even fewer of these have been conducted on model plant species with well-annotated genomes (Chang et al., 2016). Only a few studies to date have adopted an RNA-seq approach for *A. thaliana* plants treated with PBs (Weeda et al., 2014; Shukla et al., 2018; Omidbakhshfard et al., 2020).

For all studies using transcriptomic (RNA-seq) methodologies, authors select whether to adopt a MapMan functional annotation or gene ontology (GO) pipeline for downstream analysis and visualization of the data. The difference between the two and the advantages of each respectively, for *Arabidopsis thaliana*, were previously compared (Klie and Nikoloski, 2012). MapMan is used to give a general overview of the gene expression changes across the genome, categorised into bins. MapMan is designed to specifically cover plant-specific pathways and processes while GO takes a broader approach in predicting gene function and was originally developed to characterize microbial systems (Klie and Nikoloski, 2012). For the purpose of the present study, a combination of both approaches was used in order to avoid oversights and discrepancies that might arise due to automated gene function predictions (Promponas et al., 2015).

Changes in gene expression were also scattered across most of the processes (bins). Using both approaches also allows for cross-validation, as used in a recent study (Xu et al., 2019). For *Arabidopsis*, and probably for most other plant species, automated gene function prediction is inevitable because only 56.6% of the genes in *Arabidopsis* have been functionally annotated based on sequence similarity to known genes, according to the most recent release of the annotated genome (Cheng et al., 2017).

3.5.5 Increased growth possibly elicited by the upregulation of genes involved in primary metabolism including cell wall synthesis, lipid metabolism and photosynthesis

As mentioned, changes in gene expression were distributed across most pathways, but seemed to be largely concentrated in primary metabolic processes. MapMan software analysis (Thimm et al., 2004) revealed that the processes most upregulated were light reactions, photorespiration, cell wall biogenesis and minor carbohydrate (CHO) metabolism (Figure 3.7, Table 3.4), although several other key processes were also upregulated. Eight genes coding for arabinogalactan proteins (AGPs) were significantly upregulated by BC204 (Table 3.4). These AGPs, although poorly characterised to date, are involved in processes including cell expansion, cell differentiation, tissue development, somatic embryogenesis, and modulating cell wall expansion under salinity stress (Olmos et al., 2017). In addition, four genes coding for cell wall pectin esterases, as well as 12 genes coding for different enzymes involved in cell wall modification, were upregulated by BC204 (Table 3.4). Pectin esterases are involved in cell wall remodelling in response to heat stress (Wu et al., 2018).

Most downregulated genes were clustered in the same bins that contained a large number of upregulated genes (Figure 3.7). With bins displaying a combination of strongly upregulated and downregulated genes, tight interactions across several pathways and feedback mechanisms could be elicited by BC204. As an extension of MapMan ontology analysis, PageMan was used to statistically evaluate responses at the pathway or process level (Usadel et al., 2009, 2006) to BC204 treatment. This analysis provided detailed descriptions of all processes up- and downregulated by genes that had a $\log_2$fold change of less than -1 and greater than 1 (Figure 3.4). Processes mostly upregulated were photosynthesis (PS), minor carbohydrate (CHO) metabolism, OPP, TCA, cell wall, lipid metabolism, hormone metabolism, co-factor and vitamin metabolism, stress (mostly biotic) and signalling. Processes mostly downregulated were major carbohydrate metabolism (CHO), mitochondrial electron transport/ ATP synthesis, N-metabolism, amino acid metabolism, S-assimilation, secondary metabolism, redox, nucleotide metabolism, biodegradation of xenobiotics, misc, protein and transport. Processes with an almost equal distribution of both up- and downregulated processes were observed in the RNA bin. Further details of all processes, including those which were unaltered in this experiment, are presented in the *Supplementary Material* (Supplementary Figure S3.5). A detailed analysis of this image indicates that up- and down-regulated processes were almost evenly distributed, suggesting that there may be cost implications for every positively regulated process which result in the downregulation of other processes.

Mercator provides a different visualization of upregulated and downregulated processes. The processes mostly upregulated by BC204 as categorized by Mercator annotation were protein (9.07 %), miscellaneous (8.30 %), RNA (7.13%), signalling (6.36%), cell wall (4.95%), stress (4.77%), transport (4.59%) and PS (4.24%) related genes (Figure 3.5). The processes mostly downregulated by BC204 were

RNA (13.25 %), miscellaneous (8.47%), transport (6.47%) and development (4.58%) (Figure 3.6). PANTHER Classification (Thomas et al., 2003) was also used to visualise all the up- and down-regulated genes grouped into either cellular component, biological process, molecular function (Figure 3.7) or protein class (Figure 3.8). Recent developments in genome coverage, coverage and accuracy, functional information and improved genomic data analysis tools (Mi et al., 2016) makes PANTHER a useful tool for RNA-seq data visualization. In the PANTHER output figures (Figure 3.7, 3.8). BC204 changed gene expression almost equally in each process. More than 100 genes were upregulated and downregulated in the cell and organelle (GO: cellular component), in metabolic and cellular processes (GO: biological process) and catalytic activity and binding (GO: molecular function). For each category where many genes were upregulated, almost as many genes were also downregulated. This contributes to the idea that BC204 elicits complex changes not only across different processes but also within these processes. The physiological and biochemical outcomes of these major changes were not analysed in the present study, but the data provided can be used as a platform for future studies to pinpoint the specific metabolic changes. In the protein class section, a similar trend was observed (Figure 3.8). Hydrolases, oxidoreductases, and transferase were mostly upregulated, containing 108, 100 and 76 up-regulated genes respectively. One category, nucleic acid binding, had 66 genes downregulated and 32 genes upregulated. Other categories with slightly more genes downregulated than upregulated were storage protein, transcription factor and signalling.

As discussed, cell wall biogenesis appears to be the process mostly involved in the BC204 mode of action (Table 3.4, Table 3.6 Figure 3.9). Even at higher foldchange cut-offs, analysis reveals that most genes upregulated by BC204 were connected to cell wall synthesis, restructuring and modification. The induction of genes related to wax production also suggests an increase in plant defence, since wax prevents dehydration and provides an extra layer of defence against microbes (Buschhaus and Jetter 2012). Cell wall biogenesis is also an important aspect in biotic stress metabolism. Lipid metabolism was also affected, with 7 genes involved in lipid metabolism being significantly upregulated and one downregulated (Table 3.4). Under favourable conditions, lipid metabolism and storage are ramped up (Fan et al., 2019). Lipids in plant leaves are needed for the biogenesis of cell membranes and serve as signal molecules and a source of carbon and energy. There is an interplay between starch and lipid metabolism, with plants significantly increasing fatty acid synthesis when starch synthesis is disrupted (Yu et al., 2018).

Genes related to photosynthesis and light reactions in the chloroplast were heavily upregulated by BC204 (Figure 3.10, Figure 3.11). Chlorophyll biosynthesis, as a branch of tetrapyrrole metabolism, was also upregulated at several key steps (Figure 3.12, Table 3.7), which supports previous observations (Chapter 2) of an increase in total chlorophyll in BC204-treated plant leaves. Downstream of photosynthesis, at the sucrose/starch level, genes responsible for starch storage and degradation were downregulated, while genes responsible for sucrose synthesis and cleavage were upregulated (Figure 3.13, Table 3.8). A similar result was observed when *A. thaliana* plants were treated with the plant growth promoting substance lumichrome. Following an increase in photosynthetic rate and capability, more carbohydrates were available to be used for growth, resulting in an increase in biomass (Pholo et al., 2018).

3.5.6 Signalling and gene regulation is central to the growth changes elicited by BC204

An overview of regulation processes influenced by BC204 treatment indicated a change in gene expression of genes coding for transcription factors, protein modification, protein degradation and hormone metabolism. A total of 676 of these regulators were affected by BC204 treatment. Interestingly, only 117 of these had a log_2fold of larger than 1 or smaller than -1 (Figure 3.14). However, as previously mentioned, small changes to the expression of regulatory elements in the genome can cause large changes in downstream metabolic processes. This indicates an extremely complex regulation response to BC204 which makes it difficult to suggest which specific processes were targeted, but it does indicate that BC204 shifts gene expression through complex regulation processes upstream of primary metabolic processes through changes in signalling and the regulation of gene expression. The large change in RNA regulation, almost equally up and downregulated (Figure 3.4), adds to the complexity of transcription and the regulation of transcription as elicited by BC204 treatment. In conjunction with transcription factors, protein modification and protein degradation were also altered with regards to upregulation and downregulation. Genes involved in regulation of all the 8 phytohormones were both up- and downregulated (Figure 3.14).

3.5.7 Processes downstream of signalling and regulation largely affects development and biotic stress metabolism

For genes coding for large enzyme families, 10 of the sub-bins displayed a combination of up and downregulated genes (Supplementary Figure S3.3). Genes coding for cytochrome P450, oxidases, nitrilases and UDP glycosyltransferases were almost equally distributed with regards to up- and downregulation, while genes coding for GDSL-lipases, peroxidases, phosphatases and β1,3 glucan hydrolases were largely upregulated in response to BC204 treatment. This links to the previous section on signalling and regulation. Signalling and regulation will elicit a change in downstream processes and several signalling factors will often target the same process. One group of receptor-like kinases, leucine-rich repeat (LRR) proteins, were mostly upregulated by BC204 (Supplementary Figure S3.4). LRR receptor-like kinases (LRR-RLKs) are one of the largest protein families (Gou et al., 2010) and are suggested to be involved in various plant processes including the perception of various signals at the plasma membrane (Xi et al., 2019) and in development and stress responses (Dievart and Clark, 2004; Dufayard et al., 2017). BC204 could therefore influence plant growth and development through LRR-RLKs.

BC204 could play a role in priming the plant for increased resistance to biotic stress, because LRR-RLKs also involved in the activation of defence against pathogens (reviewed by Dufayard et al., 2017). This hypothesis is further strengthened by the increase in cell wall biogenesis and pathogenesis-related (PR) proteins (Figure 3.16). PR proteins are primarily induced by both pathogenic infections and by jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and brassinosteroids (BR) (Seo et al., 2008). This could also explain the increase in salt tolerance observed in Chapter 2, since overexpression of *PR10* in rice resulted in an increase in salt tolerance, possibly by activating the expression of stress-related proteins (Wu et al., 2016). Early studies hinted towards the involvement of PR-proteins in both biotic and abiotic stress responses. Other studies similarly reported that the induction of PR protein gene expression resulted in an increase in salt tolerance (Ali et al., 2018). PBs have also been shown to induce the expression of PR proteins, which forms part of a larger systemic acquired resistance (Le Mire et al., 2016). Plant growth promoting

rhizobacteria and algal polysaccharides have also been shown to induce the expression and accumulation of PR proteins (Maksimov et al., 2011; Przybysz et al., 2014; Stadnik and Freitas, 2014). Despite the absence of biotic stressors in this study, it is interesting that so many genes involved in biotic stress responses were upregulated upon BC204 treatment. The strong biotic stress response observed here could be due to the co-extraction of endophytic microbitotic elicitors that are present in certain fruit species (Glassner et al., 2015). Endophyte communities are widely present in several citrus varieties (Munir et al., 2020)

For the RT-qPCR validation of RNA-seq data, 3 out of 4 of the genes used for verification had similar expression levels as the predicted fold$_2$change calculated from the RNA-seq output data. A study using melatonin on *Arabidopsis thaliana* revealed a discrepancy of 7 genes that were up or downregulated according to RNA-seq analysis but not significantly altered according to qPCR analysis (Weeda et al., 2014). RNA-seq and qPCR analysis fundamentally determines changes in gene expression in completely different ways. RNA samples from 3 replicates were also pooled, and this complicates the matter further since statistical significance are calculated differently. Also, large variations in transcripts in different samples resulted in an average that would not reflect the RNA-seq results. This is evident in the big error bars (Weeda et al., 2014).

3.6 Conclusion

The treatment of *A. thaliana* with BC204 resulted in a major change at the transcriptomic level. In primary metabolism, genes involved in light reactions and photosynthesis were upregulated. These changes resulted in an increase in total carbon influx into the plant's metabolism. This increase in sugars was then most likely channelled towards minor carbon metabolism, cell wall biogenesis and lipid metabolism, as genes involved in these processes were mostly upregulated. Although these were the main processes that were upregulated, several other important metabolic processes were also enhanced or suppressed. Considerable induction of genes involved in priming the plant towards increased resistance towards environmental stress was observed. Interestingly, a large of number of genes related to biotic stress were upregulated, even though the plants in this study were not exposed to any biotic stressors. Within secondary metabolism, the observation of genes being up- and down-regulated, both within and between different pathways, suggests that some trade-offs between growth and chemical defences may have been made. In addition to these changes, an overwhelming number of genes coding for regulatory role-players such as transcription factors were both up- and downregulated. This points to additional major changes to regulatory networks being activated and modified by BC204.

In conclusion, these major changes across many metabolic processes and regulatory mechanisms means that BC204 has an almost holistic effect across the entire range of *A. thaliana* gene expression, ultimately resulting in an enhancement of shoot growth of *A. thaliana.* Trying to pin-point one or even a few specific mechanisms responsible for the observed increase in plant growth would be impossible due to the complex mixture of extracts present in BC204. Further exploration into each of the individual pathways would illuminate the more specific changes downstream of expression.

Author contributions

JL and PH designed the research, JL prepared and PH edited and revised the manuscript. JL conducted all the experimental work and also conducted all further gene expression analysis after DEG determination. BC and APC aided in the RNA-seq analysis, writing the scripts for the analysis. JK provided partial funding and further guidance.

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Supplementary Material

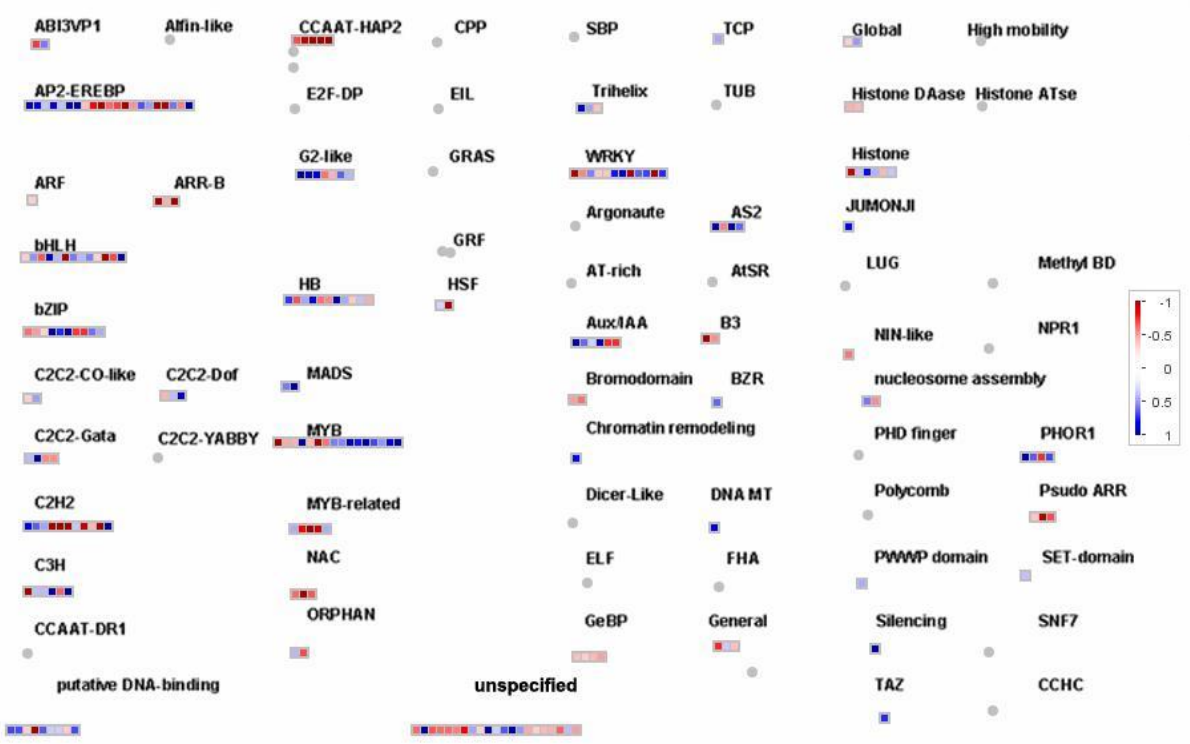


Figure S3.1 Effect of BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue on genes coding for transcription factors. 2730 of 26868 data points were mapped, with 224 visible here.

Table S3.1 *Arabidopsis thaliana* genes differentially expressed assigned to BinCodes associated with transcription factors as annotated and assigned by MapMan

BinCode	BinName	Gene ID	Type	Log_2fold
27.3.1	RNA.regulation of transcription.ABI3/VP1-related B3-domain-containing transcription factor family	*AT3G11580*	DNA-binding protein, putative	-0.714
27.3.1	RNA.regulation of transcription.ABI3/VP1-related B3-domain-containing transcription factor family	*AT3G61970*	Symbols: NGA2 | NGA2 (NGATHA2); transcription factor	0.585
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT1G21910*	AP2 domain-containing transcription factor family protein	4.231
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT1G22190*	AP2 domain-containing transcription factor, putative	0.854
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT1G25560*	Symbols: TEM1 | TEM1 (TEMPRANILLO 1); transcription factor	0.377
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT1G64380*	AP2 domain-containing transcription factor, putative	0.907
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT1G68840*	Symbols: RAV2, RAP2.8, TEM2 | RAV2 (REGULATOR OF THE ATPASE OF THE VACUOLAR MEMBRANE); DNA binding / transcription factor/ transcription repressor	0.391
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT1G77640*	AP2 domain-containing transcription factor, putative	2.605
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element	*AT2G20880*	AP2 domain-containing transcription factor,	1.211

	binding protein family		putative	
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT2G44940*	AP2 domain-containing transcription factor TINY, putative	-0.396
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT2G46310*	Symbols: CRF5 | CRF5 (CYTOKININ RESPONSE FACTOR 5); DNA binding / transcription factor	-0.798
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT3G11020*	Symbols: DREB2B, DREB2 | DREB2B (DRE/CRT-BINDING PROTEIN 2B); DNA binding / transcription activator/ transcription factor	-0.993
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT3G25890*	AP2 domain-containing transcription factor, putative	-0.624
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT3G61630*	Symbols: CRF6 | CRF6 (CYTOKININ RESPONSE FACTOR 6); DNA binding / transcription factor	-0.706
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT4G06746*	Symbols: RAP2.9 | RAP2.9 (related to AP2 9); DNA binding / transcription factor	-1.736
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT4G16750*	DRE-binding transcription factor, putative	-0.524
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT4G23750*	Symbols: CRF2 | CRF2 (CYTOKININ RESPONSE FACTOR 2); DNA binding / transcription factor	0.668
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT4G25470*	Symbols: CBF2, DREB1C, FTQ4, ATCBF2 | CBF2 (C-REPEAT/DRE BINDING FACTOR	0.505

			2); DNA binding / transcription activator/ transcription factor	
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT4G25480*	Symbols: DREB1A, CBF3, ATCBF3 | DREB1A (DEHYDRATION RESPONSE ELEMENT B1A); DNA binding / transcription activator/ transcription factor	-1.304
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT4G25490*	Symbols: CBF1, DREB1B, ATCBF1 | CBF1 (C-REPEAT/DRE BINDING FACTOR 1); DNA binding / transcription activator/ transcription factor	-0.977
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT4G32800*	AP2 domain-containing transcription factor TINY, putative	0.595
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT5G05410*	Symbols: DREB2A, DREB2 | DREB2A; DNA binding / transcription activator/ transcription factor	-0.567
27.3.3	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family	*AT5G25810*	Symbols: tny | tny (TINY); DNA binding / transcription factor	1.087
27.3.4	RNA.regulation of transcription.ARF, Auxin Response Factor family	*AT5G60450*	Symbols: ARF4 | ARF4 (AUXIN RESPONSE FACTOR 4); transcription factor	-0.348
27.3.5	RNA.regulation of transcription.ARR	*AT1G19050*	Symbols: ARR7 | ARR7 (RESPONSE REGULATOR 7); transcription regulator/ two-component response regulator	-1.269
27.3.5	RNA.regulation of transcription.ARR	*AT2G40670*	Symbols: ARR16, RR16 | ARR16	-0.458

			(ARABIDOPSIS RESPONSE REGULATOR 16); transcription regulator/ two-component response regulator	
27.3.5	RNA.regulation of transcription.ARR	*AT5G62920*	Symbols: ARR6 | ARR6 (RESPONSE REGULATOR 6); transcription regulator/ two-component response regulator	-1.18
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT1G03040*	basic helix-loop-helix (bHLH) family protein	-0.351
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT1G10120*	DNA binding / transcription factor	0.529
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT1G59640*	Symbols: ZCW32, BPEP | ZCW32; DNA binding / transcription factor	-0.657
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT1G73830*	Symbols: BEE3 | BEE3 (BR ENHANCED EXPRESSION 3); DNA binding / transcription factor	0.942
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT2G18300*	basic helix-loop-helix (bHLH) family protein	0.422
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT2G43140*	DNA binding / transcription factor	-1.072
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT3G07340*	basic helix-loop-helix (bHLH) family protein	0.584
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT3G57800*	basic helix-loop-helix (bHLH) family protein	0.418
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT3G59060*	Symbols: PIL6, PIF5 | PIL6 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 6); DNA binding / transcription factor	0.567
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT4G17880*	basic helix-loop-helix (bHLH) family protein	-0.334

27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT4G36930*	Symbols: SPT | SPT (SPATULA); DNA binding / transcription factor	-1.054
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT5G10570*	basic helix-loop-helix (bHLH) family protein	-0.682
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	*AT5G50915*	basic helix-loop-helix (bHLH) family protein	1.146
27.3.7	RNA.regulation of transcription.C2C2(Zn) CO-like, Constans-like zinc finger family	*AT2G47890*	zinc finger (B-box type) family protein	-0.34
27.3.7	RNA.regulation of transcription.C2C2(Zn) CO-like, Constans-like zinc finger family	*AT5G57660*	Symbols: ATCOL5, COL5 | zinc finger (B-box type) family protein	0.494
27.3.8	RNA.regulation of transcription.C2C2(Zn) DOF zinc finger family	*AT1G28310*	Dof-type zinc finger domain-containing protein	-0.434
27.3.8	RNA.regulation of transcription.C2C2(Zn) DOF zinc finger family	*AT5G60850*	Symbols: OBP4 | OBP4; DNA binding / transcription factor	0.411
27.3.8	RNA.regulation of transcription.C2C2(Zn) DOF zinc finger family	*AT5G62430*	Symbols: CDF1 | CDF1 (CYCLING DOF FACTOR 1); DNA binding / protein binding / transcription factor	0.96
27.3.9	RNA.regulation of transcription.C2C2(Zn) GATA transcription factor family	*AT3G24050*	GATA transcription factor 1 (GATA-1)	0.416
27.3.9	RNA.regulation of transcription.C2C2(Zn) GATA transcription factor family	*AT3G54810*	Symbols: BME3-ZF, BME3 | zinc finger (GATA type) family protein	1.854
27.3.9	RNA.regulation of transcription.C2C2(Zn) GATA transcription factor family	*AT4G34680*	GATA transcription factor 3, putative (GATA-3)	-0.554
27.3.9	RNA.regulation of transcription.C2C2(Zn) GATA transcription factor family	*AT5G66320*	zinc finger (GATA type) family protein	-0.537
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT1G27730*	Symbols: STZ, ZAT10 | STZ (salt tolerance zinc finger); nucleic acid binding / transcription factor/ transcription repressor/	0.862

			zinc ion binding	
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT1G75710*	zinc finger (C2H2 type) family protein	0.644
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT3G07940*	zinc finger and C2 domain protein, putative	0.481
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT3G28210*	Symbols: PMZ | PMZ; zinc ion binding	-1.766
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT3G42860*	zinc knuckle (CCHC-type) family protein	-1.177
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT3G46080*	zinc finger (C2H2 type) family protein	-2.232
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT3G52800*	zinc finger (AN1-like) family protein	0.379
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT3G60580*	zinc finger (C2H2 type) family protein	-0.92
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT5G16470*	zinc finger (C2H2 type) family protein	-0.422
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT5G59820*	Symbols: RHL41, ZAT12 | RHL41 (RESPONSIVE TO HIGH LIGHT 41); nucleic acid binding / transcription factor/ zinc ion binding	-1.363
27.3.11	RNA.regulation of transcription.C2H2 zinc finger family	*AT5G67450*	Symbols: AZF1 | AZF1 (ARABIDOPSIS ZINC-FINGER PROTEIN 1); DNA binding / nucleic acid binding / transcription factor/ transcription repressor/ zinc ion binding	1.477
27.3.12	RNA.regulation of transcription.C3H zinc finger family	*AT2G19810*	zinc finger (CCCH-type) family protein	-1.319

27.3.12	RNA.regulation of transcription.C3H zinc finger family	*AT2G40140*	Symbols: CZF1, ZFAR1, SZF2, ATSZF2 | CZF1; transcription factor	0.402
27.3.12	RNA.regulation of transcription.C3H zinc finger family	*AT3G02830*	Symbols: ZFN1 | ZFN1 (ZINC FINGER PROTEIN 1); DNA binding / nuclease/ nucleic acid binding	0.406
27.3.12	RNA.regulation of transcription.C3H zinc finger family	*AT3G55980*	Symbols: SZF1, ATSZF1 | SZF1 (SALT-INDUCIBLE ZINC FINGER 1); transcription factor	1.427
27.3.12	RNA.regulation of transcription.C3H zinc finger family	*AT4G29190*	zinc finger (CCCH-type) family protein	-0.636
27.3.12	RNA.regulation of transcription.C3H zinc finger family	*AT5G44260*	zinc finger (CCCH-type) family protein	1.045
27.3.14	RNA.regulation of transcription.CCAAT box binding factor family, HAP2	*AT1G54160*	Symbols: NFYA5, NF-YA5 | NF-YA5 (NUCLEAR FACTOR Y, SUBUNIT A5); specific transcriptional repressor/ transcription factor	-0.679
27.3.14	RNA.regulation of transcription.CCAAT box binding factor family, HAP2	*AT1G72830*	Symbols: HAP2C, ATHAP2C, NF-YA3 | NF-YA3 (NUCLEAR FACTOR Y, SUBUNIT A3); transcription factor	-0.921
27.3.14	RNA.regulation of transcription.CCAAT box binding factor family, HAP2	*AT3G05690*	Symbols: UNE8, ATHAP2B, HAP2B, NF-YA2 | NF-YA2 (NUCLEAR FACTOR Y, SUBUNIT A2); transcription factor	-1.238
27.3.14	RNA.regulation of transcription.CCAAT box binding factor family, HAP2	*AT3G14020*	Symbols: NF-YA6 | NF-YA6 (NUCLEAR FACTOR Y, SUBUNIT A6); transcription	-0.991

			factor	
27.3.14	RNA.regulation of transcription.CCAAT box binding factor family, HAP2	*AT5G06510*	Symbols: NF-YA10 | NF-YA10 (NUCLEAR FACTOR Y, SUBUNIT A10); transcription factor	-1.377
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	*AT1G25550*	myb family transcription factor	1.381
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	*AT1G68670*	myb family transcription factor	0.95
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	*AT2G38300*	DNA binding / transcription factor	0.884
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	*AT3G04030*	myb family transcription factor	-0.601
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	*AT3G46640*	Symbols: PCL1, LUX | PCL1 (PHYTOCLOCK 1); DNA binding / transcription factor	-0.443
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	*AT4G37180*	myb family transcription factor	0.636
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	*AT5G05090*	myb family transcription factor	0.425
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT1G46480*	Symbols: WOX4 | WOX4 (WUSCHEL RELATED HOMEOBOX 4); transcription factor	0.721
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT1G62360*	Symbols: STM, BUM1, SHL, WAM1, BUM, WAM | STM (SHOOT MERISTEMLESS); transcription factor	-0.666
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT1G69780*	Symbols: ATHB13 | ATHB13; DNA binding / sequence-specific DNA binding /	0.47

			transcription factor	
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT3G17050*	transposable element gene	0.857
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT3G60390*	Symbols: HAT3 | HAT3 (HOMEOBOX-LEUCINE ZIPPER PROTEIN 3); transcription factor	-0.597
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT3G61890*	Symbols: ATHB-12, ATHB12 | ATHB-12 (ARABIDOPSIS THALIANA HOMEOBOX 12); transcription activator/ transcription factor	-0.555
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT4G17460*	Symbols: HAT1 | HAT1; DNA binding / transcription factor	1.87
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT4G34610*	Symbols: BLH6 | BLH6 (BELL1-LIKE HOMEODOMAIN 6); DNA binding / transcription factor	0.469
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT5G02030*	Symbols: LSN, PNY, HB-6, BLR, RPL, BLH9, VAN | RPL (REPLUMLESS); DNA binding / sequence-specific DNA binding / transcription factor	-0.372
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT5G25220*	Symbols: KNAT3 | KNAT3 (KNOTTED1-LIKE HOMEOBOX GENE 3); transcription activator/ transcription factor	0.39
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	*AT5G65310*	Symbols: ATHB5, ATHB-5 | ATHB5 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 5); protein homodimerization/ sequence-specific DNA binding /	-0.459

			transcription activator/ transcription factor	
27.3.23	RNA.regulation of transcription.HSF,Heat-shock transcription factor family	*AT1G12800*	S1 RNA-binding domain-containing protein	0.336
27.3.23	RNA.regulation of transcription.HSF,Heat-shock transcription factor family	*AT3G51910*	Symbols: AT-HSFA7A, HSFA7A | AT-HSFA7A; DNA binding / transcription factor	-1.131
27.3.24	RNA.regulation of transcription.MADS box transcription factor family	*AT2G45660*	Symbols: AGL20, SOC1 | AGL20 (AGAMOUS-LIKE 20); transcription factor	0.582
27.3.24	RNA.regulation of transcription.MADS box transcription factor family	*AT5G65080*	Symbols: MAF5, AGL68 | MAF5 (MADS AFFECTING FLOWERING 5); transcription factor	1.76
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT1G66390*	Symbols: ATMYB90, PAP2, MYB90 | MYB90 (MYB DOMAIN PROTEIN 90); DNA binding / transcription factor	-1.213
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT1G74650*	Symbols: ATY13, ATMYB31, MYB31 | MYB31 (MYB DOMAIN PROTEIN 31); DNA binding / transcription factor	-0.469
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT2G02060*	transcription factor	-0.447
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT2G23290*	Symbols: AtMYB70 | AtMYB70 (myb domain protein 70); DNA binding / transcription factor	1.2
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT2G37630*	Symbols: ATPHAN, AS1, ATMYB91, MYB91 | AS1 (ASYMMETRIC LEAVES 1); DNA binding / protein homodimerization/ transcription factor	-0.411
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT2G47190*	Symbols: ATMYB2, MYB2 | MYB2 (MYB	-1.037

			DOMAIN PROTEIN 2); DNA binding / calmodulin binding / transcription activator/ transcription factor	
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT3G04030*	myb family transcription factor	-0.601
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT3G28910*	Symbols: ATMYB30, MYB30 | MYB30 (MYB DOMAIN PROTEIN 30); DNA binding / transcription factor	0.578
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT3G47600*	Symbols: MYB94, ATMYBCP70, ATMYB94 | ATMYB94 (MYB DOMAIN PROTEIN 94); DNA binding / transcription factor	0.554
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT3G50060*	Symbols: MYB77 | MYB77; DNA binding / transcription factor	2.689
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT4G01680*	Symbols: MYB55 | MYB55 (myb domain protein 55); DNA binding / transcription factor	0.775
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT4G37260*	Symbols: MYB73, ATMYB73 | MYB73 (MYB DOMAIN PROTEIN 73); DNA binding / transcription factor	1.423
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT5G11510*	Symbols: MYB3R-4, AtMYB3R4 | MYB3R-4 (myb domain protein 3R-4); DNA binding / transcription coactivator/ transcription factor	0.687
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT5G15310*	Symbols: ATMYB16, ATMIXTA | ATMYB16 (MYB DOMAIN PROTEIN 16); DNA binding / transcription factor	0.522

27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT5G62470*	Symbols: MYB96 | MYB96 (myb domain protein 96); DNA binding / transcription factor	0.879
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	*AT5G67300*	Symbols: ATMYBR1, ATMYB44, MYBR1 | MYBR1 (MYB DOMAIN PROTEIN R1); DNA binding / transcription factor	1.612
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	*AT1G71030*	Symbols: ATMYBL2, MYBL2 | MYBL2 (ARABIDOPSIS MYB-LIKE 2); DNA binding / transcription factor	0.461
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	*AT2G21650*	Symbols: MEE3, ATRL2 | MEE3 (MATERNAL EFFECT EMBRYO ARREST 3); DNA binding / transcription factor	-0.816
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	*AT4G36570*	Symbols: ATRL3 | ATRL3 (ARABIDOPSIS RAD-LIKE 3); DNA binding / transcription factor	-1.28
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	*AT5G56840*	DNA-binding family protein	-0.904
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	*AT5G58900*	myb family transcription factor	0.457
27.3.27	RNA.regulation of transcription.NAC domain transcription factor family	*AT3G04070*	Symbols: anac047 | anac047 (Arabidopsis NAC domain containing protein 47); transcription factor	-0.622
27.3.27	RNA.regulation of transcription.NAC domain transcription factor family	*AT3G15500*	Symbols: ATNAC3, ANAC055 | ANAC055 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 55); transcription	-2.446

			factor	
27.3.27	RNA.regulation of transcription.NAC domain transcription factor family	*AT3G15510*	Symbols: ATNAC2, ANAC056, NARS1 | ATNAC2 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 2); transcription factor	-0.659
27.3.29	RNA.regulation of transcription.TCP transcription factor family	*AT2G31070*	Symbols: TCP10 | TCP10 (TCP DOMAIN PROTEIN 10); transcription factor	0.475
27.3.30	RNA.regulation of transcription.Trihelix, Triple-Helix transcription factor family	*AT1G33240*	Symbols: AT-GTL1, AT-GTL2 | AT-GTL1 (GT2-LIKE 1); DNA binding / transcription factor	0.955
27.3.30	RNA.regulation of transcription.Trihelix, Triple-Helix transcription factor family	*AT1G76890*	Symbols: GT2, AT-GT2 | GT2; transcription factor	0.487
27.3.30	RNA.regulation of transcription.Trihelix, Triple-Helix transcription factor family	*AT3G11100*	transcription factor	-0.399
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT1G29280*	Symbols: WRKY65, ATWRKY65 | WRKY65; transcription factor	-0.984
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT1G62300*	Symbols: WRKY6, ATWRKY6 | WRKY6; transcription factor	-0.56
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT1G80840*	Symbols: WRKY40, ATWRKY40 | WRKY40; transcription factor	0.573
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT2G23320*	Symbols: WRKY15 | WRKY15; calmodulin binding / transcription factor	-0.372
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT2G40750*	Symbols: WRKY54, ATWRKY54 | WRKY54; transcription factor	-0.386
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT3G56400*	Symbols: WRKY70, ATWRKY70 | WRKY70; transcription factor/ transcription repressor	0.777

27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT4G01250*	Symbols: WRKY22, AtWRKY22 | WRKY22; transcription factor	0.902
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT4G04450*	Symbols: WRKY42, AtWRKY42 | WRKY42; transcription factor	-1.13
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT4G31550*	Symbols: WRKY11, ATWRKY11 | WRKY11; calmodulin binding / transcription factor	0.647
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT4G31800*	Symbols: WRKY18, ATWRKY18 | WRKY18; transcription factor	0.7
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT5G13080*	Symbols: WRKY75, ATWRKY75 | WRKY75; transcription factor	-1.748
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	*AT5G49520*	Symbols: WRKY48, ATWRKY48 | WRKY48; transcription factor	0.743
27.3.34	RNA.regulation of transcription.Orphan family	*AT3G16000*	Symbols: MFP1 | MFP1 (MAR BINDING FILAMENT-LIKE PROTEIN 1); DNA binding	0.429
27.3.34	RNA.regulation of transcription.Orphan family	*AT5G66630*	Symbols: DAR5 | DAR5 (DA1-RELATED PROTEIN 5); zinc ion binding	-0.691
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT1G77920*	bZIP family transcription factor	-0.61
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT2G36270*	Symbols: ABI5, GIA1 | ABI5 (ABA INSENSITIVE 5); DNA binding / transcription activator/ transcription factor	-0.508
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT2G40950*	Symbols: BZIP17 | BZIP17; DNA binding / transcription activator/ transcription factor	-0.334
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT2G42380*	Symbols: ATBZIP34, BZIP34 | bZIP transcription factor family protein	1.733

27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT2G46270*	Symbols: GBF3 | GBF3 (G-BOX BINDING FACTOR 3); sequence-specific DNA binding / transcription factor	0.742
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT3G58120*	Symbols: ATBZIP61, BZIP61 | BZIP61; DNA binding / transcription activator/ transcription factor	1.524
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT4G34590*	Symbols: ATB2, GBF6, AtbZIP11, BZIP11 | GBF6 (G-BOX BINDING FACTOR 6); DNA binding / protein heterodimerization/ transcription factor	-0.709
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT4G35900*	Symbols: FD, FD-1, atbzip14 | FD; DNA binding / protein binding / transcription activator/ transcription factor	-0.731
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT4G36730*	Symbols: GBF1 | GBF1; sequence-specific DNA binding / transcription factor	0.601
27.3.35	RNA.regulation of transcription.bZIP transcription factor family	*AT5G28770*	Symbols: BZO2H3 | BZO2H3; DNA binding / protein heterodimerization/ transcription factor	0.456
27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	*AT2G28500*	Symbols: LBD11 | LBD11 (LOB DOMAIN-CONTAINING PROTEIN 11)	1.223
27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	*AT3G11090*	Symbols: LBD21 | LBD21 (LOB DOMAIN-CONTAINING PROTEIN 21)	-0.563

27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	*AT3G49940*	Symbols: LBD38 | LBD38 (LOB DOMAIN-CONTAINING PROTEIN 38)	1.101
27.3.37	RNA.regulation of transcription.AS2,Lateral Organ Boundaries Gene Family	*AT5G67420*	Symbols: LBD37 | LBD37 (LOB DOMAIN-CONTAINING PROTEIN 37)	0.627
27.3.40	RNA.regulation of transcription.Aux/IAA family	*AT1G04240*	Symbols: SHY2, IAA3 | SHY2 (SHORT HYPOCOTYL 2); transcription factor	1.261
27.3.40	RNA.regulation of transcription.Aux/IAA family	*AT1G04250*	Symbols: AXR3, IAA17 | AXR3 (AUXIN RESISTANT 3); transcription factor	0.625
27.3.40	RNA.regulation of transcription.Aux/IAA family	*AT2G22670*	Symbols: IAA8 | IAA8; transcription factor	0.336
27.3.40	RNA.regulation of transcription.Aux/IAA family	*AT3G15540*	Symbols: IAA19, MSG2 | IAA19 (INDOLE-3-ACETIC ACID INDUCIBLE 19); transcription factor	0.942
27.3.40	RNA.regulation of transcription.Aux/IAA family	*AT3G16500*	Symbols: PAP1, IAA26 | PAP1 (PHYTOCHROME-ASSOCIATED PROTEIN 1); transcription factor	-0.733
27.3.40	RNA.regulation of transcription.Aux/IAA family	*AT5G25890*	Symbols: IAA28, IAR2 | IAA28 (INDOLE-3-ACETIC ACID INDUCIBLE 28); transcription factor	-0.736
27.3.41	RNA.regulation of transcription.B3 transcription factor family	*AT1G49475*	DNA binding / transcription factor	-1.336
27.3.41	RNA.regulation of transcription.B3 transcription factor family	*AT3G53310*	transcriptional factor B3 family protein	-0.534

27.3.42	RNA.regulation of transcription.Bromodomain proteins	*AT1G76380*	DNA-binding bromodomain-containing protein	-0.5
27.3.42	RNA.regulation of transcription.Bromodomain proteins	*AT3G60110*	DNA binding	-0.607
27.3.44	RNA.regulation of transcription.Chromatin Remodeling Factors	*AT5G66750*	Symbols: DDM1, CHR01, CHR1, CHA1, SOM4, SOM1, ATDDM1 | CHR1 (CHROMATIN REMODELING 1); ATPase/ helicase	0.805
27.3.46	RNA.regulation of transcription.DNA methyltransferases	*AT1G69770*	Symbols: CMT3 | CMT3 (chromomethylase 3); DNA (cytosine-5-)-methyltransferase	0.847
27.3.49	RNA.regulation of transcription.GeBP like	*AT1G44810*	transcription regulator	-0.405
27.3.49	RNA.regulation of transcription.GeBP like	*AT3G04930*	transcription regulator	-0.356
27.3.49	RNA.regulation of transcription.GeBP like	*AT4G00238*	DNA-binding storekeeper protein-related	-0.422
27.3.49	RNA.regulation of transcription.GeBP like	*AT4G00270*	DNA-binding storekeeper protein-related	-0.491
27.3.50	RNA.regulation of transcription.General Transcription	*AT3G25940*	transcription factor S-II (TFIIS) domain-containing protein	-0.743
27.3.50	RNA.regulation of transcription.General Transcription	*AT3G61420*	FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: cellular_component unknown; CONTAINS InterPro DOMAIN/s: Kelch related (InterPro:IPR013089), BSD (InterPro:IPR005607); BEST Arabidopsis	0.367

			thaliana protein match is: transcription factor-related (TAIR:AT1G55750.1); Has 247 Blast hits to 247 proteins in 115 species: Archae - 1; Bacteria - 0; Metazoa - 116; Fungi - 84; Plants - 26; Viruses - 0; Other Eukaryotes - 20 (source: NCBI BLink).	
27.3.50	RNA.regulation of transcription.General Transcription	*AT4G10920*	Symbols: KELP | KELP; DNA binding / transcription coactivator/ transcription regulator	-0.401
27.3.52	RNA.regulation of transcription.Global transcription factor group	*AT3G01770*	Symbols: ATBET10 | ATBET10 (Arabidopsis thaliana BROMODOMAIN AND EXTRATERMINAL DOMAIN PROTEIN 10); DNA binding	-0.355
27.3.52	RNA.regulation of transcription.Global transcription factor group	*AT5G65630*	Symbols: GTE7 | GTE7 (Global transcription factor group E 7); DNA binding	0.515
27.3.55	RNA.regulation of transcription.HDA	*AT2G27840*	Symbols: HDT4, HDA13, HDT04 | HDT4; histone deacetylase	-0.452
27.3.55	RNA.regulation of transcription.HDA	*AT3G44750*	Symbols: HD2A, ATHD2A, HDA3, HDT1 | HDA3 (HISTONE DEACETYLASE 3); histone deacetylase/ nucleic acid binding / zinc ion binding	-0.425
27.3.57	RNA.regulation of transcription.JUMONJI family	*AT5G46910*	transcription factor jumonji (jmj) family protein	0.84

27.3.60	RNA.regulation of transcription.NIN-like bZIP-related family	*AT2G43500*	RWP-RK domain-containing protein	-0.594
27.3.62	RNA.regulation of transcription.Nucleosome/chromatin assembly factor group	*AT1G76110*	high mobility group (HMG1/2) family protein / ARID/BRIGHT DNA-binding domain-containing protein	0.59
27.3.62	RNA.regulation of transcription.Nucleosome/chromatin assembly factor group	*AT5G23405*	high mobility group (HMG1/2) family protein	-0.544
27.3.64	RNA.regulation of transcription.PHOR1	*AT1G66160*	Symbols: ATCMPG1, CMPG1 | U-box domain-containing protein	2.038
27.3.64	RNA.regulation of transcription.PHOR1	*AT2G35930*	Symbols: PUB23 | PUB23 (PLANT U-BOX 23); ubiquitin-protein ligase	0.648
27.3.64	RNA.regulation of transcription.PHOR1	*AT3G11840*	Symbols: PUB24 | PUB24 (PLANT U-BOX 24); binding / ubiquitin-protein ligase	-0.734
27.3.64	RNA.regulation of transcription.PHOR1	*AT3G19380*	Symbols: PUB25 | PUB25 (PLANT U-BOX 25); binding / ubiquitin-protein ligase	0.686
27.3.66	RNA.regulation of transcription.Psudo ARR transcription factor family	*AT2G46790*	Symbols: APRR9, PRR9, TL1 | APRR9 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 9); protein binding / transcription regulator/ two-component response regulator	-0.388
27.3.66	RNA.regulation of transcription.Psudo ARR transcription factor family	*AT5G24470*	Symbols: APRR5, PRR5 | APRR5 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 5); transcription regulator/ two-component response regulator	-0.966

27.3.66	RNA.regulation of transcription.Psudo ARR transcription factor family	*AT5G60100*	Symbols: APRR3, PRR3 | APRR3 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 3); transcription regulator/ two-component response regulator	-0.675
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT1G13790*	XH/XS domain-containing protein / XS zinc finger domain-containing protein	0.691
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT1G19340*	methyltransferase MT-A70 family protein	0.68
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT1G56110*	Symbols: NOP56 | NOP56 (Arabidopsis homolog of nucleolar protein Nop56)	-0.348
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT1G67370*	Symbols: ASY1, ATASY1 | ASY1 (ASYNAPTIC 1); DNA binding	-1.532
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT1G67590*	remorin family protein	0.656
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT1G68580*	agenet domain-containing protein / bromo-adjacent homology (BAH) domain-containing protein	0.36
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT3G26910*	hydroxyproline-rich glycoprotein family protein	0.349
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT5G27120*	SAR DNA-binding protein, putative	-0.361
27.3.67	RNA.regulation of transcription.putative transcription regulator	*AT5G52890*	AT hook motif-containing protein	0.685

27.3.68	RNA.regulation of transcription.PWWP domain protein	*AT5G40340*	PWWP domain-containing protein	0.461
27.3.69	RNA.regulation of transcription.SET-domain transcriptional regulator family	*AT1G26761*	unknown protein	0.395
27.3.70	RNA.regulation of transcription.Silencing Group	*AT3G17185*	Symbols: TASIR-ARF, TAS3, ATTAS3 | TAS3/TASIR-ARF (TRANS-ACTING SIRNA3); other RNA	1.066
27.3.72	RNA.regulation of transcription.Transcriptional Adaptor Zinc Bundle (TAZ) domain family	*AT5G67480*	Symbols: BT4 | BT4 (BTB AND TAZ DOMAIN PROTEIN 4); protein binding / transcription regulator	0.743
27.3.80	RNA.regulation of transcription.zf-HD	*AT2G18350*	Symbols: AtHB24 | AtHB24 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 24); DNA binding / transcription factor	-0.759
27.3.80	RNA.regulation of transcription.zf-HD	*AT5G39760*	Symbols: AtHB23 | AtHB23 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 23); DNA binding / transcription factor	-0.686
27.3.84	RNA.regulation of transcription.BBR/BPC	*AT1G14685*	Symbols: BPC2, BBR/BPC2, ATBPC2 | BPC2 (BASIC PENTACYSTEINE 2); DNA binding / transcription factor	-0.379
27.3.85	RNA.regulation of transcription.sigma like plant	*AT1G08540*	Symbols: SIGB, SIG1, SIG2, SIGA, ATSIG1, ABC1, ATSIG2 | SIG2 (RNA POLYMERASE SIGMA SUBUNIT 2); DNA binding / DNA-directed RNA polymerase/ sigma factor/ transcription factor	0.435

27.3.99	RNA.regulation of transcription.unclassified	*AT1G07950*	surfeit locus protein 5 family protein / SURF5 family protein	-0.621
27.3.99	RNA.regulation of transcription.unclassified	*AT1G09950*	transcription factor-related	2.349
27.3.99	RNA.regulation of transcription.unclassified	*AT1G21000*	zinc-binding family protein	-0.661
27.3.99	RNA.regulation of transcription.unclassified	*AT1G32700*	zinc-binding family protein	-0.621
27.3.99	RNA.regulation of transcription.unclassified	*AT1G70810*	C2 domain-containing protein	-0.627
27.3.99	RNA.regulation of transcription.unclassified	*AT1G76590*	zinc-binding family protein	-0.572
27.3.99	RNA.regulation of transcription.unclassified	*AT1G78930*	mitochondrial transcription termination factor-related / mTERF-related	-0.808
27.3.99	RNA.regulation of transcription.unclassified	*AT2G02170*	remorin family protein	0.477
27.3.99	RNA.regulation of transcription.unclassified	*AT2G28450*	zinc finger (CCCH-type) family protein	-0.339
27.3.99	RNA.regulation of transcription.unclassified	*AT2G34620*	mitochondrial transcription termination factor-related / mTERF-related	1.116
27.3.99	RNA.regulation of transcription.unclassified	*AT3G52150*	RNA recognition motif (RRM)-containing protein	0.35
27.3.99	RNA.regulation of transcription.unclassified	*AT3G54400*	aspartyl protease family protein	0.658
27.3.99	RNA.regulation of transcription.unclassified	*AT3G59080*	aspartyl protease family protein	1.072
27.3.99	RNA.regulation of transcription.unclassified	*AT3G63140*	Symbols: CSP41A | CSP41A	0.517

			(CHLOROPLAST STEM-LOOP BINDING PROTEIN OF 41 KDA); mRNA binding / poly(U) binding	
27.3.99	RNA.regulation of transcription.unclassified	*AT4G00380*	XH/XS domain-containing protein / XS zinc finger domain-containing protein	-0.468
27.3.99	RNA.regulation of transcription.unclassified	*AT4G12040*	zinc finger (AN1-like) family protein	-0.351
27.3.99	RNA.regulation of transcription.unclassified	*AT5G10760*	aspartyl protease family protein	-0.43
27.3.99	RNA.regulation of transcription.unclassified	*AT5G10770*	chloroplast nucleoid DNA-binding protein, putative	-0.444
27.3.99	RNA.regulation of transcription.unclassified	*AT5G11470*	DNA binding / nucleic acid binding	-0.634
27.3.99	RNA.regulation of transcription.unclassified	*AT5G37540*	aspartyl protease family protein	0.406
27.3.99	RNA.regulation of transcription.unclassified	*AT5G50450*	zinc finger (MYND type) family protein	-0.505

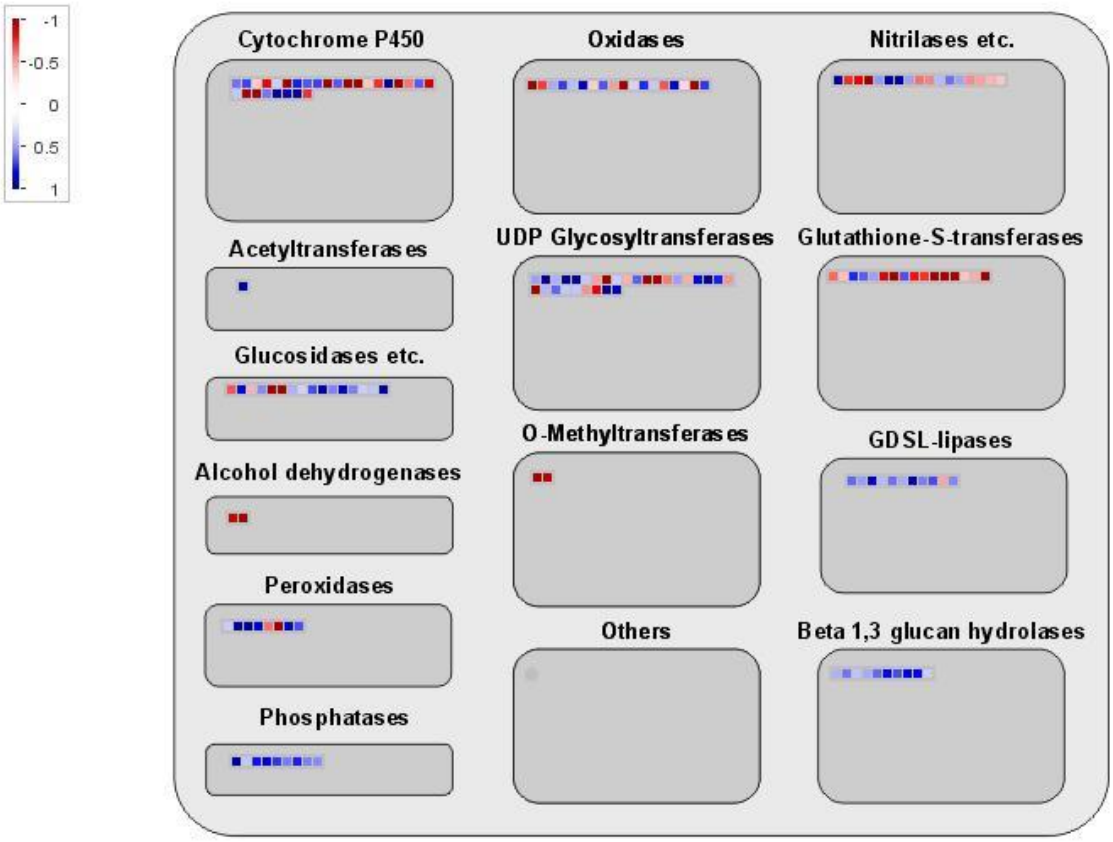


Figure S3.2 MapMan overview of *Arabidopsis thaliana* differentially expressed genes coding for large enzyme families in above-ground shoot tissue following BC204 (0.01% [v/v]) treatment Genes that were shown to be differentially expressed using $p < 0.05$ as a cut-off value were imported. Blue represents genes that were upregulated while red indicates those that were downregulated by BC204. Intensity of the colours are indicative of the levels of expression (scale adjusted to 0.1).

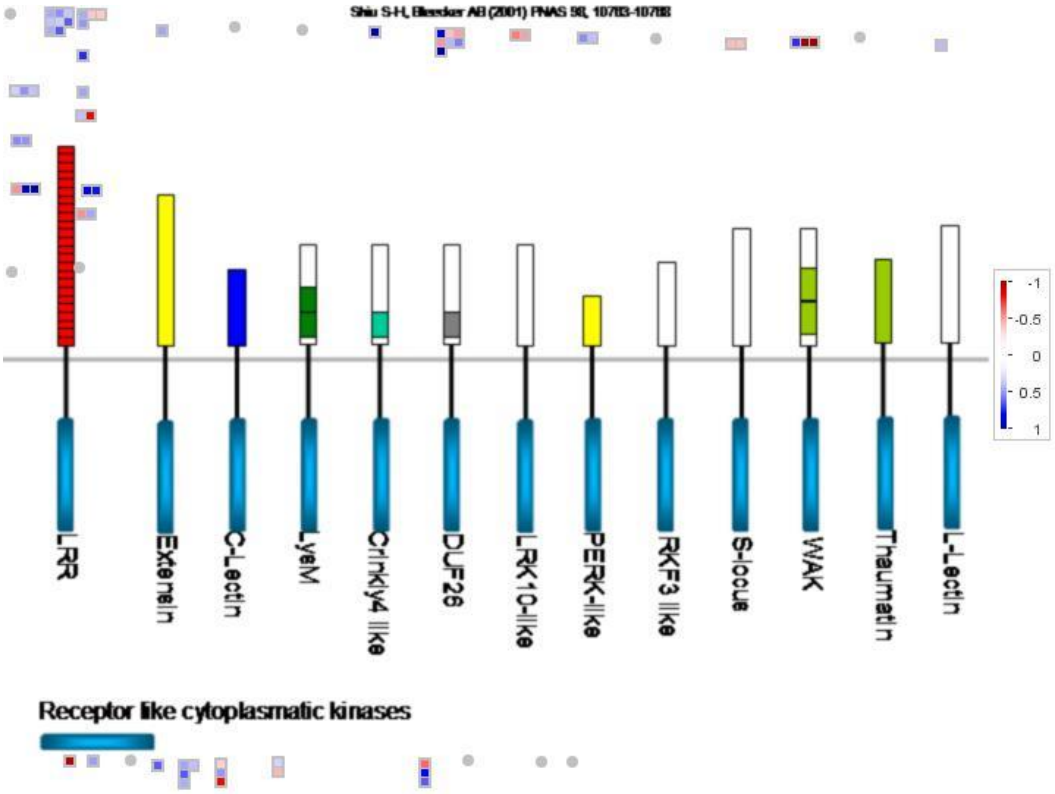


Figure S3.3 MapMan overview of BC204-treated *Arabidopsis thaliana* differentially expressed genes coding for receptor like kinases in above-ground shoot tissue relative following BC204 (0.01% [v/v]) treatment. Genes that were shown to be differentially expressed using $p < 0.05$ as a cut-off value were imported. Blue represents genes that were upregulated while red indicates those that were downregulated by BC204. Intensity of the colours are indicative of the levels of expression (scale adjusted to 0.1).

Table S3.2 Primers used in RT-qPCR validation of RNA-seq data in BC204-treated *Arabidopsis thaliana* shoot tissue

Gene ID	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size
AT2G28390	*MONENSIN SENSITIVITY1 (MON1)*	CAAGGCAGGAAATCACCAGGTTG	CTGTACAGCTGATGCAGACCAG	71 bp
AT1G50040	*Putative formin-like protein (DUF1005)*	TTCTTCATCTGGACCGTCTG	CAGAACGGGAACAGAAACAA	103 bp
AT1G58340	*ARABIDOPSIS ABNORMAL SHOOT4*	GATGACCGGGCTTCTTATGT	GGCAAAGCCTATGGAGAGAG	106 bp
AT3G50060	*MYB DOMAIN PROTEIN 77*	TCTCCTGTTGCTCAGCTGTT	TAGGTGGATCCTCCGAAGAC	106 bp

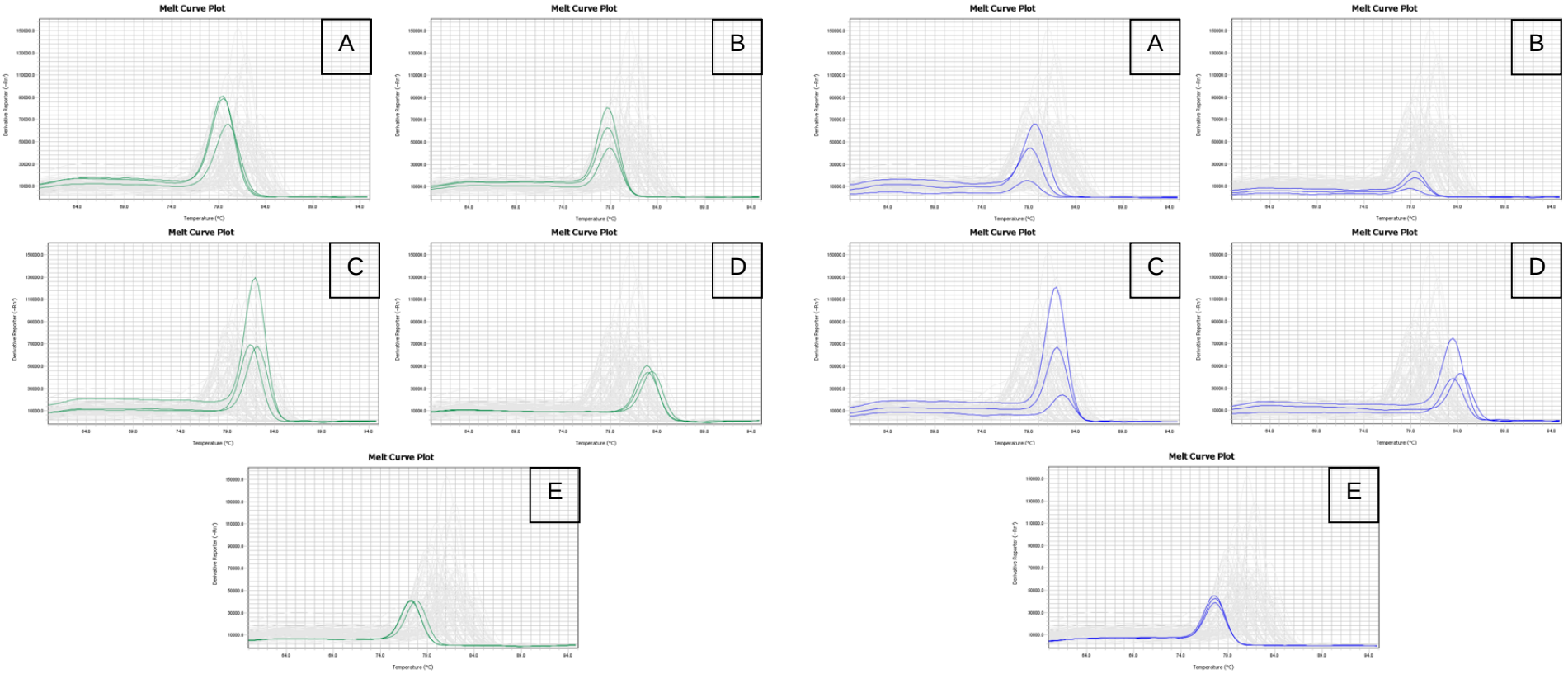


Figure S3.4 RT-qPCR Melt curves for *MON1* (A), *At1g50040* (B), *At1g58340* (C), *At3g50060* (D) and *At3g60140* (E) in control (left) and BC204-treated (right) samples.

Table S3.3 List of genes significantly upregulated by a log_2fold value greater than 1 in *Arabidopsis thaliana* shoot tissue, elicited by BC204 (0.01% [v/v]) treatment, functional classified and annotated by the DAVID database

TAIR ID	Function	log_2fold change	q_value
AT1G22470	hypothetical protein(AT1G22470)	100 (inf)	0.000776133
AT1G21910	Integrase-type DNA-binding superfamily protein(DREB26)	4.23074	0.000776133
AT5G57560	Xyloglucan endotransglucosylase/hydrolase family protein(TCH4)	3.97935	0.000776133
AT1G66760	MATE efflux family protein(AT1G66760)	3.78785	0.000776133
AT4G28040	nodulin MtN21 /EamA-like transporter family protein(UMAMIT33)	3.72004	0.00477784
AT2G20670	sugar phosphate exchanger, putative (DUF506)(AT2G20670)	3.60024	0.000776133
AT5G52300	CAP160 protein(LTI65)	3.55512	0.000776133
AT4G08950	Phosphate-responsive 1 family protein(EXO)	3.32798	0.000776133
AT1G50040	formin-like protein. putative (DUF1005)(AT1G50040)	3.2814	0.000776133
AT1G49500	transcription initiation factor TFIID subunit 1b-like protein(AT1G49500)	3.20134	0.000776133
AT1G35140	Phosphate-responsive 1 family protein(PHI-1)	3.12155	0.000776133
AT1G13650	hypothetical protein(AT1G13650)	2.96248	0.000776133
AT4G30290	xyloglucan endotransglucosylase/hydrolase 19(XTH19)	2.8336	0.00205608
AT5G44430	plant defensin 1.2C(PDF1.2c)	2.8268	0.000776133
AT4G12490	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein(AT4G12490)	2.81933	0.0477632
AT4G37240	HTH-type transcriptional regulator(AT4G37240)	2.81067	0.000776133
AT2G05440	GLYCINE RICH PROTEIN 9(GRP9)	2.80051	0.000776133
AT5G04950	nicotianamine synthase 1(NAS1)	2.78398	0.000776133
AT3G50060	myb domain protein 77(MYB77)	2.68942	0.000776133
AT4G35770	Rhodanese/Cell cycle control phosphatase superfamily protein(SEN1)	2.65439	0.000776133
AT1G77640	Integrase-type DNA-binding superfamily protein(AT1G77640)	2.60537	0.00321958
AT2G23130	arabinogalactan protein 17(AGP17)	2.60036	0.000776133
AT3G19680	hypothetical protein (DUF1005)(AT3G19680)	2.57809	0.000776133
AT5G19120	Eukaryotic aspartyl protease family protein(AT5G19120)	2.55537	0.000776133
AT4G27280	Calcium-binding EF-hand family protein(AT4G27280)	2.49459	0.000776133
AT4G08040	1-aminocyclopropane-1-carboxylate synthase 11(ACS11)	2.48933	0.00142905
AT3G28915	hypothetical protein(AT3G28915)	2.46268	0.000776133
AT3G25180	cytochrome P450. family 82. subfamily G. polypeptide 1(CYP82G1)	2.43951	0.0163121
AT5G20250	Raffinose synthase family protein(DIN10)	2.43782	0.000776133
AT1G62510	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein(AT1G62510)	2.42603	0.000776133
AT1G09950	RESPONSE TO ABA AND SALT 1(RAS1)	2.3494	0.035393
AT3G49340	Cysteine proteinases superfamily protein(AT3G49340)	2.29666	0.0170065
AT4G36850	PQ-loop repeat family protein / transmembrane family protein(AT4G36850)	2.28408	0.000776133
AT2G40400	DUF399 family protein. putative (DUF399 and DUF3411)(AT2G40400)	2.23411	0.000776133
AT5G19190	hypothetical protein(AT5G19190)	2.2285	0.000776133
AT5G49360	beta-xylosidase 1(BXL1)	2.2019	0.000776133
AT4G32480	sugar phosphate exchanger. putative (DUF506)(AT4G32480)	2.20074	0.000776133
AT5G25190	Integrase-type DNA-binding superfamily protein(ESE3)	2.19667	0.000776133

AT5G24030	SLAC1 homologue 3(SLAH3)	2.18426	0.000776133
AT3G45960	expansin-like A3(EXLA3)	2.14889	0.000776133
AT5G45340	cytochrome P450. family 707. subfamily A. polypeptide 3(CYP707A3)	2.13558	0.000776133
AT2G01300	mediator of RNA polymerase II transcription subunit(AT2G01300)	2.13301	0.0129178
AT3G07350	sulfate/thiosulfate import ATP-binding protein. putative (DUF506)(AT3G07350)	2.13034	0.0212862
AT5G15530	biotin carboxyl carrier protein 2(BCCP2)	2.12101	0.0166443
AT4G33666	hypothetical protein(AT4G33666)	2.1058	0.0348088
AT4G01140	transmembrane protein. putative (DUF1191)(AT4G01140)	2.06775	0.0291562
AT5G45830	delay of germination 1(DOG1)	2.06562	0.000776133
AT5G51190	Integrase-type DNA-binding superfamily protein(AT5G51190)	2.05794	0.000776133
AT2G26020	plant defensin 1.2b(PDF1.2b)	2.04246	0.000776133
AT1G69260	ABI five binding protein(AFP1)	2.04044	0.0297925
AT1G66160	CYS. MET. PRO. and GLY protein 1(CMPG1)	2.03768	0.000776133
AT2G23690	HTH-type transcriptional regulator(AT2G23690)	2.03654	0.00142905
AT1G10550	xyloglucan:xyloglucosyl transferase 33(XTH33)	2.03384	0.000776133
AT4G13340	Leucine-rich repeat (LRR) family protein(LRX3)	2.02657	0.000776133
AT4G24570	dicarboxylate carrier 2(DIC2)	2.01358	0.000776133
AT1G77765	transmembrane protein(AT1G77765)	2.01093	0.0342918
AT1G72430	SAUR-like auxin-responsive protein family(AT1G72430)	2.00853	0.000776133
AT5G25240	stress induced protein(AT5G25240)	1.99105	0.000776133
AT5G42110	hypothetical protein(AT5G42110)	1.98177	0.00846178
AT2G47440	Tetratricopeptide repeat (TPR)-like superfamily protein(AT2G47440)	1.96361	0.000776133
AT2G41190	Transmembrane amino acid transporter family protein(AT2G41190)	1.95761	0.000776133
AT3G44550	fatty acid reductase 5(FAR5)	1.95302	0.0252101
AT5G50950	FUMARASE 2(FUM2)	1.94869	0.000776133
AT2G34430	light-harvesting chlorophyll-protein complex II subunit B1(LHB1B1)	1.94367	0.000776133
AT3G60290	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein(AT3G60290)	1.8981	0.00884598
AT4G17460	Homeobox-leucine zipper protein 4 (HB-4) / HD-ZIP protein(HAT1)	1.86989	0.000776133
AT3G54810	Plant-specific GATA-type zinc finger transcription factor family protein(BME3)	1.85359	0.000776133
AT1G03870	FASCICLIN-like arabinoogalactan 9(FLA9)	1.8533	0.000776133
AT2G26010	plant defensin 1.3(PDF1.3)	1.84091	0.000776133
AT3G06070	hypothetical protein(AT3G06070)	1.83887	0.000776133
AT3G50800	hypothetical protein(AT3G50800)	1.83111	0.000776133
AT3G45970	expansin-like A1(EXLA1)	1.82572	0.000776133
AT2G41800	imidazolonepropionase (Protein of unknown function. DUF642)(AT2G41800)	1.82132	0.0426343
AT2G44500	O-fucosyltransferase family protein(AT2G44500)	1.81429	0.000776133
AT1G12080	Vacuolar calcium-binding protein-like protein(AT1G12080)	1.78203	0.000776133
AT3G19030	transcription initiation factor TFIID subunit 1b-like protein(AT3G19030)	1.77783	0.019346
AT5G65080	K-box region/MADS-box transcription factor family protein(MAF5)	1.76021	0.0144361
AT3G26760	NAD(P)-binding Rossmann-fold superfamily protein(AT3G26760)	1.74695	0.0439959
AT2G42380	Basic-leucine zipper (bZIP) transcription factor family protein(BZIP34)	1.73284	0.000776133
AT5G44420	plant defensin 1.2(PDF1.2)	1.73242	0.000776133

AT5G07840	Ankyrin repeat family protein(PIA1)	1.69372	0.000776133
AT4G16563	Eukaryotic aspartyl protease family protein(AT4G16563)	1.6893	0.000776133
AT5G44130	FASCICLIN-like arabinogalactan protein 13 precursor(FLA13)	1.67589	0.000776133
AT5G40890	chloride channel A(CLC-A)	1.6718	0.000776133
AT1G69140	miscRNA(AT1G69140)	1.65865	0.000776133
AT5G60270	Concanavalin A-like lectin protein kinase family protein(AT5G60270)	1.6412	0.000776133
AT4G38400	expansin-like A2(EXLA2)	1.63438	0.000776133
AT3G15620	DNA photolyase family protein(UVR3)	1.62602	0.0264352
AT1G23030	ARM repeat superfamily protein(AT1G23030)	1.6259	0.000776133
AT1G66100	Plant thionin(AT1G66100)	1.62282	0.000776133
AT5G67300	myb domain protein r1(MYBR1)	1.61199	0.000776133
AT4G01950	glycerol-3-phosphate acyltransferase 3(GPAT3)	1.6104	0.000776133
AT2G35290	hypothetical protein(AT2G35290)	1.60799	0.000776133
AT2G21210	SAUR-like auxin-responsive protein family(AT2G21210)	1.60286	0.00266002
AT4G33905	Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein(AT4G33905)	1.59962	0.000776133
AT5G61600	ethylene response factor 104(ERF104)	1.59916	0.000776133
AT1G01120	3-ketoacyl-CoA synthase 1(KCS1)	1.58396	0.000776133
AT1G32170	xyloglucan endotransglucosylase/hydrolase 30(XTH30)	1.57695	0.000776133
AT4G22470	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein(AT4G22470)	1.56454	0.00884598
AT1G72920	Toll-Interleukin-Resistance (TIR) domain family protein(AT1G72920)	1.54329	0.000776133
AT2G17230	EXORDIUM like 5(EXL5)	1.5399	0.000776133
AT1G02380	transmembrane protein(AT1G02380)	1.5325	0.000776133
AT3G58120	Basic-leucine zipper (bZIP) transcription factor family protein(BZIP61)	1.52445	0.000776133
AT1G54660	miscRNA(AT1G54660)	1.50986	0.000776133
AT1G65450	HXXXD-type acyl-transferase family protein(GLC)	1.49734	0.000776133
AT2G41170	F-box family protein(AT2G41170)	1.48839	0.000776133
AT2G37870	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein(AT2G37870)	1.48567	0.0232568
AT4G17490	ethylene responsive element binding factor 6(ERF6)	1.48415	0.000776133
AT5G67450	zinc-finger protein 1(ZF1)	1.47718	0.000776133
AT1G67910	hypothetical protein(AT1G67910)	1.46173	0.000776133
AT4G12690	DUF868 family protein (DUF868)(AT4G12690)	1.45631	0.000776133
AT1G07135	glycine-rich protein(AT1G07135)	1.45362	0.000776133
AT3G24420	alpha/beta-Hydrolases superfamily protein(AT3G24420)	1.44986	0.000776133
AT1G65310	xyloglucan endotransglucosylase/hydrolase 17(XTH17)	1.44245	0.00142905
AT5G40380	cysteine-rich RLK (RECEPTOR-like protein kinase) 42(CRK42)	1.43967	0.000776133
AT5G12940	Leucine-rich repeat (LRR) family protein(AT5G12940)	1.43269	0.000776133
AT4G34760	SAUR-like auxin-responsive protein family(AT4G34760)	1.43191	0.000776133
AT3G55980	salt-inducible zinc finger 1(SZF1)	1.42723	0.000776133
AT4G37260	myb domain protein 73(MYB73)	1.42342	0.000776133
AT3G03840	SAUR-like auxin-responsive protein family(SAUR27)	1.41956	0.0406758
AT2G36790	UDP-glucosyl transferase 73C6(UGT73C6)	1.41911	0.000776133
AT4G11280	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6(ACS6)	1.41699	0.000776133
AT3G62570	Tetratricopeptide repeat (TPR)-like superfamily protein(AT3G62570)	1.40557	0.000776133

AT3G62720	xylosyltransferase 1(XT1)	1.40435	0.000776133
AT1G13700	6-phosphogluconolactonase 1(PGL1)	1.40179	0.0302652
AT4G04745	hypothetical protein(AT4G04745)	1.3981	0.038791
AT3G52720	alpha carbonic anhydrase 1(ACA1)	1.39672	0.000776133
AT1G24170	Nucleotide-diphospho-sugar transferases superfamily protein(LGT9)	1.39558	0.000776133
AT4G37610	BTB and TAZ domain protein 5(BT5)	1.39124	0.0206488
AT3G05640	Protein phosphatase 2C family protein(AT3G05640)	1.39044	0.000776133
AT1G53100	Core-2/I-branching beta-1.6-N-acetylglucosaminyltransferase family protein(AT1G53100)	1.38221	0.000776133
AT1G25550	myb-like transcription factor family protein(AT1G25550)	1.38122	0.000776133
AT1G76650	calmodulin-like 38(CML38)	1.37648	0.000776133
AT5G44572	transmembrane protein(AT5G44572)	1.36402	0.0285646
AT3G28200	Peroxidase superfamily protein(AT3G28200)	1.35792	0.000776133
AT1G61100	disease resistance protein (TIR class)(AT1G61100)	1.35487	0.000776133
AT5G54380	protein kinase family protein(THE1)	1.35284	0.000776133
AT1G54740	FANTASTIC four-like protein (DUF3049)(AT1G54740)	1.35013	0.000776133
AT1G19940	glycosyl hydrolase 9B5(GH9B5)	1.34483	0.000776133
AT3G02170	longifolia2(LNG2)	1.33498	0.000776133
AT4G02290	glycosyl hydrolase 9B13(GH9B13)	1.32966	0.000776133
AT1G74670	Gibberellin-regulated family protein(GASA6)	1.32727	0.000776133
AT4G05170	basic helix-loop-helix (bHLH) DNA-binding superfamily protein(AT4G05170)	1.31505	0.00672516
AT4G03400	Auxin-responsive GH3 family protein(DFL2)	1.3128	0.000776133
AT5G59070	UDP-Glycosyltransferase superfamily protein(AT5G59070)	1.31218	0.0252101
AT5G03545	expressed in response to phosphate starvation protein(AT5G03545)	1.31165	0.000776133
AT3G01500	carbonic anhydrase 1(CA1)	1.30878	0.000776133
AT3G27690	photosystem II light harvesting complex protein 2.3(LHCB2.3)	1.30625	0.000776133
AT2G28210	alpha carbonic anhydrase 2(ACA2)	1.29738	0.0397997
AT4G28190	Developmental regulator. ULTRAPETALA(ULT1)	1.29683	0.000776133
AT4G29780	nuclease(AT4G29780)	1.29677	0.000776133
AT5G47550	Cystatin/monellin superfamily protein(AT5G47550)	1.29559	0.000776133
AT2G36050	ovate family protein 15(OFP15)	1.28607	0.00142905
AT3G43960	Cysteine proteinases superfamily protein(AT3G43960)	1.28567	0.00142905
AT3G48260	with no lysine (K) kinase 3(WNK3)	1.28425	0.010532
AT1G77855	BPS1-like protein(AT1G77855)	1.27456	0.00762101
AT2G46780	RNA-binding (RRM/RBD/RNP motifs) family protein(AT2G46780)	1.27451	0.000776133
AT1G04240	AUX/IAA transcriptional regulator family protein(SHY2)	1.26057	0.000776133
AT1G14700	purple acid phosphatase 3(PAP3)	1.25868	0.000776133
AT4G37450	arabinogalactan protein 18(AGP18)	1.25602	0.000776133
AT3G59880	hypothetical protein(AT3G59880)	1.24457	0.00884598
AT4G26690	PLC-like phosphodiesterase family protein(SHV3)	1.2426	0.000776133
AT3G44260	Polynucleotidyl transferase. ribonuclease H-like superfamily protein(CAF1a)	1.24085	0.000776133
AT5G10430	arabinogalactan protein 4(AGP4)	1.23634	0.000776133
AT1G61795	PAK-box/P21-Rho-binding family protein(AT1G61795)	1.23089	0.00579781
AT1G02710	glycine-rich protein(AT1G02710)	1.22724	0.00926146

AT5G45280	Pectinacetylesterase family protein(AT5G45280)	1.22705	0.000776133
AT2G28500	LOB domain-containing protein 11(LBD11)	1.22278	0.000776133
AT3G61060	phloem protein 2-A13(PP2-A13)	1.2209	0.000776133
AT1G02660	alpha/beta-Hydrolases superfamily protein(AT1G02660)	1.21892	0.000776133
AT3G06770	Pectin lyase-like superfamily protein(AT3G06770)	1.21884	0.000776133
AT5G41900	alpha/beta-Hydrolases superfamily protein(AT5G41900)	1.21576	0.000776133
AT3G27540	beta-1.4-N-acetylglucosaminyltransferase family protein(AT3G27540)	1.21419	0.00477784
AT2G28570	hypothetical protein(AT2G28570)	1.21143	0.000776133
AT2G20880	Integrase-type DNA-binding superfamily protein(ERF53)	1.2114	0.019346
AT3G61750	Cytochrome b561/ferric reductase transmembrane with DOMON related domain-containing protein(AT3G61750)	1.20915	0.000776133
AT2G41330	Glutaredoxin family protein(AT2G41330)	1.20874	0.000776133
AT2G23100	Cysteine/Histidine-rich C1 domain family protein(AT2G23100)	1.20794	0.000776133
AT4G31000	Calmodulin-binding protein(AT4G31000)	1.20512	0.000776133
AT5G67270	end binding protein 1C(EB1C)	1.2028	0.0155674
AT1G69900	Actin cross-linking protein(AT1G69900)	1.20238	0.000776133
AT2G23290	myb domain protein 70(MYB70)	1.20027	0.000776133
AT2G35710	Nucleotide-diphospho-sugar transferases superfamily protein(PGSIP7)	1.19837	0.00142905
AT2G39800	delta1-pyrroline-5-carboxylate synthase 1(P5CS1)	1.1965	0.000776133
AT4G15800	ralf-like 33(RALFL33)	1.19502	0.000776133
AT4G27520	early nodulin-like protein 2(ENODL2)	1.19268	0.000776133
AT5G22940	glucuronoxylan glucuronosyltransferase. putative(F8H)	1.19213	0.000776133
AT5G37770	EF hand calcium-binding protein family(TCH2)	1.19111	0.000776133
AT1G33610	Leucine-rich repeat (LRR) family protein(AT1G33610)	1.18907	0.000776133
AT5G38410	Ribulose bisphosphate carboxylase (small chain) family protein(RBCS3B)	1.18767	0.000776133
AT4G35320	hypothetical protein(AT4G35320)	1.18464	0.000776133
AT1G20190	expansin 11(EXPA11)	1.18247	0.000776133
AT3G55500	expansin A16(EXPA16)	1.18083	0.000776133
AT5G15350	early nodulin-like protein 17(ENODL17)	1.18036	0.000776133
AT2G20750	expansin B1(EXPB1)	1.17931	0.000776133
AT1G70985	hydroxyproline-rich glycoprotein family protein(AT1G70985)	1.17395	0.0232568
AT2G01918	PsbQ-like 3(PQL3)	1.17034	0.0132726
AT3G28180	Cellulose-synthase-like C4(CSLC04)	1.16982	0.000776133
AT4G04410	hypothetical protein(AT4G04410)	1.16855	0.000776133
AT1G22330	RNA-binding (RRM/RBD/RNP motifs) family protein(AT1G22330)	1.16433	0.000776133
AT5G28450	Chlorophyll A-B binding family protein(AT5G28450)	1.15446	0.000776133
AT5G47500	Pectin lyase-like superfamily protein(PME5)	1.1539	0.000776133
AT2G28120	Major facilitator superfamily protein(AT2G28120)	1.15281	0.000776133
AT1G70090	glucosyl transferase family 8(LGT8)	1.1526	0.000776133
AT1G14280	phytochrome kinase substrate 2(PKS2)	1.15162	0.000776133
AT5G50915	basic helix-loop-helix (bHLH) DNA-binding superfamily protein(AT5G50915)	1.14635	0.000776133
AT1G11260	sugar transporter 1(STP1)	1.14603	0.000776133
AT2G26695	Ran BP2/NZF zinc finger-like superfamily protein(AT2G26695)	1.14426	0.000776133
AT1G64640	early nodulin-like protein 8(ENODL8)	1.1432	0.000776133

AT3G19850	Phototropic-responsive NPH3 family protein(AT3G19850)	1.14233	0.000776133
AT4G18760	receptor like protein 51(RLP51)	1.14202	0.000776133
AT4G29905	hypothetical protein(AT4G29905)	1.1408	0.000776133
AT5G51600	Microtubule associated protein (MAP65/ASE1) family protein(PLE)	1.14067	0.00530611
AT5G06860	polygalacturonase inhibiting protein 1(PGIP1)	1.1402	0.000776133
AT3G28220	TRAF-like family protein(AT3G28220)	1.14002	0.000776133
AT3G05490	ralf-like 22(RALFL22)	1.12298	0.000776133
AT5G27290	stress regulated protein(AT5G27290)	1.12277	0.0494053
AT5G57100	Nucleotide/sugar transporter family protein(AT5G57100)	1.12089	0.000776133
AT1G61120	terpene synthase 04(TPS04)	1.11993	0.000776133
AT2G34620	Mitochondrial transcription termination factor family protein(AT2G34620)	1.11602	0.000776133
AT2G01850	endoxyloglucan transferase A3(EXGT-A3)	1.10825	0.000776133
AT3G10720	Plant invertase/pectin methylesterase inhibitor superfamily(AT3G10720)	1.10791	0.000776133
AT4G25830	Uncharacterized protein family (UPF0497)(AT4G25830)	1.10634	0.00266002
AT1G50590	RmlC-like cupins superfamily protein(AT1G50590)	1.10617	0.0129178
AT4G12730	FASCICLIN-like arabinogalactan 2(FLA2)	1.10237	0.000776133
AT3G49940	LOB domain-containing protein 38(LBD38)	1.10141	0.000776133
AT1G65490	transmembrane protein(AT1G65490)	1.10115	0.000776133
AT1G10020	formin-like protein (DUF1005)(AT1G10020)	1.10045	0.000776133
AT2G43290	Calcium-binding EF-hand family protein(MSS3)	1.09534	0.000776133
AT5G36002	ncRNA(AT5G36002)	1.09511	0.000776133
AT1G37130	nitrate reductase 2(NIA2)	1.09465	0.000776133
AT3G23170	hypothetical protein(AT3G23170)	1.09344	0.000776133
AT1G55330	arabinogalactan protein 21(AGP21)	1.09262	0.000776133
AT2G14610	pathogenesis-related protein 1(PR1)	1.09173	0.000776133
AT3G28340	galacturonosyltransferase-like 10(GATL10)	1.08982	0.0042563
AT2G29300	NAD(P)-binding Rossmann-fold superfamily protein(AT2G29300)	1.08908	0.000776133
AT5G25810	Integrase-type DNA-binding superfamily protein(tny)	1.08654	0.0389862
AT3G01550	phosphoenolpyruvate (pep)/phosphate translocator 2(PPT2)	1.08033	0.000776133
AT4G33550	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein(AT4G33550)	1.07699	0.0062571
AT3G59080	Eukaryotic aspartyl protease family protein(AT3G59080)	1.07196	0.000776133
AT4G02540	Cysteine/Histidine-rich C1 domain family protein(AT4G02540)	1.07118	0.000776133
AT5G03350	Legume lectin family protein(AT5G03350)	1.07053	0.000776133
AT5G65920	ARM repeat superfamily protein(AT5G65920)	1.06955	0.000776133
AT3G17185	ncRNA(TAS3)	1.0657	0.00762101
AT1G20070	hypothetical protein(AT1G20070)	1.06374	0.000776133
AT4G23440	Disease resistance protein (TIR-NBS class)(AT4G23440)	1.06369	0.000776133
AT5G20270	heptahelical transmembrane protein1(HHP1)	1.06207	0.000776133
AT2G29290	NAD(P)-binding Rossmann-fold superfamily protein(AT2G29290)	1.06136	0.000776133
AT2G47485	hypothetical protein(AT2G47485)	1.0526	0.0117267
AT1G80440	Galactose oxidase/kelch repeat superfamily protein(AT1G80440)	1.05179	0.000776133
AT3G29575	ABI five binding protein 3(AFP3)	1.05021	0.000776133
AT5G03360	cysteine/histidine-rich C1 domain protein(AT5G03360)	1.04927	0.000776133
AT2G04780	FASCICLIN-like arabinoogalactan 7(FLA7)	1.04554	0.000776133
AT5G44260	Zinc finger C-x8-C-x5-C-x3-H type family protein(AT5G44260)	1.04535	0.000776133

AT1G14250	GDA1/CD39 nucleoside phosphatase family protein(AT1G14250)	1.04471	0.000776133
AT5G10150	UPSTREAM OF FLC protein (DUF966)(AT5G10150)	1.04326	0.000776133
AT4G11650	osmotin 34(OSM34)	1.04204	0.00375011
AT5G04660	cytochrome P450. family 77. subfamily A. polypeptide 4(CYP77A4)	1.04181	0.0404428
AT1G19970	ER lumen protein retaining receptor family protein(AT1G19970)	1.03736	0.000776133
AT4G02330	Plant invertase/pectin methylesterase inhibitor superfamily(ATPMEPCRB)	1.03702	0.000776133
AT4G29610	Cytidine/deoxycytidylate deaminase family protein(AT4G29610)	1.03616	0.000776133
AT1G15550	gibberellin 3-oxidase 1(GA3OX1)	1.03545	0.00804994
AT2G28630	3-ketoacyl-CoA synthase 12(KCS12)	1.03229	0.000776133
AT2G23810	tetraspanin8(TET8)	1.03147	0.000776133
AT1G49160	Protein kinase superfamily protein(WNK7)	1.02996	0.00142905
AT1G11380	PLAC8 family protein(AT1G11380)	1.02538	0.000776133
AT5G53870	early nodulin-like protein 1(ENODL1)	1.0252	0.000776133
AT1G72790	hydroxyproline-rich glycoprotein family protein(AT1G72790)	1.02344	0.000776133
AT2G33570	glycosyltransferase family protein (DUF23)(GALS1)	1.02314	0.000776133
AT5G65470	O-fucosyltransferase family protein(AT5G65470)	1.02279	0.000776133
AT4G30440	UDP-D-glucuronate 4-epimerase 1(GAE1)	1.02192	0.000776133
AT1G12990	beta-1.4-N-acetylglucosaminyltransferase family protein(AT1G12990)	1.02132	0.000776133
AT1G03055	beta-carotene isomerase D27-like protein(D27)	1.02042	0.00375011
AT5G52900	membrane-associated kinase regulator(MAKR6)	1.01575	0.000776133
AT2G29130	laccase 2(LAC2)	1.01515	0.0260878
AT4G30280	xyloglucan endotransglucosylase/hydrolase 18(XTH18)	1.01218	0.00142905
AT3G54000	TIP41-like protein(AT3G54000)	1.00694	0.00970734
AT2G36120	Glycine-rich protein family(DOT1)	1.00458	0.000776133
AT2G25200	hypothetical protein (DUF868)(AT2G25200)	1.00379	0.000776133
AT4G34150	Calcium-dependent lipid-binding (CaLB domain) family protein(AT4G34150)	1.0025	0.000776133
AT3G18773	RING/U-box superfamily protein(AT3G18773)	1.00122	0.0042563

Table S3.4 List of genes significantly downregulated by a log_2fold value smaller than -1 elicited by BC204 (0.01% [v/v]) treatment in *Arabidopsis thaliana* shoot tissue, functionally classified and annotated by the DAVID database.

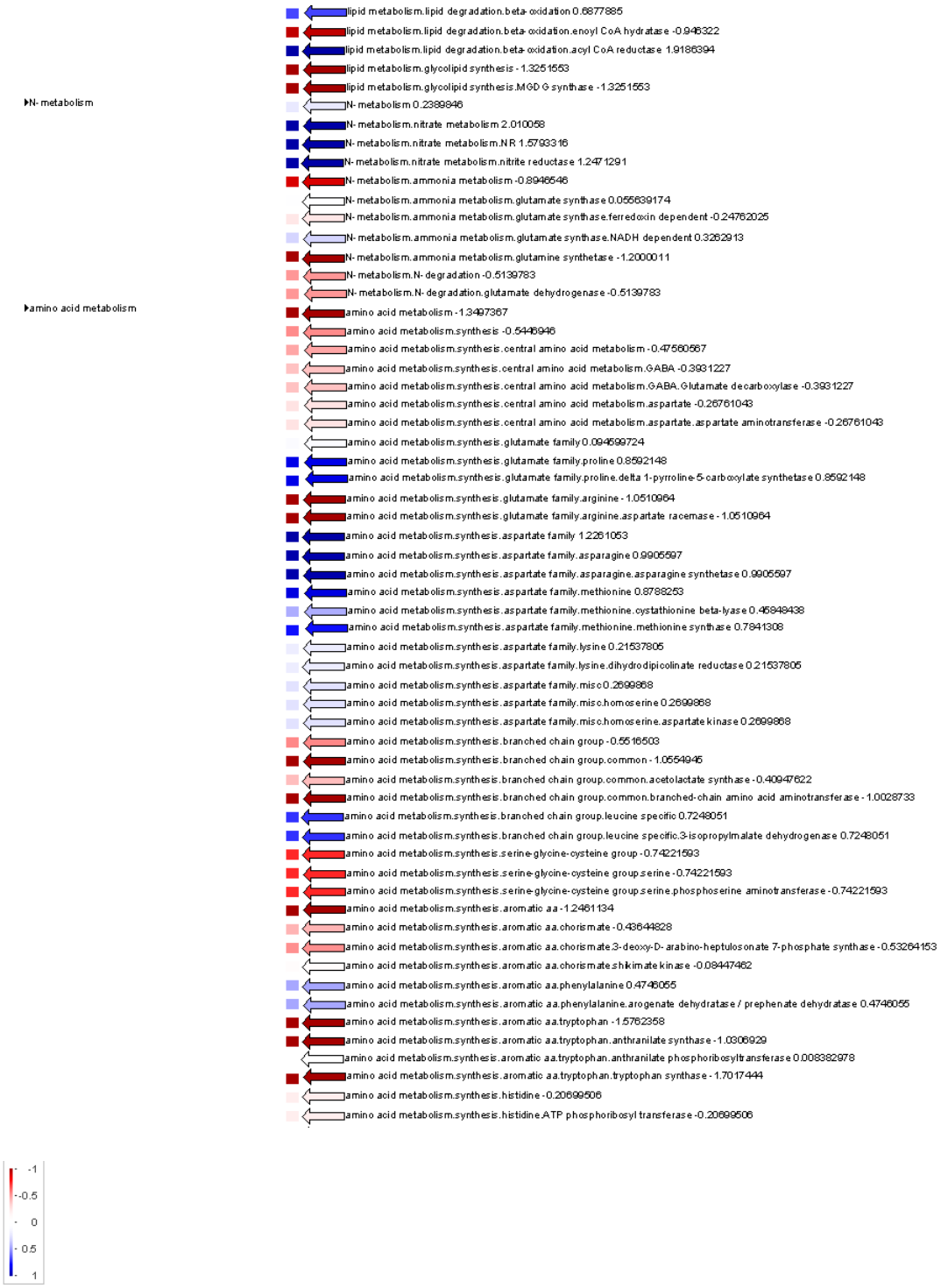
TAIR ID	Gene description	Fold change	q value
AT1G27565	hypothetical protein(AT1G27565)	-100	0.00077613
AT4G16640	Matrixin family protein(AT4G16640)	-3.80444	0.0219843
AT4G12735	hypothetical protein(AT4G12735)	-3.20858	0.0494053
AT2G17660	RPM1-interacting protein 4 (RIN4) family protein(AT2G17660)	-3.11634	0.0062571
AT1G20180	transmembrane protein (DUF677)(AT1G20180)	-3.03657	0.00142905
AT2G36780	UDP-Glycosyltransferase superfamily protein(AT2G36780)	-3.02382	0.00077613
AT2G41850	polygalacturonase ADPG2-like protein(PGAZAT)	-2.84489	0.00077613
AT5G45890	senescence-associated gene 12(SAG12)	-2.84315	0.00077613
AT3G60140	Glycosyl hydrolase superfamily protein(DIN2)	-2.79986	0.00077613
AT4G21490	NAD(P)H dehydrogenase B3(NDB3)	-2.77958	0.0189815
AT1G12940	nitrate transporter2.5(NRT2.5)	-2.69322	0.00077613
AT1G80160	Lactoylglutathione lyase / glyoxalase I family protein(GLYI7)	-2.61274	0.00142905
AT2G14620	xyloglucan endotransglucosylase/hydrolase 10(XTH10)	-2.54967	0.00077613
AT3G21520	transmembrane protein. putative (DUF679 domain membrane protein 1)(DMP1)	-2.51733	0.00077613
AT1G54020	GDSL-like Lipase/Acylhydrolase superfamily protein(AT1G54020)	-2.45332	0.00142905
AT3G15500	NAC domain containing protein 3(NAC3)	-2.4462	0.00077613
AT1G30700	FAD-binding Berberine family protein(AT1G30700)	-2.43258	0.00077613
AT3G61930	hypothetical protein(AT3G61930)	-2.42162	0.0121406
AT1G17030	hypothetical protein(AT1G17030)	-2.40726	0.00142905
AT1G11190	bifunctional nuclease i(BFN1)	-2.39102	0.00077613
AT4G33980	hypothetical protein(AT4G33980)	-2.35382	0.00077613
AT1G07050	CCT motif family protein(AT1G07050)	-2.31065	0.00077613
AT1G58340	MATE efflux family protein(ZF14)	-2.24741	0.00077613
AT5G28237	Pyridoxal-5'-phosphate-dependent enzyme family protein(AT5G28237)	-2.23704	0.00717506
AT3G46080	C2H2-type zinc finger family protein(AT3G46080)	-2.23158	0.0189815
AT4G18425	transmembrane protein. putative (DUF679)(AT4G18425)	-2.23122	0.00205608
AT2G18193	P-loop containing nucleoside triphosphate hydrolases superfamily protein(AT2G18193)	-2.16268	0.00077613
AT2G29470	glutathione S-transferase tau 3(GSTU3)	-2.15245	0.0166443
AT4G01360	BPS1-like protein(BPS3)	-2.14286	0.00077613
AT5G35407	ncRNA(MIR396b)	-2.13512	0.00077613
AT2G46950	cytochrome P450. family 709. subfamily B. polypeptide 2(CYP709B2)	-2.10967	0.0101294
AT2G45570	cytochrome P450. family 76. subfamily C. polypeptide 2(CYP76C2)	-2.07939	0.00077613
AT3G01420	Peroxidase superfamily protein(DOX1)	-2.04249	0.00077613
AT1G17665	CA-responsive protein(AT1G17665)	-1.9728	0.00142905
AT1G09500	NAD(P)-binding Rossmann-fold superfamily protein(AT1G09500)	-1.95189	0.00077613
AT4G26950	senescence regulator (Protein of unknown function. DUF584)(AT4G26950)	-1.95131	0.00077613
AT5G42900	cold regulated protein 27(COR27)	-1.91929	0.00077613
AT3G25250	AGC (cAMP-dependent. cGMP-dependent and protein kinase C) kinase family protein(AGC2-1)	-1.8923	0.00375011

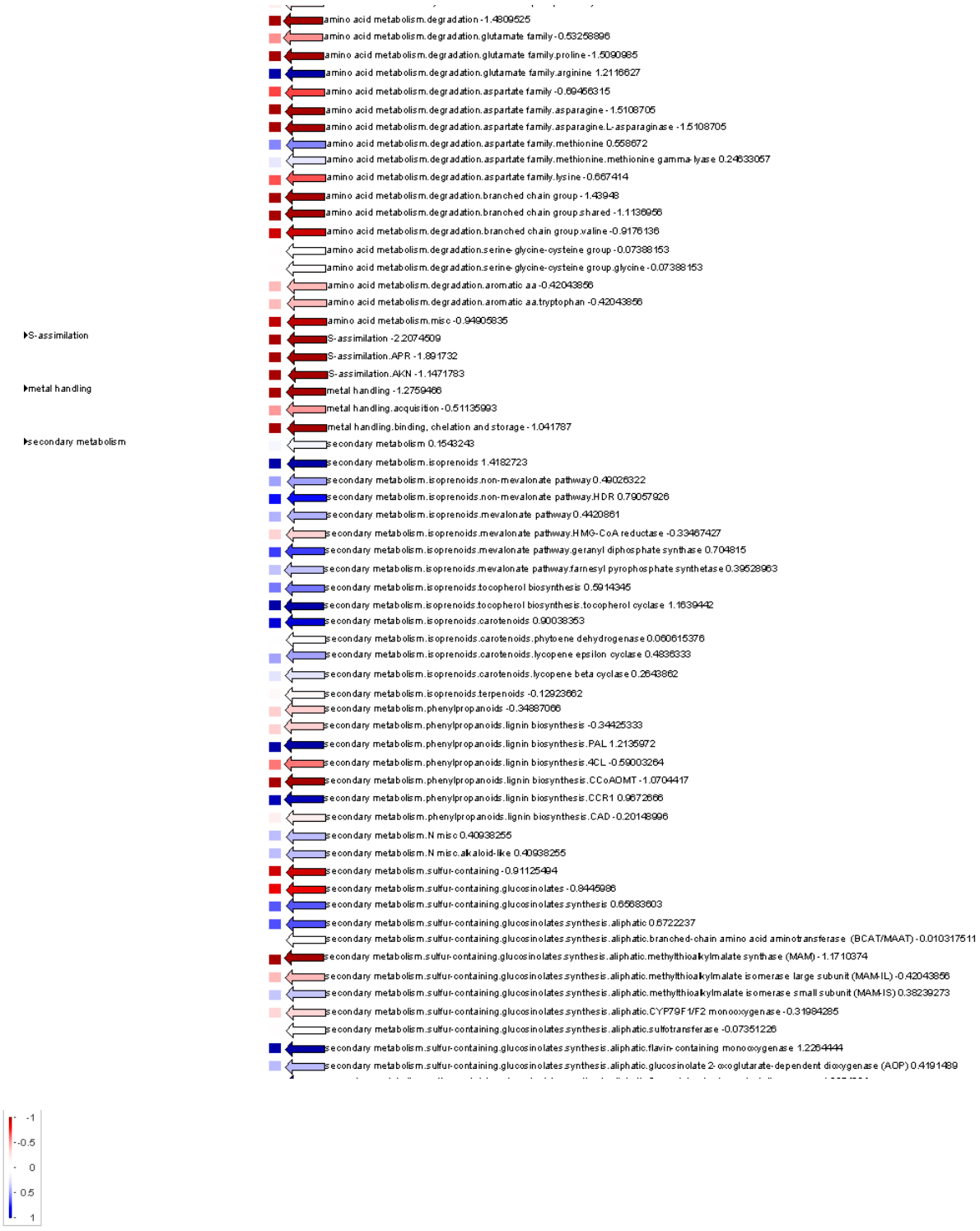
AT4G37410	cytochrome P450. family 81. subfamily F. polypeptide 4(CYP81F4)	-1.78992	0.00077613
AT4G37990	cinnamyl alcohol dehydrogenase 8(ELI3-2)	-1.78443	0.00077613
AT3G28210	zinc finger (AN1-like) family protein(PMZ)	-1.76642	0.00077613
AT5G13080	WRKY DNA-binding protein 75(WRKY75)	-1.74814	0.00530611
AT5G40690	histone-lysine N-methyltransferase trithorax-like protein(AT5G40690)	-1.74785	0.00077613
AT4G06746	related to AP2 9(RAP2.9)	-1.73633	0.00077613
AT2G29490	glutathione S-transferase TAU 1(GSTU1)	-1.7253	0.00077613
AT2G04050	MATE efflux family protein(AT2G04050)	-1.72348	0.00077613
AT3G44300	nitrilase 2(NIT2)	-1.70681	0.00077613
AT1G62370	RING/U-box superfamily protein(AT1G62370)	-1.706	0.0129178
AT4G24000	cellulose synthase like G2(CSLG2)	-1.69874	0.00077613
AT1G25054	UDP-3-O-acyl N-acetylglycosamine deacetylase family protein(LpxC3)	-1.67665	0.00077613
AT1G66700	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein(PXMT1)	-1.67567	0.00077613
AT1G43800	Plant stearoyl-acyl-carrier-protein desaturase family protein(FTM1)	-1.67119	0.00077613
AT1G62760	Plant invertase/pectin methylesterase inhibitor superfamily protein(AT1G62760)	-1.64254	0.00077613
AT5G65040	senescence-associated family protein (DUF581)(AT5G65040)	-1.617	0.00077613
AT1G15380	Lactoylglutathione lyase / glyoxalase I family protein(GLYI4)	-1.60386	0.00077613
AT1G68050	flavin-binding. kelch repeat. f box 1(FKF1)	-1.60248	0.00077613
AT5G02580	argininosuccinate lyase(AT5G02580)	-1.59925	0.0273592
AT5G24270	Calcium-binding EF-hand family protein(SOS3)	-1.5865	0.00077613
AT5G02260	expansin A9(EXPA9)	-1.58229	0.00077613
AT4G16000	hypothetical protein(AT4G16000)	-1.58012	0.00142905
AT1G21890	nodulin MtN21 /EamA-like transporter family protein(UMAMIT19)	-1.57133	0.00884598
AT2G30540	Thioredoxin superfamily protein(AT2G30540)	-1.55863	0.00142905
AT5G13170	senescence-associated gene 29(SAG29)	-1.55816	0.0385317
AT5G14730	hypothetical protein(AT5G14730)	-1.54316	0.00077613
AT5G26731	hypothetical protein(AT5G26731)	-1.53781	0.00205608
AT1G67105	ncRNA(AT1G67105)	-1.53471	0.00077613
AT1G67370	DNA-binding HORMA family protein(ASY1)	-1.53191	0.0125425
AT1G59670	glutathione S-transferase TAU 15(GSTU15)	-1.5034	0.0144361
AT1G48330	SsrA-binding protein(AT1G48330)	-1.48328	0.00077613
AT1G11220	cotton fiber. putative (DUF761)(AT1G11220)	-1.46744	0.0042563
AT4G19430	hypothetical protein(AT4G19430)	-1.45416	0.00077613
AT1G79900	Mitochondrial substrate carrier family protein(BAC2)	-1.4487	0.0239164
AT5G48850	Tetratricopeptide repeat (TPR)-like superfamily protein(ATSDI1)	-1.44303	0.00077613
AT2G25460	EEIG1/EHBP1 protein amino-terminal domain protein(AT2G25460)	-1.44172	0.00077613
AT2G36750	UDP-glucosyl transferase 73C1(UGT73C1)	-1.43997	0.0166443
AT1G07430	highly ABA-induced PP2C protein 2(HAI2)	-1.43127	0.00077613
AT1G12200	Flavin-binding monooxygenase family protein(FMO)	-1.4309	0.00077613
AT1G31760	SWIB/MDM2 domain superfamily protein(AT1G31760)	-1.42971	0.0487249
AT1G78780	pathogenesis-related family protein(AT1G78780)	-1.42133	0.00804994
AT3G49570	response to low sulfur 3(LSU3)	-1.41822	0.0276757
AT5G62480	glutathione S-transferase tau 9(GSTU9)	-1.41588	0.00579781
AT1G06830	Glutaredoxin family protein(AT1G06830)	-1.41194	0.00077613

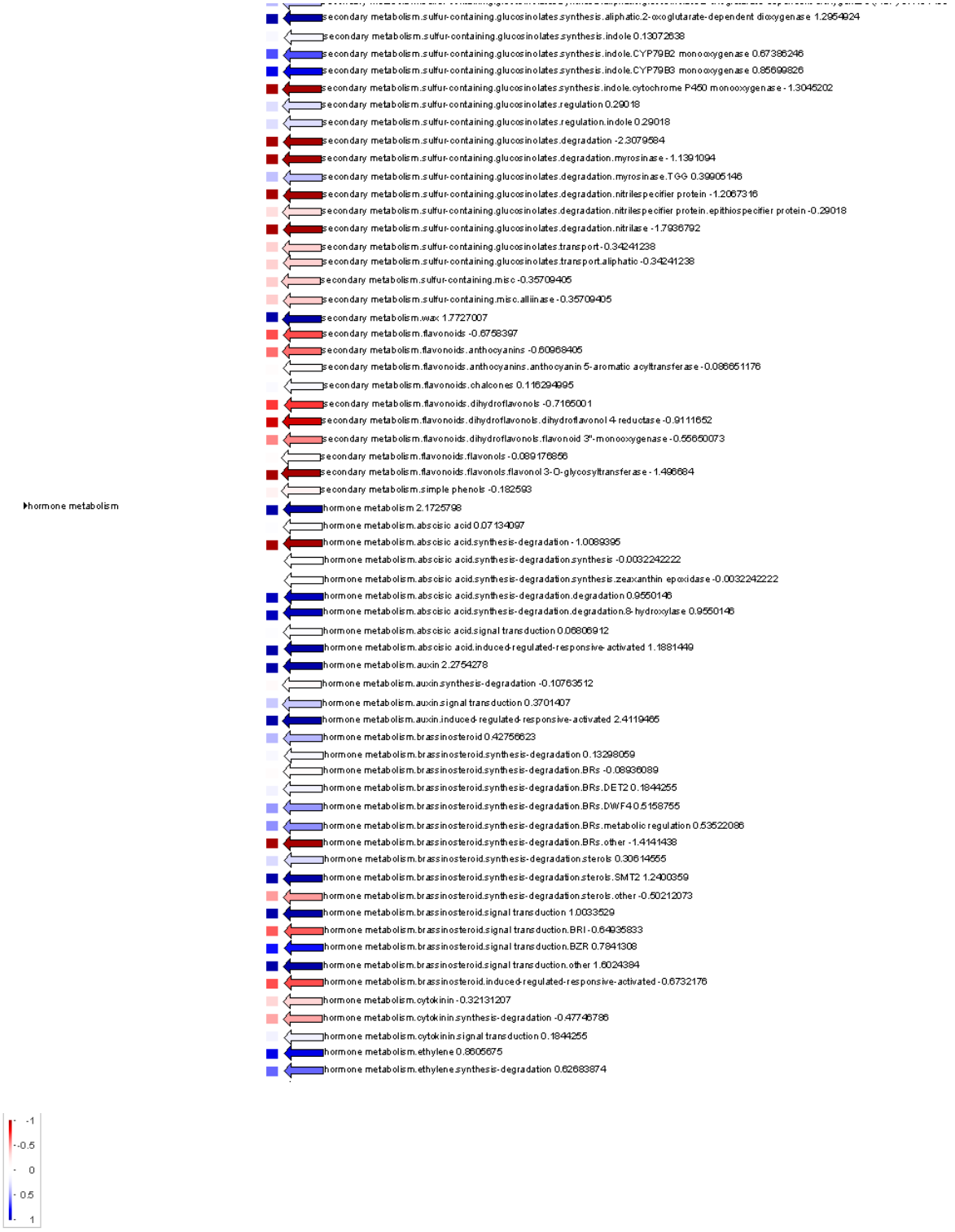
AT5G09540	Chaperone DnaJ-domain superfamily protein(AT5G09540)	-1.39688	0.00077613
AT1G27020	plant/protein(AT1G27020)	-1.38558	0.00077613
AT4G16740	terpene synthase 03(TPS03)	-1.38489	0.00077613
AT5G06510	nuclear factor Y. subunit A10(NF-YA10)	-1.37669	0.00077613
AT1G02460	Pectin lyase-like superfamily protein(AT1G02460)	-1.37232	0.00077613
AT5G59820	C2H2-type zinc finger family protein(RHL41)	-1.36306	0.00077613
AT5G16570	glutamine synthetase 1;4(GLN1;4)	-1.35544	0.00077613
AT1G06160	octadecanoid-responsive AP2/ERF 59(ORA59)	-1.35154	0.00077613
AT1G52890	NAC domain containing protein 19(NAC019)	-1.34763	0.00077613
AT5G03210	E3 ubiquitin-protein ligase(DIP2)	-1.34471	0.00077613
AT2G37770	NAD(P)-linked oxidoreductase superfamily protein(ChlAKR)	-1.33982	0.00077613
AT4G37430	cytochrome P450. family 91. subfamily A. polypeptide 2(CYP91A2)	-1.3396	0.00077613
AT4G21990	APS reductase 3(APR3)	-1.33935	0.00077613
AT1G49475	AP2/B3-like transcriptional factor family protein(AT1G49475)	-1.33574	0.00579781
AT1G33030	O-methyltransferase family protein(AT1G33030)	-1.32595	0.00077613
AT2G19810	CCCH-type zinc finger family protein(OZF1)	-1.31888	0.00077613
AT5G46960	Plant invertase/pectin methylesterase inhibitor superfamily protein(AT5G46960)	-1.30661	0.0125425
AT4G30370	RING/U-box superfamily protein(AT4G30370)	-1.30602	0.0257742
AT3G23550	MATE efflux family protein(AT3G23550)	-1.30463	0.00077613
AT4G25480	dehydration response element B1A(DREB1A)	-1.30436	0.00077613
AT2G29460	glutathione S-transferase tau 4(GSTU4)	-1.2859	0.00077613
AT4G36570	RAD-like 3(RL3)	-1.27984	0.00077613
AT4G15270	glucosyltransferase-like protein(AT4G15270)	-1.27412	0.0113268
AT4G08555	hypothetical protein(AT4G08555)	-1.27008	0.00579781
AT1G19050	response regulator 7(ARR7)	-1.26906	0.00077613
AT4G18360	Aldolase-type TIM barrel family protein(GOX3)	-1.26783	0.00077613
AT3G16660	Pollen Ole e 1 allergen and extensin family protein(AT3G16660)	-1.26425	0.00077613
AT2G46660	cytochrome P450. family 78. subfamily A. polypeptide 6(CYP78A6)	-1.26193	0.00142905
AT1G61930	senescence regulator (Protein of unknown function. DUF584)(AT1G61930)	-1.25666	0.00321958
AT3G03910	glutamate dehydrogenase 3(GDH3)	-1.2547	0.0356982
AT2G39705	ROTUNDIFOLIA like 8(RTFL8)	-1.24051	0.00077613
AT3G05690	nuclear factor Y. subunit A2(NF-YA2)	-1.23846	0.00077613
AT2G36970	UDP-Glycosyltransferase superfamily protein(AT2G36970)	-1.23713	0.00077613
AT3G52770	binding protein(ZPR3)	-1.23624	0.0183362
AT3G05727	S locus-related glycoprotein 1 (SLR1) binding pollen coat protein family(AT3G05727)	-1.22339	0.00077613
AT1G71000	Chaperone DnaJ-domain superfamily protein(AT1G71000)	-1.21427	0.00375011
AT1G66390	myb domain protein 90(MYB90)	-1.21307	0.00077613
AT1G19630	cytochrome P450. family 722. subfamily A. polypeptide 1(CYP722A1)	-1.20393	0.00077613
AT2G41730	calcium-binding site protein(AT2G41730)	-1.19499	0.00077613
AT3G14770	Nodulin MtN3 family protein(SWEET2)	-1.18427	0.00205608
AT3G28640	Tetratricopeptide repeat (TPR)-like superfamily protein(AT3G28640)	-1.18278	0.00077613
AT5G37980	Zinc-binding dehydrogenase family protein(AT5G37980)	-1.18236	0.0186608
AT5G62920	response regulator 6(ARR6)	-1.18021	0.00077613

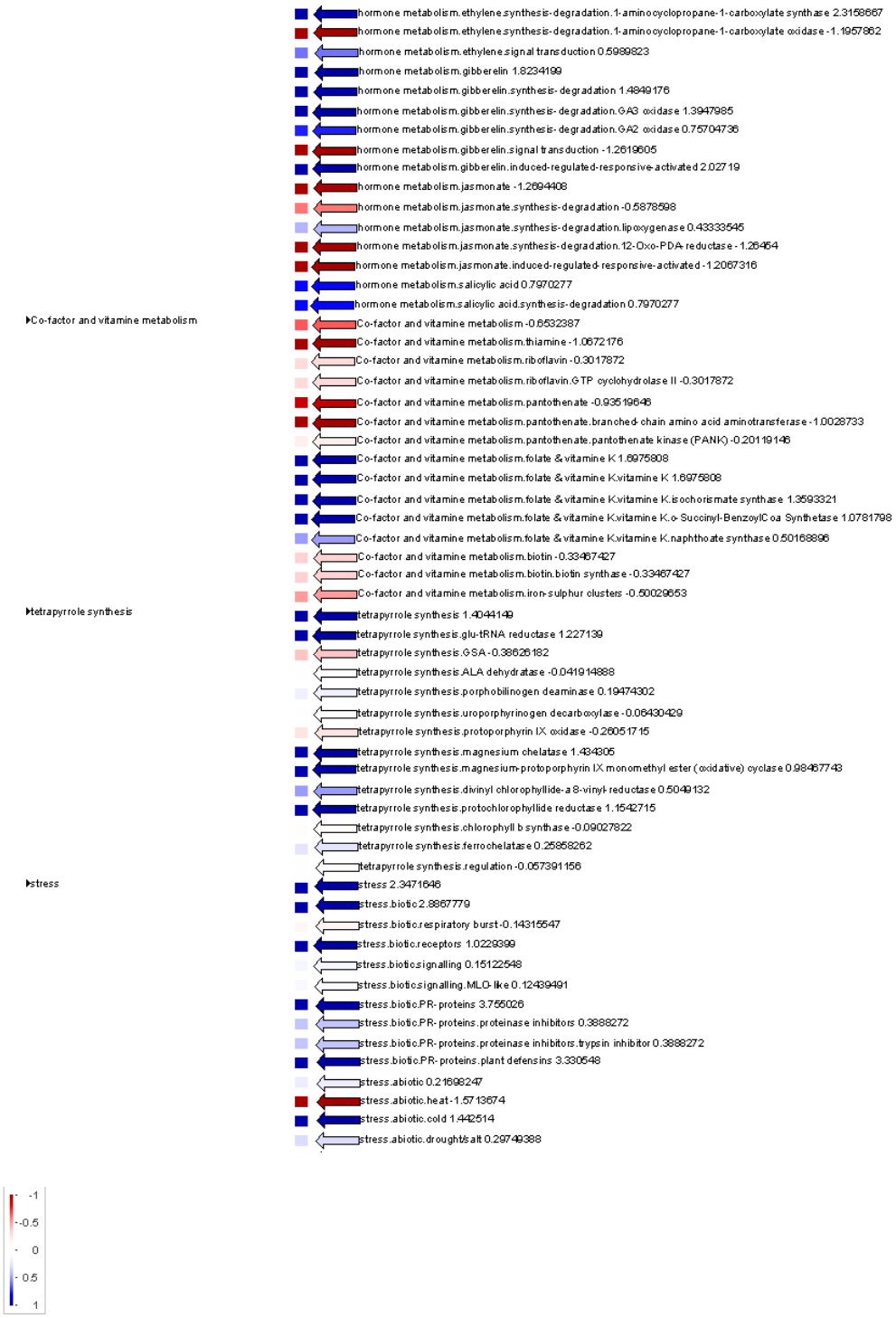
AT1G54575	hypothetical protein(AT1G54575)	-1.17829	0.00077613
AT3G42860	zinc knuckle (CCHC-type) family protein(AT3G42860)	-1.17668	0.0479824
AT1G32350	alternative oxidase 1D(AOX1D)	-1.17457	0.00804994
AT1G52030	myrosinase-binding protein 2(MBP2)	-1.16205	0.00077613
AT5G59310	lipid transfer protein 4(LTP4)	-1.16083	0.00077613
AT4G04190	transmembrane protein(AT4G04190)	-1.15934	0.00846178
AT1G15125	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein(AT1G15125)	-1.15797	0.00077613
AT5G05340	Peroxidase superfamily protein(PRX52)	-1.15119	0.0463561
AT1G76760	thioredoxin Y1(TY1)	-1.14987	0.0468407
AT1G54000	GDSL-like Lipase/Acylhydrolase superfamily protein(GLL22)	-1.14886	0.0125425
AT1G05340	cysteine-rich TM module stress tolerance protein(AT1G05340)	-1.14688	0.00077613
AT1G05300	zinc transporter 5 precursor(ZIP5)	-1.13993	0.00077613
AT1G32690	DUF740 family protein(AT1G32690)	-1.13974	0.0125425
AT1G79680	WALL ASSOCIATED KINASE (WAK)-LIKE 10(WAKL10)	-1.1394	0.00077613
AT3G03440	ARM repeat superfamily protein(AT3G03440)	-1.1389	0.00077613
AT1G09486	miscRNA(AT1G09486)	-1.13478	0.0435046
AT4G12290	Copper amine oxidase family protein(AT4G12290)	-1.13105	0.00077613
AT3G51910	heat shock transcription factor A7A(HSFA7A)	-1.13055	0.00579781
AT4G04450	WRKY family transcription factor(WRKY42)	-1.13032	0.00077613
AT4G22517	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein(AT4G22517)	-1.12293	0.00077613
AT2G22122	hypothetical protein(AT2G22122)	-1.12136	0.00077613
AT3G53690	RING/U-box superfamily protein(AT3G53690)	-1.12112	0.0364263
AT3G30720	qua-quine starch(QQS)	-1.11327	0.00321958
AT2G21640	marker for oxidative stress response protein(AT2G21640)	-1.10493	0.00077613
AT5G65300	hypothetical protein(AT5G65300)	-1.10485	0.00375011
AT1G28190	hypothetical protein(AT1G28190)	-1.10218	0.00077613
AT4G29230	NAC domain containing protein 75(NAC075)	-1.0982	0.00205608
AT5G26749	C2H2 and C2HC zinc fingers superfamily protein(AT5G26749)	-1.09593	0.0109642
AT1G53035	transmembrane protein(AT1G53035)	-1.09094	0.00077613
AT5G43420	RING/U-box superfamily protein(AT5G43420)	-1.08963	0.00077613
AT4G32810	carotenoid cleavage dioxygenase 8(CCD8)	-1.08039	0.048995
AT5G10625	flowering-promoting factor-like protein(AT5G10625)	-1.08036	0.00077613
AT1G13810	Restriction endonuclease. type II-like superfamily protein(AT1G13810)	-1.0773	0.00077613
AT3G48850	phosphate transporter 3;2(PHT3;2)	-1.07611	0.0206488
AT3G30122	miscRNA(AT3G30122)	-1.07415	0.00077613
AT2G43140	basic helix-loop-helix (bHLH) DNA-binding superfamily protein(AT2G43140)	-1.07239	0.0435046
AT5G42380	calmodulin like 37(CML37)	-1.06637	0.0132726
AT1G11180	Secretory carrier membrane protein (SCAMP) family protein(SCAMP2)	-1.05885	0.00077613
AT5G66440	tRNA-methyltransferase non-catalytic subunit trm6MTase subunit(AT5G66440)	-1.05818	0.00077613
AT2G34960	cationic amino acid transporter 5(CAT5)	-1.05481	0.00077613
AT1G10990	transmembrane protein(AT1G10990)	-1.05449	0.00205608
AT4G36930	basic helix-loop-helix (bHLH) DNA-binding superfamily protein(SPT)	-1.05436	0.00077613

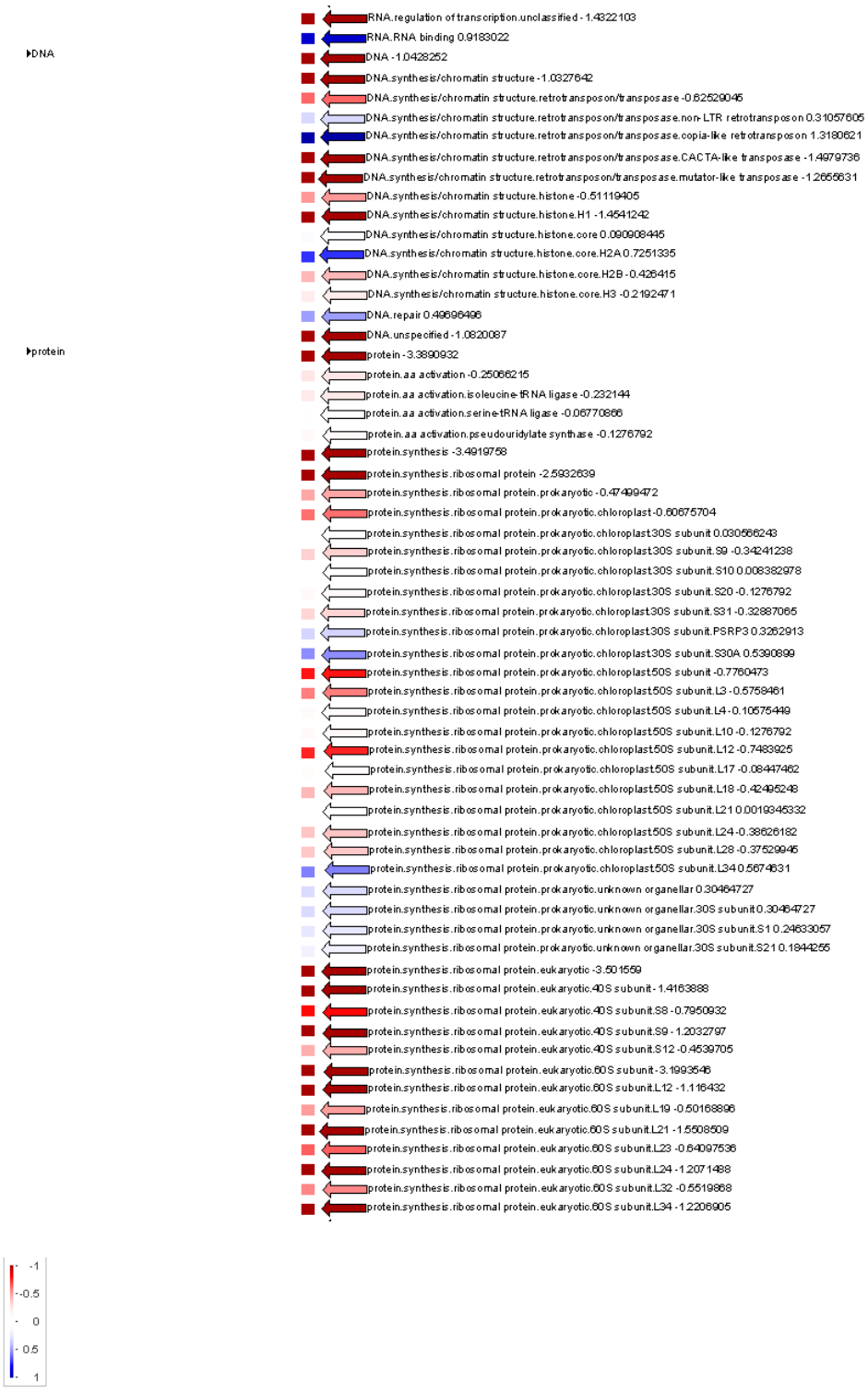
AT5G04200	metacaspase 9(MC9)	-1.05021	0.00142905
AT1G54570	Esterase/lipase/thioesterase family protein(PES1)	-1.04491	0.00077613
AT1G63440	heavy metal atpase 5(HMA5)	-1.04315	0.0109642
AT5G26220	ChaC-like family protein(AT5G26220)	-1.04255	0.00077613
AT3G16150	N-terminal nucleophile aminohydrolases (Ntn hydrolases) superfamily protein(ASPGB1)	-1.04239	0.0369341
AT1G21240	wall associated kinase 3(WAK3)	-1.03876	0.00077613
AT2G47190	myb domain protein 2(MYB2)	-1.03742	0.00077613
AT1G12210	RPS5-like 1(RFL1)	-1.03345	0.00077613
AT3G22910	ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase family protein(AT3G22910)	-1.03102	0.00077613
AT3G13950	ankyrin(AT3G13950)	-1.02765	0.00077613
AT2G15480	UDP-glucosyl transferase 73B5(UGT73B5)	-1.02027	0.00077613
AT5G63790	NAC domain containing protein 102(NAC102)	-1.01856	0.00077613
AT3G26200	cytochrome P450. family 71. subfamily B. polypeptide 22(CYP71B22)	-1.01635	0.00077613
AT3G52490	Double Clp-N motif-containing P-loop nucleoside triphosphate hydrolases superfamily protein(AT3G52490)	-1.01455	0.00530611
AT3G43828	hypothetical protein(AT3G43828)	-1.01255	0.0195926
AT4G34135	UDP-glucosyltransferase 73B2(UGT73B2)	-1.01189	0.00077613
AT3G59140	multidrug resistance-associated protein 14(ABCC10)	-1.01122	0.00077613
AT5G42210	Major facilitator superfamily protein(AT5G42210)	-1.00747	0.0232568
AT2G06255	ELF4-like 3(ELF4-L3)	-1.0045	0.00321958
AT2G44480	beta glucosidase 17(BGLU17)	-1.00068	0.00717506











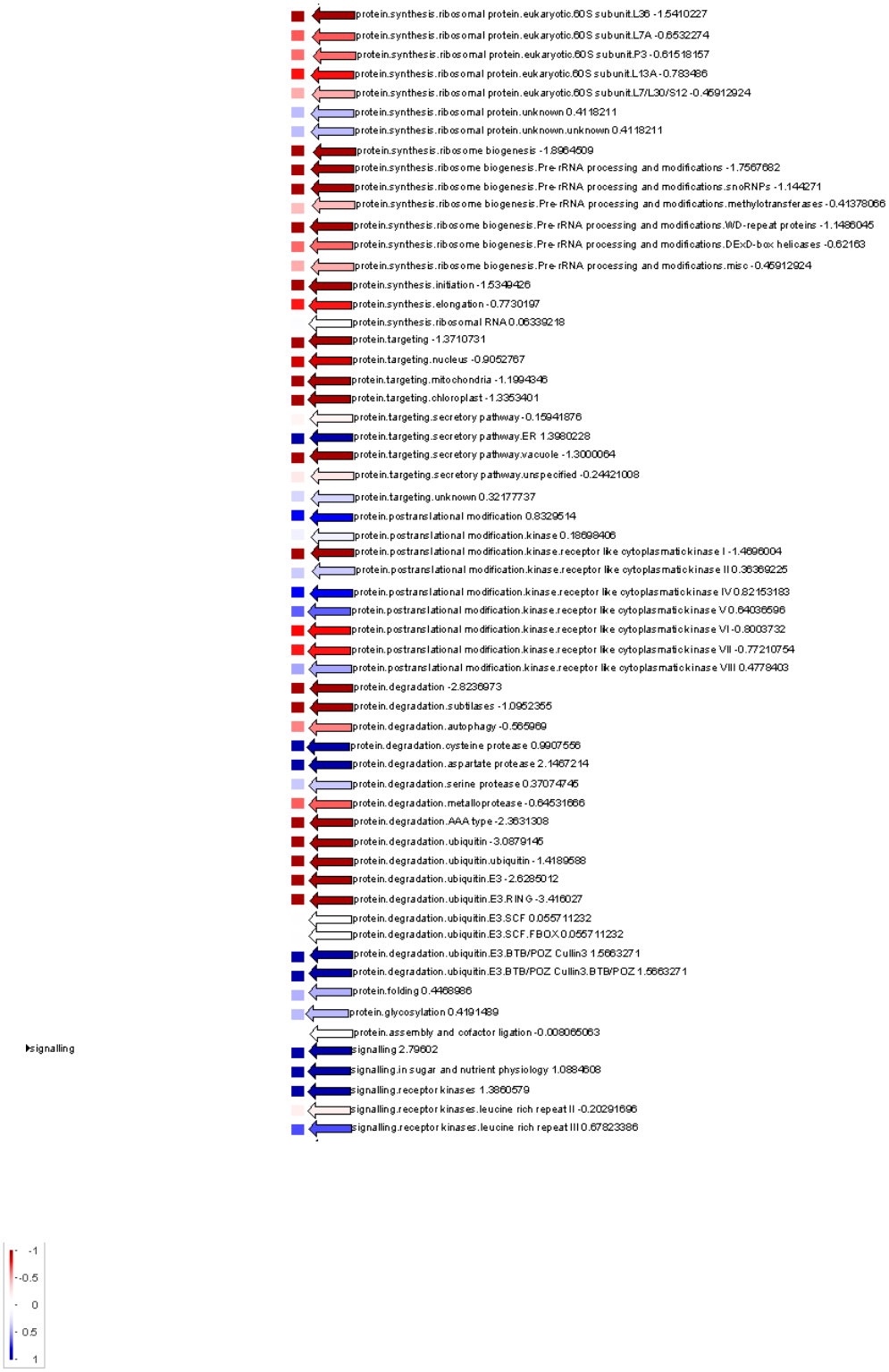


Figure S3.5 PageMan functional characterization of all processes upregulated (blue) and downregulated (red) in *Arabidopsis thaliana* shoot tissue elicited by BC204 (0.01% [v/v]) treatment.

Additional supplementary data

- Figures on the determination of optimal concentration and mode of treatment for BC204
- Outputs from analysis using the AgriGOv2 (SEA analysis) and KEGG.
- Sequence data and list of all genes up- or downregulated in response to BC204

Available on request from phills@sun.ac.za

Chapter 4: A citrus-based plant extract induces genes involved in hormone, secondary metabolism and stress responses in *Solanum lycopersicum* shoot tissue

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4.1 Abstract

Plant biostimulants have been earmarked as one of the major groups of new plant growth promoting substances to drive a much-needed revolution in agriculture. One such PB, BC204, has been used to great success, but there is no peer-reviewed data to explain the possible mechanisms by which it exerts its effects. In this study, an RNA-seq approach was adopted to elucidate the effects of BC204 on shoot tissues of hydroponically-grown *Solanum lycopersicum* seedlings at the molecular level. BC204, applied via foliar spray at a concentration of 0.05% (v/v), stimulated root and shoot biomass production, root and shoot length, and stem width compared to the untreated control plants. Out of the 33308 transcripts analysed, a total of 18.059% of all genes were significantly differentially expressed between the control and treated groups, of which 8.776% were upregulated and 9.283% downregulated. Most notably, genes involved in signalling, stress and protein metabolism were upregulated, which could explain the observed increases in growth. Additionally, hormone metabolism and genes involved in transcription and other regulation processes were also upregulated. Genes involved in protein metabolism were mostly downregulated.

Keywords: BC204, *Solanum lycopersicum*, RNA-seq, gene ontology, gene expression, signalling, secondary metabolism, hormone metabolism

4.2 Introduction

Agriculture has greatly benefited from the use of plant growth promoting substances (PGPS) (Arteca, 1996). PGPS, which include fertilizers, insecticides, fungicides and other pesticides (Carvalho, 2006), have been pivotal in contributing most notably to the Green Revolution (Pingali, 2012), in conjunction with genetic trait improvements (Hedden, 2003). A group of PGPS, known as plant biostimulants (PB), have been used increasingly in agriculture because of their broad range of benefits, including increases in crop yield and priming plants towards increased levels of tolerance environmental stresses (Brown and Saa, 2015; Calvo et al., 2014; Casadesús et al., 2019; Yakhin et al., 2017). PBs are derived for example from seaweeds, algae, plant material, industrial processing and other sources, as extensively described in previous reviews (du Jardin, 2015, 2012). Furthermore, PBs could be an environmentally-sustainable solution to agricultural challenges as they are derived from natural/organic sources, which reduces the dependency of agriculture on chemical fertilizers and pesticides (Xu and Geelen, 2018). PBs have the potential to optimise plant growth and agricultural output in an inexpensive manner. They are a cheap collection of natural and synthetic compounds, usually extracted and obtained from plant/algae material or waste-products produced by industrial processes. Unlike other PGPS, PBs do not directly provide plants with nutrients, nor do they directly protect the plant against environmental stresses such as insects, viruses or abiotic stress conditions (du Jardin, 2015, 2012). Rather, PBs are hypothesized to stimulate the plant's endogenous metabolism to elicit a large shift in metabolism which makes the plant more tolerant to stress and grow faster and more vigorously. Importantly, a PB cannot be termed a ''fertilizer", since its role and function in plant growth and development is independent of its nutrient content (Calvo et al., 2014).

PBs which are derived from plant and other food products are of particular interest for use in organic farming, due to their natural and organic origin (De Pascale et al., 2017). The use of these PBs can almost be viewed as a form of biotic recycling. This has been shown in organic tomatoes (*Solanum lycopersicum*), where fennel and lemon processing residues and spent brewer's grain significantly increased vitamin C and phenol contents in the plants (Chehade et al., 2018).

One such PB, BC204, is a commercially available product manufactured in South Africa and used in several countries. BC204 is a citrus-based PB containing extracts from *Citrus aurantium* and other plant extracts and acids. The exact formula is proprietary and known only to the company. Non peer-reviewed communications reported a variety of benefits of BC204 for plant growth. These include total improved plant growth and optimal output, an increase in tolerance towards environmental stresses, enhanced root growth and high-quality fruit production combined with an increase in yield. Additionally, a compound closely related to BC204, produced by the same manufacturer, positively influenced water use efficiency in certain table grape cultivars (Van Zyl, 2007). A lack of molecular data that could explain these observed effects therefore stimulated the initiation of this study. Although we have previously analysed the effects of BC204 in *Arabidopsis thaliana* using an RNA-seq-based approach, it is important to also study the effects of this PB on a crop species such as *S. lycopersicum*, due to the major differences between the two different plant models. *S. lycopersicum* is a model crop for fruit-bearing plants (Kimura and Sinha, 2008), but also an economically important crop plant, whereas *A. thaliana* is only used for research purposes. Although both are dicotyledonous plants, *A. thaliana* is also a non-mycotrophic plant, while tomato plants are mycotrophic and known for their close beneficial relationships with various species of arbuscular mychorrhizal fungi (Chitarra

et al., 2016) and beneficial bacteria (Harman and Uphoff, 2019). *S. lycopersicum* has been used with great success in transcriptomic studies (Chang et al., 2016) and has also been suggested as a model plant to be used for the discovery of new PBs (Povero et al., 2016). Similarly to *A. thaliana*, a rich bed of genomic data is also available for *S. lycopersicum*, further enhancing its value as a model species.

The effect of PBs has been widely studied in several tomato cultivars. PBs have been shown to increase overall plant growth in leaves and roots (Ali et al., 2019; Bulgari et al., 2019; Drobek et al., 2019; Kavipriya and Boominathan, 2018; Kim et al., 2019), tomato fruit yield (Saraswathi and Praneetha, 2013; Zodape et al., 2011) and fruit quality (Castro et al., 1988; Chehade et al., 2018; Grabowska et al., 2012) and to alter flowering patterns (Polo and Mata, 2018). PBs have been also shown to aid and possibly prime tomato plants (Hayat et al., 2018) to mitigate certain environmental stresses such salt stress (Arroussi et al., 2018), drought stress (Goñi et al., 2018; Paul et al., 2019; Petrozza et al., 2014), nutrient stress (Sestili et al., 2018) and biotic stress cause by several pathogens (Agarwal et al., 2016; Disciglio et al., 2016).

Molecular characterisation of PBs on *S. lycopersicum* has been reported, but not as extensively as their effects on basic physiology and biochemical changes. Since molecular characterisation of PBs on plant metabolism is important to elucidate the mechanisms which they induce, several studies used gene expression in efforts to gain a deeper understanding of how the various PBs improve plant growth. The determination that *Ascophyllum nodosum* extracts applied as a PB increased drought tolerance in tomato plants was investigated by using RT-qPCR analysis in conjunction with several other basic physiological measurements (Goñi et al., 2018). The effect of Megafol® on the expression of drought-responsive genes in tomato *S. lycopersicum* was also analysed using RT-qPCR (Petrozza et al., 2014). RT-qPCR is a useful tool to study changes in gene expression but is somewhat limited since only a small number of genes can be investigated at once. Therefore, transcriptomic approaches are preferable since they can provide a holistic overview of almost all genes. In a microarray study it was shown that a PB known as EXPANDO® altered the expression of genes involved in transcription, signal transduction, stress responses, carbohydrate metabolism, protein metabolism, transport and secondary metabolism in *S. lycopersicum* (Contartese et al., 2016). Another microarray study revealed that *Alfalfa*-based protein hydrolysates triggered a signal transduction pathway by modulating intracellular levels of ethylene, abscisic acid and jasmonic acid. The genes induced by this PB suggest that both kinases and transcription factors are involved in complex crosstalk between abiotic and biotic signalling pathways (Ertani et al., 2017).

Next-generation sequencing, such a mRNA sequencing (RNA-seq), is an alternative transcriptomic approach with several additional benefits compared to microarray analysis. Unlike with microarray analysis, RNA-seq uses deep-sequencing technologies and does not require species or transcript-specific probes (Wang et al., 2009). Additionally, RNA-seq provides the full transcriptome sequence which can be used to generate a more complete mechanistic understanding of the changes in gene expression (Rao et al., 2019). More comparisons between microarrays and RNA-seq have been reviewed elsewhere (Wang et al., 2009). In one such RNA-seq study, gelatin altered the expression of 620 genes in cucumber seedlings. These genes code for transcription factors, transporter genes and S-transferases (Wilson et al., 2015). In another RNA-seq study, a PB known as APR® elicited a total of 1006 differentially expressed genes involved in stress responses in the lateral roots of maize seedlings (Trevisan et al., 2017). RNA-seq has been used

successfully to study a variety of metabolic processes and responses to environmental stress in *S. lycopersicum* (Zhou et al., 2019), However, except for the effect of arbuscular mycorrhizal fungi on the tomato fruit tissue transcriptome (Zouari et al., 2014), no other peer-reviewed report has been published utilizing this method to characterize the possible mechanism of a PB compound/extract at the molecular level in tomatoes.

With regards to the production, marketing and broad usage of PBs, regulatory bodies in the European Union recently implemented regulations forcing companies to provide evidence for any claims that are made (Ricci et al., 2019). Although Europe is currently the largest market for PBs and has the best-defined regulations to date, countries on other continents are expected to follow suit in terms of such regulations. Many scientists also consider PB research to be lacking in robust, peer-reviewed scientific evaluation (Calvo et al., 2014). Due to the absence of any data for BC204 that could reveal its mode of action, this study aimed to reveal the effects of BC204 at the molecular level. We adopted a next generation sequencing (RNA-seq) approach in *S. lycopersicum* shoot tissue to reveal the effects of BC204 on gene expression after three weeks of foliar spray treatment with 0.05% (v/v) BC204. We propose that BC204 induces a large shift in gene expression towards signalling, stress, secondary metabolism and hormone metabolism, which leads to an increase in plant growth. The number of genes coding for secondary metabolism elements were more or less evenly distributed in terms of up- and down-regulation. A likely explanation would be that enhanced availability of photosynthates increases the energy imported into the plant and reallocates the carbon towards energy expensive processes including secondary and hormone metabolism. This is purely speculative and needs to be confirmed with extensive photosynthetic rate measurements. The results of this study provide a solid platform that can be used to further investigate specific biochemical pathways in order to confirm the effects seen at the molecular level. It also highlights the importance of using next-generation sequencing as an important analytic tool to characterise the effect of PBs on plant growth.

4.3 Materials and Methods

4.3.1 Plant Material and growth conditions

Solanum lycopersicum (cv. Moneymaker) seeds were surface sterilized in 1% (v/v) sodium hypochlorite, containing one drop of Tween20 per 100 mL solution, for 20 min (Schwarz et al., 2014). The seeds were then washed three times with sterile ddH_20. Rockwool was saturated with water and the pH adjusted with HCl to 5.8 and then packed into the plant support tubes. Four nutrient tanks (215 mm x 385 mm x 290 mm) with 8 wells per growth tray were utilised per treatment. Tanks were continuously oxygenated using an aquarium pump, with one airstone per nutrient container. Several seeds were sown onto the rockwool in each of the plant support tubes, which were then placed into the wells of each container and allowed to germinate in water. The growth tanks were placed in a greenhouse with a combination of natural lighting and high-pressure sodium lights simulating a 14 h:10 h light:dark period. Seven days after germination the containers were emptied and filled with ¼ strength Hoagland solution (Hoagland and Arnon, 1950). After the second week ½ strength Hoagland solution was used, which was replaced weekly with fresh solution. Treatment was initiated 3 weeks after germination. Control plants were sprayed with 10 mL water and treated plants with 10 mL 0.05% (v/v) BC204 once per week for three weeks. All experiments were conducted using the same batch of BC204 extract. All data was collected 90 min after a fourth and final treatment, which was applied 5 h after the start of the light period at the end of the third week. Root and shoot length and stem width were manually measured. Plants were photographed with a Canon E0S 550D camera. Shoot and root fresh weights were determined, and samples individually dried at 70°C for 2 d before determining dry weights.

4.3.2 Data and statistical analysis

All physiological experiments were independently replicated at least three times to ensure reproducibility. Statistical significance between control and treated groups was determined by the one-way ANOVA function in Microsoft Excel, followed by the Fischer's least significant difference (LSD) test at the 0.05 probability level.

4.3.3 RNA extractions for RNA-seq and Quantitative RT-PCR

Solanum lycopersicum leaf tissue was harvested 90 min after the fourth BC204 treatment, 3 weeks after the first treatment. Total RNA was extracted for 3 individual samples, each containing homogenised leaf tissue from 3 plants (n=9), per treatment. The frozen tissue was ground in liquid nitrogen using a pre-chilled mortar and pestle and total RNA extracted in a Maxwell® 16 AS2000 Instrument with the Maxwell® 16 Total RNA Purification Kit, as per the manufacturer's protocol. RNA integrity was determined using an Agilent 2100 Bioanalyzer at The Central Analytical Facility at Stellenbosch University, with only RNA samples with RIN scores of 9 or above being used for library construction.

4.3.4 Library preparation and Illumina sequencing

Library preparation and sequencing were performed at the Agricultural Research Council Biotechnology Platform (South Africa). Sample preparation was conducted using 1 µg of RNA, quantified using an Invitrogen Qubit fluorometer. Library preparation utilised the Illumina TruSeq Stranded mRNA library Kit which preferentially amplifies polyA RNA, according to the manufacturer's instructions. The quality

of the constructed libraries was confirmed using a PerkinElmer LabChip® GX system. The libraries were then sequenced on the Illumina HiSeq 2500 Platform using the version 4 sequencing chemistry, which resulted in the generation of 10 million paired end reads of 125 nucleotides (nt) in length.

4.3.5 Processing and Mapping of sequencing reads

Raw sequencing reads were processed by removing adaptor sequences. Then, low-quality bases at the read ends were trimmed (20 Phred score over a 3 nt window, minimum read length 20 nt) using Trimmomatic v. 0.33. The Tuxedo software suite v.2.2 (Bowtie, TopHat, Cufflinks, Cuffdiff; Trapnell et al. 2012) was used to compare samples and calculate differential expression. Trimmed sequencing reads were aligned against the wild type *Solanum lycopersicum* (S_lycopersicum_chromosomes.2.31.fa.gz) genome and gene expression was quantified as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Differential expression was calculated based on Cuffdiff statistical tests of three replicates of treated relative to untreated samples, using a statistical significance of q (adjusted P value) < 0.05.

4.3.6 Gene Ontology (GO) and gene enrichment analysis

Several online software platforms were used to obtain a visual representation of the differentially expressed genes. Sol Genomics Network (https://solgenomics.net; 1 November 2019) was consulted for gene descriptions. The agriGO v.2 (https://systemsbiology.cau.edu.cn/agriGOv2/) analysis tool Singular Enrichment Analysis (SEA) was used for gene ontology enrichment using tomato transcriptome (ITAG3.2 version) as background. The online accessible Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System Version 14.1 (www.pantherdb.org; Thomas et al., 2003) was also used to identify transcript function and UniProt accession identifiers.

4.3.7 Quantitative RT-qPCR analysis

One microgram of total RNA was used to obtain complementary DNA (cDNA) *via* reverse transcription using at oligo(dT)$_{18}$ primer and RevertAid reverse transcriptase (Thermo Scientific™, United States), according to manufacturer's protocol. The PowerUp™ SYBR™ Green Master Mix kit and the QuantStudio 3 Real-Time PCR System was used for quantitative RT-qPCR analysis and the relative expression calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Ribosomal protein L2 (*Solyc10g006580*) was used for as an internal control as it has previously been shown to be a suitable reference gene for *S. lycopersicum* (Harel et al., 2014) and its expression in the current study was also stable for each sample. For each sample, 1 µL of cDNA and 0.8 µL of each primer (10 µM, Supplementary Table S4.1) was added to the PowerUp™SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) and each reaction mixture transferred to a 0.1 mL, MicroAmp™, optical 96-well clear reaction plate (Applied Biosystems, USA). The reactions were performed by a Quantstudio™ 3 Real-Time PCR system (Thermo Fisher Scientific, USA). The incubations for the RT-qPCR reactions were as follows: 95°C for 10 min to activate the Dual-Lock *Taq* DNA polymerase, followed by 40 cycles of 95°C for 15 s (Denaturation) and 60°C for 1 min (Annealing and Extension).

4.4 Results

4.4.1 Growth response of Solanum lycopersicum *following prolonged BC204 treatment in a hydroponic growth system*

To determine the physiological effects of BC204 treatment, *S. lycopersicum* seedlings were cultivated in a custom-built hydroponic system for three weeks before being treated with BC204. The control group were sprayed with 10 mL water and the treated group sprayed with 10 mL 0.05% (v/v) BC204 once weekly for three weeks. Care was taken to only spray the foliage and a plastic film was used to prevent any of the solution flowing into the hydroponic solution. BC204-sprayed plants had visibly larger root and shoot systems (Figure 4.1). An increase in shoot and root fresh and dry weights was recorded (Figure 4.2 A-D), as well as an increase in shoot and root length (Figure 4.2E, F). Stem width (Figure 4.2G) also increased at the base of the topmost and lowermost leaves for the BC204-treated plants.

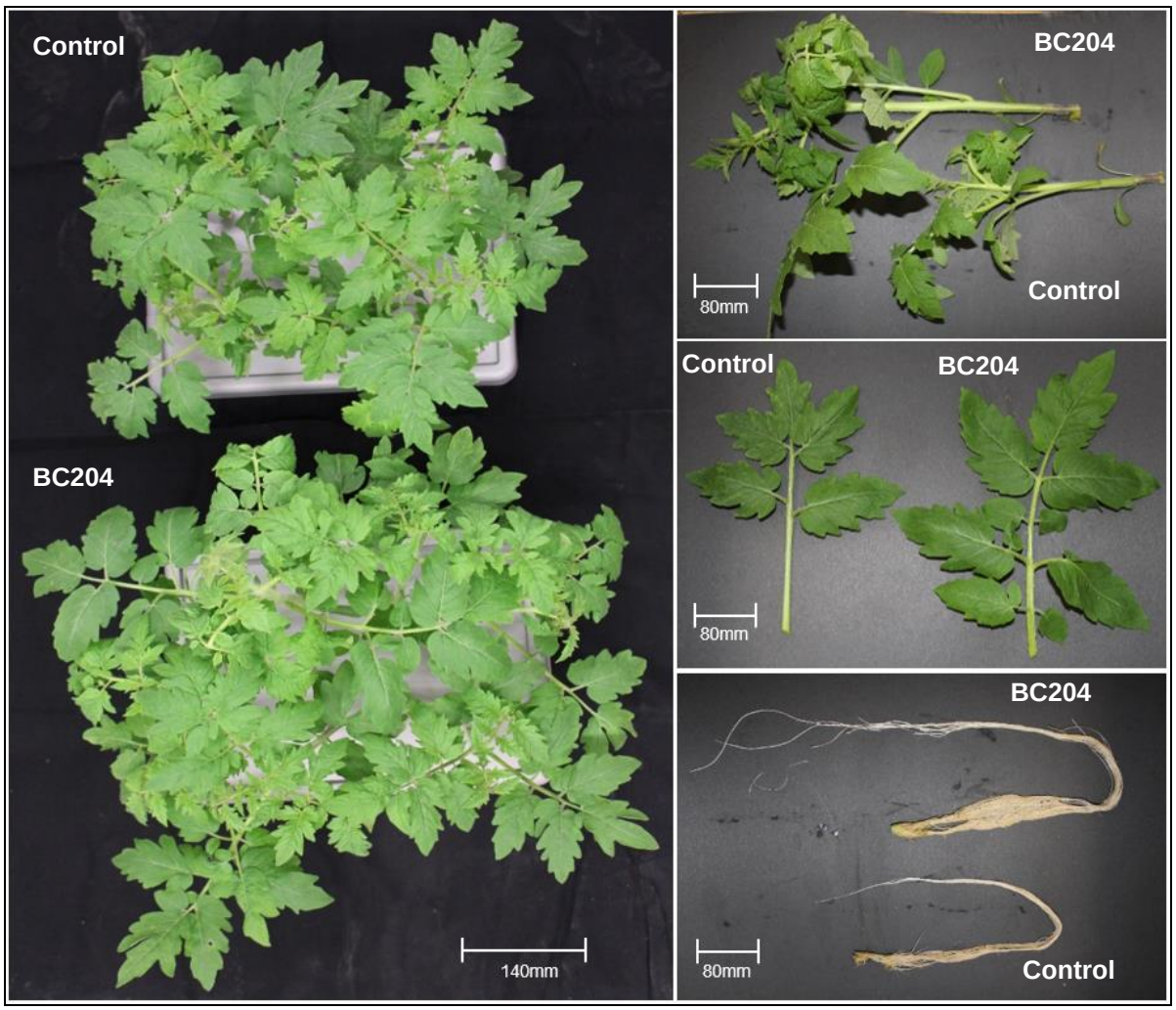

Figure 4.1 Root and shoot biomass production of *Solanum lycopersicum* plants sprayed with 0.05% (v/v) BC204. BC204-treated plants were visibly larger than the control plants at both shoot and root level.

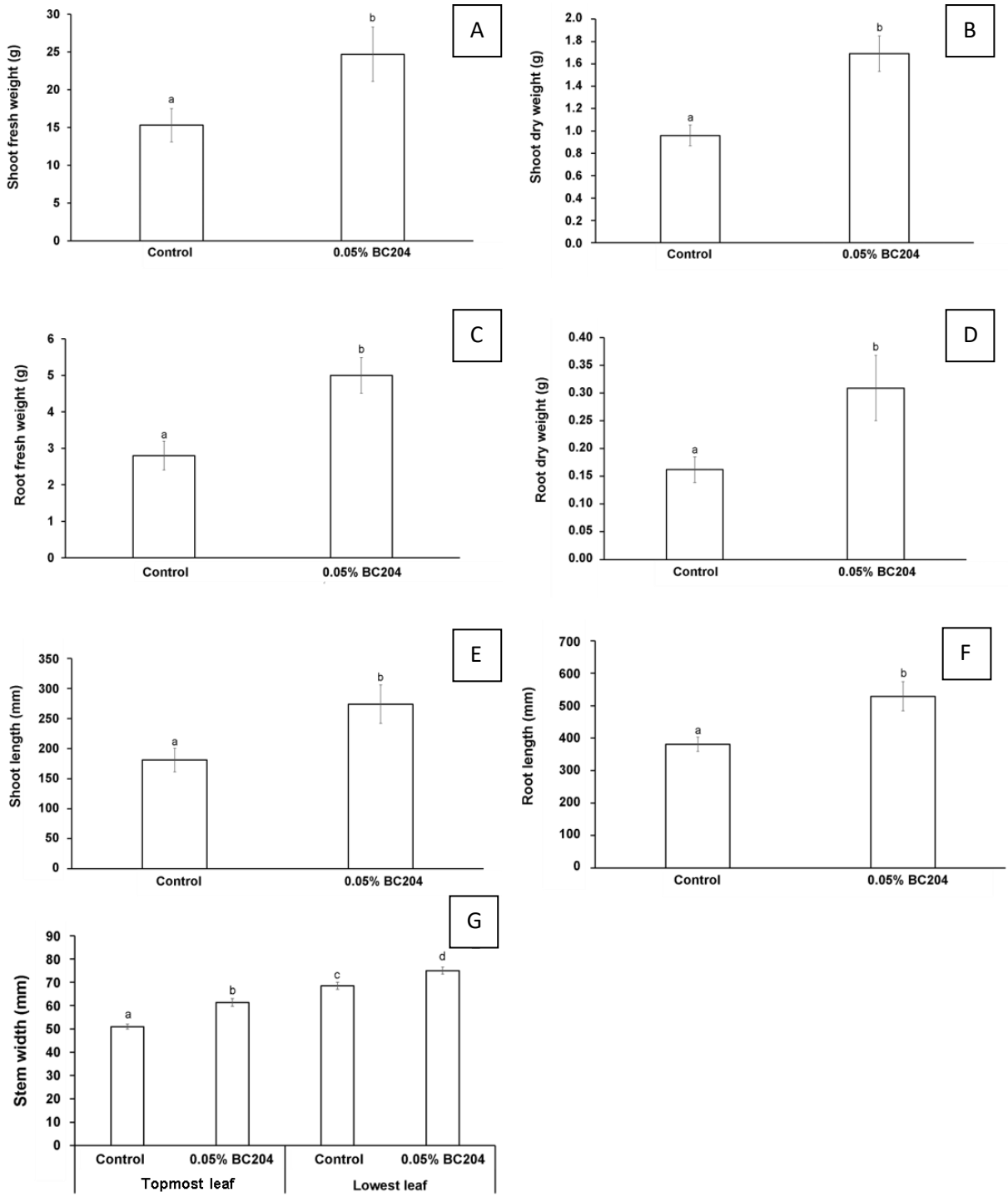

Figure 4.2 Fresh and dry weight biomass of shoot and root tissue, shoot length, root length and stem width of hydroponically grown *Solanum lycopersicum* plants treated with BC204. BC204 treatment (0.05% [v/v]) increased shoot fresh (**A**) and dry (**B**) weight, root fresh (**C**) and dry weight (**D**), shoot length (**E**), root length (**F**) and stem width at both the base of the topmost and lowermost leaf (**G**). Bars represent the mean of 16 (n=16) replicates ± standard error. Different letters indicate values that were determined by one-way ANOVA with Fisher's LSD *post-hoc* test to be significantly different (P<0.05) from the control.

4.4.2 Transcriptome analysis of untreated and BC204-treated Solanum lycopersicum *shoot tissue samples*

Illumina RNA sequencing of the 6 pooled samples (two different growth conditions and three biological replicates, with three plants pooled into each replicate) generated a total of 45.241187 million reads, translating to a mean of 7.540198 million reads per sample. Trimming the data reduced this to a total of 41.368357 million reads, translating to a mean of 6.894726 million reads per sample. Mapping of the 41.368357 million reads against the reference genome (S_lycopersicum_chromosomes.2.31.fa.gz) indicated that 98% of reads were mapped successfully.

4.4.3 Differentially expressed genes in Solanum lycopersicum *shoot tissue following BC204 foliar spray treatment*

Out of the 33308 transcripts analysed, a total of 6015 (18.059%) significantly differentially expressed genes (DEGs) were obtained between the treatment and control groups, of which 2923 (8.776%) were upregulated and 3092 (9.283%) downregulated (Table 4.1). Most upregulated genes, 2263, had a $\log_2$fold value ranging between 0 and 1. A total of 829 genes were upregulated by a log2fold value of at least 1, 130 by at least 2, 33 by at least 3 and 13 genes were upregulated by a $\log_2$fold value of at least 4. Most downregulated genes, 2923, also had a negative $\log_2$fold value ranging between 0 and 1. A total of 169 genes were downregulated by a log2fold value of at least -1, 9 genes by a value of at least -2, and two genes were downregulated by a $\log_2$value of -4. The infinite (inf) value calculated from the analysis was changed to a value of a 100 in order to ease further analysis. Gene descriptions were obtained from the Sol Genomics Network database (https://solgenomics.net/search/locus; 1 November 2019). Of the ten most upregulated genes, 7 coded for unknown proteins. Of the ten most downregulated genes, 3 coded for unknown proteins. A complete list of genes that were upregulated (Table S4.2) and downregulated (Table S4.3) by a $\log_2$fold value of at least 1 can be found in the *Supplementary Material* section of this chapter. Many of the transcripts in these lists also code for unknown and uncategorised proteins.

The 2923 significantly upregulated genes were subjected to AgriGO v2.0 Singular Enrichment Analysis (SEA) GO annotation (http://systemsbiology.cau.edu.cn/agriGOv2/index.php) using *S. lycopersicum* ITAG3.2 version as backround reference, which recognised 2096 annotated genes. A total of 55 were significant GO terms, categorised into either Biological Process (P), Molecular Function (F) or Cellular Component (C) (Table 4.4). The 3092 significantly downregulated genes were also subjected to SEA analysis and a total of 203 GO terms were obtained and categorised the same way (Table 4.5).

Table 4.1 Differentially expressed genes (DEG) significantly altered by 0.05% (v/v) BC204 foliar spray treatment compared to the control in *Solanum lycopersicum* shoot tissue

	Number of DEG	Number of upregulated genes	Number of downregulated genes
All	6015	2923	3092
Filtered (<1 log_2fold)	829	660	169
Filtered (<2 log_2fold)	130	121	9
Filtered (<3 log_2fold)	35	33	2
Filtered (<4 log_2fold)	15	13	2

Table 4.2 Ten most upregulated genes (based on log_2fold values) elicited by 0.05% (v/v) BC204 treatment in *Solanum lycopersicum* shoot tissue

Gene ID (ITAG2.3)	Gene descriptions Sol Genomics Network	Log_2ratio
Solyc01g106630	Unknown Protein (AHRD V1)	4.39178
Solyc00g026160	Ferric reductase oxidase (AHRD V1 **** D6RVS5_HORVU); contains Interpro domain(s) IPR013121 Ferric reductase, NAD binding	4.23484
Solyc10g076190	Peroxidase 1 (AHRD V1 ***- A0SWU6_SESRO); contains Interpro domain(s) IPR002016 Haem peroxidase, plant/fungal/bacterial	4.13349
Solyc09g005000	Receptor like protein kinase (AHRD V1 **** Q39139_ARATH); contains Interpro domain(s) IPR001220 Legume lectin, beta chain	4.07042
Solyc03g116690	Blue copper protein (AHRD V1 **-- D1MWY8_CITLA); contains Interpro domain(s) IPR003245 Plastocyanin-like	4.0455
Solyc08g079900	Subtilisin-like protease (AHRD V1 **-- Q9LWA4_SOLLC); contains Interpro domain(s) IPR015500 Peptidase S8, subtilisin-related	3.63573
Solyc01g009810	LRR receptor-like serine/threonine-protein kinase, RLP	3.61381
Solyc10g081970	HIN1-like protein (Fragment) (AHRD V1 **-- Q32ZJ1_SOLTU); contains Interpro domain(s) IPR010847 Harpin-induced 1	3.53659
Solyc07g040960	Os07g0175100 protein (Fragment) (AHRD V1 *--- Q0D898_ORYSJ)	3.39393
Solyc08g068680	Decarboxylase family protein (AHRD V1 ***- B1ILJ6_CLOBK); contains Interpro domain(s) IPR002129 Pyridoxal phosphate-dependent decarboxylase	3.29521

Table 4.3 Eight most downregulated genes (based on log_2fold values) elicited by 0.05% (v/v) BC204 treatment (0.05% [v/v]). in *Solanum lycopersicum* shoot tissue

Gene ID	Gene descriptions Sol Genomics Network	Log_2ratio
Solyc09g089520.3	Proteinase inhibitor I (AHRD V1 ***- Q3S492_SOLTU); contains Interpro domain(s) IPR000864 Proteinase inhibitor I13, potato inhibitor I	-2.65401
Solyc05g018850.1	Unknown Protein (AHRD V1)	-2.30756
Solyc05g005100.3	Os06g0207500 protein (Fragment) (AHRD V1 ***- Q0DDQ9_ORYSJ); contains Interpro domain(s) IPR004253 Protein of unknown function DUF231, plant	-2.26547
Solyc09g089540.3	Proteinase inhibitor I (AHRD V1 ***- Q3S492_SOLTU); contains Interpro domain(s) IPR000864 Proteinase inhibitor I13, potato inhibitor I	-2.17884
Solyc00g071180.3	Cysteine proteinase inhibitor (AHRD V1 *-** Q2VY67_9ERIC); contains Interpro domain(s) IPR000010 Proteinase inhibitor I25, cystatin	-2.13305
Solyc12g033060.1	Photosystem I P700 chlorophyll a apoprotein A2 (AHRD V1 ***- A7Y3D1_IPOPU); contains Interpro domain(s) IPR001280 Photosystem I psaA and psaB	-2.07468
Solyc01g058100.2	Unknown Protein (AHRD V1)	-2.0276
Solyc04g058010.3	Unknown Protein (AHRD V1)	-1.9946

Table 4.4 Singular Enrichment Analysis (SEA) from agriGO v.2 for all genes upregulated in *Solanum lycopersicum* shoots by BC204 treatment. Genes were categorized into either Biological Process (P) or Molecular Function (F).

GO term	Ontology	Description	Number of genes involved in the specific metabolic process		p-value	False Discovery Rate (FDR)
			In input list	In BG/Ref list		
GO:0006468	P	protein phosphorylation	237	1270	9.70E-16	2.30E-12
GO:0006464	P	cellular protein modification process	301	1773	5.30E-15	4.20E-12
GO:0036211	P	protein modification process	301	1773	5.30E-15	4.20E-12
GO:0006796	P	phosphate-containing compound metabolic process	302	1800	1.80E-14	1.10E-11
GO:0006793	P	phosphorus metabolic process	302	1804	2.30E-14	1.10E-11
GO:0016310	P	Phosphorylation	248	1403	4.30E-14	1.70E-11
GO:0043412	P	macromolecule modification	301	1850	5.60E-13	1.90E-10
GO:0008152	P	metabolic process	1072	9091	2.70E-07	7.90E-05
GO:0019538	P	protein metabolic process	391	3004	8.70E-06	0.0023
GO:0006629	P	lipid metabolic process	82	460	1.40E-05	0.0033
GO:1901565	P	organonitrogen compound catabolic process	23	71	1.60E-05	0.0036
GO:0071704	P	organic substance metabolic process	806	6832	4.10E-05	0.0081
GO:0009856	P	Pollination	21	68	6.70E-05	0.0094
GO:0008037	P	cell recognition	21	68	6.70E-05	0.0094
GO:0044706	P	multi-multicellular organism process	21	68	6.70E-05	0.0094
GO:0048544	P	recognition of pollen	21	68	6.70E-05	0.0094

GO:0009875	P	pollen-pistil interaction	21	68	6.70E-05	0.0094
GO:0044710	P	single-organism metabolic process	377	2968	8.80E-05	0.012
GO:0044703	P	multi-organism reproductive process	21	76	0.00025	0.03
GO:0044702	P	single organism reproductive process	22	82	0.00025	0.03
GO:0016567	P	protein ubiquitination	29	128	0.00035	0.04
GO:1901136	P	carbohydrate derivative catabolic process	13	35	0.00038	0.04
GO:0005975	P	carbohydrate metabolic process	114	768	0.00037	0.04
GO:0044267	P	cellular protein metabolic process	313	2470	0.00045	0.044
GO:0044238	P	primary metabolic process	751	6456	0.00047	0.045
GO:0016773	F	phosphotransferase activity, alcohol group as acceptor	260	1352	1.10E-18	6.20E-16
GO:0016301	F	kinase activity	270	1415	5.40E-19	6.20E-16
GO:0003824	F	catalytic activity	1098	8492	1.30E-18	6.20E-16
GO:0004672	F	protein kinase activity	238	1233	2.80E-17	1.00E-14
GO:0016772	F	transferase activity, transferring phosphorus-containing groups	281	1612	3.00E-15	8.60E-13
GO:0016740	F	transferase activity	447	3098	1.10E-11	2.50E-09
GO:0005524	F	ATP binding	318	2203	2.10E-08	4.30E-06
GO:0032559	F	adenyl ribonucleotide binding	342	2462	1.90E-07	3.40E-05
GO:0030554	F	adenyl nucleotide binding	342	2467	2.30E-07	3.60E-05

GO:0035639	F	purine ribonucleoside triphosphate binding	325	2451	1.60E-05	0.0023
GO:0005509	F	calcium ion binding	53	263	2.90E-05	0.0037
GO:0004674	F	protein serine/threonine kinase activity	27	97	3.10E-05	0.0037
GO:0017076	F	purine nucleotide binding	351	2720	5.30E-05	0.0058
GO:0032555	F	purine ribonucleotide binding	349	2710	6.50E-05	0.0058
GO:0032550	F	purine ribonucleoside binding	349	2710	6.50E-05	0.0058
GO:0001883	F	purine nucleoside binding	349	2710	6.50E-05	0.0058
GO:0001882	F	nucleoside binding	349	2727	0.0001	0.0081
GO:0032549	F	ribonucleoside binding	349	2726	9.90E-05	0.0081
GO:0032553	F	ribonucleotide binding	352	2757	0.00011	0.0082
GO:0097367	F	carbohydrate derivative binding	357	2802	0.00011	0.0082
GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds	68	394	0.00017	0.012
GO:0042578	F	phosphoric ester hydrolase activity	49	261	0.00025	0.016
GO:0016798	F	hydrolase activity, acting on glycosyl bonds	69	416	0.00041	0.026
GO:0004190	F	aspartic-type endopeptidase activity	27	119	0.00054	0.031
GO:0003700	F	transcription factor activity, sequence-specific DNA binding	88	570	0.00058	0.031
GO:0070001	F	aspartic-type peptidase	27	119	0.00054	0.031

		activity				
GO:0001071	F	nucleic acid binding transcription factor activity	88	570	0.00058	0.031
GO:0004568	F	chitinase activity	10	23	0.00068	0.035
GO:0005216	F	ion channel activity	19	74	0.001	0.048
GO:0022838	F	substrate-specific channel activity	19	74	0.001	0.048

Table 4.5 Singular Enrichment Analysis (SEA) from AgriGO v.2 for all genes downregulated in *Solanum lycopersicum* shoot tissue by BC204 treatment. Genes were categorized into either Biological Process (P), Molecular Function (F) or Cellular Component (C).

GO term	Ontology	Description	Number of genes involved in the specific metabolic process		p-value	FDR
			In input list	In BG/Ref list		
GO:1901566	P	organonitrogen compound biosynthetic process	416	920	5.10E-118	1.50E-114
GO:1901564	P	organonitrogen compound metabolic process	456	1240	1.70E-104	2.50E-101
GO:0006412	P	Translation	316	580	3.00E-104	3.00E-101
GO:0043043	P	peptide biosynthetic process	316	583	8.50E-104	6.50E-101
GO:0043604	P	amide biosynthetic process	316	584	1.20E-103	7.30E-101
GO:0006518	P	peptide metabolic process	317	596	1.70E-102	8.80E-100
GO:0043603	P	cellular amide metabolic process	317	604	2.60E-101	1.10E-98
GO:0006807	P	nitrogen compound metabolic process	686	3302	2.80E-65	1.10E-62
GO:0034641	P	cellular nitrogen compound metabolic process	638	3021	4.90E-62	1.60E-59
GO:0044249	P	cellular biosynthetic process	561	2639	5.00E-54	1.50E-51
GO:1901576	P	organic substance biosynthetic process	558	2637	4.00E-53	1.10E-50
GO:0010467	P	gene expression	494	2216	8.00E-52	2.00E-49
GO:0044271	P	cellular nitrogen compound biosynthetic process	469	2055	2.40E-51	5.50E-49

GO:0009058	P	biosynthetic process	572	2777	2.50E-51	5.50E-49
GO:0009059	P	macromolecule biosynthetic process	420	2034	2.30E-36	4.40E-34
GO:0034645	P	cellular macromolecule biosynthetic process	420	2034	2.30E-36	4.40E-34
GO:0044237	P	cellular metabolic process	868	6041	1.00E-24	1.80E-22
GO:0009987	P	cellular process	1008	7332	6.00E-24	1.00E-21
GO:0044267	P	cellular protein metabolic process	423	2470	1.30E-21	2.10E-19
GO:0071704	P	organic substance metabolic process	921	6832	1.90E-18	2.80E-16
GO:0022613	P	ribonucleoprotein complex biogenesis	49	81	4.00E-18	5.80E-16
GO:0044260	P	cellular macromolecule metabolic process	648	4501	4.20E-17	5.80E-15
GO:0019538	P	protein metabolic process	467	3004	8.30E-17	1.10E-14
GO:0034660	P	ncRNA metabolic process	72	193	9.90E-17	1.20E-14
GO:0044238	P	primary metabolic process	861	6456	1.30E-15	1.60E-13
GO:0042254	P	ribosome biogenesis	39	63	5.90E-15	6.80E-13
GO:0044281	P	small molecule metabolic process	186	930	9.80E-15	1.10E-12
GO:0043170	P	macromolecule metabolic process	693	5077	9.90E-14	1.10E-11
GO:0006396	P	RNA processing	95	360	1.30E-13	1.30E-11
GO:0006520	P	cellular amino acid metabolic process	66	210	1.30E-12	1.30E-10

GO:0009451	P	RNA modification	34	67	2.10E-11	2.00E-09
GO:0016072	P	rRNA metabolic process	25	36	7.30E-11	6.90E-09
GO:0006418	P	tRNA aminoacylation for protein translation	29	52	1.10E-10	9.80E-09
GO:0043038	P	amino acid activation	29	52	1.10E-10	9.80E-09
GO:0043039	P	tRNA aminoacylation	29	52	1.10E-10	9.80E-09
GO:0006364	P	rRNA processing	24	35	2.10E-10	1.80E-08
GO:0006457	P	protein folding	48	143	2.40E-10	2.00E-08
GO:0044711	P	single-organism biosynthetic process	126	632	2.90E-10	2.30E-08
GO:0006399	P	tRNA metabolic process	50	156	3.90E-10	3.00E-08
GO:0044085	P	cellular component biogenesis	79	337	1.70E-09	1.30E-07
GO:0006082	P	organic acid metabolic process	112	559	2.30E-09	1.70E-07
GO:0019752	P	carboxylic acid metabolic process	99	475	3.50E-09	2.50E-07
GO:0043436	P	oxoacid metabolic process	99	478	4.60E-09	3.30E-07
GO:0006414	P	translational elongation	21	32	5.00E-09	3.40E-07
GO:0034470	P	ncRNA processing	44	142	9.90E-09	6.70E-07
GO:0044283	P	small molecule biosynthetic process	64	260	1.20E-08	8.20E-07
GO:0008152	P	metabolic process	1079	9091	1.50E-07	9.70E-06
GO:0046483	P	heterocycle metabolic process	344	2475	2.50E-07	1.50E-05
GO:1901360	P	organic cyclic compound	349	2539	5.20E-07	3.20E-05

		metabolic process				
GO:0055086	P	nucleobase-containing small molecule metabolic process	62	281	5.70E-07	3.50E-05
GO:0016053	P	organic acid biosynthetic process	51	213	7.40E-07	4.40E-05
GO:0008380	P	RNA splicing	24	62	9.50E-07	5.50E-05
GO:0000375	P	RNA splicing, via transesterification reactions	22	55	1.70E-06	9.70E-05
GO:0000377	P	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	22	55	1.70E-06	9.70E-05
GO:0006725	P	cellular aromatic compound metabolic process	339	2503	2.70E-06	0.00015
GO:0000398	P	mRNA splicing, via spliceosome	21	54	4.20E-06	0.00023
GO:0008652	P	cellular amino acid biosynthetic process	23	66	6.60E-06	0.00035
GO:0046394	P	carboxylic acid biosynthetic process	39	162	1.30E-05	0.00067
GO:0071840	P	cellular component organization or biogenesis	127	798	1.40E-05	0.0007
GO:0001522	P	pseudouridine synthesis	13	24	2.00E-05	0.001
GO:0006139	P	nucleobase-containing compound metabolic process	315	2364	2.10E-05	0.0011
GO:0006753	P	nucleoside phosphate metabolic process	51	248	2.90E-05	0.0014

GO:0072521	P	purine-containing compound metabolic process	44	203	3.40E-05	0.0016
GO:0009066	P	aspartate family amino acid metabolic process	13	26	3.80E-05	0.0018
GO:0006397	P	mRNA processing	27	98	3.90E-05	0.0018
GO:0072522	P	purine-containing compound biosynthetic process	25	87	4.10E-05	0.0019
GO:0009165	P	nucleotide biosynthetic process	30	117	4.60E-05	0.0021
GO:1901293	P	nucleoside phosphate biosynthetic process	30	117	4.60E-05	0.0021
GO:0009117	P	nucleotide metabolic process	50	247	4.80E-05	0.0021
GO:0000387	P	spliceosomal snRNP assembly	10	15	5.10E-05	0.0022
GO:0009067	P	aspartate family amino acid biosynthetic process	12	23	5.50E-05	0.0023
GO:0009085	P	lysine biosynthetic process	8	10	0.00012	0.005
GO:0006553	P	lysine metabolic process	8	10	0.00012	0.005
GO:1901605	P	alpha-amino acid metabolic process	30	125	0.00013	0.0052
GO:0000154	P	rRNA modification	7	7	0.00013	0.0053
GO:0006400	P	tRNA modification	12	26	0.00014	0.0054
GO:0016071	P	mRNA metabolic process	32	141	0.00019	0.0074
GO:0033014	P	tetrapyrrole biosynthetic process	13	32	0.0002	0.0077
GO:0046112	P	nucleobase biosynthetic	9	15	0.00022	0.0083

		process				
GO:0006413	P	translational initiation	13	33	0.00025	0.0093
GO:0009116	P	nucleoside metabolic process	41	204	0.00025	0.0093
GO:1901657	P	glycosyl compound metabolic process	41	204	0.00025	0.0093
GO:0072527	P	pyrimidine-containing compound metabolic process	15	43	0.00026	0.0094
GO:0043933	P	macromolecular complex subunit organization	49	263	0.00032	0.011
GO:0072528	P	pyrimidine-containing compound biosynthetic process	14	39	0.00032	0.011
GO:0033013	P	tetrapyrrole metabolic process	14	39	0.00032	0.011
GO:0006164	P	purine nucleotide biosynthetic process	21	78	0.00035	0.012
GO:0022618	P	ribonucleoprotein complex assembly	11	25	0.00036	0.012
GO:1901607	P	alpha-amino acid biosynthetic process	21	78	0.00035	0.012
GO:0042364	P	water-soluble vitamin biosynthetic process	11	25	0.00036	0.012
GO:0009089	P	lysine biosynthetic process via diaminopimelate	7	9	0.00037	0.012
GO:0046451	P	diaminopimelate metabolic process	7	9	0.00037	0.012
GO:0006163	P	purine nucleotide metabolic process	38	188	0.00038	0.012

GO:0009112	P	nucleobase metabolic process	9	17	0.00043	0.014
GO:0009119	P	ribonucleoside metabolic process	37	183	0.00045	0.014
GO:0009110	P	vitamin biosynthetic process	11	26	0.00046	0.014
GO:0071826	P	ribonucleoprotein complex subunit organization	11	26	0.00046	0.014
GO:0009259	P	ribonucleotide metabolic process	37	185	0.00053	0.016
GO:0009081	P	branched-chain amino acid metabolic process	9	18	0.0006	0.018
GO:0006767	P	water-soluble vitamin metabolic process	11	27	0.0006	0.018
GO:0009082	P	branched-chain amino acid biosynthetic process	8	14	0.00062	0.019
GO:0019693	P	ribose phosphate metabolic process	39	201	0.00064	0.019
GO:0009141	P	nucleoside triphosphate metabolic process	33	161	0.00072	0.021
GO:0006766	P	vitamin metabolic process	11	28	0.00076	0.022
GO:0009260	P	ribonucleotide biosynthetic process	20	78	0.0008	0.023
GO:0046390	P	ribose phosphate biosynthetic process	20	78	0.0008	0.023
GO:0042026	P	protein refolding	7	11	0.00086	0.024
GO:0090407	P	organophosphate biosynthetic process	33	165	0.001	0.029
GO:0034622	P	cellular macromolecular	31	153	0.0012	0.034

		complex assembly				
GO:0065003	P	macromolecular complex assembly	33	167	0.0012	0.034
GO:0001510	P	RNA methylation	9	21	0.0014	0.038
GO:0051186	P	cofactor metabolic process	39	212	0.0015	0.041
GO:0009163	P	nucleoside biosynthetic process	18	71	0.0016	0.041
GO:0042455	P	ribonucleoside biosynthetic process	18	71	0.0016	0.041
GO:0042278	P	purine nucleoside metabolic process	34	177	0.0016	0.041
GO:1901659	P	glycosyl compound biosynthetic process	18	71	0.0016	0.041
GO:0046128	P	purine ribonucleoside metabolic process	34	177	0.0016	0.041
GO:0019856	P	pyrimidine nucleobase biosynthetic process	6	9	0.0017	0.043
GO:0009199	P	ribonucleoside triphosphate metabolic process	31	157	0.0017	0.043
GO:0016070	P	RNA metabolic process	223	1727	0.0017	0.043
GO:0009220	P	pyrimidine ribonucleotide biosynthetic process	7	13	0.0018	0.043
GO:0006206	P	pyrimidine nucleobase metabolic process	6	9	0.0017	0.043
GO:0046132	P	pyrimidine ribonucleoside biosynthetic process	7	13	0.0018	0.043
GO:0009218	P	pyrimidine ribonucleotide metabolic process	7	13	0.0018	0.043

GO:0046134	P	pyrimidine nucleoside biosynthetic process	7	13	0.0018	0.043
GO:0071103	P	DNA conformation change	17	66	0.0018	0.044
GO:0009150	P	purine ribonucleotide metabolic process	34	180	0.002	0.048
GO:0003735	F	structural constituent of ribosome	248	444	9.40E-83	1.20E-79
GO:0005198	F	structural molecule activity	255	499	6.30E-79	4.10E-76
GO:0003723	F	RNA binding	175	472	7.30E-39	3.20E-36
GO:0008135	F	translation factor activity, RNA binding	47	88	7.20E-16	2.40E-13
GO:0016874	F	ligase activity	54	160	1.60E-11	4.10E-09
GO:0004812	F	aminoacyl-tRNA ligase activity	29	52	1.10E-10	1.90E-08
GO:0016875	F	ligase activity, forming carbon-oxygen bonds	29	52	1.10E-10	1.90E-08
GO:0016876	F	ligase activity, forming aminoacyl-tRNA and related compounds	29	52	1.10E-10	1.90E-08
GO:0032561	F	guanyl ribonucleotide binding	66	250	6.90E-10	9.00E-08
GO:0005525	F	GTP binding	66	250	6.90E-10	9.00E-08
GO:0019001	F	guanyl nucleotide binding	66	255	1.40E-09	1.60E-07
GO:0003743	F	translation initiation factor activity	25	50	1.20E-08	1.30E-06
GO:0003746	F	translation elongation factor activity	18	26	3.40E-08	3.50E-06
GO:0008173	F	RNA methyltransferase	21	41	1.20E-07	1.20E-05

		activity				
GO:0003676	F	nucleic acid binding	423	3148	2.30E-07	2.10E-05
GO:0051082	F	unfolded protein binding	29	82	3.40E-07	2.80E-05
GO:0016741	F	transferase activity, transferring one-carbon groups	67	313	5.20E-07	4.00E-05
GO:0019843	F	rRNA binding	20	45	1.40E-06	0.0001
GO:0008168	F	methyltransferase activity	61	297	5.10E-06	0.00036
GO:0003924	F	GTPase activity	30	107	1.10E-05	0.00073
GO:0000049	F	tRNA binding	8	11	0.00019	0.012
GO:0009982	F	pseudouridine synthase activity	10	21	0.0004	0.024
GO:0008649	F	rRNA methyltransferase activity	6	7	0.0007	0.04
GO:0030529	C	intracellular ribonucleoprotein complex	286	527	1.30E-93	3.60E-91
GO:1990904	C	ribonucleoprotein complex	286	527	1.30E-93	3.60E-91
GO:0005737	C	Cytoplasm	421	1259	4.50E-85	8.40E-83
GO:0005840	C	Ribosome	251	446	2.70E-84	3.70E-82
GO:0043232	C	intracellular non-membrane-bounded organelle	311	764	1.10E-77	1.00E-75
GO:0043228	C	non-membrane-bounded organelle	311	764	1.10E-77	1.00E-75
GO:0032991	C	macromolecular complex	424	1429	1.30E-72	1.10E-70
GO:0005622	C	Intracellular	621	2728	1.00E-70	7.20E-69

GO:0044424	C	intracellular part	599	2587	4.00E-70	2.50E-68
GO:0044444	C	cytoplasmic part	342	1012	6.00E-69	3.40E-67
GO:0044464	C	cell part	632	2892	3.10E-66	1.50E-64
GO:0005623	C	Cell	632	2892	3.10E-66	1.50E-64
GO:0043229	C	intracellular organelle	456	1905	1.00E-54	4.00E-53
GO:0043226	C	Organelle	456	1905	1.00E-54	4.00E-53
GO:0044422	C	organelle part	174	735	8.10E-20	2.80E-18
GO:0044446	C	intracellular organelle part	174	735	8.10E-20	2.80E-18
GO:0044391	C	ribosomal subunit	42	65	1.80E-16	5.80E-15
GO:0015934	C	large ribosomal subunit	22	27	1.20E-10	3.60E-09
GO:0005739	C	Mitochondrion	38	99	8.80E-10	2.60E-08
GO:0044429	C	mitochondrial part	34	81	1.10E-09	3.00E-08
GO:0031967	C	organelle envelope	35	93	6.10E-09	1.60E-07
GO:0031975	C	Envelope	35	98	1.80E-08	4.40E-07
GO:0031966	C	mitochondrial membrane	29	69	1.70E-08	4.40E-07
GO:0005740	C	mitochondrial envelope	29	73	4.60E-08	1.10E-06
GO:0015935	C	small ribosomal subunit	20	38	1.80E-07	4.00E-06
GO:0098798	C	mitochondrial protein complex	17	32	1.30E-06	2.80E-05
GO:0032993	C	protein-DNA complex	32	105	1.30E-06	2.80E-05
GO:0005852	C	eukaryotic translation initiation factor 3 complex	14	22	2.40E-06	4.80E-05
GO:0019867	C	outer membrane	15	26	2.60E-06	5.00E-05
GO:0031090	C	organelle membrane	37	139	3.10E-06	5.80E-05

GO:0044455	C	mitochondrial membrane part	17	35	3.40E-06	6.20E-05
GO:0098800	C	inner mitochondrial membrane protein complex	15	27	3.70E-06	6.40E-05
GO:0000786	C	Nucleosome	30	101	4.30E-06	7.30E-05
GO:0044815	C	DNA packaging complex	30	103	5.90E-06	9.80E-05
GO:0019866	C	organelle inner membrane	19	47	7.70E-06	0.00012
GO:0005743	C	mitochondrial inner membrane	18	46	1.90E-05	0.00029
GO:0031968	C	organelle outer membrane	12	20	1.90E-05	0.0003
GO:0030684	C	Preribosome	9	10	2.40E-05	0.00034
GO:0000785	C	Chromatin	31	118	2.30E-05	0.00034
GO:0005694	C	Chromosome	40	174	2.50E-05	0.00035
GO:0043234	C	protein complex	137	901	4.40E-05	0.00061
GO:0044427	C	chromosomal part	35	156	0.00012	0.0016
GO:0005741	C	mitochondrial outer membrane	10	18	0.00016	0.002
GO:0032040	C	small-subunit processome	7	8	0.00022	0.0029
GO:0043231	C	intracellular membrane-bounded organelle	176	1271	0.00033	0.004
GO:0043227	C	membrane-bounded organelle	176	1271	0.00033	0.004
GO:0005681	C	spliceosomal complex	11	26	0.00046	0.0055
GO:0005730	C	Nucleolus	8	14	0.00062	0.0073

GO:0044428	C	nuclear part	43	232	0.00079	0.0091
GO:1990204	C	oxidoreductase complex	13	39	0.00092	0.01
GO:0005753	C	mitochondrial proton-transporting ATP synthase complex	7	14	0.0024	0.027
GO:0098803	C	respiratory chain complex	5	7	0.0035	0.037
GO:0070469	C	respiratory chain	6	11	0.0036	0.038

Mercator (Schwacke et al., 2019) was used to functionally group significantly up and downregulated genes in bins. Apart of the genes not assigned to a bin (29.71%), signalling (10.88 %), protein (10.67%, RNA metabolism (9.41%), stress (9.00%), miscellaneous (8.00 %) and hormone metabolism (5.65%) related genes were upregulated by BC204-treatment (Figure 4.4). The downregulated processes include not assigned (46.87%), protein (15.11%) and RNA metabolism (5.47%) (Figure 4.5). As an extension of this analysis, up and downregulated genes were assigned into categories by the Protein Analysis THrough Evolutionary Relationships (PANTHER) Classification System Version 14.1 (Thomas et al., 2003; www.pantherdb.org) based on cellular component, biological process, molecular function (Figure 4.6) and protein class (Figure 4.7). More than 400 genes were upregulated and downregulated in the cell and organelle (GO: cellular component), metabolic and cellular processes (GO: biological process) and catalytic activity and binding (GO: molecular function) categories. In the protein class category, hydrolases were almost equally up- and downregulated. A total of 440 genes in the nucleic acid binding category were downregulated and 60 upregulated. Genes associated with hydrolases, enzyme modulator, oxidoreductase, and transferase had both more than 100 upregulated and downregulated genes.

In addition to the Mercator, PANTHER and agriGO analysis, the Sol Genomics website was used to obtain information on all genes with a log_2fold value of larger than 2 and smaller than -2 (Table 4.6). A brief description on gene function and the predicted GO associated with the gene was obtained. The genes falling within this log2fold range code for a variety of proteins involved in many metabolic processes.

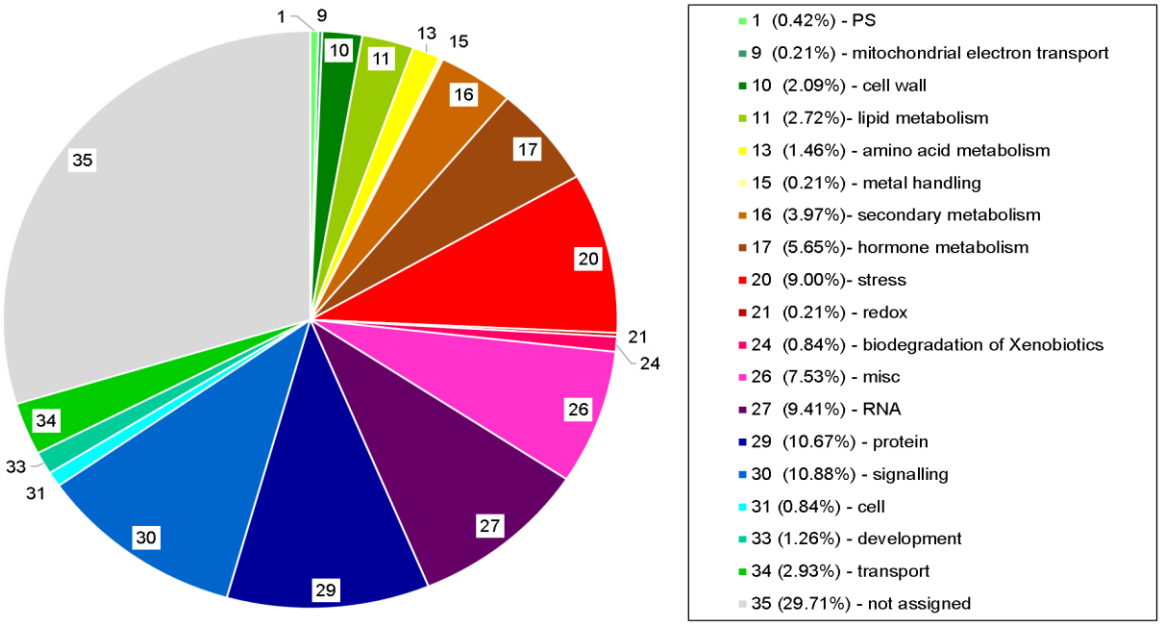

Figure 4.3 Functional biological classification by Mercator of significantly upregulated genes in *Solanum lycopersicum* shoot tissue treated with 0.05% (v/v) BC204 as a foliar spray. Upregulated genes were assigned by the Mercator functional annotation tool into different bins. The differentially expressed transcripts are indicated by percentiles.

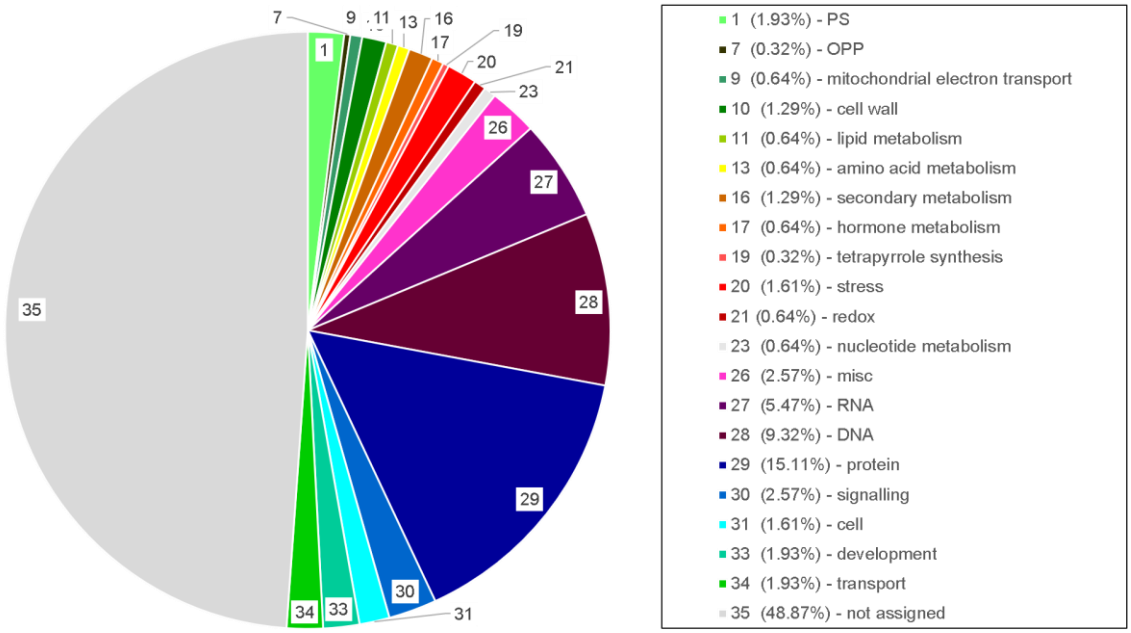

Figure 4.4 Functional biological classification by Mercator of significantly downregulated genes in *Solanum lycopersicum* shoot tissue treated with 0.05% (v/v) BC204 as a foliar spray. Upregulated genes were assigned by the Mercator functional annotation tool into different bins. The differentially expressed transcripts are indicated by percentiles.

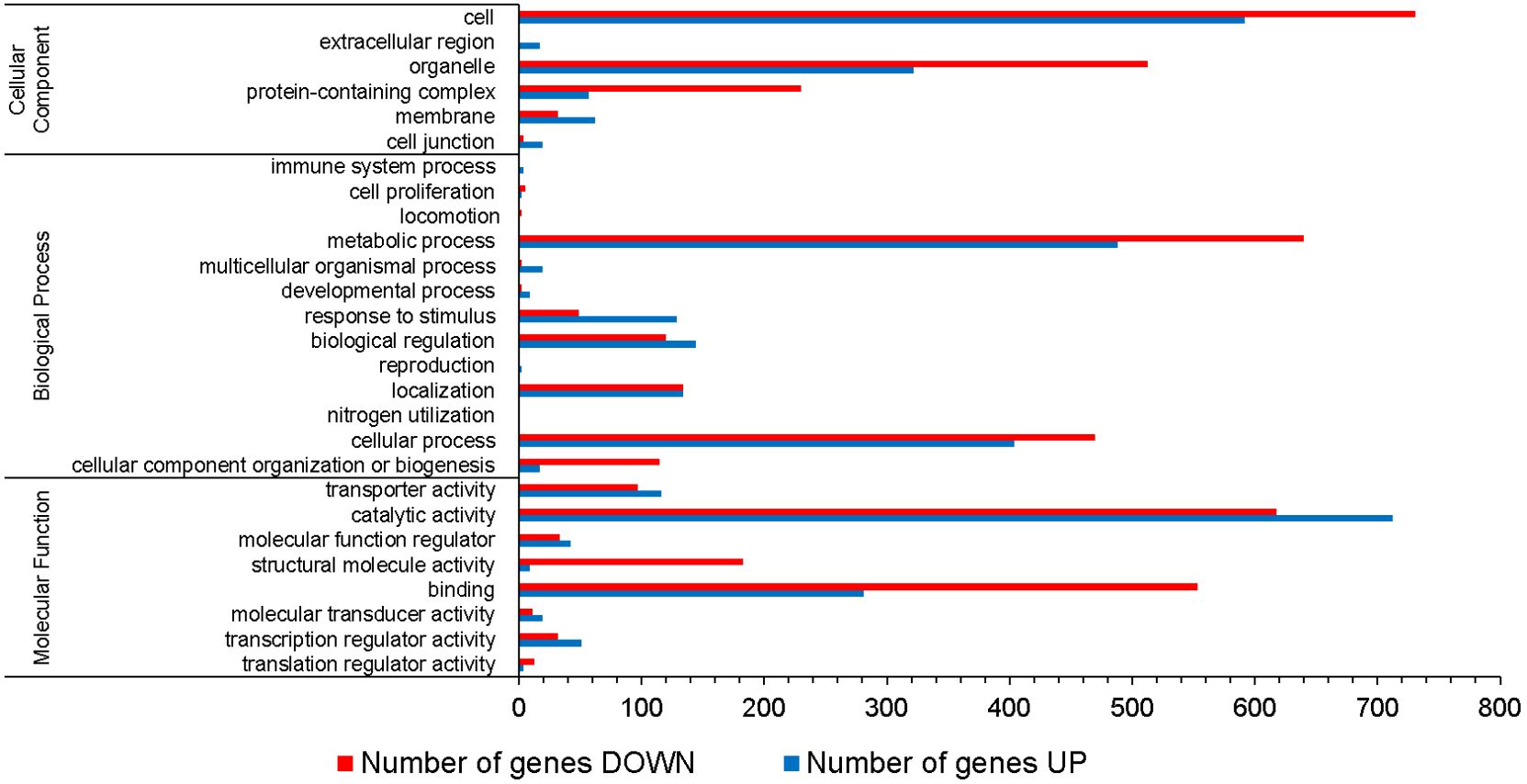

Figure 4.5 Up and downregulated genes in *Solanum lycopersicum* shoot tissue elicited by BC204 soil drench treatment functionally categorised into bins by the PANTHER database into either cellular component, biological process or molecular function gene ontologies. Red bars represent total downregulated genes while blue bars indicate total upregulated genes.

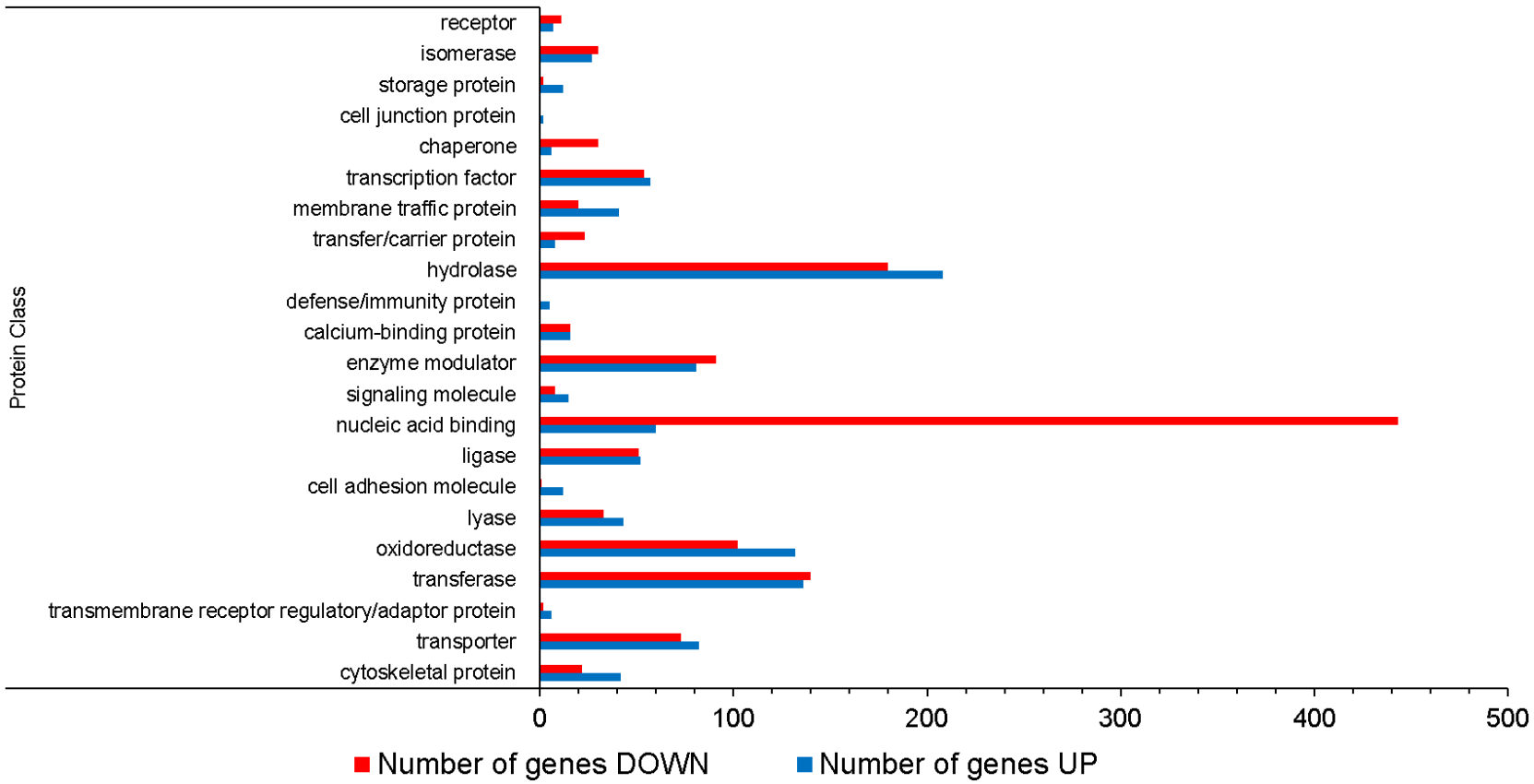

Figure 4.6 Up and downregulated genes in *Solanum lycopersicum* shoot tissue elicited by BC204 foliar spray treatment functionally categorised into bins by the PANTHER database into protein class. Red bars represent total downregulated genes while blue bars indicate total upregulated genes.

Table 4.6 *Solanun lycopersicum* genes induced/repressed by BC204 with a log_2fold change value larger than 2 or smaller than -2. Gene information obtained from Sol Genomics website and GO information obtained from agriGO SEA (version 2) compare analysis

Gene ID	Gene description proposed function from Sol Genomics Website	Log_2fold value	Associated GO (agriGO v2)	q value
Solyc01g106630	Unknown Protein (AHRD V1)	4.39178	not assigned	0.00344522
Solyc00g026160	Ferric reductase oxidase (AHRD V1 **** D6RVS5_HORVU); contains Interpro domain(s) IPR013121 Ferric reductase, NAD binding	4.23484	GO:0000293	0.000335047
Solyc10g076190	Peroxidase 1 (AHRD V1 ***- A0SWU6_SESRO); contains Interpro domain(s) IPR002016 Haem peroxidase, plant/fungal/bacterial	4.13349	GO:0055114	0.00522926
Solyc09g005000	Receptor like protein kinase (AHRD V1 **** Q39139_ARATH); contains Interpro domain(s) IPR001220 Legume lectin, beta chain	4.07042	GO:0016301	0.00656273
Solyc03g116690	Blue copper protein (AHRD V1 **-- D1MWY8_CITLA); contains Interpro domain(s) IPR003245 Plastocyanin-like	4.0455	GO:0009055	0.000335047
Solyc08g079900	Subtilisin-like protease (AHRD V1 **-- Q9LWA4_SOLLC); contains Interpro domain(s) IPR015500 Peptidase S8, subtilisin-related	3.63573	GO:0004252	0.000335047
Solyc01g009810	LRR receptor-like serine/threonine-protein kinase, RLP	3.61381	GO:0004675	0.000335047
Solyc10g081970	HIN1-like protein (Fragment) (AHRD V1 **-- Q32ZJ1_SOLTU); contains Interpro domain(s) IPR010847 Harpin-induced 1	3.53659	not assigned	0.0116064
Solyc07g040960	Os07g0175100 protein (Fragment) (AHRD V1 *--- Q0D898_ORYSJ)	3.39393	GO:0016042	0.000335047
Solyc08g068680	Decarboxylase family protein (AHRD V1 ***- B1ILJ6_CLOBK); contains Interpro domain(s) IPR002129 Pyridoxal phosphate-dependent decarboxylase	3.29521	GO:0030170 GO:0019752	0.000335047

Solyc10g050880	Gibberellin receptor GID1L2 (AHRD V1 **-- B6TIA5_MAIZE); contains Interpro domain(s) IPR013094 Alpha/beta hydrolase fold-3	3.27487	not assigned	0.0161671
Solyc12g049030	Fatty acid desaturase (AHRD V1 ***- B3SP99_CAPAN); contains Interpro domain(s) IPR005804 Fatty acid desaturase, type 1	3.26819	GO:0055114	0.000335047
Solyc01g106620	Pathogenesis-related protein 1a (AHRD V1 **-- Q00MX6_MALDO); contains Interpro domain(s) IPR018244 Allergen V5/Tpx-1 related, conserved site	3.26215	GO:0005515	0.000335047
Solyc09g064820	Circadian clock coupling factor ZGT (AHRD V1 ***- Q94FM9_TOBAC)	3.25341	not assigned	0.0161671
Solyc08g029000	Lipoxygenase (AHRD V1 **** Q43191_SOLTU); contains Interpro domain(s) IPR001246 Lipoxygenase, plant	3.21867	GO:0016165	0.000335047
Solyc02g069960	NAC domain protein IPR003441 (AHRD V1 *-*- B9HH05_POPTR); contains Interpro domain(s) IPR003441 No apical meristem (NAM) protein	3.19646	not assigned	0.0161671
Solyc12g100270	CER1 (AHRD V1 **-- B6TFH3_MAIZE); contains Interpro domain(s) IPR006694 Fatty acid hydroxylase	3.19351	GO:0006508 GO:0005783	0.000335047
Solyc12g005720	Cysteine-rich receptor-like protein kinase (AHRD V1 ***- C6ZRS1_SOYBN); contains Interpro domain(s) IPR002902 Protein of unknown function DUF26	3.16129	not assigned	0.000335047
Solyc10g078220	Cytochrome P450	3.1584	GO:0046409	0.000335047
Solyc07g055400	Receptor-like kinase (AHRD V1 **** B6EB06_NICGU); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase	3.1247	GO:0016301 GO:0005515	0.010267
Solyc04g040130	Omega-6 fatty acid desaturase (AHRD V1 **** Q461Q1_HEVBR); contains Interpro domain(s) IPR005804 Fatty acid desaturase, type 1	3.07993	GO:0042389	0.000335047

Solyc00g174330	Pathogenesis related protein PR-1 (AHRD V1 ***- Q9SC15_SOLTU); contains Interpro domain(s) IPR001283 Allergen V5/Tpx-1 related	3.06599	GO:0005576	0.000335047
Solyc04g007580	cDNA clone J100026I16 full insert sequence (AHRD V1 **-- B7FA06_ORYSJ)	3.05011	not assigned	0.00600167
Solyc09g007010	Pathogenesis related protein PR-1 (AHRD V1 **-- Q9SC15_SOLTU); contains Interpro domain(s) IPR002413 Ves allergen	3.03326	GO:0005515	0.0272986
Solyc10g055820	Chitinase (AHRD V1 ***- B9VRK7_CAPAN); contains Interpro domain(s) IPR000726 Glycoside hydrolase, family 19, catalytic	3.02484	GO:0005975 GO:0008061 GO:0016998	0.00872364
Solyc01g073820	CHP-rich zinc finger protein-like (AHRD V1 **-- Q9FNE5_ARATH); contains Interpro domain(s) IPR011424 C1-like	2.92957	not assigned	0.000335047
Solyc04g071600	Abscisic stress ripening (Fragment) (AHRD V1 *--- D1MEA2_MUSAC); contains Interpro domain(s) IPR003496 ABA/WDS induced protein	2.92503	GO:0006950	0.0316174
Solyc02g036480	Solyc02g036480.1.1 Harpin-induced protein-like (Fragment) (AHRD V1 **-- D2CFH8_COFAR); contains Interpro domain(s) IPR010847 Harpin-induced 1	2.91691	not assigned	0.000335047
Solyc03g026280	CRT binding factor 2 (AHRD V1 *-*- B3TPN7_SOLHA); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding	2.90875	GO:0016563	0.000335047
Solyc08g067630	Unknown Protein (AHRD V1)	2.89806	not assigned	0.000335047
Solyc08g067360	WRKY transcription factor 9 (AHRD V1 **** C9DHZ8_9ROSI); contains Interpro domain(s) IPR003657 DNA-binding WRKY	2.88183	GO:0003700 GO:0042802	0.000335047

Solyc09g009985	Unknown	2.86741	not assigned	0.000335047
Solyc07g048070	Membrane protein (AHRD V1 **-- B6U5U8_MAIZE); contains Interpro domain(s) IPR017214 Uncharacterised conserved protein UCP037471	2.82501	not assigned	0.000335047
Solyc06g066420	Cold induced protein-like (AHRD V1 *-*- Q94JH8_ORYSJ)	2.82187	not assigned	0.000335047
Solyc09g091000	Major allergen Mal d 1 (AHRD V1 ***- Q84LA7_MALDO); contains Interpro domain(s) IPR000916 Bet v I allergen	2.80846	GO:0009607	0.00889424
Solyc08g080650	Osmotin-like protein (Fragment) (AHRD V1 **-- Q8S4L1_SOLNI); contains Interpro domain(s) IPR001938 Thaumatin, pathogenesis-related	2.80355	GO:0005515	0.000335047
Solyc06g062420	Unknown	2.79258	not assigned	0.0381674
Solyc02g078150	Glutathione S-transferase (AHRD V1 **** D3Y4H6_9ROSI); contains Interpro domain(s) IPR004046 Glutathione S-transferase, C-terminal	2.78811	not assigned	0.0104308
Solyc01g097240	Pathogenesis-related protein 4B (Fragment) (AHRD V1 **-- Q6LBM4_TOBAC); contains Interpro domain(s) IPR018226 Barwin, conserved site IPR001153 Barwin	2.75196	GO:0050832 GO:0016998	0.000335047
Solyc07g056510	Unknown	2.75182	GO:0004364 GO:0043295	0.000335047
Solyc01g080570	Inosine-uridine preferring nucleoside hydrolase family protein (AHRD V1 *-** D7LYI5_ARALY); contains Interpro domain(s) IPR001910 Inosine/uridine-preferring nucleoside hydrolase	2.71831	GO:0016787	0.000335047
Solyc12g045020	Cytochrome P450	2.71579	GO:0019825	0.000335047
Solyc10g085010	PAR-1c protein (AHRD V1 ***- Q43589_TOBAC); contains Interpro domain(s) IPR009489 PAR1	2.67567	not assigned	0.000335047

Solyc02g068680	CHP-rich zinc finger protein-like (AHRD V1 **-- Q9FK70_ARATH); contains Interpro domain(s) IPR011424 C1-like	2.6723	GO:0008270	0.000335047
Solyc01g080790	PBSP domain-containing protein (AHRD V1 **-- C9S7G7_VERA1); contains Interpro domain(s) IPR007541 Plant Basic Secretory Protein	2.67059	not assigned	0.000335047
Solyc02g067730	RNA exonuclease 4 (AHRD V1 ***- B6T4V3_MAIZE); contains Interpro domain(s) IPR006055 Exonuclease	2.66363	GO:0003676 GO:0005622	0.00280317
Solyc08g082110	WRKY transcription factor-30 (AHRD V1 **** B6VB04_CAPAN); contains Interpro domain(s) IPR003657 DNA-binding WRKY	2.63559	GO:0003700 GO:0019900	0.000335047
Solyc04g008100	U-box domain-containing protein (AHRD V1 ***- D7MID4_ARALY); contains Interpro domain(s) IPR003613 U box domain	2.63364	GO:0005488 GO:0000151	0.010267
Solyc08g078650	Glycosyl transferase family 8 glycogenin (AHRD V1 *--- D3TM56_GLOMM); contains Interpro domain(s) IPR002495 Glycosyl transferase, family 8	2.61978	GO:0016757	0.000902265
Solyc02g086700	Beta-1 3-glucanase (AHRD V1 ***- Q9SYX6_TOBAC); contains Interpro domain(s) IPR000490 Glycoside hydrolase, family 17	2.61208	GO:0008810 GO:0005515	0.000335047
Solyc08g080640	Osmotin-like protein (Fragment) (AHRD V1 **-- Q8S4L1_SOLNI); contains Interpro domain(s) IPR017949 Thaumatin, conserved site IPR001938 Thaumatin, pathogenesis-related	2.60426	GO:0005515	0.000335047
Solyc02g077060	RPW8.2 (AHRD V1 **-- C4P0N9_ARALP); contains Interpro domain(s) IPR008808 Mildew-resistance, broad-spectrum	2.58282	not assigned	0.0376806
Solyc07g053230	Myb-related transcription factor (AHRD V1 *--- B5RHV2_MUSBA); contains Interpro domain(s) IPR015495 Myb transcription factor	2.57699	GO:0003677	0.0038445
Solyc11g011330	Cinnamyl alcohol dehydrogenase (AHRD V1 **** D1FWZ8_SOYBN); contains Interpro domain(s)	2.57329	GO:0045551	0.000335047

	IPR002085 Alcohol dehydrogenase superfamily, zinc-containing			
Solyc08g067610	ATP-binding cassette transporter (AHRD V1 ***- D8RL77_SELML); contains Interpro domain(s) IPR013525 ABC-2 type transporter	2.51234	GO:0008559	0.000335047
Solyc06g074030	CCR4-NOT transcription complex subunit 7 (AHRD V1 ***- B4FG48_MAIZE); contains Interpro domain(s) IPR006941 Ribonuclease CAF1	2.50885	GO:0000175	0.000335047
Solyc05g052280	Peroxidase (AHRD V1 ***- B9VRK9_CAPAN); contains Interpro domain(s) IPR002016 Haem peroxidase, plant/fungal/bacterial	2.47875	GO:0005515	0.000335047
Solyc09g011870	Prephenate dehydrogenase family protein (AHRD V1 ***- D7KDI5_ARALY); contains Interpro domain(s) IPR016040 NAD(P)-binding domain	2.46464	GO:0006571	0.000335047
Solyc04g079250	Patatin-like protein 1 (AHRD V1 ***- Q9FZ09_TOBAC); contains Interpro domain(s) IPR002641 Patatin	2.426	GO:0016298 GO:0045735	0.000335047
Solyc04g025530	Glutamate decarboxylase (AHRD V1 **** Q1I1D8_CITSI); contains Interpro domain(s) IPR010107 Glutamate decarboxylase	2.41623	GO:0005516 GO:0004351	0.000335047
Solyc07g006420	Unknown Protein (AHRD V1)	2.40988	not assigned	0.000335047
Solyc01g109330	Cytochrome c biogenesis protein ccsB (AHRD V1 *-*- D8G1N6_9CYAN); contains Interpro domain(s) IPR007816 ResB-like	2.40446	not assigned	0.0242138
Solyc03g083480	Receptor-like kinase (AHRD V1 *-*- Q9LL53_ORYSA)	2.39834	not assigned	0.000335047
Solyc10g055800	Chitinase (AHRD V1 ***- B9VRK7_CAPAN); contains Interpro domain(s) IPR016283 Glycoside hydrolase, family 19	2.38108	GO:0005975 GO:0008061 GO:0016998	0.000335047

Solyc10g081980	Harpin-induced protein-like (Fragment) (AHRD V1 **-- D2CFH8_COFAR); contains Interpro domain(s) IPR010847 Harpin-induced 1	2.3782	not assigned	0.000335047
Solyc04g077980	Zinc-finger protein (AHRD V1 ***- Q40899_PETHY); contains Interpro domain(s) IPR007087 Zinc finger, C2H2-type	2.35719	GO:0003700 GO:0016564	0.000335047
Solyc02g071560	Subtilisin-like protease (AHRD V1 ***- Q9FK77_ARATH); contains Interpro domain(s) IPR015500 Peptidase S8, subtilisin-related	2.34552	GO:0004252	0.000335047
Solyc10g008400	RING finger protein 5 (AHRD V1 *--- B6TI40_MAIZE); contains Interpro domain(s) IPR018957 Zinc finger, C3HC4 RING-type	2.33051	GO:0004842	0.000335047
Solyc06g005820	Copper transporter (AHRD V1 **** A9PEN3_POPTR); contains Interpro domain(s) IPR007274 Ctr copper transporter	2.32482	GO:0015089	0.000335047
Solyc01g057680	Hcr2-p4.1 (AHRD V1 ***- Q4G2V7_SOLPI); contains Interpro domain(s) IPR013210 Leucine-rich repeat, N-terminal	2.3104	not assigned	0.000335047
Solyc04g013200	Unknown Protein (AHRD V1)	2.30703	not assigned	0.00423648
Solyc10g006700	Calcium-binding EF hand family protein (Fragment) (AHRD V1 **-* D7MEH5_ARALY); contains Interpro domain(s) IPR011992 EF-Hand type	2.30415	GO:0031683	0.000335047
Solyc04g079760	Os07g0656700 protein (Fragment) (AHRD V1 *--- Q0D403_ORYSJ); contains Interpro domain(s) IPR005134 Uncharacterised protein family UPF0114	2.30365	not assigned	0.000625873
Solyc12g099790	Calcium-dependent protein kinase 17 (AHRD V1 ***- Q6KC53_NICPL); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase	2.28664	GO:0006468 GO:0005509	0.000335047
Solyc01g105630	Calmodulin (AHRD V1 ***- Q39890_SOYBN); contains Interpro domain(s) IPR011992 EF-Hand type	2.28092	GO:0005509	0.0142322

Solyc02g071475	Unknown	2.27384	not assigned	0.000902265
Solyc01g087580	Unknown Protein (AHRD V1)	2.26701	not assigned	0.0210018
Solyc01g089890	Unknown Protein (AHRD V1); contains Interpro domain(s) IPR004895 Prenylated rab acceptor PRA1	2.25568	not assigned	0.000335047
Solyc06g051940	Protein phosphatase 2C (AHRD V1 **** A1IGC7_TOBAC); contains Interpro domain(s) IPR015655 Protein phosphatase 2C	2.24378	GO:0008022 GO:0004722	0.000335047
Solyc05g012430	LRR receptor-like serine/threonine-protein kinase, RLP	2.24349	GO:0004675	0.000335047
Solyc02g091180	cDNA clone J100026I16 full insert sequence (AHRD V1 **-- B7FA06_ORYSJ)	2.24033	not assigned	0.0380541
Solyc01g107770	UDP-glucosyltransferase family 1 protein (AHRD V1 **** C6KI43_CITSI); contains Interpro domain(s) IPR002213 UDP-glucuronosyl/UDP-glucosyltransferase	2.22664	GO:0080044	0.000335047
Solyc03g115040	Xylanase inhibitor (Fragment) (AHRD V1 **-- Q53IQ4_WHEAT); contains Interpro domain(s) IPR001461 Peptidase A1	2.22491	GO:0006508	0.0038445
Solyc01g097470	Neurogenic locus notch protein-like (AHRD V1 ***- B6SSE6_MAIZE)	2.20919	not assigned	0.0230469
Solyc07g045030	NAC domain transcription factor (AHRD V1 *-** B6U2D4_MAIZE); contains Interpro domain(s) IPR003441 No apical meristem (NAM) protein	2.20624	GO:0003700	0.000335047
Solyc12g036320	Serine/threonine protein kinase B (Fragment) (AHRD V1 *-*- B1AEX8_POPTN)	2.20461	not assigned	0.000335047
Solyc10g007280	AAA-ATPase (AHRD V1 **-* C3TX92_BRASY); contains Interpro domain(s) IPR003959 ATPase, AAA-type, core	2.17055	GO:0016887	0.000335047

Solyc01g073890	CHP-rich zinc finger protein-like (AHRD V1 *--- Q9FJG5_ARATH); contains Interpro domain(s) IPR011424 C1-like	2.1697	GO:0019992 GO:0020037	0.000335047
Solyc07g056170	Subtilisin-like protease (AHRD V1 ***- Q6WNU4_SOYBN); contains Interpro domain(s) IPR015500 Peptidase S8, subtilisin-related	2.15919	GO:0004252	0.000335047
Solyc04g051690	WRKY transcription factor 16 (AHRD V1 ***- C9DI05_9ROSI); contains Interpro domain(s) IPR003657 DNA-binding WRKY	2.15237	not assigned	0.0167989
Solyc08g068730	N-acetyltransferase (AHRD V1 ***- B6SUK9_MAIZE); contains Interpro domain(s) IPR000182 GCN5-related N-acetyltransferase	2.14434	GO:0008152	0.000335047
Solyc11g005630	Receptor-like protein kinase (AHRD V1 ***- Q39202_ARATH); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase	2.14328	GO:0006468	0.000335047
Solyc07g049530	1-aminocyclopropane-1-carboxylate oxidase (AHRD V1 ***- Q94F66_SOLTU); contains Interpro domain(s) IPR005123 Oxoglutarate and iron-dependent oxygenase	2.13661	GO:0005507	0.000335047
Solyc08g068770	N-acetyltransferase (AHRD V1 ***- B6SUK9_MAIZE); contains Interpro domain(s) IPR000182 GCN5-related N-acetyltransferase	2.13356	GO:0008152	0.000335047
Solyc04g005050	Matrix metalloproteinase (AHRD V1 **-- B7TVN4_PINTA); contains Interpro domain(s) IPR001818 Peptidase M10A and M12B, matrixin and adamalysin	2.13114	GO:0004222	0.000335047
Solyc03g093560	Ethylene-responsive transcription factor 2 (AHRD V1 ***- B6U860_MAIZE); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding	2.1184	GO:0005515 GO:0003677	0.000335047
Solyc10g079930	UDP-glucosyltransferase HvUGT5876 (AHRD V1 ***- D3WYW1_HORVD); contains Interpro domain(s) IPR002213 UDP-glucuronosyl/UDP-glucosyltransferase	2.11199	GO:0008152	0.000335047

Solyc01g103650	Hydrolase alpha/beta fold family (AHRD V1 **-- A4VRL9_PSEU5); contains Interpro domain(s) IPR012020 AB-hydrolase YheT, putative	2.10547	GO:0003824	0.000335047
Solyc03g117590	Chaperone protein dnaJ (AHRD V1 *-*- C5UZI2_CLOBO); contains Interpro domain(s) IPR001623 Heat shock protein DnaJ, N-terminal	2.1054	GO:0031072 GO:0006457	0.000335047
Solyc08g079230	Cortical cell-delineating protein (AHRD V1 *--- B6T836_MAIZE); contains Interpro domain(s) IPR013770 Plant lipid transfer protein and hydrophobic protein, helical	2.10499	GO:0008289	0.000335047
Solyc02g031790	Receptor like kinase, RLK	2.08887	GO:0019199 GO:0005515	0.000335047
Solyc08g078190	Ethylene responsive transcription factor 1a (AHRD V1 *-*- C0J9I9_9ROSA); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding	2.08591	GO:0006355 GO:0003677	0.00344522
Solyc10g081040	Unknown Protein (AHRD V1)	2.07951	not assigned	0.0455745
Solyc06g034340	NAC domain protein IPR003441 (AHRD V1 ***- B9ICS8_POPTR); contains Interpro domain(s) IPR003441 No apical meristem (NAM) protein	2.07286	GO:0003700	0.00542908
Solyc12g096960	Major allergen Mal d 1.0502 (AHRD V1 ***- Q4VPK6_MALDO); contains Interpro domain(s) IPR000916 Bet v I allergen	2.0654	GO:0009607	0.000902265
Solyc08g062360	Ankyrin repeat protein (AHRD V1 **-- B1Q4U3_9ROSI); contains Interpro domain(s) IPR002110 Ankyrin	2.06335	GO:0015276	0.000335047
Solyc01g097270	Chitinase (Fragment) (AHRD V1 *--- Q38777_ALLSA); contains Interpro domain(s) IPR000726 Glycoside hydrolase, family 19, catalytic IPR001153 Barwin	2.05203	GO:0005515	0.000335047
Solyc04g074400	Os06g0220000 protein (Fragment) (AHRD V1 ***- Q0DDJ2_ORYSJ); contains Interpro domain(s) IPR006766 Phosphate-induced protein 1 conserved region	2.05194	not assigned	0.0359487

Solyc05g008980	Receptor-like protein kinase (AHRD V1 ***- D3G6F0_CAPAN); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase	2.05001	GO:0006468	0.000335047
Solyc01g021600	Disease resistance response protein (AHRD V1 ***- A8IXC7_BRACM); contains Interpro domain(s) IPR004265 Plant disease resistance response protein	2.02298	not assigned	0.00975289
Solyc10g083690	Cytochrome P450	2.01385	GO:0020037 GO:0055114	0.000335047
Solyc02g082920	Endochitinase (Chitinase) (AHRD V1 **** Q43184_SOLTU); contains Interpro domain(s) IPR000726 Glycoside hydrolase, family 19, catalytic	2.00936	GO:0008843	0.000335047
Solyc03g116700	Blue copper protein (AHRD V1 **-- D1MWY8_CITLA); contains Interpro domain(s) IPR003245 Plastocyanin-like	2.00431	GO:0005515	0.000335047
Solyc09g089520	Proteinase inhibitor I (AHRD V1 ***- Q3S492_SOLTU); contains Interpro domain(s) IPR000864 Proteinase inhibitor I13, potato inhibitor I	-2.65401	GO:0009611	0.000335047
Solyc05g018850	Unknown Protein (AHRD V1)	-2.30756	not assigned	0.00600167
Solyc05g005100	Os06g0207500 protein (Fragment) (AHRD V1 ***- Q0DDQ9_ORYSJ); contains Interpro domain(s) IPR004253 Protein of unknown function DUF231, plant	-2.26547	not assigned	0.0336936
Solyc09g089540	Proteinase inhibitor I (AHRD V1 ***- Q3S492_SOLTU); contains Interpro domain(s) IPR000864 Proteinase inhibitor I13, potato inhibitor I	-2.17884	GO:0009611	0.000335047
Solyc00g071180	Cysteine proteinase inhibitor (AHRD V1 *-** Q2VY67_9ERIC); contains Interpro domain(s) IPR000010 Proteinase inhibitor I25, cystatin	-2.13305	GO:0004869 GO:0050897	0.000335047

Solyc12g033060	Photosystem I P700 chlorophyll a apoprotein A2 (AHRD V1 ***- A7Y3D1_IPOPU); contains Interpro domain(s) IPR001280 Photosystem I psaA and psaB	-2.07468	GO:0016021	0.00165512
Solyc01g058100	Unknown Protein (AHRD V1)	-2.0276	not assigned	0.0441481

4.4.4 Quantitative RT-PCR (RT-qPCR) validation of RNA-seq data

To validate RNA-seq results, we randomly selected two upregulated and two downregulated genes. The upregulated transcripts (*Solyc01g106630.2*; *Solyc09g005000.1*) and downregulated transcripts (*Solyc04g011800.1*; *Solyc12g033060.1*) were analysed by RT-qPCR using *Solyc10g006580*, which was unchanged in the RNA-seq experiment, as a reference. Comparisons between the RNA-seq and RT-qPCR analysis showed partial positive correlation between the two approaches, indicating moderate reliability (Table 4.7). However, such discrepancies are not uncommon between RNA-seq and Rt-qPCR analyses. A study using melatonin on *Arabidopsis thaliana* revealed a discrepancy of 7 genes that were up or downregulated according to RNA-seq analysis but not significantly altered according to qPCR analysis (Weeda et al., 2014).

Table 4.7 RT-qPCR validation of differentially up- and downregulated genes in *Solanum lycopersicum* shoot tissue obtained from RNA-seq data following BC204 treatment. Log_2fold change of transcript levels was determined from replicates (n=3) of each sample while for quantitative RT-qPCR, the Ct values were averaged and normalized to *Solyc10g006580* according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the relative transcript expression was significantly different at $p \leq 0.05$. For RT-qPCR, values lower than 1 represent downregulation of the transcript.

Gene	RNA-seq	RT-qPCR
Solyc01g106630.2	4.392	31.008 ± 12.927
Solyc09g005000.1	4.07042	8.954 ± 2.7808
Solyc04g011800.1	-1.927	2.368 ± 0.71983
Solyc12g033060.1	-2.075	2.954 ± 1.060

4.5 Discussion

PBs have been used to great success in agriculture with regards to an overall increase in plant growth and mitigating environmental stress, but major questions remain on their molecular modes of action (Yakhin et al., 2017). Molecular characterisation has been extensively conducted on the effect of *Ascophyllum nodosum* extracts, one the best studied PBs, in plants (De Saeger et al., 2019; Jithesh et al., 2018; Shukla et al., 2018). Limited studies on PBs have been conducted using transcriptomic analysis (Hoeberichts et al., 2017; Santi et al., 2017; Trevisan et al., 2019, 2017; Wilson et al., 2018), and of these, only a very small number have been conducted in model plants such as *A. thaliana* (Blaszczak et al., 2016; Goñi et al., 2016; Nair et al., 2012; Povero et al., 2011; Santaniello et al., 2012; Shukla et al., 2018; Trevisan et al., 2011; Weeda et al., 2014) or *S. lycopersicum* (Cervantes-Gámez et al., 2015; Contartese et al., 2016; Ertani et al., 2017). Our results confirm that BC204 increases *S. lycopersicum* shoot and root growth, shoot and root length and stem width. This prompted RNA-seq analysis as the main methodology to elucidate the effect of BC204 on total gene expression in *S. lycopersicum* shoot tissue.

4.5.1 BC204 increased shoot and root growth, shoot and root length and stem width

BC204, applied as a foliar spray, resulted in an increase in shoot and root tissue of hydroponically-grown *S. lycopersicum* plants (Figure 4.1). Measurements of fresh and dry biomass from both shoot and root tissue reflected weight increases in BC204-treated plants compared with control plants (Figure 4.2). Shoot length, root length and stem width at both the uppermost and lowest leaf of BC204-treated plants were also increased compared to the control plants (Figure 4.2). Several other PBs have also been shown to similarly increase tomato biomass production (De Pascale et al., 2017; Farouk et al., 2012; Koleška et al., 2017) and increase shoot and root growth (Bulgari et al., 2019; Parađiković et al., 2018; Paul et al., 2019).

4.5.2 BC204 elicited a major change in gene expression across many metabolic processes

After processing the RNA-seq outputs, a mean of 7.540198 million reads per sample revealed that deep sequencing was successful. Of the 6015 significantly differentially expressed transcripts, 2923 were upregulated and 3092 downregulated. The overwhelming majority of these differentially expressed genes (DEGs) (86.22 %) had a $\log_2$fold value between -1 and 1. Of the DEGs with higher log2fold values (larger than 1 and smaller than -2), 660 were induced and 169 repressed. Most studies that use transcriptomics as methodology routinely uses a $\log_2$fold cut-off in efforts to pin-point more specifically which genes and metabolic processes are altered/involved. A popular choice is using a fold-change (FC) cutoff of 2 (Brunskill and Steven Potter, 2012; Contartese et al., 2016; Ertani et al., 2017; Weeda et al., 2014; Zhang et al., 2014). Some studies use different FC cut-offs such as 1 (Omidbakhshfard et al., 2020), 1.5 (Cordovez et al., 2018; Gu et al., 2010; Trevisan et al., 2017) or 3 (Briglia et al., 2019).

A total of 55 gene ontology terms related to upregulated genes were obtained from SEA analysis through the AgriGO v.2 analysis tool (Table 4.4). Of the GO terms associated with upregulation, 20 terms had an input of more than 300 genes. The five terms most associated with upregulation were catalytic activity (GO:0003824, 1098), metabolic process (GO:0008152, 1072 genes), organic substance metabolic process (GO:0071704, 806 genes), primary metabolic process (GO: 0044238, 751 genes) and transferase activity (GO:0016740, 447 genes).

For downregulated genes, 204 GO terms were obtained (Table 4.5), which is almost 5 times more than for the upregulated terms. This is interesting, as the total number of upregulated and downregulated genes was almost equal (2923 UP and 3092 down). The five terms most associated with downregulated GO terms were cellular process (GO:0009987, 1008 genes), metabolic process (GO:0008152, 1079 genes), organic substance metabolic process (GO:0071704, 921 genes), cellular metabolic process (GO:0044237, 868 genes) and primary metabolic process (GO:0044238, 861 genes). Three of these terms occur in both the up- and downregulated groups, pointing to a large shift in basic or primary plant metabolism by BC204.

Several upregulated and downregulated processes with the keyword ‘’protein’’ had more than 300 genes in the input list. From this analysis it is almost impossible to flag a few processes as the most influenced by BC204. However, key terms obtained from the analysis were RNA, nucleic acid binding, intracellular, protein, peptide, phosphorylation, macromolecule biosynthesis and nitrogen metabolism.

Mercator is routinely used to characterize DEGs obtained from RNAseq experiments into functionally annotated ‘’bins’’ (Lohse et al., 2014). For Mercator analysis, except for the bin called not assigned (29.71%), signalling (10.88%), protein (10.67%), RNA (9.41%), stress (9.00%) and hormone metabolism were mostly upregulated (Figure 4.4). Further upregulated processes with smaller percentiles were secondary metabolism (3.97%), transport (2.93%), lipid metabolism (2.72%) and amino acid metabolism (1.46%). Processes largely downregulated were protein (15.11%), DNA (9.32%), RNA (5.47%) and signalling (2.57%) (Figure 4.5). There was also some overlap with regards to both up- and downregulation in processes such photosystem (PS), cell wall, lipid metabolism, protein, DNA, RNA and several others. This already points towards an extremely complex shift in metabolism elicited by BC204. Moreover, several processes both up -and downregulated with small percentiles (< 1%) were also predicted. Even so, most genes were not assigned to a specific process. This is mostly due to limited or no information available about these genes.

PANTHER classification reflected the classification of AgriGO v2 to some extent. The majority of clusters reflected more genes downregulated than upregulated according to cellular component, biological process and molecular function (Figure 4.6). The categories cell, organelle, metabolic process, cellular process, catalytic activity and binding had the most genes and, except for catalytic activity, contained mostly downregulated genes. Categories that had more upregulated than downregulated genes were extracellular region, membrane, cell junction, multicellular organismal process, developmental process, response to stimulus, biological regulation, transporter activity, molecular function regulator, molecular transducer activity and transcription regulator activity. Protein class categories were more evenly distributed with regards to up- and downregulation, except for nucleic acid binding (Figure 4.7). A total of 440 genes associated with nucleic acid binding were downregulated, compared to 60 upregulated in the same category. This large downregulation of genes associated with nucleic acid binding adds to the hypothesis that BC204 plays a major role in transcriptional regulation. Nucleic acid binding enzymes can be DNA-binding transcriptional regulators involved in repressing the expression of certain genes (Rojo, 2001). Specific transcription factors related to downstream responses are discussed later in this chapter. Categories with slightly more upregulated than downregulated genes were storage protein, cell junction protein, transcription factor, membrane traffic protein, hydrolase, defence/immunity protein, signalling molecule, ligase, cell adhesion

molecule, lyase, oxidoreductase, transmembrane receptor/adaptor protein, transporter and cytoskeletal protein.

Already, from these three gene ontology prediction outputs, it is clear that BC204 affects major parts of the plant metabolism. The major downregulation in nucleic acid binding proteins (Figure 4.6) suggests a large shift in gene expression at DNA and RNA level. Nucleic acid binding elements can either induce/enhance transcription or alternatively suppress the transcription of certain genes (Leung et al., 2018). Moreover, many of the outputs also suggest post-transcriptional and post-translational changes which would affect protein synthesis and formation.

4.5.3 BC204 induces major at the regulation level, signalling and hormone metabolism

BC204 induces a complex network of genes at several levels of plant metabolism ranging from transcription factors to hormone signalling to the activation of genes involved in secondary metabolism and stress responses. The broad induction and repression of genes across many metabolic processes suggests a complex change in the metabolism activated by BC204, also evident from the major changes at RNA and protein level. Based on the broad contents of the BC204 extract this already indicates that no one or two specific mechanisms are involved, but rather a broad, non-specific metabolic shift.The trade-off between up- and downregulation of genes also seems to take place within metabolic processes, rather than between processes, with a few exceptions. Those processes which were largely upregulated were stress, signalling and hormone metabolism (Figure 4.3) while protein metabolism was largely downregulated (Figure 4.4). Signalling and hormone metabolism in plants are not as well characterised as other processes, largely due to the complexity of the interplay in regulatory components between the pathways (Qu and Zhao, 2011). Plant hormones integrate several metabolic processes through tight crosstalk between the different hormone signalling pathways.

As mentioned, RNA-binding proteins play a major role in transcription regulation as they affect RNA availability for translation, RNA stability and turn-over, all of which affects gene and protein expression (Marondedze et al., 2019). Additionally, RNA metabolism in plants plays a major role in growth, development and stress responses (Jung et al., 2013). Upregulation of a gene in this study coding for a subunit involved in mRNA polyA shortening through the CCR4-NOT transcription complex (Albert et al., 2002), *Solyc06g074030*, is one example of mRNA processing possible elicited by BC204. This would result in a change in many metabolic pathways, as evident from our data-set. The major changes observed at the protein level also suggests that BC204 elicited post-transcriptional changes, as exemplified by the strong induction of *Solyc10g007280*, which codes for AAA-ATPase. This enzyme plays a key role in the protein degradation machinery at the folding level of enzyme synthesis (Yedidi et al., 2017).

Of the most highly induced genes (Table 4.6), 121 were upregulated and 9 downregulated. A few of these code for transcription factors involved in plant stress responses and are discussed in the next section. Two of these, *Solyc06g03440* and *Solyc02g069960*, code for NAC domain proteins, which are involved in a broad range of processes including responses to salinity, drought, cold shock, viral infection and mechanical wounding (reviewed by Hu et al., 2010). Several other strongly induced genes suggest a significant hormonal response downstream of the changes in transcription.

BC204 possibly activated the involvement of gibberellin metabolism, with the observed induction of *Solyc10g050880,* which codes for the gibberellin receptor GID1L2. This receptor plays a pivotal role in gibberellin signalling and gibberellin-mediated growth. Four genes coding for cytochrome 450 were also highly induced by BC204 (*Solyc10g078220, Solyc12g045020, Solyc10g083690* and *Solyc10g078220*). The cytochrome P450 superfamily, the largest enzymatic protein family in plants, is involved in multiple metabolic processes including hormone metabolism, plant development and responses to abiotic and biotic stresses (Xu et al., 2015). Several highly induced genes coded for protein kinases (Table 4.5), which phosphorylate threonine, serine and tyrosine residues in proteins. Such protein kinases are major components of signalling networks involved in plant responses to a variety of environmental stimuli. The gene with the highest log_2fold value, *Solyc00g026160.3*, codes for ferric reductase oxidase, which plays an important role in Fe homeostasis in plant tissue (Li et al., 2019). Two genes coding for blue copper proteins, *Solyc03g116690* and *Solyc03g116700,* were also highly induced by BC204. Blue copper proteins are essential role players in electron transport and are predicted to be involved in Cu accumulation in the cell wall (Printz et al., 2016). Additionally, *Solyc06g005820*, coding for a copper transporting enzyme was also highly induced by BC204.

One highly induced gene, *Solyc04g071600*, codes for abscisic acid stress ripening protein (Table 4.6). These hormonal responsive genes are mostly involved in response to drought and salinity stress (Golan et al., 2014). Another ABA-related gene induced by BC204 is *Solyc06g051940*. This gene codes for protein phosphatase 2C and is suggested to directly regulated protein kinases activated by ABA (Umezawa et al., 2009). *Solyc01g105630*, also induced by BC204, codes for a calmodulin protein. These secondary messengers have been shown to positively regulate ROS production and ABA responses in *Arabidopsis* (Dai et al., 2018). Ethylene-responsive genes and transcription factors are also induced by BC204 and discussed in the next section.

4.5.4 BC204 induces a shift towards secondary metabolism and ultimately a stress priming response

Although *S. lycopersicum* plants in this study were not subjected to any environmental stresses, a strong upregulation of genes specifically involved in stress metabolism was observed, with 9% of the induced genes being related to stress-responses in plants (Figure 4.3). Additionally, a number of other processes that could indirectly aid in this priming response, including cell wall and lipid metabolism, were upregulated. Cell wall synthesis is associated with an increase in plant defence against biotic stress (Malinovsky et al., 2014), while lipid-mediated signalling is also important in plant defence (Lim et al., 2017), specifically salicylic acid-mediated defence (Zhang and Xiao, 2015). Other indirect responses to BC204 which could also be classified as a stress response include hormone metabolism (discussed in the previous section) and secondary metabolism. Secondary metabolites usually aid in plant defence (Isah, 2019). The major increase in secondary metabolism in response to BC204 is interesting since secondary metabolism is very taxing to the plant and is tightly regulated (Yang et al., 2012).

Nine genes coding for pathogenesis-related (PR) proteins/enzymes/transcription factors, *Solyc01g106620*, *Solyc00g174330, Solyc09g007010, Solyc03g026280, Solyc08g080650, Solyc01g097240, Solyc08g080640, Solyc08g080640* and *Solyc08g078190*, were upregulated by BC204 treatment by a log_2fold value higher than 2 (Table 4.6). Since the BC204-treated plants did not display any physical

symptoms of stress and appeared larger than their untreated counterparts, this could mean that BC204 activates the plants immune system, also known as priming (Ugena et al., 2018). A similar priming effect has been observed in *A. thaliana* plants treated with the PB melatonin (Weeda et al., 2014). In addition, two genes coding for peroxidases, *Solyc10g076190* and *Solyc05g052280*, were strongly induced by BC204. Peroxidases in plants are involved in responses to abiotic and biotic stresses as well as lignin biosynthesis, which strengthens plant structures by making cell walls more hydrophobic (Veitch, 2004). Another group of genes, *Solyc10g055820, Solyc10g055800, Solyc01g097270* and *Solyc02g082920,* coding for pathogen defence-related chitinases, were also induced by BC204. Chitinases are well-characterised enzymes that are known to provide plants with resistance to particularly fungal pathogens, since they catalyse the hydrolysis of glycosidic bonds in chitin, a major part of fungal cell walls, thereby inactivating the pathogen. In addition, chitinases have also been shown to improve plant growth and yield (Kumar et al., 2018).

Two genes, *Solyc01g009810* and *Solyc05g012430*, coding for LRR receptor-like serine/threonine-protein kinases were also upregulated by BC204. These enzymes are involved in signalling processes related to defence against pathogens (Afzal et al., 2008). Furthermore, three genes coding for WRKY transcription factors, *Solyc08g067360* (*WRKY9*), *Solyc08g082110* (*WRKY30*) and *Solyc04g051690* (*WRKY16*) were also highly induced by BC204. These transcription factors are important role players in plant defence against biotic and abiotic stresses while also contributing to secondary metabolism and plant development. WRKY transcription factors are involved both up- and downstream of jasmonic acid, salicylic acid, ethylene, cytokinins, auxins and brassinosteroid metabolism (Bakshi and Oelmüller, 2014). In *Nicotiana benthamiana,* it was demonstrated that the binding of *WRKY9* to the W-box in the promoter of the *RBOHB* gene led to a ROS burst. In rice, *WRKY30* was shown to be involved in drought tolerance (Bai et al., 2018). In *A. thaliana*, *AtWRKY16* was predicted to be involved in the activation of proteins in the defence-related ET1 pathway (Phukan et al., 2016). Another family of proteins involved in abiotic and biotic stress responses, zinc finger-related transcription factors, were also stimulated by BC204 treatment. *Solyc01g073820, Solyc02g068680, Solyc04g077980, Solyc10g008400* and *Solyc01g073890* code for zinc finger protein or zinc finger protein-like enzymes. These transcription factors are also involved in plant disease resistance (Gupta et al., 2012).

Solyc08g062360, which codes for an ankyrin repeat protein, was also strongly induced by BC204. These proteins play an important role in plant defence in against the rice leaf blight pathogen *Xanthomonas oryzae*. Exogenous application of salicylic acid or methyl jasmonate also induced the expression of this gene (Mou et al., 2013). Following pathogen infection, substilisin-like protease-coding genes like the induced *Solyc07g056170* are activated and directly involved in a stress-priming response in plants (Figueiredo et al., 2014). *Solyc02g071560*, strongly induced, also codes for a subtilisin-like protease which could play a similar role in plants. A patatin-like protein, coded for by *Solyc04g079250* and involved in plant defence against pathogens (La Camera et al., 2009), was also induced by BC204.

Of the 9 most highly repressed genes (log_2fold<-2), three coded for proteinase inhibitors (Table 4.5). Proteinase inhibitors are largely involved in defence mechanisms against herbivores and insects (Srikanth and Chen, 2016). Consequently, although BC204 appears to have a priming effect protecting the plant from pathogens, it is possible that this is at the cost of defence against herbivory. Furthermore, *Solyc04g040130*

and *Solyc12g049030*, which codes for fatty acid desaturase, was strongly induced by BC204 treatment. Fatty acid desaturases are key role players in a plant's response and acclimation to environmental stresses (Dong et al., 2016).

Although *S. lycopersicum* is a well-defined model species for fruit-bearing crops, certain information on many genes is absent or inferred from studies where their homologues were characterised. Moreover, the overwhelming majority of differentially expressed transcripts were not individually discussed due to the sheer number of DEG. For these reasons, several mechanisms could also be involved. The involvement one or two specific mechanisms activated by BC204 seems unlikely, which is evident from the broad changes in gene expression and the complex mixture of extracts in BC204. As more genomic data become available for tomato, this data-set should be revisited in order to illuminate further possible mechanisms.

Similarly, to what was observed in Chapter 3, there were large discrepancies between the RNA-seq and qPCR results. As explained, RNA-seq and qPCR analysis fundamentally determines changes in gene expression in different ways. Pooling of the RNA samples for RNAseq analysis also determined an average abundance of each transcript whereas for the qPCR analysis, individual RNA samples were used for relative quantification.

Conclusion

Despite the overall advancements made by science with regards to gene function and annotation, there are still major gaps in the characterisation of the genomes of even well-defined plant model species. This data-set should therefore be constantly revisited in the future as the annotation and characterisation of gene function in *S. lycopersicum* develops and the annotation of the genome becomes more complete. The results of this study highlight the essence of PB mode of action. PBs are characterised as substances that activate the innate metabolic processes of plants. This could illuminate further possible mechanisms involved in the effect(s) of BC204 on the plant. Furthermore, tissue could be harvested at several time points during BC204-treatment in order to elucidate the specific mechanisms. The addition of an external biotic stressor could also be used to clarify the possible priming response observed here.

It is clear from these results that BC204 treatment resulted in a considerable shift in gene expression patterns across the transcriptome of tomato seedlings. Most upregulated genes were involved in stress metabolism, signalling, hormone metabolism and protein metabolism. This suggests that BC204 activates a a broad combination of mechanisms by changing the expression of transcription factors and signalling-related genes to activate hormone and secondary metabolism, ultimately leading to an increase in the expression of genes related to a stress response. This is in line with what has been observed with many PBs, where an increased tolerance towards a variety of environmental stressors has been reported.

Author contributions

JL and PH designed the research, JL prepared and PH edited and revised the manuscript. JL conducted all the experimental work and also conducted all further gene expression analysis after DEG determination. BC and APC aided in the RNA-seq analysis, writing the scripts for the analysis. JK provided partial funding and further guidance.

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Supplementary Material

Table S4.1 Primers used in RT-qPCR validation of RNA-seq data generated from *Solanum lycopersicum* shoot tissue

Gene ID	Gene name (ITAG2.4 gene models)	Forward primer (5'-3')	Reverse primer (5'-3')	Product size
Solyc10g006580	Ribosomal protein L2	TGGAGGGCGTACTGAGAAAC	TCATAGCAACACCACGAACC	101 bp
Solyc01g106630.2	Unknown protein	ATGGACGTTGTCCTCTCCAG	ACTCGGTACGTCTTGGTTGT	102 bp
Solyc09g005000.1	Receptor like protein kinase	GGAACATTTTGCTCCGTCGT	ATCCAGCTCCCAGTCCTCTA	96 bp
Solyc04g011800.1	Glutaredoxin	TCTCACAGCATCGAAACCCT	GCCTTCTCCATTTGCTTCCC	95 bp

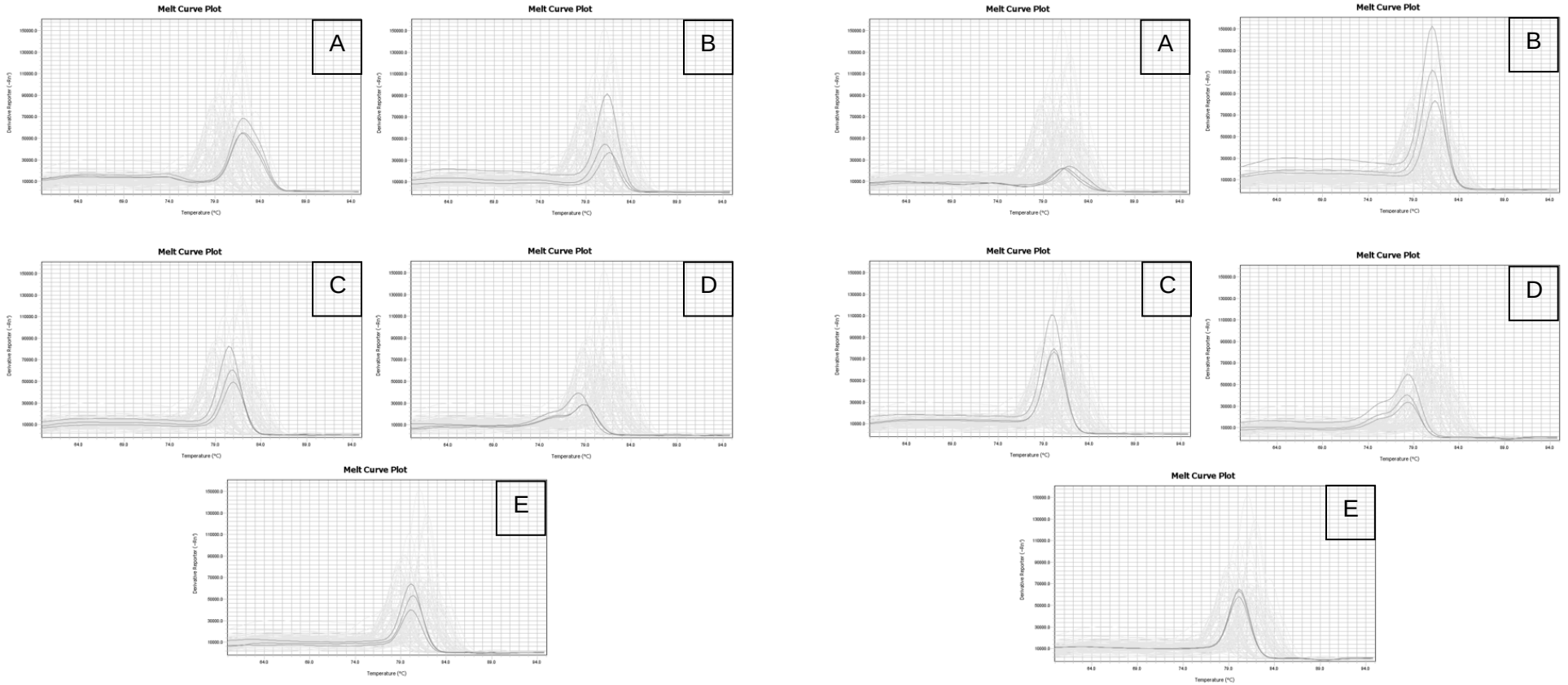

Figure S4.1 Melt curves of *Solyc10g006580* (A), *Solyc01g106630.2* (B), *Solyc09g005000.1* (C), *Soly04g011800.1* (D) and *Solyc12g033060.1* (E) for both control (left) and BC204-treated (right) samples

Table S4.2 *Solanum lycopersicum* genes significantly upregulated by BC204 treatment as annotated by PANTHER Classification

Gene ID	Uniprot identifier	log_2fold change	q_value
Solyc05g007650	K4BWU0	100	0.0381674
Solyc09g008760	K4CQR1	100	0.000335047
Solyc09g057900	K4CTH9	100	0.0381674
Solyc10g006950	Unknown	100	0.0325622
Solyc10g018340	K4CYV8	100	0.000335047
Solyc11g013250	K4D6C9	100	0.000335047
Solyc12g005000	K4DB38	100	0.00636728
Solyc12g027630	Unknown	100	0.0183165
Solyc01g106630	Unknown	4.39178	0.00344522
Solyc00g026160	Unknown	4.23484	0.000335047
Solyc10g076190	K4D1W1	4.13349	0.00522926
Solyc09g005000	K4CPZ0	4.07042	0.00656273
Solyc03g116690	K4BLH1	4.0455	0.000335047
Solyc08g079900	K4CNZ0	3.63573	0.000335047
Solyc01g009810	Unknown	3.61381	0.000335047
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Solyc07g040960	K4CDX3	3.39393	0.000335047
Solyc08g068680	Q1KSC6	3.29521	0.000335047
Solyc10g050880	K4D0P2	3.27487	0.0161671
Solyc12g049030	K4DFI4	3.26819	0.000335047
Solyc01g106620	B2LW68	3.26215	0.000335047
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Solyc08g029000	K4CJW3	3.21867	0.000335047
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Solyc12g100270	K4DI48	3.19351	0.000335047
Solyc12g005720	K4DBB0	3.16129	0.000335047
Solyc10g078220	Unknown	3.1584	0.000335047
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Solyc00g174330	Unknown	3.06599	0.000335047
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Solyc09g007010	Q04108	3.03326	0.0272986
Solyc10g055820	K4D1H1	3.02484	0.00872364
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Solyc02g036480	K4B5U1	2.91691	0.000335047
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Solyc08g067360	K4CLW2	2.88183	0.000335047
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Solyc08g080650	K4CP63	2.80355	0.000335047

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Solyc01g080790	K4AXX2	2.67059	0.000335047
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Solyc08g082110	K4CPK7	2.63559	0.000335047
Solyc04g008100	K4BNW6	2.63364	0.010267
Solyc08g078650	K4CNM4	2.61978	0.000902265
Solyc02g086700	K4BBH7	2.61208	0.000335047
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Solyc11g011330	K4D5T8	2.57329	0.000335047
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Solyc04g025530	K4BQZ8	2.41623	0.000335047
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Solyc03g083480	K4BIC4	2.39834	0.000335047
Solyc10g055800	K4D1H0	2.38108	0.000335047
Solyc10g081980	K4D384	2.3782	0.000335047
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Solyc02g071560	K4B8D2	2.34552	0.000335047
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Solyc06g005820	K4C364	2.32482	0.000335047
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Solyc04g013200	Unknown	2.30703	0.00423648
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Solyc04g079760	K4BV55	2.30365	0.000625873
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Solyc02g071475	Unknown	2.27384	0.000902265
Solyc01g087580	K4AYE8	2.26701	0.0210018
Solyc01g089890	K4AYT0	2.25568	0.000335047
Solyc06g051940	K4C5Y0	2.24378	0.000335047
Solyc05g012430	K4BXW6	2.24349	0.000335047
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Solyc01g107770	Unknown	2.22664	0.000335047

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Solyc01g097470	K4B0D4	2.20919	0.0230469
Solyc07g045030	K4CER1	2.20624	0.000335047
Solyc12g036320	K4DE89	2.20461	0.000335047
Solyc10g007280	K4CXQ7	2.17055	0.000335047
Solyc01g073890	K4AXD4	2.1697	0.000335047
Solyc07g056170	K4CGF0	2.15919	0.000335047
Solyc04g051690	K4BSC8	2.15237	0.0167989
Solyc08g068730	Q8RXB8	2.14434	0.000335047
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Solyc03g093560	K4BIN3	2.1184	0.000335047
Solyc10g079930	Unknown	2.11199	0.000335047
Solyc01g103650	K4B1U0	2.10547	0.000335047
Solyc03g117590	E2DDU6	2.1054	0.000335047
Solyc08g079230	K4CNT1	2.10499	0.000335047
Solyc02g031790	K4B5C6	2.08887	0.000335047
Solyc08g078190	K4CNH8	2.08591	0.00344522
Solyc10g081040	Unknown	2.07951	0.0455745
Solyc06g034340	K4C4Q5	2.07286	0.00542908
Solyc12g096960	K4DHG9	2.0654	0.000902265
Solyc08g062360	K4CL24	2.06335	0.000335047
Solyc01g097270	K4B0B4	2.05203	0.000335047
Solyc04g074400	K4BTX8	2.05194	0.0359487
Solyc05g008980	K4BX72	2.05001	0.000335047
Solyc01g021600	K4AV41	2.02298	0.00975289
Solyc10g083690	Unknown	2.01385	0.000335047
Solyc02g082920	Q05539	2.00936	0.000335047
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Solyc02g087540	K4BBR1	1.99001	0.000335047
Solyc10g080010	K4D2P2	1.98571	0.000335047
Solyc01g008497	Unknown	1.98489	0.000335047
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Solyc04g054950	K4BSP3	1.97154	0.000335047
Solyc01g005040	K4AS71	1.96691	0.00855758
Solyc03g098740	K4BJT7	1.96665	0.0181551
Solyc12g007050	K4DBP2	1.96656	0.0409569
Solyc08g006740	Q1KSC4	1.95215	0.000335047
Solyc01g098590	K4B0P6	1.95132	0.000335047
Solyc08g075550	Q84V46	1.95027	0.000335047
Solyc06g007580	K4C3D9	1.94013	0.0394604
Solyc06g062920	Q94FU1	1.93769	0.000335047
Solyc10g085860	Unknown	1.93357	0.0439106
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Solyc02g068040	Unknown	1.91998	0.000335047
Solyc02g092860	Unknown	1.9185	0.0172039
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Solyc08g068690	K4CM91	1.90889	0.000335047
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Solyc02g062550	K4B6E3	1.89734	0.000335047
Solyc08g007460	K4CIN3	1.89654	0.000335047
Solyc11g006730	K4D4T4	1.8936	0.0480598
Solyc10g006710	Unknown	1.88619	0.000335047
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Solyc06g082440	K4CAA3	1.85848	0.000335047
Solyc04g011480	K4BPK1	1.8526	0.00116207
Solyc05g012180	K4BXU1	1.84711	0.0199167
Solyc10g086280	K4D460	1.84518	0.000335047
Solyc04g074000	K4BTT8	1.84325	0.000335047
Solyc04g074440	K4BTY2	1.84227	0.000335047
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Solyc03g120900	K4BMN9	1.82859	0.000335047
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Solyc03g124110	Q674Z8	1.78846	0.0226178
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Solyc03g006550	K4BE78	1.78672	0.000335047
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Solyc08g068600	K4CM83	1.78352	0.000335047
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Solyc04g016230	Unknown	1.66656	0.000335047
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Solyc10g085880	Unknown	1.64263	0.00235717
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Solyc05g052670	K4C1U8	1.64167	0.0176198
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Solyc07g053420	K4CFN9	1.6028	0.0427282
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Solyc02g089130	K4BC67	1.55057	0.000625873
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Solyc01g010060	Unknown	1.49191	0.0264869
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Solyc09g007520	K4CQE1	1.47671	0.00189539
Solyc07g055560	K4CG90	1.47613	0.000335047
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Solyc09g075920	Unknown	1.4676	0.000335047
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Solyc07g008140	K4CBT3	1.46069	0.000335047
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Solyc12g044950	K4DFH9	1.45087	0.000335047
Solyc08g080670	K4CP65	1.44815	0.000335047
Solyc12g009220	A7XXZ0	1.44762	0.000335047
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Solyc11g007400	K4D501	1.44535	0.000625873
Solyc07g045530	K4CEV9	1.44355	0.000335047
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Solyc03g082690	K4BI45	1.44272	0.000335047
Solyc12g008380	K4DBS6	1.44036	0.0378074
Solyc07g055660	K4CGA0	1.43948	0.000335047
Solyc06g050440	K4C5I8	1.43928	0.0127594
Solyc01g009690	Q9ZS79	1.43669	0.0302179
Solyc11g072470	K4DAV4	1.43655	0.0332875
Solyc12g005940	K4DBD2	1.43636	0.000335047
Solyc08g076230	H1ZN90	1.43479	0.0243188
Solyc07g040890	K4CDW6	1.43454	0.000625873
Solyc09g011045	Unknown	1.43178	0.000335047
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Solyc03g111793	Unknown	1.43107	0.000335047
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Solyc07g006480	K4CBB9	1.42875	0.000335047
Solyc07g045100	K4CER8	1.42469	0.000335047
Solyc07g042520	K4CEB3	1.42178	0.000335047
Solyc12g006840	Unknown	1.42025	0.000335047

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Solyc02g077050	K4B8T2	1.41468	0.000335047
Solyc01g080260	K4AXS0	1.41301	0.000335047
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Solyc12g096350	K4DHA8	1.41054	0.00323466
Solyc10g078720	K4D2B4	1.40949	0.000902265
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Solyc02g081970	K4BA67	1.4008	0.000335047
Solyc04g014900	K4BPZ3	1.40009	0.000335047
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Solyc10g084340	K4D3M0	1.38717	0.000335047
Solyc08g006750	K4CIG3	1.38712	0.000335047
Solyc07g064870	K4CHG6	1.38642	0.000335047
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Solyc05g009040	K4BX78	1.38467	0.000335047
Solyc03g007290	K4BEF2	1.38168	0.00958883
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Solyc02g085010	K4BB15	1.37547	0.0336936
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Solyc04g050570	K4BS26	1.36585	0.000335047
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Solyc05g009000	K4BX74	1.35206	0.000335047
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Solyc01g109110	K4B3C5	1.33068	0.000335047
Solyc12g087940	K4DGL5	1.33063	0.000335047
Solyc07g005100	K4CAY2	1.33042	0.000335047
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Solyc03g043700	K4BFZ1	1.30207	0.0107718
Solyc12g096710	K4DHE4	1.30087	0.000335047
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Solyc04g048900	K4BRL8	1.28806	0.000335047
Solyc02g072240	K4B8J8	1.28786	0.000335047
Solyc05g041540	K4C0D5	1.28756	0.00212886
Solyc08g062330	K4CL21	1.28624	0.000335047
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Solyc08g081620	Q42871	1.26654	0.000335047
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Solyc09g091600	K4CWI6	1.26074	0.000335047
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Solyc10g075090	K4D1V1	1.24273	0.000335047
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Solyc01g107170	I3NN78	1.23798	0.00165512
Solyc02g061770	K4B667	1.23699	0.0038445
Solyc02g092750	K4BD71	1.23624	0.000335047
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Solyc07g053740	K4CFR9	1.23061	0.000335047
Solyc08g079700	K4CNX0	1.2293	0.000335047
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Solyc11g007770	K4D538	1.22376	0.000335047
Solyc01g006790	K4ASP5	1.21983	0.000902265
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Solyc03g123370	K4BN36	1.21361	0.000335047
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Solyc05g005150	K4BW46	1.2046	0.000335047
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Solyc09g075580	Unknown	1.05316	0.0397226
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Solyc07g005840	K4CB55	1.05132	0.000625873
Solyc06g073830	K4C9A1	1.05003	0.00820511
Solyc09g075820	Q9STA8	1.04982	0.000335047
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Solyc06g050920	K4C5N6	1.03875	0.00618561
Solyc01g006300	K4ASJ6	1.03852	0.000335047
Solyc08g041860	K4CK46	1.03804	0.000335047
Solyc12g019705	Unknown	1.03716	0.00404789
Solyc08g005560	K4CI47	1.03699	0.000335047
Solyc11g005720	K4D4I5	1.0365	0.00165512
Solyc02g063020	K4B6J0	1.03644	0.000902265
Solyc11g042650	Unknown	1.03597	0.0354353
Solyc08g081240	K4CPC1	1.03584	0.000335047
Solyc02g093230	K4BDB9	1.0355	0.000335047
Solyc01g112220	K4B483	1.033	0.000335047
Solyc04g015970	K4BQ98	1.03256	0.000335047
Solyc02g078320	K4B956	1.03253	0.00258211
Solyc03g123460	K4BN45	1.02836	0.000335047
Solyc01g106820	K4B2P9	1.02737	0.000335047
Solyc02g078810	K4B9A5	1.02708	0.00116207
Solyc10g084930	K4D3S7	1.02659	0.000335047
Solyc03g034370	Unknown	1.0262	0.00235717
Solyc07g054080	K4CFV3	1.02217	0.000335047
Solyc03g119250	K4BM74	1.02162	0.000335047
Solyc06g066170	K4C7P5	1.01873	0.000335047
Solyc11g045460	K4D8L0	1.01873	0.0111187
Solyc09g090470	K4CW73	1.01784	0.000335047
Solyc11g008250	K4D585	1.01711	0.000335047
Solyc02g085110	K4BB25	1.01694	0.000335047
Solyc10g018907	Unknown	1.01635	0.0142322

Solyc10g086390	K4D471	1.0142	0.0461112
Solyc08g068780	Q8RXB6	1.01368	0.000335047
Solyc01g007010	K4ASR7	1.01266	0.00323466
Solyc11g044450	K4D8B3	1.01173	0.00116207
Solyc06g075170	K4C9N3	1.0099	0.000335047
Solyc01g105070	K4B277	1.00961	0.000335047
Solyc10g079420	K4D2I3	1.00943	0.000335047
Solyc02g077780	K4B903	1.00771	0.000335047
Solyc12g094700	K4DH42	1.00667	0.000335047
Solyc01g095030	K4AZP3	1.00659	0.000335047
Solyc08g080940	K4CP92	1.00571	0.000335047
Solyc04g078630	K4BUU8	1.00499	0.00482596
Solyc05g052570	K4C1T8	1.00499	0.000335047
Solyc06g005650	Unknown	1.00134	0.000335047
Solyc04g082270	K4BVV4	1.00095	0.000335047
Solyc11g010330	K4D5J4	1.00089	0.000335047
Solyc01g060130	P52884	1.00055	0.000335047
Solyc10g086710	K4D4A2	1.00023	0.00364626

Table S4.3 Genes significantly downregulated by BC204 treatment in *Solanum lycoperscum* shoot tissue as annotated by PANTHER Classification

Gene ID	UniProt identifier	log_2fold change	q_value
Solyc03g058990	K4BGU6	-100	0.00600167
Solyc10g031550	Unknown	-100	0.00600167
Solyc09g089520	K4CVX9	-2.65401	0.000335047
Solyc05g018850	Unknown	-2.30756	0.00600167
Solyc05g005100	K4BW41	-2.26547	0.0336936
Solyc09g089540	K4CVY1	-2.17884	0.000335047
Solyc00g071180	Unknown	-2.13305	0.000335047
Solyc12g033060	K4DDX4	-2.07468	0.00165512
Solyc01g058100	Unknown	-2.0276	0.0441481
Solyc04g058010	Unknown	-1.9946	0.000335047
Solyc04g011800	K4BPN3	-1.92744	0.0251191
Solyc03g098760	K4BJT9	-1.86993	0.000625873
Solyc00g203660	Unknown	-1.85484	0.0363054
Solyc01g060085	Unknown	-1.84275	0.0465509
Solyc10g017890	Unknown	-1.754	0.00906969
Solyc08g013758	Unknown	-1.68105	0.000335047
Solyc01g011010	Unknown	-1.6728	0.00116207
Solyc10g048050	K4D064	-1.65381	0.00344522
Solyc03g115950	K4BL97	-1.62774	0.00522926
Solyc12g005310	K4DB69	-1.58708	0.00189539
Solyc11g021240	Unknown	-1.5095	0.0385582
Solyc12g096780	K4DHF1	-1.50205	0.000335047
Solyc02g072020	Unknown	-1.48834	0.0170627
Solyc11g071480	K4DAK5	-1.47676	0.00141231
Solyc01g109220	K4B3D5	-1.47107	0.000335047
Solyc04g050621	Unknown	-1.45407	0.0330313
Solyc01g008420	K4AT40	-1.45376	0.0244462
Solyc10g008180	K4CXZ5	-1.43905	0.0210018
Solyc01g110605	Unknown	-1.4323	0.000335047
Solyc09g055950	K4CT90	-1.40112	0.0400946
Solyc09g089530	K4CVY0	-1.39893	0.000335047
Solyc11g010420	K4D5K3	-1.39858	0.000335047
Solyc02g092510	K4BD47	-1.39747	0.000335047
Solyc03g098640	K4BJS7	-1.39722	0.000335047
Solyc04g057993	Unknown	-1.39654	0.0122667
Solyc01g005590	K4ASC5	-1.38762	0.000335047
Solyc01g095960	K4AZY5	-1.38088	0.000335047
Solyc03g005900	K4BE13	-1.36915	0.000335047
Solyc06g083050	K4CAG0	-1.36261	0.00958883
Solyc04g008810	K4BP37	-1.35462	0.000335047
Solyc07g007440	K4CBL4	-1.35025	0.00189539
Solyc05g007830	K4BWV8	-1.34978	0.000335047
Solyc08g077190	Unknown	-1.34239	0.00116207

Solyc03g098790	Q9LEG1	-1.33816	0.000335047
Solyc04g014857	Unknown	-1.33646	0.0153493
Solyc08g081960	K4CPJ2	-1.32837	0.000335047
Solyc11g071470	K4DAK4	-1.32669	0.000335047
Solyc12g089230	K4DGZ4	-1.32444	0.000335047
Solyc09g092320	K4CWQ8	-1.30813	0.000335047
Solyc03g098720	K4BJT5	-1.30732	0.00141231
Solyc11g044600	Unknown	-1.30687	0.0397226
Solyc01g098400	K4B0M7	-1.2982	0.000335047
Solyc11g012520	K4D656	-1.29479	0.0379341
Solyc07g049270	K4CF30	-1.27892	0.00141231
Solyc08g062620	K4CL49	-1.27878	0.000335047
Solyc04g005770	Unknown	-1.26902	0.0256691
Solyc06g035760	Unknown	-1.26585	0.00404789
Solyc11g020890	K4D740	-1.25038	0.000335047
Solyc03g005180	K4BDU2	-1.24937	0.0170627
Solyc03g025700	Unknown	-1.24868	0.000335047
Solyc04g005460	Unknown	-1.23658	0.0263505
Solyc09g066430	K4CUR8	-1.23194	0.000335047
Solyc08g079690	K4CNW9	-1.2266	0.000335047
Solyc06g071530	K4C8M5	-1.22127	0.000335047
Solyc03g116170	K4BLB9	-1.22029	0.000335047
Solyc05g052880	K4C1W8	-1.21161	0.0450916
Solyc11g064800	K4D993	-1.21043	0.000625873
Solyc12g014500	K4DCY5	-1.20759	0.0492335
Solyc02g080320	K4B9Q3	-1.20438	0.000335047
Solyc01g016470	Unknown	-1.20422	0.000335047
Solyc06g008260	K4C3K6	-1.19961	0.000335047
Solyc08g066610	K4CLN7	-1.19646	0.000335047
Solyc02g062240	K4B6B3	-1.17957	0.000335047
Solyc10g085900	K4D424	-1.17732	0.000335047
Solyc08g006900	K4CIH8	-1.17583	0.000335047
Solyc10g054470	K4D145	-1.1708	0.00165512
Solyc12g010350	K4DCC2	-1.16805	0.000335047
Solyc02g092200	Unknown	-1.15635	0.000902265
Solyc09g007350,Solyc10g0	K4D3L7	-1.15592	0.000335047
84310,Solyc10g086020			
Solyc06g083650	K4CAM0	-1.15583	0.000335047
Solyc01g097670	Unknown	-1.15088	0.000335047
Solyc12g088470	K4DGR8	-1.14979	0.0083703
Solyc11g042820	K4D849	-1.14829	0.00820511
Solyc09g090360	K4CW62	-1.14739	0.00141231
Solyc11g042610	K4D831	-1.1445	0.000335047
Solyc10g084265	Unknown	-1.14274	0.000335047
Solyc03g034206	Unknown	-1.13946	0.000335047
Solyc01g067030	K4AWV2	-1.13895	0.0038445

Solyc08g007140	Unknown	-1.13411	0.000335047
Solyc03g080170	K4BHZ3	-1.13112	0.00323466
Solyc04g072150	K4BTK2	-1.13054	0.000335047
Solyc04g072660	K4BTQ3	-1.13011	0.000335047
Solyc04g078460	K4BUT1	-1.12927	0.000335047
Solyc02g091300	K4BCS8	-1.12794	0.0242138
Solyc12g010960	Unknown	-1.12241	0.000335047
Solyc03g006100	K4BE33	-1.11914	0.0167989
Solyc02g089150	K4BC69	-1.11053	0.0327973
Solyc11g067030	K4D9W0	-1.11022	0.00258211
Solyc08g014550	K4CJ85	-1.10706	0.000335047
Solyc03g096360	K4BJ57	-1.10581	0.000335047
Solyc11g068420	K4DA00	-1.10581	0.000335047
Solyc11g071490	K4DAK6	-1.10568	0.000335047
Solyc06g073300	K4C949	-1.10507	0.000335047
Solyc04g008110	K4BNW7	-1.10484	0.000335047
Solyc07g065610	K4CHN9	-1.10213	0.00820511
Solyc11g008530	K4D5B3	-1.10177	0.000335047
Solyc03g078290	K4BHQ8	-1.10076	0.000335047
Solyc08g077480	K4CNA7	-1.09901	0.000335047
Solyc01g097970	K4B0I4	-1.09815	0.000335047
Solyc12g044420	K4DFD0	-1.09791	0.00116207
Solyc02g088740	K4BC29	-1.09751	0.0433286
Solyc09g075590	Unknown	-1.09355	0.000335047
Solyc01g098090	K4B0J6	-1.09246	0.000335047
Solyc04g076260	K4BU64	-1.09196	0.00280317
Solyc11g066270	K4D9N5	-1.08641	0.000335047
Solyc06g074430	K4C9G1	-1.08168	0.000335047
Solyc07g062440	K4CGS7	-1.08092	0.0132394
Solyc01g111080	E5KBY0	-1.08057	0.000335047
Solyc09g061340	Unknown	-1.07999	0.000335047
Solyc10g006570	K4CXI8	-1.07903	0.0177619
Solyc05g053440	K4C224	-1.07446	0.000335047
Solyc01g006170	K4ASI3	-1.07411	0.000335047
Solyc03g111050	K4BJW8	-1.07366	0.000335047
Solyc02g071100	K4B886	-1.0706	0.000335047
Solyc06g066660	K4C7U1	-1.06651	0.000335047
Solyc01g080100	Unknown	-1.06295	0.000335047
Solyc06g008220	K4C3K2	-1.06283	0.000335047
Solyc01g095160	Unknown	-1.06203	0.00802297
Solyc05g005690	K4BW97	-1.05871	0.000335047
Solyc06g075180	K4C9N4	-1.05719	0.000335047
Solyc10g086133	Unknown	-1.05452	0.000335047
Solyc09g097880	K4CWW0	-1.05393	0.000335047
Solyc09g082450	Unknown	-1.05376	0.000335047
Solyc11g012195	Unknown	-1.05154	0.00463898

Solyc11g056410	K4D8V2	-1.04845	0.0210018
Solyc05g052810	K4C1W1	-1.04551	0.000335047
Solyc07g008800	K4CBZ9	-1.04543	0.000335047
Solyc06g071870	K4C8Q9	-1.04486	0.000335047
Solyc09g082660	K4CVP2	-1.04472	0.000335047
Solyc09g008290	K4CQL7	-1.04421	0.0238138
Solyc10g084610	K4D3P5	-1.04349	0.000335047
Solyc01g110560	K4B3R9	-1.04188	0.0393225
Solyc01g088770	Unknown	-1.04027	0.000335047
Solyc03g120775	Unknown	-1.0393	0.000335047
Solyc06g005600	K4C342	-1.0393	0.000335047
Solyc05g012270	K4BXV0	-1.03618	0.000335047
Solyc08g008590	K4CIZ3	-1.03255	0.000335047
Solyc04g079310	K4BV15	-1.02944	0.000335047
Solyc02g070640	K4B846	-1.02608	0.000335047
Solyc02g079730	K4B9J4	-1.02527	0.000335047
Solyc12g056150	K4DFU6	-1.02223	0.000335047
Solyc12g009630	Unknown	-1.02206	0.00212886
Solyc12g039120	K4DEQ8	-1.01935	0.000335047
Solyc09g083370	K4CVW3	-1.01761	0.000335047
Solyc05g014652	Unknown	-1.01422	0.000335047
Solyc03g116020	K4BLA4	-1.01253	0.000335047
Solyc11g012670	Unknown	-1.01155	0.000335047
Solyc03g080160	K4BHZ2	-1.01	0.000335047
Solyc10g086510	K4D482	-1.00858	0.000335047
Solyc07g007760	K4CBP6	-1.00617	0.000335047
Solyc01g111070	K4B3X0	-1.00443	0.00189539
Solyc04g009190	K4BP75	-1.00228	0.000335047
Solyc06g082140	Unknown	-1.00207	0.000335047
Solyc02g069070	K4B7N9	-1.00194	0.000335047
Solyc02g082360	K4BAA6	-1.00173	0.000625873
Solyc09g065330	K4CUG0	-1.00132	0.000335047

Additional supplementary data

- Sequence data and list of all genes up- or downregulated in response to BC204

Available on request from phills@sun.ac.za

CHAPTER 5: General Discussion and Conclusion

5.1 Background

Plant biostimulants (PBs) are a relatively novel group of plant growth promoting substances from mostly biotic sources (Bulgari et al., 2015). PBs have broad effects on plant growth and physiology, which includes having the ability to enhance plant growth and increase plant stress tolerance, activating or stimulating their innate metabolism (du Jardin, 2015). PBs are usually a much cheaper alternative or supplement to current agricultural products and provide a sustainable solution to increase outputs and subsequent profit (De Pascale et al., 2017). A wide variety of beneficial effects have been reported. These include an increase in yield, improved nutrient usage (Halpern et al., 2015) and improved fruit quality (Drobek et al., 2019). Other important benefits elicited by PBs are increased tolerance towards abiotic stresses such drought and salinity (Van Oosten et al., 2017) and reduced susceptibility to a variety of pathogens. There is, however, consensus among the scientific community that there is not enough verifiable, independent, and peer-reviewed data available to explain the mechanisms underlying these positive effects elicited by PBs.

BC204 is a commercially-available PB consisting of a citrus-based plant extract and has reportedly been shown to improve the growth of a variety of crops whilst increasing their tolerance to environmental stress. However, as with many available PBs, very little data, none of which is peer-reviewed, is available that could explain and verify these effects. Molecular data is especially scarce amongst studies on PB modes of action. In one postgraduate study, it was determined that a product closely related to BC204 improved water utilisation efficiency in certain table grape cultivars (Van Zyl, 2007), although no mechanism/s for how this occurred was presented.

5.2 General Discussion

The aim of this study was to elucidate the molecular mechanisms responsible for the enhancements in plant growth and physiology observed following treatment with BC204 in the model species *Arabidopsis thaliana* and *Solanum lycopersicum,* through a combination of basic plant physiological measurements, biochemical characterisation and transcriptomic analyses. Large datasets like this would very valuable not only to the company that manufactures the product, but also to the scientific community studying PB modes of action.

In this study, we used *A. thaliana* as model plant species in physiological experiments where unstressed and NaCl-stressed plants were treated with BC204 via a soil drench. Unstressed plants, when treated with BC204, grew significantly larger than the plants in the untreated control group. The addition of either 50 mM NaCl or 100 mM NaCl resulted in smaller, stressed plants. However, plants receiving both NaCl and BC204 grew significantly larger than those only stressed with NaCl, resembling the phenotype of the unstressed control plants. Under NaCl-stress conditions, BC204-treated plants had increased fresh and dry biomasses in comparison to the untreated plants. A similar observation was made with the *in vitro* experiments. Again, NaCl-stressed seedlings were visible smaller and struggled to grow, while the addition

of BC204 reverted the phenotype back to that of the unstressed, untreated control plants. At the biochemical level, BC204 increased chlorophyll content and Fv/Fm values, while reducing stomatal conductance in source leaves. Furthermore, while NaCl significantly increased the levels of anthocyanin, proline and MDA in the shoots of untreated plants, BC204 treatment reverted the levels of these stress markers to almost the same levels observed in the untreated, unstressed control plants. Additionally, BC204 induced the expression of two NaCl-responsive genes, *RD29A* and *SOS1,* in unstressed plants, suggesting that BC204 primes the plants against stress conditions, even in the absence of a stress factor.

For the second part of the study, three-week-old *A. thaliana* plants were treated with BC204 for three weeks via a soil drench. An increase in biomass was again observed in BC204-treated plants. RNA-seq analysis was conducted on both the untreated and BC204-treated plants to determine differential gene expression elicited by BC204. A total of 8.212% of all genes were differentially expressed at various levels between the untreated and treated groups. Of these DEG, 1680 were upregulated and 1006 were downregulated. Upregulated genes were mostly involved in protein metabolism, signalling, stress, transport, cell wall biogenenis and photosynthesis. Genes involved in many other processes were also upregulated, particularly those involved in regulatory processes such as transcription. Downregulated genes were mostly involved in protein metabolism, RNA metabolism, transport, development and signalling.

The increase in cell wall metabolism, signalling, secondary metabolism and induction of genes involved in photosynthesis already suggested why the NaCl-stressed plants treated with BC204 had an increase in salt tolerance. Changes in cell wall metabolism elicited by BC204 could partly explain the increased resistance to the NaCl, although the consequences of stress to plant cell walls remain largely unstudied (Le Gall et al., 2015). Loosening of cell wall polysaccharides and other restructuring responses in the cell wall seems to be important in under abiotic stress conditions, since it enables cells and organs to expand (reviewed by Tenhaken, 2015). Although genes specifically involved in salt tolerance were not overwhelmingly induced by BC204, there are several clues in the RNA-seq data that could explain the physiological response observed in the first part of this study. In Chapter 2, the induction of *SOS1* was suggested to infer some tolerance to the salt stress. Although the RNA-seq analysis did not confirm the up-regulation of this gene, one group of transcription factors, bHLH, that were induced by BC204 have been linked to increased salt tolerance (Diray-Arce et al., 2019). Other groups of transcription factors linked to salt tolerance were also induced by BC204, including WRKY (Gao et al., 2020) and MYB (Tang et al., 2019) transcription factors. One specific transcription factor induced by BC204 is a C2H2 zinc finger family nucleic acid binding transcription factor, coded for by *AT1G27730*. Constitutive overexpression of this transcription factor enhanced the tolerance of *A. thaliana* to salinity, heat and osmotic stress (Mittler et al., 2006). In fact, many transcription factor families, including WRKY and MYB TFs, have diverse roles spanning several developmental processes in the plant as well as roles in a variety of abiotic and biotic stresses (Hoang et al., 2017). At least some of these up-regulated TFs therefore are most likely to play a role in the enhanced salinity tolerance observed in *A. thaliana* plants following BC204 treatment.

Lastly, the same RNA-seq approach was adopted for hydroponically grown *S. lycopersicum* plants treated with BC204 via a foliar spray. The BC204-treated plants were visibly larger and had more biomass in both the shoots and roots. Primary root length also increased, while the stems were thicker than those of

untreated plants. After RNA-seq analysis of the shoot tissue, a total of 18.059% of all genes were differentially expressed. Of these, 2923 were upregulated and 3092 downregulated. A wide variety of different processes were altered by BC204. The upregulated processes were signalling, protein metabolism, RNA metabolism, stress, hormone metabolism and secondary metabolism. Other upregulated processes, albeit at a smaller scale, were cell wall, lipid and amino acid metabolism.

The RNA-seq analyses reported in Chapter 3 and Chapter 4 did not identify identical patterns of changing gene expression in response to BC204, but large overlaps in the major processes that are elicited by BC204 were observed. Generally, in both *A. thaliana* and *S. lycopersicum*, a large fraction of the transcriptome was altered. The *S. lycopersicum* genome is approximately 7 times larger than the *A. thaliana* genome and it was therefore expected that the number of differentially expressed genes would be greater. The major difference between the two transcriptomes, however, was that 62.55% of the differentially expressed genes in *A. thaliana* were upregulated, whereas in *S. lycopersicum* there was a more even distribution between up- and down-regulation of genes, with 48.6% and 51.4% of genes being up- and down-regulated respectively. Overall, similar processes were induced in both species, including regulatory components such as transcription factors, signalling, hormone metabolism and protein metabolism, as well as a major overlap that was observed in stress responsive genes. Differences between the two plant species were mainly related to photosynthesis and cell wall metabolism. In *A. thaliana*, a major increase in genes related to photosynthesis was observed, while in *S. lycopersicum* these represented only a small fraction of the differentially expressed genes that were altered and a slight repression of photosynthesis-related gene expression was observed. The same was true for genes involved in cell wall metabolism, which was not one of the major processes elicited by BC204 in tomato plants, where only 2.09% of all upregulated genes and 1.29% of all downregulated genes were involved in cell wall metabolism. The differences in photosynthesis and cell wall metabolism can most likely be explained by the differences in growth conditions utilised for the two plant species. Three-week old *A. thaliana* and 6-week old *S. lycopersicum* plants are at completely different growth and developmental stages and would therefore have completely different baseline transcriptomes. The *Arabidopsis* plants were grown on Jiffy™ peat disks, without additional nutrient supplementation, under relatively low light conditions (50 µmol photons.m^{-2}.s^{-1}), whereas the tomato plants were grown in a nutrient-rich hydroponics environment under supplemented daylight conditions. The *Arabidopsis* plants were treated via a soil-drench at a concentration of 0.01% (v/v), whilst *S. lycopersicum* plants were treated via a foliar spray at a concentration of 0.05% (v/v). The *Arabidopsis* plants were harvested at a point nearing the end of their vegetative growth stage, whereas the *S. lycoperosicum* plants were still at the seedling stage upon harvest. The metabolic priorities of the plants from these two experiments were therefore likely to be very different and it is therefore not particularly surprising that differing effects on the transcriptomes from these two species would have been observed.

For both species, interestingly, large clusters of genes related to biotic stress responses were elicited by BC204. This priming response is predicted to be a downstream response to large changes in transcription, protein metabolism, signalling and hormone metabolism. It is widely reported that many PBs acts as priming agents in plants, effectively making them more resistant to a variety of environmental stresses such as abiotic and oxidative stress (Kerchev et al., 2020) and biotic stresses such as pathogenesis (Ugena et al., 2018). Although certain processes were overwhelmingly influenced by BC204 treatment, the

transcriptomic changes in both species were very broad. This is not uncommon, since broad changes in physiology are usually reported and described in PBs modes of action. The few microarray and RNA-seq studies conducted on plants treated with PBs have all reported similarly broad effects at the molecular level (Trevisan et al., 2017; Weeda et al., 2014), although not at the magnitude observed in this study, where we observed considerably greater numbers of differentially-expressed genes than have been reported in all previous studies. In the root tissue of maize treated with protein hydrolysates, transport processes, cell wall organization, hormone metabolism and stress responses were observed at the transcriptomic level (Santi et al., 2017).

Based on the results of the three chapters, the BC204 mode of action, even though it is impossible to assign this to specific genes, appears to involve major changes in a complex network involving transcription regulation, signalling, hormone metabolism and stress-responsive genes, presumably controlled and driven by a large number of transcription factors, driving a broad priming response in both *A. thaliana* and *S. lycopersicum* which improves general plant growth and health.

5.3 Study strengths and limitations

A major limitation of this study was determining the ideal time to harvest the plant material following BC204 treatment. Since there was no molecular data available to establish a protocol for harvesting time, it was decided to treat plants once a week for three weeks, and to harvest the plant tissue 90 min after the fourth treatment at the end of this period. The goal of this harvesting procedure was to enable the detection of long-term changes in gene expression, as well as short-term changes happening rapidly post treatment. However, differences in their growth forms and life cycles meant that the *A. thaliana* and *S. lycopersicum* plants were at different developmental stages during these treatments. Regardless of the differences in growth conditions and method of treatment, the major changes elicited by BC204 were relatively similar in both plant species. The fact that these overlaps in effects were observed even under vastly different conditions highlights the efficacy of BC204 and suggests that it has similar effects on widely different plant species under a variety of different conditions. These observed changes include an increase in primary metabolism and large changes in gene expression and signalling, ultimately leading to a broad priming response at the genetic level.

Another limitation is that we were unable to identify critical individual genes and confirm their involvement in growth enhancement and/or enhancement of stress tolerance *via* reverse genetics. Due to the huge numbers of differentially-expressed genes that were identified, it would have been impossible within the confines of this study to have undertaken such a study. Nonetheless, the physiological results reported in Chapter 2 enabled us to directly compare the transcriptomic results with the observed physiology of plants under similar growth and environmental conditions. Overall, the physiological changes induced through BC204 expression overlapped well with the changes in gene expression that were observed during the transcriptomic analysis, which increases our confidence in the validity of the observed results.

5.4 Future prospects

The results of this study provided a broad overview of transcriptomic changes occurring in tomato and *Arabidopsis* plants treated with BC204. These results provide an excellent basis for further investigation

into the effects of BC204 on plant growth. To strengthen the arguments made in this thesis, a reverse genetics approach could be used to verify the involvement of key genes induced/repressed by BC204 treatment. This could be done in both *A. thaliana* and *S. lycopersicum*. Of particular interest in this regard are the pantheon of transcription factors, from a variety of different families, whose expression was significantly affected by BC204 treatment. A number of projects investigating transcription factors can be initiated from these datasets. Overexpressing or silencing certain transcription factors could aid in determining the effects of these transcriptional modulators on plant growth and stress tolerance, ultimately leading to the generation of GM crop varieties with enhanced growth and/or stress-tolerance. Transcription factors have been described as being key role players in salinity stress (Franzoni et al., 2020), drought stress (Joshi et al., 2016) and pathogen or pest stresses (Das et al., 2019). Transcription factors involved in abiotic and biotic stress have great potential for improved GM crop varieties (Baillo et al., 2019).

Since such a major priming response at the gene expression level was observed, experiments with biotic stresses, such as pathogenic fungi or bacteria, should be conducted to determine if the priming response also manifests at the physiological and phenotypic levels. Abiotic stress experiments should also be continued and extended to other stressors such as heat, drought and chilling, since several genes involved in these responses were induced in both *A. thaliana* and *S. lycopersicum,* and these three stresses all share a common basis relating to water availability. Additionally, proteomic and metabolomic approaches could also be utilised to illuminate the finer responses to BC204. Although proteomic results rarely correlate well with RNA-seq outputs, due to factors including RNA half-life and post-transcriptional levels of gene expression regulation (Haider and Pal, 2013), a proteomic investigation could reveal further valuable information on the BC204 mode of action. Both the *Arabidopsis* and the tomato RNA-seq datasets revealed major changes at both the RNA and protein levels, a proteomic analysis consequently would be invaluable towards unravelling the BC204 mechanisms of action. The RNA-seq results from these two species also indicated that considerable changes were enacted in secondary metabolism, and these should be further investigated using targeted metabolomic analyses, which would generate greater insight into possible mechanisms underlying biotic stress tolerance, since secondary metabolism is often related to plant defence mechanisms.

In this study, shoot tissue was used for both RNA-seq analyses, whilst only changes in root biomass were investigated, and this only in tomato. Studying the transcriptomic, proteomic and metabolomic responses of roots to BC204 treatment would provide important information that could explain the relationship between the roots and the rhizosphere. Studying root morphology such as lateral root number and length in conjunction with gene expression and possible root exudates would elucidate the BC204 mechanisms involved at the root level. The hydroponic system developed in this study for tomato could be easily adapted to collect the root exudates produced by *S. lycopersicum* after BC204 treatment. This would give insight into whether BC204 contributes to the establishment of symbiotic relationships with arbuscular mycorrhizal fungi and beneficial rhizobacteria. Although *A. thaliana* is a non-mycotrophic plant, analysing their root exudates could also be valuable since this could help to elucidate if and how BC204 contributes towards soil nutrient mobilization (Canarini et al., 2019). For *S. lycopersicum*, flower development, flowering patterns and fruit development should also be investigated in the future. This would be of particular benefit in helping to understand how BC204 affects vegetable, grain and fruit yields and quality, as has been

reported for a wide variety of crop species, including lettuce, potato, sugarcane, soybean, tomato, plum, apples and grapes, on which BC204 has been tested by its producers (N Hanekom, unpublished results).

Despite the rich bed of genetic information available for the *A. thaliana* and *S. lycopersicum* genomes, major gaps in the annotation of these genomes still remain and a considerable fraction of the transcripts from both species were poorly characterised. Reassessing the two datasets obtained here in the future would refine our current understanding of, as well as illuminate further possible mechanisms by which BC204 enhances growth and stress tolerance. Additionally, further bioinformatic analysis could be conducted to identify possible novel transcripts, since only the expression of characterised transcripts were discussed in this thesis.

5.5 Final conclusions

To our knowledge, this was the first study in which next-generation sequencing (RNA-seq) was used as the major methodological approach to elucidate the mechanisms underpinning the beneficial effects of a PB, although a few microarray-studies have previously been undertaken. This study has identified a shared, major change in gene expression patterns across numerous important metabolic processes in both *A. thaliana* and *S. lycopersicum*, despite differing experimental conditions and modes of treatment. Metabolic processes elicited by BC204 include signalling, protein metabolism, hormone metabolism and major changes in transcription factors, all of which drives a broad stress priming response. The results obtained in this study provides a perfect basis for many future projects to further elucidate and understand the mechanisms by which BC204 enhances plant growth and development. The results of this study will be invaluable to the company which produces this PB and an important addition to the current literature.

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