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The biochemical consequences of ascorbate deficiency in *Arabidopsis thaliana*

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I certify that all material in this thesis which is not my own work has been identified and that no material is included for which a degree has been previously approved from this or any other University

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Table of Contents

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Dedication

I would like to dedicate this thesis to my mother whose wonderful support and encouragement enabled me to complete my task successfully Alhamdulillah.

List of Figures

List of Tables

List of abbreviations

AsA	Ascorbic acid
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
JA	Jasmonic acid
SA	Salicylic acid
ABA	Abscic acid
Prx	Peroxidase
AOX	Ascorbate oxidase
CW	Cell wall
vtc	Ascorbate deficient mutants
LC-MS	Liquid chromatography mass spectrometry
ESI	Electrospray ionisation
CE	Collision Energy
FW	Fresh weight
TIC	Total ion chromatogram
EIC	Extracted ion chromatogram
m/z	Mass to charge ratio
Rt	Retention time
KMPA	Kernal mass peak algorithm
HL	High light
LL	Low light
NCED	9-cis-epoxicarotenoid dioxygenase
F3H	Flavanone 3-hydroxylase
FLS	Flavonol synthase
LDOX	Leucoanthocyanidin dioxygenase

Abstract

Biochemical consequences of ascorbate deficiency were studied in the leaf tissue of *Arabidopsis thaliana* ascorbate-deficient *vtc* mutants with a view of understanding the relationship between ascorbate, stress response and metabolism. Ascorbate is an important antioxidant and is also a cofactor for 2-oxoglutarate-dependent dioxygenases, which are involved in the biosynthesis of a number of metabolites. The response of wild type (Col-0) and *vtc1*, *vtc2-1*, *vtc2-2* and *vtc3-1* mutants to high light intensity, wounding and salinity was investigated using a metabolomics and proteomics approach. Metabolite profiling and comparative proteomics were performed by liquid chromatography-quadrupole time of flight mass spectrometry (LC-QToF MS) and targeted analysis of plant hormones and flavonoids by liquid chromatography triple-quadrupole mass spectrometry (LC-QQQ-MS). These combined analyses revealed the effect of ascorbate deficiency and stress on metabolites and cell wall proteins.

LC-QToF-MS based untargeted metabolite profiling methodologies were developed for analysis of metabolites on a large scale. Using this method about 3000-5000 metabolites (mass-retention time pairs) could be reproducibly detected in *A. thaliana* leaf extract and aligned between samples. Approximately 1000 metabolites were differentially expressed between WT and *vtc* mutants in different experiments. Of these, twenty eight compounds were confirmed to be differentially expressed by LC-QQQ-MS between WT and *vtc* mutants, and eight of these compounds were positively identified and validated with standards.

The plant hormones abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) have all been implicated in plant stress responses and differences in their accumulation in some of the *vtc* mutants have been reported. A systematic study of the response to stress of these hormones in several *vtc* mutants was carried out using LC- QQQ- MS. While some of the mutants showed increased SA and SA-glycoside accumulation, stress-induced ABA and JA accumulation was generally unaffected. Methods for identifying the metabolites in a targeted manner by LC- QQQ-MS was developed and were shown that all *vtc* mutants were impaired in the accumulation of anthocyanin in response to HL treatment. In strong contrast to anthocyanin, flavonol glycosides were not affected by ascorbate deficiency. Therefore, ascorbate deficiency has a specific effect on the anthocyanin biosynthesis.

Ascorbate occurs in the plant cell wall and isolation of apoplastic fluid showed that all *vtc* mutants have decreased apoplastic ascorbate compared to WT. Ionically-bound proteins were from the cell wall of *A. thaliana* leaves. Peroxidase specific activity in this fraction tended to be higher in *vtc* mutants than WT. High light intensity also increased peroxidase activity in WT and *vtc* mutants. To determine which peroxidase isoenzyme caused increased peroxidase activity, ionically-bound cell wall N-glycosylated proteins were isolated by Concanavalin A chromatography and analysed by LC-QToF-MS. Comparison of WT and *vtc2-2* grown in low light and high light identified 937 peptides significantly different between WT and *vtc2-2* and some are also affected by light intensity. Specifically, peroxidases 33 and 34 had increased abundance in *vtc2-2*.

The results show that ascorbate deficiency causes a detectable change in the metabolome of *A. thaliana* leaves, with specific effects on anthocyanin accumulation being detected. Ascorbate deficiency also influences the expression of cell wall proteins. Peroxidase activity is increased, and this response could be related to the increased pathogen resistance reported in *vtc* mutants.

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1: General Introduction

1.1 Introduction

Plants are sessile and so are unable to escape unfavourable local conditions. As a result they must be able to respond promptly and precisely in order to survive. An important aspect of these responses involves alteration of flux through different metabolic pathways, achieved through transcriptional, translational and post-translational regulation. Adverse environmental conditions such as biotic (herbivores and pathogens) and abiotic stresses (high light, mechanical wounding, drought, salinity, low and high temperature) cause perturbations in the normal redox state of the cell by the production of harmful reactive oxygen species (ROS). ROS are involved in the oxidative damage of the cell (Yang *et al.*, 2004; Jambunathan, 2010; Hancock *et al.*, 2009; Parent *et al.*, 2008; Gill and Tuteja, 2010a). Plants are able to defend themselves against these stresses by scavenging ROS using their efficient defence system which consists of a network of antioxidant enzymes and small antioxidant and signalling molecules.

In addition to the well-characterised role of the antioxidants, plants produce a wide variety of secondary metabolites (secondary = not essential for growth) which also have protective effects against biotic and abiotic stress. Secondary metabolites are species specific and play a very important role in plant adaptation to adverse environmental conditions. The very common examples of secondary metabolites are phenylpropanoids, flavonoids, glucosinolates, terpenoids and alkaloids.

Metabolomic approaches (e.g. metabolite profiling by NMR, GC-MS and LC-MS) are being increasingly used to analyse secondary metabolites in a complex biological mixture of different tissues.

In this thesis, I focused on characterising the accumulation of secondary metabolites and ionically-bound cell wall proteins in response to stresses in *Arabidopsis thaliana vtc* mutants (these have low ascorbate (vitamin C) levels, a key defence metabolite of plants). Plants were treated with different abiotic stresses and analysed for their biochemical changes as affected by both stress and ascorbate-deficiency.

1. 2 Reactive oxygen species and oxidative stresses

Various abiotic stresses lead to a state of "oxidative stress" in plants, characterised by the overproduction of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals. ROS are versatile molecules mediating various cellular responses, including programmed cell death (PCD), hormonal signalling, metabolic activation, growth and development. ROS generation in certain tissues needs to be kept in certain limits because ROS are not only involved in oxidative damages but also act as a signal which is important for physiological functioning (Apel and Hirt, 2004; Mittler *et al.*, 2004; Mori and Schroeder, 2004). For example, ROS generation seems to be closely linked with calcium fluxes, which are involved in the activation of signalling proteins such as kinases (Salmeen *et al.*, 2003). In the guard cells, H_2O_2 activates the calcium-permeable cation channels leading to stomatal closure (Pei *et al.*, 2000). Under physiological conditions, ROS production and ROS scavenging mechanisms are balanced. It causes an imbalance when the

production/scavenging system is perturbed by stresses such as changed temperature, light intensity, UV radiation, drought, salinity, pathogens and heavy metals (Banfi *et al.*, 2004; Segmuller *et al.*, 2008; Bowler *et al.*, 1994).

In either case, controlled synthesis of ROS, biochemical response and rapid utilisation or removal is essential. Overproduction of ROS in response to different stresses is very toxic to the cells, causing widespread oxidation of carbohydrates, lipids, proteins and nucleic acids leading to cellular damage and ultimately, death. Understanding the plant defence mechanisms in response to environmental stresses is a challenging yet essential task in plant biology. Production of ROS during environmental stresses is one of the major causes of reduced crop productivity, so, if we understand more, maybe we could minimise crop losses and increase food availability (Mittler, 2002; Mahajan and Tuteja, 2005).

1. 3 ROS and their generation

ROS is a term used for the oxygen species relatively more reactive than molecular oxygen. These are the species which are produced by sequential reduction of molecular oxygen (O_2) during oxidative stresses, as well as under physiological conditions in different parts of the plant cell. They are categorised into free-radical and non-radical species. The free radicals are: superoxide radical ($O_2^{\cdot}$), the protonated perhydroxyl radical ($HO_2^{\cdot}$), and the hydroxyl radical ($HO^{\cdot}$) which is the most harmful ROS. The non-radical species are hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Gill and Tuteja, 2010b; Smirnoff, 2005). The major sites of ROS production are the mitochondrial electron transport chain (MET) and the

photosynthetic electron transport chain (PET) in the chloroplasts. ROS are also produced in the apoplast by NADPH oxidase and peroxidase of the cell wall (Navrot *et al.*, 2007; Pfannschmidt *et al.*, 2009; Del Rio *et al.*, 2002; Bolwell *et al.*, 2002; Apel and Hirt, 2004, Foreman *et al.*, 2003). During biotic and abiotic stresses these enzymes are more active in generating ROS, especially H_2O_2 and superoxide. The major oxygen consuming processes are associated with photosynthesis. Here, ROS are produced by the direct reduction of molecular oxygen to yield superoxide radicals (Pfannschmidt *et al.*, 2009). Although H_2O_2 is less reactive than superoxide its removal is critical because it inactivates the thiol-dependent bisphosphatase enzymes in the Calvin cycle (Tanaka *et al.*, 1982). It is also converted into the hydroxyl radical *via* the Fenton reaction (Equation 1) (Halliwell and Gutteridge, 1999) which is the most powerful oxidising species reported in biological systems. In addition to these, over reduction of photosystem II components causes oxidative damage directly by conversion of molecular oxygen to high-energy singlet oxygen (Asada, 2006). Light dependent ROS production is very harmful in the chloroplast where irreversible damage to photosynthetic components can occur (Foyer, 2010).

Atmospheric oxygen is used as an electron acceptor in the respiratory mechanisms, leading to the production of superoxide through single electron reduction of O_2. Further protonation of superoxide leads to the production of $HO_2\cdot$ and H_2O_2 species. Metals such as copper and iron are also responsible for the production of ROS (Gill and Tuteja, 2010b) by the Fenton reaction (Equation 1) in which ferrous ion is oxidised by H_2O_2 to ferric ion, a hydroxyl radical and a hydroxyl anion (Fenton, 1894).

Equation 1

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$

ROS synthesis can also be produced enzymatically as a by-product of various reactions such as photorespiratory glycolate oxidase in the peroxisomes (del Rio *et al.*, 2002), amine oxidase and oxalate oxidase in the apoplast (Bolwell and Wojtaszek, 1997), xanthine oxidase and other enzymes involved in the oxidation of fatty acids in the peroxisomes (Szarka *et al.*, 2007; Corpas *et al.*, 2001). A schematic diagram for the ROS generation and their site of production is shown in Figure 1.1.

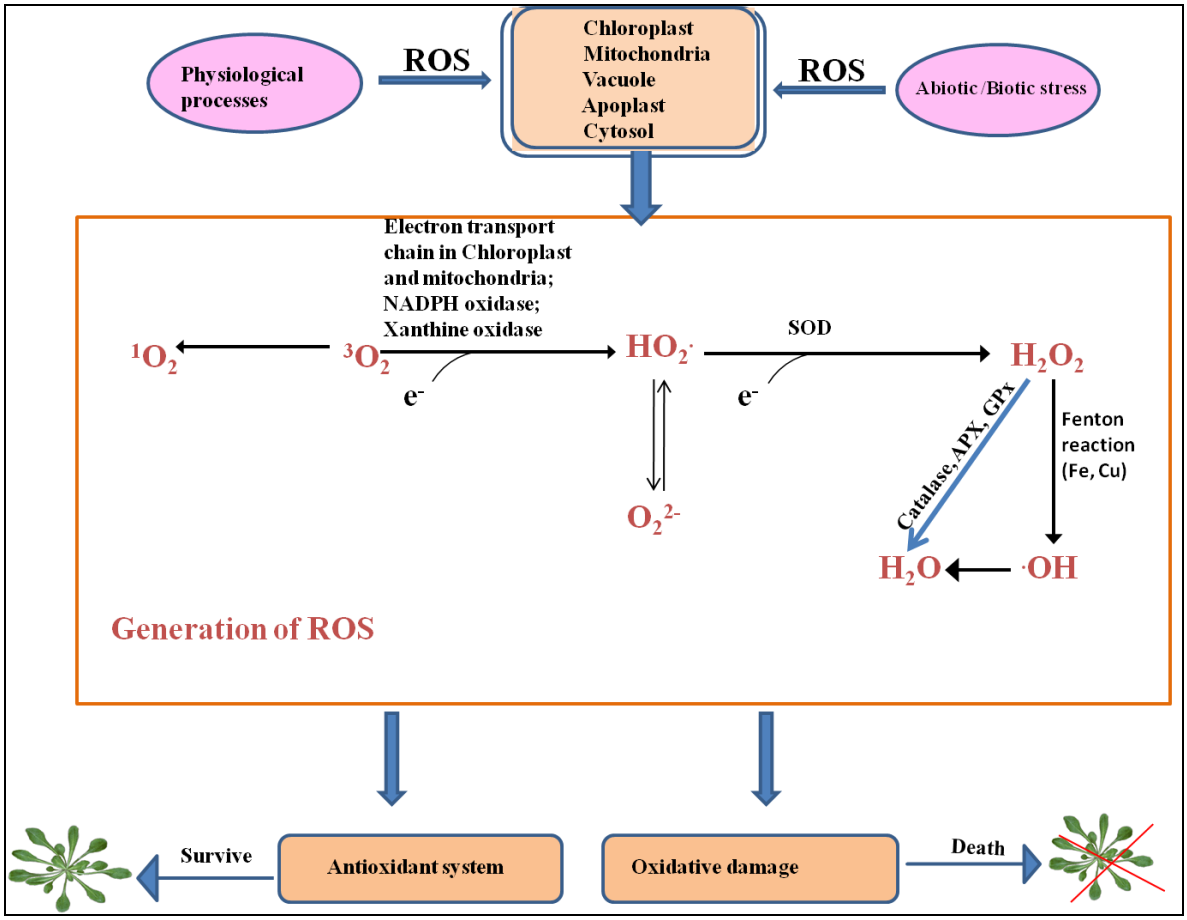


Figure 1.1 Schematic diagram representing ROS production. Some of the important enzymes involved in ROS production and scavenging are illustrated. SOD = Superoxide dismutase, APX = Ascorbate peroxidase, GPx = Glutathione peroxidase (Mori and Schroeder, 2004).

1. 4 Antioxidant system

Plants adjust their capacity in antioxidants activities in response to prevailing environmental conditions (oxidative stress). They have signal transduction mechanisms which can activate antioxidant system and also induce programmed

cell death (PCD). The Plant's antioxidant system is very efficient and it can protect them by coping with oxidative species. These antioxidants are enzymes or small molecular weight metabolites localised in different parts of the plant cells. For example, superoxide dismutase is a metal-dependent enzyme which disproportionates superoxide to molecular oxygen and hydrogen peroxide (Hu *et al.*, 2008). This enzyme is localised in the cytosol, mitochondria, chloroplast, apoplast and peroxisome (Del Rio *et al.*, 2003; Del Rio *et al.*, 2002). A significant increase in the activity of this enzyme was found after salt and drought treatments (Pan *et al.*, 2006). The peroxidases, including ascorbate peroxidase (APX), guaiacol peroxidase (GPOX) and glutathione peroxidase (GPX) are another most important group of antioxidant enzymes found in the chloroplast, cytosol, vacuole and apoplast of the plant cell (Asada, 1992; Asai *et al.*, 2002; Biehler and Fock, 1996; Bindschedler *et al.*, 2006; Chang *et al.*, 2009). They remove hydrogen peroxide (H_2O_2) by reducing it to water (Agrawal *et al.*, 2003; Allison and Schultz, 2004). Catalase (CAT) is involved in removing H_2O_2 generated during oxidative stresses, β-oxidation of fatty acid in the peroxisome, photorespiration and purine catabolism (Polidoros and Scandalios, 1999). Monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione oxidase and glutathione S-transferase also have important roles in the antioxidant system in *A.thaliana* (Gill and Tuteja, 2010b; Smith *et al.*, 2003). Non-enzymatic components are also crucial in the plant antioxidant system. For example, ascorbate (AsA) (known as vitamin C) and glutathione (GSH) are small molecules in the plant which act as powerful antioxidants. AsA is a reductant molecule which protects the cell from oxidative damage. It accumulates in response

to many stresses such as high light and is involved in reducing ROS and other small molecules. The oxidised form of AsA is dehydroascorbate (DHA) which is recycled by glutathione (GSH) (Figure 1.3). GSH is a disulfide reductant of many enzymes, involved in the regeneration of AsA through the ascorbate-glutathione cycle (Foyer-Halliwell-Asada cycle) and it is found in the chloroplast, mitochondria and cytosol (Chew *et al.*, 2003; Walker and McKersie, 1993). Other small molecules such as α-tocopherol, carotenoids and flavonoids are also involved in plant antioxidant systems. Similarly, proline (an amino acid) is also an elite non-enzymatic antioxidant accumulated in response to salt, high light and microbes (Fabro *et al.*, 2004; Hoque *et al.*, 2007; Tripathi and Gaur, 2004). In addition to these, plant hormones such as abscisic acid, jasmonic acid and salicylic acid are known as important signalling molecules, which provide protection against various, biotic and abiotic stresses.

1. 5 AsA: an antioxidant

AsA is a monosaccharide-derived sugar acid found in plants and many animals. Some animals like primates (monkey and apes), Guinea pigs and some species of birds cannot synthesise vitamin C (Smirnoff et al., 2004). Humans also have no capability to synthesise vitamin C, due to a lack of L-gulonolactone oxidase, the last enzyme of the AsA biosynthesis pathway. The absence of this enzyme is caused by the hereditary lack of or a defect in the gene required for its synthesis (Harris, 1996) while all other genes involved in this biosynthesis are present in the human liver (Chatterjee *et al.*, 1961). The deficiency of AsA in humans causes a disease called

scurvy. Therefore, it is called 'ascorbic acid' the name being derived from 'a' (meaning no) and *scorbuticus* (means scurvy).

Ascorbate is an important low molecular weight antioxidant present in millimolar concentration, which plays a major role in defence against biotic and abiotic stress and is proposed to be involved in the regulation of cell division (Conklin and Barth, 2004). AsA is present in all plant tissue, except dry seeds (DeGara *et al.*, 1997). It occurs in many compartments of the cell such as chloroplasts (Foyer and Lelandais, 1996); mitochondria (Jimenez *et al.*, 1997); vacuoles (Rautenkranz *et al.*, 1994); peroxisomes (Jimenez *et al.*, 1997); and apoplasts (Pignocchi and Foyer, 2003; Pignocchi *et al.*, 2006; Zechmann *et al.*, 2011). The foliar concentration of AsA is high, particularly in the chloroplast. It is transported by phloem from source to sink (Franceschi and Tarlyn, 2002).

AsA is a very important ROS scavenger because it has the ability to donate an electron in an enzymatic or non-enzymatic manner. It is perhaps the most important plant antioxidant involved in removing H_2O_2 with the help of other antioxidant enzymes and small molecules (Smirnoff, 2005; Athar *et al.*, 2008). Ascorbate is oxidised when acting as a reductant for dioxygenases (Smirnoff, 2000).

AsA is a strong reducing agent; its stable oxidized form is L-dehydroascorbate (DHA). The structure of ascorbic acid and its oxidised forms are shown in Figure 1.2. Under physiological conditions, ascorbate is predominately present as the ascorbate anion. It readily loses an electron from the ene-diol group to produce the monodehydroascorbate (MDHA) radical which is also called ascorbyl radical. MDHA is converted into ascorbate and its oxidised form dehydroascorbate (DHA)

by disproportionation of the two molecules. Alternatively, MDHA is reduced to ascorbate in the presence of pyridine-nucleotide dependent monodehydroascorbate reductase (Smirnoff, 2005; Smirnoff, 2011). DHA can be converted to AsA by DHAR, a GSH-dependent enzyme. In this reaction, GSH is oxidised to GSSG which is recycled by GR, using NADPH shown in Figure 1.3. AsA recycling is important in both protections from oxidative damage by H_2O_2 and also in peroxide-related signalling (Foyer and Noctor, 2011). Enzymes involved in recycling of ascorbate and glutathione have not been reported in the apoplast (Castillo and Greppin, 1988). There is also evidence that reducing power in the form of NADPH is not available in the apoplast (Vanacker *et al.*, 1998); therefore, recycling of AsA and GSH takes place using the cytoplasmic machinery. In the apoplast AsA is converted into its semi-oxidised form MDHA, by the action of ascorbate peroxidase (APX) (De Pinto and De Gara, 2004). MDHA is a very unstable compound and cannot exist in the cell for long, so it accepts electrons from membrane-bound cytochrome b and is converted into AsA and DHA by the action of MDHAR (Drazkiewicz *et al.*, 2003). The DHA is transported to the cytoplasm where it is reduced to AsA by glutathione with the incorporation of DHA reductase as mentioned earlier (Horemans *et al.*, 2000).

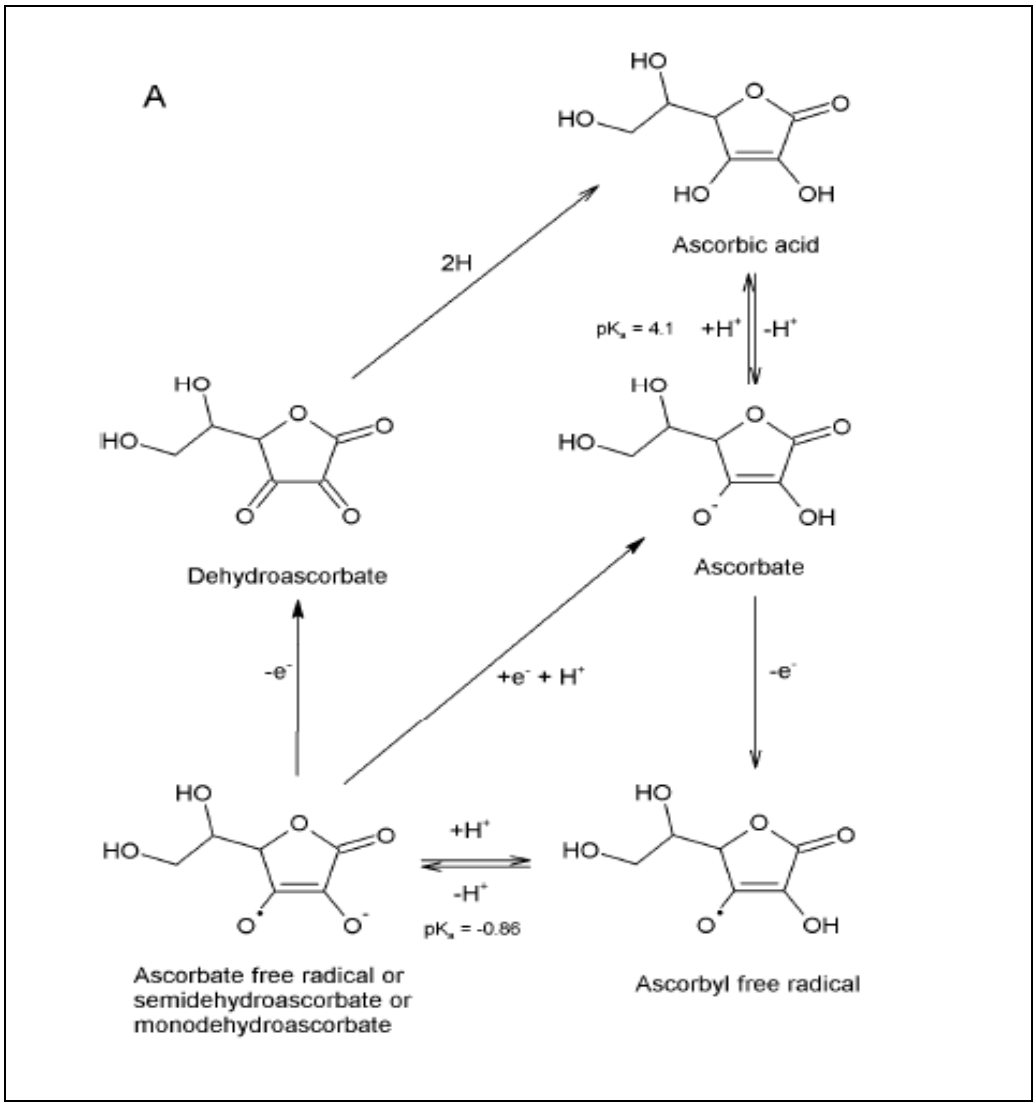


Figure 1. 2 The structure of ascorbic acid (vitamin C) and its oxidation products (Smirnoff, 2011)

AsA can protect membranes by detoxifying O_2^{2-} and HO^- non-enzymatically (Helliwell and Gutteridge, 1999), especially at lower temperature when enzymes are inactive. It is also involved in the regeneration of lipophilic antioxidant α-tocopherol from tocopheroxyl radical, which is produced by singlet oxygen-induced lipid peroxidation (Figure 1.3) (Colville and Smirnoff, 2008). In the chloroplasts in reponse to high light xanthophyll cycle is activated which involved in the dissipation of excess excitation energy as heat and protects the cell from oxidative damage. AsA acts as a cofactor for violaxanthin-de-epoxidase (VDE), the first enzyme of the xanthphyll cycle which converts violaxanthin to zeaxanthin (Neubauer and Yamamoto, 1992) (Figure 1.3). It can also protect the activity of enzymes, which contain prosthetic transition metals ions, and it is an emergency electron donor to the photosynthesis electron transport (PET) chain (Noctor and Foyer, 1998; Foyer and Noctor, 2011). AsA may also act as an emergency electron donor to the PSII when the oxygen evolving complex is inactivated by stresses (Smirnoff, 2011).

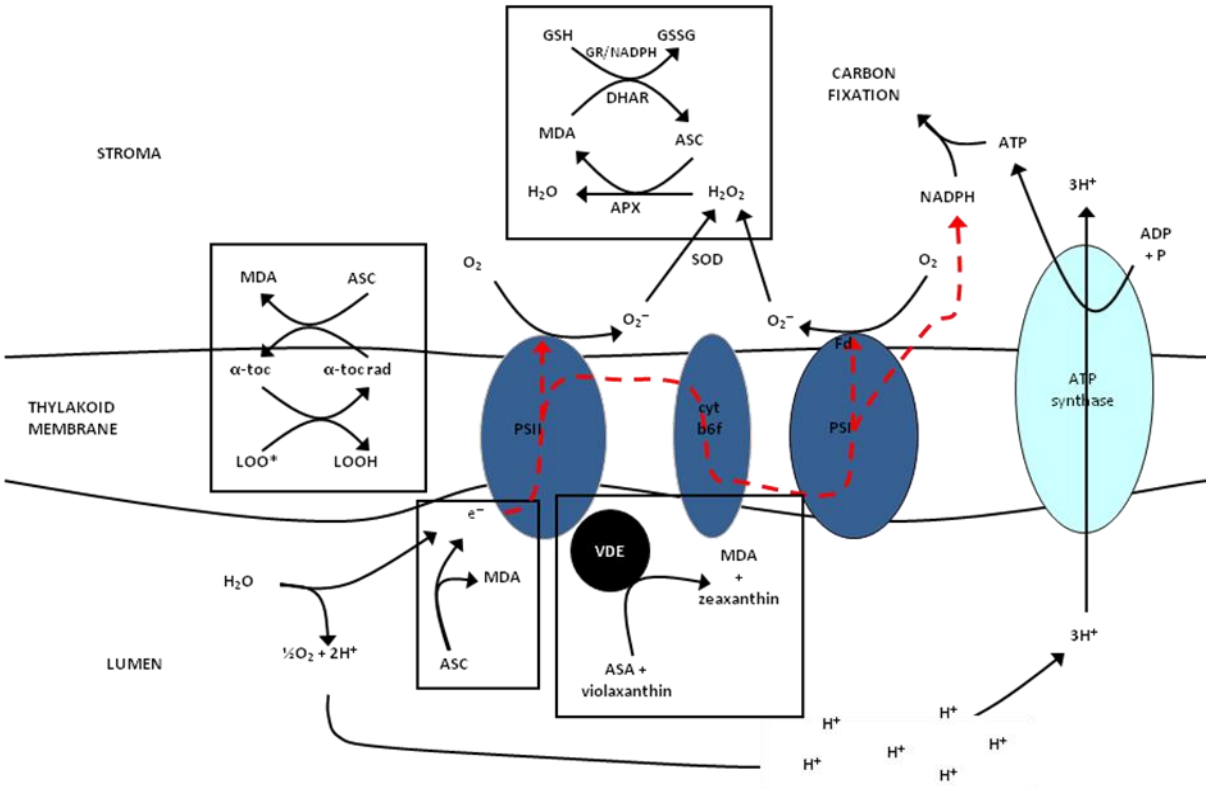


Figure 1. 3 A schematic shows the ROS scavenging mechanism of ascorbate in the chloroplast and ascorbate-glutathione cycle (Prepared by Mike Page). The model is based on results described by different researchers (Valero *et al.*, 2009, Smirnoff, 2011, Foyer and Noctor, 2011, Polle, 2001).

1. 6 2-Oxoglutrate dependent dioxygenases (2-ODD)

Ascorbate deficient (*vtc*) mutants are not only useful for studying the ascorbate biosynthesis pathway, but they are very important tools for investigating the function of ascorbate. AsA is a substrate for ascorbate peroxidase (Lad *et al.*, 2002). AsA is required for the efficient activity of enzymes designated as 2-oxoglutarate dependent dioxygenases (2-ODD) which are involved in diverse metabolic processes, including flavonoid, hormone, hydroxyproline and carotenoid biosynthesis (Prescott, 1996). As previously stated, humans cannot synthesise AsA and must obtain their requirement through their diet. Ascorbate is required for prolyl hydroxylase activity (Qian *et al.*, 1993; Murad *et al.*, 1981), a 2-ODD enzyme employed in hydroxyproline rich glycoprotein (HPRG) synthesis. HRGP is an important structural component of the cell wall (Cassab, 1998; Kieliszewski and Shpak, 2001) and mammalian collagen is also a hydroxyproline containing protein and prolyl hydroxylase is a key enzyme of its biosynthesis pathway, shedding light on the human symptoms of AsA deficiency (Myllyharju, 2003).

ASA may also be involved in plant growth. There is evidence suggesting that AsA modulates cell growth by the regulation of hydroxyproline-rich glycoproteins, which are required for the progress from the G1 to the G2 phase of the cell cycle (Smirnoff and Wheeler, 2000). Its redox state enhances the elongation mechanism at the plasma membrane (Cordoba and Gonzalez-Reyes, 1994). Flowering and senescence are also accelerated in the *vtc* mutants, suggesting a role in these major developmental processes (Barth *et al.*, 2004).

1. 7 AsA biosynthesis: the D-mannose/L-galactose (D-Man/L-Gal) pathway

The D-mannose/L-galactose (D-Man/L-Gal) biosynthesis pathway of AsA was proposed by Wheeler *et al* (1998). This pathway is supported by evidence provided by the isolation of AsA-deficient mutants (*vtc1*, *vtc2,* and *vtc4*) (Conklin *et al.*, 1999; Lukowitz *et al.*, 2001; Dowdle *et al.*, 2007; Conklin *et al.*, 2006). These mutants are compromised in their ability to produce AsA due to genetic defects in pathway genes. The AsA biosynthesis pathway in *Arabidopsis thaliana* is shown in Figure 1.4. The first step of this pathway is the conversion of glucose-6-phosphate into fructose-6-phosphate by the glucose-6-phosphate isomerase (GPI). The fructose-6-phosphate is then converted into mannose-6-phosphate. This reaction is catalysed by phosphomannose isomerase (PMI). Mannose-6-phosphate is converted into mannose-1-phosphate; a phosphate from C6 is transferred to C1 by phosphomannose mutase (PMM) (Qian *et al.*, 2007). Dephosphorylation of mannose-1-phosphate takes place and produces GDP-mannose. This reaction is catalysed by GDP-mannose phosphorylase (GMP) in the presence of GTP (Conklin *et al.*, 1999). GDP-mannose undergoes a double epimerisation yielding GDP-L-galactose by the action of GDP-mannose-3, 5-epimerase (Wolucka *et al.*, 2001). GDP-L-galactose is then converted into L-galactose-1-phosphate by GDP-L-galactose phosphorylase (GDP-L-Gal: orthophosphate guanylyltransferase), the first dedicated enzyme in the AsA biosynthesis (Dowdle *et al.*, 2007; Ishikawa *et al.*, 2006). L-Galactose-1-P is hydrolysed to L-galactose catalysed by L-galactose-1-phosphatase. In the penultimate steps of AsA biosynthesis, L-galactose dehydrogenase (LGalDH) catalyses the NAD^+-dependent oxidation of L-galactose to give galactono-1-4-lactone

(L-GalL) (Conklin *et al.*, 2006). The last step is the conversion of L-galactono-1, 4-lactone into AsA. This is an oxidation reaction catalysed by the mitochondrial-localised galactono-1, 4-lactone dehydrogenase (L-GalDH) enzyme (Mapson and Breslow, 1958). The galactose/mannose pathway is the major AsA biosynthesis pathway found in *Arabidopsis thaliana*. Some other pathways have also been identified, as in mammals (D-glucuronic acid) and Euglena (D-galacturonic acid). It has also been proposed that in addition to using the L-galactose/mannose pathway, plants can also synthesise AsA from myo-inositol /D-glucuronic acid, L-gulose or D-galactouronic acid (Smirnoff, 2001; Dowdle *et al.*, 2007; Conklin *et al.*, 1999; Conklin *et al.*, 2000; Smirnoff, 2011).

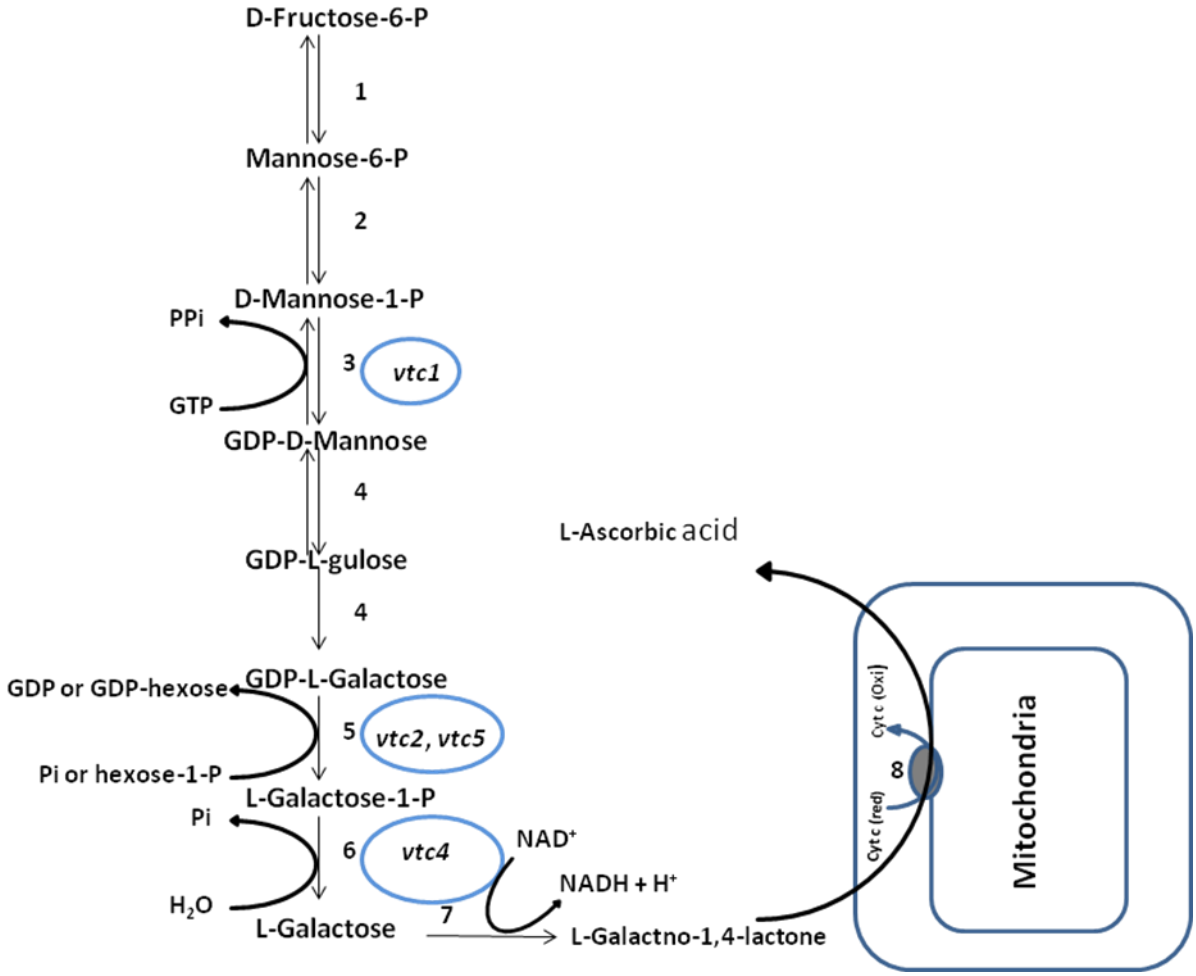


Figure 1. 4 Ascorbic acid biosynthesis pathway (Smirnoff and Wheeler, 1999, Smirnoff et al, 2001, Smirnoff, 2011), The D-mannose/L-galactose ascorbate biosynthesis pathway enzymes: 1. phosphomannose isomerase, 2. phosphomannose mutase, 3. GDP-mannose phosphorylase, 4. GDP-mannose-3, 5-epimerase, 5. GDP-galactose phosphorylase.guanolyltransferase, 6. L-galactose-1-phosphatase, 7. L-galactose dehydrogenase, 8. galactono-1-4 lactone-dehydrogenase (mitochnodrial).

1. 8 AsA-deficient mutants (*vtc*) of *Arabidopsis thaliana*

AsA-deficient mutants of *Arabidopsis thaliana* (*vtc1*, *vtc2-1*, *vtc2-2* and *vtc3-1*) are valuable tools for understanding the physiological role of AsA in plants. These mutants have less AsA than wild type (WT) plants. The *vtc1* plants contain 20-30% of the WT level, *vtc2-1* and *vtc2-2* contains 10-20% and *vtc3* contains about 50% of the normal concentration of the AsA (Conklin *et al.*, 1999; Dowdle *et al.*, 2007). The *vtc1* and *vtc2* mutants have defects in different ascorbate biosynthesis genes (Figure 1.4) where, *vtc1* has a mutation in GDP-mannose-pyrophosphorylase. Mapped-based cloning showed that there is a point mutation (miss-sense, C64T) located in the At2g39770 gene which results in the conversion of a highly conserved proline (P) residue to serine (S). This mutation was found not to alter the transcript level, but the activity and stability of the protein were found to be compromised at about 35% of the WT (Conklin *et al.*, 1999). The phenotypes of *vtc1* mutants may not be solely due to ascorbate-deficiency. GDP mannose phosphorylase (GMPase) mutation causes alteration in the level of protein glycosylation. GMPase is important for GDP-mannose biosynthesis, a nucleotide sugar necessary for protein N-glycosylation, and therefore, involved in the synthesis of functional cell wall polysaccharide (Lukowitz *et al.*, 2001; Helenius and Aebi, 2001; Keller *et al.*, 1999). The *vtc2* mutation is found in the first dedicated enzyme in AsA synthesis, GDP-L-galactose phosphorylase. Map-based cloning suggested the mutation for this enzyme is in At4g26850 (Dowdle *et al.*, 2007). Four different mutant alleles for *vtc2* were identified (*vtc2-1*, *vtc2-2*, *vtc2-3*, *vtc2-4*) (Conklin *et al.*, 2000). The *vtc2-1* plants have a single base pair change (G to T) at the predicted 3' splice site of intron 5 producing a low abundance and truncated

message. This shorter transcript produces a smaller protein about two thirds of the size of WT VTC2. The *vtc2-2* contains a point mutation (G to A) which results in an amino acid change (Gly223Asp) (Dowdle *et al.*, 2007). In the *vtc2-2* mutants, GDP-L-galactose phosphorylase activity is reduced (Linster and Clarke, 2008; Dowdle *et al.*, 2007). The genetic defect for the *vtc3* gene is not yet published. Preliminary investigations suggested that VTC3 is a kinase protein (P. Conklin, pers. comm.). With the completion of the *A. thaliana* genome sequence, newly created T-DNA knockout mutants deficient in AsA biosynthesis enzymes became rapidly available, further aiding the efforts to understand the role of AsA.

1. 9 *Arabidopsis thaliana*: a model system

Arabidopsis thaliana is a member of the Brassicaceae family, and is a dicotyledonous flowering plant with a broad natural distribution throughout Europe, Asia and North America. It has numerous advantages that make it an ideal model organism. *A. thaliana* has a relatively small genome size (five pairs of chromosomes comprising approximately 30,000 genes) of around 120 MB (Meinke *et al.*, 1998). In addition, A *thaliana* is a small size, has a short life cycle (about 6-8 week), and produces an abundance of seeds (thousands of seeds per plant). Its diploid nature makes it an ideal organism for genetic research (Meinke *et al.*, 1998).

An international collaborative research consortium was established by plant scientists in 1996 to sequence the whole genome of *Arabidopsis thaliana* (The *Arabidopsis thaliana* Initiative). The first two chromosomes were sequenced, and data

were published in 1999 (Mayer *et al.*, 1999; Lin *et al.*, 1999). Sequencing of the remaining three chromosomes and a comprehensive genome overview were completed in 2000 (Tabata *et al.*, 2000; Salanoubat *et al.*, 2000).

Coupled with the completion of the genome sequence, many gene knockouts mutants possessing defects in critical enzymes of different metabolic/signalling pathways were generated, and these are used as valuable tools to assess the importance of specific genes in various processes. The genetic resources available with the completion of the *A. thaliana* genome sequence in combination with other genetic and biochemical tools helped plant researchers to understand fundamental aspects of plant metabolism.

1. 10 Aims of thesis

The aim of the work described in this thesis was to determine the effect of AsA deficiency on secondary metabolites and signalling compounds, and determine their relationship with ascorbate during stress. More specifically, I aimed to: develop a rapid and robust methodology and implement new LC-MS approaches to study plant secondary metabolites in both a targeted and an untargeted manner. The method development aspects described hereafter were driven by the need to develop sensitive LC-MS based approaches and data handling strategies, able to separate and identify wide ranges of metabolites in a complex biological sample. The objectives of the current research were to:

- Analyse the effects of stress and AsA deficiency on phytohormone levels.
- Analyse the effects of stress and AsA deficiency on flavonoid accumulation in response to abiotic stress.
- Analyse the effects of ascorbate deficiency on metabolic changes in the apoplast.
- Analyse the effects of ascorbate deficiency on the activity of ionically-bound cell wall peroxidases and quantify other cell wall proteins affected by ascorbate deficiency.

CHAPTER 2

EXPERIMENTAL METHODS

Chapter 2: Experimental Method

2. 1 Plant Material

Arabidopsis thaliana vtc mutants were obtained from Patricia Conklin (State University of New York, Cortland) and all were in the Col-0 background (Conklin *et al.*, 2000).The purity of the vtc mutants seed stock was assessed. Fragments containing the single molecule polymorphism described in the introduction were amplified by PCR and sequenced.

2. 2 Growth and maintenance of seeds

2.2. 1 Surface sterilisation and plant growth

An aliquot (approximately 0.2 g) of seeds of *Arabidopsis thaliana* WT and the *vtc* mutants were transferred into 1.5 ml Eppendorf tubes for surface sterilisation. 1 ml of 70 % ethanol was added in each tube containing seeds and shaken for 5 min. The ethanol was decanted and then 1 ml mixture of 10 % sodium hypochlorite (bleach) and 0.1 % of Tween-20 were added in each tube and shaken for 5 min. The tubes were centrifuged for few seconds, and the bleach solution decanted. The seeds were extensively washed with sterilised water. The washing steps were repeated 6 times to remove all of the bleach and ethanol. Finally, 0.1% agar solution was added in the seeds and incubated at 4 ^{o}C for 3 days.

2.2. 2 Sowing seeds on soil and growth room conditions

Surface sterilised seeds were cold treated at 4 °C and imbibed in the dark for 3-4 days. After incubation, the seeds were sown onto Levington F2 compost (Scott, Marysville, USA) (4:1 compost: vermiculite) in shallow trays consisting of 4×4 pots with several seeds per pot. The pots were covered with cling film or a propagator lid and transferred to the growth room set to a short day photoperiod (8 h light/ 16 h dark, 23 °C, 70 % relative humidity) and a photosynthetic photon flux density (PPFD) of 120 μmol $m^{-2}s^{-1}$. Small seedlings (2 weeks old) were thinned out to one per pot and watered with intercept 70WG (Scott) at 1.6 g/8 L water to control sciarid fly larvae. The pots were kept covered with a propagator lid for 2-3 d to maintain the humidity. Plants were watered normally 2-3 times (after every 2-3 days) in a week with tap water.

2.2.3 Harvesting and storage of seeds

2.2.3.1 Tissue harvesting

Leaf tissue from 7-8 week old plants was harvested 1 h after the beginning of the light period. For the leaf extracts, the fully expanded middle aged leaves were selected from each strain (WT, *vtc1*, *vtc2-1*, *vtc2-2*, and *vtc3-1*) and were immediately frozen in liquid nitrogen and then stored at -80 ^{o}C. For apoplastic fluid extraction, fresh tissues were used on the day of extraction.

2.2.3.1 Seed collection

When siliques of *Arabidopsis thaliana* began to turn brown, plants were covered with a waxed paper bag. When plants had completely dried they were cut off at the base. Dried material was gently hand pressed from the outside, and seeds were poured into the small fine strainer positioned on a clean piece of paper to remove all plant debris. Cleaned seeds were transferred into a labelled tube and stored in the dark at 4 °C.

2. 3 Metabolite extractions and assays

2.3. 1 Inactivation of the metabolic pathways

Tissues were harvested and immediately stored in liquid nitrogen. Ice was used throughout the extraction process, and samples were stored at -80 ^{o}C for the inactivation of metabolic pathways prior to extraction and analysis.

2.3. 2 Ascorbate assay

2-3 fully expanded rosette leaves (0.1 g) were excised weighed and then flash frozen in liquid nitrogen. Leaf samples were ground in 1 mL of 1 % metaphosphoric acid (MPA). The homogenate was centrifuged for 5 min at 13200 x g at 4 °C. The supernatant was collected in a clean, labelled Eppendorf tube and stored at -80 ^{o}C until analysis.

The apoplastic fluid was extracted using a vacuum diaphragm pump system (ILMVAC, UK). Fresh 6-7 week old leaf tissue (WT, *vtc*1, *vtc2-1*, *vtc2-2*, and *vtc3-1*)

was excised, weighed and cut into 3 roughly equal pieces and vacuum infiltrated at -90 kPa, (3×1 min) in a phosphate buffer (25 mM K_2HPO_4 + 12.5 mM citric acid (4:1), 10 mM sorbitol, pH 3). Sorbitol was used as internal standard to measure the dilution of the apoplastic fluid. Infiltrated leaves were blotted dry, placed in a perforated tube and centrifuged for 10 min, 88 g at 4 °C to extract the buffer mixed with apoplastic fluid. The apoplastic fluid was weighed and immediately frozen and stored at -80 °C until analysis.

100 µl of each sample was combined with an equal volume of either 1% MPA (to assay reduced AsA) or 20 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 1% MPA (to assay AsA + dehydroascorbate (DHA) – referred to as "total AsA"). DHA was reduced to AsA within 30 min under the conditions used, and the AsA remained stable for at least 24 h at 21 °C. The assay mixture was passed through 0.2 µm polyvinyl difluoride (PVDF) syringe filters (Chromacol, Welwyn Garden City, UK) and AsA was concentration was measured using HPLC. Mobile phases consisted of (A) 95% water, 5% acetonitrile, 0.1% formic acid (B) 95% acetonitrile, 5% water, 0.1% formic acid (B). 20 µl of sample was injected onto a Phenomenex (Torrance, CA, USA) Luna C_{18} column (10 µm particle size, 250 x 4.6 mm) and subjected to the following gradient using a Dionex DX500 HPLC system (Dionex, Sunnyvale, USA): 0 min – 0 % B; 4 min – 40 % B; 7 min – 100 % B; 9 min – 100 % B; 10 min – 0 % B; 2 min post time. The flow rate was maintained at 1 mL min^{-1} and the assay was carried out at room temperature. AsA was detected using a SPD-10A dual wavelength detector (Shimadzu, Japan) at 265 nm and 280 nm and had a retention time of approximately 3.5 min. Data were analysed with Chromeleon software

(Dionex, Sunnyvale, USA). The peak purity of AsA was determined by monitoring the 265/280 nm signal ratio, which is ~10 for pure AsA. Interfering compounds were not found under the chromatographic conditions used. AsA was quantified using the ascorbate standard curve using the peak areas at 265 nm.

2.3. 3 Metabolite extraction and analysis from whole leaf

For metabolite profiling, (1) fully expanded leaves were harvested and flash frozen in liquid nitrogen. They were then freeze dried at -100 ^{o}C temperature and ~ 0.05 kPa for 3-4 days and ground in a Tissuelyser (Qiagen) with 3 mm tungsten beads at 25 Hz/s for 2 min. 10 mg of freeze dried tissue was extracted with 800 µl of extraction medium (80 % LC-MS) grade methanol + internal standards (4, 5, 7-trihydroxy flavanone and L-azetidine-2-carboxylic acid) and sonicated for 15 min followed by centrifugation at high speed (13000 g) for 10 min at 4 ^{o}C. The supernatant was carefully pipetted off and stored at -80 ^{o}C until analysis. (2) Apoplastic fluid was extracted using the same method as described above for ascorbate only with a slight modification in the pH of the extraction buffer (25 K_2HPO_4, 10 mM sorbitol, pH 5). The apoplastic fluid was weighed and immediately frozen and stored at -80 °C until analysis.

The samples were filtered through a 0.2 µm PVDF syringe filter (Chromacol, Welwyn, and Garden City, UK). Metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. 5 µl of sample extract was loaded onto a Zorbax StableBond C_{18} (1.8 µm, 2.1 x 100 mm) reverse-phase analytical column

(Agilent Technologies, Palo Alto, USA). Mobile phase: A was composed of 5% acetonitrile with 0.1 % formic acid in water and B was 95 % acetonitrile with 0.1 % formic acid in water. The following gradient was used: 0 min – 0 % B; 1 min – 0 % B; 5 min – 20 % B; 20 min – 100 % B; 25 min – 100 % B; 26 min – 0 % B; 9 min post time. The flow rate was 0.25 ml min^{-1} and the column temperature was held at 35 °C for the duration of the run. The source conditions for electrospray ionisation were as follows: gas temperature was 350 °C with a drying gas flow rate of 11 l min^{-1} and a nebuliser pressure of 55 psig. The capillary voltage was 3.5 kV in both positive and negative ion mode. The fragmentor voltage was 115 V and the skimmer voltage was 70 V. Scanning was performed using the auto MS/MS function at 3 scans sec^{-1} with a sloped collision energy of 3.5 V/100 Da with an offset of 5 V.

2.3. 4 Glucose-6-P assay (an extracellular marker)

Glucose-6-phosphate (G6P) does not occur in the apoplast and can be used as a marker of contamination by intracellular contents (Burkey, 1999). The apoplastic fluid (5 μl) was analysed for glucose-6-phosphate on LC-ESI-MS/MS in a targeted manner. The sample was injected onto a ZIC- HILIC column (Merck. 150 × 2.1 cm, 3.5 um particle size) with a flow rate of 0.2 mL/min of mobile phase. Mobile phase A was 100 % acetonitrile plus 0.1 % formic acid and B was 5 mM ammonium acetate plus 0.1 % formic acid in LC-MS grade water (pH = 4) using the linear gradient A/B, 0 min (90/10), 19 min (5/95) and 20 min (90/10). Glucose-6-phosphate was detected using an Agilent 6410 triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode using a transition of *m/z* 259 > 97 and *m/z* 259 >138.9. The fragmentor voltage was 50 V and the collision energy was 30 V in a negative

ionisation mode. No glucose-6-phosphate was detected suggesting that the leaf cells remained intact during extraction of apoplastic fluid.

2.3. 5 Extraction of hormones and flavonoids

For LC-MS/MS analysis of flavonoids and hormones, 10 mg of freeze dried leaf powder was mixed with 800 µl of 10 % methanol + 1 % acetic acid containing deuterated standards for hormones (0.5 ng of $^{2}H_{6}$ ABA, 5 ng of $^{2}H_{2}$ JA and 14.2 ng of $^{2}H_{4}$ SA) (Forcat *et al.*, 2008) and unlabelled umbelliferone (3.6 µg/µl) was used as an internal standard for flavonoids. The homogenate was incubated in ice for 30 min and centrifuged at 13000 g for 10 min at 4 ^{o}C. The supernatant was carefully collected in a new clean Eppendorf tube and immediately stored at -80 ^{o}C until analysis.

2. 4 Mass Spectrometry method development

2.4. 1 Development and optimisation of method for hormone and flavonoid analysis

Hormone and flavonoid analyses were performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. Optimisation of hormone separation was carried out using the deuterium labelled hormone standards and A mobile phase composed of acetonitrile with the addition of 0.1 % formic acid. The separation of phenolic compounds (flavonol glycosides and anthocyanins) on a reverse-phase column has been reported previously (Tohge *et al.*, 2005, Stobiecki *et*

al., 2006). In the light of this consideration, the C_{18} reverse-phase separation was optimised using linear gradients. Mass spectrometry conditions were also optimised in both negative and positive modes using different combinations of collision energy and fragmentor voltage. The most suitable conditions for the compounds of interest were chosen and used for further analysis and identification.

Chromatographic separation was carried out on a reverse-phase column (Agilent Eclipse plus C_{18}, 3.5 µm, 2.1 × 150 mm) at 35 ^{o}C. The solvent gradient was used as 100 % A (5 % CH_3CN: 94.9 % H_2O: 0.1 HCOOH) to 100 % B (4.9 % H_2O: 95 % CH_3CN: 0.1 % HCOOH) over 35 min for hormones and flavonoids. The following gradient was used: 0 min-0 % B; 1 min-0 % B; 5 min-20 % B; 20 min-100 % B; 25 min-100 % B; 26 min-0 % B; 9 min post-time, the flow rate was 0.25 ml/min. To avoid contamination of the MS, the first 5 min run was directed to waste as hormones/flavonoids elute after 7 min. Labelled and un-labelled hormones standards were run to optimise the conditions (collision energy and fragmentor voltage). Previously, hormones such as jasmonic acid (JA), abscisic acid (ABA), salicylic acid (SA) and SA glycosides (SA gly) were analysed by LC-MS and it was reported that they produced very high signals in negative mode with the transitions for both labelled and unlabelled $^{2}H_2$-JA, 211 > 61; JA, 209 > 59, $^{2}H_4$-SA, 141> 97; SA, 139 > 93; $^{2}H_6$-ABA, 269 > 159; ABA, 263 > 153; SA-gly, 299 > 93 (Forcat *et al.*, 2008). The collision energy and fragmentor voltage were optimised by injecting 20-40 µl of both labelled and unlabelled standards.

Due to unavailability of standards for flavonoids, endogenous flavonoid was used to optimise the conditions (CE and fragmentor). As described in Tohge *et al* (2008)

flavonoids ionised most effectively in positive mode. The optimised conditions which were common for all flavonoids and hormones are as follows: temperature for nebuliser was 350 ^{o}C; desolvation gas flow was 11 l/min and pressure was 35 psi; capillary voltage was 4000 V in positive and negative mode and dwell time (DT) was 200 ms. Collision energy (CE) and fragmentor voltage was specific for each hormone or flavonoid. The optimised collision energy and fragmentor voltage for hormones were: ABA, CE-5 and fragmentor-95 V; JA, CE-15 and fragmentor 90 V; SA and SA glycoside, CE-30 and fragmentor 135. Anthocyanin (A1-A9) fragmentor voltage was 100 V but the collision energy was different for some of the compounds. The CEs for anthocyanins (A1-A9) were as follows: A1, A2, A4 and A5 were 25 V, A3 was 15 V, A6 and A7 were 35 V and A8 and A9 were 40 V. Similarly for all flavonol glycosides (F1-F5) the fragmentor voltage was 100 V and CE was 40 V. The injection volume was 40 µl for hormones and 10 µl for flavonoids. All the metabolites were eluted in a single run depending on the ionisation mode using all of the above conditions and data were analysed and quantified using Agilent MassHunter qualitative analysis (Version B.03.01) and quantitative analysis software (Version B.03.02/build 3.2.170.21).

2. 5 Plants stress treatments

2.5. 1 Optimisation of growth chamber conditions for high light stress experiments

Plant growth conditions were as described in section 2.2.2. Light stress was imposed by transferring 7 week old plants to a high light cabinet under a PPFD of 550 µmol

m^{-2} s^{-1} and photoperiod of 8 h and temperature 23 ^{0}C (day/night). Humidity was 60 %. Plants were transferred to a high light cabinet 2-3 days prior to treatment and grown under normal growth conditions such as PPFD 120 µmol photon m^{-2} s^{-1} and a photoperiod of 8 h and temperature 23 ^{0}C (day/night). Humidity was 60 %, to settle down the metabolic pathways. In the preliminary experiment, different light conditions were tested, and the most suitable conditions were selected. Plants were grown under high light conditions for 4 d (Microclima 1000E; Snijders, Tilburg, the Netherlands) and watered every day during stress and pots were shuffled around regularly to equalise the conditions. After 4 d of stress leaf tissues were harvested and immediately frozen in liquid nitrogen.

2.5.2 Mechanical wounding

Plants were grown in normal growth conditions as described in section 2.2.2 until they were 6-7 weeks old. Plants were then wounded with a small syringe needle at 4 places on each side of the midrib. After wounding, plants were incubated under normal growth conditions for 2 h before harvesting.

2.5.3 Salt stress treatment

Plants were grown under normal growth conditions as described in section 2.2.2 until they were 6-7 weeks old. Plants were then watered with 200 mM NaCl solution. 60 ml of 200 mM NaCl was added underneath each plant which were then grown under normal growth conditions. The same procedure was repeated every 24 h for 3 days. Tissues were harvested for the analysis of hormones and other related metabolites after 72 h.

2. 6 Proteomic analysis

2.6.1 Ionically-bound cell wall protein extraction

0.1 g of 6-7 week old fresh leaf tissues was homogenised in 1 ml of ice- cold extraction buffer (50 mM HEPES + 1 mM EDTA + 2 mM DTT + One Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche, Basel, Switzerland), which was added to every 10 ml buffer). The homogenates were incubated in ice for 5 min and centrifuged at 12,000 g for 5 min at 4 ^{o}C. The supernatant containing all soluble fractions was discarded, and the pellet was washed 2-3 times with washing buffer (50 mM HEPES + 1 mM EDTA, pH 7.0) following the centrifugation each time at 12,0000 g for 10 min. The supernatant was discarded, and the pellet was re-suspended in 500 µl of extraction buffer (50 mM HEPES + 1 mM EDTA + 200 mM $CaCl_2$, pH 7) and centrifuged at 12,000 g for 10 min. The supernatant was collected in a clean, labelled Eppendorf tube and stored at -20 ^{o}C until analysis.

Alternatively, 1 g leaf tissue was extracted with 5 ml acetate buffer (5 mM potassium acetate with 0.5 M sucrose + 1 mM EDTA + protease inhibitor, pH = 4.6). The homogenate was centrifuged at 13000 g for 10 min at 4 ^{o}C. The supernatant containing soluble protein was discarded and the pellet was washed 4-6 times with washing buffer (acetate buffer pH = 4.6) to remove sucrose and protease inhibitor, following the centrifugation each time at 13,000 g for 10 min. The supernatant was discarded and the pellet was re-suspended in 2.5 ml of 0.2 M $CaCl_2$ buffer in 2 successive steps and centrifuged at 13,000 g for 10 min. The supernatant was collected in a clean, labelled Eppendorf tube and stored in ice. The pellet was re-

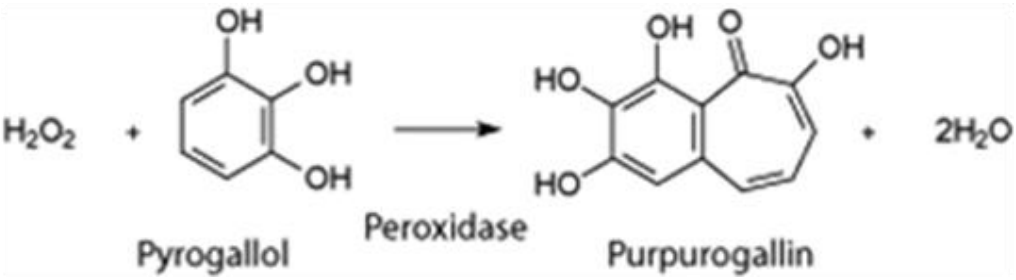
suspended in 2.5 ml 2M LiCl in 2 successive steps, and centrifuged at 13000 g for 10 min at 4 ^{o}C. The supernatant was collected and mixed with previous extracted in $CaCl_2$ and stored at -20 ^{o}C until analysis.

2.6. 2 Total protein assay

Total proteins contents were measured by Bradford assay (Bradford, 1976). 10 µl of sample was mixed with Bradford reagent in 96 well plate. The absorption of the sample was measured by a VersaMax microplate reader (Molecular devices, USA) and quantified using a standard curve. Six different dilutions of γ-globulin protein (stock 1.5 mg/ml) were used for the standard curve.

2.6.3 Peroxidase assay

Peroxidase assay was performed on a microplate reader. The assay mixture consisted of 230 µl of 50 mM HEPES (pH 7.0) containing 1 mM EDTA, 1M pyrogallol, 10 µl of extract, and 5 µl of 30 % H_2O_2 in a total volume of 250 µl. Pyrogallol and H_2O_2 react with each other in the presence of the peroxidase enzyme and produced purpurogallin (yellow colour) and H_2O. The absorbance was recorded and peroxidase specific activity was calculated using total protein contents. The extinction coefficient for pyrogallol is 1.953 mM^{-1} corrected for path length in the plate assay.



2.6. 4 Lectin affinity chromatography

A Supelclean™ LC-NH_2 SPE tube (SUPELCO, Bellefonte, USA) was emptied of its orginal contents and then filled with 1 ml of ConA Sepharose (Sigma chemical Co., St Louis, MO. USA) (Minic *et al.*, 2007) and washed with 3 ml of washing buffer (20 mM TRIS, pH 7.4 (HCl), 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). The ionically-bound cell wall protein extracts (1 ml) were loaded onto the gel and then washed with 3 ml of washing buffer. The proteins were eluted with 3 ml of 0.2 M methyl-α-glucopyranoside in the same buffer. The elute was collected and concentrated by Spin-X UF 500 concentrators (10 kDa molecular weight cut-off) (Corning, UK).

2.6. 5 Tryptic Digestion

10 µl of concentrated sample was mixed with 4 µl of 10 mM DTT (reducing agent) and 2 µl of RapiGest MS-compatible detergent (Waters corporation, Milfork, USA), and incubated for 20 min at 50 ^oC (to break disulphide bonds). 4 µl of 50 mM iodoacetamide (to donate a functional group which stops the formation of disulfide bridges) was added in the same mixture. This mixture was incubated at room temperature for 20 min in the dark. Proteins were digested with 0.1 µg trypsin (Trypsin Gold, Mass spectrometry Grade, Promega, Madison WI) and incubated at 37 ^oC for 16 h. 1 µl of 1 % trifluoroacetic acid (TFA) was added to stop the reaction and the samples were stored at -20 ^oC until analysis.

2.6. 6 Proteins analysis by HPLC-Chip

The trypsinised samples were analysed with an Agilent 6520 QToF coupled to a 1200 series HPLC-Chip interface system and the samples were loaded onto a large capacity micro C_{18} reverse-phase analytical column (Agilent Protein Identification Chip), comprising of a 160 nl enrichment column, and a 150 mm x 75 µm analytical column packed with Zorbax 300SB- C_{18} 5 µm material. The enrichment column flow rate was 3 µl min^{-1} and the analytical column flow rate was 0.4 µl min^{-1}. Mobile phase A was 2 % methanol with 0.1 % formic acid in water and B was 95 % methanol with 0.1 % formic acid in water. Complex digested mixtures were eluted with a linear gradient, 0 min – 5 % B, 10 min – 10 % B, 65 min – 30 % B, 100 min – 60 % B, 120 min – 100 % B, 122 min – 100 % B, 123 min – 5 % B with a re-equilibration time of 9 minutes.

The instrument was operated with fixed conditions: the gas temperature was 300 °C and gas flow rate was 4 l min^{-1}. The capillary voltage was 1850 V with all analysis being carried out in positive ion mode. The fragmentor voltage was 175 V and the skimmer 70 V. Scanning was performed using the auto MS/MS function at 5 scans sec^{-1} with a sloped collision energy of 3.7 V/100 Da and an offset of -2.5 V.

Protein identification was carried out using Progenesis LC-MS (Non-Linear Dynamics, Newcastle, UK), and Mascot and Spectrum Mill MS Proteomics Workbench software (Agilent, Technologies, USA). The spectra were searched against the NCBI proteins database and TAIR database (http://www.arabidopsis.org/) (See Chapter 5 for further details).

2. 7 Statistical Analyses

All experiments were repeated three times and data from a representative experiment are shown in different Chapters. Metabolite and protein data were subjected to Analysis of Variance (ANOVA) using SPSS v.16 (IBM, Chicago, USA), Progenesis LC-MS (non-linear dynamics) and Genespring GX (Agilent). Where significant effects ($p < 0.05$, $p < 0.0001$) were found by ANOVA, the treatments means were compared with the Tukey test.

CHAPTER 3

METABOLITE PROFILING OF *ARABIDOPSIS THALIANA* *vtc* MUTANTS

Chapter 3: Metabolite Profiling of *Arabidopsis thaliana vtc* mutants

3. 1 Introduction

Metabolomic analysis has emerged as the next challenge for researchers after the establishment of high-throughput DNA sequencing (genomics) and protein analysis (proteomics), to accelerate the understanding of complex biochemical interactions in biological systems. Plant cells produce a large number of metabolites, which play an important role in growth, development and defence by interacting with different biotic and abiotic stresses (Gao and Zhang, 2008; Garcia-Plazaola and Becerril, 1999; Sudha and Ravishankar, 2002; Jwa *et al.*, 2006). The overall aim of metabolomic studies is to produce comprehensive and unbiased metabolite profiling in a biological sample under certain conditions. No single analytical technique is capable of analysing the whole metabolite profile in a single biological sample due to diversity in their chemical structure. Metabolomic approaches seek to profile as many compounds as possible in a single run in a non-targeted way. Over the past few decades, the application of non-targeted metabolite analysis in plant science has increased the interest of researchers in developing tools for studying metabolomics. The most popular techniques applicable to studying the wide ranges of metabolites are proton-nuclear magnetic resonance (H-NMR) spectroscopy, gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS).

NMR is able to detect a large number of metabolites in a given biological sample. It has the advantage over other analytical techniques is that it produces a signal directly related to metabolite abundance, but it lacks the sensitivity for detecting less abundant metabolites, which can be detected by MS linked approaches (von Roepenack-Lahaye *et al.*, 2004a). GC-MS, used for the detection of volatile compounds, can detect large primary metabolites when converted to trimethylsyl (TMS) derivatives. The addition of TMS groups improved volatility of the metabolites enabling the detection with high accuracy and sensitivity (Zuo *et al.*, 2002; Zhang and Zuo, 2004).

During the last few decades, LC-MS has become a more widely used and useful technique. It offers the combination of high selectivity and sensitivity and therefore, is imperative in all metabolomic approaches. It covers a wide range of metabolite classes from very small to large molecules by both targeted and untargeted analysis (De Vos *et al.*, 2007; t'Kindt *et al.*, 2008). LC-MS, the most versatile of the three techniques is very popular in current plant metabolomic and proteomic studies. It offers advantages over GC-MS and NMR methods by being able to analyse non-volatile, polar and low and high molecular weight compounds without the need of derivatisation (Basu *et al.*, 2006; t'Kindt *et al.*, 2008; Theodoridis *et al.*, 2008; Lu *et al.*, 2008). For metabolite profiling, the development of analytical techniques and establishment of data processing methodologies are of utmost importance and a technically challenging task (von Roepenack-Lahaye *et al.*, 2004b; Zhang and Zuo, 2004; Chauton *et al.*, 2003). Recent advances in high-throughput technologies such as mass spectrometry techniques provide a fundamental platform for large scale

metabolomic studies. Previously, most analytical techniques were used for detecting specific classes of metabolites in a targeted manner. Now, untargeted metabolomic approaches have breached those limits, and it is now possible to study the large numbers of metabolites in a single sample extract in a reduced time frame. Consequently, the amount of data generated through untargeted manners is vast, and the integration of large multi-variant data sets can be very complicated. The remaining major challenge is the ability to interpret the data sets to generate statistically meaningful biological outcomes. Therefore, bioinformatic tools to handle these data sets continue to grow. Combining these new bioinformatic tools with experimental techniques discussed here provide the characterisation of the metabolites which leads to annotation and ultimately results in identification.

The following tools are necessary for metabolite profiling: (1) An algorithm for alignment of multiple samples, (2) Software for statistical tests such as Student t-test, analysis of variance (ANOVA), hierarchical clustering analysis (HCA), and principal component analysis (PCA), (3) Mass spectrometric interpretation software such as MassHunter (Agilent) for the extraction of mass spectra, retention time, isotopic distributions and empirical formulae, (4) Database search engines (e.g. Massbank, Pubchem, MetaCyc, AraCyc, Knapsack and Chemspider), (5) MS/MS fragment interpretation (e.g. ChemSketch, Sirius) and (6) Literature review.

This Chapter aims to develop a strategy for the analysis of untargeted LC-MS and LC-MS/MS generated datasets to find and potentially identify, unknown compounds differentially expressed between *Arabidopsis thaliana* wild type (WT) and ascorbate-deficient mutants (*vtc*). Metabolite profiling of both apoplasts and the

whole leaf extracts of the *vtc* mutants was the focus of this study. Until recently, very little was known about the apoplastic metabolites of *Arabidopsis thaliana*. The strategy adopted here has the potential for the putative identification of metabolites, which require further investigations to validate the structural elucidation of metabolites using NMR technology.

3. 2 Analytical techniques

3.2. 1 High Performance Liquid Chromatography-Mass Spectrometry (LC-MS)

High Performance Liquid Chromatography (HPLC) coupled to mass spectrometry (MS) is an obvious approach for the analysis of metabolites and proteins in a targeted and untargeted manner. LC-MS has proved to be a rapid and robust technology with unique capabilities for metabolomic and proteomic analysis for many years. In particular, the benefits augmented were the combination of MS and tandem MS/MS spectrometry with well accepted and commonly used 'soft' ionisation techniques such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI).

HPLC is simply defined as a technique used for separating a complex mixture of compounds based on their polarities and interaction with a stationary phase, across a mobile phase gradient between aqueous and organic mobile phase varies with time. Column composition is very important in developing methods for the analysis of different metabolite classes depending on their nature. The majority of

compounds are separated using a reverse-phase (RP) C_{18} column where, hydrophobic compounds are eluted at the end using a predominantly organic phase (acetonitrile/methanol) (Kuhlmann *et al.*, 1995). Polar compounds are not readily retained on such stationary phases. The reverse phase (RP) or non-polar stationary phase are packed with porous silica particles coated with non-polar material (styrene-divinylbenzene copolymers) (Bakry *et al.*, 2006). The limitation of the silica-based column is the mobile phase pH range of 2-7 due to limited pH stability of the silica particles (Agilent datasheet). In contrast, porous graphite carbon (PGC) is stable over the whole pH range of 0-14 (Agilent datasheet). Very polar molecules like sugars and amino acids can be separated using a column packed with porous graphite carbon or other hydrophilic interaction chemistries. Thus, it is not possible to separate the whole metabolite profile on a single LC-chromatographic system/column. Consequently, to get the most out of metabolite profiling, a combination of methods is needed to identify different classes of metabolites (Pan et al., 2006; Grumbach et al., 2004; Garcia-Plazaola and Becerril, 1999; De Vos et al., 2007). In this study, for both targeted and untargeted metabolite analysis, a reverse-phase (C_{18}) column for the large-scale separation of compounds was used.

Mass spectrometry is an analytical technique which in combination with GC or LC is used for measuring a multitude of molecules in biological and non-biological samples. The advantage of LC-MS over other spectroscopic techniques (such as NMR) is that it putatively annotates the spectral components of a compound based on their mass and retention time. The molecular mass of the small lower molecular weight organic compounds can be measured within an accuracy of 5 ppm or less

which is sufficient for the characterisation and putative identification of a compound (Venter *et al.*, 2002). Metabolites must be charged for MS analysis. The charge enables their entry into the mass analyser; fragments of desired ions are separated based on their mass to charge ratio (*m/z*). The separated ions are detected as an electronic signal converted into a digital output in the format of *m/z* and abundance. These are presented as mass spectra. The mass analyser is maintained under high vacuum, so the analytes can move from one end to other without any hindrance.

Many ionisation methods are available (Figure 3.1). The selection of the ionisation technique depends on the nature of the analysis. The most commonly used ionisation techniques for biological mass spectrometry are atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) (Hoffmann and Stroobant, 2008). These ionisation methods work in both positive and negative mode, depending on the chemical nature of the compounds under investigation. ESI was used in this study.

3.2. 2 Electrospray Ionisation

ESI was invented in the early 1980s (Yashmita and Fenn, 1984; Fenn *et al*, 1980) and has become one of the most popular ionisation methods, which is capable of ionising the metabolites and proteins in a complex biological mixture. ESI was initially developed to ionise proteins, but later it was applied to other small molecules (Hoffmann and Stroobant, 2008). ESI is described as a 'soft ionisation' technique, as it produces ions with very low internal energy so that there are minimal chances of analyte fragmentation. This method is good for polar, charged molecules and ions. It

allows the mass determination of molecules with high relative molecular masses (RMM), the size constraint being dependent on the analyser. The ions generated in the source contain multiple charges to produce a charge state distribution which is deconvoluted to calculate the RMM. It produces low background noise compared to other ionisation methods, which leads to excellent detection. It is also compatible with tandem mass spectrometry (MS/MS) (Venter *et al.*, 2002).

There are, unsurprisingly, some limitations as well. Ionisation is very sensitive to contaminants such as basic alkali and metal ions forming adducts. ESI is not good for uncharged, low polarity compounds. Ions are selected using the voltage applied to the capillary. For example, when run in positive mode, negative ions are repelled away from the source so that only positive ions are directed into the analyser. Ionisation efficiency is dependent on the voltage applied to the capillary. This is dictated by the size and functionality of the molecule.

ESI is a very sensitive ionisation method and compatible with HPLC. LC flow rates ranging from 0.05-1 ml min^{-1} for HPLC and 0.1-10 µl min^{-1} for capillary electrophoresis are eluted into the ES ionisation chamber *via* a nebuliser needle containing a surrounding nitrogen gas flow to create a plume of droplets. The needle is held at ground potential and is surrounded by a semi-cylindrical electrode (counter electrode) to which a high voltage (3-6 kV) is supplied. The potential difference between nebuliser and the counter electrode produces a strong electric field that charges the surface of the emerging liquid to form a fine spray of charged droplets. The charged droplets are then attracted towards the capillary through a counter flow of heated nitrogen drying gas. Evaporation of the solvent takes place

which shrinks the droplets through a process called desolvation. The droplets continue to shrink until the repulsive electrostatic forces of the ions exceed the droplet cohesive forces (surface tension) leading to droplet explosions (Columbic repulsion) (Ho *et al.*, 2003). This process is repeated until analyte ions are ultimately desorbed into the gas phase. The emerging gas ions are then passed through the capillary into the lower pressure region of the ion source and entre into the mass analyser (Ho *et al.*, 2003)

Once in the gas phase the ions species generated are either protonated $[M+nH]^{n+}$] in positive mode or de-protonated $[M-nH]^{n-}$ in negative ion mode. The number of protons gained or lost determines the *m/z*. ESI can result in the formation of different adducts. In positive ion mode, a proton may be replaced by Na^+, K^+ or NH_4^+. In negative ion mode, the charge may be formed by association with anion such as Cl^-, Br^-, and CH_3COO^-. Some compounds preferentially ionise as $[M + Na]^+$, $[M + K]^+$ and $[M + NH_4]^+$ are the most common positively charged adducts while $[M + CH_3COO]^-$ is the most common negatively charged adduct (Hoffmann and Stroobant, 2008).

3.2. 3 Analyser

The mass analyser filters the ions generated based on their mass to charge ratio (*m/z)*. The separation of ions according to *m/z* is based on different working principles. A number of mass analysers are available (Figure 3.1). The quadrupole time-of-flight (QToF) and triple quadrupole (QQQ) were used in this study. The QToF was used to scan a wide range of metabolites in an untargeted manner while

the QQQ mass analyser was applied in a targeted manner on metabolites of interest. The five main characteristics which can affect the performance and robustness of a mass analyser are the (a) mass range limit, (b) the duty cycle (scan rate), (c) ion transmission, (d) mass accuracy and (e) resolution.

Tandem mass spectrometry is the fragmentation of a precursor ion selected in the first quadrupole of the analyser (Q1). Collision induced dissociation (CID) is the most commonly used, and the one used here. In CID precursor ions collide with neutral gas (N_2 or He) in the collision cell and fragment into product ions. These are scanned into the analyser which may comprise a quadrupole (Q3) or TOF tube. The *m/z*, Rt and abundance of these product ions are recorded by the detector. There are a number of ion fragmentation methods shown in Figure 3.1 (Hoffmann and Stroobant, 2008).

3.2. 4 Detector

The type of detector used must have compatibility with the type of analyser. The most commonly available detectors are the Faraday Cup (FC), Electron Multiplier (EM) and the Electro-Optical Ion Detector (EOID) (Figure 3.1). The choice of the detector depends on the design of the instrument and the type of the analysis that will be performed. An electron multiplier, a widely used detector, is generally connected to quadrupoles. The most commonly used EM detectors are Channeltron, Microchannel plate and microsphere plate. In EM detectors ions from the analyser are accelerated at high frequency causing them to collide into the conversion electrode also called a dynode. Positive or negative ions striking the dynode result in

the emission of secondary electrons which in turn collide forming a cascade, thus amplifying the signal. A microchannel plate is the most commonly used and suitable of TOF analysers due to fast response time because the secondary electron path inside the channel is very short. It is a thin plate at the surface of the detector containing a set of microscopic tubes that pass from the front surface to the rear of the plate (Hoffmann and Stroobant, 2008).

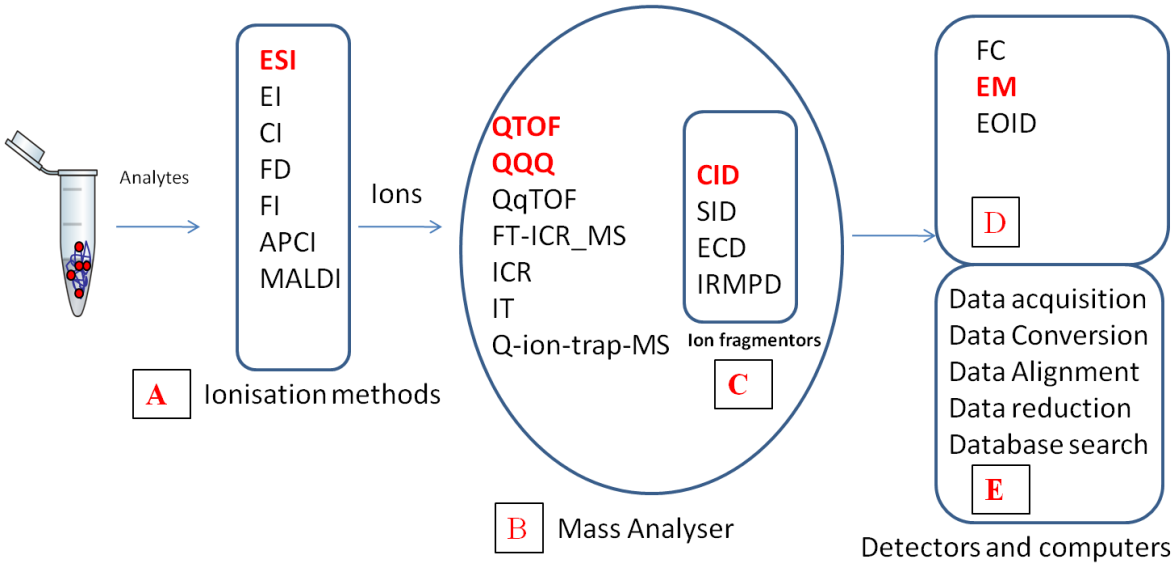


Figure 3. 1 Configurations of mass spectrometers: Mass spectrometer is based on the ionisation source, type of analyser and detector. (A) Different ionisation sources for the production of ions are: Electrospray Ionisation (ESI), Electron Impact (EI), Chemical Ionisation (CI), Field Desorption (FD), Field Ionisation (FI), Atmospheric Pressure Chemical Ionisation (APCI) and Matrix Assisted Laser Desorption Ionisation (MALDI) (B) The available mass analysers are: Quadrupole time of flight (QToF), Triple quadrupole (QQQ), Quadrupole-quadrupole Time of Flight (QqToF), Fourier Transform-Ion Cyclotron Resonance-MS (FT-ICR-MS) , Ion Trap (IT) and Q-ion-trap-MS. (C) The ion fragmentor techniques are: Collision induced dissociation (CID) surface induced dissociation (SID), infrared multiphoton dissociation (IRMPD) and Electron Capture Dissociation (ECD). ECD and CID are used for singly and multiply charged molecules such as metabolites and polypeptides. (D) Detectors are: Faraday Cup (FC), Electron multiplier (EM) and Electro-Optical Ion detectors (EOID). (E) Sets of software packages are used in data processing to complete the analysis. Labels in red text are used in the current study.

3. 3 Untargeted Metabolites Analysis by LC-MS

Untargeted metabolomic approaches lead to a complete metabolite profile in a biological sample, while targeted analyses can be applied for the detection of a specific group of metabolites. Quadrupole and triple quadrupole mass spectrometers are commonly used analysers for the metabolite profiling. Unlike primary metabolites such as amino acids, carbohydrates and lipids, the availabilities of internal standards for secondary metabolite are sparse. A literature survey suggested that developments in metabolomic studies over the last several years now offer a more holistic approach for the analysis of metabolites on a large scale. A numbers of bioinformatic tools have been developed for unbiased mass peak deconvolution and alignment of LC-MS based data, which are aimed at collating information for as many metabolites as possible in a single biological sample. These are either commercially available or open source packages, including MassHunter, Genespring MS and Mass Profiler Professional (Agilent Technologies, USA), Progenesis LC-MS, MetAlign (Lommen, 2009; Bino *et al.*, 2004; Vorst *et al.*, 2005), XCMS (Nordstrom *et al.*, 2006), MZmine (Katajamaa et al., 2006) and KMPA (personal communication-Venura Perera). Previously, a number of studies has been performed using metabolomic analysis. An example of prominent research by Illijma *et al* (2008) claimed to have identified almost 500 novel metabolites in tomato (*Solanum* l*ycopersicum)*. Similarly other untargeted metabolomic studies include: comparison of the secondary metabolites in the roots and leaves *of Arabidopsis thaliana* (von Roepenack-Lahaye *et al.*, 2004b), metabolic alteration in light hypersensitive tomato mutants (Bino *et al.*, 2005), metabolic comparison of different

developmental stages of potato (Vorst *et al.*, 2005), establishing gene-to metabolite networks in *Catharanthus roseus* (Rischer *et al.*, 2006), determining tissue-specificity of metabolic pathways in tomato fruit and a metabolomic database for tomato (Moco *et al.*, 2006; De Vos *et al.*, 2007), metabolic profiling in Arabidopsis tissue culture (t'Kindt *et al.*, 2008) and metabolite profiling of Arabidopsis biosynthetic mutants (Bottcher *et al.*, 2008).

In metabolite profiling, compound identification is not the first step of data processing. It requires a combination of strategies to achieve data alignment, followed by compound filtering, statistical and multivariate analyses to identify differential mass peaks correlating to a specific strain or treatment, which leads to the identification to some extent on the basis of accurate mass, retention time, fragmentation pattern, isotopic distributions and available standards.

3. 4 Material and Methods

Multiple strategies were developed for the LC-MS based metabolite profiling of *Arabidopsis thaliana* WT and the *vtc* mutants under normal growth and stressed conditions. The plant growth conditions, sample extraction methods and LC and MS conditions used for profiling are described in Chapter 2. The data generated using LC-QToF-MS from all strains were aligned and subjected to statistical analysis to determine the significantly expressed metabolites in different strains. The analysis of variance (ANOVA) was performed together with hierarchical clustering to produce a list of significant compounds. The data processing for differential profiling followed several steps.

3.4. 1 Compound deconvolution and alignment

(a) Deconvolution was the first step of the data processing which reduced the total ion chromatogram (TIC) into an extracted compound list. The MassHunter Molecular feature extraction (MFE) identifies co-eluting compounds with *m/z* values that represent adducts in positive (Na^+, K^+, NH_4^+) or negative (H^-, $CHOO^-$, Cl^-, and Br^-) ionisation mode. These are combined to form 'compounds' with predicted neutral mass and Rt. If no adduct was found it is assumed (possibly not always correctly) that it was $[M+H]^+$ or $[M-H]^-$. An example of deconvolution using a threshold abundance of 100 counts is shown in Figure 3.2.

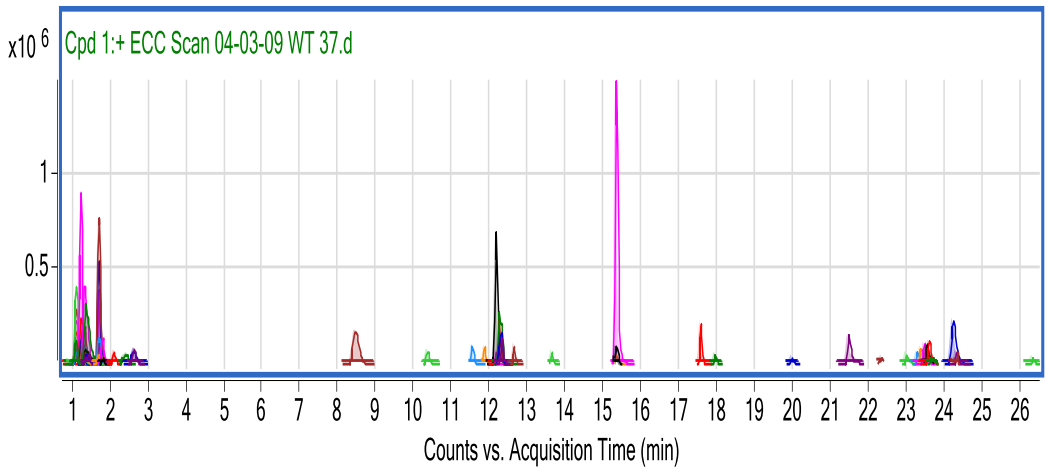


Figure 3. 2 The annotated image shows the overlay of Extracted Compound Chromatograms (ECC). Produced by deconvolution of the Extracted Ion Chromatograms (EIC) of a single metabolite and its isotopes and adducts where present, each metabolite peak is represented by a single colour.

(b) Alignment

After deconvolution of TICs to produce a list of features defined by predicted neutral mass and Rt, it is necessary to align them to identify which features are common across samples. Alignment methods searched for corresponding peaks across different LC-MS runs and aligned them. Peaks for the same features usually match *m/z* values closely (< 10 ppm) across the samples, but there can be variation in retention times between the runs. The former depends on mass accuracy and resolution of the mass spectrometer while the latter largely depends on the chromatographic system used. Two commercial alignment packages were available for the alignment and statistical analysis (1) GeneSpring MS (Agilent Technologies) and Progenesis LC-MS (Non-Linear dynamics, Newcastle, UK). Both programs are applicable for metabolomics, proteomics and genomic data processing.

GeneSpring MS and Progenesis LC-MS were tested with different data sets (unpublished data). They were not used due to many problems. The false discovery rate (FDR) was found to be very high and there were many false-positive entities in the data set (i.e. features, which were aligned as a genuine feature not present in the raw data or it selected the background noise or other redundant informations). Due to compatibility issues, these packages were unable to remove redundant peaks of isotopes, adducts and other non-metabolite peaks from the data set during deconvolution and considered them to be a positive independent feature. As Progenesis LC-MS was originally designed for proteomic analysis, it not only picks low abundance peaks within the noise region, but it is also designed to use multiple isotopes for each feature.

To avoid the above mentioned problems, the data were deconvoluted using MassHunter (see section 3.4.1a) and aligned using the Kernel Mass Profile Algorithm (KMPA), a new program designed by Venura Perera, University of Exeter (V.Perera and R. Yang personal communication). Data sets comprising 4 biological replicates for each strain of *Arabidopsis thaliana* WT and the *vtc* mutants were obtained using the LC-QToF-MS/MS. Parameters such as baseline correction (performed by MassHunter in pre-aligned data), mass and Rt drifts were tested on a few samples to obtain default settings for the extraction and subsequent alignment algorithm. The output was designed to be compatible with the statistical packages, Genespring GX (Agilent) and MultiExperiment Viewer (MEV-T4). This algorithm offered an easy approach for automated noise calculation, noise filters and $\log_2$ normalisation. The retention time correction factor for aligning all masses across all samples was 18 s

(0.3 min) on average, and the mass correction was less than 10 ppm. This software is capable of aligning large data sets in a single run (V.Perera and R. Yang personal communication). The extracted raw data files contained lists of features where each row represents a single feature with its own unique ID, mass, Rt, *m/z*, scan number and abundance.

KMPA uses a sequential alignment method; during this process, any feature which is found not to have any alignment against the consensus sets (reference or control data set) is then selected as unique and added to the consensus set. The algorithm selects one peak at a time from the control peak list and aligns the peak to either the best matching existing row of the control peak list or appends a new row for a match which cannot be found. After aligning, it is likely that peak lists will contain empty gaps (missing values), because not every peak is detected and aligned in a narrow range of RT and mass (RT variance = 0.3 and mass variance < 10ppm) in every sample. These missing values correspond to features, which are present in one strain and absent or found only < 50 % in other. Post alignment was performed where these missing values were filled with calculated background.

The alignment accuracy was checked manually using the raw data files where, 8 out of 12 (67%) features selected from apoplast spectra and 7 out of 10 (70%) from leaf extract spectra were in total agreement with the KMPA output. Subsequently, samples from stress experiments (high light, mechanical wounding, pathogen and salinity) were processed as above across all strains. Consequently, the data generated from KMPA produced 60-70 % alignment accuracy.

In this study, it was possible to align more than 200 samples in a single batch from independent experiments of apoplast or leaf extracts. However, these experiments were collated over a period of three years causing chances of misalignment and misclassification. For this reason care was taken to decide which files to align together to minimise the chances of this happening. All aligned features were normalised on a $\log_2$ scale.

3.4. 2 Noise filtering and Analysis of Variance

Spectral filtering aims to reduce the complexity of spectra and reduce noise. There are many filters applied to remove noise and generate a data set that can be easily handled.

- MassHunter deconvolution removed all initially redundant information such as isotopes, adducts, fragment ions and other low abundance ions which existed by chance in the data.
- Data collected during the first 1 min and last 3 minutes were discarded during data deconvolution.
- Post-alignment noise filters were applied to minimise noise from the data set. If a feature to be classified is not considered to be noise then it must be present at a significant number of times (> 50%) across the biological replicates.
- We could calculate the background noise values for each chromatogram in order to remove missing values. Any feature that was selected as a missing value non-randomly (feature present < 50 % in all samples) was filled with background noise. Such missing values often complicate further statistical analyses, (V. Perera and R.

Yang personal communication). Therefore these were removed by applying cut-off filters with GeneSpring GX (Agilent Technologies).

- An ANOVA based filter was used to remove the non-significant signals from the data. ANOVA allows multiple group comparisons. It also allows the detection of differences between groups of related compounds, and isolates differentially expressed features in each group, to produce a quality data set for further analysis. The ANOVA filters were applied to all samples against WT (control) at different probability levels (Table 3.2) to evaluate the size of data sets calculated as significant. ANOVA filtering demonstrated a relevant effect of discrimination between different genotypes, i.e. between WT and the *vtc* mutants. A $p < 0.05$ was used for the best compromise for the relevant metabolite detection. The remaining 70-80 % features were excluded from the lists was probably those shared by all strains. The features with $p < 0.0001$ were selected for HCA (Figure 3.8) and the features with $p < 0.05$, and the abundance was > 5 fold between WT and *vtc* mutants, and stress and control samples were selected for identification.
- The low abundant compounds do not produce clear isotope and fragment ions so were also removed from the detection list because they could result in false detection.

3. 5 Metabolite Identification

3.5. 1 Visualisation of the raw data

Visualisation of the raw data is very important to ensure selection of truly existing compounds before going further with identification. Both biological and analytical variation can affect the reproducibility of data so these were frequently monitored throughout the data processing. It also produces information regarding the reliability of data processing such as alignment accuracy. In large data sets, there is an increased chance of mis-alignments due to drift in Rt and mass, depending on instrumental conditions.

Firstly, the raw data was inspected for the masses of interest in the filtered peak list ($p < 0.05$) and Rt, mass and relative heights were compared. Secondly, the fragment ions for each compound produced by auto-MS/MS were extracted manually and added to an inclusion list. The fragmentation pattern of a metabolite is highly efficacious in the identification of metabolites as it provides information, which helps in identifying the functional group and the structural elucidation of a metabolite. The metabolites which were not fragmented efficiently during analysis were not processed for identification at this stage. These metabolites needed to be reanalysed in a targeted manner which can produce the fragmentation in selected parameters and validate further identification. Thirdly, the isotopic distribution is important for accurate identification. Isotope filters efficiently reduce the number of potential elemental compositions (Kind and Fiehn, 2006).

3.5. 2 Generation of Molecular Formulae and Database Searches

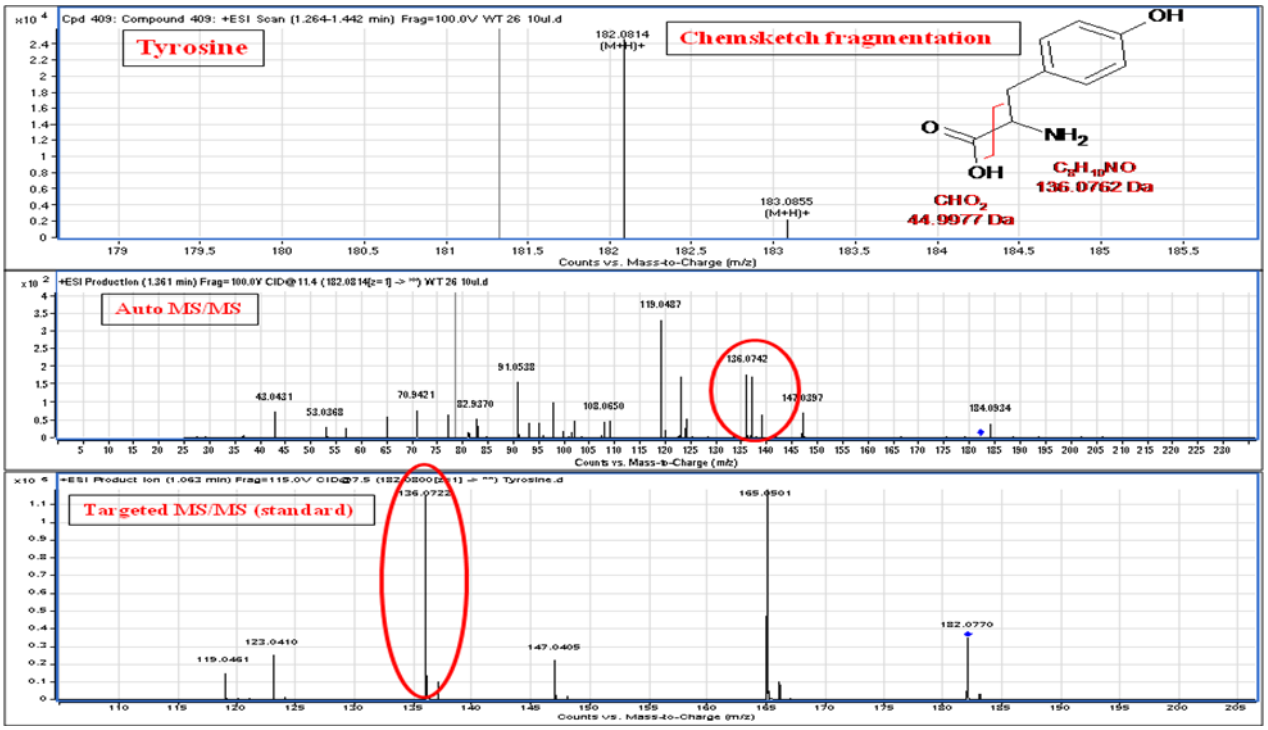
The calculation of chemical formulae which best fit the accurate mass produced by the high-resolution mass spectrometer with high mass accuracy (< 5 ppm) was the first step of the compound identification. Molecular formulae based on isotopic distribution can be calculated using many tools. In the current study, MassHunter has the capability of generating molecular formulae through the combination of the elements which were the best fit for the molecular mass and its isotopic distribution. As Kind and Fiehn (2006) suggested, the core elements (carbon (C), hydrogen (H), oxygen (O), nitrogen (N) sulphur (S) and phosphorus (P) should be included in the list because these are the basic components of most naturally occurring compounds. Animal and plant metabolites mostly lack metals ions which are only present in the form of adducts (K^+, Na^+) that are produced during ionisation (Moco, 2007). The elements which are rarely present or absent in species under the study should be excluded from the list. For example, secondary metabolites in *Arabidopsis thaliana* most likely lack the halogens (Cl, Br and I) so, these were excluded from the list. The ionisation mode was considered during parameter setup for formula generation. The number of rings and double bonds can be calculated by the number of C, H, N and O atoms existing in the molecule (Moco, 2007). For small organic molecules, the intensity of the second isotope was determined by elemental composition such as C, H, N, S, P and O. For example, the natural abundances of the first isotope of these elements are $^{13}C_1$ is 1.11; ^{2}H is 0.015; ^{15}N is 0.366, S is 0.750; O is 0.038 % (Bocker *et al.*, 2009). The molecular formulae generated using MassHunter were accepted or rejected using ppm difference between theoretical and observed mass (selected < 1

ppm) in combination of MS/MS fragmentation and retention time. All the above strategies can remove approximately 95 % of the false positive and can outperform the identification of an accurate mass using a mass spectrometer which produces mass accuracies of less than 0.1 ppm (Kind and Fiehn, 2006; Moco, 2007).

Freely available metabolite databases (AraCyc, MetaCyc, Knapsack, Massbank, PubChem and ChemSpider) were used to search the molecular formulae calculated from the isotopic distribution of each compound. Massbank was used for metabolite identification http://www.massbank.jp/en/database.html (Horai *et al.*, 2010). This database not only produces hits for possible metabolites, but also gives expected product ions resulting from fragmentation. The fragment ions of the metabolites of interest from the current study were compared with the fragment ion library in the Massbank database to validate the identifications; however one must consider that fragmentation can differ subtly between mass spectrometers. Most of the features derived from untargeted LC-MS data are still unknown, and for many standards are not available (von Roepenack-Lahaye *et al.*, 2004b; Vorst *et al.*, 2005; Moco *et al.*, 2006). Therefore, many of the putatively annotated structures cannot yet be unambiguously identified without performing further analysis. Most of the compounds were overlapping in mass and Rt but due to diversity in chemical composition they have different isotopic distributions and in some cases unique fragmentation patterns which are helpful in identification to some extent.

To strengthen the identification of metabolites it is useful to use a combination of techniques. Previously, most studies used NMR in combination with LC-MS or GC-

MS, LC-ultraviolet/visible (UV/Vis) chromatography for the authentication of their metabolite identifications (Moco 2007; Ward *et al.*, 2011). As many secondary metabolites contain chromophores, the wavelength at which they absorb light can provide clues about the possible classes of compound. In addition, the most reliable method for the confirmation of these metabolites is to test them against commercially available standards using the same analytical conditions. The ideal method is to purify unknown metabolites for NMR analysis, which provides their structural elucidation. In this study, the masses of interest were re-analysed using the QToF-MS/MS and QQQ-MS/MS in a targeted manner to obtain quality fragmentation spectra for quality control purposes. Formulae were generated and searched against metabolite databases as described above. At each stage, a comparison was made with the auto MS/MS results. The identified structure was fragmented manually using ChemSketch (by bond breakage at the correct positions) (Spessard, 1998) and automatically on the Massbank database (Horai *et al.*, 2010). Figure 3.3 provides a comparison of fragments produced by auto MS/MS and targeted MS/MS. The best matching metabolites were selected for validation by running standards corresponding to the compounds of interest and comparing Rts, isotopic distributions and fragmentation patterns against the endogenous metabolites. Twenty eight compounds were putatively identified as being differentially expressed between the WT and the *vtc* mutants in leaf tissue; some of them were light responsive. The study demonstrated the feasibility of the approach and the potential of LC-ESI-MS to contribute a significant role in investigating the metabolic changes in ascorbate-deficient mutants.



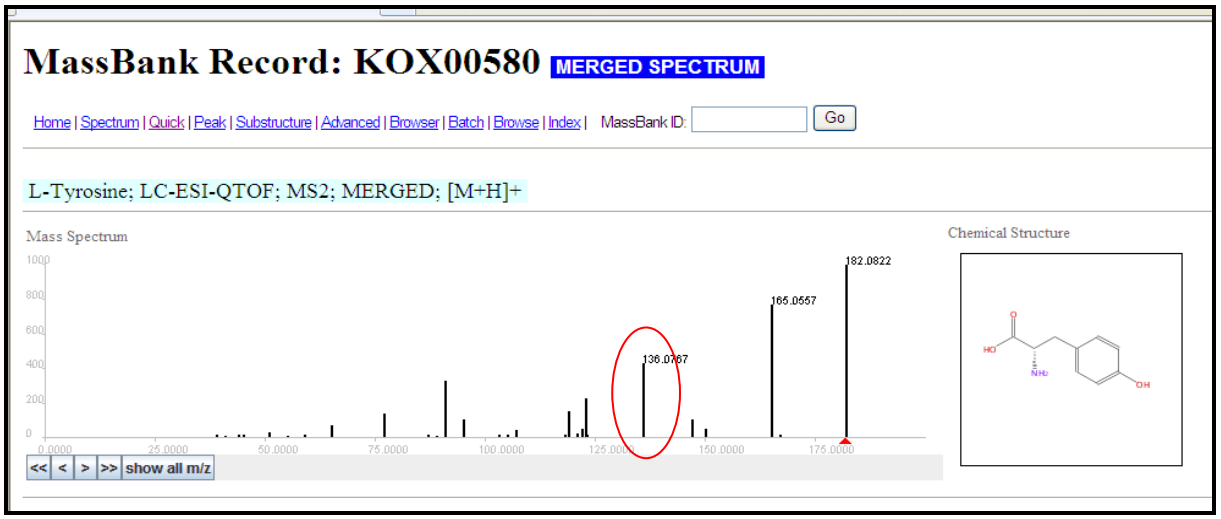


Figure 3.3 Comparison of fragment ions of metabolites from LC-MS and Massbank database. An example of tyrosine precursor ion *m/z* 182.07 and fragment ion *m/z* 136.07 (A) ChemSketch (manually fragmented) (B) QTOF auto MS/MS (C) QTOF Targeted MS/MS of tyrosine standard (0.05 mM) and (D) Massbank database recorded fragment.

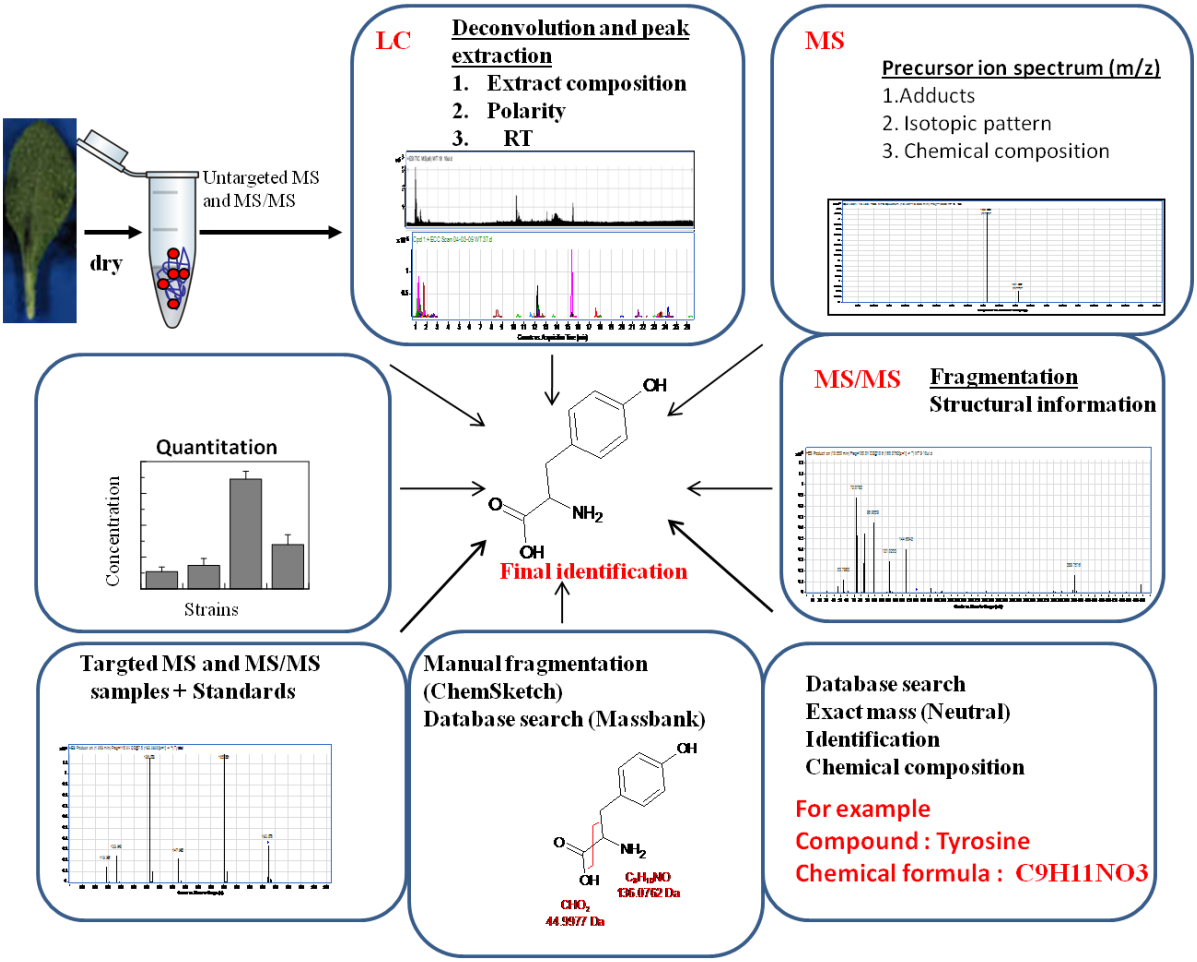


Figure 3.4 A schematic diagram of analytical technique and data processing steps that lead to the identification of a metabolite. Here this present an example of tyrosine, Neutral mass = 181.07, molecular formula $C_9H_{11}NO_3$. Clockwise from top left: Liquid Chromatography (LC), Mass Spectrometry (MS), fragmentation pattern analysis (MS/MS), database search on the basis of mass spectral information (e.g. MassBank), Manual fragmentation using ChemSketch, validation by standard compounds and information present in the literature and in databases and finally, quantitation of identified compounds.

3. 6 Results

3.6. 1 Ascorbate concentration in the apoplastic fluid

The ascorbate concentration in the leaf apoplast of *Arabidopsis thaliana* WT and the *vtc* mutants was quantified using a standard curve. The total ascorbate concentration observed in the leaf apoplast of *vtc* mutants was significantly lower than WT (Figure 3.5). Therefore, the previously reported decrease in total leaf ascorbate in these mutants (Conklin *et al.*, 2000) also occurs in the apoplastic fluid. The pattern of accumulation of ascorbate in the apoplast was identical to the whole leaf ascorbate of WT and the *vtc* mutants but the concentration was low in the apoplast compared to leaf extract (Figure 3.5 and 4.5). Glucose-6-phosphate (G6P) was not detected (method explained in Chapter 2) in the apoplastic fluid (data not shown) suggesting little or no leakage of intracellular contents because G6P is considered to be an exclusively intracellular metabolite (Burkey, 1999). Sorbitol was added in the extraction buffer (described in Chapter 2) and used to measure the dilution of apoplastic fluid. The final concentration of ascorbate in the apoplastic fluid was calculated using sorbitol, which is used as internal standard in the extraction buffer (described in Chapter 2).

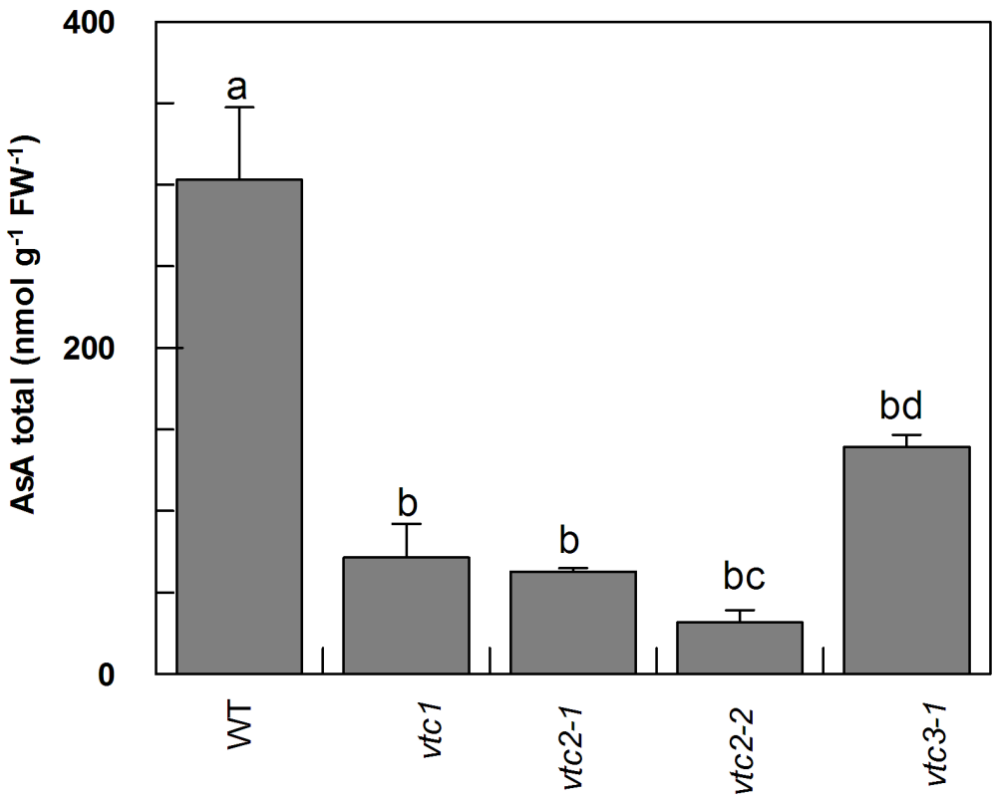


Figure 3.5 Total ascorbate (ascorbate + dehydroascorbate) concentrations in the apoplastic washing fluid (AWF) of *Arabidopsis thaliana* WT and *vtc* mutants. Ascorbate concentration was measured in nmol g-1 FW-1 of leaf tissue. Error bars indicate standard error (+ SE). The experiment comprised 3 independent biological replicates (2 technical replicates). Lower case letters above the bars indicate that the values are significantly different calculated using ANOVA ($p < 0.01$). The column labelled 'a' is significantly different from the corresponding columns labelled with the letter 'b'. The column labelled with 'bc' is significantly different from the column labelled with 'bd'.

3.6. 2 Metabolite profiling

To compare the metabolite profile between the WT and the *vtc* mutants from apoplastic fluid and the whole leaf extract of 7-8 week old *Arabidopsis thaliana* (n=4) plants from normal and stressed conditions (described in Chapter 2) were used.

3.6.2. 1 Total ion Chromatogram (TIC)

In order to check the reproducibility and the technical error of the LC-MS, chromatograms of leaf apoplastic fluid and the whole leaf extracts, collected over a period of 2-3 years, were manually compared for Rt and mass drift using the internal standard and the endogenous sinapoyl malate peak (*m/z* = 341.08) (one of the abundant metabolites in *Arabidopsis thaliana*). These comparisons were performed within and between experiments. Within an experiment 5-8 biological replicates were randomly picked from different strains and the observed RT and mass variation was very small such as RT and mass drifts of < 0.1 min and < 5ppm respectively (listed in Table 3.1). The same samples were analysed between independent experiments (n=2), where the variation in retention time was between 1-2 min and the mass variation was < 10ppm (listed in Table 3.1). The same was observed for other metabolites selected for identification.

Representative TICs of 2 independent experiments from leaf extracts are presented in Figure 3.6 to show the reproducibility of data between independent experiments. Figure 3.7 show the EIC of sinapoyl malate peak for 6 biological replicates from each experiment. These showed the total ion current (signal intensity) plotted against chromatographic retention time.

Total Ion Chromatograms (TICs)

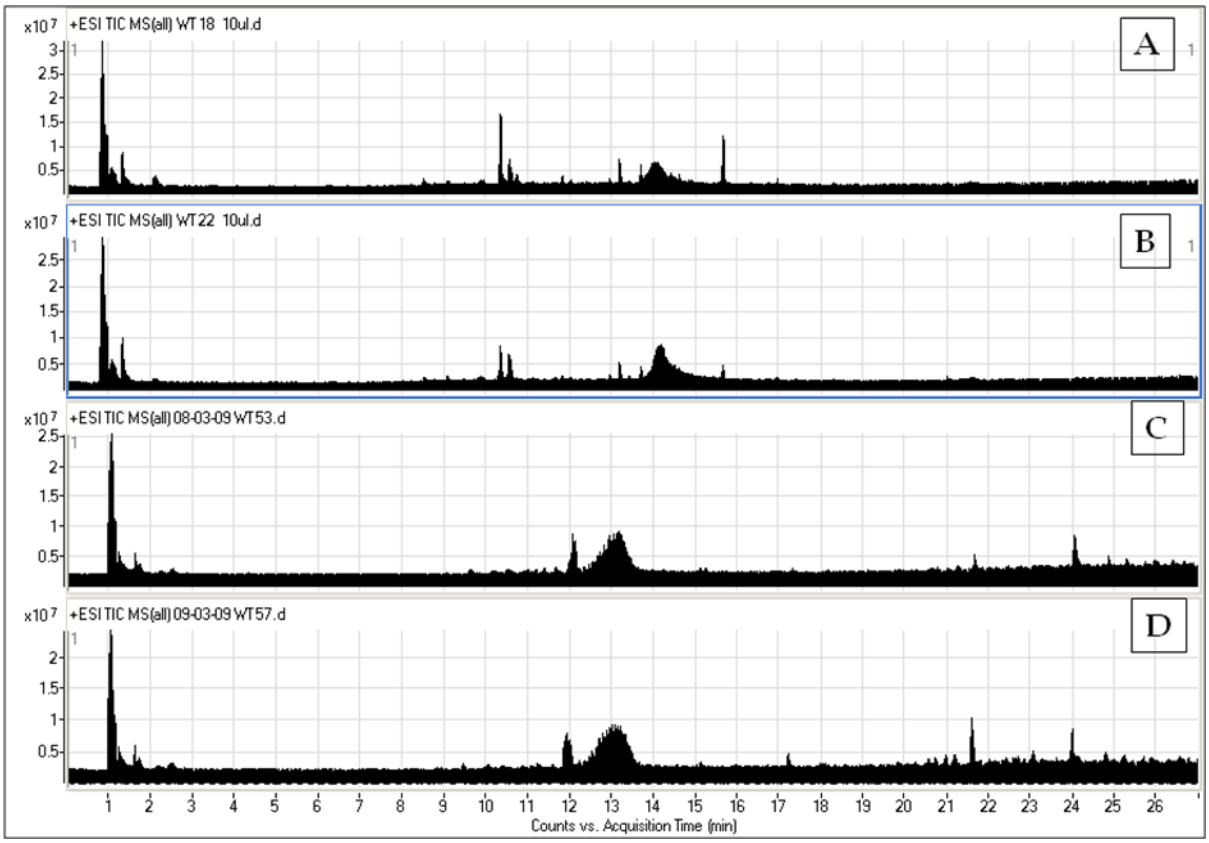


Figure 3. 6 Representation of TICs of two independent experiments: Two biological replicates from each experiment of *Arabidopsis thaliana* wild type leaf extract. TIC A & B are from experiment 1. TIC C & D are from experiment 2. Low Rt drift was seen in the biological replicates within the same experiment but there was a drift of 1-2 min between two experiments.

Table 3.1 Retention time and mass shift observed during LC-QToF–MS/MS analysis in *Arabidopsis thaliana* within and between experiments. Abbreviations used are: n = number of biological replicates, RT = retention time, m/z = mass to charge ratio, Ave = average and SE = standard error

Sinapoyl malate						
Samples	**Rt Ave (min)**	**SE**	**Rt drift (min)**	***m/z* ave**	**SE**	***m/z* drift**
Within Experiment 1 (n=6)	10.60	0.0003	0.02	341.0863	3.00E-04	0.0005
Within Experiment 2 (n=6)	12.10	0.008	0.05	341.0867	3.00E-04	0.0001
Between Experiments (n=12)	11.354	0.225	1.5	341.0865	0.0001	0.0003

Extracted ion chromatograms (EICs) for Sinapoyl malate

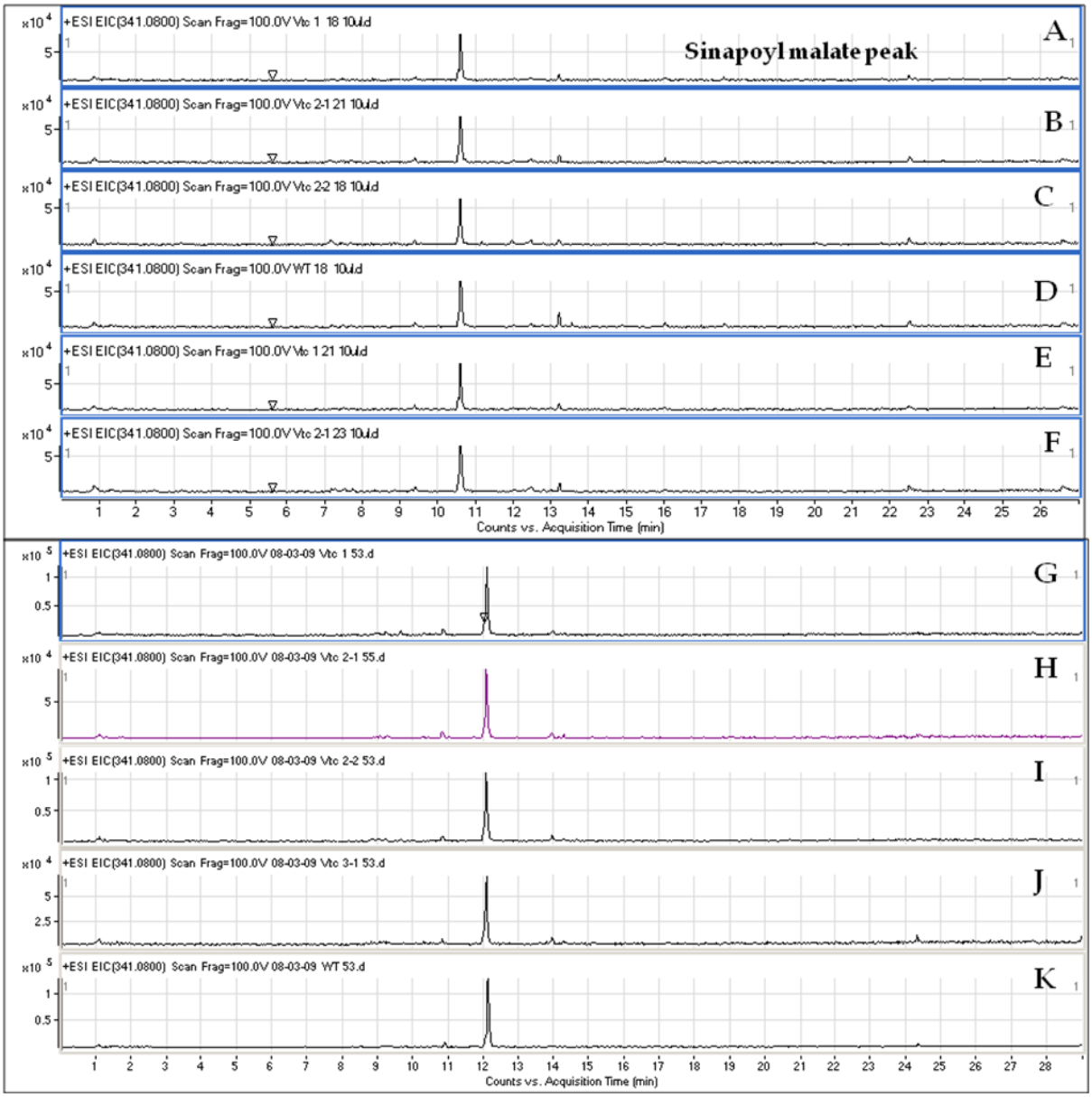


Figure 3.7 Sinapoyl malate peak compared within and between two independent experiments. (A-F) Experiment 1, (G-K) Experiment 2 (where each letter represents abiological replicate). The x-axis of the chromatogram represents the RT peak intensity while on the y-axis it represents the peak intensity.

3.6.2. 2 Deconvolution and Alignment

The deconvolution of each sample produced 3500-5000 features (varies between strains). After alignment of 20 samples comprising 4 biological replicates from each of the 5 strains approximately 7617 mass spectral features were aligned in the apoplast experiment 1 and 10263 in experiment 2. Similarly, 8837, 9495 and 10979 features were aligned in the leaf extract experiments 1, 2 and 3 respectively. 3500-5000 features are higher than would be expected, so, it is probable that many of these are either minor components; errors generated during the alignment or deconvolution, or they could be contaminants that are not found in all samples. Thus low peak intensity features were discarded during filtering, as were many redundant compounds (as described in section 3.4.2). The significant data with different probability values ($p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$) from all experiments undertaken in the current study are listed in Table 3.2.

Table 3.2 Number of features significantly different between WT and the *vtc* mutants as determined by 1-way ANOVA (1) Apoplast Exp 1 ESI^+, (2) Apoplast Exp 2 ESI^+ (3) Apoplast Exp 3 ESI^+ (4) Leaf Extract Exp 1 ESI^+ (5) Leaf Extract Exp 2 ESI^+(6) Leaf Extract Exp 3 ESI^+ and 2-Way ANOVA (7) High Light in ESI^+ (8) High Light in ESI^- (9) *P.syringae* in ESI^+ (10) *P. syringae* in ESI^- (11) Wounding in ESI^+ (12) Wounding in ESI^-. The noise filter varied from experiment to experiment. ANOVA was performed on all strains/conditions against WT control and *Benjamin Hochberg FDR* was used, ESI^+ = Positive Ionisation mode, ESI^- = Negative ionisation mode.

S. No	Experiments	ESI	Total aligned after noise filter	No. Of features at different probability values			
				$p < 0.05$	$p < 0.01$	$p < 0.001$	$p < 0.0001$
1	Apoplast Exp1	ESI+	2118	118	118	74	44
2	Apoplast Exp2	ESI+	2309	1204	1093	957	869
3	Apoplast_Exp3	ESI+	2563	2015	1896	1735	1587
4	Leaf Extract Exp1	ESI+	821	217	210	164	126
5	Leaf Extract Exp2	ESI+	1161	849	755	629	507
6	Leaf Extract Exp3	ESI+	2247	1609	1455	1241	1102

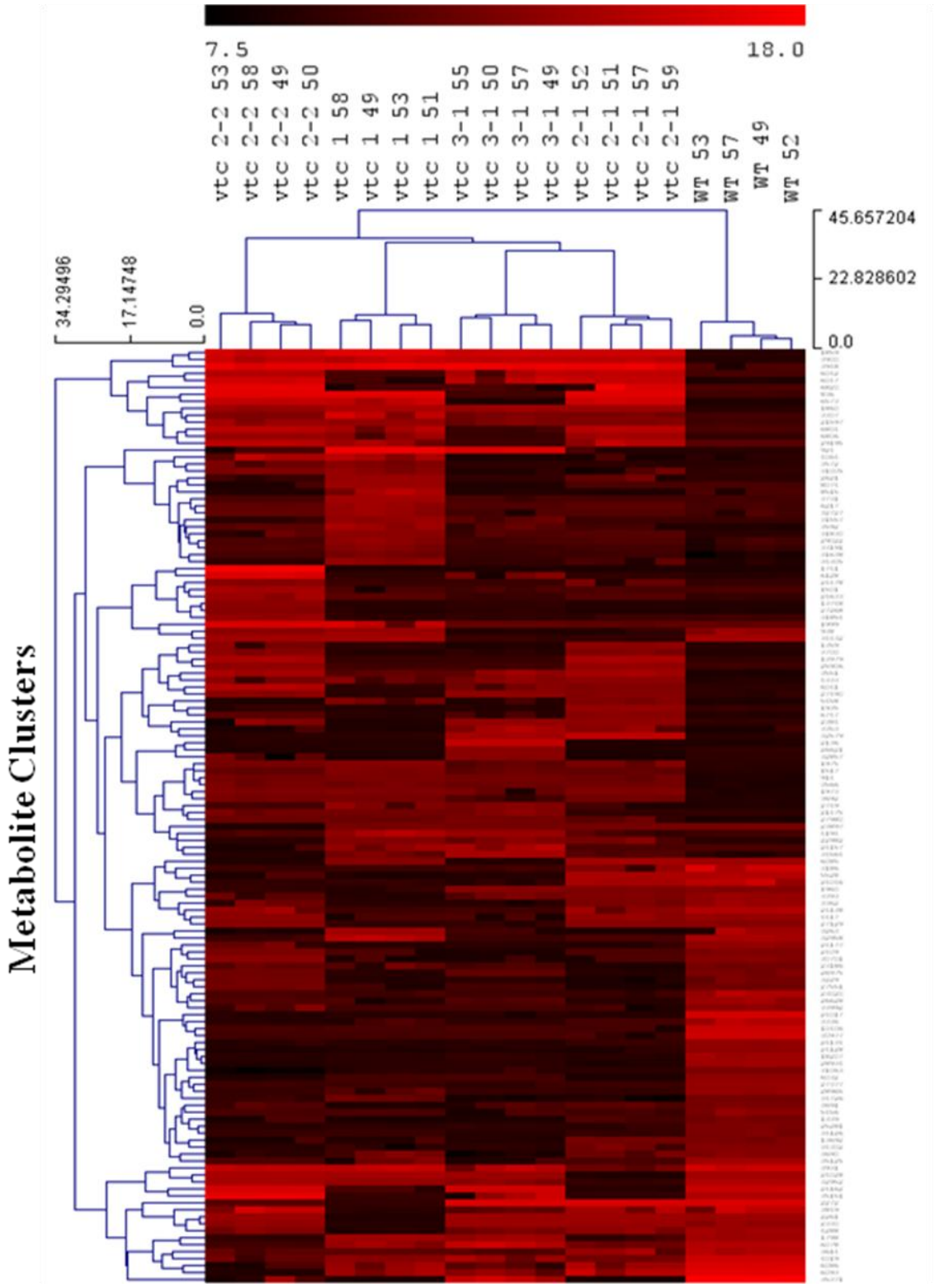
Table 3.2 continued.....

S. No	Experiments	ESI	Total aligned after noise filter	No. Of features at different probability values			
				p< 0.05	p < 0.01	p < 0.001	p < 0.0001
7	High Light treated	ESI+	1670	991	703	441	264
8	High Light treated	ESI-	631	439	335	215	139
9	*P. syringae*	ESI+	8795	5673	4097	2772	1720
10	*P.syringae*	ESI-	2791	1511	957	534	330
11	Wounding	ESI+	636	379	272	167	100
12	Wounding	ESI-	1128	718	542	353	212

3.6.2. 3 Hierarchical Clustering Analysis (HCA)

The log_2 normalized data were imported into the statistical package MultiExperiment Viewer or MEV (Version 4.6) (Saeed *et al.*, 2003) for hierarchical clustering. Hierarchical clustering was performed to check data quality, reproducibility and to investigate the relationship between strains at a biochemical level. HCA was performed using an agglomerative method commonly used in metabolomics (Boccard *et al.*, 2007). HCA Euclidean distances and complete linkage clustering were used in this study. HCA was performed on ANOVA-filtered data ($p < 0.0001$). The results showed little variation between biological replicates across all strains but highlighted differences between strains. However, overalls all *vtc* mutants clustered together and showed similar behaviour under control and stressed conditions. All strains, WT and the *vtc* mutants accumulated large number of metabolites in response to different treatments (biotic and abiotic stresses). Heatmaps are shown in Figure 3.8 for all experiments under both control and stressed conditions. The horizontal (X) axis of the heatmap represents the different strains and biological replicates. In all experiments, all biological replicates for each strain were clustered together, which showed the reproducibility of the data. The y-axis (left) it represents the clustering of metabolites, which were clustered together on the basis of response to ascorbate deficiency (as higher or lower in *vtc* mutants) and in response to environmental stimuli.

A


7.5
18.0
vtc 2-2 53
vtc 2-2 58
vtc 2-2 49
vtc 2-2 50
vtc 1 58
vtc 1 49
vtc 1 53
vtc 1 51
vtc 3-1 55
vtc 3-1 50
vtc 3-1 57
vtc 3-1 49
vtc 2-1 52
vtc 2-1 51
vtc 2-1 57
vtc 2-1 59
WT 53
WT 57
WT 49
WT 52
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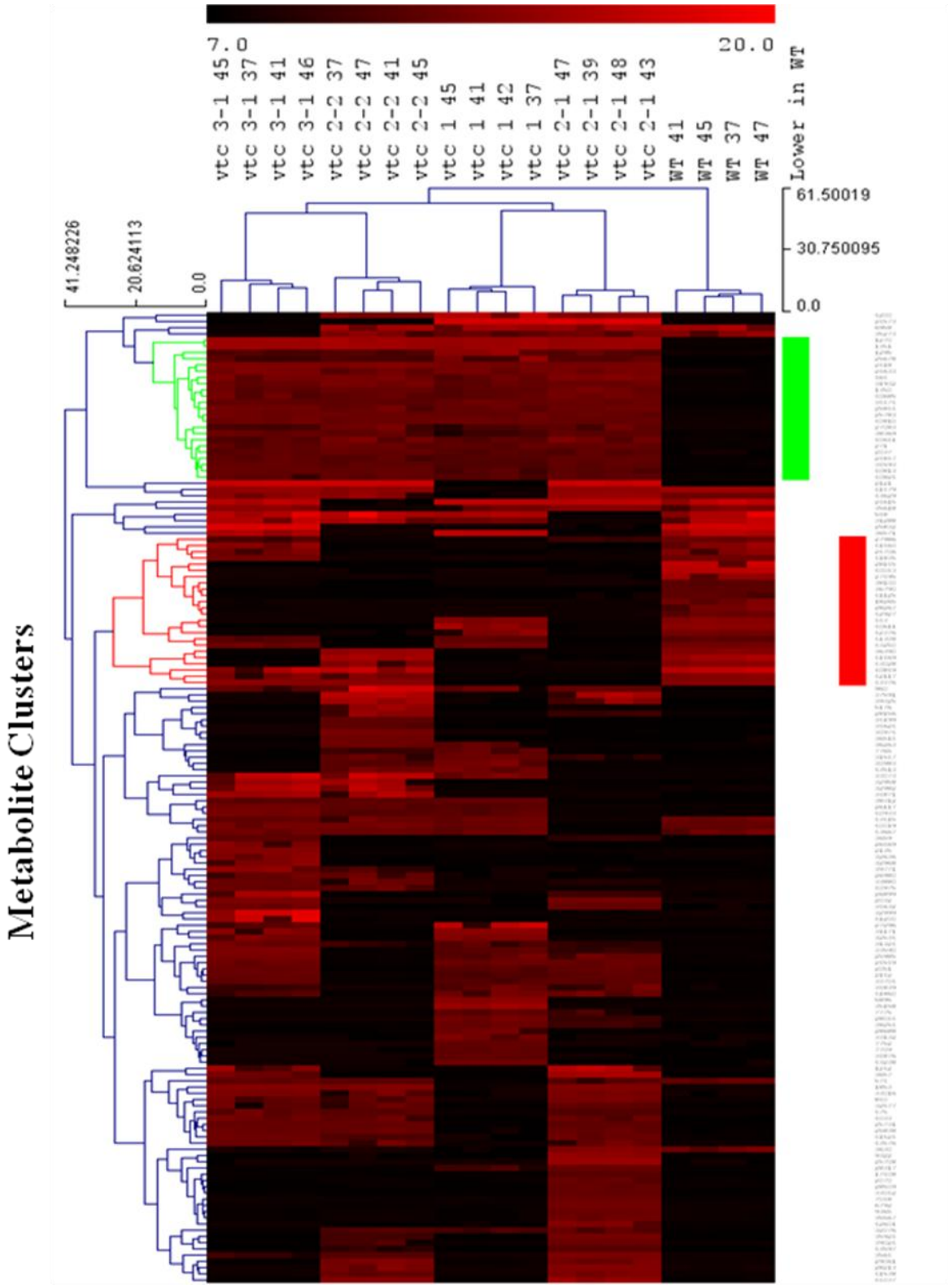
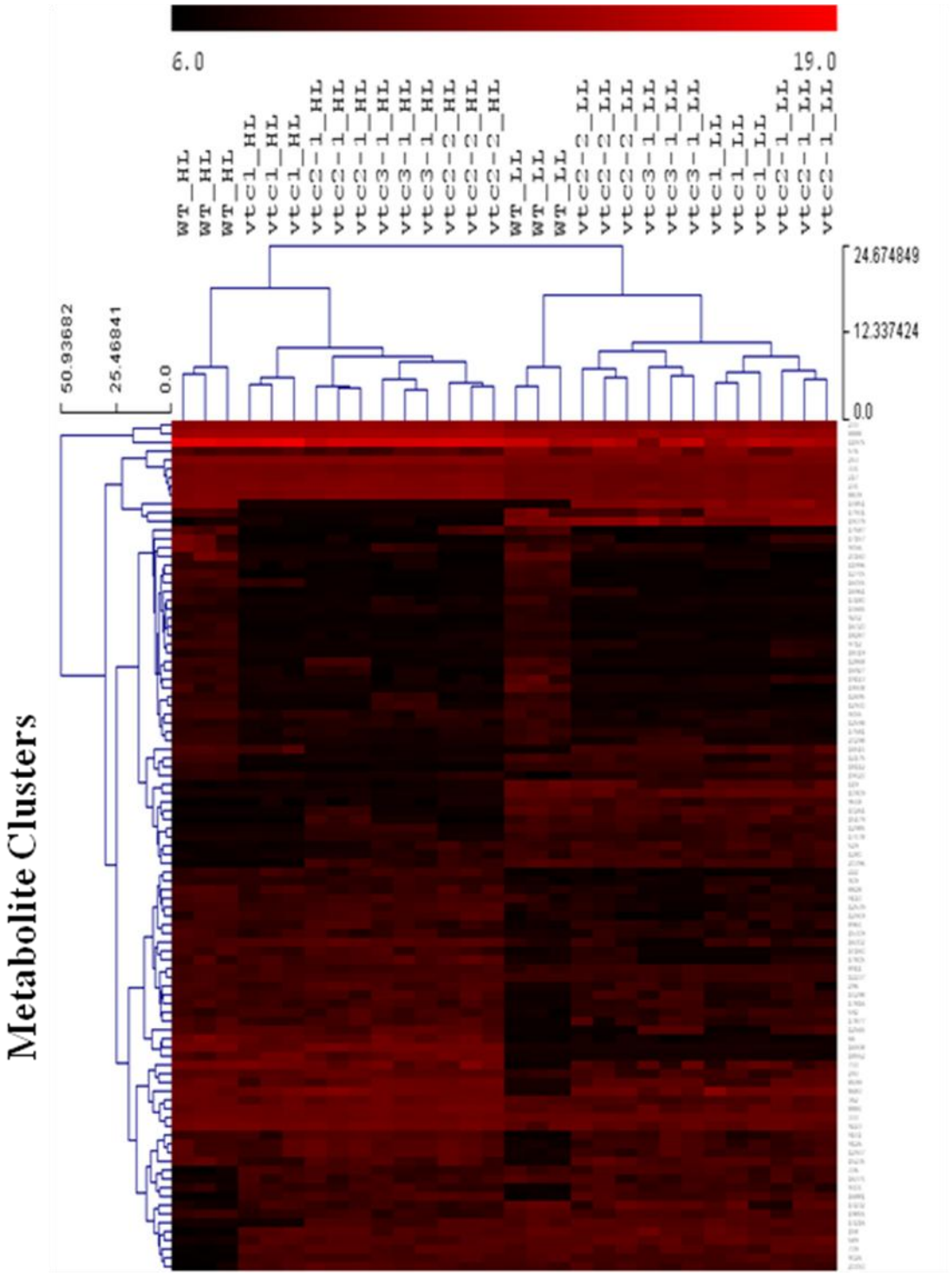
22.828602
0.0
34.29496
17.14748
0.0
Metabolite Clusters

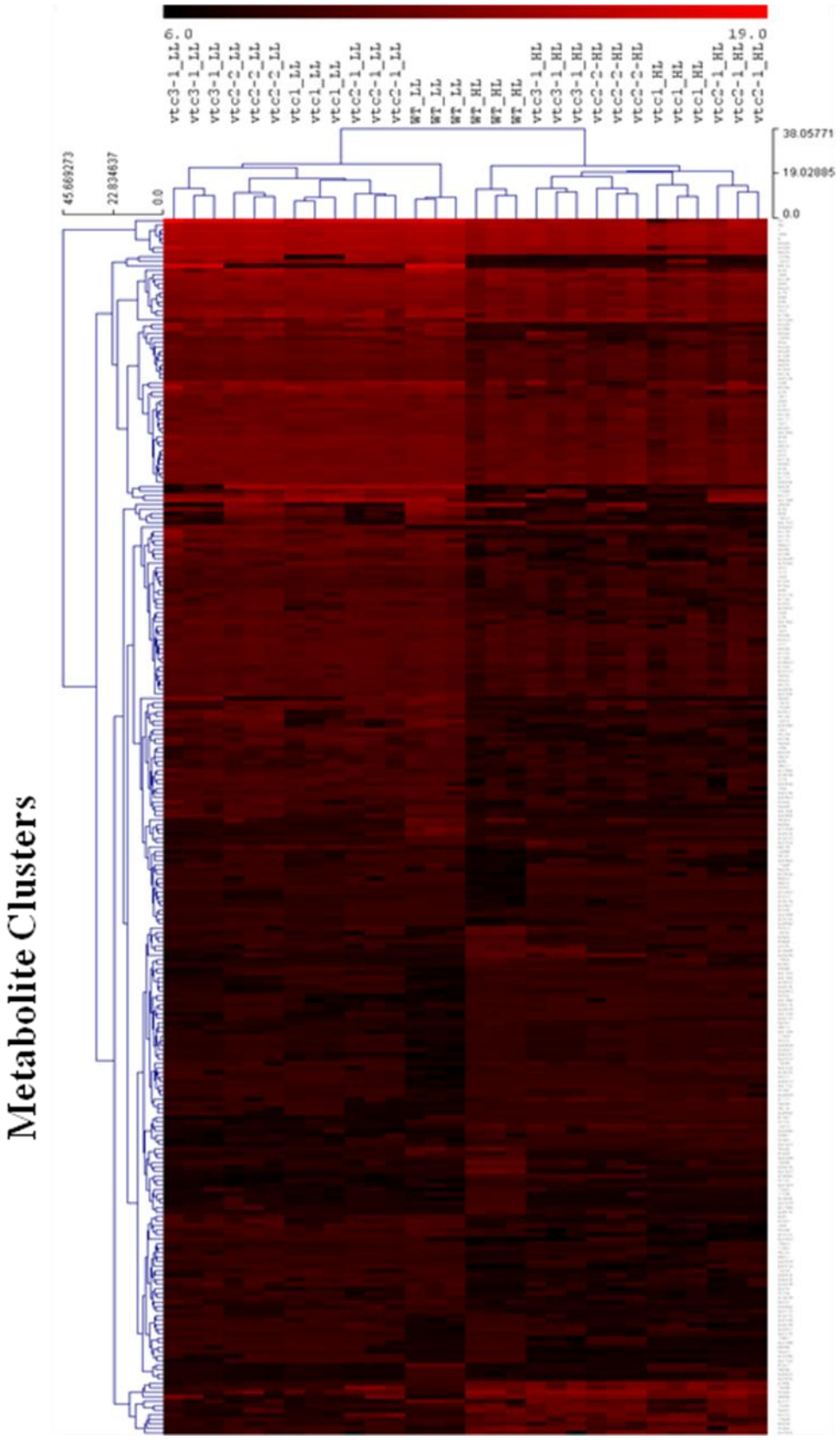
B


7.0
20.0
vtc 3-1 45
vtc 3-1 37
vtc 3-1 41
vtc 3-1 46
vtc 2-2 37
vtc 2-2 47
vtc 2-2 41
vtc 2-2 45
vtc 1 45
vtc 1 41
vtc 1 42
vtc 1 37
vtc 2-1 47
vtc 2-1 39
vtc 2-1 48
vtc 2-1 43
WT 41
WT 45
WT 37
WT 47
Lower in WT
61.50019
30.750095
0.0
41.248226
20.624113
0.0
Metabolite Clusters

C


6.0
19.0
WT_HL
WT_HL
WT_HL
vtc1_HL
vtc1_HL
vtc1_HL
vtc2-1_HL
vtc2-1_HL
vtc2-1_HL
vtc3-1_HL
vtc3-1_HL
vtc2-2_HL
vtc2-2_HL
vtc2-2_HL
WT_LL
WT_LL
WT_LL
vtc2-2_LL
vtc2-2_LL
vtc2-2_LL
vtc3-1_LL
vtc3-1_LL
vtc1_LL
vtc1_LL
vtc1_LL
vtc2-1_LL
vtc2-1_LL
vtc2-1_LL
50.93682
25.46841
0.0
24.674849
12.337424
0.0
Metabolite Clusters

D


6.0
19.0
vtc3-1_LL
vtc3-1_LL
vtc3-1_LL
vtc2-2_LL
vtc2-2_LL
vtc2-2_LL
vtc1_LL
vtc1_LL
vtc1_LL
vtc2-1_LL
vtc2-1_LL
vtc2-1_LL
WT_LL
WT_LL
WT_LL
WT_HL
WT_HL
WT_HL
vtc3-1_HL
vtc3-1_HL
vtc3-1_HL
vtc2-2-HL
vtc2-2-HL
vtc2-2-HL
vtc1_HL
vtc1_HL
vtc1_HL
vtc2-1_HL
vtc2-1_HL
vtc2-1_HL
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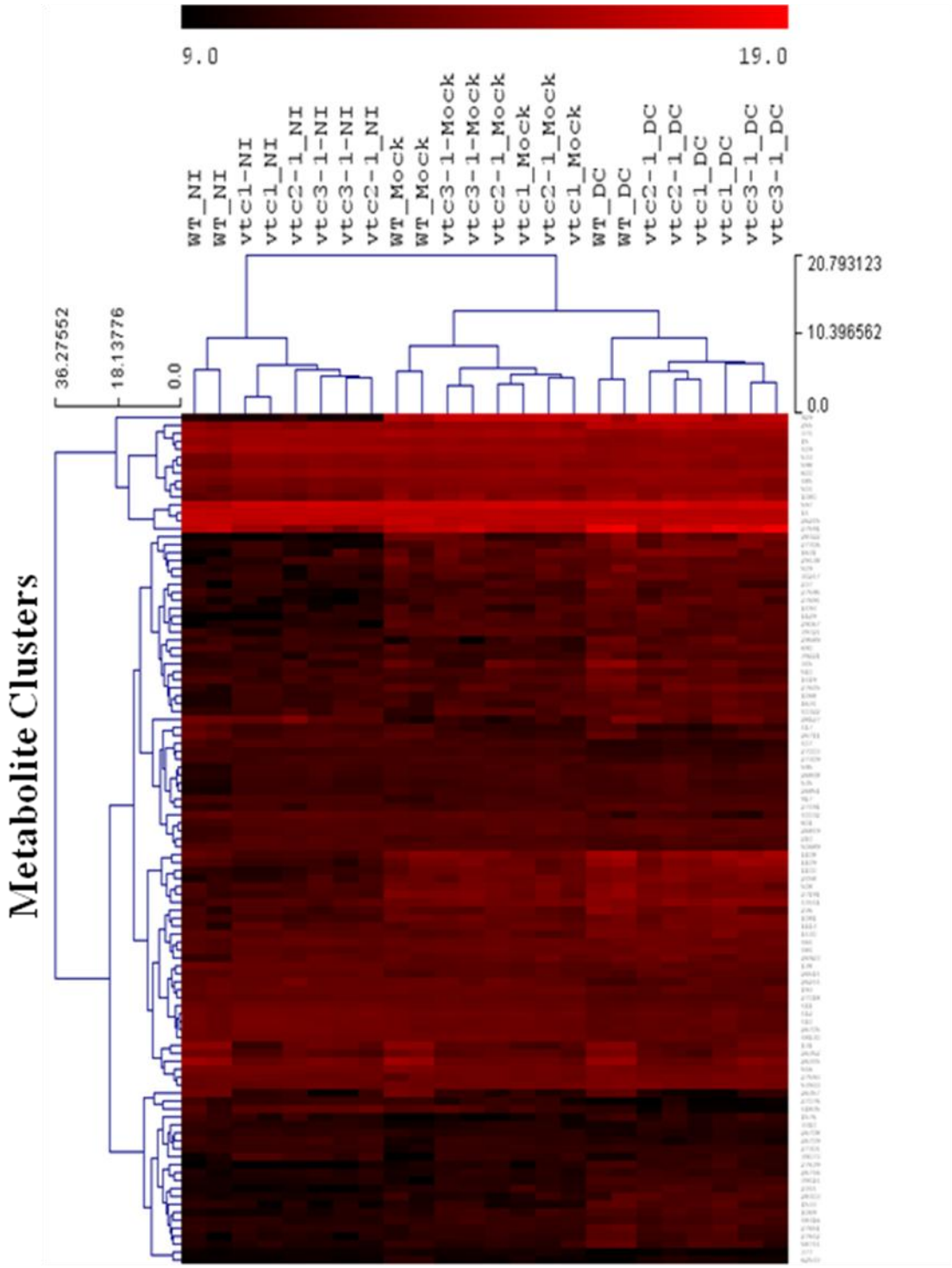
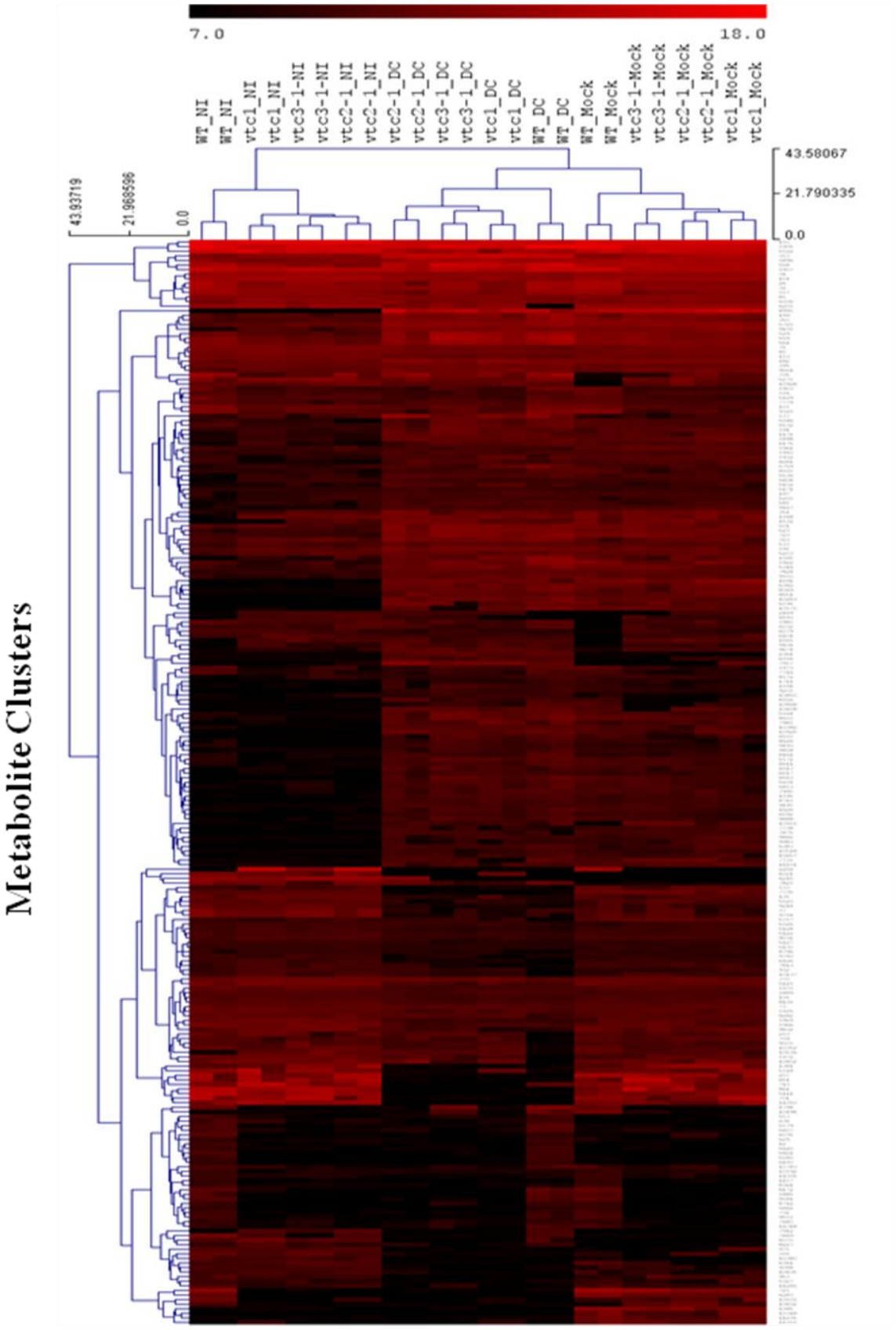
19.02885
0.0
45.669273
22.834637
0.0
Metabolite Clusters

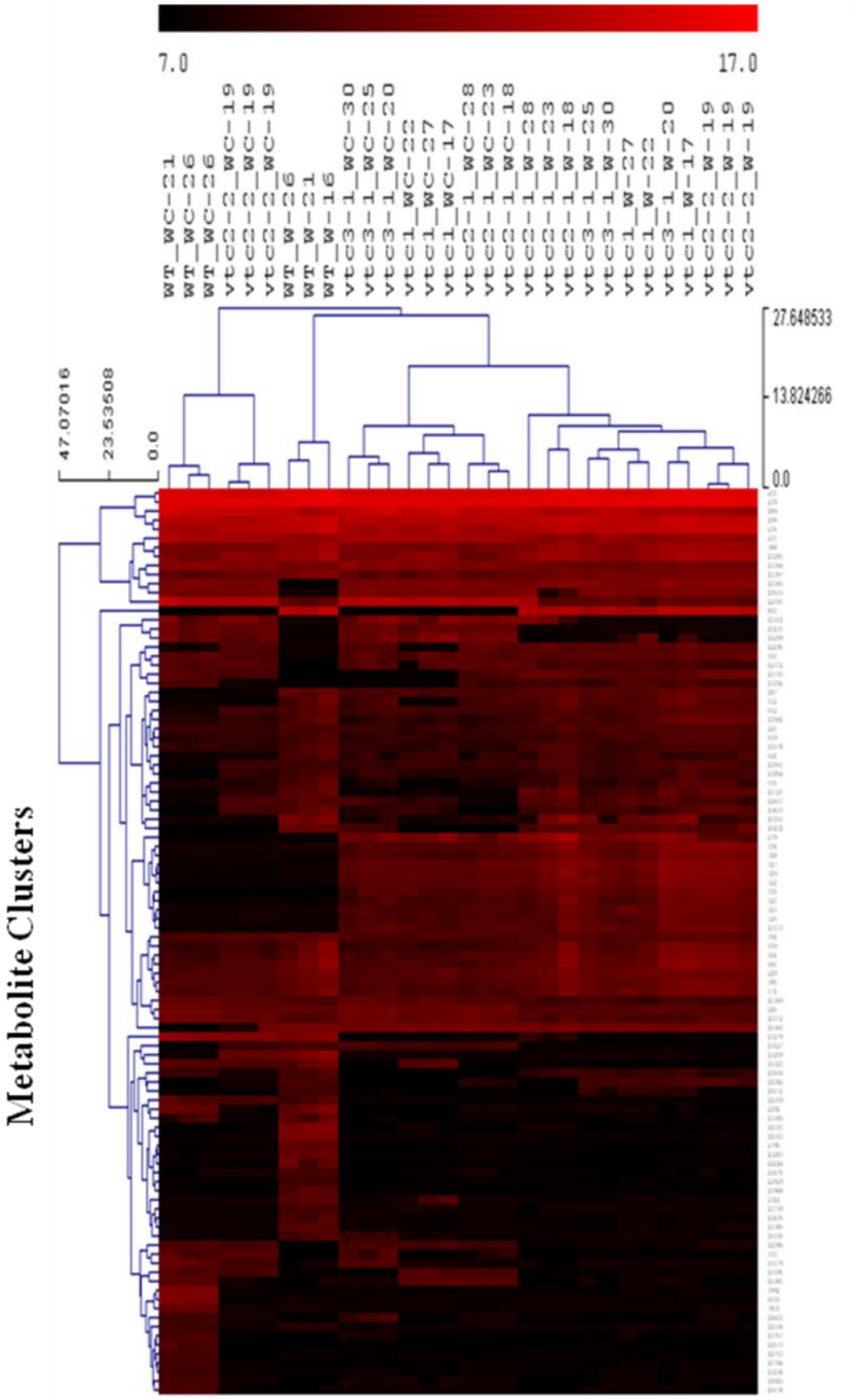
E


9.0
19.0
WT_NI
WT_NI
vtc1-NI
vtc1_NI
vtc2-1_NI
vtc3-1-NI
vtc3-1-NI
vtc2-1_NI
WT_Mock
WT_Mock
vtc3-1-Mock
vtc3-1-Mock
vtc2-1_Mock
vtc1_Mock
vtc2-1_Mock
vtc1_Mock
WT_DC
WT_DC
vtc2-1_DC
vtc2-1_DC
vtc1_DC
vtc1_DC
vtc3-1_DC
vtc3-1_DC
20.793123
10.396562
0.0
36.27552
18.13776
0.0
Metabolite Clusters

F


7.0
18.0
WT_NI
WT_NI
vtc1_NI
vtc1_NI
vtc3-1-NI
vtc3-1-NI
vtc2-1_NI
vtc2-1_NI
vtc2-1_DC
vtc2-1_DC
vtc3-1_DC
vtc3-1_DC
vtc1_DC
vtc1_DC
WT_DC
WT_DC
WT_Mock
WT_Mock
vtc3-1-Mock
vtc3-1-Mock
vtc2-1_Mock
vtc2-1_Mock
vtc1_Mock
vtc1_Mock
43.58067
21.790335
0.0
43.93719
21.968596
0.0
Metabolite Clusters

G


7.0
17.0
WT_WC-21
WT_WC-26
WT_WC-26
vtc2-2_WC-19
vtc2-2_WC-19
vtc2-2_WC-19
WT_W-26
WT_W-21
WT_W-16
vtc3-1_WC-30
vtc3-1_WC-25
vtc3-1_WC-20
vtc1_WC-22
vtc1_WC-27
vtc1_WC-17
vtc2-1_WC-28
vtc2-1_WC-23
vtc2-1_WC-18
vtc2-1_W-28
vtc2-1_W-23
vtc2-1_W-18
vtc3-1_W-25
vtc3-1_W-30
vtc1_W-27
vtc1_W-22
vtc3-1_W-20
vtc1_W-17
vtc2-2_W-19
vtc2-2_W-19
vtc2-2_W-19
47.07016
23.53508
0.0
27.648533
13.824266
0.0
Metabolite Clusters

H

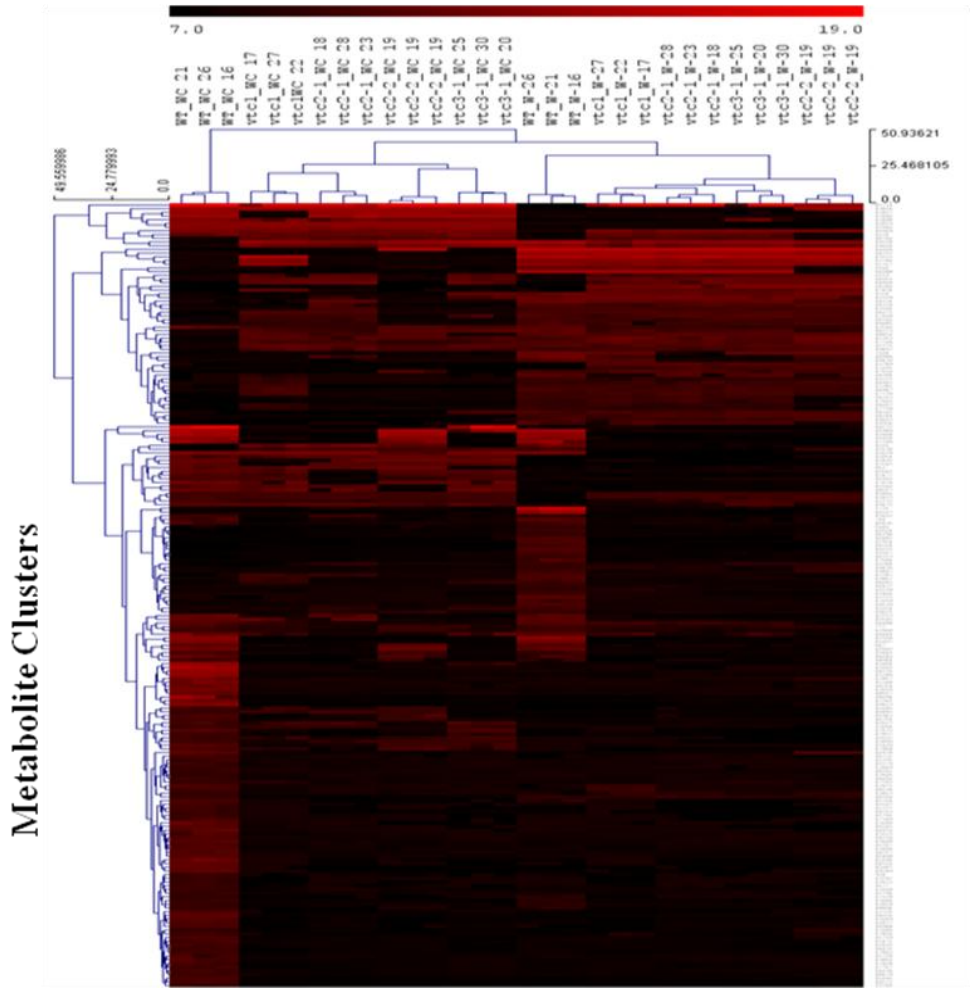
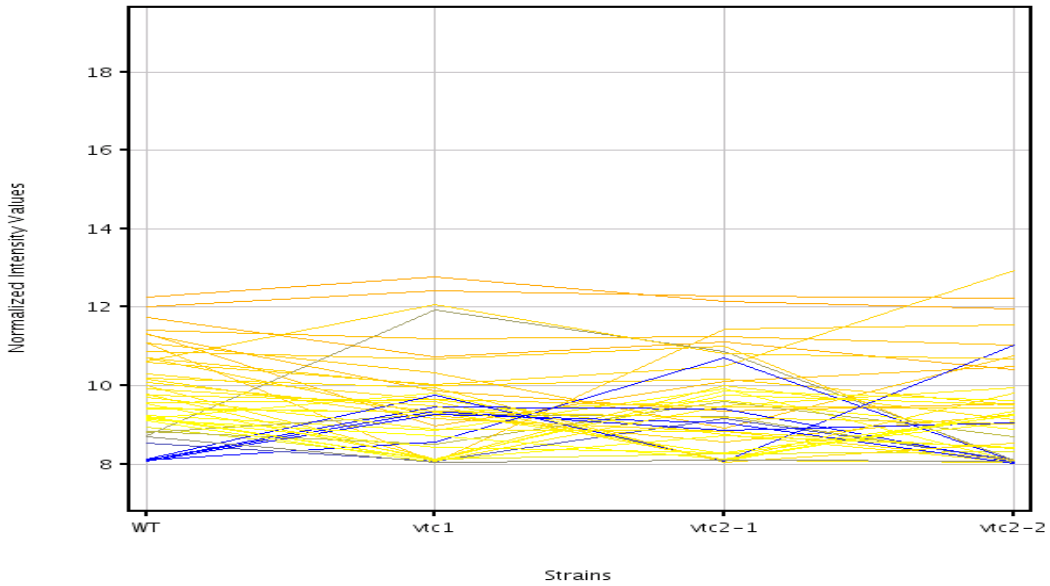


Figure 3. 8 Representation of unsupervised hierarchical clustering. Hierarchical clustering of metabolites was performed on log_2 normalised features which were differentially expressed ($p < 0.0001$) between WT and *vtc* mutants or treatments (stressed and control). The heatmaps visualise metabolite abundance in each sample with the colour scale given in the upper panel ranging from high (red) to low (black). Heatmap (dendograms) showed all the *vtc* mutants clustered close to each other in both control and stressed conditions. (A) Leaf extract in positive mode, (B) Apoplast in positive mode, (C) High light in positive mode, (D) High light in negative mode, (E) Pathogen in positive mode, (F) Pathogen in negative mode, (G) wounding in positive mode, (H) wounding in negative mode.

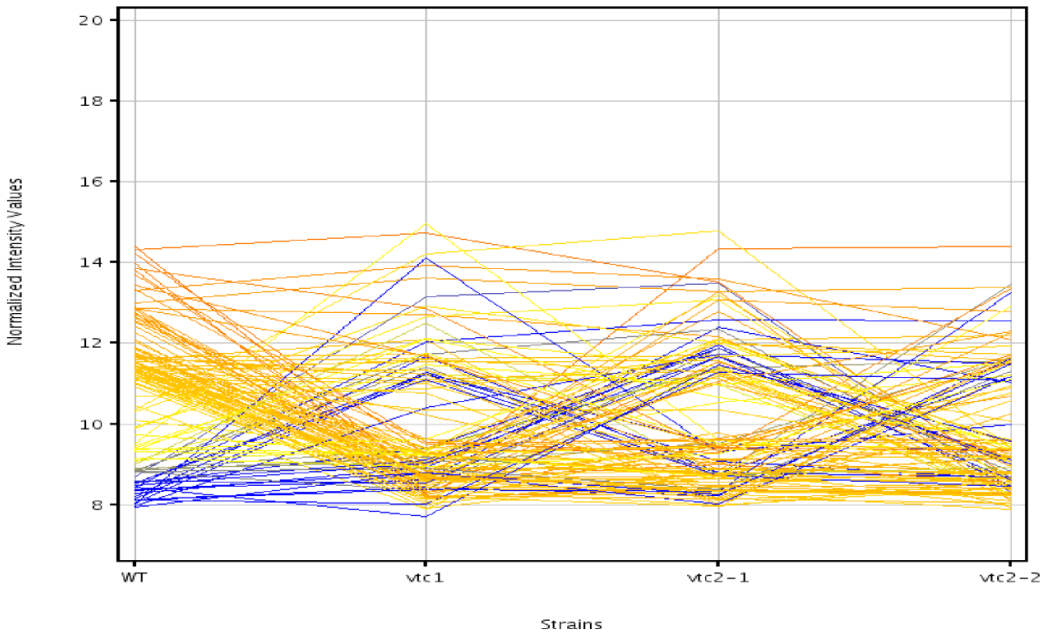
3.6.2. 4 Profile plot

Significantly different metabolites ($p < 0.0001$) from WT and the *vtc* mutants are displayed in a profile plot view using GeneSpring GX (Figure 3.9). In the plot, each continuous line corresponds to a single metabolite's normalised log ratio intensity value (Y-axis) in WT and the *vtc* mutants of *Arabidopsis thaliana*. In KMPA, data were generally normalised on $\log_2$ during alignment. The normalisation was not undertaken when the data were processed for statistical analysis. The noise level detected on KMPA was filtered out by Genespring GX (Agilent, Technologies) where the lowest values of normalised intensity were considered as the plot baseline. These results showed that in each experiment, distinct patterns of metabolites differentiate the strains. Overall, approximately half of these masses were significantly different between WT and the *vtc* mutants. There are therefore, a considerable number of masses specifically affected by either ascorbate deficiency or the mutations.

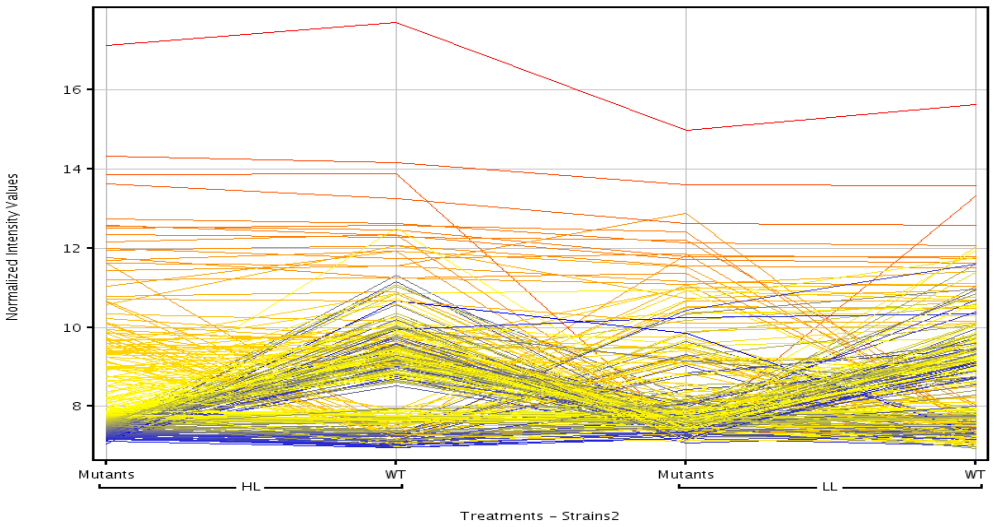
A


Normalized Intensity Values
18
16
14
12
10
8
WT
vtc1
vtc2-1
vtc2-2
Strains

B


Normalized Intensity Values
20
18
16
14
12
10
8
WT
vtc1
vtc2-1
vtc2-2
Strains

C



D

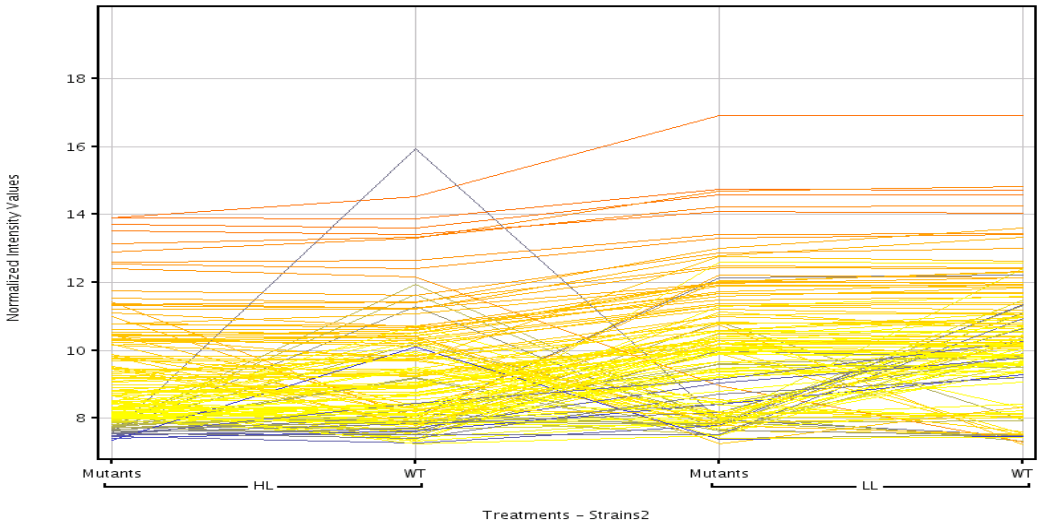


Figure 3.9 Profile plots depicting masses which are significantly different between WT and the *vtc* mutants as determined by ANOVA. (A) Leaf extract in positive ionisation mode (B) Apoplast in positive ionisation mode (C) High Light treated in positive ionisation mode (D) HL treated in negative ionisation mode. Masses shown were significantly different between strains or significantly affected by stress treatment (Analysis of variance $p < 0.0001$). Each line in the graph view represents a metabolite intensity value (Y-axis) and strains (X-axis). The graph shows the average values of biological replicates for each strain.

3.6. 3 Metabolite Identification

3.6.3. 1 Identification of selected metabolites differentially expressed between WT and *vtc* mutants

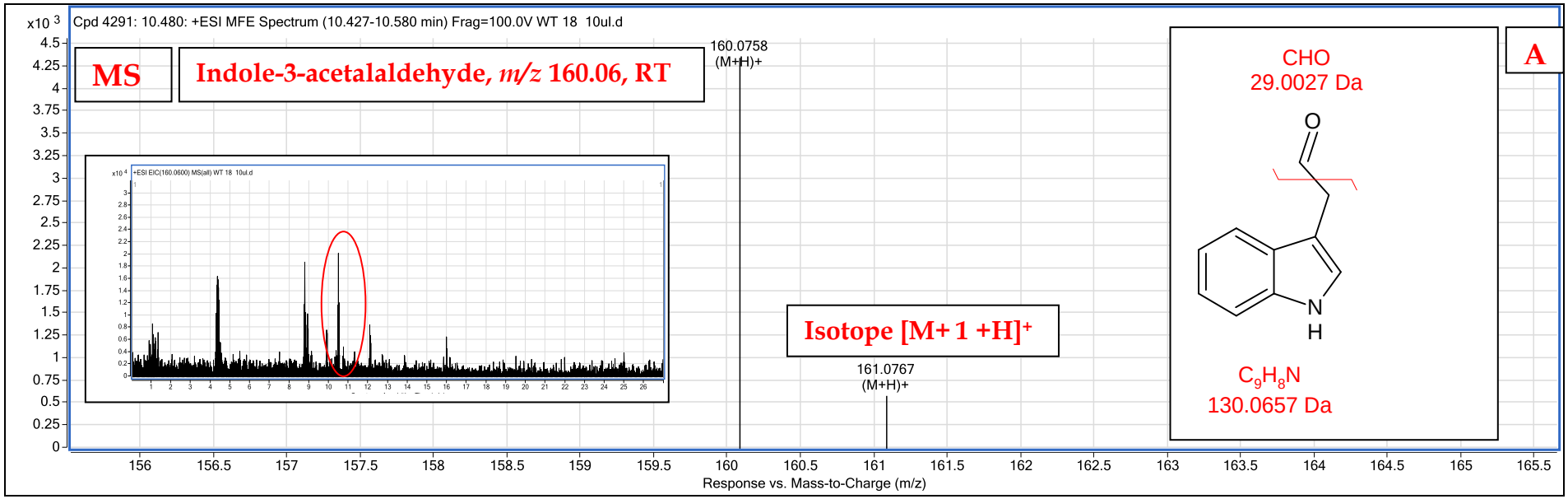
The analysis shown in the previous sections reveals that a large number of metabolites are affected by ascorbate deficiency and abiotic stresses. Twenty eight metabolites were selected for further investigation using isotopic distributions and fragmentation. Five metabolites were validated using standards. The Rt and *m/z* values of precursor and product ions were compared with the endogenous metabolites identified from the sample extract. Employing this method scopoletin, tyrosine, pyroglutamate, glutathione and proline were identified. Three metabolites such as indole-3-acetaldehyde, scopolin and 5-hydroxyferulic acid were identified using information from previous literature (Page *et al.*, 2011; Kai *et al.*, 2006) and the Massbank database (Horai *et al.*, 2010) because standards for these metabolites were unavailable. Theoretical mass and % abundances of isotopes of each metabolite are shown in brackets next to the detected *m/z* and isotopic abundance. The RT and *m/z* of observed precursor and product ion for all identified metabolites are listed in Table 3.3. Extracted ion chromatograms, mass spectra of isotopes and fragment ions are shown in Figure 3.10 and peak heights are shown in Figure 3.11. The positively identified metabolites are as follows (1) Metabolite, *m/z* 160.075 (159.074) with a first isotope abundance of 12.4 % (11.8 %) was identified as indole-3-acetaldehyde from the apoplast and the whole leaf extract under normal growth conditions. It was significantly higher in WT than the *vtc* mutants. (2) Tyrosine was identified in the

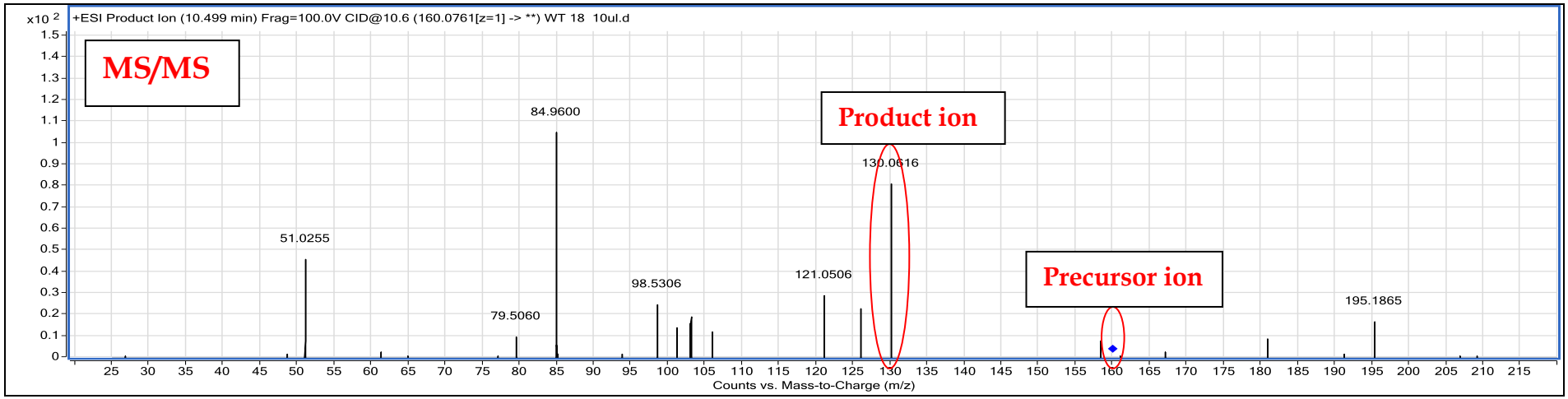
leaf extract, which was significantly higher in *vtc2* than WT and *vtc1*. The *m/z* for tyrosine was 182.081 (181.0809) with a first isotope abundance of 10.11% (9.73%). (3) Scopoletin and its β-glucopyranoside scopolin (glycosylated form of scopoletin) were identified in leaf tissue of *Arabidopsis thaliana* WT and the *vtc* mutants. The *m/z* for scopoletin was 193.049 (192.042) with a first isotope abundance of 10.7 % (10.8 %). The quantity of scopoletin was significantly higher in WT than the *vtc* mutants. It was also identified in the apoplast and showed an identical pattern of accumulation as in the whole leaf extracts but showed no response to HL treatment. Interestingly, in strong contrast to scopoletin, the quantity scopolin, *m/z* 355.10 (354.095) with a first isotope abundance of 17.68 % (17.30 %) was significantly lower in WT than the *vtc* mutants and also showed no significant changes in HL. (5) Glutathione (GSH) was also identified in WT and the *vtc* mutants after light treatment. GSH significantly increased in all strains in response to HL, but no significant difference was found between WT and *vtc* mutants in both light conditions. The *m/z* for glutathione was 308.06 (307.083) with a first isotope abundance of 12.21 % (13.2). (6) Proline, an amino acid, *m/z* 116.07 (115.063) with an isotope abundances of 5.81 % (5.41%) and 0.3 % (0.4 %) was identified. Its quantity was significantly increased in HL acclimated plants. WT and the *vtc1* accumulated significantly higher proline than *vtc2* and *vtc3*. The quantity of hydroxyferulic acid, a phenylpropanoid, *m/z* 209.045 (210.051) with an isotope abundance of 11.81 % (10.8 %) was significantly higher in WT than the *vtc* mutants in both light conditions, it slightly decreased in HL but no significant difference was found between HL and LL. All the above metabolites were

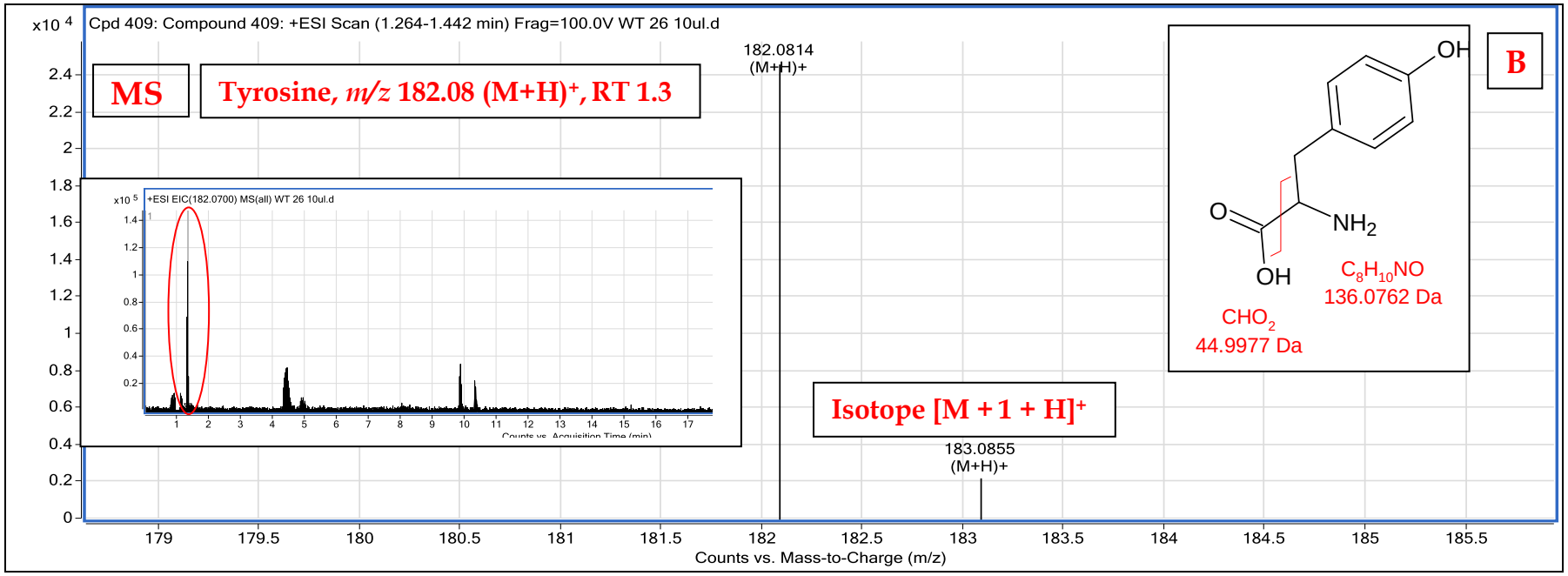
analysed in positive ionisation mode except hydroxyfeulic acid, which was analysed in negative ionisation mode.

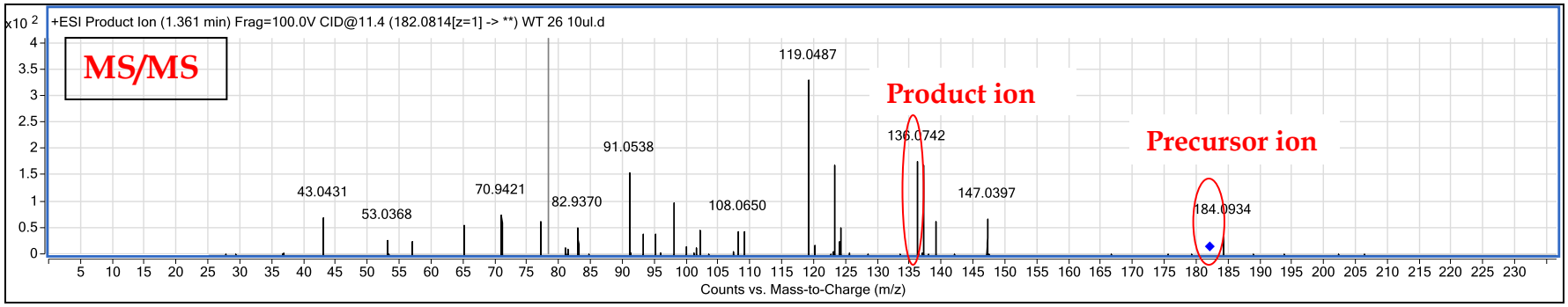
Table 3. 3 Metabolites identified in leaf apoplast and the whole leaf extract of *Arabidopsis thaliana* under normal growth conditions and after light treatments in positive and negative ionisation mode. Cpd = Compound (metabolite), Rt = Retention time, SE = standard Error, *m/z* = mass to charge ratio, ESI = Electrospray Ionisation, Treatment = stress, HL = High light, No = under normal growth conditions.

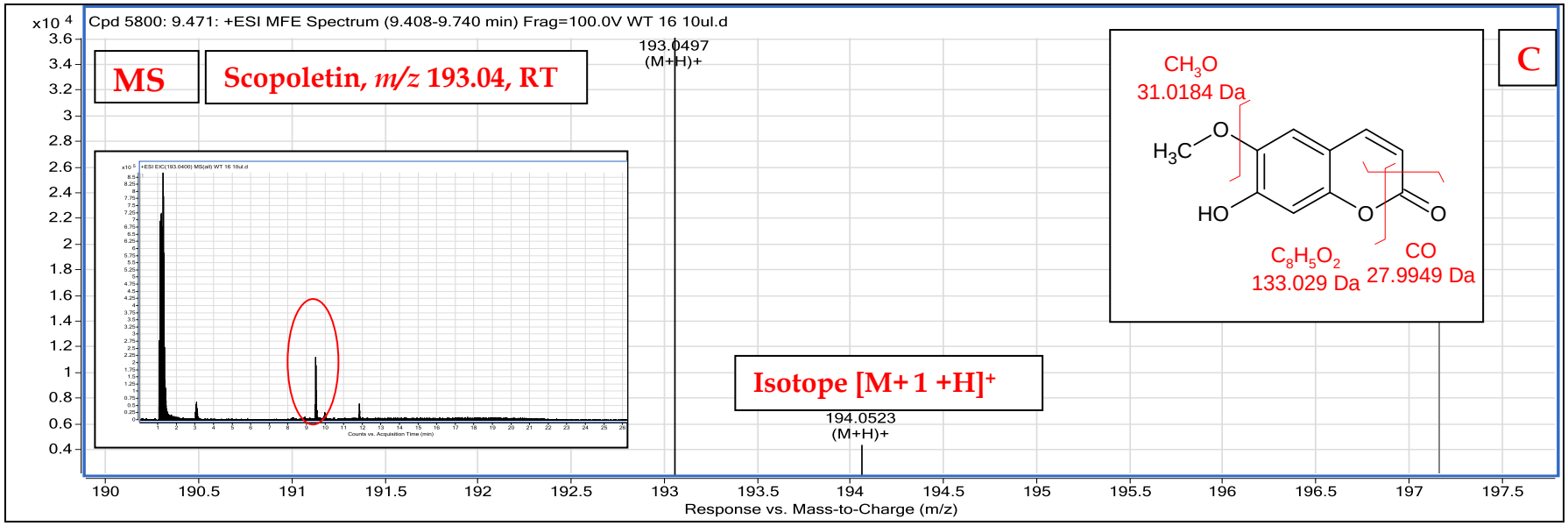
Compound ID	cellular location	Mode	Rt (mins)	Rt SE	Precursor Ion (*m/z*)	Neutral mass	Mass SE	Product Ion (*m/z*)	Identified compounds	Treatment
Cpd1	Apoplast	ESI^+	10.7	0.04	160.0756 $[M+ H]^+$	159.067	0.0001	77.03 130.06 144.04	Indole-3-acetaldehyde	No
Cpd 11	Leaf Extract	ESI^+	1.3	0.002	182.08 [M+ H]+	181.07	0.000005	136.07	Tyrosine	No
Cpd 18	Leaf Extract	ESI^+	9.1	0.010	193.08 [M + H]+	192.07	0.0002	114 103 137	Scopoletin	HL
Cpd 13	Leaf Extract	ESI^+	8.2	0.01	355.1 [M + H]+	354.09	0.0002	133.02 193.04 114.04	Scopolin	No
Cpd 15	Leaf Extract	ESI^+	1.4	0.006	130.05 [M+ H]+	129.04	0.001	84.04 69.5	Pyroglutamate	HL
Cpd 19	Leaf Extract	ESI^+	1.04	0.0007	308.06 [(M+ H]+	307.05	0.0004	84.9 116.06 162.02	Glutathione	HL
Cpd 17	Leaf Extract	ESI^+	1.01	0.001	116.06 [M+ H]+	115.06	0.000006	70.05	Proline	HL
Cpd 22	Leaf Extract	ESI^-	7.9	0.004	209.04 $[M -H]^-$	210.04	0.0003	119.03 107.9 164	5-Hydroxyferulic acid	HL

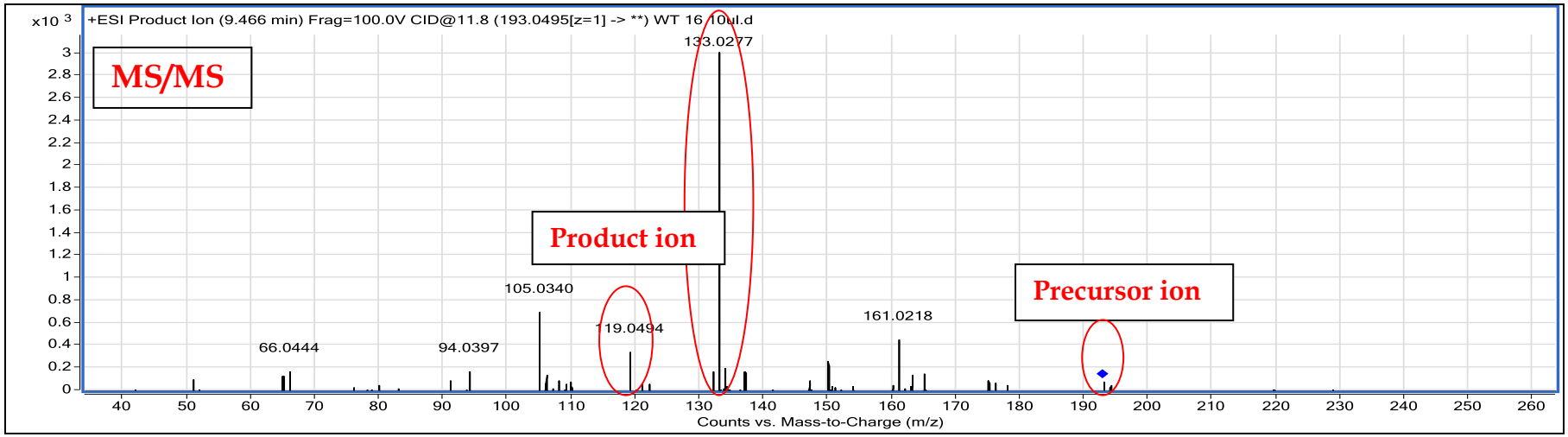

Cpd 4291: 10.480: +ESI MFE Spectrum (10.427-10.580 min) Frag=100.0V WT 18 10ul.d
MS
Indole-3-acetalaldehyde, m/z 160.06, RT
160.0758
(M+H)+
Isotope [M+1 +H]+
161.0767
(M+H)+
CHO
29.0027 Da
C9H8N
130.0657 Da
A
Response vs. Mass-to-Charge (m/z)

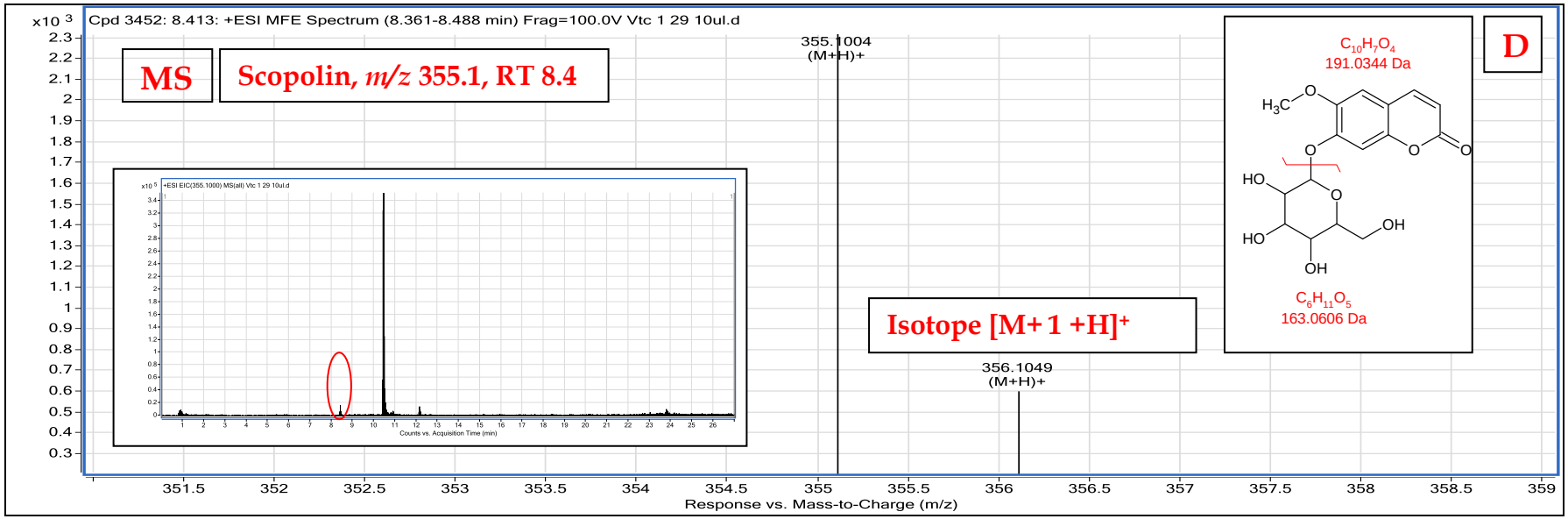

+ESI Product Ion (10.499 min) Frag=100.0V CID@10.6 (160.0761[z=1] -> **) WT 18 10ul.d
MS/MS
Product ion
Precursor ion
84.9600
130.0616
51.0255
98.5306
121.0506
79.5060
195.1865
Counts vs. Mass-to-Charge (m/z)

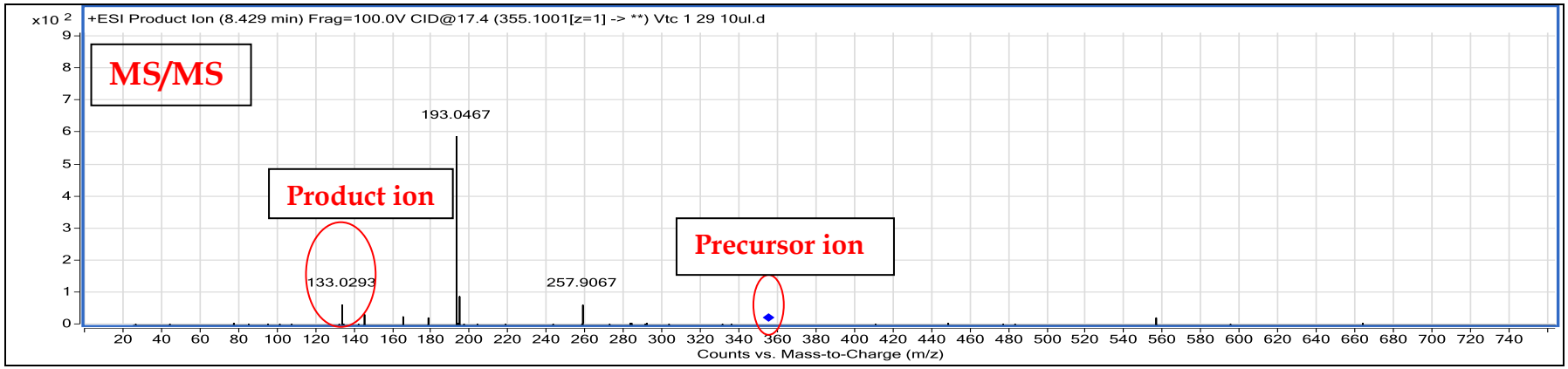

Cpd 409: Compound 409: +ESI Scan (1.264-1.442 min) Frag=100.0V WT 26 10ul.d
MS
Tyrosine, m/z 182.08 (M+H)+, RT 1.3
182.0814
(M+H)+
+ESI EIC(182.0700) MS(all) WT 26 10ul.d
B
OH
O
NH2
OH
C8H10NO
136.0762 Da
CHO2
44.9977 Da
Isotope [M +1 + H]+
183.0855
(M+H)+
Counts vs. Mass-to-Charge (m/z)

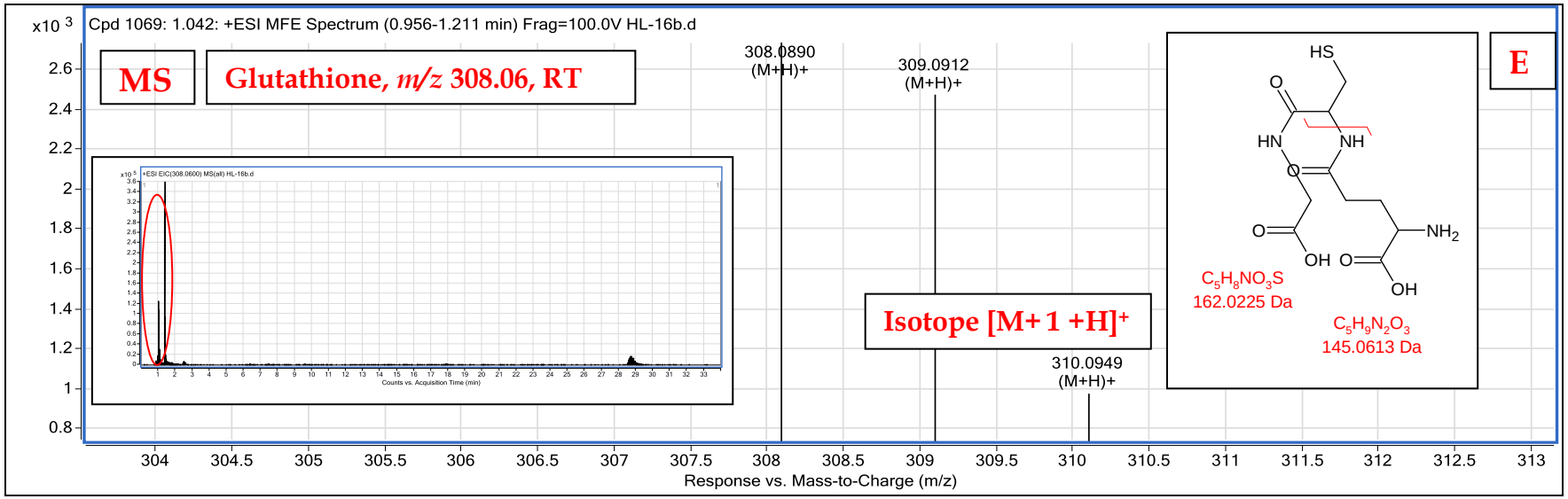

+ESI Product Ion (1.361 min) Frag=100.0V CID@11.4 (182.0814[z=1] -> **) WT 26 10ul.d
MS/MS
119.0487
Product ion
136.0742
Precursor ion
91.0538
43.0431
53.0368
70.9421
82.9370
108.0650
147.0397
184.0934
Counts vs. Mass-to-Charge (m/z)

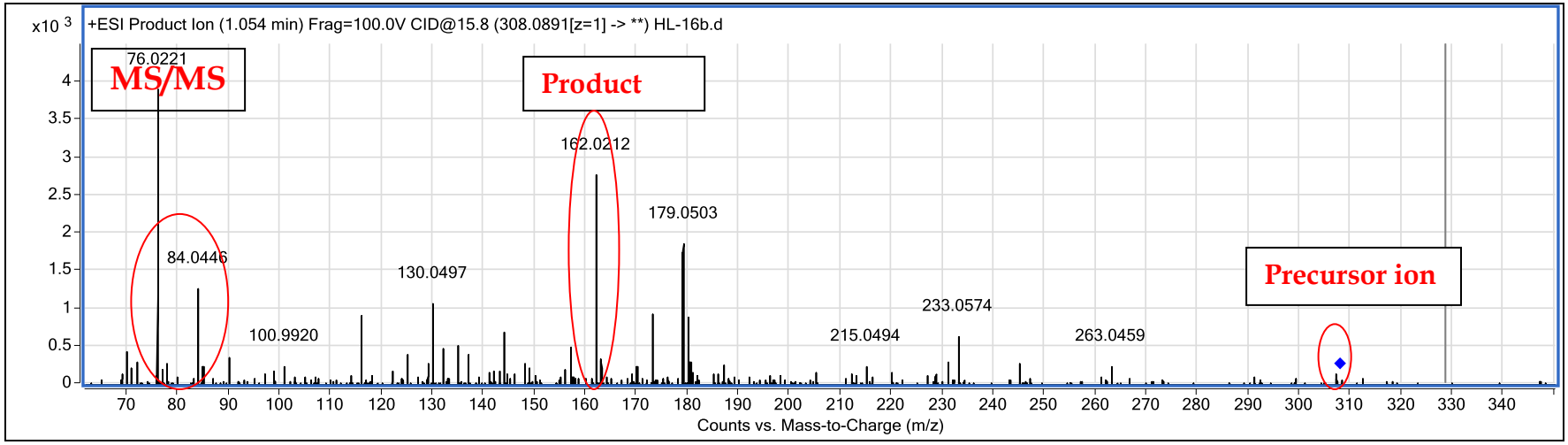

Cpd 5800: 9.471: +ESI MFE Spectrum (9.408-9.740 min) Frag=100.0V WT 16 10ul.d
MS
Scopoletin, m/z 193.04, RT
193.0497
(M+H)+
C
CH3O
31.0184 Da
C8H5O2
133.029 Da
CO
27.9949 Da
Isotope [M+1 +H]+
194.0523
(M+H)+
Response vs. Mass-to-Charge (m/z)

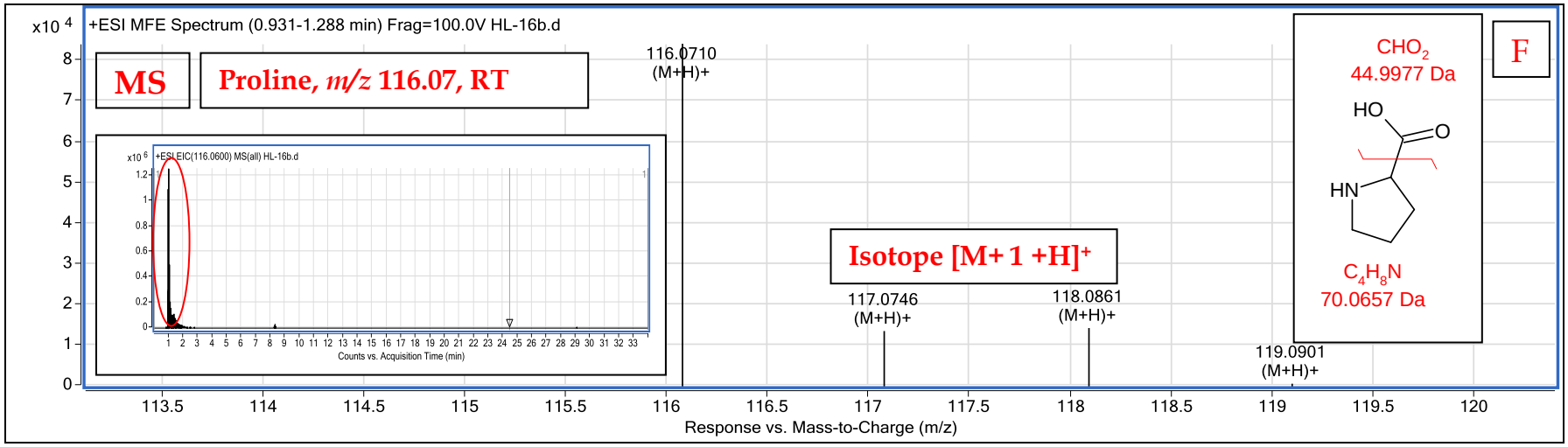

+ESI Product Ion (9.466 min) Frag=100.0V CID@11.8 (193.0495[z=1] -> **) WT 16 10ul.d
MS/MS
133.0277
Product ion
105.0340
119.0494
66.0444
94.0397
161.0218
Precursor ion
Counts vs. Mass-to-Charge (m/z)

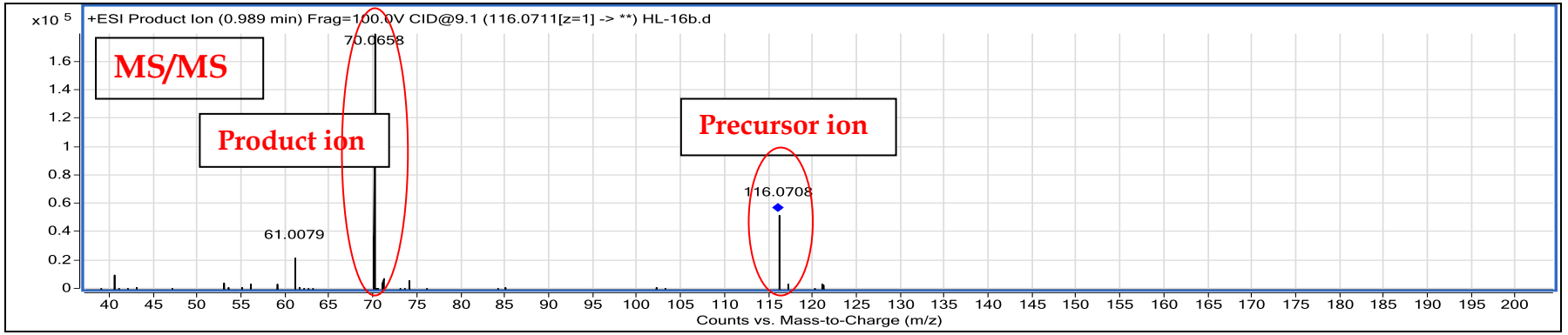

Cpd 3452: 8.413: +ESI MFE Spectrum (8.361-8.488 min) Frag=100.0V Vtc 1 29 10ul.d
MS
Scopolin, m/z 355.1, RT 8.4
355.1004
(M+H)+
356.1049
(M+H)+
Isotope [M+1 +H]+
C10H7O4
191.0344 Da
C6H11O5
163.0606 Da
D
Response vs. Mass-to-Charge (m/z)


+ESI Product Ion (8.429 min) Frag=100.0V CID@17.4 (355.1001[z=1] -> **) Vtc 1 29 10ul.d
MS/MS
193.0467
Product ion
133.0293
257.9067
Precursor ion
Counts vs. Mass-to-Charge (m/z)


Cpd 1069: 1.042: +ESI MFE Spectrum (0.956-1.211 min) Frag=100.0V HL-16b.d
MS
Glutathione, m/z 308.06, RT
308.0890
(M+H)+
309.0912
(M+H)+
Isotope [M+1 +H]+
310.0949
(M+H)+
E
C5H8NO3S
162.0225 Da
C5H9N2O3
145.0613 Da
Response vs. Mass-to-Charge (m/z)


+ESI Product Ion (1.054 min) Frag=100.0V CID@15.8 (308.0891[z=1] -> **) HL-16b.d
MS/MS
76.0221
Product
162.0212
179.0503
84.0446
130.0497
100.9920
233.0574
215.0494
263.0459
Precursor ion
Counts vs. Mass-to-Charge (m/z)


x10 4
+ESI MFE Spectrum (0.931-1.288 min) Frag=100.0V HL-16b.d
MS
Proline, m/z 116.07, RT
116.0710
(M+H)+
+ESI EIC(116.0600) MS(all) HL-16b.d
Counts vs. Acquisition Time (min)
Isotope [M+1 +H]+
117.0746
(M+H)+
118.0861
(M+H)+
119.0901
(M+H)+
CHO2
44.9977 Da
HO
O
HN
C4H8N
70.0657 Da
F
Response vs. Mass-to-Charge (m/z)


x10 5
+ESI Product Ion (0.989 min) Frag=100.0V CID@9.1 (116.0711[z=1] -> **) HL-16b.d
MS/MS
70.0658
Product ion
Precursor ion
116.0708
61.0079
Counts vs. Mass-to-Charge (m/z)

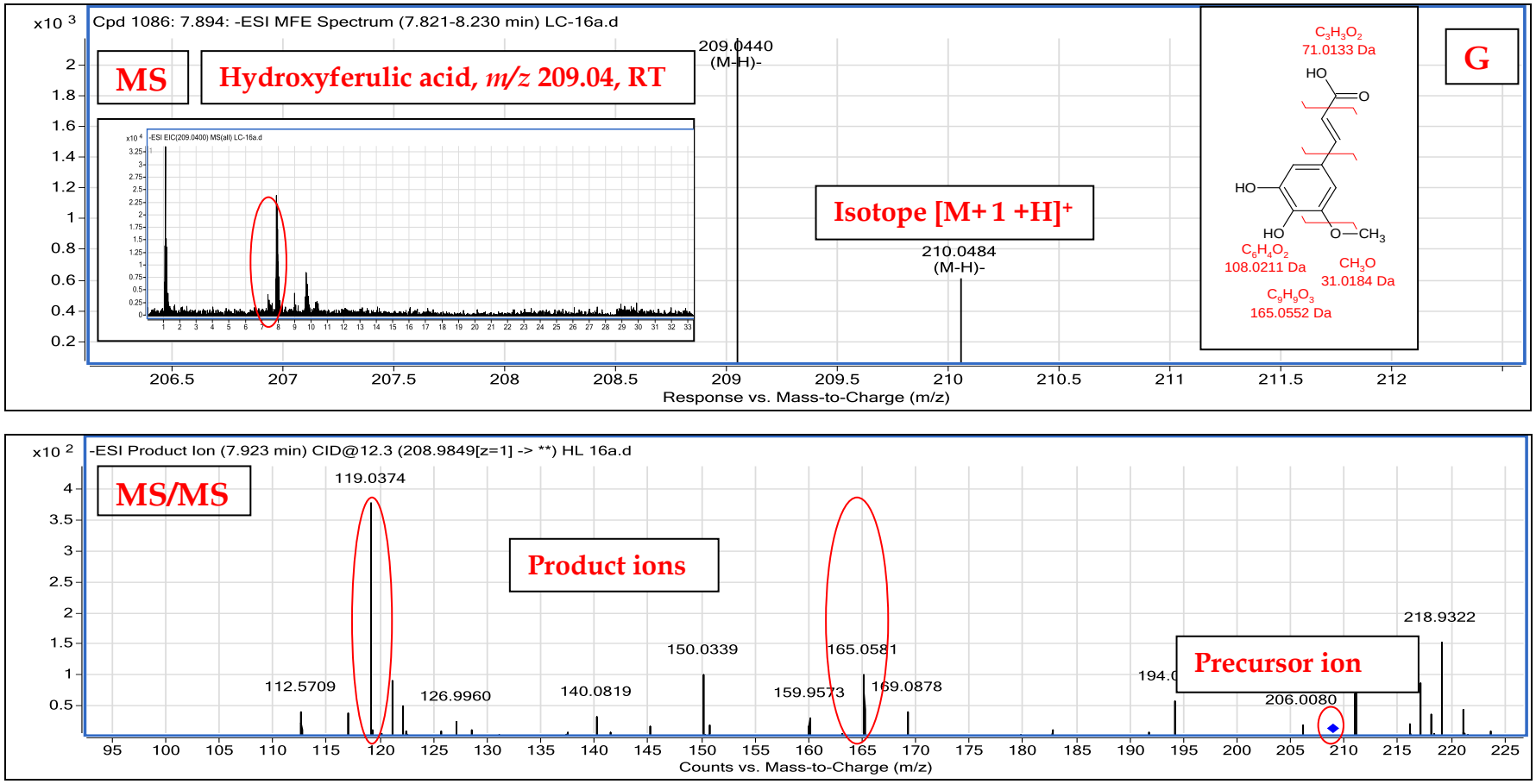


Figure 3.10 Chromatograms and mass spectra of positively identified metabolites from *Arabidopsis thaliana*. Upper panel show extracted ion chromatogram, detected accurate masses of [M+H]+ ions, isotopes structure and chemical formula of relevant metabolites. Lower panel show the MS/MS spectra of the identified metabolite. (A) Indole-3-acetaldehyde, (B) Tyrosine, (C) Scopoletin, (D) Scopolin, (E) Glutathione, (F) Proline (G) 5-Hydroxyferulic acid.

3.6.3.2 Other metabolites affected by ascorbate deficiency

Twenty metabolites shown to be differentially expressed between the WT and the *vtc* mutants were identified by comparing their Rt, mass, fragmentation and isotopic distributions from QToF auto MS/MS (untargeted fragmentation), QToF-targeted MS/MS and QQQ-targeted MS/MS. Due to unavailability of standards these compounds were not identified with certainty. Important information such as Rt and mass for these possible hits are listed in Table 3.4 and peak heights are shown in Figure 3.11. These compounds were significantly different between the WT and the *vtc* mutants. Plots of higher or lower expression relative to the WT are shown in Figure 3.11.

3.6.3.2.1 Metabolites differentially expressed in the apoplastic fluid

Four metabolites identified in the leaf apoplast which were significantly different between WT and the *vtc* mutants. These metabolites were *m/z* 385.2352 $[M+H]^+$ with a first isotope abundance of 5.41 %, *m/z* 393.0472 $[M+H]^+$ with the first and second isotope abundance of 11.20 % and 6.35 % respectively, *m/z* 342.175 $[M+H]^+$ and isotope abundance of 11.23 % and *m/z* 308.523 $[M+H]^+$ with isotopes abundances of 6.83 % and 1.50 %. All were present at significantly higher levels in the WT compared to the *vtc* mutants. The Rt, fragment ions and possible hits from database searches with identities known to be present in *Arabidopsis thaliana* are listed in Table 3.4 A and peak heights are shown in Figure 3.11 A. All these compounds were detected in positive ionisation mode.

3.6.3.2. 2 Metabolites differentially expressed in the whole leaf extract

In the whole leaf extracts under normal growth conditions, Rt, mass, fragmentation, and isotopic distributions for seven metabolites were extracted and compared across different samples. Metabolites, *m/z* 283.174 $[M+H]^+$ with first isotope abundance of 14.15 %, *m/z* 338.265 $[M+H]^+$ with a first isotope abundance of 18.74 %, *m/z* 502.371 $[M+H]^+$ with a first isotope abundance of 27.09 % and *m/z* 263.017 with an isotope abundance of 9.063 % were present at a significantly higher level in WT than the *vtc* mutants while the metabolite *m/z* 669.188 $[M+H]^+$ with a first isotope abundance of 30.84 % was present at a significantly lower level in the WT. All other information related to these compounds such as Rt, fragmentation pattern and possible hits are listed in Table 3.4 B and peak heights are shown in Figure 3.11 B. All these compounds were ionised in positive ionisation mode.

3.6.3.2.3 Metabolites differentially expressed in HL acclimated plants

(A) Identification in positive ionisation mode

In high light treated plants, a few compounds affected by light intensity were identified as being significantly different between the WT and *vtc* mutants and affected by light intensity. Proline and glutathione were positively identified and validated using standards as described above and listed in Table 3.3, and Figures 3.10 and 3.11 C. Similarly, Rt, mass, fragmentation, isotopic distributions and peak height were recorded for the rest of the compounds. Metabolites, *m/z* 147.020 $[M+H]^+$ (no isotope found), *m/z* 584.473 $[M+H]^+$ with a first isotope abundance of 29.369 %, *m/z* 855.150 $[M+H]^+$ with isotope abundance of 47.7 % were identified. The

metabolite with *m/z* 147.02 did not show a significant difference in LL across all strains but when acclimated from LL to HL it started to deplete. The *vtc* mutants showed a slight decrease, but it completely vanished in the WT after HL treatment. Similarly, a compound with *m/z* 855.15 was absent in the *vtc* mutants when grown in LL, but an easily detectable concentration was found in the WT. It increased dramatically in the *vtc* mutants when acclimated from LL to HL, but no significant difference was found between the WT and the *vtc* mutants after HL. The metabolite with *m/z* 584.473 was similar in WT and the *vtc* mutants in LL. A significant increase was observed after light treatment in the WT and the *vtc* mutants, but the WT accumulated significantly less than in the *vtc* mutants. All other information related to these metabolites such as Rt, fragment ions and possible hits are listed in Table 3.4 C and peak heights are shown in Figure 3.11 C. All these metabolites were ionised in positive ionisation mode.

(B) Identification in negative ionisation mode

In light treated samples, metabolites were also analysed in negative ionisation mode. Most of these metabolites were detected as a formate adduct (formic acid was used as a mobile phase modifier). The following metabolites were differentially expressed between the WT and the *vtc* mutants. Metabolite, *m/z* 390.037 with first and second isotope abundances of 14.61 % and 10.74 % respectively, was present in significantly higher amounts in the WT than the *vtc* mutants when acclimated from LL to HL, but with no difference between strains in LL. The metabolite with *m/z* 369.175 and isotope abundances of 16.84 % and 3.07 % was slightly reduced in the WT but not

significantly different between LL and HL.The amount was reduced in the *vtc* mutants when treated with HL being significantly different between HL and LL. It was also significantly lower in the *vtc* mutants compared to WT in HL but there was no difference in LL across all strains. Metabolite *m/z* 209.948 with a first isotope abundance of 12.812 % was present at a higher level in the *vtc* mutants in LL but dramatically reduced in all strains when treated with HL, but there was no significant difference between the WT and the *vtc* mutants in HL. Similarly, the metabolite *m/z* 182.990 with its isotope abundances of 7.03 % and 4.26 % was the same in all strains in LL. A dramatic decrease occurred in all strains when acclimated to HL, but the quantity was always significantly lower overall in the WT. Metabolite *m/z* 325.054 with a first isotope abundance of 17.691 % was not affected by HL but was present in significantly higher amount in the WT than the *vtc* mutants. Another metabolite, *m/z* 217.990 with a first isotope abundance of 11.156 % was shown to have no difference in quantity between strains in LL and HL. A significant decrease occurred in all strains when acclimated from LL to HL. The auto MS/MS data were combined with targeted MS/ MS for further identification and confirmation. All other information related to these compounds such as Rt, fragmentation pattern and possible hits are listed in Table 3.4 D and peak heights are shown in Figure 3.11 D. All these compounds were ionised in negative ionisation mode.

Table 3. 4 Metabolites identified in *Arabidopsis thaliana.* (A) leaf apoplast (B) whole leaf extract under normal growth conditions (C) High Light light in positive mode (D) High Light in negative mode. Cpd = Compound, Rt = Retention time, SE = standard Error, *m/z* = mass to charge ratio, ESI = Electrospray ionisation (positive or negative), Treatment = Stress, HL = High Light

A

Compound ID	Cellular location	Mode	Rt (min)	Rt SE	Precursor Ion (*m/z*)	Neutral Mass	Mass SE	Product Ion (*m/z*)	Possible hits	Treatment
Cpd 2	Apoplast	ESI^+	9.9	0.01	193.123 [M + H]+	192.131	0.05	42.03 67.1 105.03 134.09	Scopoletin	No
Cpd 3	Apoplast	ESI^+	26.2	0.0003	385.235 [M + H]+	384.228	0.01	98.9 77 187.9 213.1	2-hydroxy-tetracosanoic acid/adenosyl-homocystiene	No
Cpd 4	Apoplast	ESI^+	1.2	0.01	393.047 [M + H]+	392.039	0.0002	129.01 139 173.02 43.02	4-Hydroxybutylglucosinolate	No
Cpd 5	Apoplast	ESI^+	4.3	0.01	342.175 [M + H]+	341.171	0.0002	117.08 176.04 123.04	Caffeic acid hexose or coumaric acid hexose	No
Cpd 6	Apoplast	ESI^+	1.3	0.008	308.523 [M + H]+	307.518	8.30E-05	76.02 84.04 115.9 179.04	No hit	No

B

Compound ID	Cellular location	Mode	Rt (mins)	Rt SE	Precursor Ion (*m/z*)	Neutral Mass	Mass SE	Product Ion (*m/z*)	Possible hits	Treatment
Cpd 7	Leaf Extract	ESI+	7.9	0.01	283.174 [M + H]+	282.167	0.0002	151.9	dihydroxyphaseic acid	No
								89	Xylobiose	
								45.03		
Cpd 8	Leaf Extract	ESI+	17	0.007	338.265 [M + H]+	337.258	0.0002	136.3	indole-3-acetyl-beta-1-D-glucose	No
								184.9		
Cpd 9	Leaf Extract	ESI+	21.7	0.006	502.371 [M + H]+	501.364	0.0001	89.05	Dihidrozeatin-riboside-O-glucoside	No
								107.05		
								128.06		
								165.08		
Cpd 10	Leaf Extract	ESI+	1.009	0.003	263.01 7 [M + H]+	262.01	0.001	38.96	no hit	No
								86.06		
								113.98		
Cpd 12	Leaf Extract	ESI+	9.1	0.01	193.085 [M + H]+	192.078	0.0002	114	Scopoletin	No
								103		
								137		
Cpd 14	Leaf Extract	ESI+	9.2	0.007	669.188 [M + H]+	668.181	0.0004	346.09	no hit	No
								226.87		
								185.04		

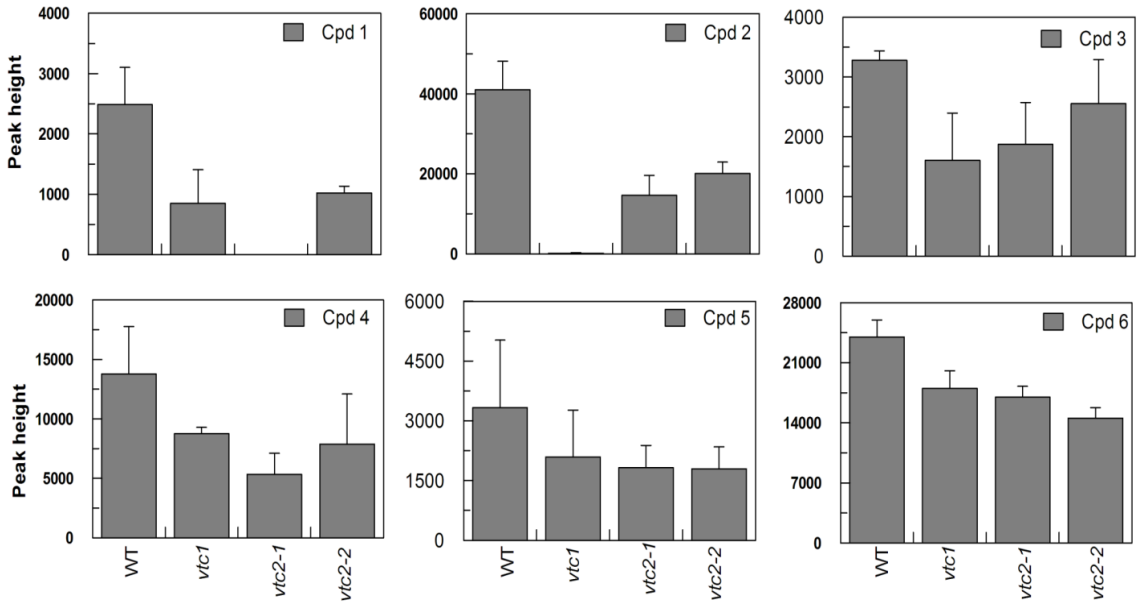
C

Compound ID	Cellular location	Mode	Rt (mins)	Rt SE	Precursor Ion (*m/z*)	Neutral mass	Mass SE	Product Ion (*m/z*)	Possible hits	Treatment
Cpd 16	Leaf Extract	ESI^+	1.3	0.0008	147.020 [M + H]+	146.013	5.96E-05	84.03	Coumarins	HL
								72.08	L-Lysine	
								55.04	L-Glutamine	
								133.02		
Cpd 18	Leaf Extract	ESI^+	7.2	0.004	193.040 [M + H]+	192.033	0.0001	107.95	Scopoletin	HL
								114.03	Isocitric acid/ citric acid	
								89.09	Quinic acid	
								90.06		
Cpd 20	Leaf Extract	ESI+	18.2	0.005	584.474 [M + H]+	583.393	0.005	89.05	N-octadecanoyl-phytosphingosine	HL
								90.06		
Cpd 21	Leaf Extract	ESI^+	11.3	0.004	855.150 [M + H]+	854.143	0.0003	100.5	No hit	HL
								712.16		
								704.56		

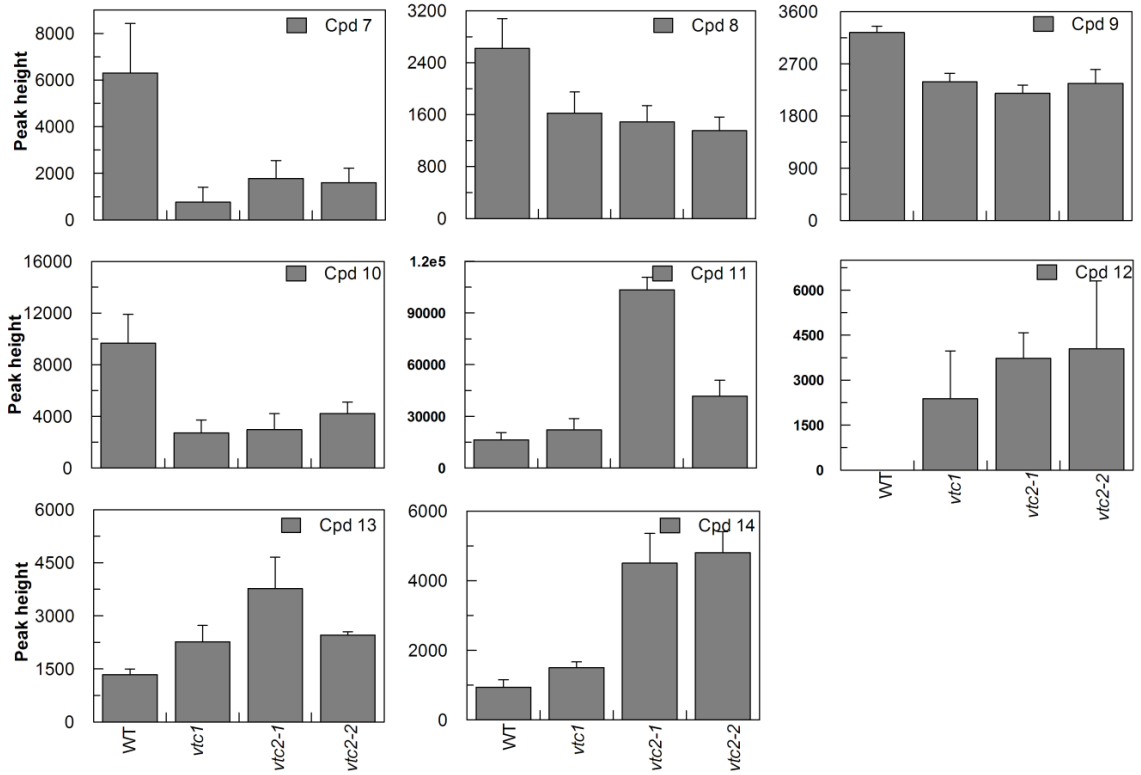
D

Compound ID	Cellular location	Mode	Rt (mins)	Rt SE	Precursor Ion (*m/z*)	Neutral mass	Mass SE	Product Ion (*m/z*)	Possible hits	Treatment
Cpd 23	Leaf Extract	ESI-	1.5	0.0025	390.037 [M + H]-	391.044	0.0006	111.008 191.01 59.01	Hydroxybutyl glucosinolate	HL
Cpd 24	Leaf Extract	ESI-	8.04	0.003	369.175 [M + H]-	370.182	0.0002	44.99 174.95 158.97	23-hydroxytricosanoic acid	HL
Cpd 25	Leaf Extract	ESI-	1.1	0.0013	209.948 [M + H]-	210.955	0.0001	61.98	No hits	HL
Cpd 26	Leaf Extract	ESI-	1.9	0.0065	182.990 [M + H]-	183.997	0.00007	85.02 111.01 115	D-3,4-Dihydroxymandelic acid	HL
Cpd 27	Leaf Extract	ESI-	7.9	0.0047	325.054 [M + H]-	326.061	0.00020	133 209.04 71.01	No hits	HL
Cpd 28	Leaf Extract	ESI-	1.4	0.0085	217.990 [M + H]-	218.997	0.0001	133.01 59.01 101.02	No hits	HL

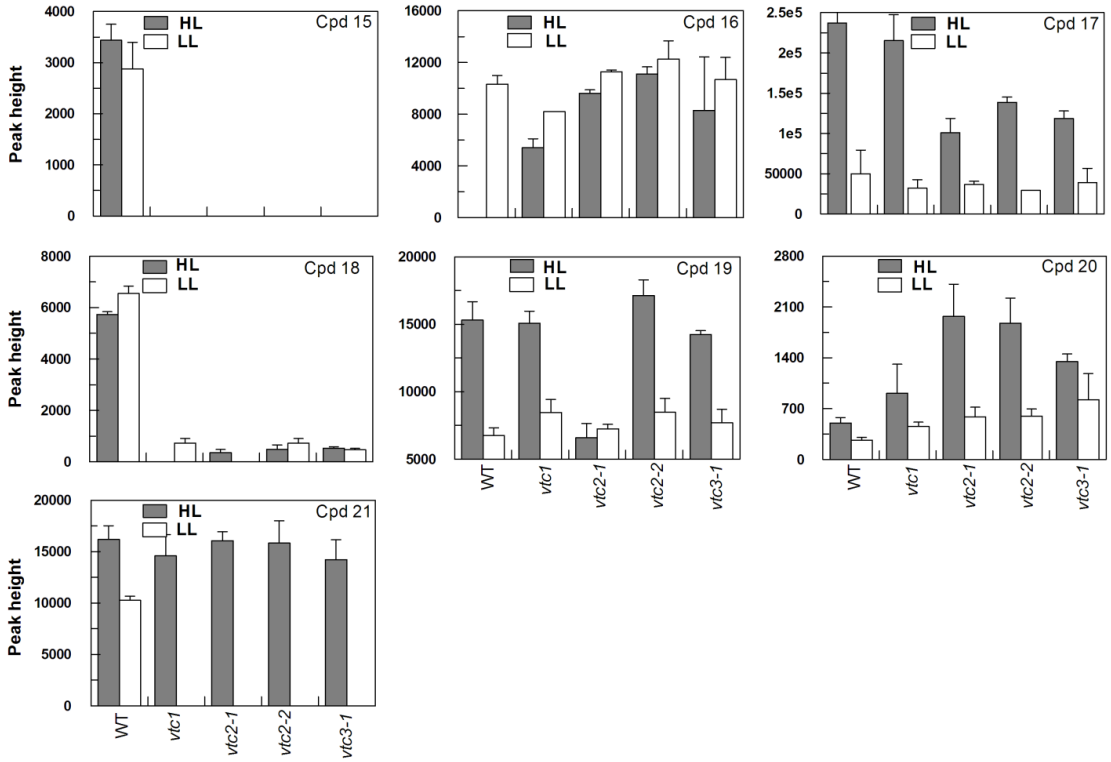
A


Cpd 1
Cpd 2
Cpd 3
Cpd 4
Cpd 5
Cpd 6
Peak height
WT
vtc1
vtc2-1
vtc2-2

B


Cpd 7
Cpd 8
Cpd 9
Cpd 10
Cpd 11
Cpd 12
Cpd 13
Cpd 14
Peak height
WT
vtc1
vtc2-1
vtc2-2

C


Peak height
HL
LL
Cpd 15
Cpd 16
Cpd 17
Cpd 18
Cpd 19
Cpd 20
Cpd 21
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

D

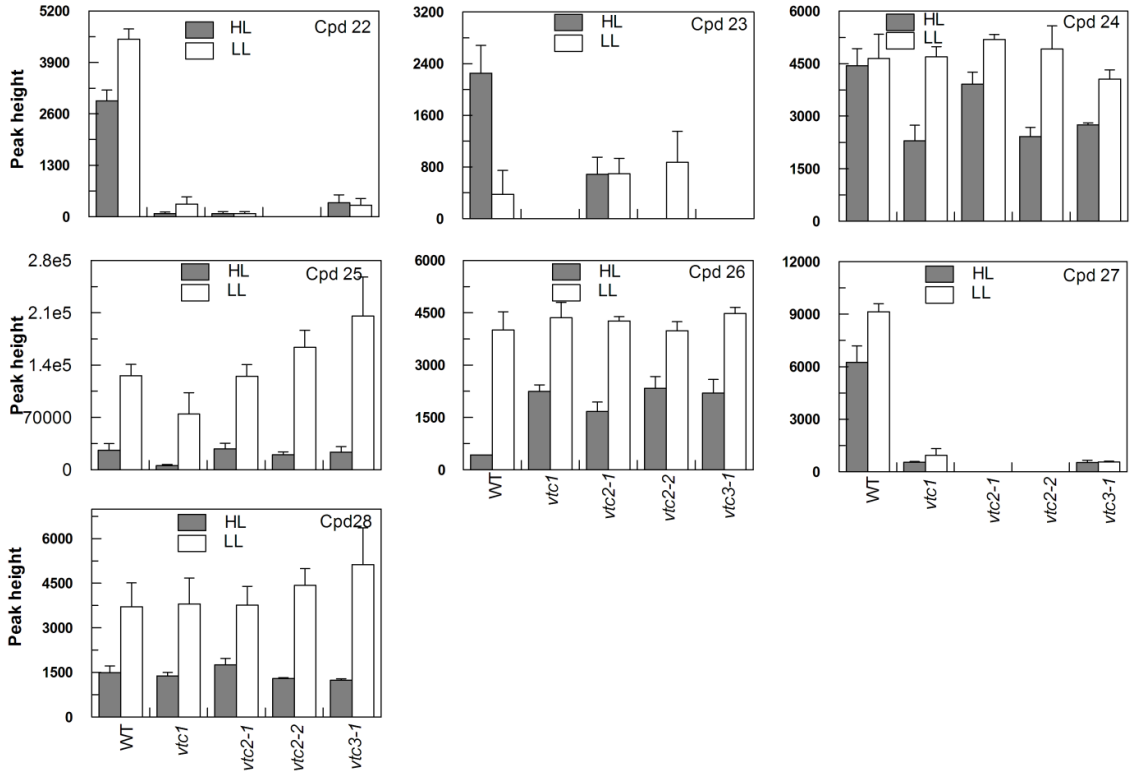


Figure 3. 11 Peak heights of metabolites identified in *Arabidopsis thaliana* are differentially expressed in WT and the *vtc* mutants (Table 3.3 and 3.4). Figures (A-D) (A) Apoplastic fluid (B) Leaf extracts (C) High Light treated (*Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 µmol m^{-2} s^{-1}) or HL (550-650 µmol m^{-2} s^{-1}) for 4 days in positive ion mode (D) High Light treated (*Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 µmol m^{-2} s^{-1}) or HL (550-650 µmol m^{-2} s^{-1}) in negative ion mode. All the compounds were identified in positive ion mode $(M+H)^+$ except 'D' which was analysed in negative ion mode. Error bars represent +1 SEM, n = 4, using one-way analysis of variance (apoplast and leaf extract) and 2-way analysis of variance for stress. The graph shows the peak height of each metabolite.

3. 7 Discussion

The aim of the research described in this chapter was to develop and evaluate a reproducible LC-MS based method for metabolite profiling in *Arabidopsis thaliana*. This was then used to identify metabolic changes in ascorbate deficient *vtc* mutants of *Arabidopsis thaliana* and to identify metabolites that respond to various abiotic stresses. The results described in this chapter established a very sensitive and reproducible method for large-scale metabolite profiling by developing an LC-QToF-MS based metabolomic method in combination with statistical tools.

The information produced by a single LC-QTOF-MS run is extensive and the selection of the dedicated software for data processing and comparison is crucial. The deconvolution of relevant mass signals, retention time and the subsequent alignment of different peaks were performed using MassHunter and KMPA (V. Perera and R. Yang personal communication) respectively. MassHunter removed all redundant signals (isotope, adducts and other un-intended in source) during data deconvolution. So, the total number of deconvoluted mass signal was counted as an independent signal which belongs to each feature in a sample but there are many chances of noise contamination, which was abstracted during the noise filtering step. In detailed visualisation of the data, an average of less than 0.1 min Rt drift was detected in a single experiment of samples but a drift of about 1-1.5 min between series, which were analysed over the last 2-3 years. This was possibly due to variations in chromatographic conditions. As the Rt drift was found to be non-linear, it was not possible to align all the experiments together. This large Rt shift can cause misalignments and misclassification of the data. The small Rt drift within the

experiment was sufficient to align all the samples together under the same chromatographic conditions which produced about 60-70% of alignment accuracy. As previously reported Rt is an important parameter during metabolite profiling studies, which can vary between runs thus it is important to take it into account when processing LC-MS data for further analysis (Moco *et al.*, 2006). To control for RT drift and *m/z* differences, both within and between experiments, the RT and *m/z* of the internal standards and sinapoyl malate (an abundant compound of *Arabidopsis*) were compared. The method enables detection and alignment of 3000-5000 molecular features per sample of *Arabidopsis* leaf extract and apoplastic fluid. On average 9500 features were aligned across all strains. This compares to well established metabolomic research by Wageningen group (Moco *et al.*, 2006; De Vos *et al.*, 2007; von Roepenack-Lahaye *et al.*, 2004a) who extracted and aligned ~5000 mass signals in tomato peel between samples. Therefore, the method is comparable with similar studies that have used with varying deconvolution and alignment methods. Previous studies have only identified a small proportion (Moco *et al.*, 2006; Matsuda *et al.*, 2009) of features detected by LC-MS profiling. The total number of features is somewhat more than would be predicted from the *Arabidopsis* metabolic map (AraCyc) which is 2814. Many of these could be formed by chemical reaction during extraction. Alternatively, a wide variety of compounds could accumulate as a result of non-specific oxidation, hydroxylation, methylation, glycosidation and other modifications reactions that occur as leave age. The number of features found in the apoplastic fluid is surprising. It is clear that many intracellular metabolites also occur in the apoplast. Glucose-6-phosphate, generally considered to be absent from the

apoplast (Burkey, 1999) was not found, indicating that these molecular features were not likely to be derived from cell damage during the preparation of apoplastic fluid. Primary metabolites (sugars, amino acids and organic acids) occurs in the apoplast (Tejera *et al.*, 2006; Song *et al.*, 2000) but the molecular feature found in this study are less polar (have retention time more than 3 minutes) than primary metabolites. There have been no other detailed reports of the metabolites profile of the apoplast, so further work is needed to characterise the compounds present and compare them with the intracellular.

3.7. 1 Identification of compounds differentially expressed between WT and *vtc* mutants

A general strategy was developed to generate a list of differentially expressed features and finally, to try to elucidate their structures for the study of biochemical changes in *Arabidopsis vtc* mutants. The mass and Rt of each metabolite from aligned data were compared to raw data having a mass accuracy (less than 5 ppm) and small Rt drift (< 0.1 min) were subjected to statistical analysis. Features which were significantly different ($p < 0.05$) between the WT and the *vtc* mutants were selected for annotation and identification.

The HCA clustering showed that the biological replicates of different samples were clustered together, which provides strong evidence that the deconvolution and alignment methods are robust. Metabolites which could be similar in their chemical nature and show similar behaviour to change environmental conditions were clustered close to each other and HCA clustering also indicated that most of the

metabolites which were affected by ascorbate deficiency were clustered together in the *vtc* mutants.

The compound identification stage, in particular, for secondary metabolites, through an untargeted metabolite profiling approach encountered many problems. The low availability of standards of secondary metabolites affected compound validity. Due to overlapping of compounds, low intensity mass signals or difficulties in the isolation of the mass signal for MS/MS fragmentation and isotopes, the extraction of useable information for identification purposes was complicated. The lack of dedicated software that integrates all the masses generated by MS limited the identification procedure to a manual level. In spite of all the above mentioned hurdles, and complexity of metabolite analysis, 28 metabolites were isolated based on Rt, mass, isotopic distributions and fragment ions from different experiments which were differentially expressed ($p < 0.05$) between the *Arabidopsis* WT and the *vtc* mutants, while leaving more to be identified. These data were analysed in untargeted and targeted manners on LC-QToF-MS. Eight metabolite identifications were validated using standards and information from previous literature, and their Rt, mass, isotopic distribution and fragmentation were compared to endogenous metabolites analysed using QToF MS and QQQ-MS in an untargeted and targeted manner. The total number of detectable compounds cannot be identified on the basis of fragmentation, isotopic distributions and adduct formation because some of the features gave more than one hit due to similarity in formula and chemical nature and unfortunately, standards for all compounds were not available.

The positively identified compounds include indole-3-acetaldehyde which is an intermediate in the synthesis of the plant hormone auxin (Tsurusaki *et al.*, 1997) which is involved in many growth and developmental stages, including cell division, cell elongation, cell differentiation and flowering (VandenBosch *et al.*, 2009; Bogre *et al.*, 2005; Campanoni *et al.*, 2003; Sakurai *et al.*, 2000; Luthen *et al.*, 2006). As we observed indole-3-acetaldehyde was detected in the apoplast and leaf extract where in both compartments, it was present at significantly higher level in the leaf apoplast and whole leaf extract of the WT than the *vtc* mutants. The reason for lower level of indole-3-acetaldehyde in the *vtc* mutants is not clear. One could speculate that this difference is associated with the small size of the *vtc* mutants compared to WT plants (Olmos *et al.*, 2006; Kerchev *et al.*, 2011). Scopoletin and its glycosylated form the scopolin synthesised from the phenylpropanoid pathway have been identified in *Arabidopsis* root tissues (Bednarek *et al.*, 2005; Rohde *et al.*, 2004). Scopoletin was also known to be accumulated in *Arabidopsis* and tobacco in response to *Streptomyces scabiei* suggesting its role in pathogen defence (Bouarab *et al.*, 2009). Previously, it was reported that scopoletin and scopolin biosynthesis were induced in the shoot and stem cells of *Arabidopsis thaliana* when treated with 2-4-dichlorophenoxyacetic acid (2-4-D) (Kai *et al.*, 2006). Kai *et al* (2008) analysed the accumulation of scopoletin and scopolin in the root tissue of *Arabidopsis thaliana* C3′H mutants (T-DNA insertion in a gene which encodes cytochrome P450 which catalysed 3′ hydroxylation of *p*-coumarate). The mutants produced lower scopoletin and scopolin than WT. They concluded that both scopoletin and scopolin are synthesised from the phenylpropanoid pathway where ferulic acid is first converted

to hydroxyferulic acid, which then produces scopoletin (Figure 3.12) (Kai *et al.*, 2008). Conversion of ferulic acid to hydroxyferulic acid used a 2-oxoglutarate-dependent dioxygenase enzyme (Shimizu *et al.*, 2008; Kai *et al.*, 2008). As reviewed in Chapter 1 (section 1.6) 2-oxo-glutarate dependent dioxygenases (2-ODD) require ascorbate for their efficient activity. Interestingly, we identified scopoletin in the leaf tissue of *Arabidopsis thaliana.* This has not been reported before and it was validated with a standard. In the leaf tissue of the *vtc* mutants, the concentration of scopoletin was significantly lower than in the WT, while in strong contrast, scopolin was present in a higher level in the *vt*c mutants than the WT. It is interesting compound 22 (Table 3.3) identified as hydroxyferulic acid, was present at much lower level in the *vtc* mutants than the WT. This is consistent with a limitation on the hydroxylation of ferulic acid. So, further investigations are required to understand the relationship between ascorbate and scopoletin metabolism.

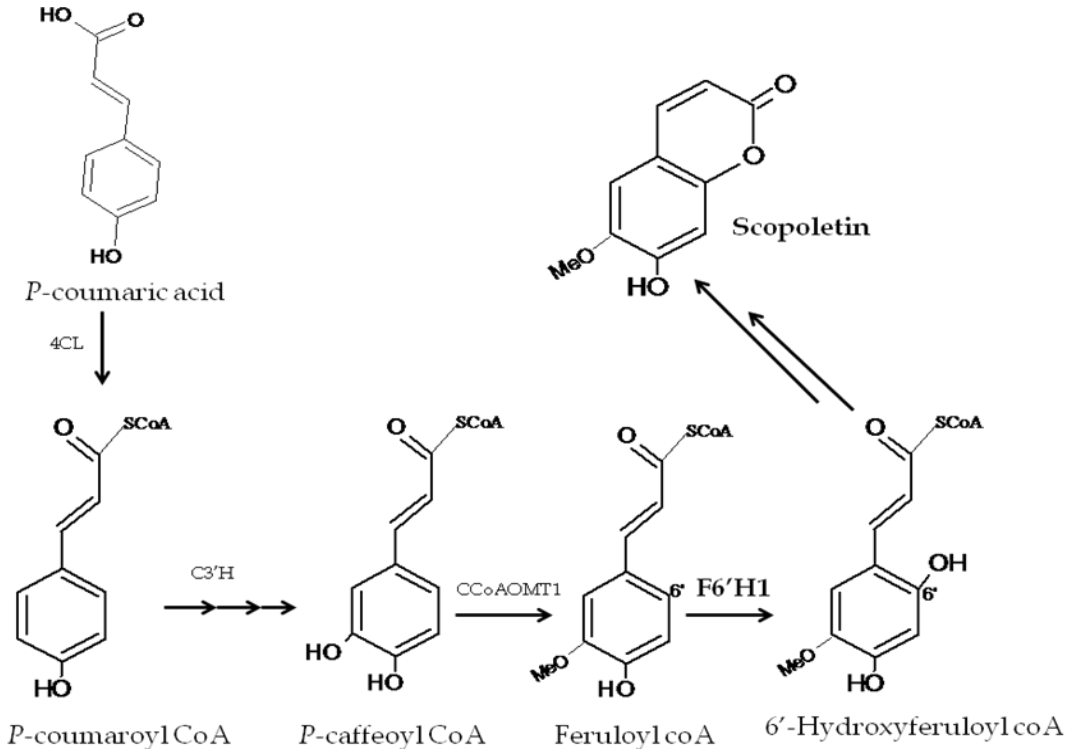


Figure 3.12 Proposed pathway of Scopoletin biosynthesis in *Arabidopsis thaliana* (Kai *et al.*, 2008). 4CL = 4-coumarate CoA ligase, C3'H = *p*-coumarate 3'-hydroxylase, CCoAOMT1 = caffeoyl CoA O-methyltransferase 1, F6'H1 = Feruloyl ortho-hydroxylase. 3'-O-methylation of the caffeate unit occurs mainly *via* CCoAOMT1 using caffeoyl CoA. Ortho hydroxylation of feruloyl CoA is catalyzed by F6'H1 (Fe(II)- and 2-oxoglutarate-dependent dioxygenase), followed by trans/cis isomerisation of the side chain and lactonisation to form scopoletin.

Some other differentially expressed metabolites are well-known and require less discussion. These include tyrosine, proline and glutathione. Tyrosine is an aromatic amino acid synthesised from the shikimate pathway. The reason for its accumulation, particularly in *vtc2-1* and *vtc2-2* is not clear. However, since anthocyanin accumulation is decreased in the *vtc* mutants (see Chapter 4), it is possible that competition between flavonoid and tyrosine biosynthesis is decreased. Proline is an amino acid that is well known to respond to a wide range of stresses including salinity and drought (Gautam and Singh 2009; Ueda *et al.* 2007). The response to high light observed in these experiments could be related to the increased drought stress under these conditions. This is supported by increased ABA accumulation in high light (See Chapter 4). ABA accumulation is induced by high light, most likely as a result of increased water loss under these conditions (Galvez-Valdivieso *et al.*, 2009). Glutathione is an important antioxidant in plants and is related to protection from abiotic stress (Szalai et al., 2009). There was no difference in glutathione concentration between WT and *vtc* mutants, which is also consistent with previous observations (Colville and Smirnoff, 2008).

3. 8 Conclusions

The LC-QToF-MS approach described in this Chapter proved to be a reproducible method for the metabolite profiling. This method identified a large number of features whose expression pattern is consistent between replicate samples, many of which are affected by ascorbate deficiency or abiotic stress. Comparison of

metabolites across different strains can elucidate the differences between metabolites, but to explore the chemical nature of each metabolite is very challenging. There is still a long way to go to completely describe the chemical nature of the metabolites and their behaviour to different external stimuli. The development of bioinformatic tools is essential in facilitating the analysis of the large amount of data produced by mass spectrometry to filter the noise and redundant information and produce the biologically useful information.

From the overall results, it can be concluded that ascorbate deficiency has a significant effect on the metabolite profile of apoplastic fluid and the whole leaf. This is the first investigation of metabolites in apoplastic fluid using LC-MS, and it is particularly surprising that such a wide range of metabolites can be detected. Previous investigations have reported sugars, amino acids and organic acids (Tejera et al., 2006) and ATP (Song et al., 2006), but our results indicate the presence of a range of secondary metabolites. We are still unable to investigate the nature of relationship between ascorbate and these metabolites. Detailed comparison between apoplast and leaf extract is underway. Further investigations are needed to elucidate the structure of the metabolites identified as differentially expressed using NMR.

CHAPTER 4

RESPONSE OF FLAVONOIDS AND HORMONES TO ABIOTIC STRESS IN *ARABIDOPSIS THALIANA* ASCORBATE-DEFICIENT MUTANTS

Chapter 4: Response of flavonoids and hormones to abiotic stress in *Arabidopsis thaliana* ascorbate-deficient mutants

4.1 Introduction

Plants frequently encounter stresses from the environment that adversely affect their growth, development or productivity. These stresses are categorised into biotic (induced by other organisms e.g. pathogens) and abiotic (arising from adverse environmental factors). Abiotic stresses include high light, salinity, drought, mechanical injuries, inadequate minerals, and anthropogenic factors (e.g. NO_2, SO_2, and O_3). Stress resistance mechanisms can be grouped into two general categories: **avoidance** mechanisms in which plants prevent exposure to stress and **tolerance** mechanisms that permit the plants to withstand the stress. Resistance and sensitivity to these stresses depend on the age, species and genotype of the plants. In response to these stresses plants can alter gene expression, cellular metabolism and growth rate. Duration, severity and rate of stress depend on the plant's response and vary from species to species. Combined effects of these stresses elicit a different response as compared to a single stress (Frischknecht *et al.*, 1987; Le Deunff *et al.*, 2004). Plants cope with these adverse conditions by responding in different steps. Firstly, they recognise the type of stress; secondly, they induce changes at a molecular and biochemical level to respond to the stress; finally, the regeneration phase occurs in which the stress is removed and plants gradually start to restore their physiological functions. The regeneration phase takes the plant back to its normal condition, depending on age, defence system, strength, duration and the type of stress.

Stress-induced biochemical changes in plants are linked to the alteration in gene expression, which is involved in different metabolic and signalling pathways (Garcia-Pineda *et al.*, 2004; Le Deunff *et al.*, 2004). Initialisation of stress responses takes place when a plant recognises a stress at the cellular level. Cell recognition activates the signal transduction pathways that transmit information within the individual cell, and throughout the plant. Ultimately, changes that occur at the cellular level are integrated within the whole plant and influence its growth, development and reproductive capability. Environmental stresses (biotic and abiotic) cause drastic changes in plants. Plants have complex and dynamic systems of response to cope with these adverse conditions for their survival, although these changes are very complex. This is in part due to the complexity of interactions between stress stimuli to various molecular, biochemical and physiological phenomena affecting plant growth and development (Lin and Kao, 2001; Hussain *et al.*, 2010; Galazka *et al.*, 2008).

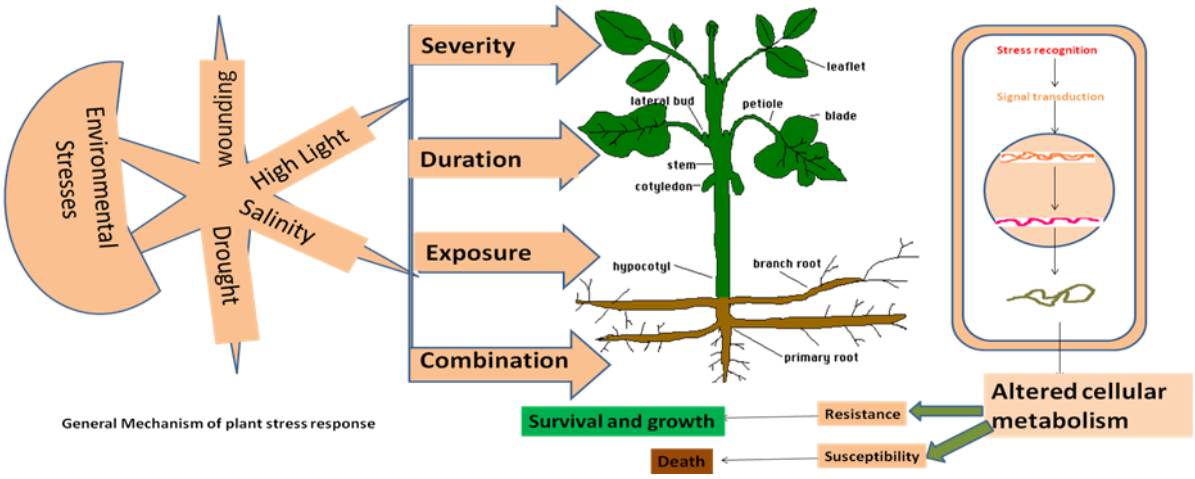


Figure 4. 1 General mechanism of plant stress response (Buchanan *et al* 2000)

4. 2 Plant hormones and their role in stress response

Plant hormones are signalling molecules, present in very low concentrations. Alteration in hormonal levels mediates a whole range of developmental processes, many of which are involved in interaction with environmental factors. The plant hormones such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) are involved in the response and resistance of plants to biotic and abiotic stresses.

4.2. 1 Abscisic acid (ABA)

Abscisic acid was first identified as a growth inhibitor in the 1960s from cotton fruit (*Gossypium herbaceum*) and the dormant sycamore leaf tissue in response to abscission (Addicott, 1983). ABA is a hormone which is ubiquitous in lower and higher plants. Since the discovery of ABA in the early 1960s, much effort has been devoted to understanding the biosynthesis and physiological roles of ABA in the living system. ABA is synthesised from carotenoids and most of the genes encoding

enzymes involved in the biosynthesis pathway have been characterised (Millar *et al.*, 2006; Seo and Koshiba, 2002; Seo *et al.*, 2003; Nambara and Marion-Poll, 2005; Zeevaart, 1983; Milborrow, 2001). Under normal growth conditions the carotenoid violaxanthin slowly converts to zeaxanthin which leads to the biosynthesis of ABA in the chloroplast (Milborrow, 2001). It has been identified in a number of plants at different stages of growth and development, such as seed dormancy, cell division and cell elongation (Lefebvre *et al.*, 2006). ABA is also involved in plant stress responses-both biotic and abiotic. It regulates many genes, which are preferentially expressed during drought stress in stomatal guard cells (Seki *et al.*, 2002). A major role of ABA is an adaptation to changing environmental conditions while retaining water by closing the stomata during water stress and salinity (Endo *et al.*, 2007; Endo *et al.*, 2008). ABA triggers the closing of stomata when soil water is insufficient to keep up with transpiration (Jacobsen *et al.*, 2009; Koshiba, 2004). ABA binds to receptors at the surface of the stomata guard cells and activates several interconnecting pathways such an increase in the cytosolic pH and transfer of Ca^{2+} ions from the vacuole to the cytosol (Li *et al.*, 2000). These changes stimulate the loss of potassium, which reduces the osmotic pressure of the cell and thus turgor which causes the closure of stomata (Bright *et al.*, 2006; Desikan *et al.*, 2004; Li *et al.*, 2000) and protects the cell from dehydration during water stress. ABA signalling turns on the expression of genes encoding proteins that protect cells in seeds as well as vegetative tissues from damage when they become dehydrated (Bray, 2002; Bright *et al.*, 2006). ABA mediates the conversion of the apical meristem into a dormant bud where, the newly developing leaves that are growing above the meristem are

converted into stiff bud scales that wrap the meristem closely and protect it from mechanical damage and drying out (Shimizu-Sato and Mori, 2001). ABA also acts to enforce dormancy in the bud and potato tuber. If an unseasonably warm spell occurs before the end of winter, the buds will not sprout prematurely (Destefano-Beltran *et al.*, 2006). ABA is essential for seed maturation and dormancy, especially during unseasonably mild conditions when there are chances of immature seed germination (Bianco *et al.*, 1997; Hu *et al.*, 2010). ABA may inhibit stem elongation and lateral root growth probably by its inhibitory effect on gibberellic acid (a growth hormone) (Graeber *et al.*, 2010; Guo *et al.*, 2009).

4.2. 2 Jasmonic acid (JA)

Jasmonic acid, a cyclopentanone metabolite, is an oxylipin hormone derived from linolenic acid a C_{18} polyunsaturated fatty acid in a lipoxygenase-dependent process in the chloroplastic membrane *via* the octadecanoic acid pathway (Feussner and Wasternack, 2002; Gobel *et al.*, 2001). JA is a signalling molecule regulated in response to developmental and environmental signals, especially in wounding and herbivore defence. In *Arabidopsis thaliana* accumulation of JA was enhanced by the wound-induced release of α-linolinic acid (precursor of JA biosynthesis) in the chloroplast by the action of many enzymes (Delker *et al.*, 2006). JA is an important component in the wound-induced intracellular signalling which accumulates transiently within 1 h of wounding (Stenzel *et al.*, 2003). JA along with other molecules such as ethylene and DNA binding proteins enhance the expression of wound-responsive genes (Gross *et al.*, 2004; Onkokesung *et al.*, 2010). Li *e*t *al* (2002) used the JA mutants (*spr2*) impaired in JA biosynthesis due to a defect in fatty acid

desaturase (*Fad*) and JA signalling mutants (*Jai-1*) in grafting experiments which revealed the importance of JA in wounding. As well as being wound responsive, JA is also involved in other biotic and abiotic stresses. For example, JA is regulated in response to salinity. The exogenous application of JA induces many JA- responsive genes, which suppress the salinity effect (Walia *et al.*, 2007; Tsonev *et al.*, 1998). It was also reported that exogenous application of JA reduced the accumulation of proline suggesting the possible role of jasmonate in salinity because proline is a well known amino acid accumulated in response to drought and salt stress (Gomez-Galera *et al.*, 2008; Liu *et al.*, 2007). JA deficient mutants *fad3-2*, *fad7-2* and *fad8* (deficient in jasmonate precursor linolenic acid) were found to be extremely susceptible to root rot caused by the fungal root pathogen *Phytium mastophorum* (Vijayan *et al.*, 1998). Some studies also reported JA involved in the inhibition of growth in wounded plants but the mechanism is still not clear (Zhang and Turner, 2008; Onkokesung *et al.*, 2010). It is also involved in senescence, root growth, tendril coiling, floral induction and secretion of nectar in the floral part. (Nectar is a mixture of sugars and amino acids, which are very important for the attraction of pollinators and help in seeds dispersion.) (Radhika *et al.*, 2010; Wasternack, 2007; Krajncic *et al.*, 2006). JA and its methylated form MeJA has been reported to be involved in the regulation of *de novo* biosynthesis of ascorbate (Shan and Liang, 2010; Sasaki-Sekimoto *et al.*, 2006; Wolucka *et al.*, 2005a).

4.2. 3 Salicylic acid (SA)

Salicylic acid (SA) is a phenolic hormone which is common throughout the plant kingdom and plays an important role in physiological processes and defence in response to external stimuli such as pathogen attack. SA is a key regulator of plant signalling and plays an important role in response to biotic and abiotic stresses. The importance of salicylic acid in pathogen defence has stimulated considerable attention in its biosynthesis. Two pathways have been proposed for SA biosynthesis. It is synthesised by phenylpropanoid pathway *via* decarboxylation of cinnamic acid and hydroxylation of benzoic acid or from isochorismate through the shikimate pathway (Metraux, 2002; Sticher *et al.*, 1997). SA is also found in a conjugated form with other molecules. The major stable and non-volatile conjugate of SA is a glucoside (SA gly) which is regarded as storage form and releases SA through hydrolysis (Kawano *et al.*, 2004). Umemura *et al* (2008) reported that SA gly is an important modulator of pathogen defence. They used RNAi-mediated silencing of glucosyl transferase required for the synthesis of SA gly, and found it to be susceptible to pathogens suggesting SA gly is also required against pathogen defence. SA also participates in different physiological processes such as growth, photosynthesis, nitrate metabolism, flowering, heat production and ethylene synthesis (Rivas-San Vicente and Plasencia, 2011; Martinez *et al.*, 2004; Rao *et al.*, 2002). A moderate level of SA is required for optimal photosynthesis and improved stability by controlling the redox state (Tavassoli *et al.*, 2010, Misra *et al.*, 2009; Gautam and Singh, 2009; Mateo *et al.*, 2006; Barba-Espin *et al*; 2011).

SA accumulation in response to pathogens induces the expression of transcripts, which encode the pathogenesis related (PR) proteins (Loake and Grant, 2007). SA also protects the plants by responding to different abiotic stresses such as metal toxicity, heat tolerance, temperature, salinity and biotic stresses (Gautam and Singh, 2009). SA is involved in the up and down-regulation of small antioxidant molecules such as ascorbate and glutathione, and the activity of many antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and dehydroascorbate reductase (DHAR) in response to different abiotic stresses (Gautam and Singh, 2009; Szepesi, 2005; Barba-Espin *et al*; 2011). SA also induces the accumulation of the free osmolyte proline and the enzymes involved in its biosynthesis (Misra and Saxena, 2009; Gautam and Singh, 2009; Wei *et al.*, 2006). Recently, it was found that SA is involved in stomatal closure by activation of ROS and NO production and Ca^{2+} oscillations and activation of K^{+} channels in *Arabidopsis* (Khokon *et al.*, 2011).

4. 3 Plant Secondary Metabolites

Plant metabolites can be classified as primary and secondary metabolites. Metabolites that are essential for plants and are involved in growth, structure, developments are primary metabolites. These include lipids, sugars and proteins. Those which are involved in the interaction of external environment are mostly secondary metabolites. Secondary metabolites are often species specific and they play a very important role in plant adaptation to adverse environmental conditions.

Common examples of secondary metabolites are flavonoids, phenylpropanoids, terpenoids and alkaloids.

4.3.1 Phenolic metabolites

The plant phenols are biogenetically synthesised from two pathways "the shikimate pathway" and "the polyketide or acetate pathway". Phenolic compounds are reported as excellent antioxidant molecules due to electron donating properties which result from their hydroxyl groups (RiceEvans *et al.*, 1997). Phenolic metabolites show their scavenging properties because of the lower electron reduction potential of phenoxyl radicals than those of oxygen anions, like superoxide (O_2^{2-}), peroxyl ($ROO^{\cdot}$), alkoxyl (RO^{-}) and hydroxyl ion (^{-}OH) (Blokhina *et al.*, 2003; Borges *et al.*, 2010). Phenolic metabolites easily scavenge toxic oxidants and inactivate them, so protecting the cell from different oxidative stresses (Borges *et al.*, 2010; Li *et al.*, 1993). Phenolic metabolites have roles in promoting health, flavouring and colouring fruits and flowers and their antioxidant activity has attracted the interest of researchers for their identification and further investigation (Markham *et al.*, 2000; Ghasemzadeh *et al.*, 2010a; Ghasemzadeh *et al.*, 2010b; Kitamura *et al.*, 2010; Kusano *et al.*, 2007; Nakabayashi *et al.*, 2009; Stracke *et al.*, 2010; Tohge *et al.*, 2005; Yonekura-Sakakibara *et al.*, 2008; Yonekura-Sakakibara *et al.*, 2007; Borges *et al.*, 2010; Page *et al.*, 2011).

Phenylpropanoids and flavonoids are widespread examples of phenolic compounds. Phenylpropanoids are the naturally occurring phenolic metabolites composed of 3 aromatic rings carrying small side chains. These are the derivatives of aromatic

amino acids such as phenylalanine and tyrosine derived from the shikimate pathway (Herrmann, 1995). Amongst the phenylpropanoids including hydroxycoumarins, phenyl propenes, suberin, tannins and lignin, some contribute substantially to the stability of plants towards environmental conditions. The most ubiquitous hydroxycoumaric acids in plants are sinapic acid, sinapoyl malate, caffeic acid and *p*-coumaric acid (Kai *et al.*, 2006; Mattana *et al.*, 2005; Page *et al.*, 2011). In Arabidopsis, genes encoding enzymes involved in phenylpropanoid pathways are well characterised (Guranowski *et al.*, 2002; Lee *et al.*, 2003).

4.3. 2 Flavonoids

Flavonoids are water soluble plant secondary metabolites, which are structurally derived from aromatic amino acids. Flavonoids are widely distributed in fruits, flowers and the vegetative part of the plants. Flavonoids are known to be involved in defence in plants either acting as antioxidants or UV-B screeners (Einbond *et al.*, 2004; Beekwilder *et al.*, 2005; Bieza and Lois, 2001). They are accumulated in plants in response to various types of oxidative stresses such as UV radiation, wounding, pathogenic infections, high light, chilling, environmental pollutants or some nutrient deficient stress (Shirley, 1998; Shirley *et al.*, 1995; Borges *et al.*, 2010; Pietta, 2000). The flavonoid composition in plants also varies between different stages of growth. A wide variety of flavonoids have been isolated and identified from different tissues, such as flowers, fruits or leaves (Markham *et al.*, 2000). The basic anthocyanin and flavonol biosynthetic pathways have been characterised and most of the genes involved in their regulatory biosynthetic pathways have been characterised (Schoenbohm *et al.*, 2000). These are the aromatic metabolites which are synthesised

by one of the two main biosynthetic pathways such as the acetate-malonate and shikimate pathways from phenylalanine (Winkel-Shirley, 2002). These metabolites share the common framework which is derived from the basic aromatic metabolites. The tricyclic skeletons consist of two benzene rings (A and B) derived from acetate and cinnamic acid units joined by third oxygenated heterocyclic ring containing a pyran group. These are C_{15} benzo- α-pyrone derivatives and are classified according to the substitution of different groups. Flavonoids differ on the basis of presence and arrangement of hydroxyl, methoxy and glycosides side chains and the conjugation of A and B rings (Vickery and Vickery, 1981). The most abundant flavonoids existing in the nature are in the glycosidic form which contributes to the complexity of their structure. During metabolism these rings structures are hydroxylated, methylated or glycosylated. 3-O-glycosides are common in all flavonoids and are important free radical scavengers (Delazar *et al.*, 2010b; Delazar *et al.*, 2010a) and contribute different colours to plants (Welch *et al.*, 2008; Martens *et al.*, 2010; Vanderauwera *et al.*, 2005; Nomura *et al.*, 2009; Kim *et al.*, 2008; Sithisarn *et al.*, 2007). The flavonoids are thought to be perhaps the most important group of phenolic compounds. The most important flavonoids are: **Anthocyanins** (cyanidin, pelargonidin and petunidin), **Flavanols** (quercetin and kaempferol), **Flavones** (luteolin and apigenin), **Flavanones** (myricetin, naringin, hesperetin and naringenin), **Flavan-3-ols** (catechin, epicatechin and gallocatechin), **and Isoflavones** (genistein and daidzein) (Vickery and Vickery, 1981). Structures of the six main flavonoids are shown in Figure 4.2.

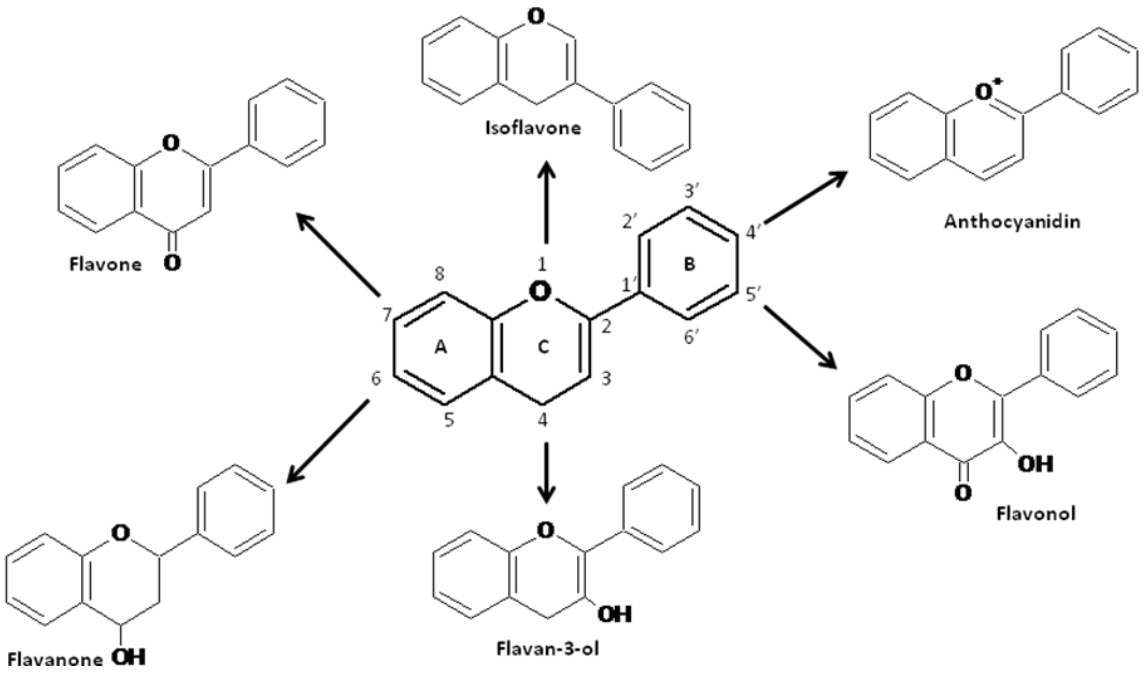


Figure 4. 2 Structure of the six main classes of flavonoids (Vickery and Vickery, 1981)

4.3.2.1 Anthocyanins

Of all the flavonoids, anthocyanins are the probably best known members of the flavonoid group, predominantly present in fruits and flowers. Because of their strong red to blue colouring, anthocyanins are the most recognised, visible members of the flavonoids. They can be found in large amounts in fruits, and at especially high levels in berries and grapes (Borges *et al.*, 2010). They are glycosylated or conjugated with malonate, coumarate or sinapate at different positions. The accumulation of anthocyanin is induced by many factors such as high light

(Giacomelli *et al.*, 2006; Page *et al.*, 2011), sucrose (Teng *et al.*, 2005), hormones such as ABA and JA (Loreti *et al.*, 2008; Shan *et al.*, 2009), low temperature (Leyva *et al.*, 1995) and nutrient deficiency (Rubin *et al.*, 2009).

Anthocyanins provide pigmentation in fruits, flowers and vegetables. Their colours are influenced by structure, pH, temperature, light, oxygen, methylation and divalent ions. The pH, hydroxylation and methylation are the important factors which switch one colour to another (Welch *et al.*, 2008; Vickery and Vickery, 1981). These colours are very important in attraction or repulsion of various herbivores and other insects and birds which cause the seed dispersion. Anthocyanins are important from a pharmacological point of view (Ghosh and Konishi, 2007; Stoner and Wang, 2008). Anthocyanins play an important role in photosynthesis during increased light intensity. They protect plants from photoinhibition effectively by reducing the internal light due to strong absorbance of blue-green light (Markham *et al.*, 2000; Giacomelli *et al.*, 2006). They are also involved in anti-herbivore defence and protect the plants from UV-B radiation (Li *et al.*, 1993; Karageorgou and Manetas, 2006).

4.3.2.2 Flavonols

Flavonols are the most widely spread flavonoids in food, mostly present in the form of quercetin and kaempferol. The basic flavonols are further decorated with different groups such as sugars (usually glucose and rhamnose). Most commonly these sugars bind at C3 but less frequently at C7. The UV absorbing properties of flavonol means that they are important in protecting plants from UV irradiation, which is sometimes ascribed to kaempferol (Tokusoglu *et al.*, 2003). Flavonol glycosides are also reported as antioxidants, which protect cells from chronic diseases (Pietta, 2000).

Fourteen different flavonoids, including nine cyanidin derivatives (anthocyanin) and five flavonols glycosides (kaempferol) were identified in this study. These flavonoids share the basic tricylic ring cynidine for anthocyanin and flavonol glycosides which were further decorated by glycosylation, malonylation and some other coumaryl and sinapoyl groups. The enzymes involved in substitution of these groups are still unknown. The basic biosynthesis pathway and structure for all these 14 flavonoids are shown in Figures 4.3 and 4.4.

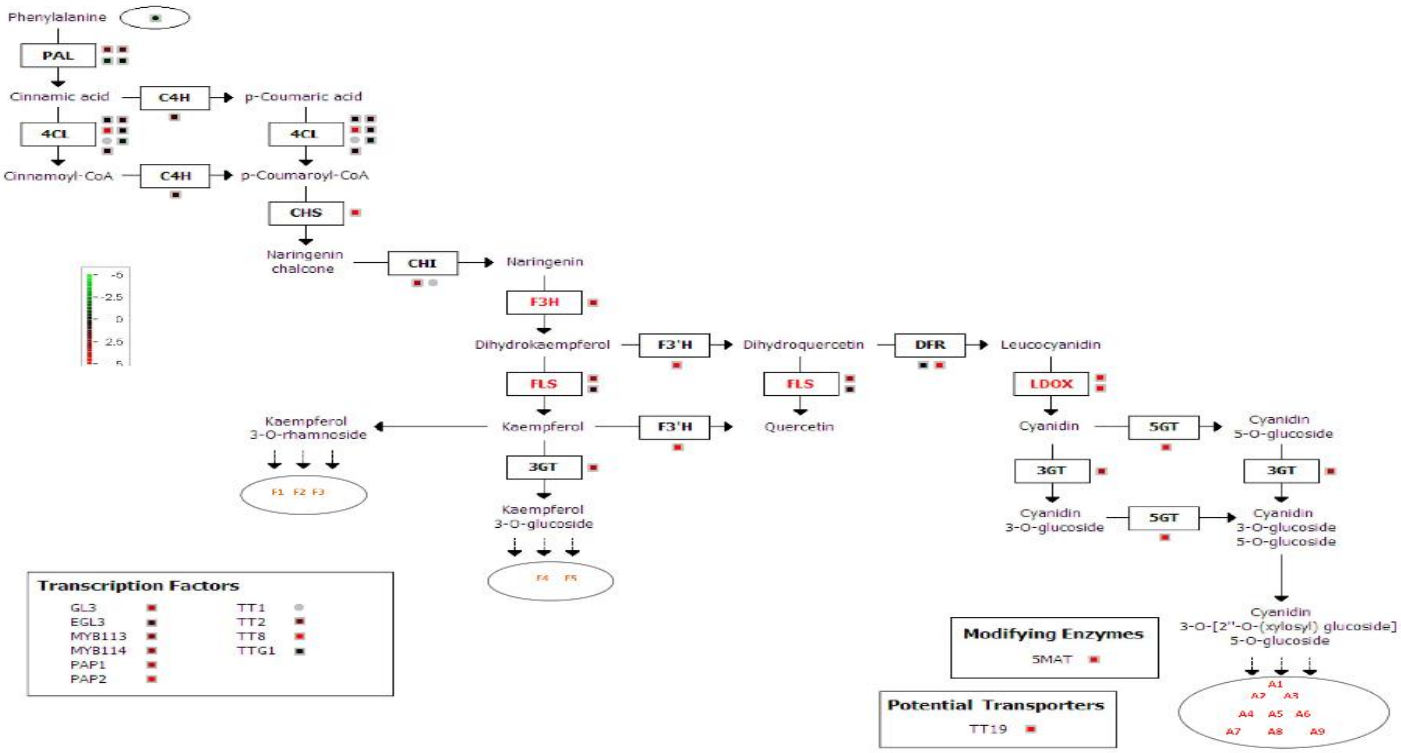
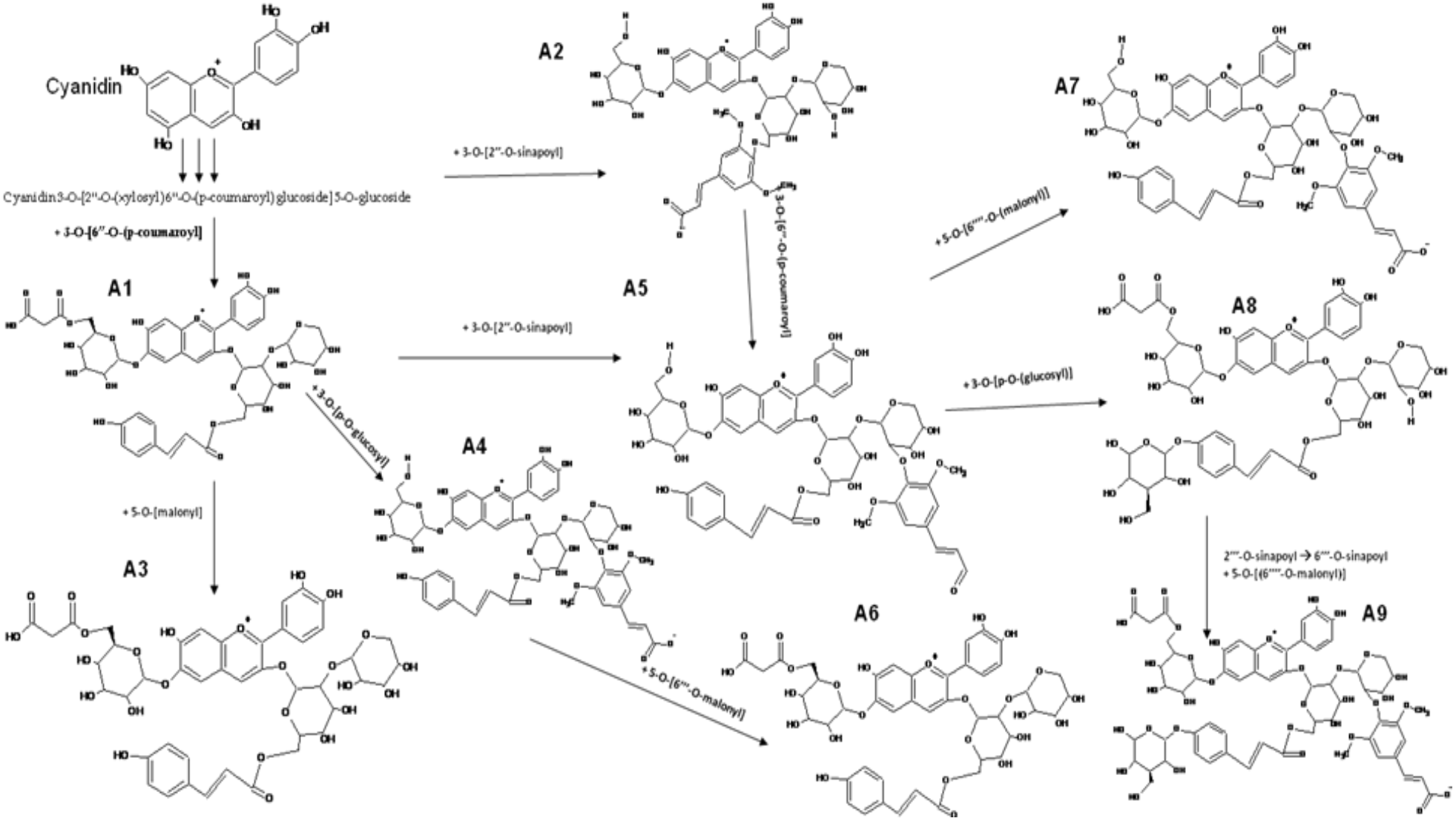


Figure 4.3 Flavonoid biosynthesis pathway showing changes in gene expression and flavonoid accumulation in response to HL (Page *et al*, 2011). *Arabidopsis thaliana* plants were subjected to 4 days of LL (100 µmol m^{-2} s^{-1}) or HL (550 µmol m^{-2} s^{-1}). Enzymes shown in red font are 2-oxoglutarate-dependent dioxygenases which may require AsA to maintain their activity. Abbreviations: PAL - phenylalanine ammonia lyase; 4CL - 4-coumarate CoA ligase; C4H - cinnamate-4-hydroxylase; CHS - chalcone synthase; CHI - chalcone flavanone isomerase; F3H - flavanone 3-hydroxylase; F3'H - flavonoid 3' hydroxylase; FLS – flavonol synthase; DFR - dihydroflavonol 4-reductase; LDOX - leucoanthocyanidin dioxygenase; 3GT - anthocyanidin 3-O-glucosyltransferase 5GT - anthocyanidin 5-O-glucosyltransferase. All anthocyanins from A1-A9 are shown in grey oval shape in red text on the right-hand side; similarly five flavonol glycosides which all share the same pathways are shown.

A


Cyanidin
Cyanidin 3-O-[2''-O-(xylosyl) 6''-O-(p-coumaroyl) glucoside] 5-O-glucoside
+ 3-O-[6''-O-(p-coumaroyl]
A1
+ 5-O-[malonyl]
A3
+ 3-O-[p-O-glucosyl]
A4
+ 3-O-[2'''-O-sinapoyl]
A2
+ 3-O-[2'''-O-sinapoyl]
A5
+ 5-O-[6''''-O-malonyl]
A6
+ 5-O-[6''''-O-(malonyl)]
A7
+ 3-O-[p-O-(glucosyl)]
A8
2''''-O-sinapoyl → 6''''-O-sinapoyl
+ 5-O-[(6''''-O-malonyl)]
A9

B

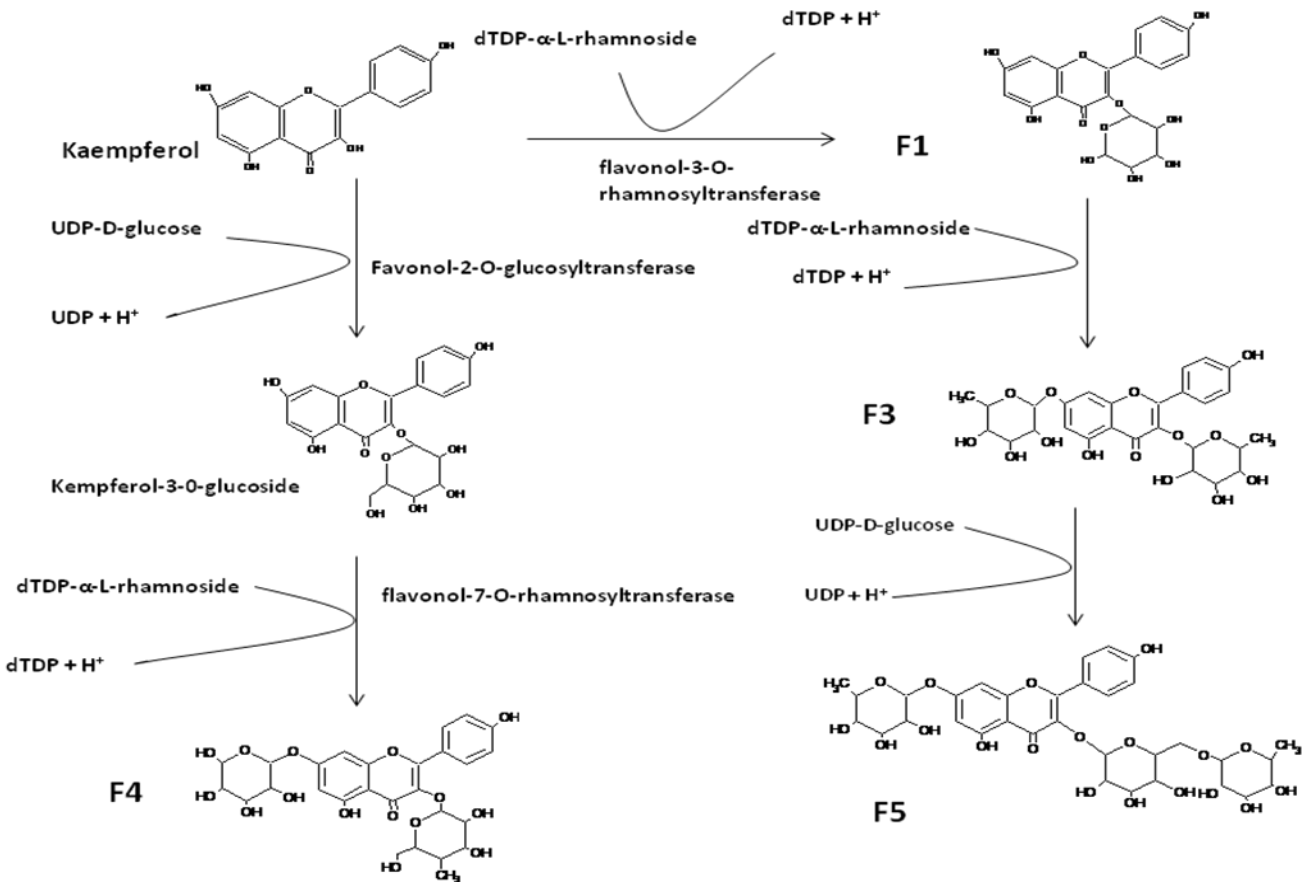


Figure 4. 4 (A) Anthocyanin and (B) Flavonol glycoside derivatives from *Arabidopsis thaliana* (Tohge *et al*, 2005)

The multiple functions of ascorbate summarised in Chapter 1 suggested ascorbate deficiency may have significant effects on plant function. Therefore, metabolite profiling (metabolomics) using mass spectrometry (MS) could be a valuable approach to identifying changes in metabolism caused by ascorbate deficiency. Elucidation of the specific roles of plant hormones and flavonoids and their interactions with ascorbate in response to different stresses (wound, high light and salinity) is a major focus of our biotic and abiotic treatments. The primary goal of this research is to utilise *Arabidopsis* ascorbate-deficient mutants (*vtc1*, *vtc 2-1*, *vtc 2-2* and *vtc 3-1)* and explore their chemical behaviour in response to different stress treatments.

The stresses addressed in this chapter are high light (HL), salinity and mechanical wounding which were given to *Arabidopsis thaliana vtc* mutants, which were analysed for their responses to hormonal regulation and flavonoid accumulation. This chapter also focuses on the development of a reverse phase-ESI-MS method for the analysis of hormones and flavonoids.

4. 4 Methods

4.4. 1 The Effect of light intensity, wounding and salinity on hormones accumulation

Plants were grown under normal growth conditions (as discussed in Chapter 2) and treated with three different stresses discussed below (1) In order to characterise the response of the *vtc*-mutants to HL stress, plants were grown in LL (PPFD 120 µmol $m^{-2}s^{-1}$) as a control and transferred to HL (PPFD 550 µmol $m^{-2}s^{-1}$) for four days. (2) To check the quick response of the *vtc* mutants to wounding, all strains were wounded with a small syringe needle as described in Chapter 2 (3) In salinity stress, plants were treated with 200 mM NaCl (as discussed in Chapter 2) to observe the hormonal response in the WT and the *vtc* mutants. The analysis of jasmonic acid, abscisic acid, salicylic acid and salicylic acid glycoside was conducted using LC-MS-QQQ. Three independent experiments were run to check the reproducibility of the data. Data for all hormones are shown in Figure 4.7- 4.9.

4. 5 Results

4.5.1 Ascorbate accumulation in HL

It has been previously reported that the foliar ascorbate pool increases in *Arabidopsis thaliana* (WT) when plants were acclimated from LL to HL (Smirnoff, 2000; Dowdle *et al.*, 2007; Bartoli *et al.*, 2006). A slight increase in ascorbate was also observed in the *vtc* mutants, but due to a genetic defect of the *vtc* mutants in the ascorbate biosynthesis pathway (discussed in Chapter 1), the *vtc* mutants did not accumulate

as much ascorbate as the WT. Unlike WT, the ascorbate pool was not significantly increased in the *vtc* mutants after light treatment (Figure 4.5).

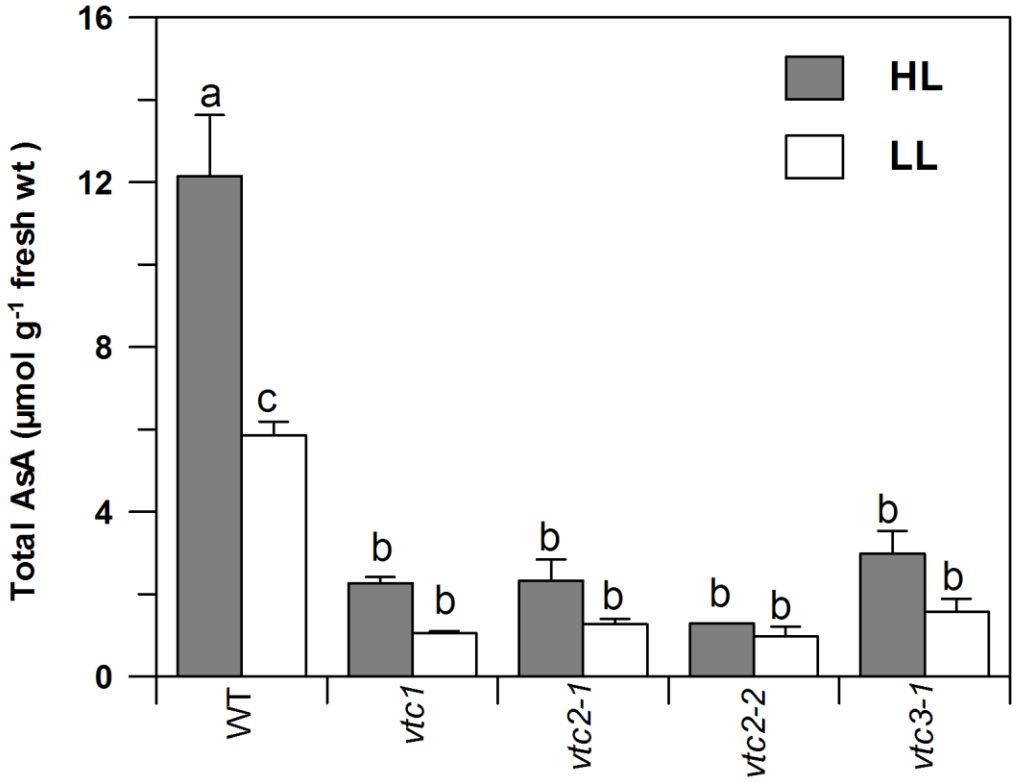


Figure 4. 5 Total leaf ascorbate contents in *Arabidopsis thaliana* WT and the *vtc* mutants acclimated in HL (550-650 µmol $m^{-2}s^{-1}$) and LL (100 umol m^{-2} s^{-1}) for 4 days. Mean values +1 SEM (n = 4) are shown. Column labelled 'a' are significantly different from labelled 'c' LL treatment from same strain. Columns labelled 'b' are significantly different ($p < 0.05$) corresponding to the WT, LL and HL. Although they look significantly different in the graph but statistically it was not the case.

4.5.2 Hormone identification by LC- QQQ-MS

The plant hormone (ABA, JA, SA and SA glycoside) analyses were carried out using LC-QQQ-MS/MS (method development and conditions are described in Chapter 2). All hormone peaks were isolated on the basis of retention time (RT) and mass to charge ratio (*m/z*) of precursor and selected product ions in negative ion mode. Analysis of the WT and the *vtc*-mutants showed that they have similar chromatograms; therefore, only fragmentation and mass spectral data obtained from the WT are presented in Figure 4.6. Four different types of hormones (ABA, JA, SA and SA glycoside) were identified based on the MRM data. The experiment was repeated three times to check the reproducibility of the data. The RT and mass spectra were the same in all samples and experiments; therefore, we selected only WT from one experiment to present chromatogram.

(a) The targeted data analysis by LC-MS-QQQ eluted the first peak (R_t = 9.2), *m/z* 299 $[M-H]^-$ with a fragment ion of *m/z* 93. The peak was identified as salicylic acid glycoside on the basis of previous literature (Forcat *et al.*, 2008) as the deuterium labelled internal standard was not available.

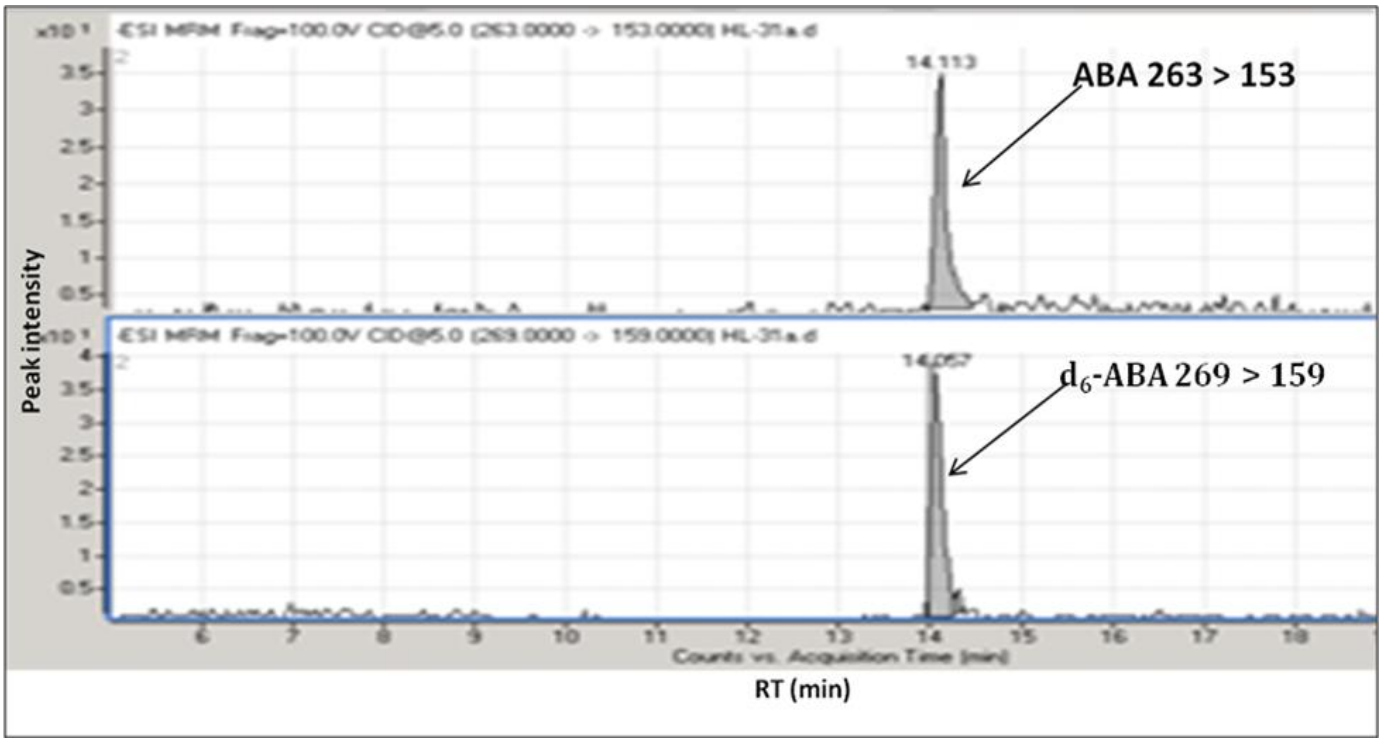
(b) Peak 2 (R_t = 13.9 min), *m/z* 137 $[M - H]^-$ produced a fragment ion of *m/z* 93. The d_4-labelled internal standard was used to quantify the data. MS analysis of standard peak revealed an *m/z* 141 $[M - H]^-$ which produced a fragment ion of *m/z* 97 (4 mass units higher than the endogenous metabolite) (Forcat *et al.*, 2008).

(c) The peak 3 (Rt = 14.05 min), *m/z* 263 $[M-H]^-$ produced two fragment ions of *m/z* 219 and 153. The most abundant peak carrying deuterium was chosen and used for further analysis. The peak was identified as abscisic acid using the deuterium-labelled internal standard (d_6-ABA). The retention time was the same for both

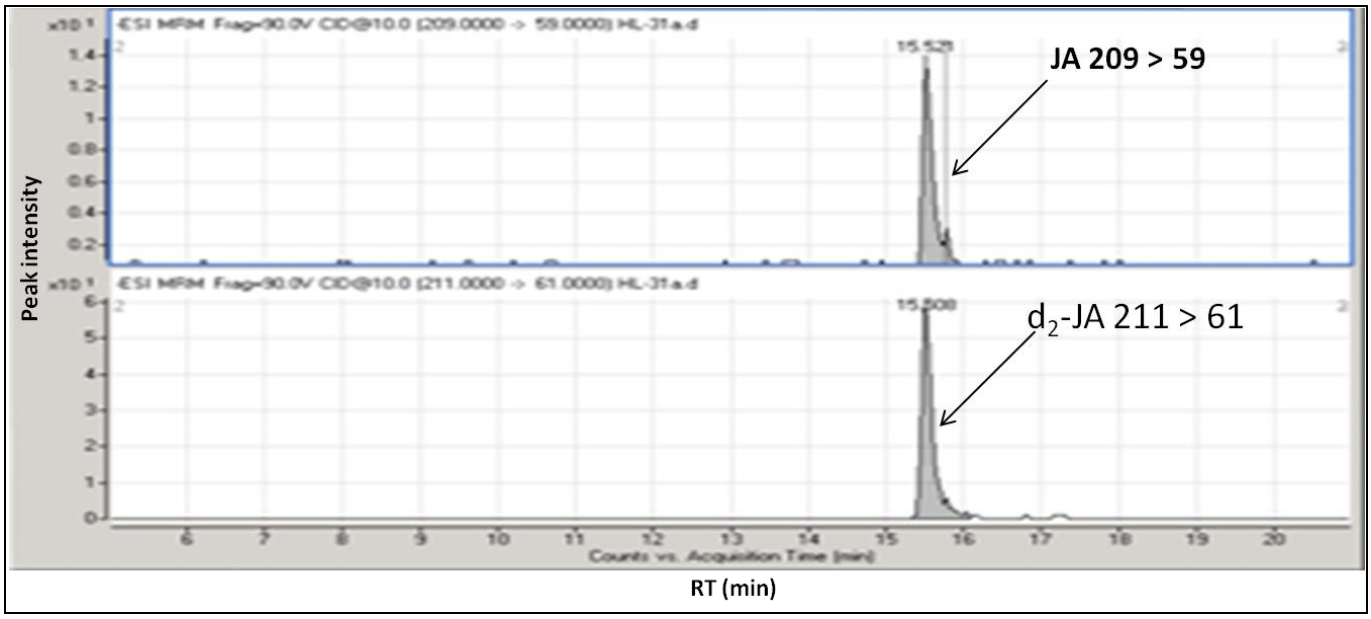
standard and endogenous ABA but the *m/z* of the standard precursor ion was 269, 6 mass units higher than endogenous ABA, and its fragment ion, *m/z* 159 (Forcat *et al.*, 2008.

(d) Peak 4 (Rt = 15.5 min), *m/z* 209 $[M\text{-}H]^-$ produced the most abundant fragment ion of *m/z* 59. Based on the mass spectral data reported previously, this metabolite was jasmonic acid (Forca*t et al.*, 2008). The d_2-labelled internal standard which was 2 mass units higher than endogeneous JA was added in the sample for the identification and quantification of endogenous JA**.**

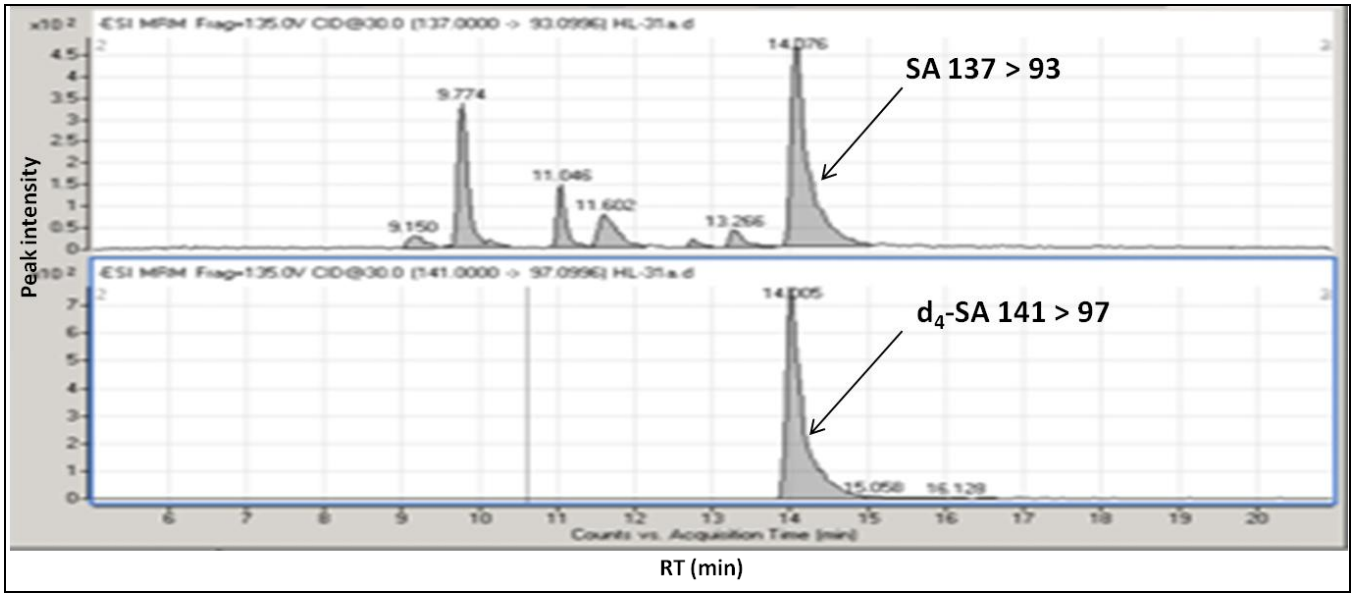
A



B


Peak intensity
JA 209 > 59
15.526
d_2-JA 211 > 61
15.508
Counts vs. Acquisition Time [min]
RT (min)

C


Peak intensity
SA 137 > 93
14.076
9.774
11.046
11.602
9.150
13.266
d_4-SA 141 > 97
14.005
15.058
16.129
Counts vs. Acquisition Time [min]
RT (min)

D

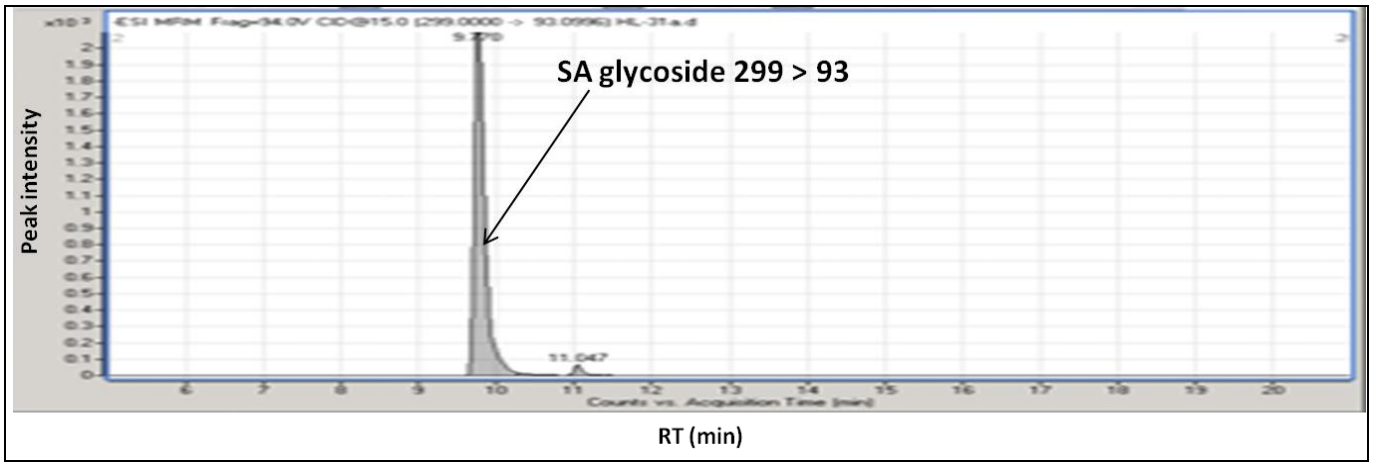


Figure 4.6 Product ion chromatograms of endogenous hormones and deuterium labelled internal standards. Chromatogram represented: (A) Absicic acid, (B) Jasmonic acid), (C) Salicylic acid and (D) Salicylic acid glycoside

4.5.3 The effect of abiotic stresses on foliar hormone concentration

The accumulation of ABA, JA, SA and SA glycoside was analysed in response to three different stresses including high light, wounding and salinity in the WT and the *vtc* mutants (*vtc1, vtc2-1, vtc2-2,* and *vtc3-1*). Data from three independent experiments for each stress are presented in Figures 4.7-4.9.

4.5.3. 1 High light (HL)

(a) Abscisic acid (ABA)

Generally, ABA concentration was increased in HL acclimated plants but the significant differences between HL and LL were not consistent between independent experiments across all strains. The WT accumulated a higher concentration of ABA in HL than in the *vtc* mutants but in LL, it was same in all strains. The pattern of ABA accumulation in LL and HL was fairly consistent in all three independent experiments, although the concentration varied between experiments (Figure 4.7). In experiment 1, the ABA concentration was significantly increased ($p < 0.05$) in HL acclimated WT plants, but no significant difference was found in mutants between HL and LL. The *vtc1* and *vtc2* mutants produced significantly lower quantity of ABA than the WT when exposed to HL but *vtc3* was in-between WT and other *vtc* mutants but not significantly different from both. In LL, the ABA concentration remained the same in all strains (Figure 4.7A).

Experiments 2 and 3 showed consistent results in ABA accumulation in response to HL. In both experiments, ABA concentration was marginally increased in HL treated

plants than LL, but no significant difference was found in both light conditions. Similarly, the WT showed a slightly higher ABA concentration in HL compared to *vtc1* and *vtc3-1* but *vtc 2-2* produced significantly lower quantity than the WT while in LL the ABA concentration remained the same in all strains. The overall results showed no significant effect of ascorbate-deficiency and high light on ABA accumulation. The results are presented in Figure 4.7 A-C.

(b) Jasmonic acid (JA)

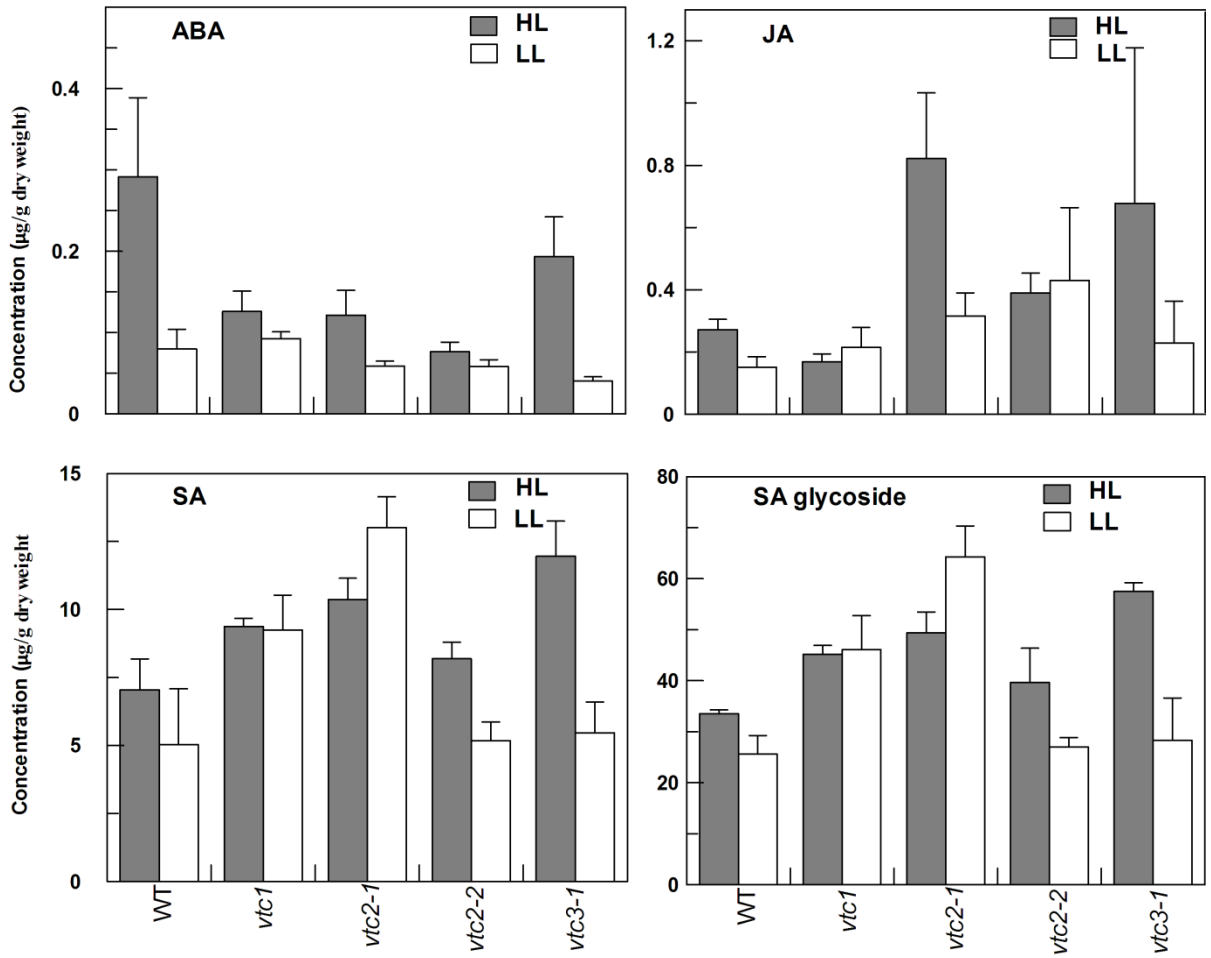
JA accumulation showed no response to light intensity in the WT and the *vtc* mutants. No difference in JA concentration was observed in the WT and the *vtc* mutants in HL and LL except *vtc2-1* in which a dramatic increase in JA concentration was observed after HL treatment in 3 independent experiments, this needs further investigation. The JA concentration in three independent experiments was fairly consistent. The overall results showed ascorbate deficiency, and light intensity had no significant effects on the accumulation of JA (Figure 4.7 A-C).

(c) Salicylic acid and Salicylic acid glycoside

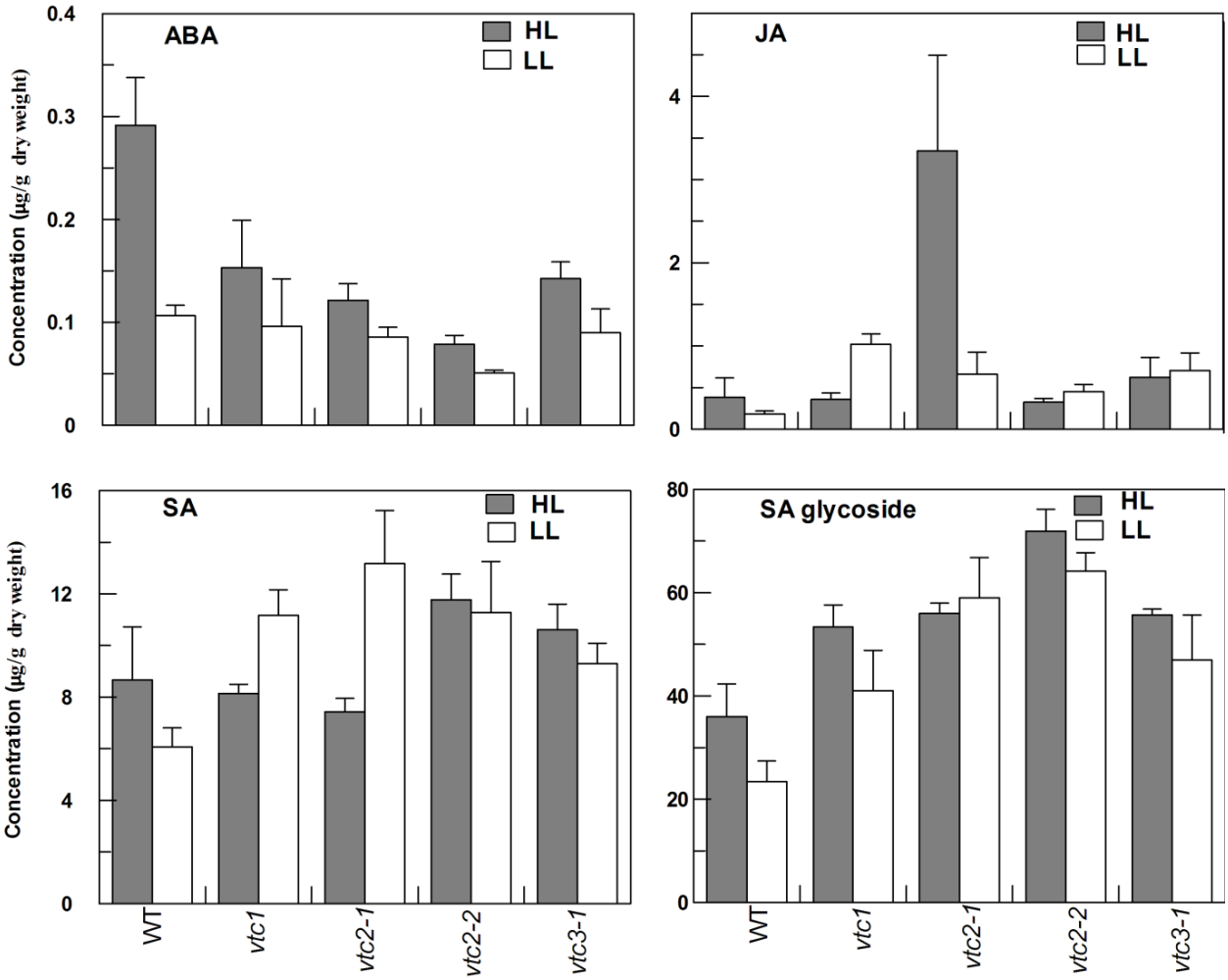
Accumulation of SA between HL and LL was not consistent in 3 independent experiments in the WT and *vtc* mutants except *vtc2-1* where it was depleted after HL treatment and consistent results were shown in three independent experiments. In other strains, there was a variation in SA accumulation in HL and LL between experiments. SA glycoside concentration was higher in *vtc* mutants in both light conditions. Unluckily, there was variation in SA and SA glycoside quantity in response to HL across all strains between different experiments; therefore, further

replication is needed. Consequently, it was difficult to interpret these hormones in response to HL. Generally, accumulation of SA and SA glycoside in response to HL was not consistent across all strains. The *vtc2-2* mutant showed a substantial increase in SA and SA glycoside in response to HL in all 3 independent experiments; in contrast *vtc2-1* showed a decrease in accumulation after HL treatment while WT, *vtc1* and *vtc3-1* showed variability in 3 independent experiments. Low concentrations of SA and SA glycoside were observed in the WT under low light conditions as compared to the mutants.

A


ABA
HL
LL
JA
HL
LL
SA
HL
LL
SA glycoside
HL
LL
Concentration (µg/g dry weight)
Concentration (µg/g dry weight
0
0.2
0.4
0.8
1.2
5
10
15
20
40
60
80
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

B


ABA
HL
LL
JA
HL
LL
SA
HL
LL
SA glycoside
HL
LL
Concentration (µg/g dry weight)
Concentration (µg/g dry weight)
0.4
0.3
0.2
0.1
0
4
2
0
16
12
8
4
0
80
60
40
20
0
WT
vtc1
vtc2-1
vtc2-2
vtc3-1
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

C

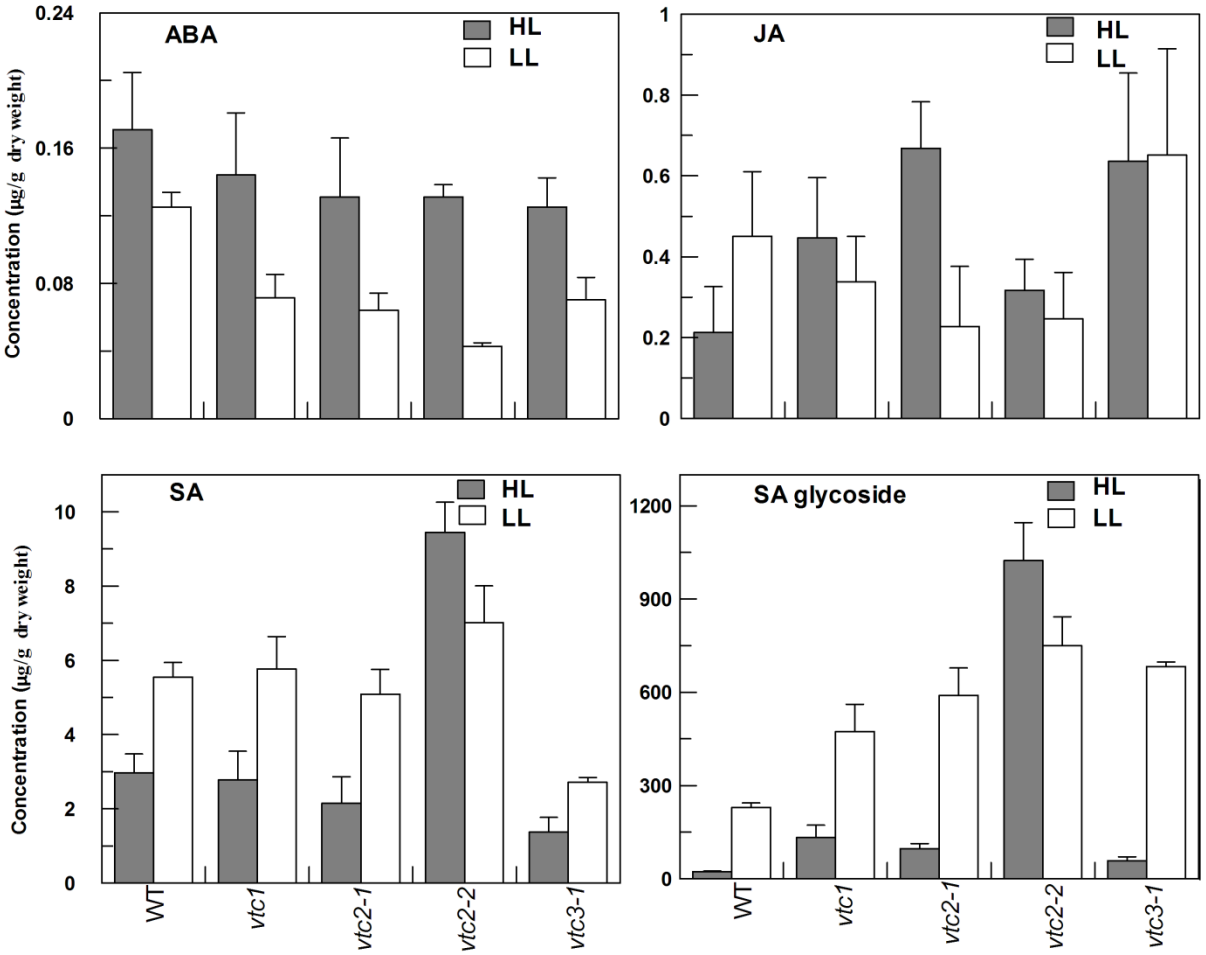


Figure 4.7 Concentrations of foliar hormones: abscisic acid (ABA), Jasmonic acid (JA), Salicylic acid (SA) and SA glycosides (SA gly) in *Arabidopsis thaliana* WT and the *vtc* mutants. Plants were acclimated to HL (550-650 µmol m^{-2} s^{-1}) and LL (100 µmol m^{-2} s^{-1}) for 4 days. Data from three independent experiments are shown in (A) Experiment 1 (B) Experiment 2 and (C) Experiment 3. The data were quantified using deuterium labelled internal standards except SA glycoside and subjected to analysis of variance (ANOVA) to identify the statistical differences between treatments and strains. All results are expressed as mean values + standard error (n = 3) where, 'n' represents the number of biological replicates.

4.5.3. 2 Wounding

(a) Abscisic acid (ABA)

The ABA concentration showed no significant response to wounding in all strains (WT and *vtc* mutants). The concentration of ABA was slightly increased in wounded plants but not significantly different compared to control plants (not-wounded). There was no difference in ABA accumulation in the WT and the *vtc* mutants in both wounded and control conditions. The pattern of accumulation of ABA in all three independent experiments was identical. Overall, results showed short term wounding had no significant effects on the accumulation of ABA in all strains (Figure 4.8).

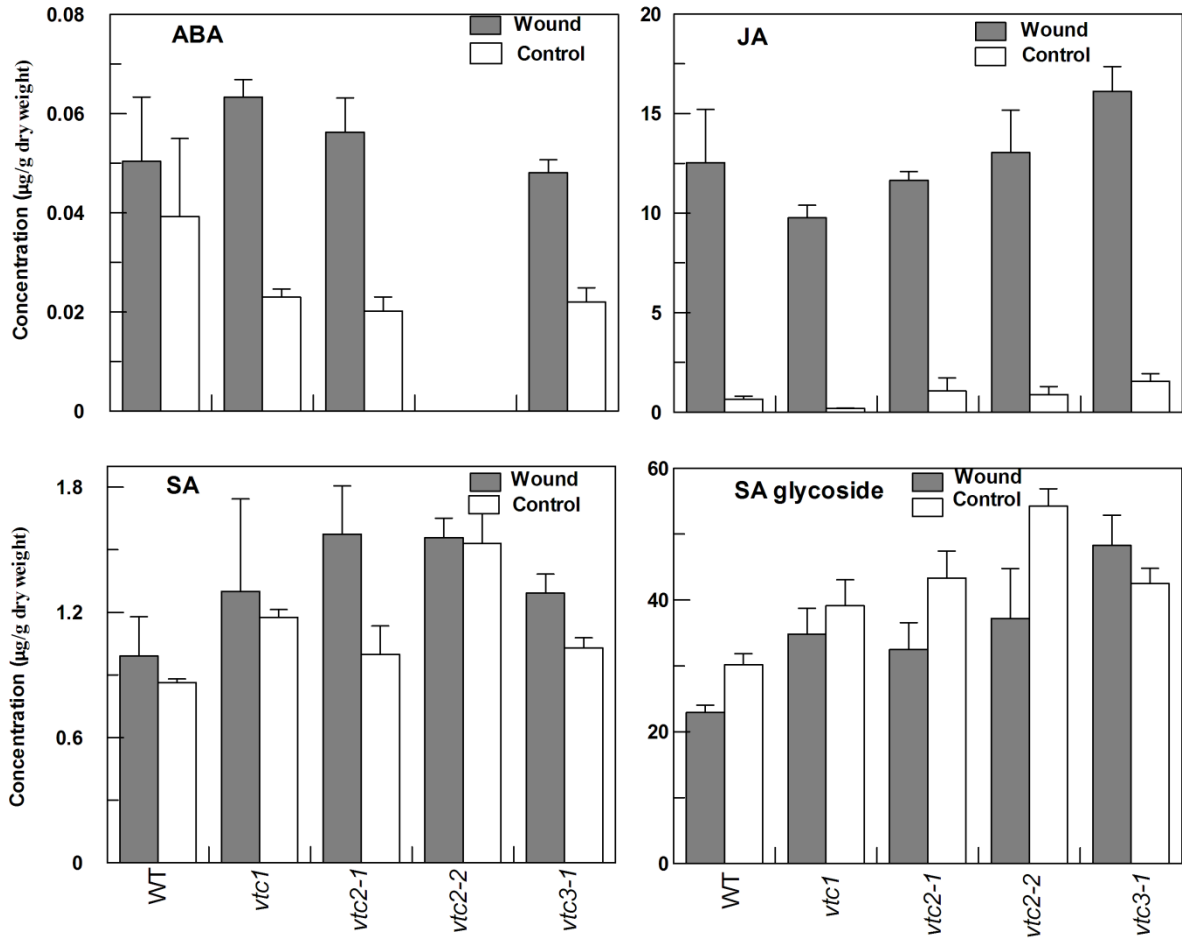
(b) Jasmonic acid (JA)

JA responded rapidly to minor wounding. A dramatic increase in JA concentration was observed in the WT and the *vtc* mutants in response to short term (2 h) wounding (method described in Chapter 2). There was a significant difference ($p < 0.05$) in the wounded and control plants in all strains. The *vtc* mutants showed the same behaviour in JA accumulation in response to wounding but no difference was observed in JA accumulation between the WT and the *vtc* mutants in both wounded and control plants. The results were consistent in three independent experiments suggesting that ascorbate deficiency had no effect on the accumulation of JA (Figure 4.8 A-C).

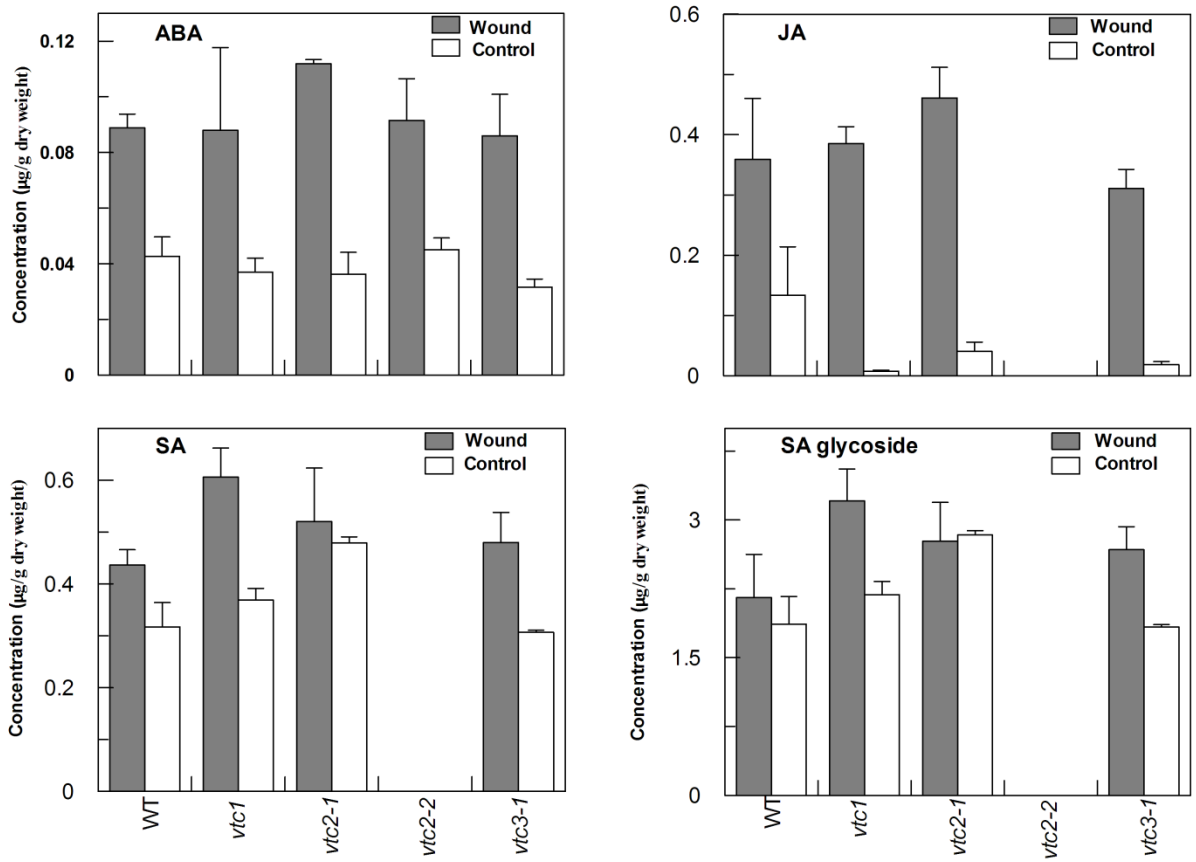
(c) Salicylic acid and SA glycoside

SA and SA glycosides showed no significant difference in short term wounded and control plants. A slight increase in SA and SA glycoside concentrations was observed in wounded plants but this was not consistent in all experiments. The *vtc* mutants produced higher SA and SA glycoside levels than the WT, but the difference was not significant. The data are shown in Figure 4.8.

A



B


ABA
Wound
Control
Concentration (µg/g dry weight)
0.12
0.08
0.04
0
JA
0.6
0.4
0.2
0
SA
0.6
0.4
0.2
0
SA glycoside
3
1.5
0
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

C

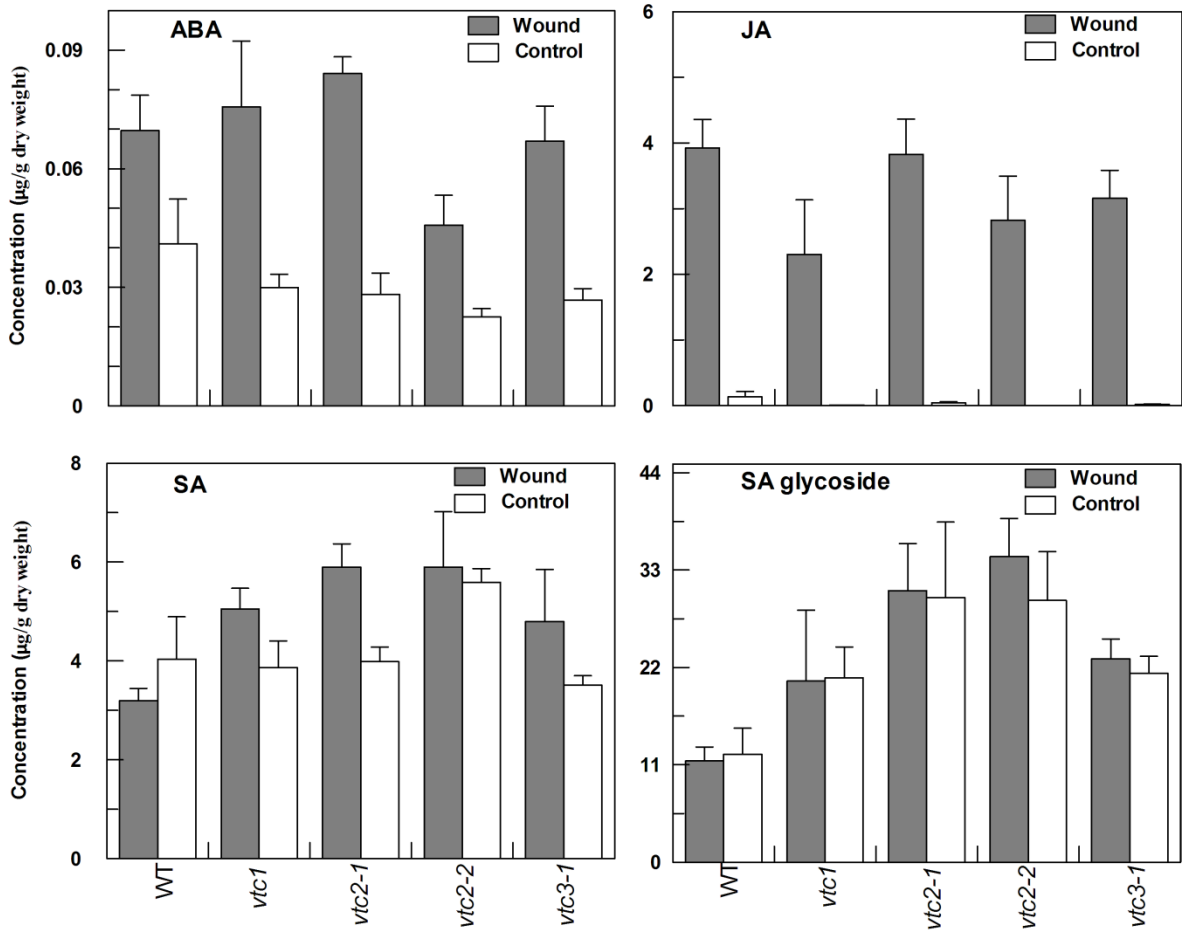


Figure 4. 8 Concentrations of foliar hormones: abscisic acid (ABA), Jasmonic acid (JA), Salicylic acid (SA) and SA glycoside in *Arabidopsis thaliana* WT and *vtc* mutants in response to mechanical wounding. Data from three independent experiments are shown in graph (A) Experiment 1 (B) Experiment 2 and (C) Experiment 3. The data were quantified using deuterium labelled internal standards and subjected to analysis of variance to identify the statistical differences between treatments and genotypes. All results are expressed as mean values + standard error (n = 3) where, 'n' represents the number of biological replicates.

4.5.3.3 Salinity

(a) Abscisic acid (ABA)

A slight increase in ABA concentration was observed in all strains in response to salinity, but the significant difference was not consistent between experiments. No significant difference in ABA concentration was observed in the WT and *vtc* mutants in salt treated and control plants, suggesting ascorbate deficiency had no effect on the accumulation of ABA as shown in Figure 4.9.

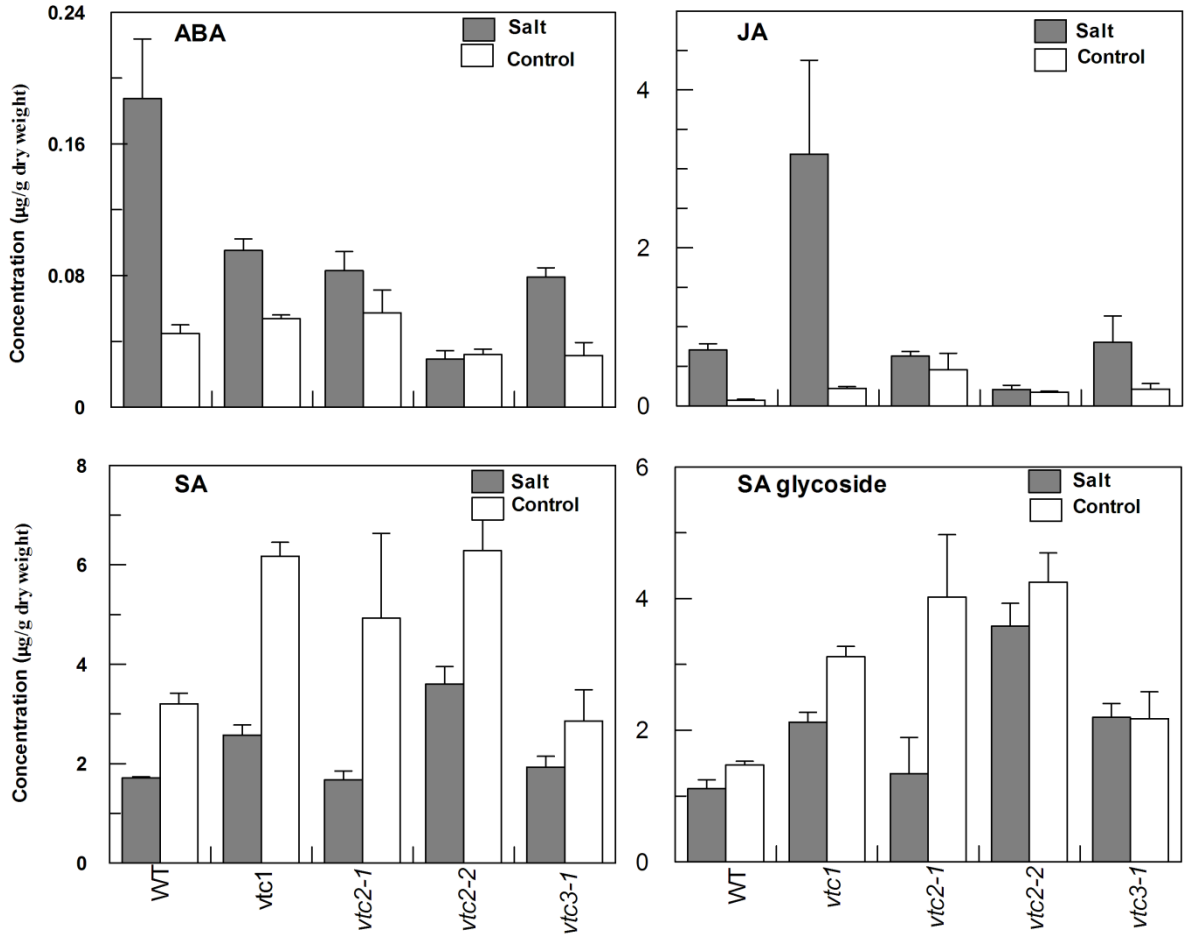
(b) Jasmonic acid (JA)

Unlike wounding, JA did not, respond to salinity. JA slightly increased after salt treatment but the difference was not significant. No difference was found in the WT and *vtc* mutants in both stressed and control conditions except, *vtc1* which showed a startling increase in JA concentration after salt treatment, which was consistent in 2 out of 3 experiments. Further work is needed to investigate the response of *vtc1* to salinity. All the results are shown in Figure 4.9.

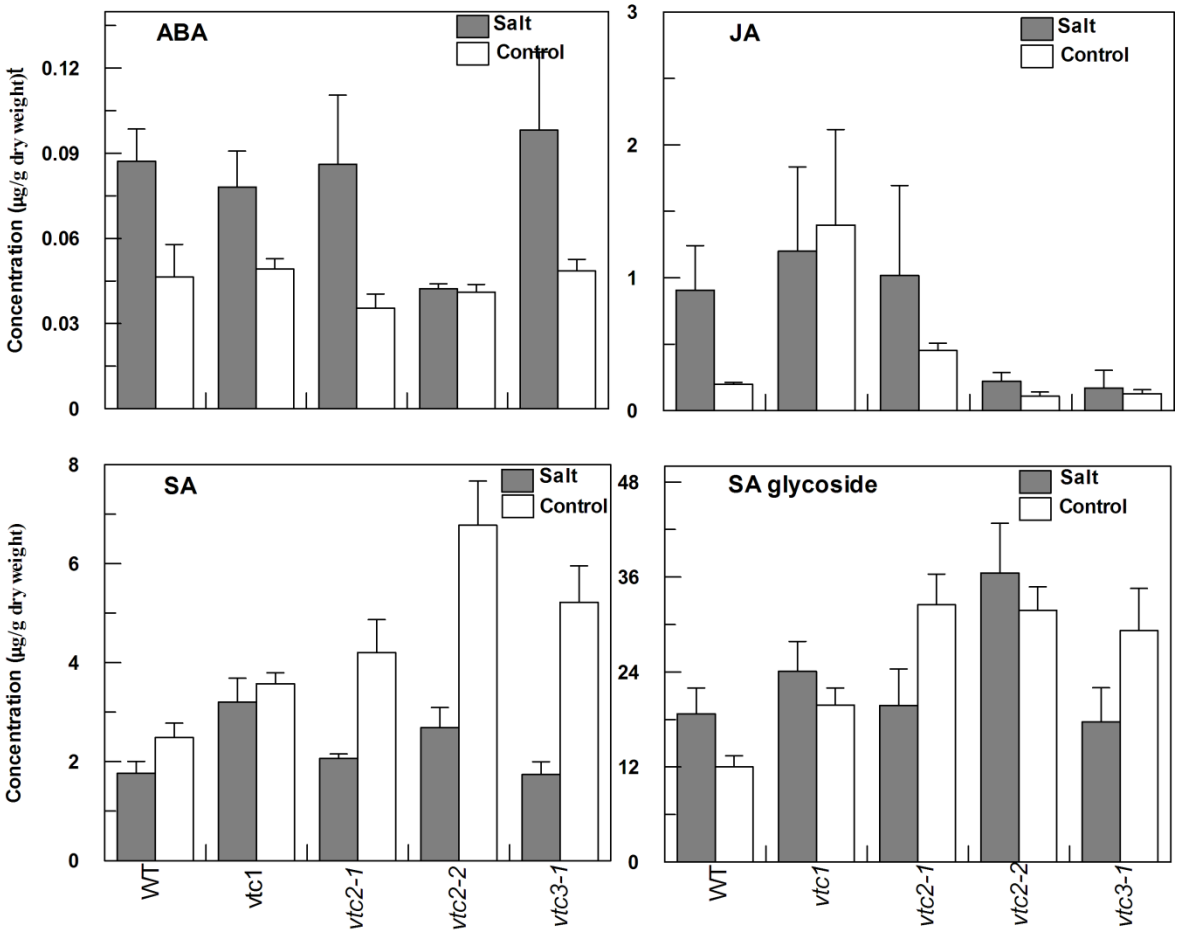
(c) Salicylic acid and SA glycoside

SA and SA glycosides were depleted in salt treated WT and the *vtc* mutants. Both SA and SA glycoside concentrations were significantly higher in *vtc2* than the WT and the *vtc1* and *vtc 3-1* mutants were in-between but not significantly different from both (Figure 4.9).

A


ABA
JA
SA
SA glycoside
Salt
Control
Concentration (µg/g dry weight)
0.24
0.16
0.08
0
4
2
8
6
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

B


ABA
Salt
Control
Concentration (μg/g dry weight)
0
0.03
0.06
0.09
0.12
JA
Salt
Control
0
1
2
3
SA
Salt
Control
Concentration (μg/g dry weight)
0
2
4
6
8
SA glycoside
Salt
Control
0
12
24
36
48
WT
vtc1
vtc2-1
vtc2-2
vtc3-1
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

C

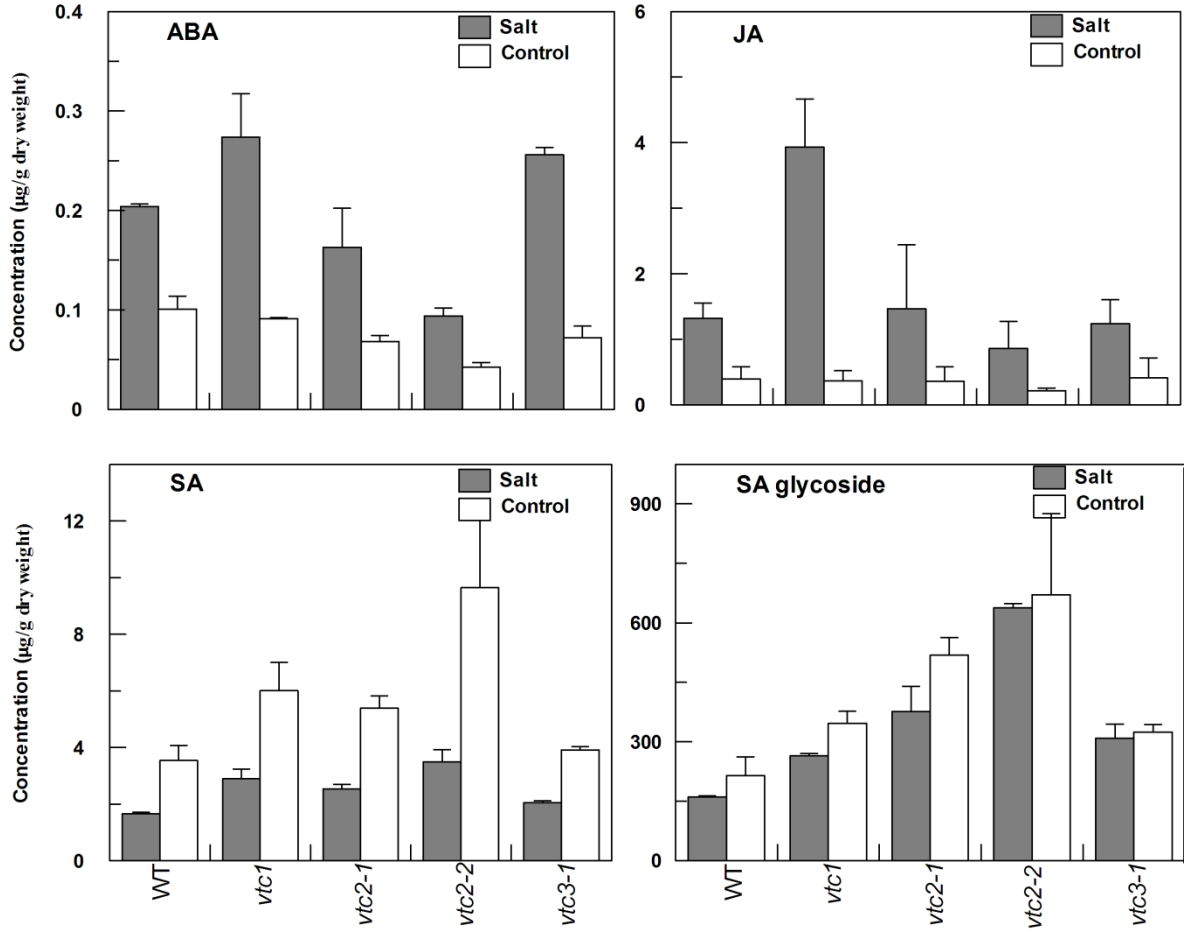


Figure 4. 9 Concentrations of foliar hormones: abscisic acid (ABA), Jasmonic acid (JA), Salicylic acid (SA) and SA glycosides in salt treated *Arabidopsis thaliana* WT and *vtc* mutants. Data from three independent experiments are shown in graph (A) Experiment 1 (B) Experiment 2 and (C) Experiment 3. The hormones were quantified using deuterium labelled internal standards. All results are expressed as mean values + standard error (n = 3) where, 'n' represents the number of biological replicates. Statistical analysis was carried out using one-way analysis of variance (ANOVA). Values at $p < 0.05$ were considered statistically significant.

4.5.3. 4 Summary of stress effects on hormones

The overall data showed hormones concentrations were unaffected in *vtc* mutants than WT in all stress experiments (ABA, JA, SA and SA glycosides). The *vtc* mutants produced more SA and SA glycoside than the WT. Accumulation of hormones was regulated by stress treatments. In our data sets we found much variation between experiments, which might be due to small differences in plant age or environmental conditions even though attempts were made to provide uniform growth conditions. There was a considerable variation between experiments even though growth conditions were kept as consistent as possible. Considering that internal standards were used from the point of extraction, it seems most likely that biological variability is to blame.

4.5. 4 The effect of high light on anthocyanin and flavonol glycoside accumulation

Accumulation of anthocyanins in response to HL have been reported in previous studies (Muller-Moule, *et al*., 2003; Dowdle et al., 2007; Giacomelli *et al*., 2006; Muller-Moule, *et al*., 2004). In the current study, we analysed the accumulation of anthocyanin and flavonol glycosides in HL exposed *vtc* mutants. The *vtc* mutants produced considerably less anthocyanin pigmentation under HL conditions (light conditions described in section 4.4.1), resulting in a light brown colour, particularly on the adaxial side of the leaf. By contrast, the WT leaves turned deep red/brown colour due to the accumulation of high anthocyanin pigmentation. The WT and *vtc*-mutants did not produce a visible level of anthocyanin pigments when grown under LL, and the *vtc* mutants appeared non-pigmented. The accumulation of anthocyanin was more intense in fully expanded leaves compared to young leaves (Figure 4.10).

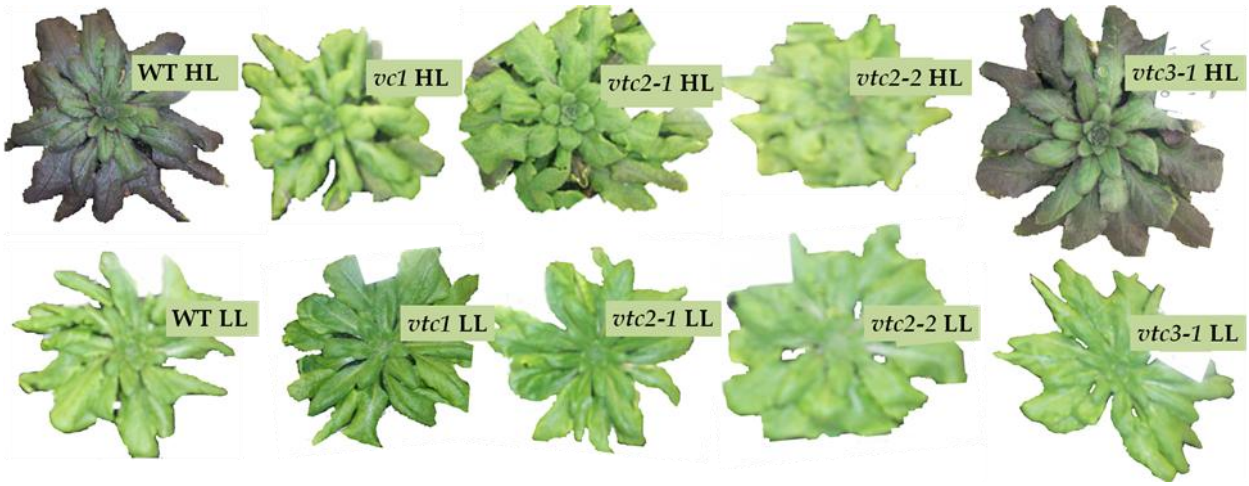


Figure 4. 10 Accumulation of pigments in *Arabidopsis thaliana* in response to HL. Light conditions used are: LL (100 µmol m^{-2} s^{-1}) to HL (550-650 µmol m^{-2} s^{-1}). WT = wild type, *vtc* = ascorbate-deficient mutants, HL = high light, LL = low light. Top left and top right can clearly see the dark pigmentation in WT and *vtc 3-1* HL. Other mutants are green and accumulated less anthocyanin in HL. No anthocyanin accumulation was seen in LL in the WT and the *vtc* mutants (bottom left to right).

The middle age (approximately 6 week old) leaf tissues were treated with HL and harvested for the extraction of flavonoids (methods discussed in Chapter 2). Flavonoid targeted analysis was carried out using liquid chromatography-mass spectrometry. Fourteen different flavonoids (cyanidin and flavonol derivatives) were identified in a single run on the basis of RT and precursor and product ion *m/z*. The metabolites putatively detected from their UV absorption spectra and comprehensive analyses of mass fragmentation pattern obtained by MS/MS spectrometry were compared with previously reported data (Tohge *et al.*, 2005). 14 different peaks were detected by LC-QQQ-MS in which nine represented cyanidin

based anthocyanin and five kaempferol based flavonol glycosides. Structures for the metabolite corresponding to each peak (Table 4.1) were determined by LC-MS/MS defined by published data (Tohge *et al.,* 2005).

Table 4.1 Flavonoids identified in *Arabidopsis thaliana* leaf tissue by liquid chromatography–electrospray ionisation–tandem mass spectrometry (ESI-LC MS/MS) analysis of product ions were used for quantification. (A) Flavonol glycosides (F1-F5), (B) Anthocyanin (A1-A9) Cy = cyanidin, Glc = glucose, Xyl = xylose, Cou = *p*-coumaroyl moiety, Mal = malonyl moiety, Sin = sinapoyl moiety, Km = kaempferol, Rha = rhamnose (Page *et al*, 2011).

A

Peak	Rt (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Metabolites
F1	11.48	433 [M + H]+	287 [Km + H]+	Kaempferol 3-*O*-rhamnoside
F2	11.28	565 [M + H]+	287 [Km + H]+	Kaempferol [(pentoside)-rhamnoside]
			433 [Km + Rha + H]+	
F3	11.47	579 [M + H]+	287 [Km + H]+	Kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside
			433 [Km + Rha + H]+	
F4	11.12	595 [M + H]+	287 [Km + H]+	Kaempferol 3-*O*-glucoside 7-*O*-rhamnoside
			433 [Km + Rha + H]+	
F5	10.48	741 [M + H]+	287 [Km + H]+ 433 [Km + Rha + H]+ 595 [Km + Rha + Glc + H]+	Kaempferol 3-*O*-[6''-*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside

B

Peak	Rt (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Metabolite
A1	11.19	889 [M]+	287 [Cy]+	Cyanidin 3-*O*-[2''-*O*-(xylosyl) 6''-*O*-(*p*-coumaroyl) glucoside] 5-*O*-glucoside
A2	10.04	949 [M]+	287 [Cy]+ 449 [Cy + Glc]+	Cyanidin 3-*O*-[2''-*O*-(2'''-*O*-(sinapoyl) xylosyl) glucoside] 5-*O*-glucoside
A3	11.46	975 [M]+	287 [Cy]+ 535 [Cy + Glc + Mal]+ 727 [Cy + Glc + Xyl + Cou]+	Cyanidin 3-*O*-[2''-*O*-(xylosyl)-6''-*O*-(*p*-coumaroyl) glucoside] 5-*O*-malonylglucoside
A4	10.23	1051 [M]+	287 [Cy]+ 449 [Cy + Glc]+ 889 [Cy + 2Glc + Xyl + Cou]+	Cyanidin 3-*O*-[2''-*O*-(xylosyl)-6''-*O*-(*p*-*O*-(glucosyl)-p-coumaroyl) glucoside] 5-*O*-glucoside
A5	11.45	1095 [M]+	287 [Cy]+ 535 [Cy + Glc + Mal]+ 975 [Cy + 2Glc + Xyl + Cou + Mal	Cyanidin 3-*O*-[2''-*O*-(2'''-*O*-(sinapoyl) xylosyl) 6''-*O*-(p-coumaroyl) glucoside] 5-*O*-glucoside

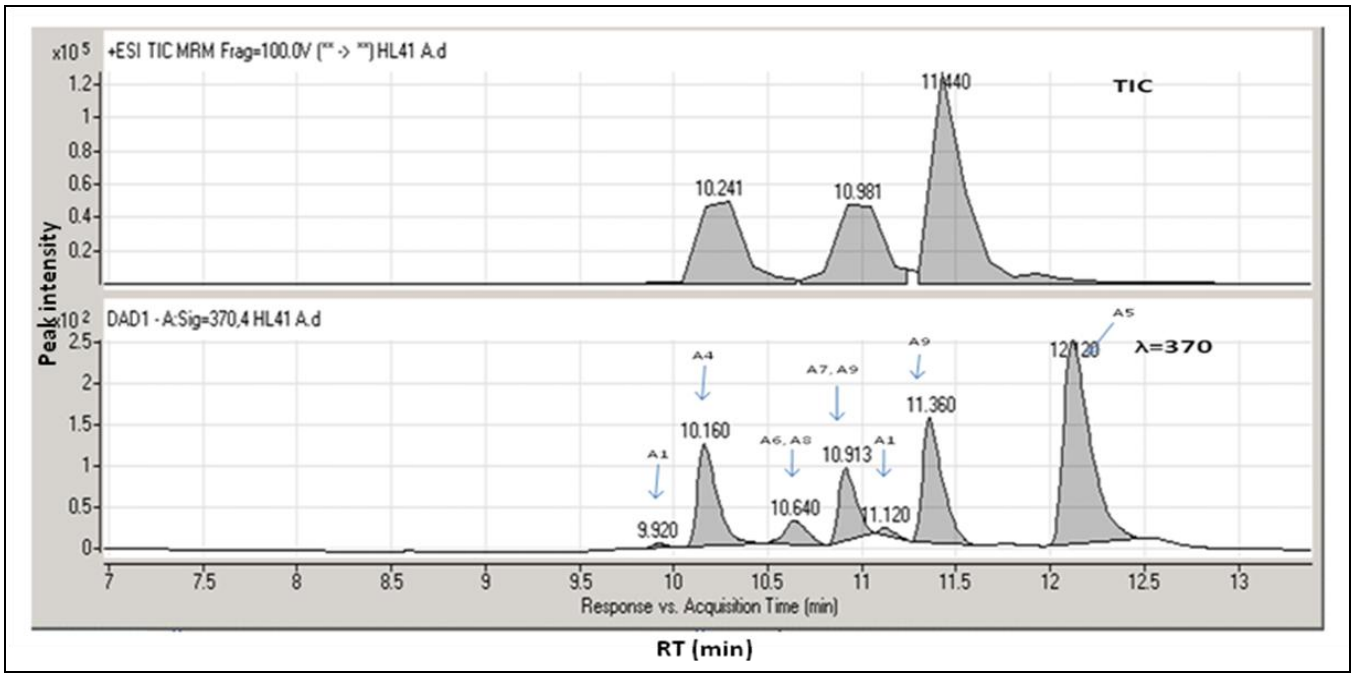
Table 4.2 B continued.....

Peak	Rt (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Metabolite
A6	10.46	1137 [M]+	287 [Cy]+	Cyanidin 3-*O*-[2''-*O*-(xylosyl) 6''-*O*-(*p*-*O*-(glucosyl) p-coumaroyl) glucoside] 5-O-[6'''-O-(malonyl) glucoside]
			535 [Cy + Glc + Mal]+	
			889 [Cy + 2Glc + Xyl + Cou]+	
A7	11.72	1181 [M]+	287 [Cy]+	Cyanidin 3-*O*-[2''-*O*-(2'''-*O*-(sinapoyl) xylosyl) 6''-*O*-(*p*-*O*-coumaroyl) glucoside] 5-O-[6''''-O-(malonyl) glucoside
			535 [Cy + Glc + Mal]+	
			933 [Cy + Glc + Xyl + Sin + Cou]+	
A8	10.68	1257 [M]+	287 [Cy]+	Cyanidin 3-*O*-[2''-*O*-(2'''-*O*-(sinapoyl) xylosyl) 6''-*O*-(*p*-*O*-(glucosyl) *p*-coumaroyl) glucoside] 5-*O*-glucoside
			449 [Cy + Glc]+	
			1095 [Cy + 2Glc + Xyl + Cou + Sin]+	
A9	10.9	1343 [M]+	287 [Cy]+	Cyanidin 3-*O*-[2''-*O*-(6'''-O-(sinapoyl) xylosyl) 6''-*O*-(*p*-*O*-(glucosyl)- *p*-coumaroyl) glucoside] 5-*O*-(6''''-*O*-malonyl) glucoside
			535 [Cy + Glc + Mal]+	
			1095 [Cy + 2Glc + Xyl + Cou + Sin]+	

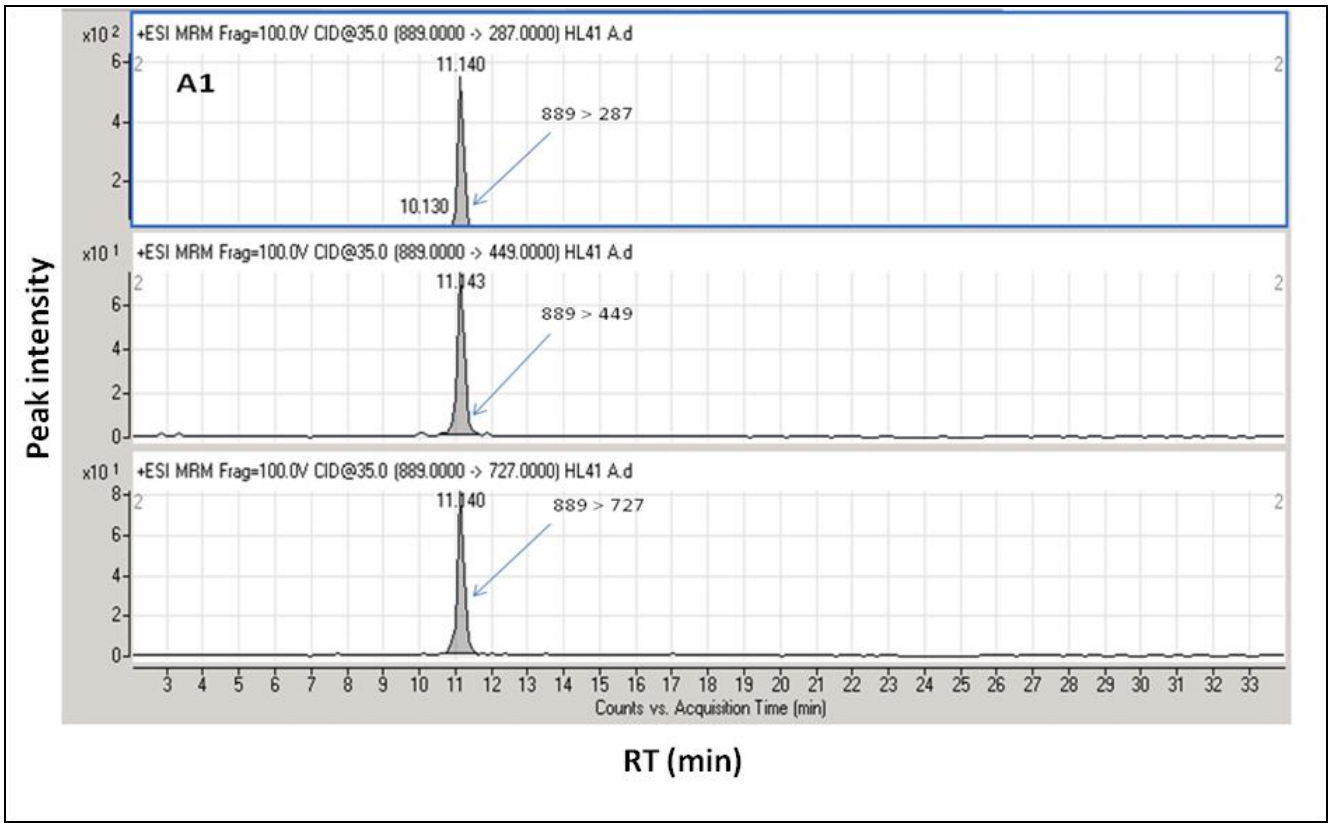
4.5.5 Fragmentation of cyanidin and kaempferol by LC- QQQ-MS

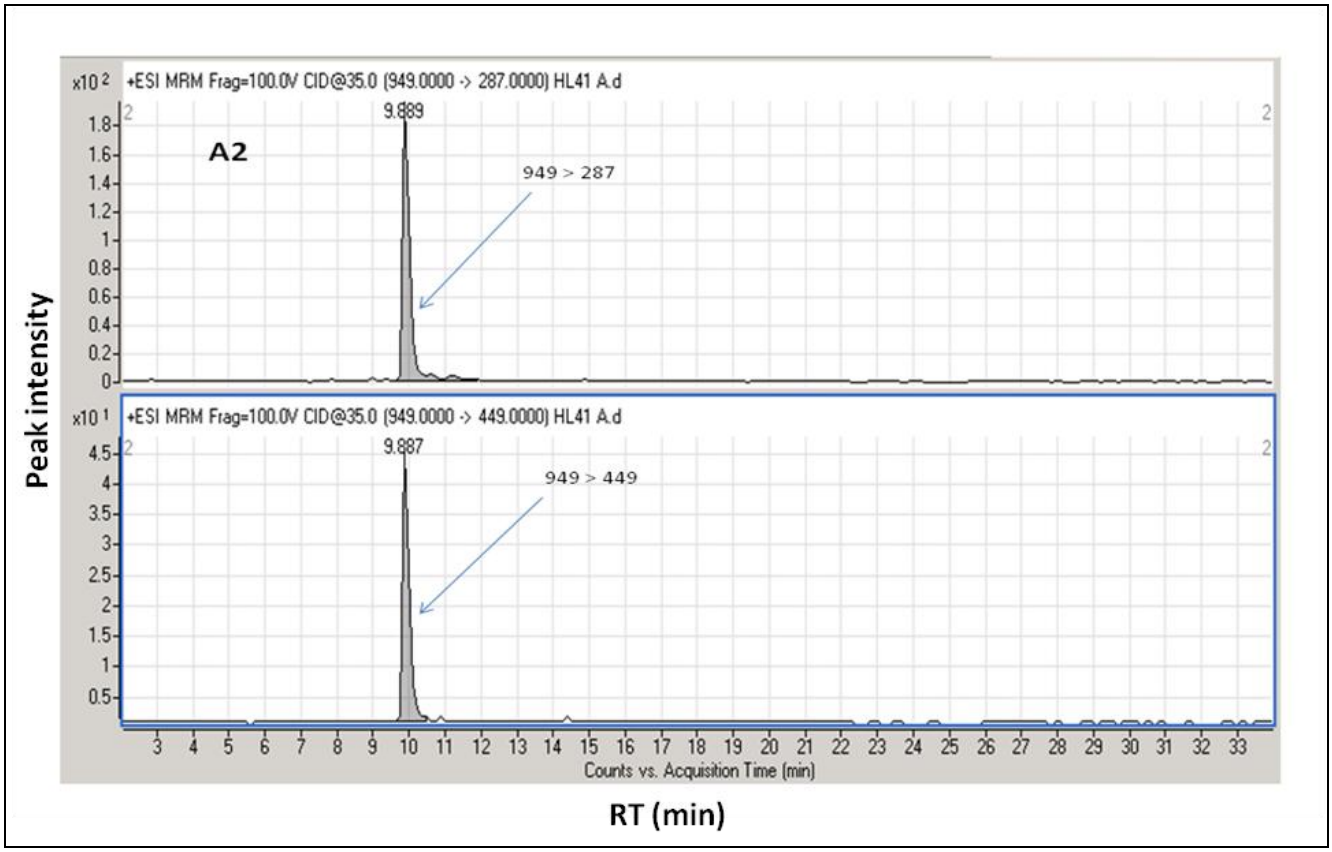
Chromatographic analysis of all samples (WT and *vtc* mutants) showed that they have similar total ion chromatograms (TIC), UV absorption spectra (λmax = 370 nm) and product ions. Therefore, only fragmentation and mass spectrometric data obtained from the WT are presented in Figure 4.11 and 4.12. 14 different closely-eluting peaks were identified as kaempferol based flavonol glycosides and cyanidin based anthocyanins. Nine anthocyanin (cyanidin derivatives) and 5 flavonol glycosides were identified using LC-MS-QQQ in positive ion mode (Table 4.2). The identification was based on information described in previous literature reports (Tohge *et al.*, 2005).

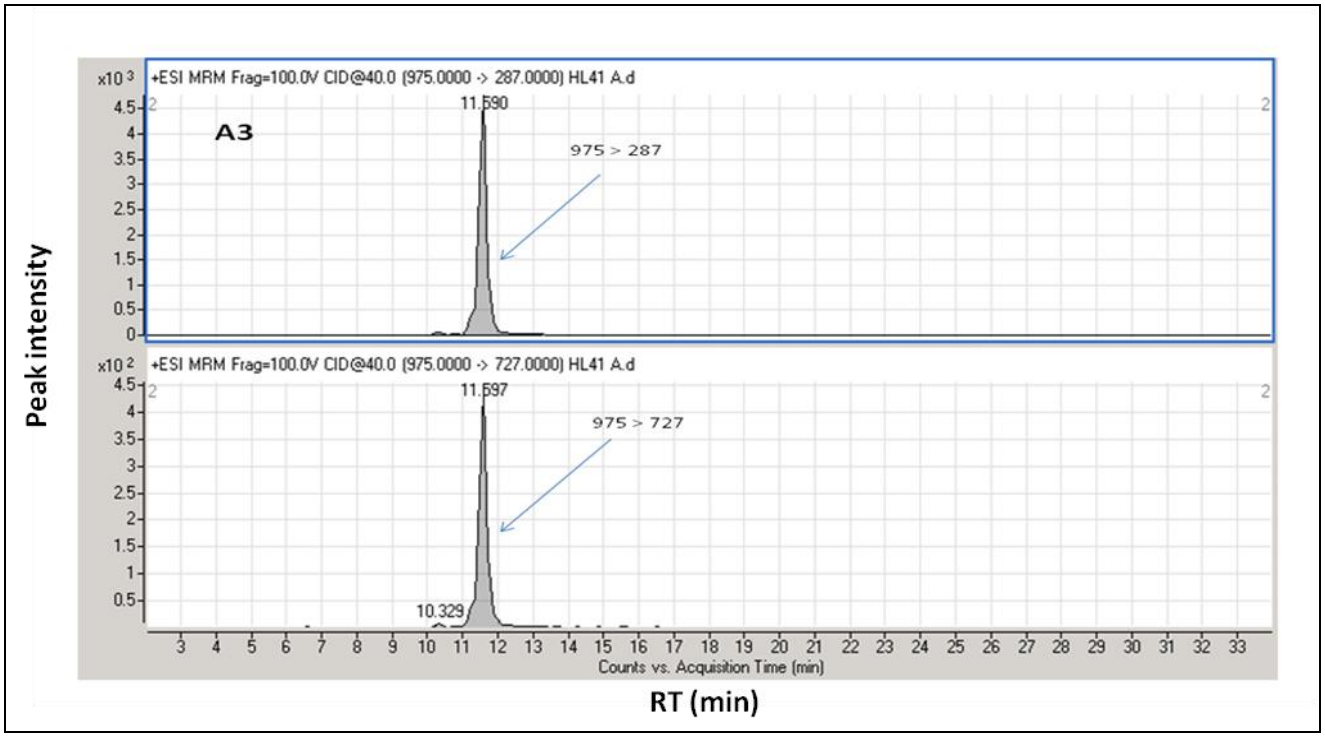
A

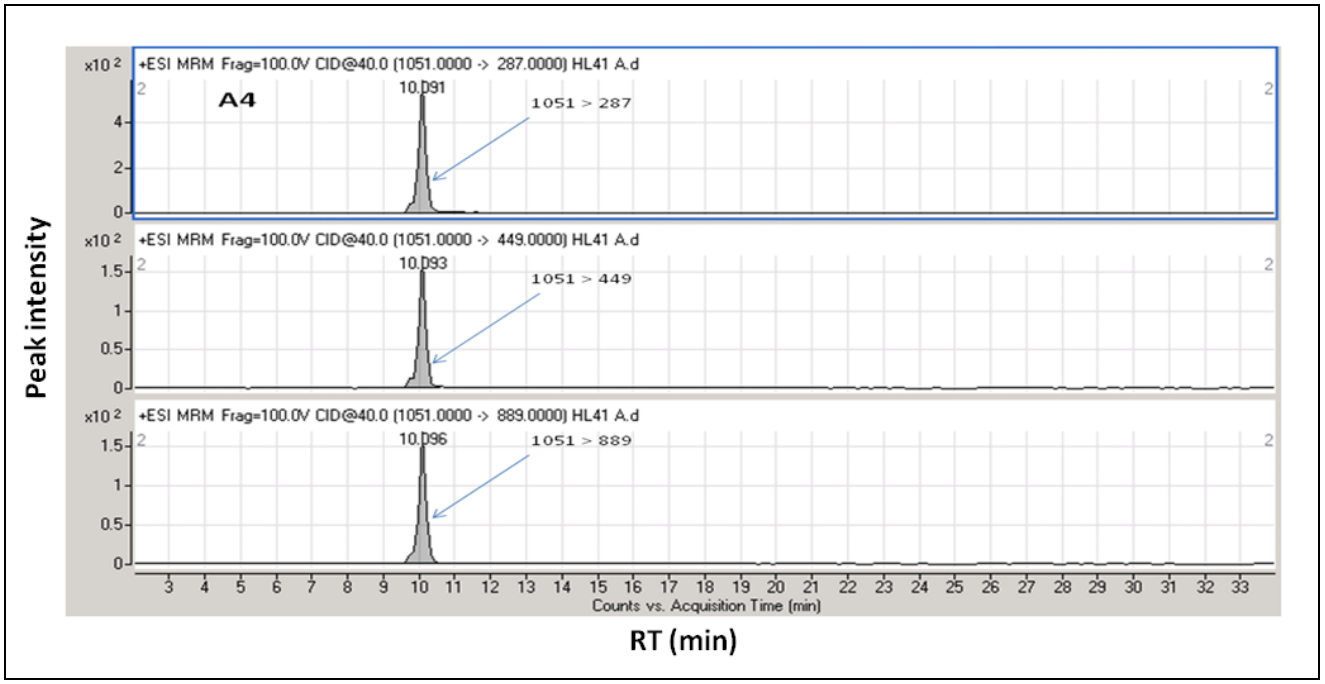


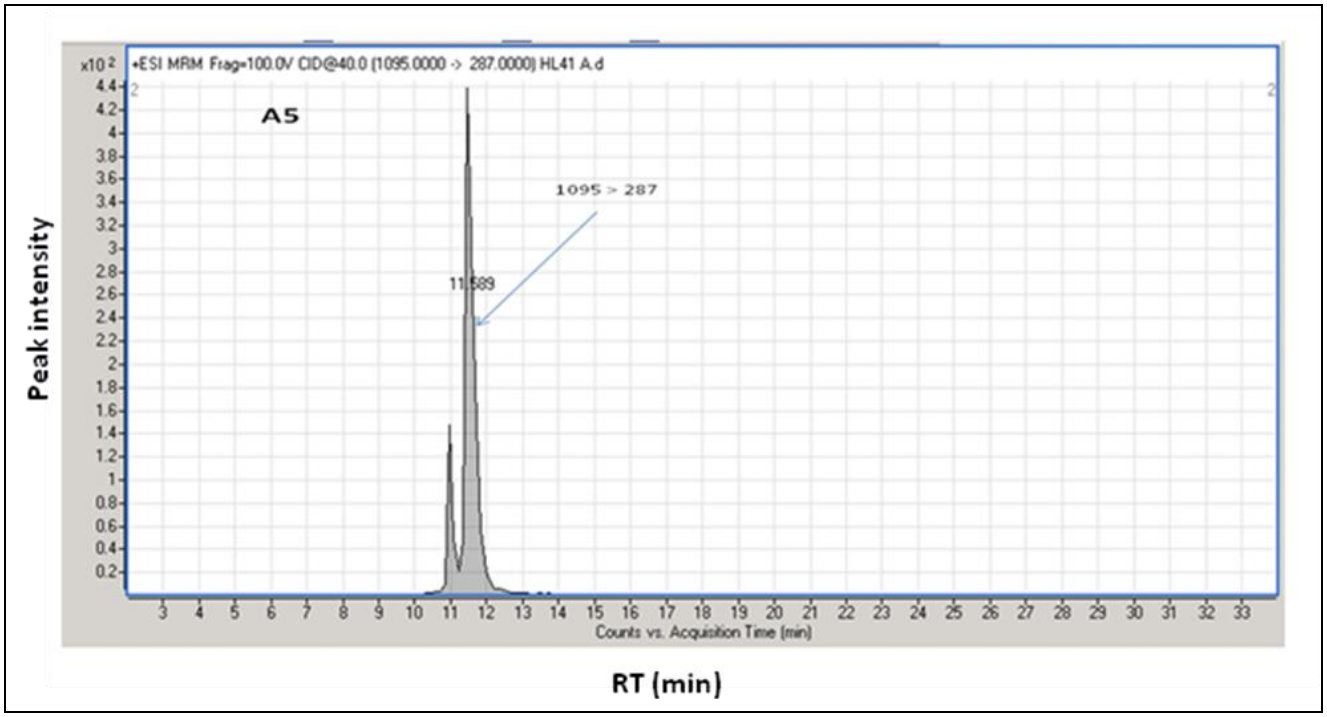
B

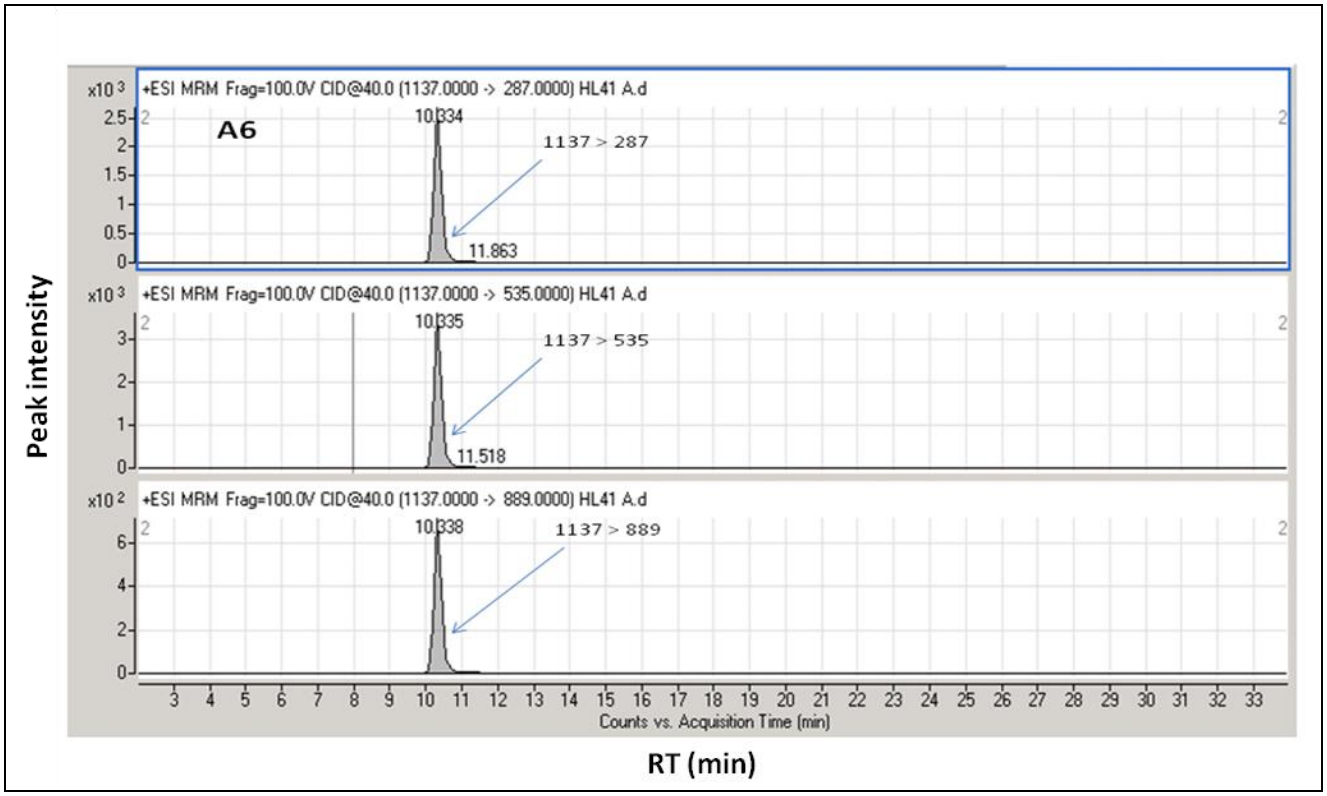

+ESI MRM Frag=100.0V CID@35.0 (889.0000 -> 287.0000) HL41 A.d
A1
11.140
889 > 287
10.130
+ESI MRM Frag=100.0V CID@35.0 (889.0000 -> 449.0000) HL41 A.d
889 > 449
+ESI MRM Frag=100.0V CID@35.0 (889.0000 -> 727.0000) HL41 A.d
889 > 727
Peak intensity
Counts vs. Acquisition Time (min)
RT (min)

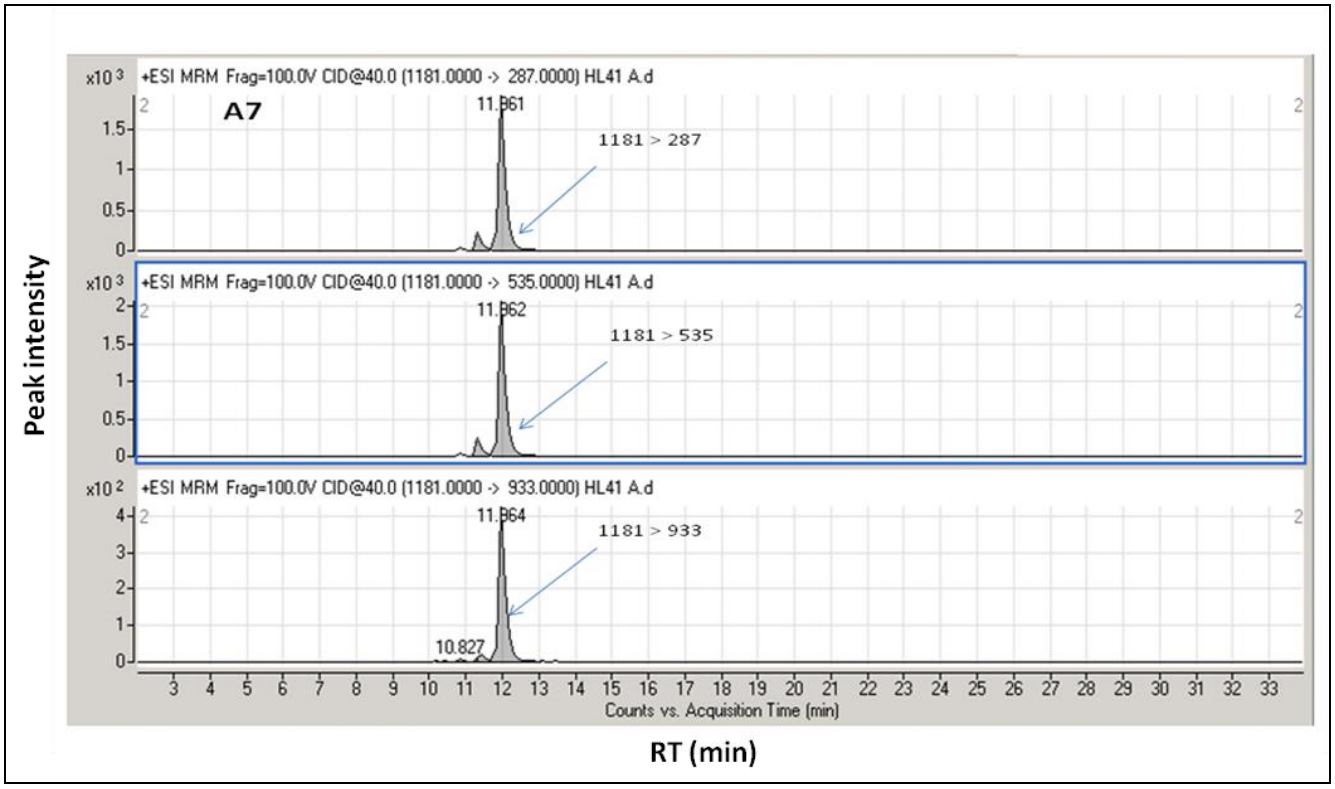

+ESI MRM Frag=100.0V CID@35.0 (949.0000 -> 287.0000) HL41 A.d
A2
9.889
949 > 287
+ESI MRM Frag=100.0V CID@35.0 (949.0000 -> 449.0000) HL41 A.d
9.887
949 > 449
Peak intensity
Counts vs. Acquisition Time (min)
RT (min)

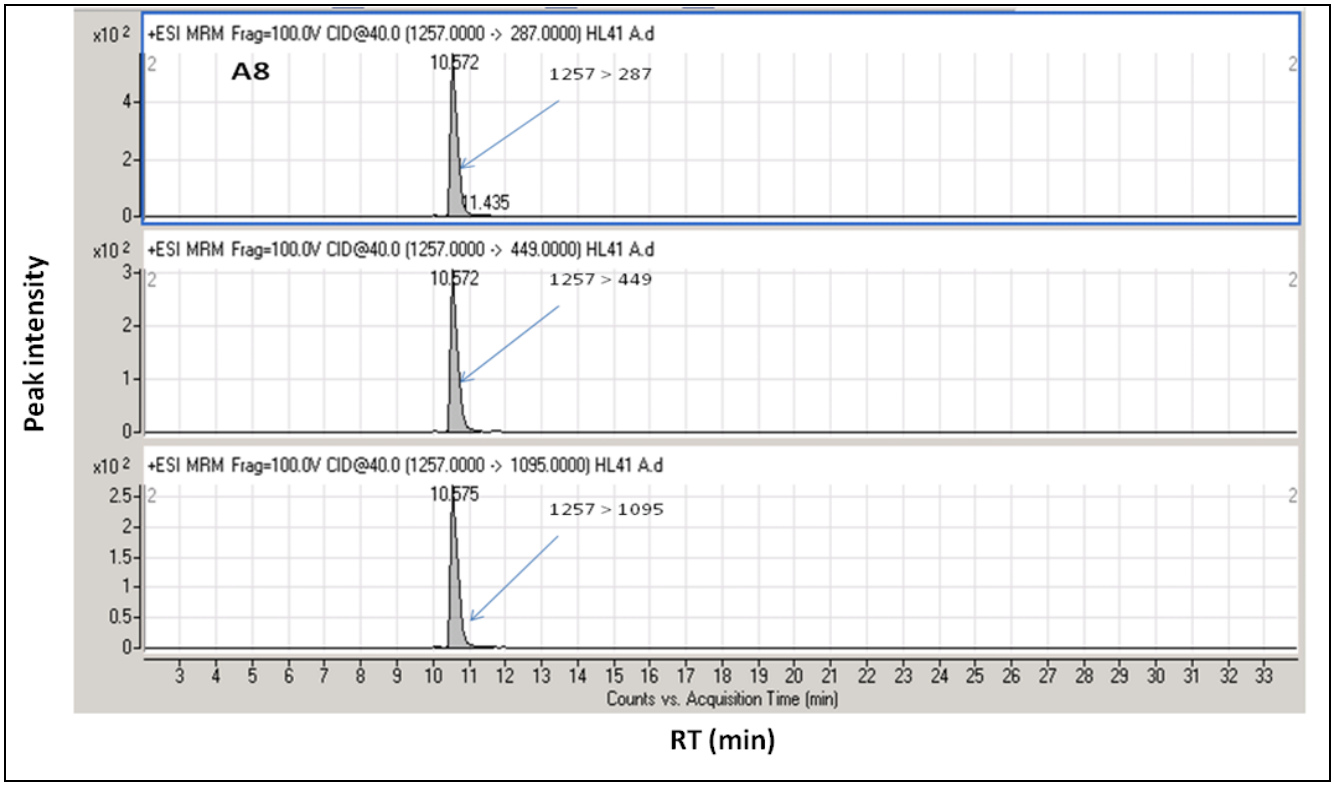

Peak intensity
+ESI MRM Frag=100.0V CID@40.0 (975.0000 -> 287.0000) HL41 A.d
A3
11.590
975 > 287
+ESI MRM Frag=100.0V CID@40.0 (975.0000 -> 727.0000) HL41 A.d
11.597
975 > 727
10.329
Counts vs. Acquisition Time (min)
RT (min)


Peak intensity
+ESI MRM Frag=100.0V CID@40.0 (1051.0000 -> 287.0000) HL41 A.d
A4
10.091
1051 > 287
+ESI MRM Frag=100.0V CID@40.0 (1051.0000 -> 449.0000) HL41 A.d
10.093
1051 > 449
+ESI MRM Frag=100.0V CID@40.0 (1051.0000 -> 889.0000) HL41 A.d
10.096
1051 > 889
Counts vs. Acquisition Time (min)
RT (min)


Peak intensity
x10 2
+ESI MRM Frag=100.0V CID@40.0 (1095.0000 -> 287.0000) HL41 A.d
A5
1095 > 287
11.589
Counts vs. Acquisition Time (min)
RT (min)


Peak intensity
x10 3
+ESI MRM Frag=100.0V CID@40.0 (1137.0000 -> 287.0000) HL41 A.d
A6
10.834
1137 > 287
11.863
x10 3
+ESI MRM Frag=100.0V CID@40.0 (1137.0000 -> 535.0000) HL41 A.d
10.835
1137 > 535
11.518
x10 2
+ESI MRM Frag=100.0V CID@40.0 (1137.0000 -> 889.0000) HL41 A.d
10.838
1137 > 889
Counts vs. Acquisition Time (min)
RT (min)


Peak intensity
x10 3
+ESI MRM Frag=100.0V CID@40.0 (1181.0000 -> 287.0000) HL41 A.d
A7
11.861
1181 > 287
x10 3
+ESI MRM Frag=100.0V CID@40.0 (1181.0000 -> 535.0000) HL41 A.d
11.862
1181 > 535
x10 2
+ESI MRM Frag=100.0V CID@40.0 (1181.0000 -> 933.0000) HL41 A.d
11.864
1181 > 933
10.827
Counts vs. Acquisition Time (min)
RT (min)


Peak intensity
x10 2
+ESI MRM Frag=100.0V CID@40.0 (1257.0000 -> 287.0000) HL41 A.d
A8
10.572
1257 > 287
11.435
x10 2
+ESI MRM Frag=100.0V CID@40.0 (1257.0000 -> 449.0000) HL41 A.d
10.572
1257 > 449
x10 2
+ESI MRM Frag=100.0V CID@40.0 (1257.0000 -> 1095.0000) HL41 A.d
10.575
1257 > 1095
Counts vs. Acquisition Time (min)
RT (min)

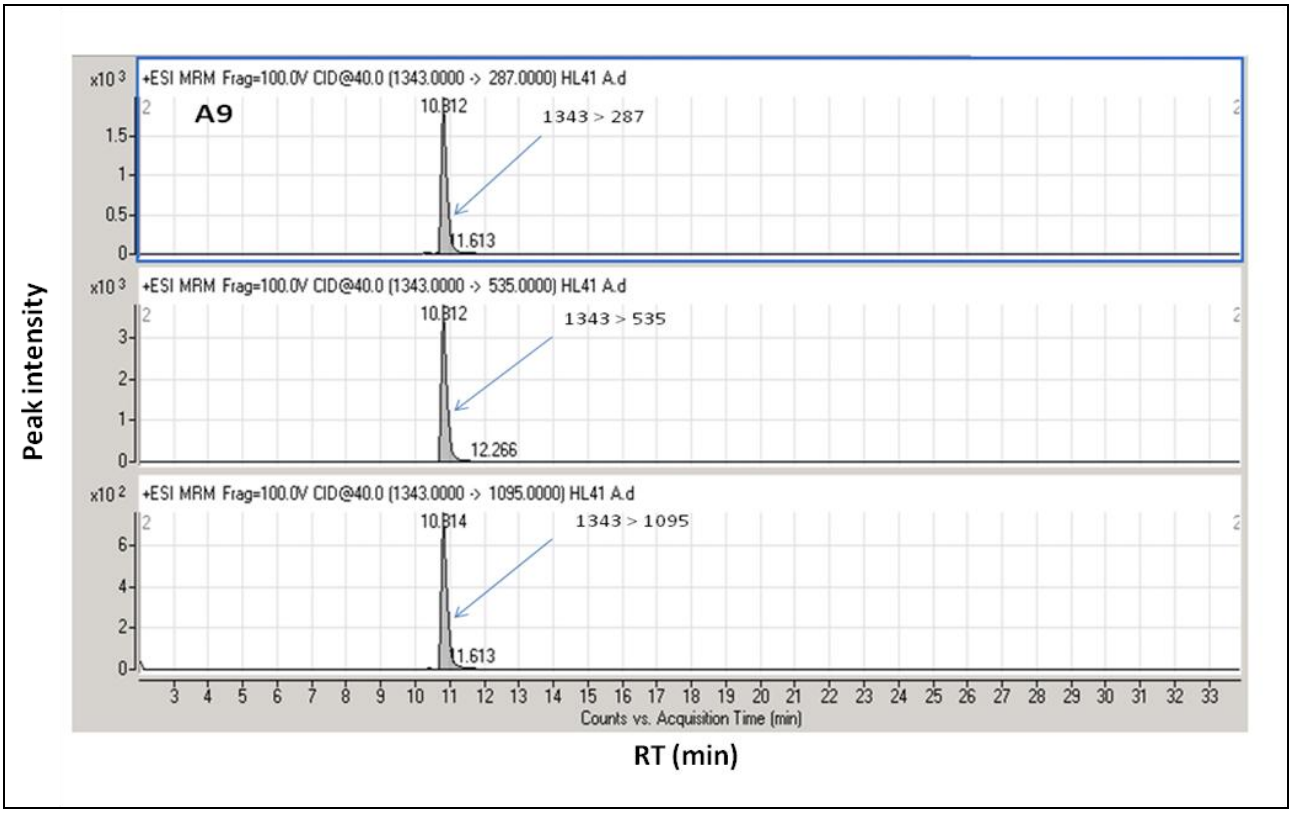
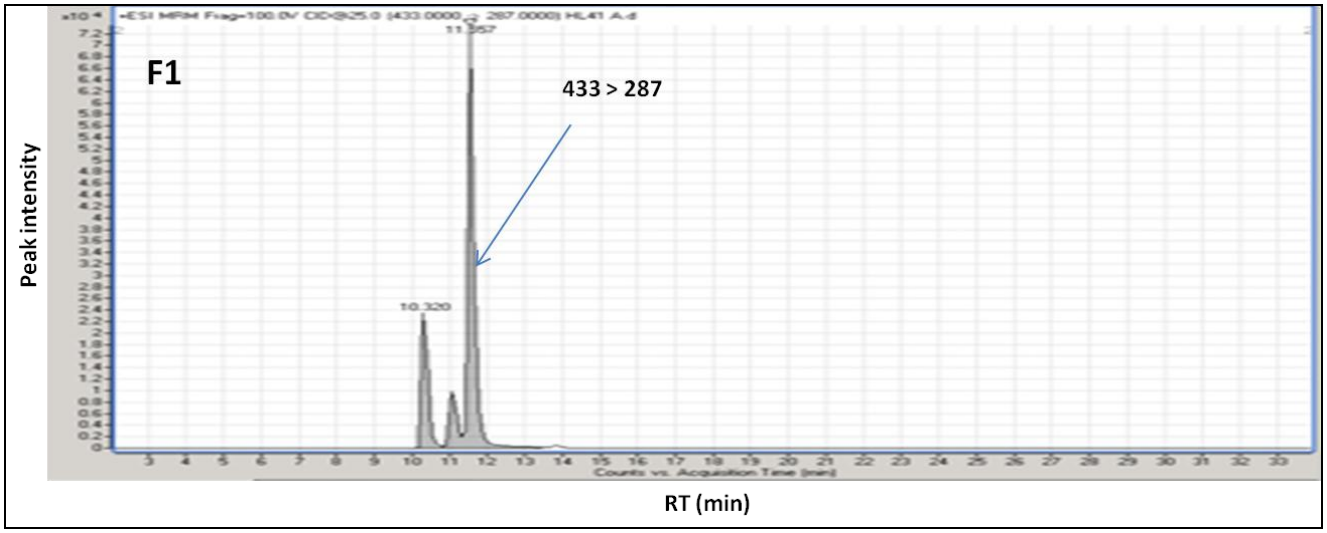
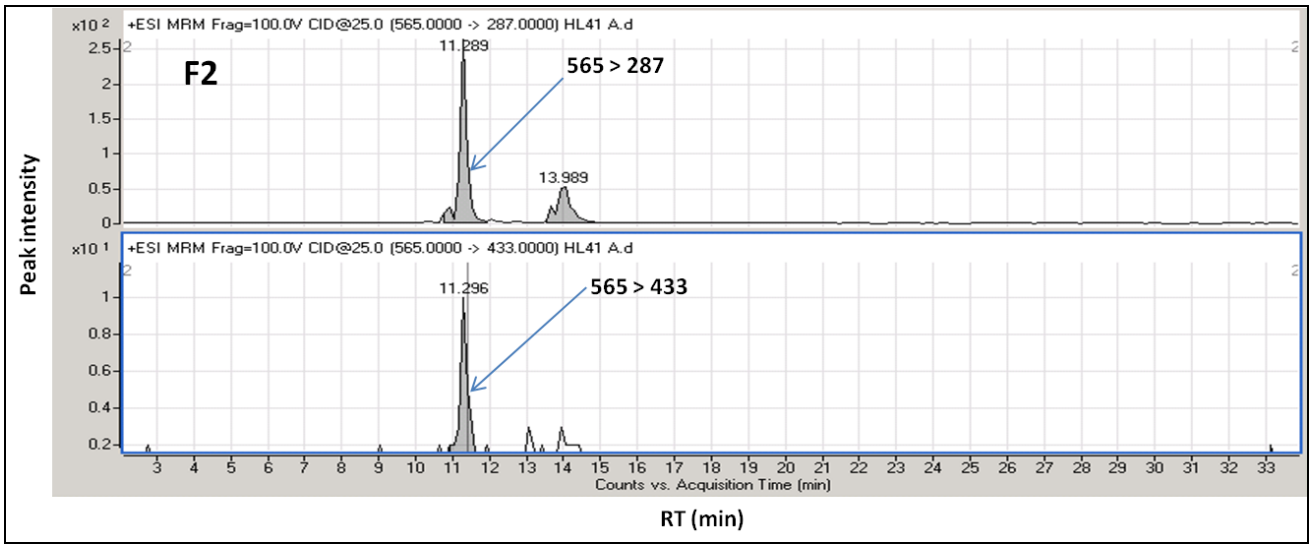
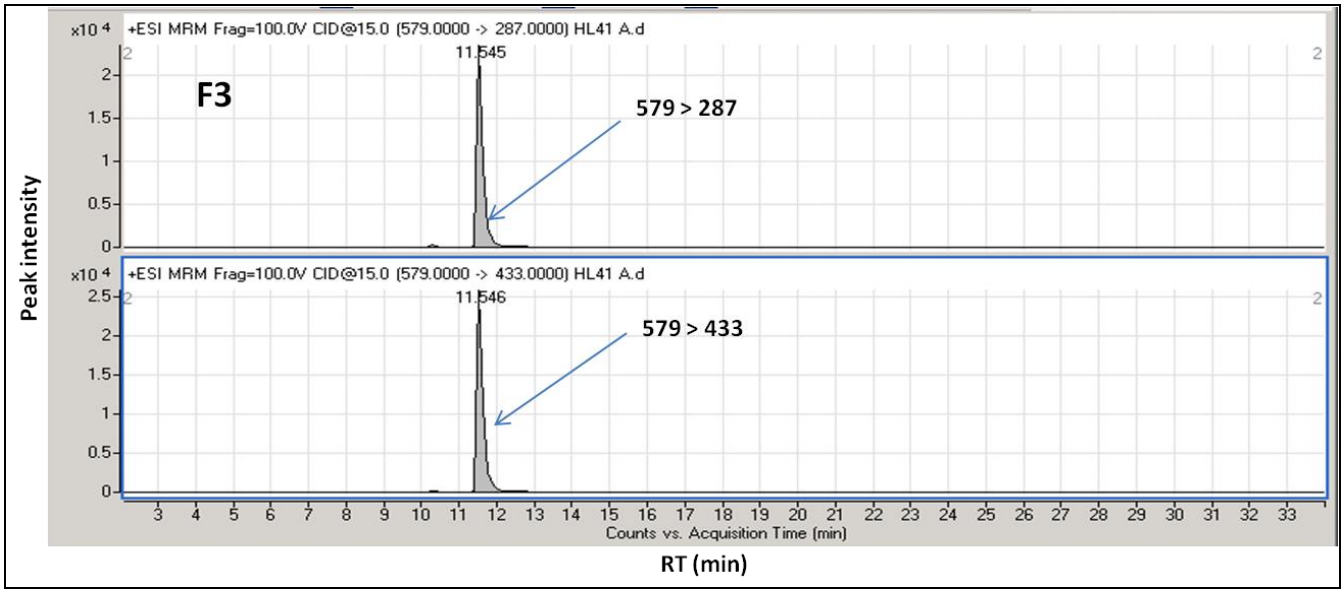
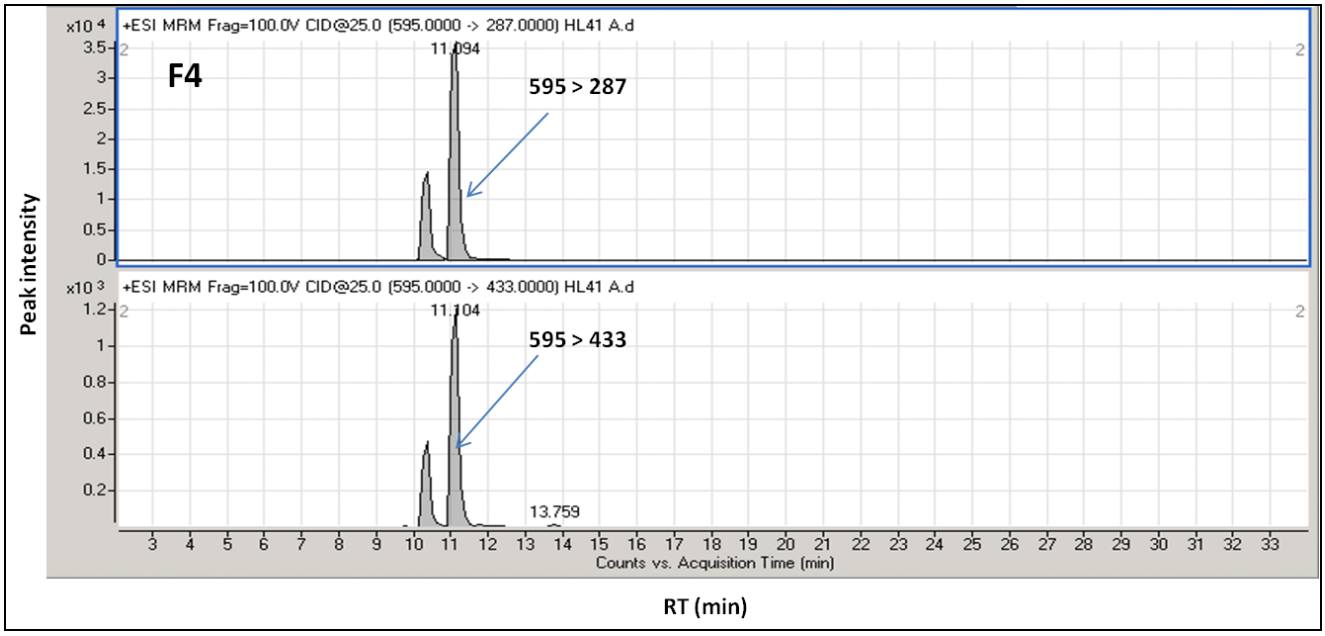


Figure 4.11 Chromatographic representation of anthocyanins identified in *Arabidopsis thaliana* (A) Total ion chromatograms and UV absorption (λ = 340 nm). (B) Product ion chromatograms of anthocyanins (A1-A9) detected in positive ion mode (See Table 4.1B).


F1
433 > 287
11.357
10.320
Peak intensity
RT (min)


F2
+ESI MRM Frag=100.0V CID@25.0 (565.0000 -> 287.0000) HL41 A.d
11.289
565 > 287
13.989
+ESI MRM Frag=100.0V CID@25.0 (565.0000 -> 433.0000) HL41 A.d
11.296
565 > 433
Counts vs. Acquisition Time (min)
Peak intensity
RT (min)


F3
+ESI MRM Frag=100.0V CID@15.0 (579.0000 -> 287.0000) HL41 A.d
11.545
579 > 287
+ESI MRM Frag=100.0V CID@15.0 (579.0000 -> 433.0000) HL41 A.d
11.546
579 > 433
Peak intensity
Counts vs. Acquisition Time (min)
RT (min)


F4
+ESI MRM Frag=100.0V CID@25.0 (595.0000 -> 287.0000) HL41 A.d
11.094
595 > 287
+ESI MRM Frag=100.0V CID@25.0 (595.0000 -> 433.0000) HL41 A.d
11.104
595 > 433
13.759
Peak intensity
Counts vs. Acquisition Time (min)
RT (min)

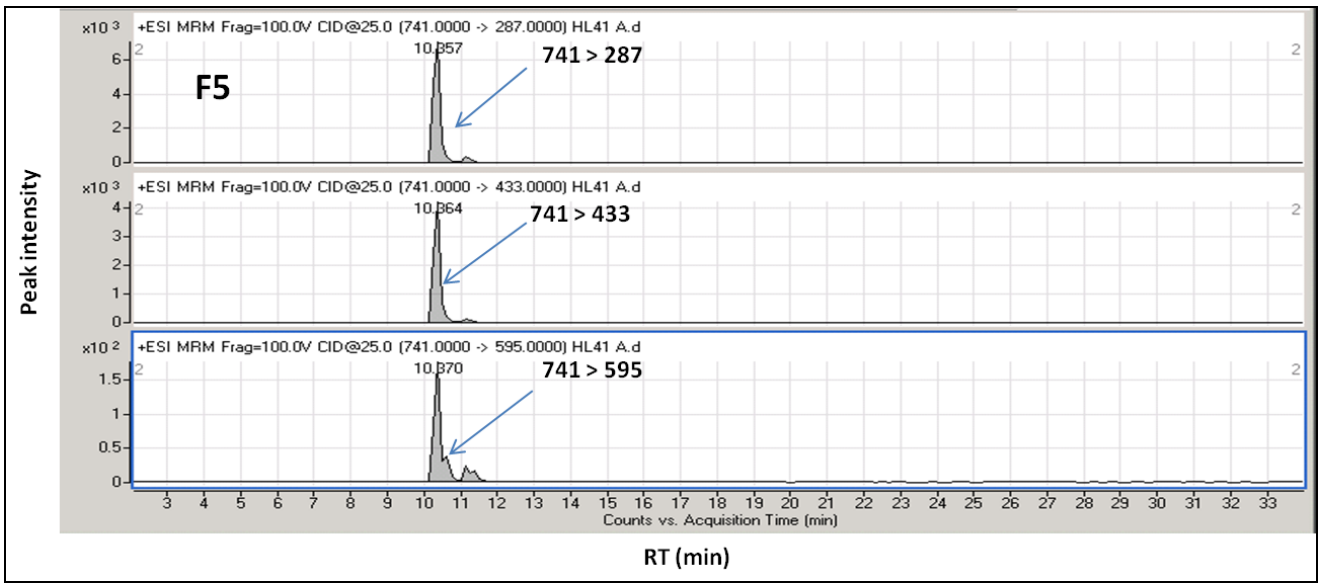


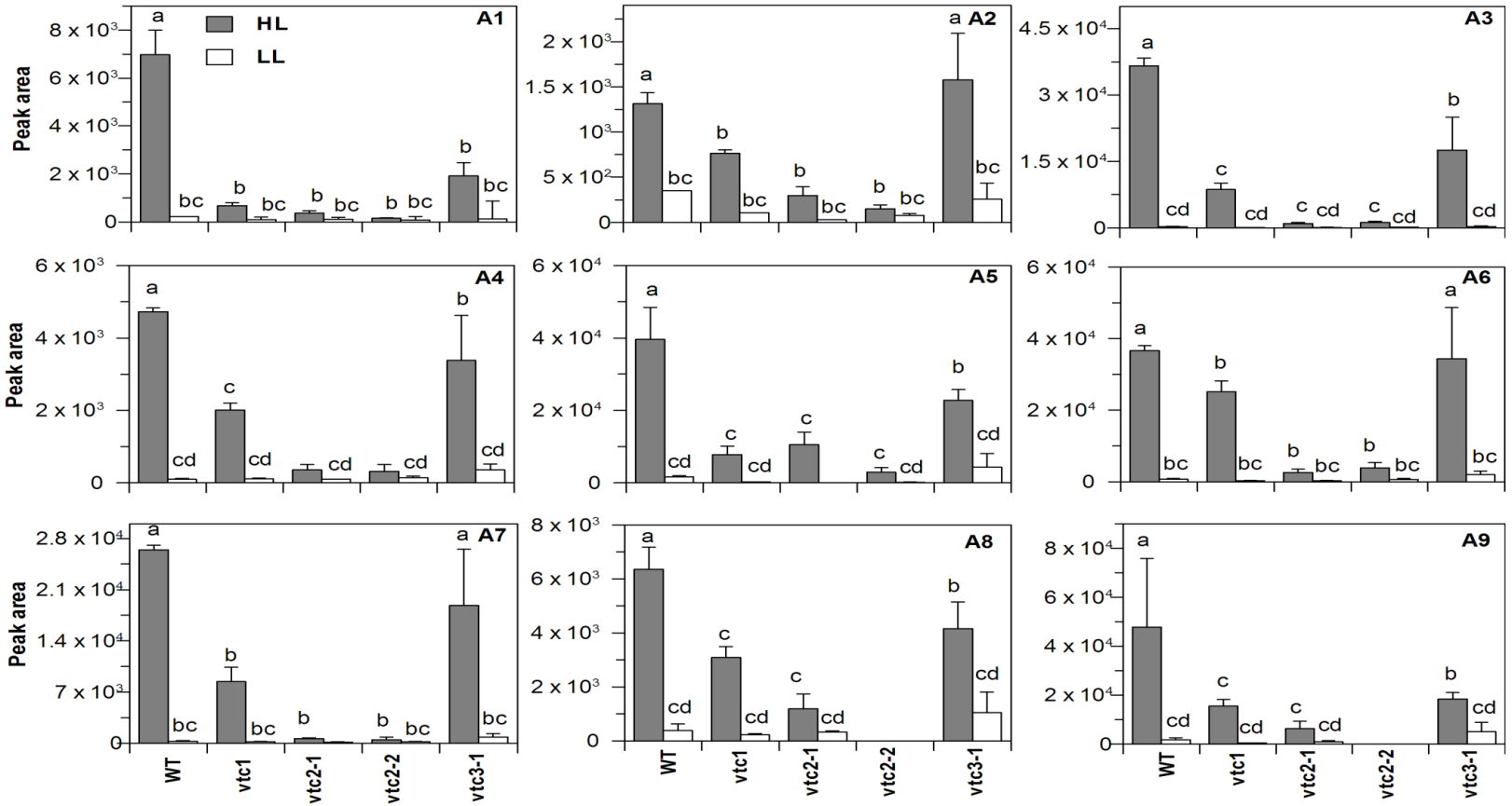
Figure 4.12 Product ion chromatograms of flavonol glycosides (F1-F5) from *Arabidopsis thaliana*. Flavonol glycosides, F1-F5 were detected in positive ionisation mode (See Table 4.1A).

4.5. 6 The effect of HL intensity on the accumulation of anthocyanin in *Arabidopsis thaliana* WT and *vtc* mutants

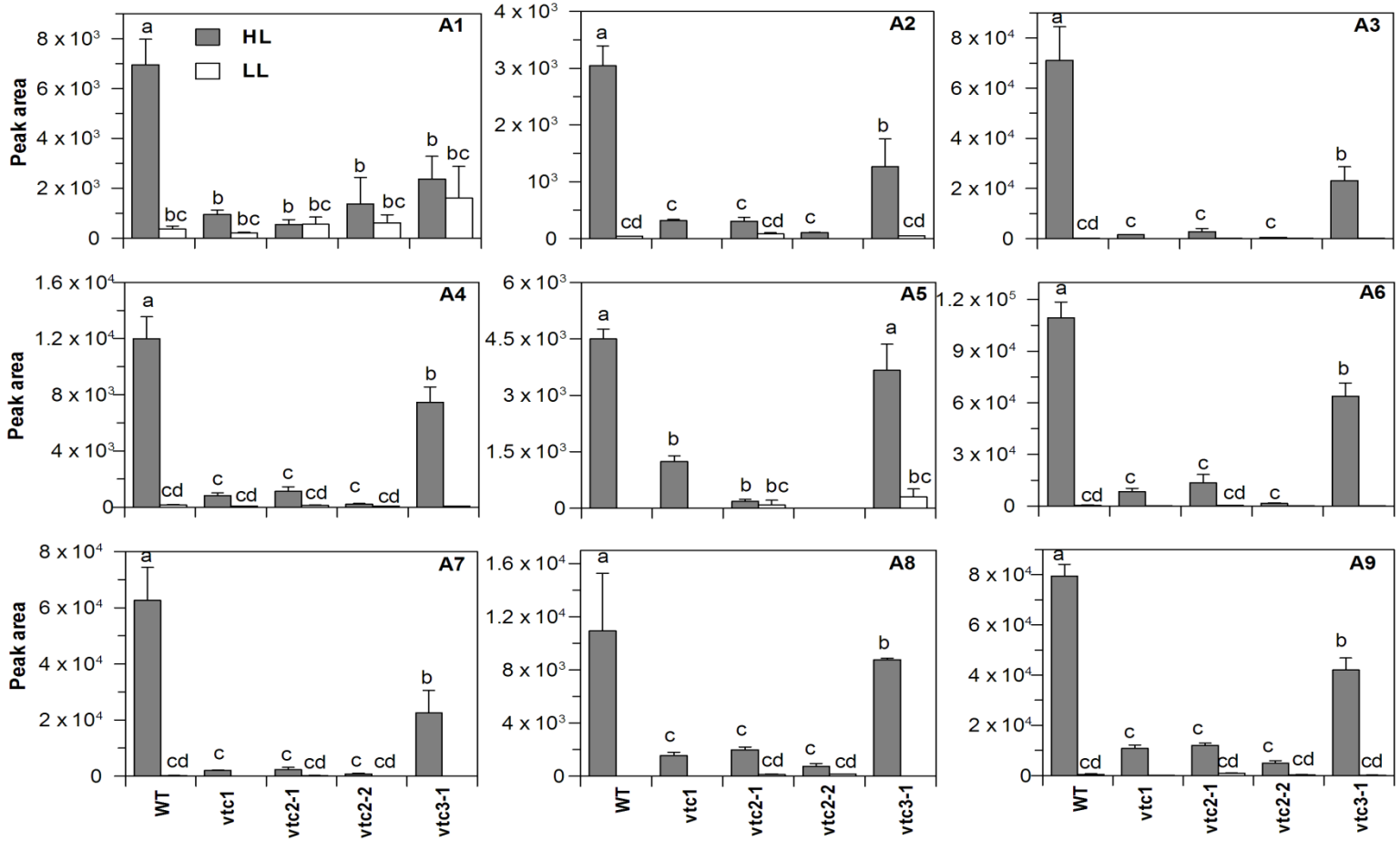
The accumulation of anthocyanin was regulated by increasing the light intensity in the WT and *vtc3-1* mutant but no increase was observed in other mutants (*vtc1* and *vtc2*) as they have less ascorbate in HL and LL (Figure 4.5). Low abundant or no peak (below the range of detection) of anthocyanin was detected in *vtc* mutants (*vtc1* and *vtc2*) in both HL and LL but *vtc 3-1* and the WT produced comparatively intense pigmentation. In LL *vtc* mutants lacked pigmentation whereas the WT produced relatively weak pigmentation in petioles and leaf tissue. The statistical analysis showed no significant difference in anthocyanin accumulation between the WT and the *vtc* mutants in LL. Increasing the light intensity gave a substantial increase in pigmentation in the WT and *vtc3-1* but this was very low in other mutants (*vtc1* and *vtc2*).

In HL LC-MS profiling detected 9 different cyanidin based anthocyanin peaks (A1-A9) in WT and *vtc3-1* but a very low signal was detected in *vtc1* and *vtc2* mutants (*vtc2* refers to *vtc2-1* and *vtc2-2,*). In LL anthocyanin accumulation was not directly observable in *vtc* mutants. Anthocyanin peaks A1-A9 were significantly higher in the WT than the *vtc* mutants ($p < 0.001$) in HL. The *vtc3-1* mutant produced in between WT and other mutants, was significantly different from both except A2 and A5 which were same in *vtc 3-1* and WT. All nine anthocyanins were significantly higher in WT acclimated in HL than low light ($p < 0.0001$). Interestingly, all strains produced the identical pattern in accumulation of all nine anthocyanins in three independent experiments as shown in Figure 4.13.

A


HL
LL
A1
A2
A3
A4
A5
A6
A7
A8
A9
Peak area
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

B


HL
LL
A1
A2
A3
A4
A5
A6
A7
A8
A9
Peak area
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

C

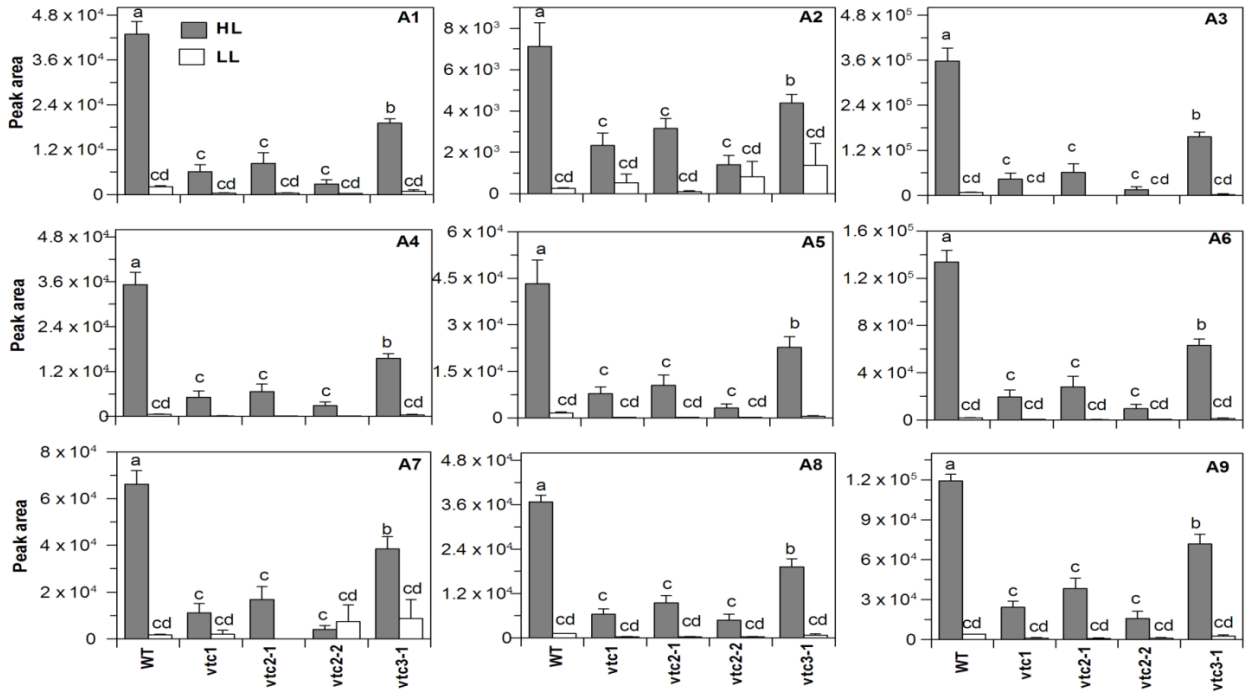
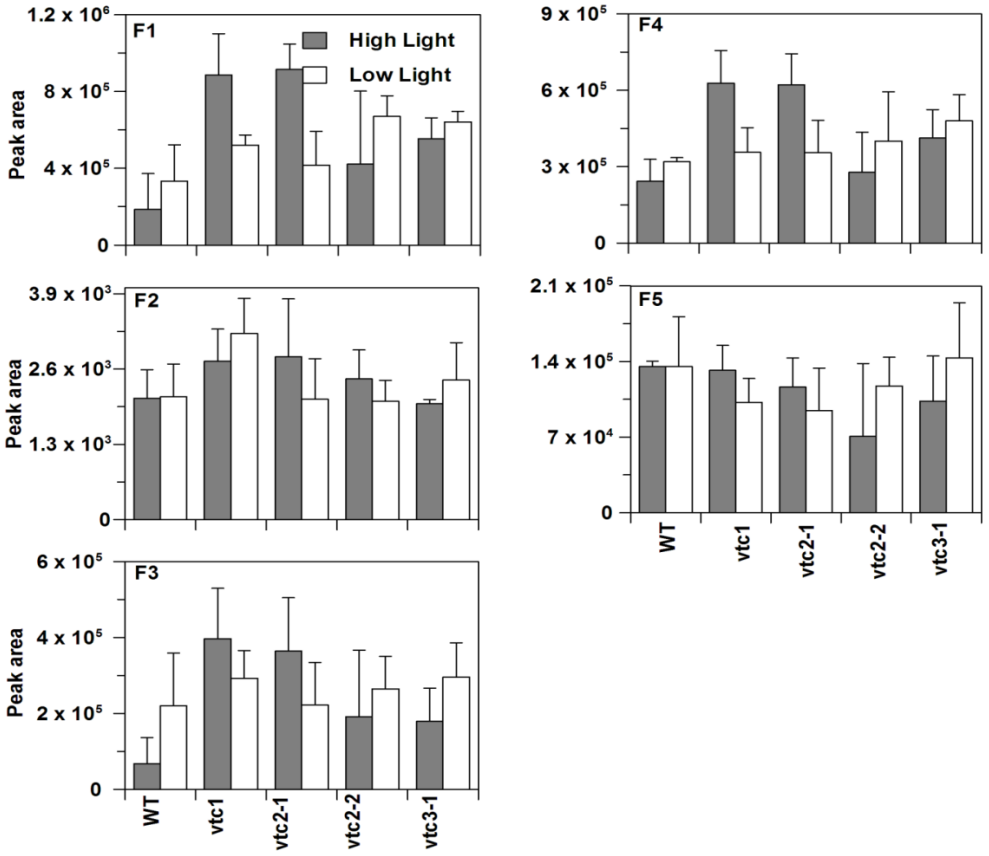


Figure 4.13 Foliar Anthocyanin profiles (A1-A9) of *Arabidopsis thaliana* WT and the *vtc* mutants acclimated to HL and LL. Light intensities used are: HL; 550-650 µmol m^{-2} s^{-1} and LL; 100 µmol m^{-2} s^{-1}. Anthocyanins A1-A9 (see Table 4.1B) were quantified by LC-ESI-QQQ MS/MS. Data from three independent experiments (A) Experiment 1 (B) Experiment 2 (C) Experiment 3. The data were quantified using peak area and subjected to analysis of variance to identify the statistical differences between treatments and strains. All results were expressed as mean values + standard error (n = 3) where, 'n' represents the number of biological replicates. Analyses were carried out using one-way analysis of variance (ANOVA). Values at $p < 0.05$ were considered statistically significant.

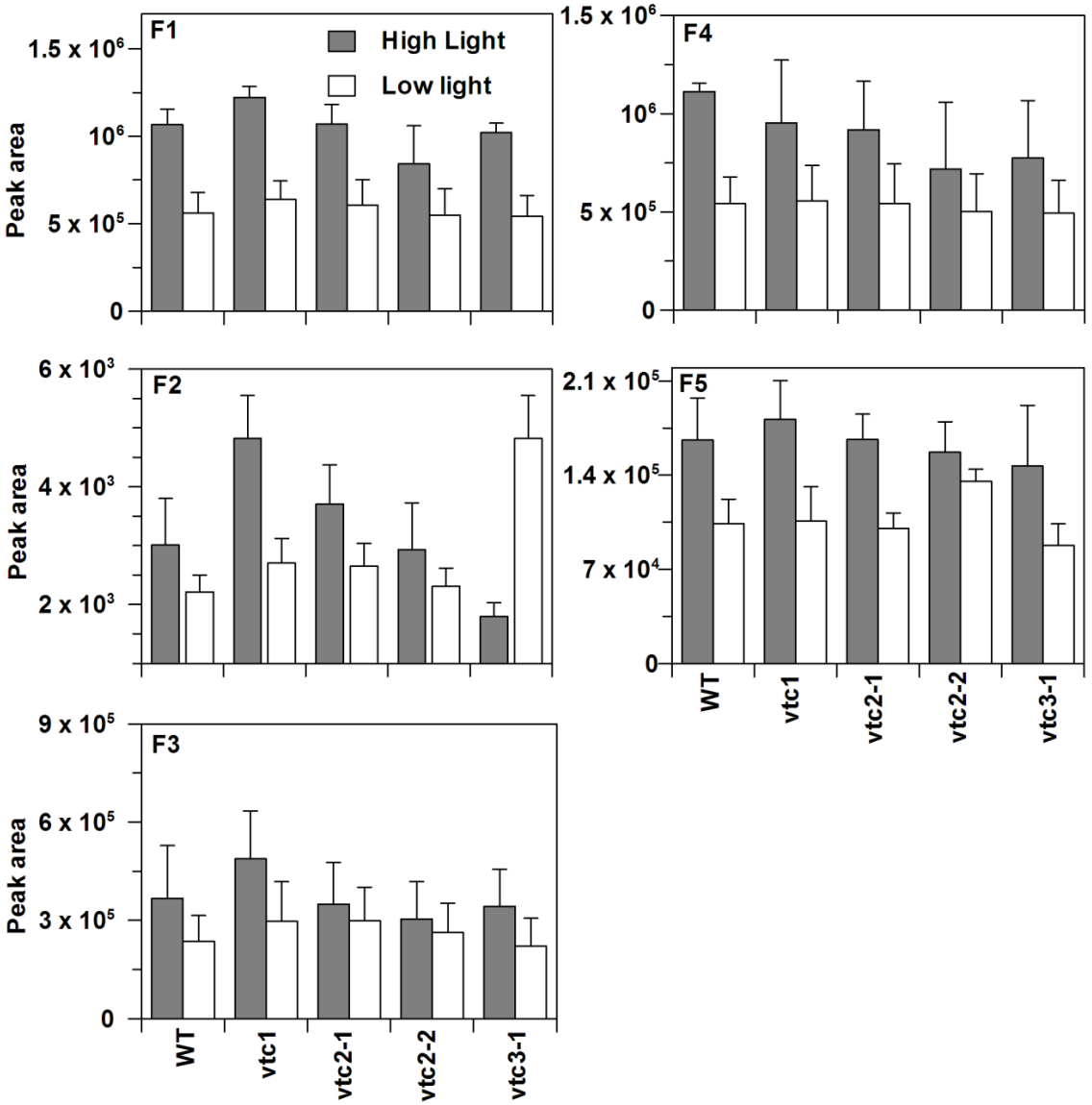
4.5. 7 The effect of light intensity on flavonol glycosides in *vtc* mutants

The five kaempferol glycosides (F1-F5) with different glycosylation pattern were detected and identified in *Arabidopsis thaliana* WT and *vtc* mutants (Table 4.1A). In strong contrast to anthocyanin, the accumulation of kaempferol seems not to be affected by the increased light intensity and no difference was observed in the WT and the *vtc* mutants in both light conditions. In summary, flavonol glycosides showed no significant difference between HL and LL and between the WT and the *vtc* mutants.

A



B


F1
High Light
Low light
Peak area
1.5 x 10^6
10^6
5 x 10^5
0
F4
1.5 x 10^6
10^6
5 x 10^5
0
F2
6 x 10^3
4 x 10^3
2 x 10^3
Peak area
F5
2.1 x 10^5
1.4 x 10^5
7 x 10^4
0
F3
9 x 10^5
6 x 10^5
3 x 10^5
0
Peak area
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

C

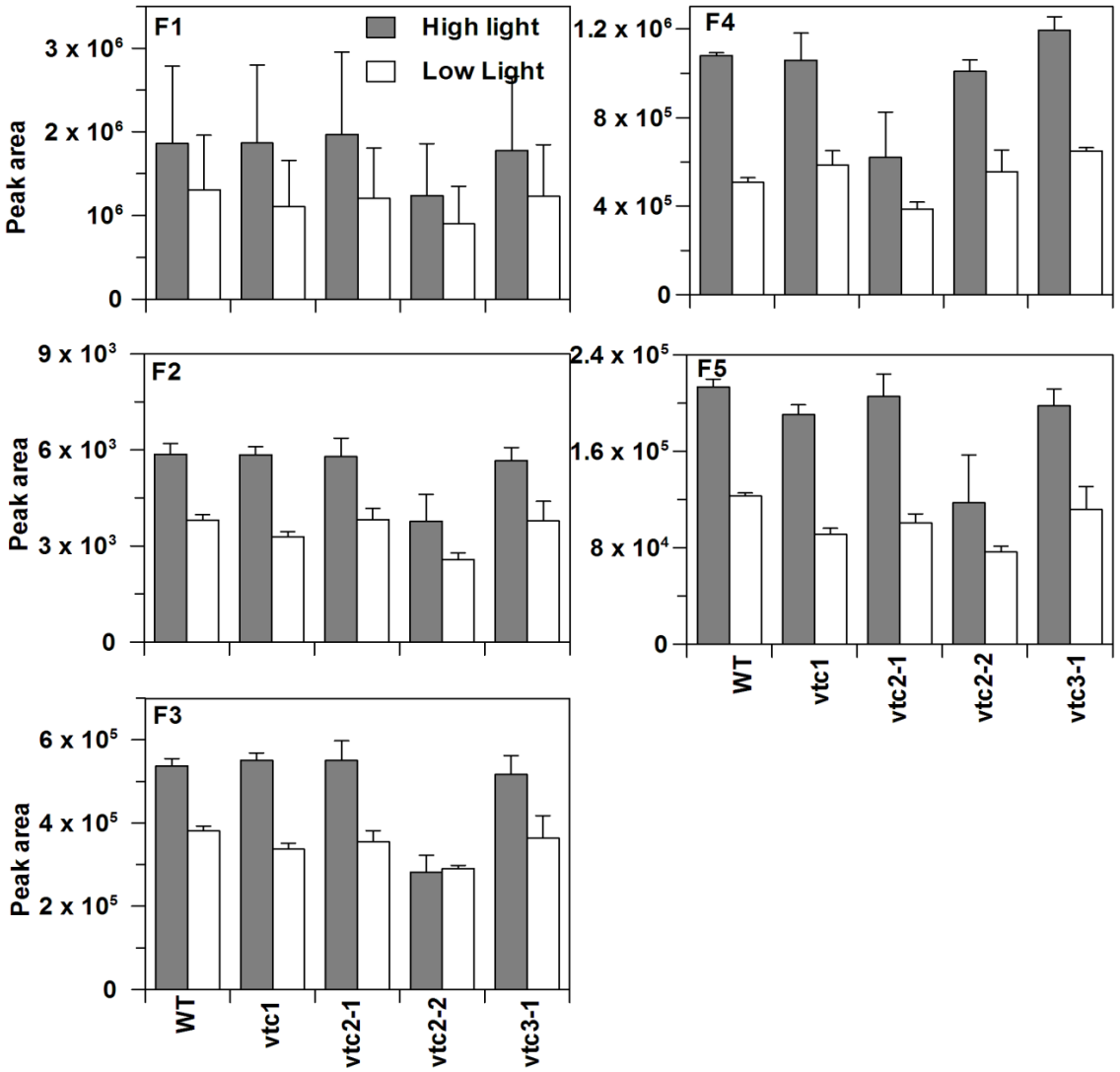


Figure 4.14 Foliar flavonol glycosides profiles (F1-F9) of *Arabidopsis thaliana* WT and the *vtc* mutants acclimated to HL and LL. Light intensities used are: HL; 550-650 µmol m^{-2} s^{-1} and LL; 100 µmol m^{-2} s^{-1}. Favonol glycosides F1-F5 (see Table 4.1B were quantified by LC-ESI-QQQ MS/MS. Data from three independent experiments are shown in graphs (A) Experiment 1 (B) Experiment 2 (C) Experiment 3. The data were quantified using peak area. All results were expressed as mean values + standard error (n = 3) where, 'n' represent number of biological replicates. Analyses were carried out using one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered statistically significant.

4. 6 Discussion

4.6. 1 AsA deficiency effect on hormone regulation

It has previously been reported that the foliar AsA pool increases in *Arabidopsis thaliana* when exposed to HL (Smirnoff, 2000; Dowdle *et al*., 2007; Bartoli *et al*., 2006). As AsA is required in different biosynthesis pathways and is also an important antioxidant, AsA deficiency might affect the accumulation of different metabolites in response to abiotic stresses. For example, AsA is involved in the biosynthesis pathways of ABA and flavonoids (discussed later in section 4.6.2). The multiple functions of ascorbate and hormones as summarised in the current study suggested hormonal regulation may be affected by ascorbate-deficiency and other stresses.

Therefore, the *Arabidopsis thaliana vtc* mutants were used to study the role of AsA in response to abiotic stresses and its deficiency effect on hormonal regulation. In the current study, the response of ABA, JA, SA and SA glycoside in the *vtc* mutants against different abiotic stresses such as HL, wounding and salinity, was investigated. ABA level was moderately increased after HL, wounding and salt treatment in all strains. Overall, in all independent experiments and treatments, no consistent significant differences were observed in stressed and control plants, but the pattern of ABA accumulation in response to different stresses (HL, wounding and salt) corresponded to previous reported studies (Galvez-Valdivieso *et al*., 2009; Kato-Noguchi, 2001). Previously, an increased level of ABA was observed in HL in combination with low humidity in *Arabidopsis thaliana* plants (Galvez-Valdivieso *et al*., 2009). Accumulation of ABA was also reported in response to wounding in maize and lettuce (Kato-Noguchi, 2001). ABA deficient *flacca* mutants (*flc*) demonstrated

that ABA is essential for the induction of systemin (a plant peptide hormone involved in wound response) and wound-induced genes in tomato (Chao *et al*., 1999). Salt treated plants were fed with exogenous ABA which was found to alleviate the salt stress by the activation of specific enzymes, which regulate RNA and protein synthesis (Jia *et al*., 2009; Zhu *et al*., 2010). Similarly, low expression of genes induced by salinity in ABA deficient mutants (*aba2-1* and *aba3-1*) and an ABA insensitive mutant, *abi1-1* suggested ABA is an important regulator in the expression of these genes in response to salinity (Shi and Zhu, 2002). There was evidence that ABA hypersensitive *ELONGATOR* mutants had increased expression of genes involved in the anthocyanin biosynthesis pathway and accumulate increased levels of anthocyanins (Zhou *et al*., 2009). As *vtc* mutants produced a low level of anthocyanin accumulation, it might affect the ABA accumulation in the *vtc* mutants (Page *et al*., 2011). Interestingly, we observed there was no significant difference in ABA accumulation in the WT and the *vtc* mutants, although, 9-cis-epoxicarotenoid dioxygenase (NCED), an important enzyme of the ABA biosynthesis pathway, requires ascorbate as a cofactor (Arrigoni and De Tullio, 2000). Current results also contradicted the previous work, which reported a high concentration of ABA in *vtc* mutants compared to the WT in response to stress and control conditions (Pastori *et al*., 2003; Conklin and Barth, 2004; Kerchev *et al*., 2011). In contrast to previous literature, after extensive analysis in the current study, we found slightly higher ABA in the WT than the *vtc* mutants in all stressed and control experiments.

Similarly, JA slightly increased in HL and with salt treatment but dramatically increased in response to wounding. These results were similar to those reported in

earlier studies (Delker *et al.*, 2006; Stenzel *et al.*, 2003) and suggested that wounding triggers JA biosynthesis. As previously reported, JA and methyl JA are wound responsive and are also involved in the *de novo* biosynthesis of ascorbate, so, it was not clear whether ascorbate-deficiency affected JA accumulation in the *vtc* mutants (Wolucka *et al.*, 2005b; Shan and Liang, 2010). Similarly, JA was reported as an important signalling molecule during wounding and pathogen infection. The *fad3*, *fad7* and *fad8* mutants of *Arabidopsis* were susceptible to necrotrophic fungal pathogens, and the feeding of JA protected the mutants by reducing the level of disease, and revealed the role of JA in plant defence against necrotrophic fungal pathogens (Browse, 2009; Vijayan *et al.*, 1998). The *vtc* mutants were also resistant to pathogens, possibly due to altered JA accumulation in response to wounding and other stresses. In the current study, there was no clear difference in JA accumulation between WT and the *vtc* mutants in all stress treatments.

There was variation between independent experiments in SA and SA glycoside accumulation in response HL and wounding. Overall, no significant differences in SA and SA glycoside accumulation were observed in response to these stresses. There are conflicting reports describing the integrated effects of SA and salinity. Gautum and Singh (2009) reported the foliar spraying of SA regulated growth and reduced lipid peroxidation by reducing the NaCl toxicity. Similarly, Karlidag *et al* (2009) suggested SA ameliorated the effect of salinity in strawberry plants while in contrast, Barba-Espin *et al* claimed SA negatively affected salt stress in pea (Karlidag *et al*; 2009; Barba-Espin *et al*; 2011). In the current study, we analysed the response of SA and SA gly to salinity, which revealed that both SA and SA glycosides showed a

negative response in accumulation in the WT and the *vtc* mutants after salt treatment. SA and SA glycosides were significantly higher in the *vtc* mutants than the WT, except *vtc 3-1,* in all stress treatments. Previously, it has been shown that *vtc* mutants exhibit constitutive priming, which enhances the resistance against pathogens by promoting the biosynthesis of SA and PR proteins (Loake and Grant, 2007; Mukherjee *et al.*, 2010). It was also demonstrated that AsA deficiency promotes expression of genes in the SA signalling pathway, and that premature senescence contributes to this enhanced pathogen resistance (Barth *et al.*, 2004; Mukherjee *et al.*, 2010).

In summary, we undertook the extensive analysis of hormones (ABA, SA, JA and SA glycosides) in response to HL, wounding and salinity in the *vtc* mutants. SA and SA glycoside showed the same behaviour in accumulation as previously reported but the unchanged ABA and JA levels showed that AsA status does not affect the accumulation of these hormones, but does not rule out decreased sensitivity. The overall data showed ascorbate deficiency had no significant effects on the accumulation of hormones even though some previous studies suggested that ABA levels are increased in the *vtc* mutants.

4.6. 2 Flavonoid accumulation regulated by high light and ascorbate deficiency

Anthocyanins have received considerable attention in the recent literature due to their proposed role as light-attenuators in leaves vulnerable to high light stress (Neill and Gould, 1999; Tohge *et al.*, 2005; Bloor and Abrahams, 2002; Doodeman *et al.*,

1982; Jiang *et al.*, 2007; Martens *et al.*, 2010; You *et al.*, 2011). Many flavonol derivatives were detected in different *Arabidopsis thaliana* (WT) tissue (Yonekura-Sakakibara *et al.*, 2008). Collective evidence from many studies has shown that plants respond to different stresses by accumulation of various phytochemicals. It was already determined that light intensity and plant age are the major factors which enhance the accumulation of these metabolites (Vanderauwera *et al.*, 2005). Other factors are also involved in the biosynthesis of these metabolites such as AsA, which is involved in maintaining the activity of many dioxygenases enzymes involved in the biosynthesis pathways of these metabolites (Martens *et al.*, 2010). The light responsiveness of AsA in *Arabidopsis* and other species is well-known (Dowdle *et al.*, 2007; Smirnoff, 2000). A time course study suggested that ascorbate accumulation responded to long exposure of plants to high light intensity (Page *et al.*, 2011). The physiological response of the *vtc* mutant plants to high light (500-1800 μmol $m^{-2}s^{-1}$) has been analysed in previous studies (Giacomelli *et al.*, 2006; Muller-Moule, *et al.*, 2002; Muller-Moule, *et al.*, 2004; Muller-Moule, *et al.*, 2003). Decreased levels of anthocyanin have also been observed in the *vtc* mutants (Giacomelli *et al.*, 2006; Wilmouth *et al.*, 2002). To find out the importance of ascorbic acid in the biosynthesis of these phenolics (anthocyanin and flavonols), *vtc* mutants were exposed to increased light intensity. The anthocyanin accumulated in WT plants acclimated to HL. Accumulation was greatly decreased in the *vtc* mutants and showed close correspondence to each other. In contrast to anthocyanin, the kaempferol glycosides tended to increase slightly in HL in all strains although the response was not significant. In strong contrast to the anthocyanin, the levels of kaempferol glycosides

were very similar in the WT and the *vtc* mutants. Thus, the repression of anthocyanin accumulation in low ascorbate *vtc* mutants compared to wild type likely resulted from the restricted availability of reduced ascorbate.

Anthocyanins and flavonol glycosides act as UV-B screens, and are synthesised by ascorbate dependent dioxygenases, flavanone-3-hydroxylase (F3H), flavonol synthase (FLS) and leucoanthocyanidin dioxygenase (LDOX). F3H is involved in the basic synthesis pathway for both anthocyanin and flavonol glycosides. FLS is exclusively involved in flavonol glycoside synthesis. LDOX is solely in the anthocyanin biosynthesis pathway. As previously discussed all these dioxygenases need AsA for efficient activity (Martens *et al.*, 2010). Flavonol glycosides were not affected in the *vtc* mutants, although, F3H and FLS both showed low gene expression (Page *et al.*, 2011). Further investigations are required to investigate the relationship of ascorbate and flavonol glycosides. The difference between flavonols and anthocyanins could be related to their rate of synthesis and breakdown. In addition, flavonol glycosides may be more stable than anthocyanins, so, possibly a low synthesis rate can maintain the pool in HL. The specific accumulation of anthocyanins suggested that they have specific properties over flavonol glycosides when acclimated from LL to HL (Gould *et al.*, 2010). The low expression of anthocyanin biosynthesis and their regulatory genes in *vtc* mutants is also a clue that anthocyanin biosynthesis is dependent on AsA and the decreased expression of these genes in *vtc* mutants is primarily caused by the lesion in the processes involved in the sensing or signalling pathways (Page *et al.*, 2011).

These results suggested the deficiency of ascorbate results in the down-regulation of anthocyanin biosynthesis in response to HL, but no effect was found on the synthesis of kaempferol glycoside. Additionally, a strong correlation between AsA and anthocyanin was also observed across six different ecotypes of Arabidopsis (Col-0, GOT1, HR5, Is-0, NFE1 and Old-2) after HL acclimation. These have natural variations in ascorbate contents. The ecotype contained low AsA accumulated low anthocyanins. AsA and anthocyanin showed very close correspondence in accumulation after HL treatment (Page *et al.*, 2011). The transcript level of the anthocyanin-related genes showed the relatively low expression of genes in the *vtc* mutants than the WT (Page *et al.*, 2011). The total AsA and anthocyanin showed a strong correlation in accumulation in HL in the A*rabidopsis* WT.

Vanderauwera *et al* (2005) showed that the genes for the complete anthocyanin biosynthetic pathway are induced by HL, including the regulatory genes. All the known genes involved in the flavonoid biosynthesis were increased in HL. They also demonstrated that *Arabidopsis thaliana* plants with low catalase activity (7 % of WT), due to RNAi suppression were impaired in the anthocyanin biosynthesis which acclimated to HL. Catalase-deficient mutants showed the decreased expression of genes involved in flavonoid biosynthesis pathways (Vanderauwera *et al.*, 2005). There is a possibility that increased level of H_2O_2 in catalase-deficient mutants suppressed the biosynthesis of anthocyanin (Martens *et al.*, 2010; Vanderauwera *et al.*, 2005). Similarly, Xiang *et al* (2001) reported glutathione-deficient mutants, antisense suppression in γ- EC synthetase, produced low anthocyanin in HL, they concluded the decreased anthocyanin accumulation in glutathione deficient mutants

is because glutathione is required in transportation of anthocyanin to the vacuole. Queval *et al* (2007) demonstrated that catalase deficiency affects the accumulation of glutathione and redox state. Interestingly catalase deficiency decreases ascorbate concentration. So, it is possible that the decreased ability of catalase mutants to accumulate anthocyanin (Vanderauwera *et al.*, 2005) is actually caused by low ascorbate. From the *vtc* mutants combined with evidence from catalase mutants affecting level of H_2O_2, which are impaired in anthocyanin accumulation (Vanderauwera *et al.*, 2005), it appears that the early signalling events of anthocyanin synthesis are redox sensitive.

4. 7 Conclusions

Slight differences in hormone accumulation in the WT and *vtc* mutants in some experiments suggested ascorbate deficiency does not have a significant effect on the ABA and JA accumulation, but these hormones are the important defence molecules in biotic and abiotic stresses. In the case of SA and SA glycoside, the levels are higher in the *vtc* mutants compare to the WT, which might be because of insufficient APX-mediated removal of H_2O_2 due to ascorbate deficiency which leads to the SA biosynthesis (Mukherjee *et al.*, 2010).

Anthocyanins are lower concentration in the *vtc* mutants than the WT suggesting that ascorbate plays an important role in anthocyanin accumulation. 2-oxoglutarate-dependent dioxygenase enzymes are involved in the anthocyanin biosynthesis might need ascorbate for their efficient activity. This decreased dioxygenase activity could contribute to impaired anthocyanin accumulation. Secondly, if the presence of

ascorbate protects the anthocyanin in the vacuole from oxidation, ascorbate deficiency may lead to degradation and therefore lower levels in *vtc* mutants. However, the current evidence suggests that the transcriptional regulation may be the most important factor because the expression of genes encoding anthocyanin biosynthesis genes and transcription factors were lower in *vtc1* and *vtc2* (Page *et al.*, 2011). The metabolomic data in *vtc* mutants in response to HL established that anthocyanin synthesis is ascorbate and irradiance-dependent, but the complete mechanism is still not understood. It was confirmed that flavonol glycoside biosynthesis is not dependent on ascorbate.

CHAPTER 5

IDENTIFICATION OF CELL WALL PROTEINS IN *ARABIDOPSIS THALIANA VTC* MUTANTS

Chapter 5: Identification of cell wall proteins in *Arabidopsis thaliana vtc* mutants

5.1 Introduction

The plant cell wall is an important part of the cell which plays structural as well as functional roles. The cell wall of higher plants is a dynamic structure. It is essential for growth, expansion, cell division, signalling and cell to cell communication (Ellis *et al.*, 2002; Vogel *et al.*, 2004). The plant cell wall is composed of polysaccharides, proteins and lignin. Polysaccharides contribute 90-95 % of the cell wall composition and the remaining 5-10 % is composed of proteins and other small molecules (Cassab and Varner; 1988, Carpita and Gibeaut, 1993). The cell wall structure depends on the arrangements of polysaccharides and proteins. The polysaccharides found in the cell wall are cellulose, hemicellulose and pectin, which interact to form a dense interwoven network (Jamet *et al.*, 2008). Cellulose, hemicellulose and cell wall structural proteins such as extensin constitute a network embedded in a pectin matrix. Apart from cellulose which is synthesised at the plasma membrane, all other polysaccharides are synthesised in Golgi bodies and transported to the cell wall (Sandhu *et al.*, 2009). The plant cell wall plays a crucial role during developmental stages of cell division, enlargement and differentiation. Cell wall proteins contribute to the structure as well as the function of the cell wall. They are involved in defence against biotic and abiotic stresses. Therefore, the cell wall produces a barrier between plant cells and adverse environmental conditions such as pathogens and other harmful factors (Jamet *et al.*, 2006; Lee *et al.*, 2004; Huckelhoven, 2007).

The extraprotoplasmic matrix of plant cells is composed of all the compartments present outside the external face of the plasma membrane is termed the 'apoplast'. The plant apoplast is a modulator of communication between the symplast and the external environment (Figure 5.1). The apoplast is important in transport of small molecules and contains inorganic ions, amino acids and sugars (Tejera *et al.*, 2006; Song *et al.*, 2006). It is also the site which contributes to defence from external stimuli (biotic and abiotic) by activating the antioxidant system (Bolwell *et al.*, 2002; Sattelmacher, 2001).

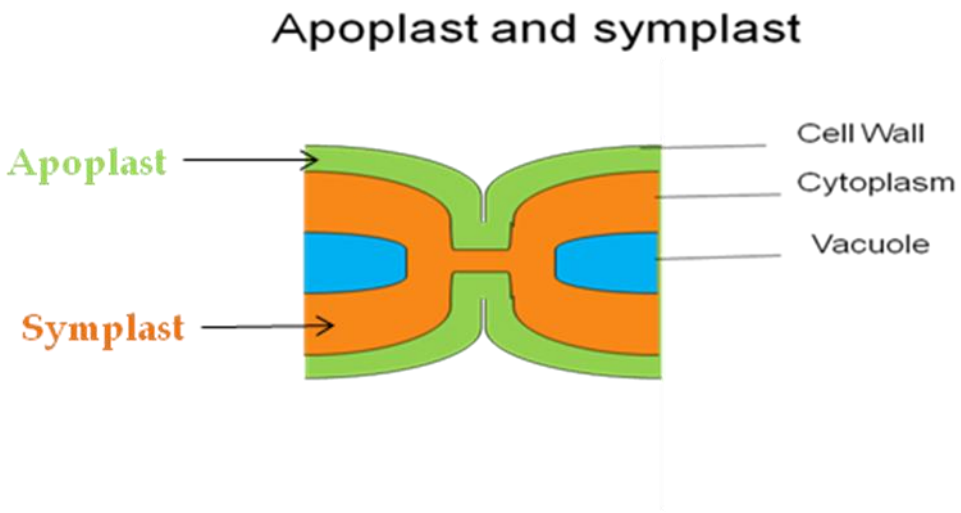


Figure 5.1 Apoplast and symplast in-between the cells. Each represented with different colours.

5. 2 Cell wall proteins (CWPs)

The plant extracellular matrix contains many structural as well as functional proteins. These proteins are linked to sugars and are called glycoproteins. Most of these proteins are secretory; some are enzymes while others have unknown functions. The nine main types of proteins identified so far, have been identified on the basis of their predicted functions (Jamet *et al.*, 2008). These proteins are classified as: (1) proteins acting on the cell wall polysaccharides, (2) oxido-reductases (3) proteases (4) proteins having domains interacting with other proteins or polysaccharides (5) structural proteins (6) proteins involved in signalling (7) proteins related to lipid metabolism (8) miscellaneous (9) unknown function. Structurally, these proteins vary on the basis of amino acid richness in the peptide sequence or networking with sugars moieties. The most abundant proteins found in the plant cell wall are hydroxyproline-rich glycoproteins (HRGPs) and the glycine-rich glycoproteins (GRPs), leucine rich proteins (LRP) are also reported. The hydroxyproline residues of HRGPs are formed by post-translational hydroxylation of proline using ascorbate-dependent peptidyl-prolyl hydroxylase (Qian *et al*; 1993) and so they could be affected by ascorbate deficiency.

CWPs were categorised into three categories on the basis of interaction with the cell wall (Jamet *et al.*, 2006). They are labile, weakly and strongly bound to the cell wall. (a) Labile proteins have no bonding with the cell wall so are soluble and freely moveable in the apoplast. These proteins can be easily extracted by vacuum infiltration. In a destructive method of extraction, it is possible to incur losses due to successive washing steps. (b) Weakly bound to the cell wall with hydrogen bonding

or Van der Waals forces. These proteins can be extracted using salts or chelating agents (c) Strongly bound proteins are cross-linked to the cell wall with covalent or ionic bonding. They are embedded in the membrane and cannot be easily removed with low concentrations of salt (Brady *et al.*, 1996; Jamet *et al.*, 2006). The cell wall contains both enzymatic and non-enzymatic proteins. The non-enzymatic proteins are mostly involved in the structural configuration of the cell wall. The enzymatic proteins are involved in controlling the cell wall assembly and remodeling during growth, development and altered environmental conditions (Passardi *et al.*, 2004b).

5. 3 Post translational modifications

Cell wall proteins are secretory proteins, synthesised in the endoplasmic reticulum (ER) and Golgi bodies and transported to the cell wall (Rose and Lee, 2010). The post-translational modifications of CWPs are crucial in understanding their structure and biological behaviour. Glycosylation is the most common modification and occurs during transportation where proteins reinforce networking with polysaccharides in the ER and Golgi bodies (Spiro, 2002; Zachara and Hart, 2004). They are either N-glycosylated or O-glycosylated.

N-glycosylation occurs as soon as the nascent secretory proteins enter the ER by transferring an oligosaccharide precursor from a lipid carrier on to a specific Asn residue. This Asn residue belongs to an N-glycosylation consensus sequence Asn-X-Ser/Thr in which X can be any amino acid except Pro or Asp (Faye *et al.*, 2005). N-glycosylated proteins are identified and isolated by affinity chromatography using

the lectin concanavalin A (ConA) (Minic *et al.*, 2007). Pattison and Amtmann (2009) reported that N-glycosylation is involved in cell wall deposition and development, growth, seed development, protein quality, salt tolerance and interaction of pathogens by the induction of pathogen-related proteins. Ionically-bound cell wall peroxidases are a common example of N-glycosylated proteins (Lige *et al.*, 2001).

Unlike N-glycosylation, **O-glycosylation** occurs at Ser/Thr or Hyp residues, which is specific to plant species. The well-known O-glycosylated proteins are HRGPs such as extensin and arabinogalactan proteins (AGPs) (Faye *et al.*, 2005; Kieliszewski, 2001). Only a limited number of studies have been done on CWPs and glycosylation in plants. Glycosylation enhances the activity and stability of proteins by protecting them from unfolding (Lige *et al.*, 2001). It was recently proposed that N-glycosylation motifs on cell wall proteins are crucial in host-pathogen-interactions (Lim *et al.*, 2009). O-glycosylation is also involved in keeping extensin (structural protein) in a rigid extended configuration, and it can also protect proteins from proteolytic degradation (Seifert and Roberts, 2007).

Unfortunately, no single method is able to isolate all CWPs from a complex mixture which contains contamination from intracellular proteins. Zhang *et al* (2010) described a method using different strategies for the separation of a significant number of proteins from the cell wall differentiated by post translational modification (Zhang *et al.*, 2010). Affinity chromatography is a wide spread method for separating proteins. For example, *Concanavali ensiformis* (ConA) was used to isolate N-glycosylated proteins (Minic *et al*; 2007). O-glycosylated proteins are

specifically recognised by β-Yariv reagent through the antigen-antibody interaction mechanism (Schultz *et al.*, 2004).

5. 4 Peroxidases

Peroxidases are a diverse group of proteins present in animals, plant and microorganisms. They are categorised into three classes: I, II and III on the basis of their primary structure. Class I peroxidases comprises 3 distinct groups (a) plant cytosolic and chloroplastic ascorbate peroxidase, (b) mitochondrial and yeast cytochrome C peroxidases and (c) catalase-peroxidases (Teixeira *et al.*, 2004; Jespersen *et al.*, 1997; Zamocky, 2004; Asada, 1992; Obinger *et al.*, 1999). They constitute a small family of 7-10 genes encoding both soluble and insoluble (membrane bound) peroxidases, which are involved in scavenging H_2O_2. Class II peroxidases are a small group of fungal peroxidases, generally extracellular, called lignin peroxidases and they are involved in secondary metabolism and the degradation of lignin (Martinez *et al.*, 2005; Piontek *et al.*, 2001).

Class III are classical plant peroxidases also called guaiacol peroxidases (EC 1.11.1.7). They belong to a multigene family. For example, 138 genes were identified for peroxidase III in rice (Passardi *et al.*, 2004a) and the analysis of the *Arabidopsis* genome revealed 73 full-length genes, two psuedogenes and 6 fragments spread evenly across 5 chromosomes (Tognolli *et al.*, 2002b; Oliva *et al.*, 2009; Koua *et al.*, 2009; Passardi *et al.*, 2007b; Bakalovic *et al.*, 2006; Passardi *et al.*, 2007a). They are mainly found in the apoplast, vacuole and ionically-bound to the cell wall (Takabe *et al.*, 2001; Tognolli *et al.*, 2002a). Class III peroxidases are N-glycosylated proteins, which possess a signal peptide which targets the secretory pathway.

Class III peroxidases play many roles in the cell. They are involved in redox reactions using H_2O_2 as an electron acceptor and various phenolic compounds as electron donors (Oliva *et al.*, 2009). These are also involved in the 'oxidative burst' because they also generate H_2O_2. Antisense plants which possess low peroxidase activity are sensitive to pathogen stress and showed decreased levels of apoplastic H_2O_2 (Bindschedler *et al.*, 2006). They may be involved in plant growth and development by hormonal regulation and also cell wall rigidification and lignification (Welinder *et al.*, 2002; Tognolli *et al.*, 2002a; Hiraga *et al.*, 2001). They are the important components of the plant antioxidant system. Generally, peroxidase first reacts with H_2O_2 to yield a highly oxidising intermediate, which reacts with an organic or inorganic substrate (guaicol, pyrogallol and ascorbate) to produce a product (Welinder *et al.*, 2002).

The aim of this study was to investigate the effect of ascorbate deficiency on the cell wall proteome of the *Arabidopsis thaliana*. This chapter describes the cell wall peroxidase activity and proteomic variation between *Arabidopsis thaliana* WT and the ascorbate deficient mutants (*vtc1*, *vtc2-1*, *vtc2-2*, *and vtc3-1*).

5. 5 Results

In the current study, ionically bound cell wall peroxidases and other cell wall proteins, which can be affected by ascorbate deficiency in HL acclimated *Arabidopsis thaliana vtc* mutants as described in Chapter 2 were analysed. Peroxidase activity was measured by hydrogen-peroxide-dependent pyrogallol. ConA chromatography was used for the separation of N-glycosylated proteins and analysis, and identification was performed by nano-LC-MS with a combination of computational approaches described in section 5.5.2.1.

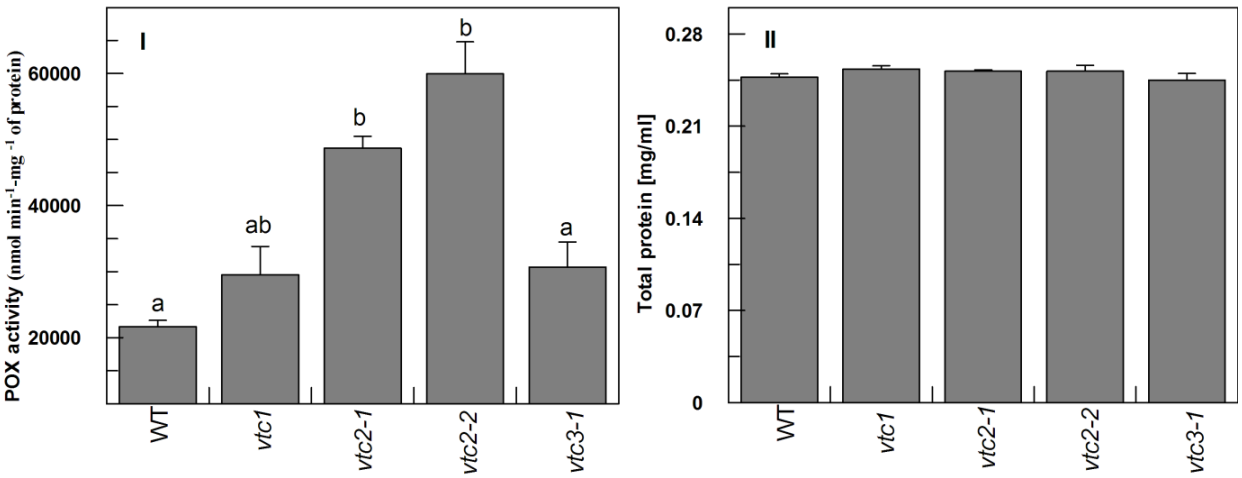
5.5. 1 Peroxidase activity and total protein contents

Peroxidase showed significantly higher activity in the *vtc* mutants compared to the WT under normal growth conditions. The *vtc1* and *vtc3-1* mutant activities were higher than the WT and lower than the *vtc2* mutant. Peroxidase activities are shown in Figure 5.2 A & B, Graph I. There was a negative correlation between ascorbate and cell wall peroxidase activity across all strains as described earlier (Colville and Smirnoff, 2008). Peroxidase activity was increased in HL acclimated plants compared to LL, but the significant differences were not consistent between independent experiments (Figure 5.2 C-D, Graph I).

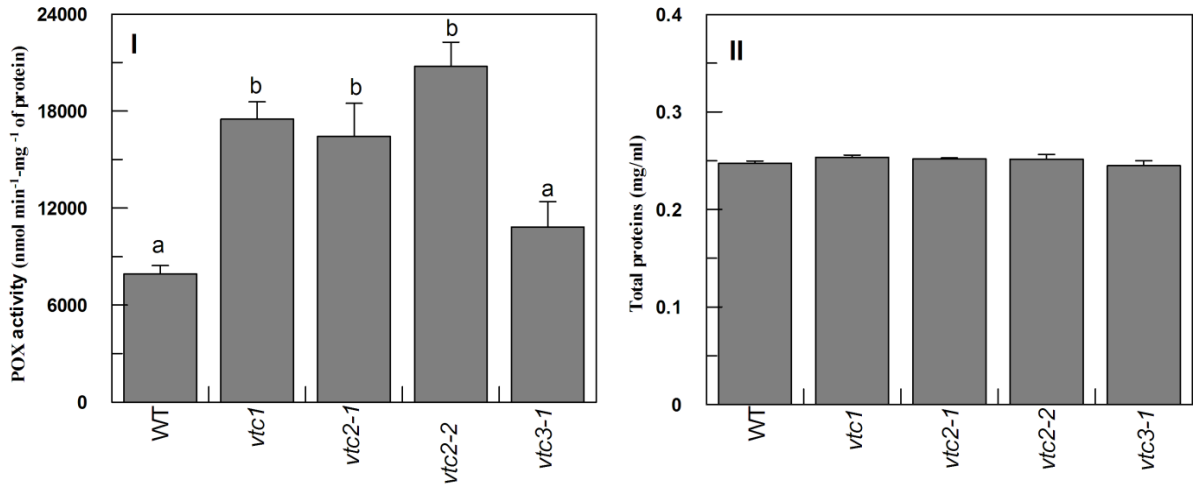
Total protein concentration was measured against a standard curve of γ-globulin protein by Bradford assay (Bradford, 1976) (Figure 5.2 C-D, Graph II). Total protein contents were similar across all strains under normal growth conditions shown in Figure 5.2 A-B, Graph II. A slight increase in total protein contents was observed

when acclimated from LL to HL with no significant differences across the strains (Figure 5.2 C-D). Concentrations ranged over 0.25-0.26 mg/ml in HL and 0.23-0.24 mg/ml in LL in experiment 1 and 0.29-0.33 mg/ml in HL and 0.27- 0.29 mg/ml in LL for experiment 2. However, there was no significant difference between WT and *vtc* mutants.

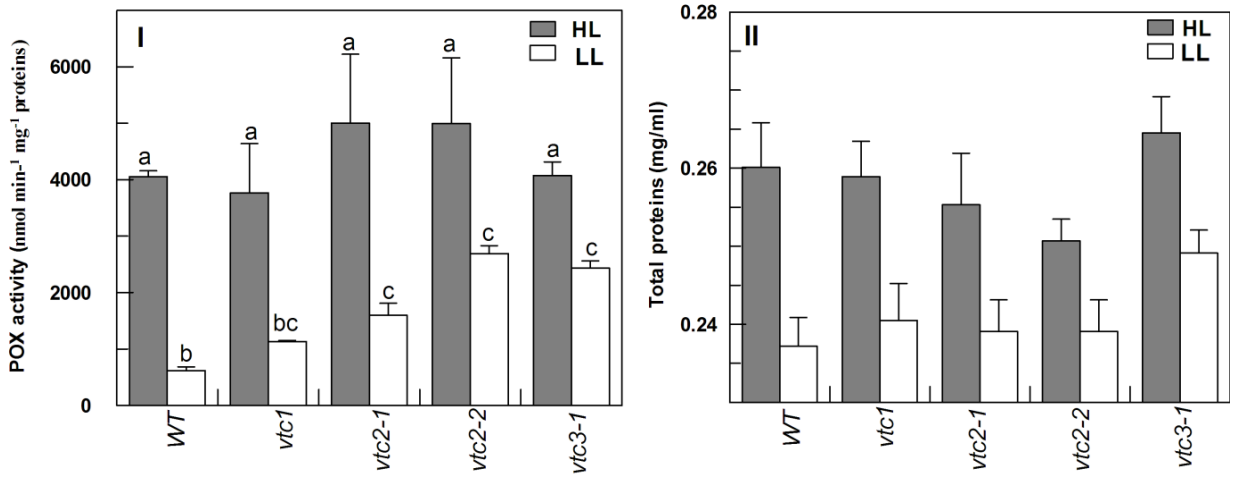
A



B


I
POX activity (nmol min-1-mg -1 of protein)
24000
18000
12000
6000
0
a
b
b
b
a
WT
vtc1
vtc2-1
vtc2-2
vtc3-1
II
Total proteins (mg/ml)
0.4
0.3
0.2
0.1
0
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

C


I
HL
LL
POX activity (nmol min-1 mg-1 proteins)
6000
4000
2000
0
a
b
a
bc
a
c
a
c
a
c
WT
vtc1
vtc2-1
vtc2-2
vtc3-1
II
HL
LL
Total proteins (mg/ml)
0.28
0.26
0.24
WT
vtc1
vtc2-1
vtc2-2
vtc3-1

D

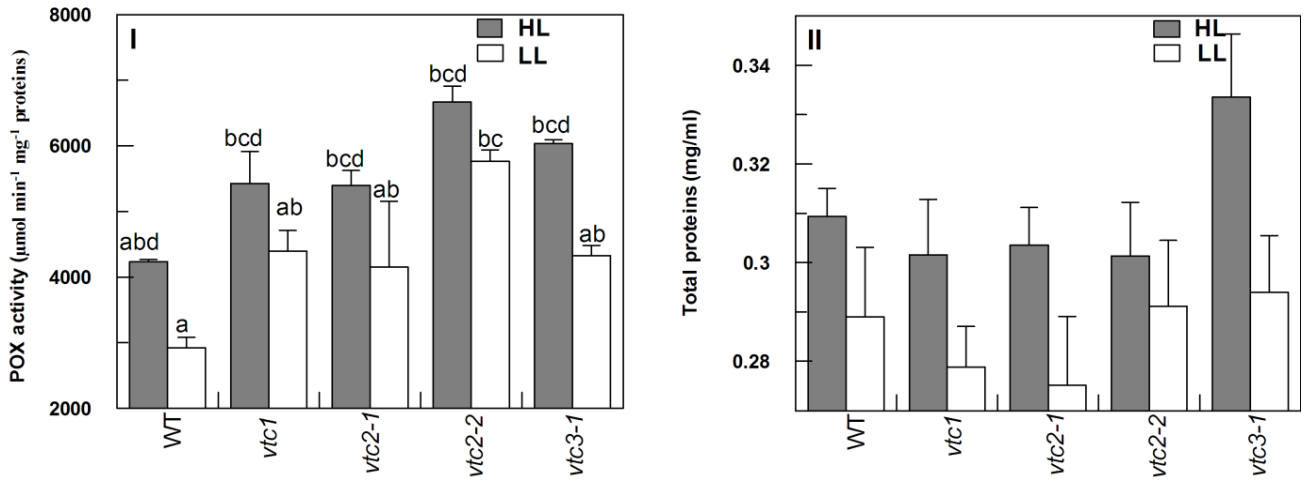


Figure 5. 2 Total protein contents and ionically-bound cell wall peroxidase activity. (A) Experiment 1 under normal growth conditions (B) Experiment 2 under normal growth conditions (C-D) Plants acclimated to HL (550-650 µmol m^{-2} s^{-1}) and LL (100 µmol m^{-2} s^{-1}) for 4 d. The total protein contents were quantified using a standard curve of γ-globulin (1.5 mg / ml stock) and subjected to analysis of variance to identify the statistical differences between treatment and strains. Peroxidase activity was normalised to total protein contents. All results were expressed as mean values + standard error (n = 3) where, 'n' represents the number of biological replicates. Analysis was carried out using one-way analysis of variance (ANOVA). Values at $p < 0.05$ were considered statistically significant. The significant differences were labelled with lower case letters. Columns labelled 'a' are significantly different from the corresponding columns labelled with letter 'b'. Columns labelled with 'b' are significantly different from columns labelled with 'c'.

5.5. 2 Identification of cell wall proteins by LC-MS

The difference in peroxidase activity between the WT and the *vtc* mutants prompted further investigation to identify which of the 73 peroxidases present in *Arabidopsis* are different. It would also be of interest to identify other proteins affected in the *vtc* mutants. HL treated samples were extracted with $CaCl_2$ and separated using ConA chromatography (described in Chapter 2) and analysed by nano-LC-MS in both centroid and profile format and identified with a variety of bioinformatic tools. A schematic of all the steps involved from extraction to identification is shown in Figure 5.3.

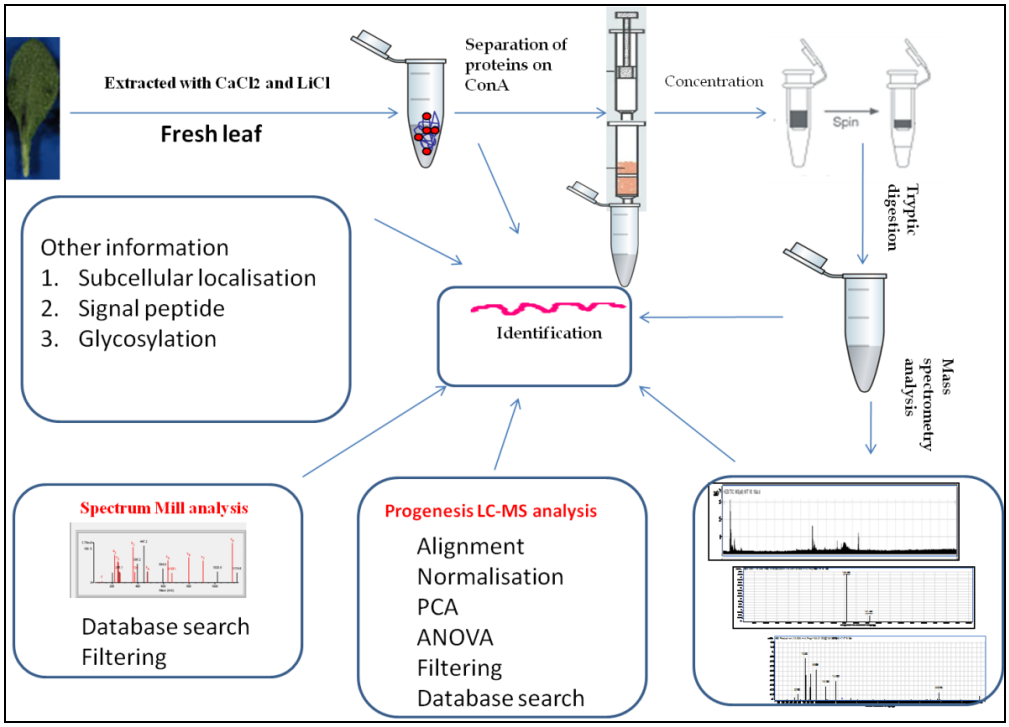


Figure 5.3 A schematic to show the extraction, isolation and analysis of proteins. Clockwise from top left: Extraction, Isolation on ConA chromatography, Concentration on mini spin concentrator, Tryptic digestion, LC-MS analysis, Progenesis LC-MS analysis, Spectrum Mill database search and other information.

5.5.2.1 Bioinformatics analysis

Classical approaches such as immunological assays are not necessarily efficient techniques for the prediction of protein structures and identifications due to the high homology in amino acid sequences. Mass spectrometry-based protein analysis is a fundamental technique in proteomic studies for the putative or absolute identification of known and unknown proteins. The selection of bioinformatic tools is critical because they are not only necessary to process the mass spectrometry data

but it is also necessary to identify the proteins and characterise their sub-cellular localisation (Clemente *et al.*, 2009).

The raw MS/MS data files were processed using the Spectrum Mill MS Proteomics Workbench (Agilent Technologies). Data extraction, MS/MS search and protein peptide summary results were performed with the following settings (**a**) Data Extraction: fixed modification carbamidomethylation, $(M+H)^+$ 300 – 4000, sequence tag length > 1, minimum MS (S/N) 25 **(b)** MS/MS search: database search TAIR 10 *Arabidopsis thaliana*, Tryptic digestion, maximum missed cleavages 2, fixed modifications: Carbamidomethylation (C), variable modifications: acetylation (K), oxidising methionine (M), pyroglutamic acid (N-term Q), Deaminated (N), N-acetylglucosamine (S), minimum scored peak intensity 50%, the precursor mass tolerance $\pm$ 20 ppm, product mass tolerance $\pm$ 50 ppm, maximum ambiguous precursor charge +2 to +8 **(c)** Protein/ peptide summary: Peptide filter score 6 and % SPI > 60, protein filter score > 11.

General parameters considered useful for protein identification are: % amino acid coverage above 20 % although this is dependent on protein size and 2 distinct peptides are considered to be a minimal requirement for accepting a hit. Similarly, the protein and peptide score and % of score peptide intensity (% SPI) are important attributes as an indication of certainty, an SPI lower than 60% is questionable, as is a peptide score $\leq$ 6 for further qualification. If these values are exceeded, this indicates well assigned fragmentation of the peptide sequence with minimal mass drift from the expected values. The protein score is the sum of the peptide scores. The stringent conditions for these scores should be applied in order to reduce the risks of false-

positive identifications (Personal communication with Hannah Florance and the Spectrum Mill manual). A limitation of Spectrum Mill is its inability to make multiple sample comparisons and perform statistical analysis. Therefore, Progenesis LC-MS, an LC-MS/MS features extraction and a statistical package (described in Chapter 3) was used to analyse multiple samples. Progenesis LC-MS allowed visualisation of complex data (multiple samples) by aligning, normalising and comparing between different set of data. It also allowed identification of these proteins by searching against an NCBI database in Mascot or exporting a suitable format for Spectrum Mill where it can be searched against the TAIR 10 database. The parameters used in Mascot were: fixed and variable modifications the same as Spectrum Mill, peptide tolerance was + 20 ppm and MS/MS tolerance was + 0.6 Da. Peptide charge states were +2, +3 and +4.

The information on subcellular localisation of proteins was obtained from information on **TAIR** (http://www.arabidopsis.org/) and **UniProtKB** (http://www.uniprot.org/). Furthermore, different tools were used to obtain useful information: **(1) Signal P** (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al.*, 2011, Emanuelsson *et al.*, 2007) was used to confirm the signal peptide **(2) Target P** (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson *et al.*, 2000), to check the presence of mitochondria or chloroplastic signal peptide; **(3) DictyOGlyc** (http://www.cbs.dtu.dk/services/DictyOGlyc/) (Gupta *et al.*, 1999) to predict O-(alpha)-GlcNAc glycosylation sites; **(5) NetOGlyc** (http://www.cbs.dtu.dk/services/NetOGlyc/) to predict O-GalNAc glycosylation

sites; **7) NetNGlyc** (http://www.cbs.dtu.dk/services/NetNGlyc/) (Gupta and Brunak, 2002) to predict N-Glycosylation sites.

5.5.2. 2 Protein identification

The LC-MS output for each individual sample (biological replicate) was processed using Spectrum Mill. Proteins which were consistently present in 3 biological replicates were selected for further analysis. All secretories, intracellular and undefined proteins identified in different samples are shown in Figure 5.4 and Table 5.1.

Table 5.1 Number of identified proteins from the cell wall extract of *Arabidopsis thaliana* WT and *vtc* mutants leaf tissue. Abbreviations used are: HL = High light, LL = Low light

Treatment	Strains	No of proteins			
		Total	Intracellular	Secretory	undefined
HL	WT	71	13	52	13
	vtc1	95	22	62	11
	vtc2-1	62	11	45	6
LL	WT	111	24	70	17
	vtc1	110	34	60	16
	vtc2-1	54	9	41	4

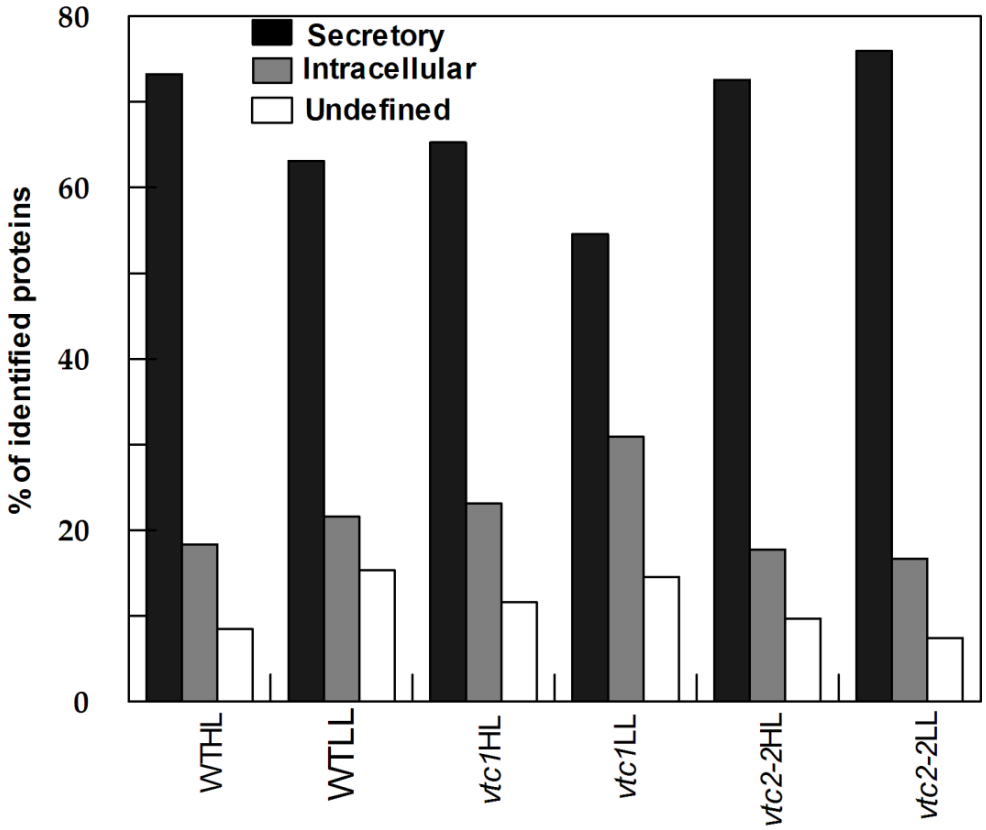


Figure 5. 4 Classification of proteins identified from the cell wall extract of foliar tissue of *Arabidopsis thaliana* WT and the *vtc* mutants. The proteins identified in each strain were isolated using ConA chromatography and analysed by nano LC-MS. Graph shows the % of each group of proteins identified from the extract.

5.5.2. 3 Secretory proteins and glycosylation

The WT and the *vtc 2* mutant centroid data from HL and LL were processed with Progenesis LC-MS and searched against the TAIR 10 database using the Spectrum Mill search engine. A total of 128 proteins were identified including 19 intracellular, 27 undefined and 82 secretory proteins present in both strains. The data were filtered on the basis of MS/MS < 2 spectra, % AA coverage < 4 and the number of distinct peptides < 4 being removed from the list. Finally, 94 proteins were considered as putatively identified of which 63 were secretory, 12 were intracellular and 18 were undefined (listed in Table 5.2-5.4). The secretory proteins contained signal peptides and are located in different cellular compartments such as the apoplast, cell wall, plasma membrane, endomembrane and vacuole (Table 5.3 and Figure 5.5). The identified secretory proteins belong to different functional classes described by Jamet *et al* (2008) such as oxidoredutases, proteases, proteins acting on polysaccharides, signalling proteins, interacting domains and proteins with unknown function. Predicted post-translational modifications showed that most of the secretory proteins were N-glycosylated, and some were also O-glycosylated listed in Table 5.3.

Table 5.2 Intracellular proteins identification from the ionically-bound cell wall protein fraction of *Arabidopsis thaliana* after ConA chromatography.

Accession no	Location	Putative function
AT1G20020	Chloroplast	Encodes a leaf-type ferredoxin:NADP(H) oxidoreductase
AT3G55800	Chloroplast	Encodes the chloroplast enzyme sedoheptulose-1,7-bisphosphatase (SBPase)
AT4G04640	Chloroplast	Encoding the gamma subunit of Arabidopsis chloroplast ATP synthase.
AT4G09650	Chloroplast	ATP synthase delta chain
AT4G24770	Chloroplast	encodes a chloroplast RNA-binding protein
AT5G66190	Chloroplast	Encodes a leaf-type ferredoxin:NADP(H) oxidoreductase
AT5G66570	Chloroplast	Encodes a protein which is an extrinsic subunit of photosystem II
AT2G06020	other (e.g. cytoplasm)	myb family transcription factor
AT3G06400	other (e.g. cytoplasm)	Encodes a SWI2/SNF2 chromatin remodeling protein
AT4G23670	other (e.g. cytoplasm)	major latex protein-related / MLP-related protein
AT5G20320	other (e.g. cytoplasm)	Encodes an RNase III-like enzyme
AT5G64330	other (e.g. cytoplasm)	Interacts with the blue light photoreceptor NPH1

Table 5.3 Proteins identified from the ionically-bound cell wall protein fraction of *Arabidopsis thaliana* after ConA chromatography. Predicted subcellular localisation and glycosylation pattern are shown. O- = O-glycosylation, N- = N-glycosylation, PM = plasma membrane, CW = cell wall, EM = endo membrane, ER = Endoplasmic reticulum

Accesion no	Glycosylation	Predicted location	Putative identification
AT4G01130	N-	ER	Acetylesterase, similar to GDSL-motif lipase/hydrolase family protein
AT4G25900	N & O	CW/CM	Aldose 1-epimerase family protein
AT3G47800	N	CW	Aldose 1-epimerase family protein
AT3G56310	N-	CW	Alpha-galactosidase/ melibiase / alpha-D-galactoside galactohydrolase
AT1G03220	N & O	CW	Aspartyl protease family protein
AT1G03230	N-	CW	Aspartyl protease family protein
AT1G09750	N & O	CW	Aspartyl-like protease protein
AT3G52500	N & O	CW	Aspartyl protease family protein
AT2G27060	N	PM	ATP binding /threonine kinase
AT1G65590	N & O	PM	beta-hexosaminidase
AT3G13790	N	CW	beta-fructofuranosidase
AT1G68560	N & O	CW/apoplast	Bifunctional alpha-l-arabinofuranosidase/beta-d-xylosidase

Table 5.3 continued....

Accesion no	Glycosylation	Location	Putative identification
AT1G56340	N-	ER	Calreticulin CRT1.
AT1G09210	N & O	ER	Calreticulin 2 (CRT2)
AT1G08450	N-	ER	Calreticulin CRT3.
AT4G01700	N	CW	Chitinase, putative
AT3G49110	N	CW	Class III peroxidase Perx33
AT3G49120	N	CW	Class III peroxidase Perx34
AT4G08770	N-	Apoplast	Class III peroxidase 37
AT4G08780	N-	CW	Class III peroxidase 38
AT1G71695	N-	Vacuole	Class III peroxidase 12 (PER12) (P12) (PRXR6)
AT4G21960	N	EM	Class III peroxidase 42
AT1G78820	N & O	Apoplast	Curculin-like (mannose-binding) lectin family protein
AT1G78830	N-	CW	Curculin-like (mannose-binding) lectin family protein
AT1G33590	N & O	CW	Disease resistance protein-related / LRR protein-related
AT1G21750	N-	ER/vacuole	Disulfide isomerase-like (PDIL) protein
AT1G77510	N & O	ER	Disulfide isomerase-like (PDIL) protein
AT2G06850	N & O	CW	Endoxyloglucan transferase (EXGT-A1)

Table 5.3 continued....

Accesion no	Glycosylation	Location	Putative identification
AT2G04780	N & O	PM	Fasciclin-like arabinogalactan-protein 7 (FLA7)
AT5G55730	N & O	CW	Fasciclin-like arabinogalactan-protein 1 (FLA1)
AT4G12730	N & O	PM	Fasciclin-like arabinogalactan-protein 2 (FLA2)
AT2G45470	N & O	CW	Fasciclin-like arabinogalactan protein 8 (FLA8)
AT3G60900	N & O	CW	Fasciclin-like arabinogalactan-protein 10 (FLA10)
AT1G72610	N-	CW	Germin-like protein (GLP1)
AT5G20630	N-	CW	Germin-like protein, suggesting some kind of circadian regulation
AT1G66970	N-	PM	Glycerophosphoryl diester phosphodiesterase family protein
AT5G20950	N & O	CW	Glycosyl hydrolase family 3 protein
AT3G07320	N	CW	Glycosyl hydrolase family 17 protein
AT4G23300	N	EM	Cysteine rich-repeat like Kinase family protein
AT5G48540	N & O	CW	Cysteine-rich repeat like kinase protein 55
AT1G28600	N-	CW	GDSL-motif lipase
AT3G14210	N-	CW	Lipase GDSL/A semidominant QTL which has an epistatic effect on the Epithiospecifier gene.
AT3G15356	N & O	CW	Legume lectin family protein
AT5G03350	N & O	CW	Legume lectin family protein
AT3G16530	N & O	CW/apoplast	Lectin like protein

Table 5.3 continued.....

Accesion no	Glycosylation	Location	Putative identification
AT1G33600	N-	CW	Leucine-rich repeat family protein
AT3G20820	N & O	CW	Leucine-rich repeat (LRR) family protein
AT5G12940	N & O	CW	Leucine-rich repeat (LRR) family protein
AT3G12145	N-	CW	Leucine-rich repeat protein
AT4G13340	N	CW	Leucine-rich extensin like protein
AT5G25980	N & O	Apoplast	Myrosinase (thioglucoside glucohydrolase)
AT3G10410	N-	Vacuole	Serine carboxypeptidase-like 49 (SCPL 49)
AT4G36195	N	CW	Serine carboxypeptidase S28 family protein
AT5G23210	N & O	CW	Serine carboxypeptidase 34 (SCPL34)
AT2G33530	N & O	CW	Serine carboxypeptidase-like 46 (SCPL 46)
AT1G17100	N-	PM	SOUL heme-binding family protein
AT1G78980	N-	TM	Strubbelig-receptor family 5 (SRF 5)
AT4G37800	N-	CW	Xyloglucosyl transferase 7 (XTH 7)
AT3G08030	N-	CW	Unknown protein
AT5G25460	N & O	CW	Unknown protein
AT5G11420	N & O	CW	Unknown protein
AT4G12420	N & O	CW	Unknown function (monocopper oxidase like protein SKU5)
AT4G32460	N & O	CW	Unknown protein

Table 5.4 Undefined proteins identification from the ionically-bound cell wall protein fraction of *Arabidopsis thaliana* after ConA chromatography. Predicted subcellular localisation and glycosylation pattern are shown. O- = O-glycosylation, N- = N-glycosylation, CW = Cell Wall.

Accession no	Glycosylation	Predicted location	Putative identification
AT1G01590	N	Membrane	Ferric-chelate reductase
AT1G27540	O	Unknown	F-box family protein
AT1G27580	no signal	Unknown	F-box family protein
AT1G49750	N	Chloroplast	Leucine-rich repeat family protein
AT1G53240	N	Mitochondria	Malate dehydrogenase (NAD)
AT1G72370	O	Cytosol, PM, chloroplast	Acidic protein associated to 40S ribosomal subunit of ribosomes.
AT2G47400	O	Chloroplast	CP12-1 encodes a small peptide found in the chloroplast stroma
AT3G14310	N	CW	Pectin methylesterase
AT3G15020	N	Mitochondria, apoplast	Malate dehydrogenase (NAD)
AT3G18070	N	Mitochondria, apoplast	Glycosyl hydrolase family 1 protein
AT3G18080	N	CW	Glycosyl hydrolase family 1 protein
AT3G25700	N	Chloroplast	Chloroplast nucleoid DNA-binding protein
AT3G44310	O	Apoplast	Nitrilase 1(NIT1) catalyzes the terminal activation step in indole-acetic acid biosynthesis
AT3G54400	N	CW	Aspartyl protease family protein
AT3G55440	N	Cytosol, CW	Triosephosphate isomerase activity.
AT4G05420	N	Nucleus	Structurally similar to damaged DNA binding proteins
AT5G05870	Unknown	Unknown	UDP-glucosyl transferase 76c1 (UGT76C1)
AT5G11660	no signal	Unknown	Unknown protein

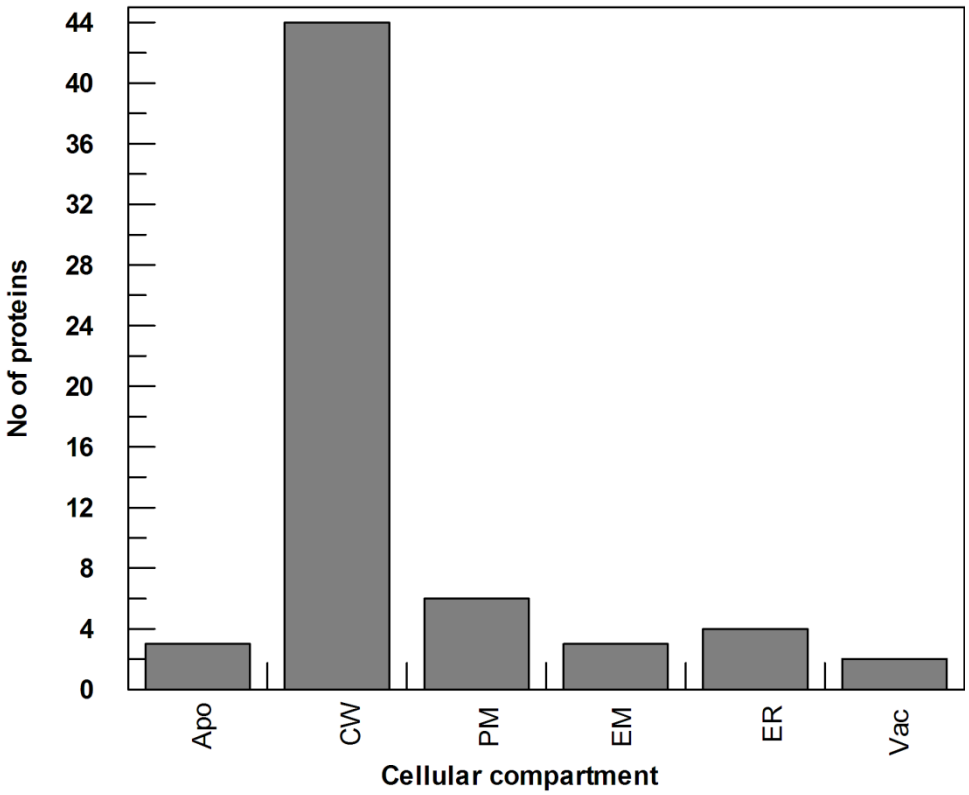


Figure 5.5 Classfication of secretory proteins identified from different cellular compartments of *Arabidopsis thaliana* leaf tissue. Abbreviations used are: Apo = apoplast, CW = Cell wall, PM = Plasma membrane, EM = Endomembrane, ER = Endoplasmic reticulum, Vac = Vacuole.

5.5. 3 Quantification of proteins

The LC-MS outputs were imported into Progenesis LC-MS for alignment and statistical analysis. The peptides which were significantly different between the WT and *vtc 2* and HL and LL at different probability values were compared using Progenesis LC-MS and GeneSpring GX, and are listed in Table 5.5. Peptides which contained MS/MS spectra > 2 and which were ranked in level 1 were searched using Mascot against the NCBI *Arabidopsis thaliana* protein database and selected for further validation.

Table 5. 5 Number of significant peptides with different probability values.
Analysis of variance (ANOVA) was calculated using GeneSpring GX and Progenesis LC-MS.

Software	**P all**	**Probability values**		
		$p < 0.05$	$p < 0.01$	$p < 0.001$
Genespring GX	5184	1187	449	90
Progenesis LC-MS	938	938	162	32

5.5.3.1 Cell wall proteins differentially expressed between WT and *vtc2*

Peptides were filtered as described in section 5.5.2.1 and analysed using Mascot against the NCBI *Arabidopsis thaliana* protein database. 16 cell wall proteins were identified as being significantly different ($p < 0.05$) between the WT and *vtc 2* or HL and the LL (Table 5.6 B). The number of unique peptides and their intensities for the proteins of most interest such as Prx 33, Prx34 and aspartyl protease-like protein are listed in Table 5.7. These proteins were identified from 2 independent experiments and showed identical patterns in protein abundance. As described earlier, peroxidase activity was higher in the *vtc* mutants than the WT. Therefore, the aim of analysing these data was to find out which peroxidase showed high enzyme activity in the *vtc* mutants. 73 genes were reported for class III peroxidases as discussed earlier. The most interesting was peroxidase 34 (Prx34) encoded by AT3G49120. The amounts of Prx33 and 34 were higher in the *vtc* mutants than theWT in both light conditions. The response of peroxidase activity and protein abundance in HL was not consistent between biological replicates and therefore, needs further experimentation. The concentration in LL corresponds to enzyme activity and it showed negative correlation to ascorbate in the *vtc* mutants. The mRNA sequence data also showed gene expression of Prx 34 was higher in the *vtc 2-1* mutant but no significant difference between HL and LL was seen (M. Page and N. Smirnoff unpublished data). Similarly, two aspartyl protease proteins identified (encoded by AT1G09750 and AT1G0320) were at higher concentration in the *vtc* mutants than the WT. The intensity of proteins was decreased when acclimated to HL (shown in Table 5.6). Furthermore, an uncharacterised protein encoded by AT5G58960 was also

identified and was significantly higher in the *vtc* mutants than the WT. It was slightly increased in level when acclimated to HL in all strains, but not significantly different between HL and LL.

In addition, some other cell wall proteins showed different protein amounts between the WT and *vtc2* and HL and LL. For example; the overall abundance of Lectin-like protein (AT5G03350) was significantly decreased in HL when compared to LL. However, it showed higher abundance in *vtc 2* than the WT in LL but remained the same in both strains in HL. Leucine rich repeat (FLR1) (gi|7637423) was increased overall in HL exposed plants with no difference observed between WT and the *vtc 2* mutant. Three Fasciclin like- arabinogalactan proteins, FLA1 (AT5G5570), FLA2 (AT4G12730) and FLA8 (AT2G45470) showed higher amounts in the WT than the *vtc2* mutant. A slight increase was observed in HL treated plants, but this was not significantly different. Similarly, disulfide isomerase (encoded by AT1G21750) was lower in HL treated plants than LL. In LL conditions, it showed the higher abundance in *vtc2* compared to the WT, whereas there was no such difference in HL. Germin 1 (GLP1) (encoded by AT1G72610) was decreased in HL acclimated plants but was higher in the WT compared to *vtc2* in LL. Levels in both strains were the same in HL. SOUL heme (encoded by AT1G17100) was higher in HL than LL, but no difference was observed between the WT and the *vtc2* mutant. Another protein, GDSL-like lipase (encoded by AT1G28590) was higher in WT than *vtc2* and was also increased in HL in both mutants. Cysteine rich secretory protein 55 was higher in *vtc2* than the WT under both light conditions, but no difference was observed between HL and LL.

Table 5.6 Ionically-bound cell wall peroxidase and other proteins which are differentially expressed between WT and *vtc* mutants. Plants were acclimated to HL (550-650 µmol m^{-2} s^{-1}) and LL (100 µmol m^{-2} s^{-1}) for 4 d. Proteins were analysed by LC-MS and subjected to analysis of variance to identify the statistical differences between treatment and strains. All results were expressed as mean normalised abundance (n = 3) where, 'n' represents the number of biological replicates. Analysis was carried out using one-way analysis of variance (ANOVA). Values at $p < 0.05$ were considered statistically significant. (A) Experiment 1 (B) Experiment 2

A

Accession	Description	Average Normalised Abundances					
		WTHL	**WTLL**	***vtc1*HL**	***vtc1*LL**	***vtc2-1*HL**	***vtc2-1*LL**
AT3G49120	Peroxidase 34	1.67E+06	4.30E+05	5.49E+06	1.10E+06	5.61E+06	1.83E+06
AT1G09750	Aspartyl protease-like protein	1.12E+04	9918.75	2.02E+04	7.45E+04	3125.44	1.36E+05
AT5G58960	Uncharacterized protein	1.76E+05	1.27E+05	3.90E+05	3.12E+05	4.17E+05	2.51E+05

B

Accession no	Peptides	Score	Anova (p)*	Fold	Description	Average Normalised Abundances			
						WTHL	WTLL	*vtc2-2*HL	*vtc2-2*LL
AT3G49120	11 (3)	294.88	1.08E-04	2.01	Peroxidase 34	1.31E+07	1.28E+07	2.57E+07	2.29E+07
AT1G0320	8	192.17	2.71E-04	2.11	Aspartyl protease-like protein	2.25E+06	3.24E+06	3.26E+06	4.74E+06
AT5G03350	6 (5)	164.2	1.35E-03	5.33	Lectin-like protein	2.84E+06	7.34E+06	4.21E+06	1.52E+07
AT2G45470	6	120.57	1.01E-04	2.6	Fasciclin-like arabinogalactan protein (FLA8)	4.14E+06	5.78E+06	2.22E+06	3.26E+06
AT3G49110	6 (1)	88.68	4.38E-03	1.87	Peroxidase 33	1.46E+05	1.58E+05	2.62E+05	2.73E+05
AT4G08780	4 (1)	86.12	0.03	2.11	Peroxidase 38	7.84E+05	9.18E+05	4.34E+05	7.14E+05
gi|7637423	3	70.8	0.03	2.03	Leucine-rich repeat protein (FLR1)	2.76E+06	1.54E+06	2.58E+06	1.36E+06
AT5G5570	3	63.96	1.85E-03	1.89	Fasciclin-like arabinogalactan protein 1	1.72E+06	1.19E+06	9.30E+05	9.13E+05
AT1G21750	3	59	2.95E-03	3.21	Protein disulfide-isomerase A1	3.30E+05	4.90E+05	3.18E+05	1.02E+06
AT3G08030	4	58.23	0.03	3.23	Uncharacterized protein	3.19E+06	1.84E+06	9.86E+05	1.88E+06
AT4G12730	4	56	1.21E-03	1.55	Fasciclin-like arabinogalactan-protein 2	1.57E+06	1.58E+06	1.02E+06	1.20E+06
AT5G45260	2	53.37	0.01	1.95	Calreticulin like protein	2.48E+05	4.58E+05	3.70E+05	4.84E+05
AT1G72610	2	45.89	5.37E-03	3.56	Germin-like protein (GLP1)	5.20E+06	1.38E+07	3.86E+06	7.90E+06
AT1G17100	2	39.07	4.59E-03	2.13	SOUL heme-binding protein	6.55E+05	3.56E+05	6.30E+05	3.08E+05
AT1G28590	2	30.5	7.40E-04	2.15	GDSL-like Lipase	1.55E+06	8.66E+05	1.04E+06	7.18E+05
AT5G48540	2	28.62	4.49E-03	1.91	Cysteine-rich repeat secretory protein 55	1.17E+05	1.18E+05	1.68E+05	2.23E+05

Table 5.7 Peptides analysed by LC-MS and identified using Progenesis LC-MS. (A) Peroxidase 34 (B) Peroxidase 33 (C) Aspartyl protease protein.

A

Peroxidase 34 (AT3G49120)								
Sequence	**Feature**	**Score**	**Mass**	**Charge**	**Average Normalised Abundances**			
					WTHL	**WT LL**	***vtc*2-2HL**	***vtc*2-2LL**
AYADGTQTFFNAFVEAMNR	537	7.35	2151.98	3	8.43E+05	5.97E+05	1.96E+06	1.42E+06
DAFGNANSAR	56	29.47	1021.46	2	1.47E+06	1.35E+06	2.45E+06	2.19E+06
GFPVIDR	25	10.12	802.435	2	2.38E+06	2.51E+06	5.80E+06	5.25E+06
IAASILR	12	38.02	742.471	2	3.13E+06	3.29E+06	6.27E+06	6.89E+06
MGNITPTTGTQGQIR	336	29.44	1573.79	2	4.65E+05	5.10E+05	9.52E+05	1.11E+06
NQCQFILDR	112	55.51	1192.57	2	1.48E+06	1.51E+06	2.61E+06	3.18E+06
NVGLDRPSDLVALSGGHTFGK	45	22.39	2139.1	3	4.80E+06	4.52E+06	9.99E+06	9.27E+06
SALVDFDLR	17	54.9	1034.54	2	3.75E+06	4.07E+06	8.97E+06	7.13E+06
TPTVFDNK	29	10.99	920.452	2	4.53E+06	4.21E+06	6.75E+06	6.47E+06
VPLGR	11	24.08	540.339	2	1.66E+06	1.65E+06	2.78E+06	2.71E+06
YYVNLK	21	12.61	798.429	2	2.45E+06	2.71E+06	5.24E+06	5.41E+06

B

Peroxidase 33 (AT3G49110)								
Sequence	**Feature**	**Score**	**Mass**	**Charge**	**Average Normalised Abundances**			
					WTHL	**WTLL**	***vtc*2-2HL**	***vtc*2-2LL**
AYADGTQTFFNAFVEAMNR	537	7.35	2151.98	3	8.43E+05	5.97E+05	1.96E+06	1.42E+06
GFPVIDR	25	10.12	802.435	2	2.38E+06	2.51E+06	5.80E+06	5.25E+06
MGNITPTTGTQGQIR	336	29.44	1573.79	2	4.65E+05	5.10E+05	9.52E+05	1.11E+06
NVGLDRPSDLVALSGAHTFGK	2810	5.08	2153.12	3	1.46E+05	1.58E+05	2.62E+05	2.73E+05
VPLGR	11	24.08	540.339	2	1.66E+06	1.65E+06	2.78E+06	2.71E+06
YYVNLK	21	12.61	798.429	2	2.45E+06	2.71E+06	5.24E+06	5.41E+06

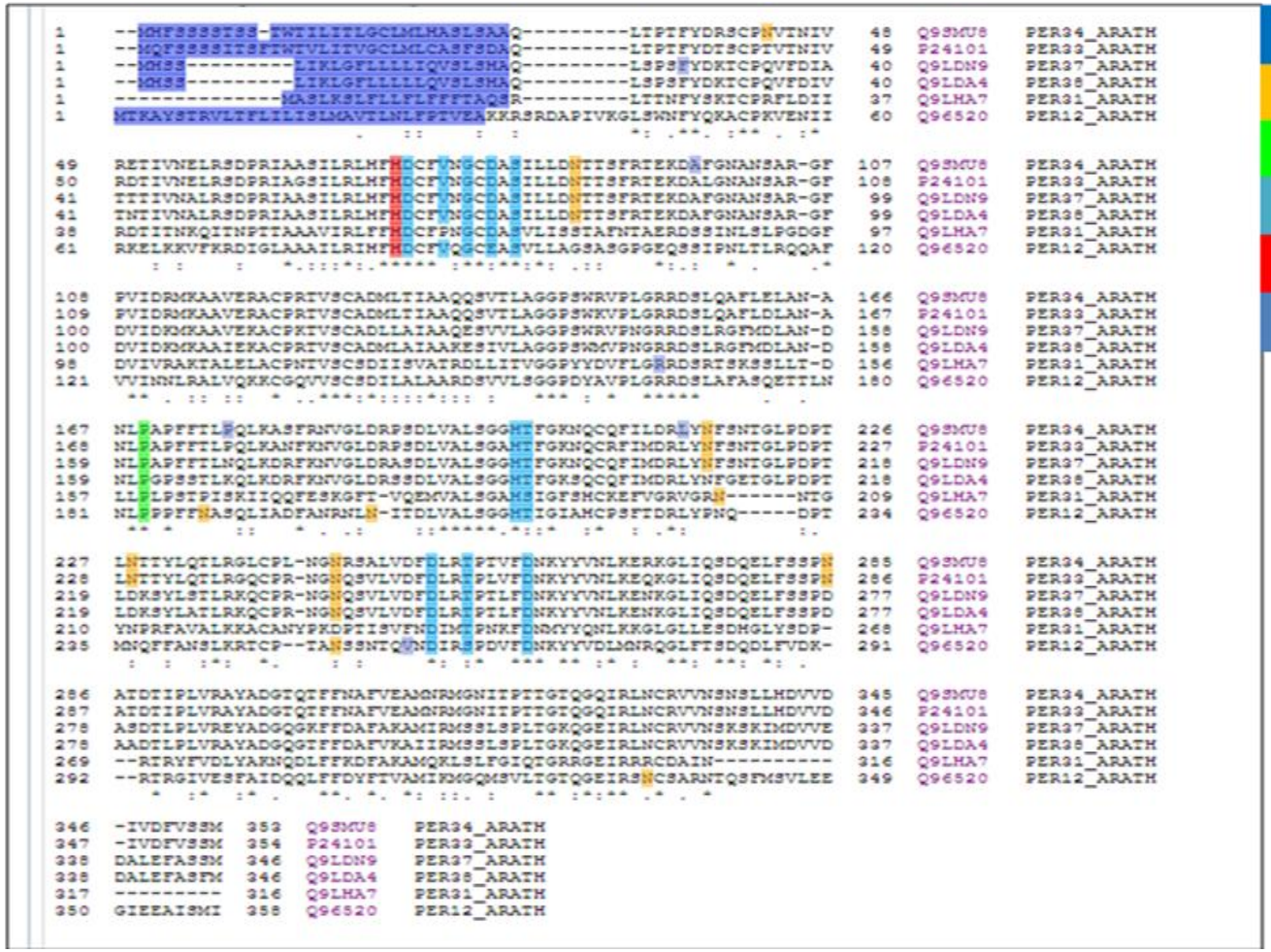
C

Aspartyl protease-like protein [AT1G0320]								
Sequence	**Feature**	**Score**	**Mass**	**Charge**	**Average Normalised Abundances**			
					WTHL	**WTLL**	***vtc*2-2HL**	***vtc*2-2LL**
ALLLPVTK	306	6.09	853.5638	2	2.87E+05	4.36E+05	4.59E+05	5.68E+05
DPSTLQYTTVINQR	4253	17.84	1634.813	2	1.07E+05	1.57E+05	1.34E+05	1.99E+05
FGFSSTLLGR	352	71.71	1083.571	2	5.19E+05	6.23E+05	7.14E+05	8.68E+05
IPNLIFSCGSTSLLK	1265	10.23	1648.86	2	3.77E+05	4.63E+05	4.89E+05	7.23E+05
ISSVNPYTVLESSIYK	1907	4.8	1798.932	2	2.03E+05	3.37E+05	2.97E+05	4.47E+05
LGYAVPEIQLVLHSK	822	11.52	1665.944	3	2.68E+05	4.11E+05	3.72E+05	6.13E+05
TLPIDPTLLK	505	26.54	1109.67	2	2.02E+05	3.22E+05	2.53E+05	5.00E+05
TPLLINPGTTVFEFSK	1168	16.26	1762.939	2	2.51E+05	4.33E+05	4.76E+05	7.06E+05
TPLLINPGTTVFEFSK	3442	27.18	1762.95	3	3.18E+04	6.25E+04	6.35E+04	1.12E+05

5.5. 4 Discrimination between proteins with high sequence similarity

Many groups of proteins belong to multigene families. They are derived from gene duplication during evolution and therefore, show high sequence homology. For example, 73 genes predicted for peroxidases in *Arabidopsis thaliana* (www.arabidopsis.org) and have high sequence similarity. Previously, Valerio *et al* (2004) reported close sequence homology in different peroxidase genes belonging to class III peroxidases. Also, proteins belonging to other multigene families have very similar amino acid sequences, which impose large constraints in identification of these proteins. LC-MS is a very powerful technique for detecting the different peptide masses, which are in turn useful for determining sequence homology. In this study, the proteins of interest belonging to the same gene family were aligned using UniProtKB (http://www.uniprot.org/align/). For example, the amino acid sequences of Prx34 (AT3G49120) and Prx33 (AT3G49110) were aligned. They are located next to each other on chromosome 3 and share 92.3 % sequence homology (Figure 5.6 A). Peptide sequences for both peroxidases were analysed by mass spectrometry and identified with Progenesis LC-MS as shown in coloured text. Similarly, six different class III peroxidases identified in this study were also aligned (shown in Figure 5.6 B). Prx33 and 34 showed 75-80% sequence homology to Prx 37 and 38 while Prx 12 showed less than 45% sequence homology to all other peroxidases detected in the current study.

A



Signal peptide
•Glycosylation
Binding site
Metal binding
Active site
Sequence conflict

B

1MHFSSSSTSSTWTILITLGCLMLHASLSAAQLTPTFYDRSCPNVTNIVRETIVNELRSD 59 **Prx 34**
1MQFSSSSITSFTWTVLITVGCLMLCASFSDAQLTPTFYDTSCPTVTNIVRDTIVNELRSD 60 **Prx33**

60 PRIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGNANSARGFPVIDRMKAAVER 119
61 PRIAGSILRLHFHDCFVNGCDASILLDNTTSFRTEKDALGNANSARGFPVIDRMKAAVER 120

120 ACPRTVSCADMLTIAAQQSVTLAGGPSWRVPLGRRDSLQAFLELANANLPAPFFTLPQLK 179
121 ACPRTVSCADMLTIAAQQSVTLAGGPSWKVPLGRRDSLQAFLDLANANLPAPFFTLPQLK 180

180ASFRNVGLDRPSDLVALSGGHTFGKNQCQFILDRLYNFSNTGLPDPTLNTTYLQTLRGLC 239
181ANFKNVGLDRPSDLVALSGAHTFGKNQCRFIMDRLYNFSNTGLPDPTLNTTYLQTLRGQC240

240 PLNGNRSALVDFDLRTPTVFDNKYYVNLKERKGLIQSDQELFSSPNATDTIPLVRAYADG 299
241 PRNGNQSVLVDFDLRTPLVFDNKYYVNLKEQKGLIQSDQELFSSPNATDTIPLVRAYADG 300

300 TQTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM 353
301 TQTFFNAFVEAMNRMGNITPTTGTQGQIRLNCRVVNSNSLLHDVVDIVDFVSSM 354

Figure 5.6 Identification and alignment of amino acid sequences of peroxidases. (A) Alignment of six peroxidase proteins. Different colours indicate different sequence features as shown on the right hand side of the sequence. (B) Alignment of Prx 33 and 34. Amino acids highlighted in yellow represent conflicts between the two proteins. Peptides with different colours in both proteins were analysed by mass spectrometry and identified using Progenesis LC-MS and Mascot

5. 6 Discussion

In the field of proteomics significant progress has been made on the analysis of proteins in different cell compartments, including chloroplasts, mitochondria and apoplast from different plant species (Lilley and Dupree, 2007; Friso *et al.*, 2004; Kleffmann *et al.*, 2004; Millar *et al.*, 2005). A number of studies have been reported on the cell wall proteomes of cell culture, stem, root, hypocotyl and fruit tissue, of tomato *Arabidopsis thaliana Cicer arietimum*, *Zea mays* and *Medicago sativa* (Catala *et al.*, 2011; Borderies *et al.*, 2003; Minic *et al.*, 2007; Feiz *et al.*, 2006; Jamet *et al.*, 2008; Jamet *et al.*, 2006; Irshad *et al.*, 2008). More than 400 cell wall proteins have been identified, which is a quarter of the expected cell wall proteome in *Arabidopsis thaliana* as reviewed by a French group (Jamet *et al.*, 2006; Jamet *et al.*, 2008). In contrast, the cell wall proteomes of leaf tissue from *Arabidopsis* and other species are far less were characterised, due to the technical challenges of isolating extracellular proteins, which were extensively contaminated with intracellular proteins such as ribulose-bisphosphate carboxylase oxygenase (Rubisco), the most abundant chloroplastic protein. Rubisco and other abundant small and large units of ribosomal proteins can suppress the signal of low abundant proteins during LC-MS detection. In addition, some intracellular proteins are non-specifically bound to the cell wall so a contamination is unavoidable during extraction. Some cell wall proteins are ionically-bound to the cell wall and are highly resistant to extraction with current protocols (Rose and Lee, 2010; Jamet *et al.*, 2008; Dunkley *et al.*, 2006; Lee *et al.*, 2004).

There are several constraints in cell wall proteome analysis (Jamet *et al.*, 2008). (i) It is very challenging to extract all of the proteins in a single method of extraction due to

different properties such as interaction with the cell wall and pI of the proteins. $CaCl_2$/LiCl/NaCl solutions were used for the extraction of cell wall proteins, but this approach may resulted in (a) the loss of many loosely- or non-specifically-bound proteins (b) Strongly bound proteins may not be removed from the cell wall (c) they were simply not detected due to the lack of sites for tryptic digestion, (d) steric hindrance of glycan groups near proteolytic sites, (e) they were not identified due to heavy glycosylation (Jamet *et al.*, 2008) (ii) Polysaccharide networking and interaction with the cell wall proteins and other components of the cell wall cause hindrance in protein separation by ConA leading to identification problems downstream. Heavily-glycosylated proteins can also cause hindrance in migration on a 2D-gel (iii) Different types of glycosylation (N- or O-) also affect protein isolation and separation from a complex biological mixture. Different strategies have been developed as discussed earlier to resolve these problems (Zhang *et al.*, 2010) (iv) Another problem in proteomic studies is the presence of keratin, a human skin protein that can contaminate the material during extraction and sample handling. This protein may suppress the detection of other low abundant proteins by LC-MS. This work describes three different processes (a) Evaluation of a protocol to extract ionically-bound cell wall proteins by a destructive method using leaf tissue as a starting material which has not been reported before. (b) The isolation and ultimate identification of 63 secretary proteins which belong to the cell wall, plasma membrane, endomembrane and apoplast. (c) A comparison of the WT cell wall proteome with *vtc* mutants in both HL and LL. Cell wall proteins were extracted from leaf tissue of *Arabidopsis thaliana* WT and the *vtc* mutants as described in

Chapter 2 and were isolated by ConA chromatography, which is specific to N-glycosylated proteins (Minic *et al.*, 2007). A significant number of the extracellular proteins separated were reported as N-glycosylated in the TAIR 10 database. Some were intracellular or undefined (described in section 5.5.2). The presence of non cell wall proteins may have two possible explanations as described earlier. These proteins either stick to the cell wall non-specifically (not experimentally tested), or they are simply intracellular (soluble) (Jamet *et al.*, 2008). Some proteins embedded in the membrane and strongly bound to the cell wall were difficult to extract with the current protocol. Over the past 10 years several studies were performed on the cell wall proteins in different tissues of *Arabidopsis thaliana*.

Table 5.8 Other studies of the cell wall proteome of *Arabidopsis thaliana* compared with the current study. Total identified = Total proteins analysed from the cell wall protein fractions.

Organs	Total identified	Cell wall	Refrences
Seedling tissue culture	97	44	(Charmont *et al.*, 2005)
Rosette	97	87	(Boudart *et al.*, 2005)
Stem	102	99	(Minic *et al.*, 2007)
Etiolated hypocotyls	147	137	(Irshad *et al.*, 2008)
Etiolated hypocotyls	127	127	(Zhang *et al.*, 2010)
Stem, root, hypocotyl, cell culture	-	~ 500	(http://www.polebio.scsv.ups-tlse.fr/WallProtDB/)

There have been no reports on the cell wall proteome from mature leaf tissue of *Arabidopsis thaliana*. In the current study, 63 secretary proteins were identified with high confidence of which 47 proteins were with a minimum of 3 distinct peptides and MS/MS > 2. These are located in the cell wall and the apoplast. 43 were previously reported in different tissues (WallProtDB) and 4 proteins with accession number AT1G72610, AT3G12145, AT3G14210 and AT4G36195 (for the further details see Table 5.3) were identified in the current study but have not been previously reported.

Foliar ascorbate concentration is lower in the *vtc* mutants as previously reported (Conklin *et al.*, 2000; Dowdle *et al.*, 2007). Ascorbate concentration was determined in both leaf extract and apoplast of theWT and *vtc* mutants as described in Chapter 3 (Figure 3.5) and Chapter 4 (Figure 4.5). Interestingly, both the apoplast and leaf extract produced the identical pattern for ascorbate concentration; however, the apoplastic ascorbate pool was very small compared to leaf extract. Therefore, the aim of the current study was to identify the cell wall and the apoplastic proteins which were affected by ascorbate deficiency in the *vtc* mutants. High activity of ionically-bound cell wall peroxidases in *Arabidopsis thaliana vtc* mutant seedlings suggested ascorbate and cell wall peroxidases show a negative correlation across all strains (Colville and Smirnoff, 2008). Increased peroxidase activity was also observed in the *vtc1* mutant (Veljovic-Jovanovic *et al.*, 2001) and *A thaliana* plants expressing antisense L-galactose dehydrogenase enzyme involved in the ascorbate biosynthesis (Gatzek *et al.*, 2002). The soil- grown plants used here showed higher activity of ionically-bound cell wall peroxidases in the *vtc* mutants than the WT under normal

growth conditions. In LL, peroxidase activity was significantly lower in the WT than the *vtc* mutants. Peroxidase activity was also measured in HL exposed plants. An increase in activity was observed, but unfortunately significant differences between WT and *vtc* mutants and HL and LL were not consistent between experiments; so further analyses are required. The *vtc* mutants were also found to be resistant to pathogens and also showed high activity of peroxidases when inoculated with *Pseudomonas syringae* (Pavet *et al.*, 2005; Barth *et al.*, 2004). Combining results from the previous literature (Colville and Smirnoff, 2008), it was suggested that peroxidase activity was affected by ascorbate deficiency, but the relationship between ascorbate and class III peroxidases is still not clear and needs further work. There is a possibility, that ascorbate deficiency in the *vtc* mutants was compensated by the peroxidases which are also involved in scavenging of ROS (see Chapter 1).

Welinder *et al* (2002) identified 73 genes for class III peroxidases expressed at different developmental stages of growth, which showed 28-94% amino acid sequence homology. As previously reported, peroxidases belonging to the oxidoreductase class and categorised in peroxidase III, play many roles in the plant defence system. Coville and Smirnoff (2009) reported peroxidase activity was higher in the *vtc* mutants in *Arabidopsis* seedlings, but individual peroxidases affected by ascorbate deficiency were unknown. The 6 peroxidases Prx33, Prx34, Prx37, Prx38, Prx42 and Perx12 identified here, all belong to class III and are located in the cell wall and are ncoded by AT3G49110, AT3G49120, AT4G08770, AT4G08780, AT4G21960 and AT1G71695 respectively. Interestingly, Prx 33 and 34 identified in 2 independent experiments, correspond to enzyme activity lower in the WT than the

vtc mutants in LL, and increased activity in HL, but not significantly different overall between HL and LL (Table 5.6 & 5.7). Bindschedler *et al* (2006) reported Prx 33 and Prx34 were highly expressed in *Arabidopsis thaliana* when inoculated with *Pseudomonas syringae*. In addition, peroxidases are also reported as biomarkers of metal stress, antioxidant defence (Almagro *et al*., 2009), controlling cell wall growth by contributing to liginification and rigidification (Fagerstedt *et al*., 2010; Passardi *et al*., 2004b) and plant hormonal regulation such as auxin and ethylene metabolism. They are also involved in ozone response. The transcript level of Prx 34 was 10-fold higher after 6 h treatment of ozone in *Arabidopsis thaliana* (Ludwikow *et al*., 2004).

Aspartyl protease-like protein and lectin like proteases were also identified as being higher in the *vtc* mutant than the WT in 2 independent experiments. Previously, it was reported that both of these proteins were associated with the pathogen response (LaPointe and Taylor, 2000; Ng *et al*., 2006). Mukherjee *et al* (2010) observed that *vtc* mutants were resistant to pathogens because they accumulated higher levels of pathogenesis-related (PR) proteins and salicylic acid than WT when exposed to *Pseudomonas syringae*. This might be due to insufficient removal of ROS in the *vtc* mutants which induces the pathogenesis-related proteins which are involved in these pathways. A few other pathogen-related proteins activate the signalling biosynthesis pathway and were also identified as slightly higher in the *vtc* mutants than the WT but not significantly different. Further work is needed to understand the functional significance of these differences in protein concentration.

5.7 Conclusions

The results show that the proposed protocol used for the extraction of the cell wall proteome from leaf tissue identified a promising number of proteins, which are extracellular and N-glycosylated. 70-80% of proteins accounted for were extracellular, despite the invasion of the chloroplastic protein Rubisco. This protocol appears efficient for the separation of N-glycosylated proteins from a complex mixture of proteins.

A striking difference between this protocol and a previous extraction protocol of cell wall proteins using non-destructive vacuum infiltration showed the latter failed to detect any peroxidases or some of the other proteins (Boudart *et al.*, 2005) identified in the current study. This suggested a destructive method for the extraction of cell wall proteins is more powerful.

This study has led to the identification of a large set of CWPs from leaf tissue for the first time in HL acclimated plants. Among the CWPs identified, a number of proteins expected to be were not detected, and this was possibly due to (a) the current protocol used for the extraction of cell wall proteins with salt has a limited efficiency for the extraction of all cell wall proteins which are tightly bound and (b) ConA chromatography showed high specificity for N-glycosylated proteins, so other proteins possessing other PTMs were lost. Some technical challenges can affect the isolation and detection of proteins, such as improper tryptic digestion, and low abundance of proteins, which were either beyond the detection limit of the mass spectrometer or suppressed by the highly abundant ones.

As previously reported, peroxidase activity is higher in the *vtc* mutants, but it was unknown which isoenzyme is affected by ascorbate deficiency. This study concluded peroxidase 33 and 34 are affected by ascorbate deficiency, unlike the other peroxidases identified here, which were the same in WT and the *vtc* mutants. Some other pathogenesis-related (PR) proteins also identified in the current study were higher in *vtc* mutants. Further investigation is needed to find out the nature of relationship between ascorbate, peroxidases and other proteins affected in *vtc* mutants.

GENERAL DISCUSSION

Chapter 6: General Discussion

At the outset of this study, the intention was to find out the effect of ascorbate deficiency on metabolites and proteins in response to abiotic and biotic stresses. For this purpose, four different *Arabidopsis thaliana vtc* mutants which contained a low concentration of ascorbate compared to the WT were used throughout the study. The previous Chapters have described the LC-MS based analysis of metabolites and proteins in response to different abiotic stresses. This chapter summarises the findings and suggests future work.

The diversity of metabolites in living system is enormous, which attributed to secondary metabolites, varies from species to species. The most prominent groups of primary and secondary metabolites are sugars, lipids, flavonoids, carotenoids, organic acids, hormones, vitamins and many others. Depending on the species total of 200,000 metabolites were estimated, which exist in plant species. To study these wide ranges of metabolites is very challenging. From the current study, it is clear that slight changes in normal growth conditions can perturb the whole biological system. In response to biotic and abiotic stresses, changes in metabolism suggest that some of these protect them from adverse environmental conditions, as stress-induced metabolic changes play a role of antioxidant or other defence molecules.

For the last few decades, the rise of robust analytical technologies has been very fast. This has attracted the interest of the researchers to explore the metabolome of plants. This work describes the development and optimisation of methodologies for the analysis of LC-MS based approaches for studying plants metabolites and proteins in

Arabidopsis thaliana. The method development aspects described in Chapter 2 were driven by the need to develop sensitive LC-MS methods for the separation and isolation of wide range of metabolites in an untargeted manner and specific class of metabolites in targeted manners. The LC-MS methods described in this thesis proved to be efficient for the detection of wide ranges of metabolites from a complex biological mixture.

In Chapter 3, the development of an LC-MS-ESI-QToF method, for the profiling of metabolites from the WT and *vtc* mutants was described. The LC-QToF-MS and LC-QQQ-MS proved to be a reproducible method for metabolite profiling in both targeted and untargeted manners. The optimised method for the analysis of metabolites provided the relevant information such as RT, mass, isotopic distributions and fragmentation for the detection and ultimately identification of metabolites, where differentially expressed across different strains. Thousands of metabolites could be deconvolved and aligned across samples, and many were affected by ascorbate deficiency and also responded to stress treatments. Although, MS alone cannot identify unknown compounds with certainty a number of compounds were identified with the aid of standards. Comparison of metabolites across different strains can elucidate the differences between metabolites, but to explore the chemical nature of each metabolite is very challenging. There is still a long way to go to completely describe the chemical nature of metabolites and their behaviour to different external stimuli.

From the overall results, it can be concluded that ascorbate deficiency has a significant effect on the metabolite profile of apoplastic fluid and the whole leaf. This

is the first investigation of metabolites in apoplastic fluid using LC-MS, and it is particularly surprising that a wide range of metabolites; mostly secondary metabolites were detected. Similarly, most of the metabolites were detected, which were up or down regulated in response to different stresses. Detailed comparison of metabolites in the apoplast and leaf extract under control and stress conditions is underway. Further investigation is needed to elucidate the structure of the metabolites identified as differentially expressed using NMR.

As described in Chapter 4, in response to HL accumulation of anthocyanins but not flavonol glycoside was inhibited by ascorbate deficiency. This suggested ascorbate is an important signal for altering plant cell metabolism. As previously reported ascorbate also affects the hormonal regulation in the *vtc* mutants such as increased levels of ABA in mutants compared over the WT. After extensive analysis of hormones in the *vtc* mutants in response to different stresses, high levels of ABA in the *vtc* mutants were not found. There is a conflict between our and previous data, which needs further investigation. There was no significant difference of JA accumulation in the WT and the *vtc* mutants but accumulation of SA and SA glycosides was higher in the *vtc* mutants compared to the WT. All these results suggested ascorbate did not have a significant effect on the accumulation of hormones. In our data set much variation was observed in the accumulation of hormones in independent experiments, which might be due to small difference in the plants age.

To investigate the proteome of the cell wall, it was decided to simplify the analysis by enriching for ionically-bound N-glycosylated proteins before MS analysis.

Interestingly, no previous study has been reported on the cell wall of mature leaf tissue of *Arabidopsis*. We isolated cell wall N-glycosylated proteins by ConA chromatography and identified number of these by MS. Most of these proteins were located in the extracellular compartment. Some of these proteins were found to be differentially expressed between the WT and *vtc* mutants as described in Chapter 5. For example, peroxidase activity was higher in *vtc* mutants than the WT. Cell wall extracts were analysed by LC-MS and normalised abundances were compared between the WT and *vtc* mutants. Six different peroxidase proteins were identified in all strains but only Prx34 corresponded to peroxidase activity. This peroxidase was previously reported to be involved in pathogen resistance.

In addition to the work described in this thesis, the extraction, isolation and further analysis of metabolites from *Arabidopsis thaliana* WT and the *vtc* mutants for structural elucidation could be performed by NMR. The complete identification of metabolites described in Chapter 3 needs further efforts in the improvement of strategies and technologies (LC-MS and NMR) with other bioinformatic tools, which help in structural elucidation and further absolute identification. Complete identification and comparison of metabolites in the apoplast and leaf extract of *Arabidopsis thaliana* WT and the *vtc* mutants is underway. Extending the analysis of flavonoids described in Chapter 4 in *vtc* mutants with the combination of flavonoid biosynthesis mutants will greatly improve our understanding of how to investigate the relationship between ascorbate and flavonoid biosynthesis.

Two crucial next steps in the cell wall proteomic work described in chapter 5 would be to correlate the identified proteins with gene expression data, and to understand

the fuctional differences of gene expression in the *vtc* mutants. Further investigation of the nature of the relationship between ascorbate and peroxidase and their role in stress response could be analsyed using peroxidase knockout mutants. The peroxidase 34 gene could also be over-expressed in *Arabidopsis thaliana* and analysed phenotypically.

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Appendix

Outcome of PhD

Conferences attended

1. Plastid Preview 2010. Imperial College London, 22nd – 23rd September, 2010.
2. Society of Experimental Biology (SEB), Annual meeting Glasgow, 1st to 4th July, 2011.

Original Publication

Page, M., Sultana, N., Paszkiewicz, K., Florance, H. & Smirnoff, N. (2011). The influence of ascorbate on anthocyanin accumulation during high light acclimation in *Arabidopsis thaliana*: further evidence for redox control of anthocyanin synthesis. *Plant Cell and Environment*, **35**, 388-404.