Metabolic changes in Arabidopsis thaliana plants overexperssing chalcone synthase

Dao, T.H.H.

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Chapter 7

^{1}H-NMR analysis of metabolic changes in *Arabidopsis thaliana* and CHS transgenic plants upon treatment with UV-A/blue light

T.T.H. Dao[1, 2], H.K. Kim[1], H.J.M. Linthorst [3], Y. H. Choi[1] and R. Verpoorte[1]

[1] Section Metabolomics, Institute of Biology, Leiden University, Leiden, The Netherlands
[2] Traditional Pharmacy Department, Hanoi Pharmacy University, Hanoi, Vietnam
[3] Section Plant Cell Physiology, Institute of Biology, Leiden University, Leiden, The Netherlands

Abstracts

The metabolic response of *Arabidopsis thaliana* Col. 0 and CHS transgenic plants upon treatment with UV-A/blue light were investigated using high resolution ^{1}H-NMR spectroscopy combined with multivariate data analysis. The investigation of the score and loading plots of partial least square (PLS) and partial least square-discriminant analysis (PLS-DA) showed a high accumulation of flavonoids, phenylpropanoids, glucose, fructose, rhamnose, and organic acids in *A. thaliana* Col. 0 whereas no significant change was obtained in CHS transgenic plants after treatments with UV-A/blue light. The control transgenic plants in fact had already similar levels of flavonoids and phenylpropanoids as the UV-A/blue light treated wild type *Arabidopsis*.

Keywords: UV-A/blue light, chalcone synthase, flavonoid, phenylpropanoid, *Arabidopsis*, metabolome.

7.1. Introduction

Plants detect and respond to a wide range of endogenous and environmental signals that control their metabolism and development. The synthesis of secondary metabolites in response to stresses has been implicated as a major defense response of higher plants [Bell, 1981]. UV radiation from the sun induces various responses in higher plants. While the greatest portion of UV-B (280–320 nm) is absorbed by the ozone layer, UV-A (320–400 nm) penetrates the atmosphere to reach the earth surface. DNA is especially sensitive to UV-B, resulting in the formation of pyrimidine dimers [Taylor *et al.*, 1997; Frohnmeyer and Staiger, 2003]. Low UV-B stimulates distinct other responses in plants, such as the accumulation of UV-absorbing pigments and expression of stress response-related genes [Hahlbrock and Scheel, 1989; Mackerness *et al.*, 2001; Brosché and Strid, 2003; Frohnmeyer and Staiger, 2003]. UV and blue light regulate the expression of various plant genes. In several species, UV-B, UV-A, and blue light stimulate the transcription of genes encoding the key phenylpropanoid and flavonoid biosynthesis enzymes like phenylalanine ammonia–lyase (PAL) and chalcone synthase (CHS) [Fuglevand *et al.,* 1996, Chappell and Hahlbrock 1984; Kubasek *et al.,* 1992; Jenkins 1997; Schäfer *et al.,* 1997]. That causes an induction of flavonoid accumulation such as kaempferol derivatives and sinapoyls in *Arabidopsis* [Hahlbrock 1981; Beggs *et al.,* 1985; Li *et al.,* 1993; Lois, 1994]. UV-B and UV-A/blue light act through separate but synergistic pathways in inducing CHS expression in *Arabidopsis* [Fuglevand *et al.,* 1996]. UV-B, UV-A and blue light each stimulate *CHS* expression up to about 10-fold in mature *Arabidopsis* leaf tissue and the combination of light treatments give even a much larger increase [Fuglevand *et al*., 1996].

Plants are thought to produce natural sunscreens, which selectively absorb photons in the UV-B and UV-A range, and flavonoid pigments are generally regarded as UV-absorbing agents. The accumulation of UV-absorbing compounds (including flavonoids such as flavones, isoflavonoids and anthocyanins) in the vacuoles of the epidermal layer provide selective attenuation of UV-B radiation [Hrazdina *et al.*, 1982, Schmelzer *et al.*, 1988, Tevini *et al.*, 1991, Strid and Porra, 1992]. UV light induces anthocyanins in for example, the swollen hypocotyls of turnip, apple fruit, Gros Colman' grapes (*Vitis vinifera* L.), petals of *Rosa hybrida*, *Arabidopsis* [Zhou *et al.,* 2007; Arakawa, 1988;

Kataoka *et al.,* 2003; Nakamura *et al.,* 1980], and camalexin in *Arabidopsis* [Mert *et al.,* 2003].

There are 3 UV/blue light photoreceptors in *Arabidopsis* identified: cryptochromes 1 and 2 (cry1 and cry2), and the phototropism photoreceptor phototropin (nph1) [Lin, 2000]. Plant nph1 is tightly associated with the plasma membrane [Reymond *et al.,* 1992] and cry1 and cry2 are nuclear proteins. Cry1 is present largely in the cytosol of light-grown plants and mediates the UV-A/blue light induction of several genes involved in flavonoid biosynthesis and anthocyanin accumulation in *Arabidopsis* [Kuhn *et al.,* 1984, Kreuzaler *et al.,* 1983]. Cry2 has a minor role in this response and it is constitutively imported to the nucleus regardless of light treatment [Wade *et al.*, 2001; Ahmad *et al.*, 1998, Lin et al., 2003]. The blue-light increases the cytoplasmic calcium concentration strongly [Baum *et al.,* 1999]. UV-B and UV-A /blue light induction of *CHS* expression involves calcium and these responses are inhibited by the calcium channel blockers nifedipine and ruthenium red [Christie and Jenkins, 1996; Frohnmeyer *et al.*, 1997]. Calcium flux of the cytosol involves Ca^{2+}–ATPases activity and some Ca^{2+}–ATPases are activated by calmodulin [Bush, 1995; Askerlund and Sommarin, 1996] and may cause a calmodulin-stimulated Ca^{2+}–ATPase which is involved in UV-B signal transduction [Long and Jenkins, 1998]. A scheme of the affects of UV-blue light in plant cells is shown in **Figure 7.1**.

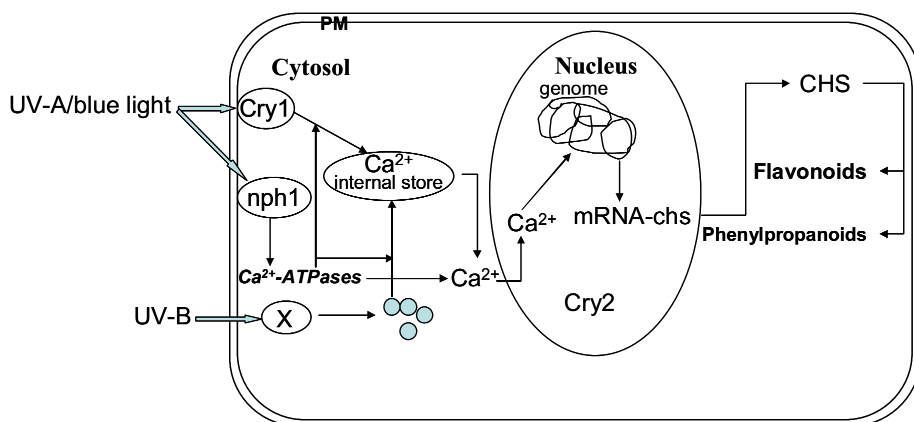

Figure 7.1. Model showing effect of UV/blue light on *Arabidopsis* cell and *CHS* expression. PM: Plasma membrane, X: UVB receptor (unknown)

So far flavonoid production has been regarded as the only major metabolic response of plants to UV-A/blue light irradiation. But flavonoids are part of the metabolic network and changes in this part of the network may affect the overall metabolic network in a cell. This study will use NMR-based metabolomics with multivariate data analysis to examine the whole of metabolic changes in Arabidopsis after UV-A/blue light stress. This allows the study of the total of metabolic networks in more detail and to understand more about the relationship between individual genes and metabolic processes.

7.2. Materials and methods

7.2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col.0) is the genetic background and 6 transgenic ACS lines were used. Seeds were surface sterilized by submersion for 1 min in 70% ethanol, 15 min in 2.5% sodium hypochlorite followed by five rinses with sterile water. Surface sterilized seeds were grown on plates containing half MS medium with 0.6% agar and supplemented with 20 mg/L hygromycin for selection of transgenic plants. After 3 days at 4^oC in the dark the seeds were germinated at 23^oC and long day condition (16/8 h light/dark cycle) in a growth chamber. Plants were routinely grown in a long day growth conditions under white light for 14 days before transfer to different light qualities. Plants were separated in two sets. One set as control was kept growing under white light. The other set was transferred to a UV-A/blue light chamber. Samples were collected after 6 hours, 24 hours and 48 hours after UV-A/blue light irradiation. Immediately after harvesting the material was frozen in liquid nitrogen and kept at −80 °C until used.

7.2.2. Light sources

Illumination was performed in controlled environment rooms at 23^oC. White light was provided by warm-white fluorescent tubes (Osram, Munich. Germany). UV-A light was provided by F35WIBI-26 blacklight-blue fluorescent tubes (GTE Sylvania, Shipley, UK), which emit light between 350 and 400 nm with a λ_{max} at 370 nm. Blue light was provided by 40W TI2 blue fluorescent tubes (GTE Sylvania) with λ_{max} at 430 nm,

covered with a UV226 filter (Lee Filters, Andover, UK) to remove wavelengths <400 nm

7.2.3. Extraction of plant materials

Plants were ground in liquid nitrogen and pooled before subjected to freeze-drying. Twenty-five milligrams of freeze-dried material were transferred to a micro-centrifuge tube before adding 600 µl of CH_3OH-d_4. The mixture was vortexed for 2 minutes and sonicated for 20 min, followed by centrifugation at 13,000 rpm for 5 minutes at room temperature. Five hundred microliters of the supernatant were then transferred into 2 ml micro-centrifuge tubes and 250 µl of KH_2PO_4 buffer, pH 6.0, containing 0.1% trimethyl silyl propionic acid sodium salt (w/v) were added. The mixture was left for 30 minutes in 4°C, and then centrifuged at 6000 rpm for 5 minutes at room temperature. Seven hundred µl of the supernatant were then transferred into 5 mm NMR tubes for analysis.

7.2.4. Solvents and chemicals

D_2O (99%) and CH_3OH-d_4 (99.8%) were obtained from Cambridge Isotope Laboratories Inc (Miami, FL, USA). NaOD was purchased from Cortec (Paris, France). Potassium dihydrogen phosphate and trimethylsilane propionic acid sodium salt (TSP) were purchased from Merck (Darmstadt, Germany). KH_2PO_4 was added to D_2O as a buffering agent. The pH of the D_2O was adjusted to 6.0 using a 1 M-NaOD solution.

7.2.5. NMR spectra measurement

^{1}H-NMR, 2D J-resolved spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany). ^{1}H-^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Kalsruhe, Germany). All the NMR parameters were the same to those of our previous reports (Jahangir *et al.*, 2008; Abdel-Farid *et al.*, 2007).

7.2.6. Data analysis

Spectral intensities of ^{1}H-NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4- δ 10.0. The regions of δ 4.8-δ 4.9 and δ 3.28-δ 3.40 were excluded from the analysis because of

the residual signal of water and CH_3OH. Principal component analysis (PCA) and partial least square regression analysis (PLS) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden).

7.3. Results

In order to exclude the separation in analysis due to the effect of the transgene and only observe the effect of UV-A/blue light, the sample data set was split into two groups: wild type and transgenic *Arabidopsis.* The results are presented below.

7.3.1. Metabolomic profiling of UV-A/blue light treated Arabidopsis thaliana Col. 0 by NMR spectroscopy

To reveal the change of metabolites after a UV-A/blue light treatment in *A. thaliana* Col. 0 the multivariate analysis partial least square (PLS) method was applied, that method can apply a time point (6, 24, 48 hrs) variable in the data analysis. Thus we can see the metabolite changes based on UV-A/blue light treatment as well as due to growth. A clear separation was obtained in the PLS score plot (**Figure 7.2**). There is no metabolomic change after 6 hrs of UV-A/blue light treatment, most differentiation was observed after 24 hrs UV-A/blue light treatment. However, during the developmental period the plant metabolome always changes and thus also affects the results of UV-A/blue light treated plants. To solve this problem the supervised method PLS-DA was applied. For PLS-DA, the data were divided into two classes: controls and UV-A/blue light treated plants. The score plot (**Figure 7.3**) of PLS-DA shows a distinct separation between the UV-A/blue light treated plants and controls. Most separation was displayed in component 1 (PC 1). The UV-A/blue light treated group (2) stays on the negative side of PLS-DA PC 1, whereas the control group is on the positive side. To interprete which metabolites correspond to each group in the PLS-DA diagram the loading plot was investigated. The loading plot of PC 1 (**Figure 7.4**) clearly shows that phenylpropanoids, flavonoids, glucose, fructose and organic acids are a higher in the UV-A/blue light treated group (**Figure 7.4 A, B, C**) and non-treated group has higher amount of sucrose and amino acids (**Figure 7.4 A, B**).

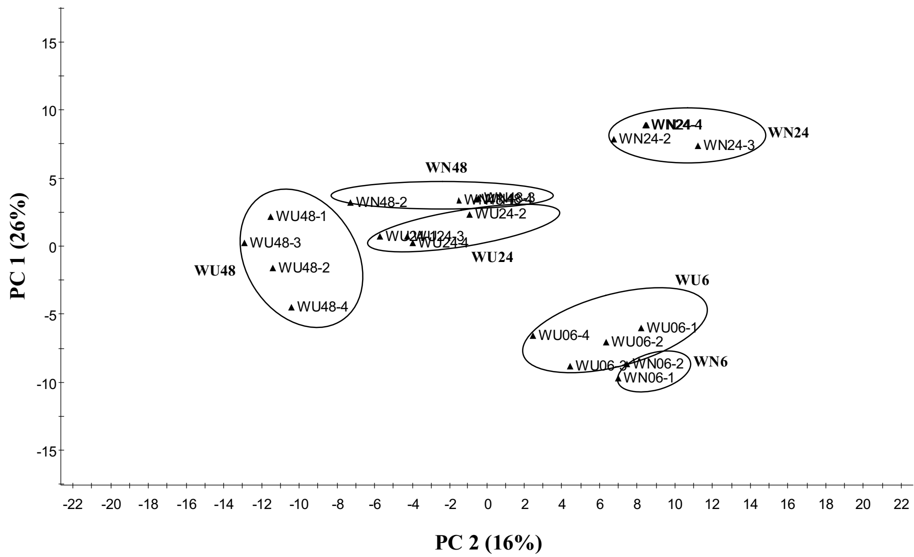

Figure 7.2. PLS score scater plot of UV-A/blue light treated *Arabidopsis* in 6 hr (WU6), 24 hr (WU24), 48 hr (WU48) and controls (WN6, WN24, WN48).

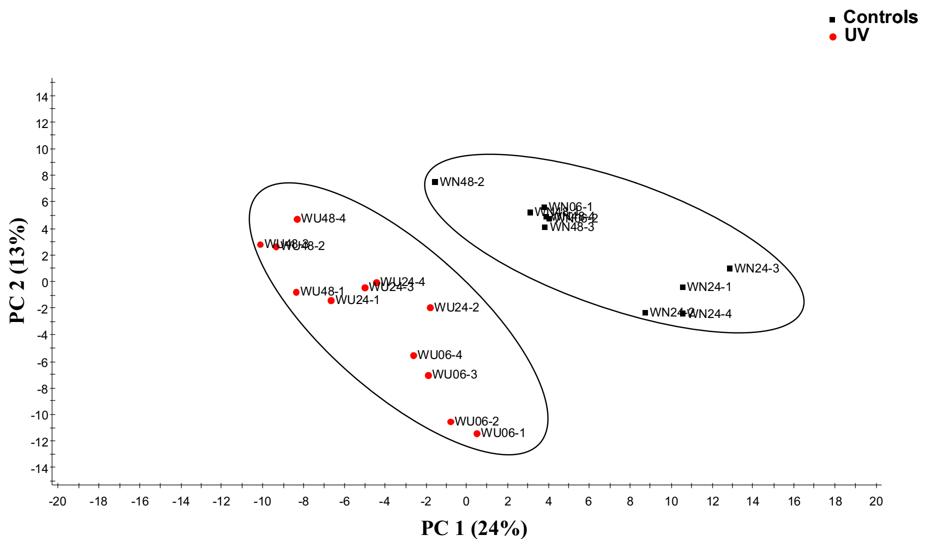

Figure 7.3. PLS-DA score scatter plot of UV-A/blue light treated *Arabidopsis* Col.0 in 6 hr (WU4), 24 hr (WU24), 48 hr (WU48) and controls (WN6, WN24, WN48)

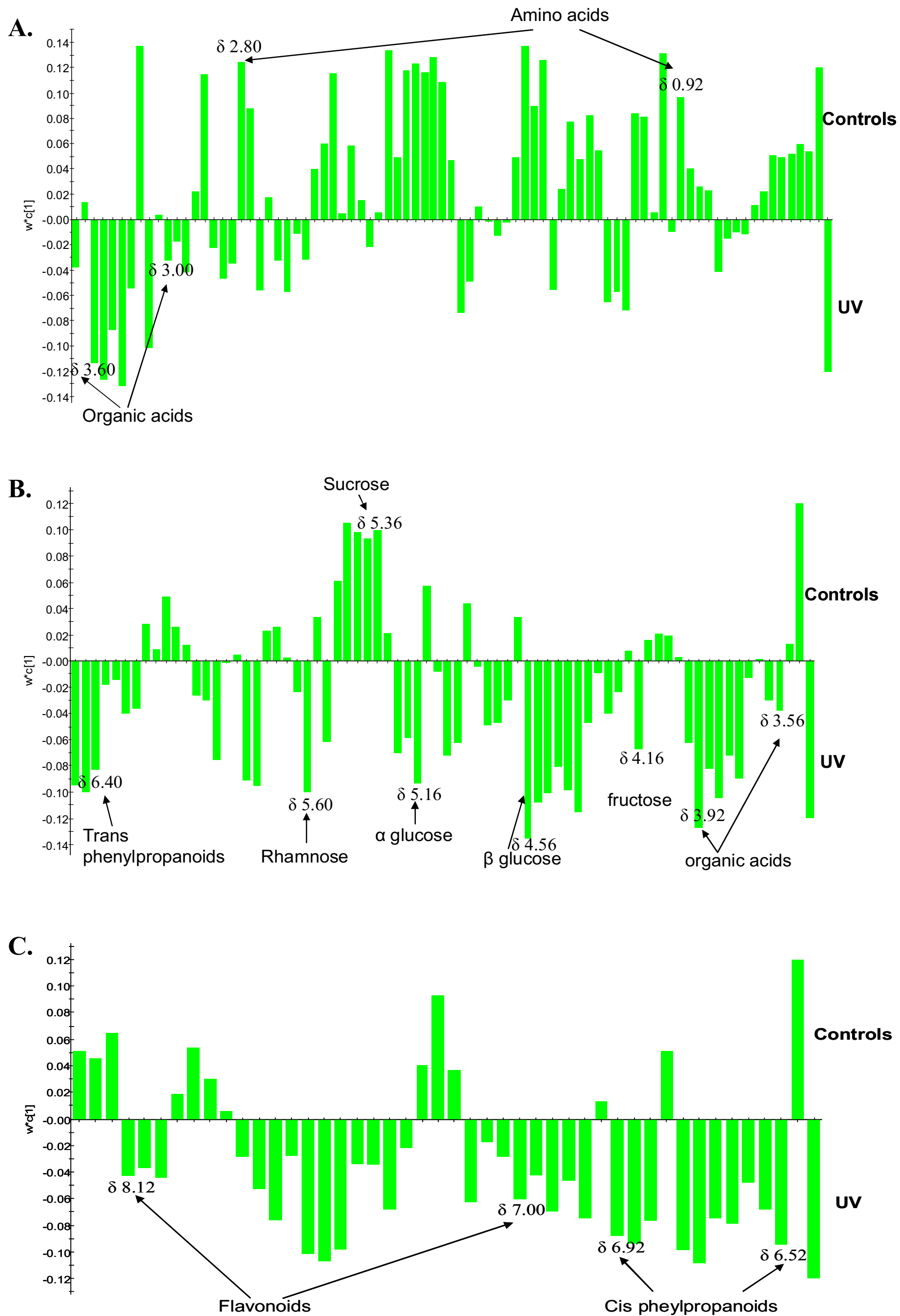

Figure 7.4. PLS-DA loadings column plot of UV-A/blue light treated *Arabidopsis* Col.0 and controls

7.3.2. Metabolic profiling of UV-A/blue light treated CHS transgenic Arabidopsis thaliana Col. 0 by NMR spectroscopy

The ^{1}H-NMR data of UV-A/blue light treated CHS transgenic *Arabidopsis* were analyzed in the same way as the control group but no clear separation was obtained between UV-A/blue light treated and none UV-A/blue light treated plants in the PLS score plot (**Figure 7.5**). So a supervised method PLS-DA was applied to see the effect of UV light on the metabolome of transgenic ACS plants. The PLS-DA score plot (**Figure 7.6**) shows only minor separation between UV-A/blue light treated ACS and control ACS in both PC1 and PC2. In order to identify the corresponding metabolites responsible for the separation we used a PLS-DA loadings Bi plot which resulted in **Figure 7.7**. **Figure 7.7** do show that there are only few metabolites which correspond to separation but those are unknown signals and none of the main identified metabolites contributes to seperation. It means there are no significant metabolome changes in ACS plants after the UV-A/blue light treatments.

In order to see the effect of *CHS* expression on the metabolome of UV-A/blue light treated *Arabidopsis* plants, PCA was applied to analyze only the CHS transgenic Arabidopsis and wild type plants at 24h UV-A/blue light treatment. That time point showed the biggest change of *Arabidopsis* metabolites under UV-A/blue light stress (**Figure 7.2**). The results of PCA analyse are presented in **Figure 7.8** which shows that the UV-A/blue light treated groups are very close to the non-treated UV-A/blue light groups in *CHS* transgenic *Arabidopsis* whereas the UV-A/blue light treated groups is very far from non-treated UV-A/blue light groups in wild type *Arabidopsis.* Also the UV-A/blue light treated groups of wild type *Arabidopsis* are very close to transgenic plants. This result means that UV-A/blue light did not have a clear effect on the CHS transgenic *Arabidopsis* in contrast it has a big effect on wild type *Arabidopsis* in which the metabolites of UV-A/blue light treated wild type plants became similar to CHS transgenic *Arabidopsis.*

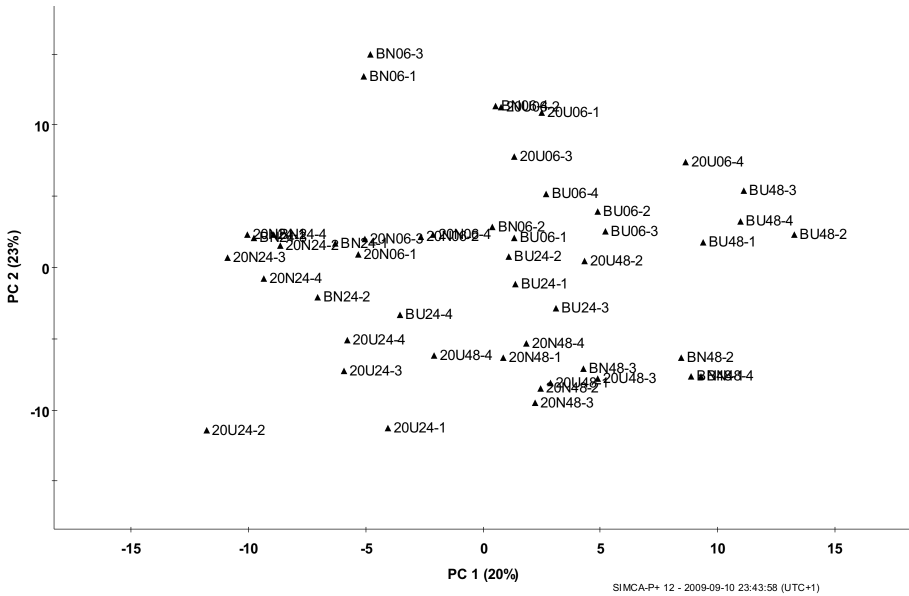

Figure 7.5. PLS score scatter plot of UV-A/blue light treated CHS transgenic *Arabidopsis* line ACS 20 (20) and ACS 2 (B) in 6 hr (20U6, BU6), 24 hr (20U24, BU24), 48 hr (20U48, BU48) and controls (20N6, 20N24, 20N48, BN6, BN24, BN48)

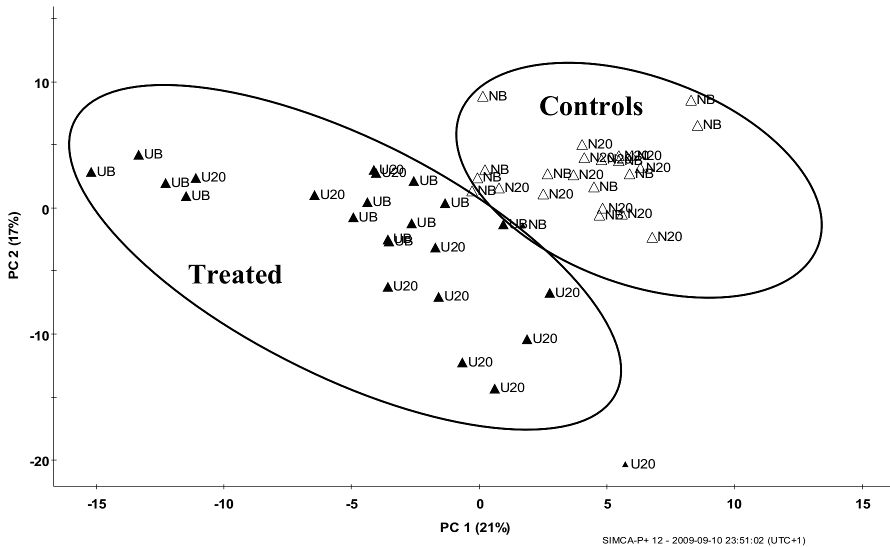

Figure 7.6. PLS-DA loading scatter plot of UV-A/blue light treated CHS transgenic *Arabidopsis* line ACS 20 (U20), ACS 2 (UB) and controls (N20, NB)

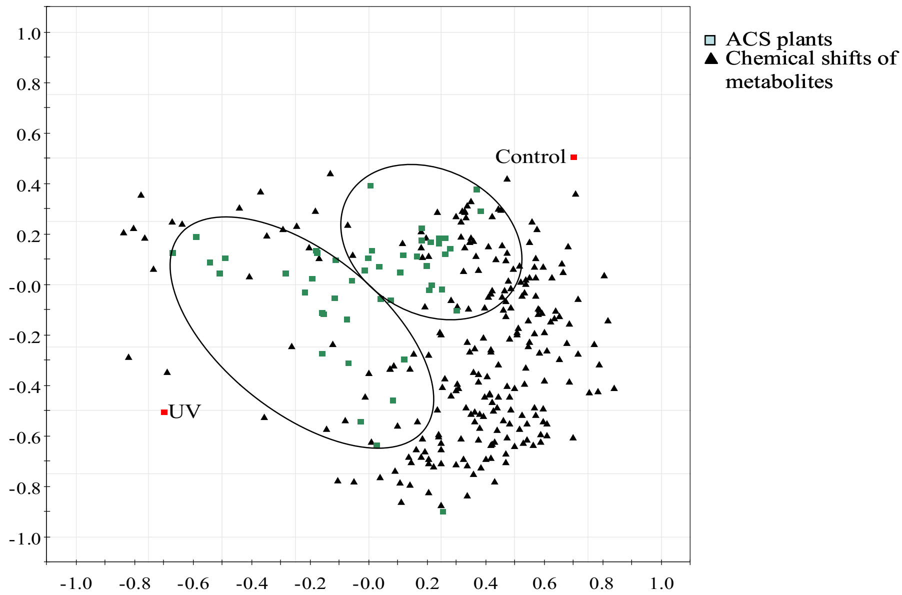

Figure 7.7. The PLS-DA loadings Bi plot of UV-A/blue light treated CHS transgenic *Arabidopsis* (ACS 20 and ACS 2) and controls

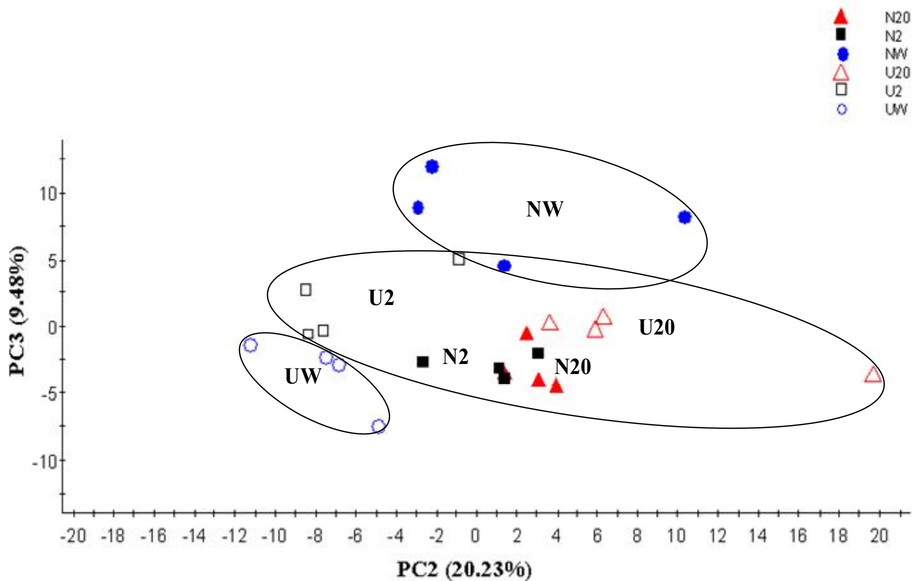

Figure 7.8. The PCA loading scatter plot of UV-A/blue light treated CHS transgenic *Arabidopsis* ACS 20 (U20), ACS 2 (U2), wild type (UW) and controls (N20, N2, NW).

7.4. Discussion

This study shows that UV-A/blue light strongly influences the metabolome of *A. thaliana* Col. 0 wild type while the metabolome of transgenic ACS does not show any specific change under UV-A/blue light treatment. The samples of 6 hr UV-A/blue light treatment showed no metabolic changes. At that time point a response at gene level may have been accomplished which causes the metabolic changes observed at 24 hr after the start of the treatment. That could explain that the biggest separation was obtained between control and UV-A/blue light treated group at 24 hr (**Figure 7.1**). The light treated group shows accumulation of flavonoids, phenylpropanoids, simple sugars such as glucose, fructose and rhamnose and a decrease of sucrose and amino acids. Flavonoids, phenylpropanoids and simple sugars can absorb UV-A/blue light. Several studies have shown that flavonoid production is induced by UV-A/blue light and occurs in the leave epidermal layers [Buchholz *et al.,* 1995, Jenkins *et al.,* 2001]. Those compounds play a role as sun filter for the plants to avoid DNA damage caused by UV-A/blue light. The decrease of sucrose and amino acids in UV-A/blue light treated plants might be due to the major metabolic changes needed from cell homeostasis to cell stress metabolism. The simple sugars are needed for energy and precursors or the biosynthesis of flavonoids and phenylpropanoids. Amino acids are possibly required for the de-novo biosynthesis of enzymes and as precursors.

We did not see any specific effect of UV-A/blue light on the transgenic ACS metabolome. There was no specific change of the metabolome observed for UV-A/blue light treated transgenic plants, if compared with transgenic controls and UV-A/blue light treated wild type (**Figure 7.8**). The ACS transgenic plants have high accumulation of flavonoids and phenylpropanoids itself (**Chapter 6**) so it might be enough to protect the plant from UV light. Very high levels of flavonoids and phenylpropanoids can be toxic for the plant and consequently there will be a limit in the level of metabolites that plants can produce without causing cell death. In the transgenic plants probably the limit of flavonoid and phenylpropanoid production is already achieved, not allowing any further increase of the biosynthesis, whereas in the wild type cells induction is possible. At what level the phenylpropanoid biosynthesis is regulated cannot be concluded from our results, but it might be at the level of CHS. At least our results support that the

phenylpropanoid and flavonoid biosynthetic pathways are the major metabolic pathways involved in the response to UV-A/blue light stress.