Analysis of CHS, MEB5.2, PDX1.3 and PR-5 expression in *Arabidopsis thaliana* ecotypes during UV-B irradiation.

Therese Östrand
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Examinator: Åke Strid
Abstract

The genes MEB5.2, CHS, PDX1.3 and PR-5 in the UV-B exposed Arabidopsis thaliana ecotypes Columbia-0, Ler, N22571, N22583, N22597, N22615, N22621, N22635, N22637, N22641, N1284, N1454, N22589, N22590, N22612, N22616, N22620, N22642, N22644, N1572 and N6038 have been analysed. The methods used were total RNA isolation, RNA electrophoresis, northern blot and CHURCH hybridization. The results indicate that all of the genes have an increased expression after the plants have been exposed to UV-B radiation. To examine the stress response of the ecotypes, measurements of hydrogen peroxide with DAB-staining have been carried out. The experiments could not indicate that any hydrogen peroxide had been produced.
1. Background

1.1 Aim

The aim with this project is to analyze the expression of the genes MEB5.2, CHS, PDX1.3 and PR-5 in different ecotypes of *Arabidopsis thaliana* during UV-B irradiation.

1.2 Arabidopsis

The *Arabidopsis* plant is a member of the *Brassicaceae* family and is an annual weed that occurs in temperate areas of North Africa, Asia and Europe. It has also been introduced to Australia and North America. The natural habitat for the *Arabidopsis* is open free draining ground, such as soil that is gravelly or sandy.¹

The *Arabidopsis* has (as can be seen in *Figure 1*) rosette leaves near the ground with a diameter of 2-5 cm. From the rosette one or more stems will be produced with a height of 20-70 cm. On the stem flowers will appear, approximately 1 mm wide and 3 mm long.

The *Arabidopsis* is self-fertile and the rapid flowering ecotypes (such as Columbia) can complete a life cycle in six weeks.

The *Arabidopsis* species that is the most famous is *Arabidopsis thaliana* which is widely used in genetic research. *Arabidopsis* is suitable as a research model because it is a diploid and has a small genome with little repetitive DNA and over 60% of the DNA is coding for some protein. Its small size and rapid life cycle makes it easy to handle and it is easily transformed.²

Most of the ecotypes used in this project come from the Nordborg collection which consists of 96 natural ecotypes.¹

1.3 UV-B

As a consequence of the thinning of the stratospheric ozone layer the levels of reaching UV-B radiation (280-320 nm) to the surface of the earth has increased. This leads to an increase in UV-B exposure to the plants in the environment which results in alteration in expression of several genes.³ When the gene expressions are altered many changes can happen to the plant such as its morphology, biochemical composition and its defence system. This makes it important to know what happens with the genes because it is the genes that control the organism. Different ecotypes can respond different depending on were they usually grow.

Ecotypes that usually grow in areas with a low UV-B radiation level are more likely to take more damage than ecotypes that are exposed to higher amounts of radiation in their natural habitat.⁴ To get a gene expression, the UV-B light has to be perceived by some kind of receptor that can transfer the information through a signalling pathway to the targets (proteins or genetic material in the nucleus).⁴

![Figure 1. Arabidopsis thaliana.³](image-url)
UV-B receptor:
There have been many discussions about which receptor perceive the UV-B light. Three types of light receptors have been considered; the phytochromes, the cryptochromes and the UV-B receptor that no one for sure knows exist.\textsuperscript{4}

Phytochromes: It is known that exposure to UV-B results in an increase of the expression of the gene chalcone synthase (CHS). Experiments have been performed on Arabidopsis thaliana mutants lacking its different types of phytochromes. The mutants still got an increase in the CHS expression which indicates that the phytochromes are not the primary UV-B receptors.\textsuperscript{6}

Cryptochromes: In Arabidopsis blue light and/or UV-A can induce CHS expression trough the CRY1 photoreceptor. Although when exposed with UV-B at the same time the yielded CHS expression was much higher than if the Arabidopsis was exposed to one wavelength only. An experiment with a mutant lacking the CRY1 receptor for UV-A and blue light showed that UV-B exposure still gave an increase in the expression of CHS. This indicate that the cryptochromes are not the primary UV-B receptor but that there in interacting signalling transduction pathways between UV-B, UV-A and blue light.\textsuperscript{2}

UV-B receptor: It is not known if a specific UV-B receptor exists. The fact that a cell contains many UV-B absorbing compounds makes the search for a UV-B receptor difficult. However is seems possible because other photoreceptors exists and none of the other examined alternatives seems to be the answer.\textsuperscript{5}

Level of UV-B radiation required to change the gene expression:
Genes can be arranged into different groups according to the level of UV-B radiation that can alter the expression of the gene. MEB5.2 which codes for a protein with unknown function needs a very low level of UV-B radiation (0.17 kJ/m\textsuperscript{2}) to affect the expression.\textsuperscript{4} PDX1.3 is a protein that is involved in the synthesis of pyroxidine also known as vitamin B\textsubscript{6} and it requires a low level of UV-B exposure (1.0 kJ/m\textsuperscript{2}).\textsuperscript{4} Chalcone synthase is an important enzyme in flavonoid synthesis\textsuperscript{9} and needs a low level of 1.5 kJ/m\textsuperscript{2} to get a changed gene expression. PR-5 need an intermediate level of exposure (7.8 kJ/m\textsuperscript{2}) and encodes proteins that are pathogenesis-related. Genes like PR-5 that need an intermediate level of radiation may also partly be regulated by ROS produced the specific UV-B-dependent way. The type of regulation can also differ between the different genes. MEB5.2 only gets a transient increase in its gene expression while PR-5 gets a continuous increase. CHS and PDX1.3 have an increase with plateau.\textsuperscript{4}

From receptor to gene expression:
CHS specific way:
It has been shown that both calcium and calmodulin are required for altering the CHS gene expression. Kinases that are able to perform phosphorylation are also needed for the induction of the CHS gene expression. It is also suggested that nitric oxide might be a second messenger in the induction of CHS gene expression. The second messengers activate different transcription factors that can induce expression or down-regulate it.\textsuperscript{4}

Wound and defence signalling:
UV-B induces the formation of different molecules that are involved in the plant’s wound and defence system. Some of them are jasmonic acid, ethylene and salicylic acid.\textsuperscript{5} Salicylic acid is thought to be needed in the formation of intermediate level of UV-B exposure genes as PR-5. Ethylene and jasmonic acid might have a role in the stimulation of genes that need a high level of UV-B radiation to be induced.\textsuperscript{4}
ROS:
Reactive oxygen species (ROS) can be produced in two ways. The first is the non-specific way. It occurs in high level UV-B radiation when molecules that can absorb UV-B transfer the energy from the radiation to oxygen molecules nearby. In this way the formation of superoxide and hydrogen peroxide can occur. The other way requires much lower levels of UV-B exposure. It is a catalytic production of ROS by NADPH oxidases or peroxidases induced by UV-B. First it generates superoxide which converts to hydrogen peroxide that can alter the expression of the PR-5 gene.[4]

A proposed model can be seen in Figure 2.

Figure 2. A modified possible model showing the signal transduction pathways during UV-B exposure in Arabidopsis thaliana with focus on the genes CHS, MEB5.2, PDX1.3 and PR-5.[4]
2. Methods

2.1 Light measurements

The light was measured with an OL754 UV-visible spectroradiometer (Optronic Laboratories Inc, Orlando, FL). Two different measurements were performed. First, the light in the room was measured (250-800 nm) and secondly the UV-B light, which was measured with a UV-B specific film (cellulose acetate) covering the detector. The film would only let light with a wavelength longer than 292 nm pass through to the detector.

The light in the room was measured on the middle of the exposure table (See E in Figure 3) and in the middle of all the tables in the growth room (See Figure 4).

The UV-B light was measured at 9 places (A-I) on the exposure table like in Figure 3. This was done twice, the first time the distance to the UV-B lamp was 109 cm and the second 74 cm.

Figure 3. The positions A-I on the exposure table where the light was measured.
From the light measurements, the spectral irradiance for each measured wavelength was received. The UV-\textsubscript{B} values for each place on the table and mean values for the whole table were then calculated.\textsuperscript{10, 11}

2.2 Growth conditions

The \textit{Arabidopsis thaliana} seeds were sowed in a soil mixture of weibulls fertilized peat, vermiculite and RHP Agra-perlite (1:1:1) and were then put in a dark cold room for 2-3 days for an even growth. They were then put in a greenhouse where the temperature was 20.5°C ±2.5°C, the air humidity 65% and the light was on 16 hours per day. After 1.5 week in the greenhouse the plants were tend to so there only were about 3 plants in each pot and after 3 weeks the plants were exposed to UV-light and harvested.

The ecotypes used in the first screening were a selection from an ecotype collection obtained from the European Arabidopsis stock centre and consisted of:

Control ecotypes: \textit{Columbia-0 (Col-0), Ler.}
Tolerant ecotypes: \textit{N22571, N22583, N22597, N22615, N22635, N22637, N22641, N1284, N1454.}
Sensitive ecotypes: \textit{N22589, N22590, N22612, N22616, N22620, N22642, N22644, N1572, N6038.}

The ecotypes used in the following experiments were a selection from the ecotypes from the first screening after their gene expressions had been analyzed and consisted of:

Control ecotypes: \textit{Col-0, Ler.}
Tolerant ecotypes: \textit{N22597 (Askot Berks, UK), N22635 (Markt, Baden, Germany), N22637 (Weitz, Germany), N1454 (Pitzal, Tyrol).}
Sensitive ecotypes: \textit{N22616 (Eifel, Germany), N22642 (Martuba, Libya), N1572 (Taynuilt, UK), N6038 (Kelsterbach, Germany).}
2.3 UV-exposure

The *Arabidopsis thaliana* plants were exposed to UV-B light for periods of 3, 6 or 9 hours or for 4 days (6 hours radiation per day). For each pot exposed to UV-B there was a control exposed to UV-A at the same time. To get some plants exposed to UV-B and some to UV-A, UV-B and UV-A specific films (mylar with a cut-off of 315 nm (UV-A) and cellulose acetate with a cut-off of 292 nm (UV-B)) were used. The exposures were always initiated at the same time on the day (09.00). The UV lamp was 74 cm from the table during the exposure and the mean UV-B$_{BE,300}$ value for the table was 0.105 W/m$^2$. After exposure, the plants were immediately harvested and kept in -80°C or directly used for DAB-staining to measure hydrogen peroxide.

The plants used for gene expression analysis were exposed to UV-light for 3 and 9 hours. The plants used for DAB-staining were exposed for 6 hours and for 4 days (6 hours radiation per day).

2.4 Total RNA isolation and concentration measurements

The RNA from all the harvested ecotypes was isolated as following:

The frozen harvested leaves were grounded in liquid nitrogen in a mortar (wrapped in aluminum foil and incubated in 200°C over night) with a pestle and transferred to microtubes, filling the tubes to approximately 0.5 ml. To the tubes, 500 µl of RNA extraction buffer (0.2 M Tris-HCl pH 8.8, 0.4 M LiCl, 0.025 M EDTA and 1% SDS in DEPC-water) was added and the tubes were rubbed against a rack to mix the material. The samples were kept on ice to the next step. Extraction was done by adding 500 µl phenol-chloroform-isoamyl alcohol (25:24:1), vortexing 30 s and centrifuging 3 min, 16100 rcf at 4°C. The water phases were transferred to fresh microtubes and the extraction was repeated with 500 µl chloroform-isoamyl alcohol (24:1). Lithium Chloride (10M) was added to a final concentration of 2M and the samples were incubated over night on ice in a cold room (4°C).

To pellet the precipitated RNA the tubes were centrifuged 20 min, 16100 rcf at 4°C. The supernatants were removed and the pellets dissolved in 100 µl sterile DEPC-water. If possible samples were pooled. By adding 2.5 volumes of absolute ethanol and 1/10 volume of 3M sodium acetate the RNA was precipitated again after 2 hours incubation at -20°C. The RNA was pelleted by centrifuging 30 min, 16100 rcf at 4°C and the supernatants were removed. The pellets were then washed with 500 µl 75% ethanol and the tubes centrifuged 30 min, 16100 rcf at 4°C. The ethanol was removed and the tubes centrifuged again so the remaining ethanol could be removed. The pellets were dried with a facial tissue on a pipette tip and then resuspended in 30 µl DEPC-water. The tubes were centrifuged for 5 minutes and the supernatants were transferred to clean microtubes.$^{12}$

The absorptions were measured with NanoVue Plus Spectrophotometer (GE Healthcare) which also calculated the concentration and the purity of the sample.

2.5 RNA electrophoresis and Northern blot

The RNA samples were separated by RNA electrophoresis and then transferred to a membrane by northern blot. RNA electrophoresis was done four times for each sample. This was done as following:
**RNA electrophoresis:**
The gel was made by boiling 0.6 g agarose in 5 ml 10 x MOPS and 36 ml milli Q water. After cooling, 9 ml formaldehyde 37% was added and the gels could be casted. After solidification, 800 ml of 1 x MOPS was poured over the gel and the combs were removed. A mastermix containing 5 µl sample buffer (40 mM MOPS, 13% formaldehyde, 5 mM EDTA and 40% formamid), 0.5 µl BFB (0.4% BFB and 15% Ficoll) and 1 µl EtBr was made per sample. The RNA samples (5 µg of each) was mixed with the mastermix in microtubes. The samples were centrifuged briefly and applied on the gel. A voltage of 100 V was put on until the samples had left the wells, then the voltage was set to 75 V. After 2 hours the electrophoresis was done and the gels were photographed.

**Northern blot:**
Before the transfer the gels were soaked in denaturation buffer (0.05 M NaOH, 0.15 M NaCl) for 10 minutes, washed with milli Q water and soaked in neutralization buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) for 30 minutes and again washed with milli Q water. The RNA was transferred to a Hybond-N membrane (GE Healthcare) by pressure blotting, 75 mmHg for 1 hour. After the transfer the RNA was fixated on the membranes with an UV Stratalinker 1800 (Stratagene). The RNA bands and the wells were marked on the backside of the membranes and the membranes were photographed. The membranes were stored in a refrigerator.

If an ecotype did not show on the gel, a complementary gel was made so all ecotypes could be represented in the gene expression analysis.

**2.6 CHURCH method for hybridization of northern blot**

**Labeling of probes:**
Probes able to hybridize with the genes CHS, MEB5.2, PDX1.3 and PR-5 were made by denaturing 25 ng of the PCR product or plasmid of respective gene mixed with 5 µl sterile milli Q water at 95°C for 5 minutes. After the denaturation 2 µl dATP, 2 µl dTTP, 2 µl dGTP, 5.4 µl random primers, 5 µl klenow buffer and 20.1 µl sterile milli Q water were added. In the hot-lab 2.5 µl [α-32P]dCTP and 0.5 µ l klenow fragment were added and everything mixed and centrifuged down. The mixtures were incubated for 2 hours in room temperature. The probes were purified with QIAquick Nucleotide Removal Kit (QIAGEN) and before hybridization the probes were denatured at 95°C for 5 minutes.

**Hybridization:**
The membranes from the northern blots were put in hybridization tubes and were prehybridized in 10-20 ml CHURCH buffer in 65°C for at least 2 hours. The probe was added to the CHURCH buffer and hybridization was done over night in 65°C. After hybridization the membranes were washed 2 x 5 minutes in 65°C with 20-25 ml wash buffer 1 (2 x SSC and 0.5% SDS) and then washed with 20-25 ml wash buffer 2 (0.2 x SSC and 0.1% SDS) in 65°C for 2 x 5 minutes + 1 x 10 minutes. The membranes were dried between two filter papers and wrapped in plastic film with only one layer film on the front. The packed membranes were put in cassettes together with Super RX films (Fuji) and placed in a -80°C freezer.
**Development of films:**
The films were developed after different amount of days and if the bands were to dark or pale, a new film was put in the cassette and the amount of days were adjusted. To make the bands visible the films were in a dark room soaked in development solution (Kodak’s processing chemicals for autoradiography films GBX developer/replenisher) for 1 minute, washed in water, soaked in fixating solution (Kodak’s processing chemicals for autoradiography films GBX fixer and replenisher) for 5 minutes, washed in water and then left to dry.

**2.7 DAB staining**

DAB staining was performed by adding 5-10 leaves to 50 ml falcon tubes (one for each ecotype and type of exposure) containing 10 ml DAB-staining buffer (0.1% DAB and 10 mM MES, pH 6.5). Vacuum infiltration was performed and the tubes were incubated under ordinary light for 30 minutes. The DAB-staining buffer was then poured off and replaced with 10 ml 95% ethanol. The falcon tubes were placed in a water bath with a temperature of 70°C for 30 minutes for decolorization. The 95% ethanol was then replaced with 10 ml 50% ethanol, in which the leaves could be stored. The leaves from the third sowing were then dried off and photographed on a petri dish. The brown spots were then quantified with Quantity One (Bio-Rad). A calculation for each ecotype and exposure time was done. The mean intensity/mm² value for UV-A was added with the one for UV-B and then divided with the value for UV-A. In the second DAB staining experiment a positive control was used by pouring 1 µM paraquat (0.1% Tween) over Col-0 leaves 6 hours before the start of the DAB staining. The Col-0 leaves were then stained in the DAB-staining buffer the same way as the rest of the ecotypes. Only the leaves from the positive control were photographed from the second experiment because the control indicated that the infiltration had not been successful.
3. Results

3.1 Light measurements:

The sum of the light between 250 and 800 nm:
On the exposure table: \( \Sigma_{(250-800 \text{ nm})} = 4.39 \times 10^{-3} \text{ W/cm}^2 \)
On Table 1 in the growth room: \( \Sigma_{(250-800 \text{ nm})} = 4.61 \times 10^{-3} \text{ W/cm}^2 \)
On Table 2 in the growth room: \( \Sigma_{(250-800 \text{ nm})} = 1.54 \times 10^{-3} \text{ W/cm}^2 \)
On Table 3 in the growth room: \( \Sigma_{(250-800 \text{ nm})} = 5.45 \times 10^{-3} \text{ W/cm}^2 \)

The sum of the UV-B light between 250 and 400 nm measured with a cellulose acetate film and the UV-B\(_{BE,300}\) from measurement 1 are shown in Figure 5.

![Table of Results]

Figure 5. The results of the first measurement of the UV-B light. The distance from the lamp was 109 cm. A: The sums, given in W/cm\(^2\). B: UV-B\(_{BE,300}\) in W/m\(^2\).

The mean of the UV-B\(_{BE,300}\) for the first measurement is: 0.0596 W/m\(^2\).

The sum of the UV-B light between 250 and 400 nm measured with a cellulose acetate film and the UV-B\(_{BE,300}\) from measurement 2 are shown in Figure 6.
Figure 6. The results of the second measurement of the UV-B light. The distance from the lamp was 74 cm. A: The sums, given in W/cm². B: UV-B$_{BE,300}$ in W/m².

The mean of the UV-B$_{BE,300}$ for the second measurement is: 0.105 W/m².

The UV-B$_{BE,300}$ mean value converted to kJ/m²:
- 3 h radiation: 1.1 kJ/m²
- 6 h radiation: 2.3 kJ/m²
- 9 h radiation: 3.4 kJ/m²
- 3 days and 6 h radiation: 9.1 kJ/m²

### 3.2 RNA electrophoresis and northern blot

All RNA electrophoresis gels with matching membranes from the northern blots can be seen in Appendix 1.

### 3.3 Gene expression analysis

The developed films from the CHURCH hybridizations of the tolerant ecotypes from the screenings of all 20 ecotypes are shown below.

In Figure 7 none of the ecotypes exposed to UV-A expressed the gene MEB5.2. The three tolerant ecotypes exposed to UV-B that had the strongest expression of the gene were N22597, N22635 and N1454 and of them N22635 was the ecotype that expressed most MEB5.2. The plants were exposed to the UV-light for 3 hours.
Figure 7. This shows the amount of MEB5.2 in the control ecotypes Col-0 and Ler and the tolerant ecotypes N22571, N22583, N22597, N22615, N22621, N22635, N22637, N22641, N1284 and N1454 exposed to both UV-A and UV-B for 3 hours. The films were developed from the membranes in Figure 1, Figure 2 and Figure 4 (Appendix 1).

In Figure 8 none of the ecotypes exposed to UV-A expressed the gene MEB5.2. The three sensitive ecotypes exposed to UV-B that had the strongest expression of the gene were N22612, N22616 and N22642 and of them N22642 was the ecotype that expressed most MEB5.2. The plants were exposed to the UV-light for 3 hours.

Figure 8. This shows the amount of MEB5.2 in the control ecotypes Col-0 and Ler and the sensitive ecotypes N22589, N22590, N22612, N22616, N22620, N22642, N22644, N1572 and N6038 exposed to both UV-A and UV-B for 3 hours. The film was developed from the membrane in Figure 9 (Appendix 1).
In Figure 9 all of the ecotypes exposed to UV-A expressed the gene MEB5.2. The gene N22597 that was exposed for 3 hours expressed less than the others which had a strong expression. The ecotypes exposed to UV-B that were exposed for 3 hours had a stronger expression than those which were exposed for 9 hours but not as strong as the ones exposed to UV-A. The plants were exposed to the UV-light for 3 and 9 hours.

In Figure 10 all of the ecotypes exposed to UV-A expressed the gene MEB5.2 strongly. The ecotypes exposed to UV-B that were exposed for 3 hours had a stronger expression than those which were exposed for 9 hours but not as strong as the ones exposed to UV-A. The plants were exposed to the UV-light for 3 and 9 hours.
Figure 10. This shows the amount of MEB5.2 in the control ecotype *Ler* and the sensitive ecotypes *N22616, N22642, N1572* and *N6038* exposed to both UV-A and UV-B for 3 and 9 hours. The film was developed from the membrane in Figure 17 (Appendix 1).

In Figure 11 none of the ecotypes exposed to UV-A expressed the gene CHS. The three tolerant ecotypes exposed to UV-B that had the strongest expression of the gene were *N22571, N22615* and *N22635* and of them *N22635* was the ecotype that expressed most CHS. The plants were exposed to the UV-light for 3 hours.

Figure 11. This shows the amount of CHS in the control ecotypes *Col-0* and *Ler* and the tolerant ecotypes *N22571, N22583, N22597, N22615, N22621, N22635, N22637, N22641, N1284* and *N1454* exposed to both UV-A and UV-B for 3 hours. The films were developed from the membranes in Figure 3, Figure 4 and Figure 5 (Appendix 1).
In *Figure 12* none of the ecotypes exposed to UV-A expressed the gene CHS. The three sensitive ecotypes exposed to UV-B that had the strongest expression of the gene were *N22612, N22616 and N22642* and of them *N22616* was the ecotype that expressed most of the gene. The plants were exposed to the UV-light for 3 hours.

![Figure 12](image)

*Figure 12*. This shows the amount of CHS in the control ecotypes *Col-0* and *Ler* and the sensitive ecotypes *N22589, N22590, N22612, N22616, N22620, N22642, N22644, N1572* and *N6038* exposed to both UV-A and UV-B for 3 hours. The film was developed from the membrane in *Figure 10* (*Appendix 1*).

In *Figure 13* none of the ecotypes exposed to UV-A expressed the gene CHS. The ecotypes exposed to UV-B expressed the gene and those that were exposed for 3 hours had a stronger expression than those which were exposed for 9 hours except for *N22635* that had a strong expression when exposed for 9 hours. The plants were exposed to the UV-light for 3 and 9 hours.
Figure 13. This shows the amount of CHS in the control ecotype Col-0 and the tolerant ecotypes N22597, N22635, N22637 and N1454 exposed to both UV-A and UV-B for 3 and 9 hours. The film was developed from the membrane in Figure 14 (Appendix 1).

In Figure 14 none of the ecotypes exposed to UV-A expressed the gene CHS. The ecotypes exposed to UV-B that were exposed for 3 hours had a stronger expression than those which were exposed for 9 hours. The plants were exposed to the UV-light for 3 and 9 hours.

Figure 14. This shows the amount of CHS in the control ecotype Ler and the sensitive ecotypes N22616, N22642, N1572 and N6038 exposed to both UV-A and UV-B for 3 and 9 hours. The film was developed from the membrane in Figure 18 (Appendix 1).
In Figure 15 most of the ecotypes exposed to UV-A expressed the gene PDX1.3 and N22641 had the strongest expression. All of the ecotypes except N22583 exposed to UV-B expressed the gene. The four tolerant ecotypes exposed to UV-B that had the strongest expression of the gene were N22597, N22615, N22635 and N1454 and of them N22615 was the ecotype that expressed most PDX1.3. The plants were exposed to the UV-light for 3 hours.

Figure 15. This shows the amount of PDX1.3 in the control ecotypes Col-0 and Ler and the tolerant ecotypes N22571, N22583, N22597, N22615, N22621, N22635, N22637, N22641, N1284 and N1454 exposed to both UV-A and UV-B for 3 hours. The film was developed from the membrane in Figure 6 (Appendix I).

In Figure 16 some of the ecotypes exposed to UV-A expressed the gene PDX1.3. All the ecotypes exposed to UV-B expressed the gene. The three sensitive ecotypes exposed to UV-B that had the strongest expression of the gene were N22616, N22642 and N1572 and of them N22642 was the ecotype that expressed most of the gene. The plants were exposed to the UV-light for 3 hours.
Figure 16. This shows the amount of PDX1.3 in the control ecotypes Col-0 and Ler and the sensitive ecotypes N22589, N22590, N22612, N22616, N22620, N22642, N22644, N1572 and N6038 exposed to both UV-A and UV-B for 3 hours. The film was developed from the membrane in Figure 11 (Appendix 1).

In Figure 17 none of the ecotypes exposed to UV-A or UV-B expressed the gene PDX1.3. The plants were exposed to the UV-light for 3 and 9 hours.

Figure 17. This shows the amount of PDX1.3 in the control ecotype Col-0 and the tolerant ecotypes N22597, N22635, N22637 and N1454 exposed to both UV-A and UV-B for 3 and 9 hours. The film was developed from the membrane in Figure 15 (Appendix 1).
In Figure 18 none of the ecotypes exposed to UV-A or UV-B expressed the gene PDX1.3. The plants were exposed to the UV-light for 3 and 9 hours.

Figure 18. This shows the amount of PDX1.3 in the control ecotype Ler and the sensitive ecotypes N22616, N22642, N1572 and N6038 exposed to both UV-A and UV-B for 3 and 9 hours. The film was developed from the membrane in Figure 19 (Appendix 1).

In Figure 19 most of the ecotypes exposed to UV-A expressed the gene PR-5 and N1454 had the strongest expression. Most of the ecotypes except exposed to UV-B expressed the gene. The four tolerant ecotypes exposed to UV-B that had the strongest expression of the gene were N22571, N22615, N22637 and N1454 and of them N1454 was the ecotype that expressed most PR-5. The plants were exposed to the UV-light for 3 hours.

Figure 19. This shows the amount of PR-5 in the control ecotypes Col-0 and Ler and the tolerant ecotypes N22571, N22583, N22597, N22615, N22621, N22635, N22637, N22641, N1284 and N1454 exposed to both UV-A and UV-B for 3 hours. The films were developed from the membranes in Figure 7 and Figure 8 (Appendix 1).
In Figure 20 none of the ecotypes exposed to UV-A or UV-B expressed the gene PR-5. The plants were exposed to the UV-light for 3 hours.

In Figure 21 none of the ecotypes exposed to UV-A or UV-B expressed the gene PR-5. The plants were exposed to the UV-light for 3 and 9 hours.
In Figure 22 none of the ecotypes exposed to UV-A or UV-B expressed the gene PR-5. The plants were exposed to the UV-light for 3 and 9 hours.

![Figure 22](image)

Figure 22. This shows the amount of PR-5 in the control ecotype Ler and the sensitive ecotypes N22616, N22642, N1572 and N6038 exposed to both UV-A and UV-B for 3 and 9 hours. The film was developed from the membrane in Figure 20 (Appendix 1).

In Table 1 the gene expression from the first gene expression analysis is shown for the eight selected ecotypes and the control ecotypes. It is the expression after 3 hours of UV-B radiation and 1 stand for no expression and 5 for a very strong expression.

Table 1. The MEB5.2, CHS, PDX1.3 and PR-5 expression from the first gene expression analysis for the selected ecotypes. It is the expression after 3 hours of UV-B radiation and 1 stand for no expression and 5 for a very strong expression.

<table>
<thead>
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<th>Ecotype</th>
<th>MEB5.2</th>
<th>CHS</th>
<th>PDX1.3</th>
<th>PR-5</th>
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<td>4</td>
<td>4</td>
<td>2</td>
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<td>5</td>
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<tr>
<td>N22616</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>N22642</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>-</td>
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<tr>
<td>N1572</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>N6038</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>

3.4 DAB-staining

Table 2 shows the (UV-A+UV-B)/UV-A values for all the selected ecotypes from the first DAB-staining experiment. The UV-A and the UV-B values are the mean values obtained from an analysis of the DAB-stained leaves with the program Quantity One from Bio-Rad.
Table 2. Shows the selected ecotypes (UV-A+UV-B)/UV-A values where the UV-A and UV-B values are the mean intensities/mm² calculated Quantity One. The values are shown for the exposure times 6 hours and for 4 days (6 hours radiation per day).

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Exposure time</th>
<th>6 hours</th>
<th>4 days (6 hours radiation per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N22597</td>
<td>(Tolerant)</td>
<td>1.91</td>
<td>1.81</td>
</tr>
<tr>
<td>N22635</td>
<td>(Tolerant)</td>
<td>2.00</td>
<td>1.87</td>
</tr>
<tr>
<td>N22637</td>
<td>(Tolerant)</td>
<td>1.96</td>
<td>1.95</td>
</tr>
<tr>
<td>N1454</td>
<td>(Tolerant)</td>
<td>2.04</td>
<td>1.93</td>
</tr>
<tr>
<td>N22616</td>
<td>(Sensitive)</td>
<td>2.02</td>
<td>1.93</td>
</tr>
<tr>
<td>N22642</td>
<td>(Sensitive)</td>
<td>2.08</td>
<td>1.86</td>
</tr>
<tr>
<td>N1572</td>
<td>(Sensitive)</td>
<td>2.12</td>
<td>1.94</td>
</tr>
<tr>
<td>N6038</td>
<td>(Sensitive)</td>
<td>1.95</td>
<td>2.01</td>
</tr>
<tr>
<td>Col-0</td>
<td>(Control)</td>
<td>2.01</td>
<td>1.96</td>
</tr>
<tr>
<td>Ler</td>
<td>(Control)</td>
<td>2.07</td>
<td>1.80</td>
</tr>
</tbody>
</table>

A calculated value over 2 indicates hydrogen peroxide production in the leaves as a result to UV-B radiation. The ecotype with the highest value is N1572 which was exposed for 6 hours. If hydrogen peroxide was produced there would be brown spots on the leaves but there were none on the leaves of N1572 (See Figure 23).

Figure 23. The DAB-stained leaves of N1572 after 6 hours UV-B exposure.

Figure 24 shows the control samples from the second DAB-staining. The other ecotypes were not analyzed because the positive controls did not turn dark brown as they should have done.
Figure 24. The positive control leaves from the second DAB-staining experiment.
4. Discussion

4.1 Light measurements, growth conditions and UV-B exposure

The light measurements were performed so the plants would be exposed to a reasonable amount of UV-B radiation. Because the radiation was not as high as wanted when the lamp was 109 cm above the table, the lamp was lowered to a distance of 74 cm from the table. The new distance gave a sufficient amount of radiation to induce the wanted gene expression. One important thing to take into consideration is that the different locations at the table differ in the amount of UV-B radiation exposure. The mean UV-B$_{BE,300}$ value for the table is 0.105 W/m$^2$ but on some part of the table the radiation get as low as 0.0397 W/m$^2$ and as high as 0.233 W/m$^2$. This gives the following UV-B radiation intervals for the different exposure times:
- 3 h radiation: 0.43-2.5 kJ/m$^2$
- 6 h radiation: 0.86-5.0 kJ/m$^2$
- 9 h radiation: 1.3-7.5 kJ/m$^2$
- 3 days and 6 h radiation: 3.4-20 kJ/m$^2$
The longer exposure time, the larger the variation is in radiation on the plants.

The plants sowed for the second gene analysis experiment and the DAB-staining experiments did not grow as large as the ones sowed for the first gene analysis experiment. This could be due to colder weather outside and the greenhouse was not able to hold the temperature as good as in the summer. It is also darker in the winter, which could have affected the plants growth rate. The gene expression in the plants can differ due to of how much they have grown. This should be taken into consideration when results from the first and second experiment are compared. The exposures were initiated at the same time in day because the gene expression in the plants can differ at different times of the day.

4.2 RNA isolation, gel electrophoresis and northern blot

The reason the RNA was isolated according to Carpenter and Simon instead of the faster way by using a RNA isolation kit was that the received concentrations were much higher then the kit could provide. According to the results from the NanoVue Plus Spectrophotometer all the samples were relatively free from proteins and carbohydrates.

In a few of the gels some of the ecotypes were missing. This was probably a result of pipetting mistakes and insufficient mixing of the samples. Due to the mistakes more gels with the missing ecotypes were made so all the ecotypes were represented in the gene expression analysis. It is not optimal, cutting out the samples that were missing but it ensured that all ecotypes were represented in the analysis of all the genes. All the northern blots were successful which can be seen in Appendix 1. Every band that can be seen on the gels can be seen on the matching Hybond-N membrane.

4.3 CHURCH hybridization

The probes used in the first experiment with all ecotypes were made of templates that consisted of PCR products of respectively gene. The templates had been used before in successful experiments. In the second experiment the same CHS template was used while the MEB5.2 template was made by amplifying the template used in the first experiment with PCR. It looked clean on a gel and the remaining nucleotides were removed. Nothing indicated
that it should not work properly. The probes binding to the genes PDX1.3 and PR-5 were made with plasmids containing the genes as templates. An explanation for a probe not working could be insufficient denaturation or a pipetting mistake while adding a component to the PCR. If no gene expression could be seen on a developed film the film was replaced with a new one and the cassette was allowed several weeks of incubation in the freezer before the next development to be sure that no gene expression had been induced in the leaves. This was done to make sure that if no band could be seen it was not because of a too short incubation time.

### 4.4 Selection of ecotypes for a second gene analysis

**Tolerant ecotypes:**
The ecotypes for further studies were selected first by checking which ones that had the strongest gene expression. In Figure 7 N22597, N22635 and N1454 had the strongest expression of MEB5.2. In Figure 11 N22571, N22615 and N22635 had the strongest CHS expression. The ecotypes with the strongest PDX1.3 expression were N22597, N22615, N22635 and N1454 (four ecotypes were selected because it was hard to make out which three that were the strongest) which can be seen in Figure 15. In Figure 19 the ecotypes N22571, N22615, N22637 and N1454 were the strongest in PR-5 expression. Adding earlier results to those above the ecotypes chosen were N22597, N22635, N22637 and N1454 which all had a strong expression of at least one of the genes.

**Sensitive ecotypes:**
The ecotypes were selected in the same way as the tolerant ecotypes. In Figure 8 and 12 N22612, N22616 and N22642 had the strongest expression of MEB5.2 respectively CHS. The ecotypes with the strongest PDX1.3 expression were N22616, N22642 and N1572 which can be seen in Figure 16. In Figure 20 none of the ecotypes expressed the gene PR-5. Adding earlier results to those above the ecotypes chosen were N22616, N22642, N1572 and N6038. N22616, N22642 and N1572 were selected from their strong expressions from the first experiment while N6038 was selected from the previous studies.

**Differences between ecotypes:**
As can be seen in Table 1 the ecotypes differ in their gene expression. In general the tolerant ecotypes seem to have a stronger response to UV-B than the sensitive ones, which is not the expected result. N22635 is the ecotype with the strongest gene expression in the UV-B specific genes while it has a weak expression in the stress related PR-5 gene. This pattern can be seen in N22597 as well but not as clearly. All the ecotypes have their strongest response in the PDX1.3 gene which encodes the protective compound vitamin B6. CHS is the gene with most variation in expression. Some ecotypes expressed it strongly while other not at all. The control ecotypes did not stand out in their expressions which were neither weak nor strong in comparison to the other ecotypes.

### 4.5 MEB5.2

The expression of MEB5.2 can be seen in Figure 7-10. Figure 7 and 8 shows that UV-B induces the expression of MEB5.3 which is the expected result. That the expression is stronger after 3 hours then after 9 hours which can be seen in Figure 10 is also expected. In Figure 9 and 10 the plants exposed to UV-A have a stronger expression than those exposed to UV-B. This can not be right because there should not be a stronger expression of MEB5.2 in plants exposed only to UV-A. If the plants accidentally had been exposed to UV-B it would
be noticeable in other figures as well because the same RNA samples were used on four different gels, one for each gene. It is hard to come up with a reasonable explanation because the same thing can be seen on both Figure 9 and 10. It is different ecotypes, the electrophoresis and northern blots were done different times and the hybridization of the probe was done in different days. The only thing they have in common is the probe which was used on both the membranes. It is not clear what could have been wrong with the probe, but it seems like the most probable explanation. According to Brosché and Strid (2003), 3 hours radiation should be enough to initiate MEB5.2 expression.

4.6 CHS

The expression of CHS can be seen in Figure 11-14. All the figures show an expression of CHS as a result to UV-B radiation. UV-A exposure gave no induction of the CHS gene. Both 3 and 9 hours exposure induced the gene and in the sensitive ecotypes the expression is strongest after 3 hours of radiation while it is harder to make out a difference in the tolerant ecotypes. According to and Strid (2003), 3 hours radiation should be enough to initiate CHS expression.

4.7 PDX1.3

The expression of PDX1.3 can be seen in Figure 15-18. In Figure 15 and 16 an increased expression of PDX1.3 can be seen as a result to UV-B exposure. The figures also show an expression of PDX1.3 as a result of UV-A radiation but not as strong as if radiated with UV-B. Figure 17 and 18 show no sign of PDX1.3 expression. That indicates that the probes made from plasmids did not work. The same probe was used on both figures. According to Brosché and Strid (2003), 3 hours radiation may not be enough to initiate PDX1.3 expression while 9 hours are more than enough.

4.8 PR-5

The expression of PR-5 can be seen in Figure 19-22. Only one of the figures showed any expression of PR-5 and that was Figure 19. Both UV-A and UV-B radiated ecotypes got an expression of PR-5. Some ecotypes got a stronger expression when exposed to UV-A than to UV-B and that was not expected. The sensitive ecotypes are assumed to react more to UV-B than the tolerant ecotypes. There is no sign of PR-5 expression in the sensitive ecotypes which is odd. It can not be anything wrong with the RNA because it would show on more than one film and that leaves the probe. It is possible that something went wrong with the hybridization. According to Brosché and Strid (2003), 3 hours radiation is not enough to initiate PR-5 expression and perhaps not even 9 hours.

4.9 DAB-staining

It can be assumed that the method for the DAB-staining was not successful due to the positive controls from the second DAB experiment. If the method had worked, the leaves of the positive control would have been brown. Instead they became more white than brown and it is not likely that the paraquat treatment did not work because the plants were covered with the solution that was poured on the leaves. Because the same method was used in both experiments it is likely that the first experiment was unsuccessful as well. During the vacuum infiltration the DAB buffer in the tubes should start to bubble if the vacuum is good enough. In neither of the experiment bubbles could be seen which indicates that the vacuum was not
sufficient. Only 25-75% of the leaves sank to the bottom of the tubes which indicates that not enough DAB buffer had been infiltrated in the leaves. To increase the vacuum the tube, the exicator and the pump was replaced with others to test if there was one of the components that gave rise to a leak that could worsen the vacuum. But no difference was noticed so the components were carefully cleaned in hope that it would increase the vacuum, which it did not noticeably. None of the ecotypes from the first experiment got calculated values significantly over 2 which indicated that the method was not successful. The picture of the leaves without the brown spots strengthens this conclusion. There could be another explanation. Perhaps no hydrogen peroxide was produced in the leaves. Although this is not likely because the plants were when they were exposed for 3 days and 6 hours exposed to a radiation of 9.1 kJ/m² which is a high dose of UV-B radiation. There could be other types of ROS production in the plants. There are also other types of ROS that could have been produced in the leaves and had an effect on the gene expression but they can not be detected with DAB-staining.
5. References

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RNA electrophoresis and northern blot

The gels from the first sowing of the tolerant ecotypes with matching membranes from the northern blots is showed in Figure 1, 2, 3, 4, 5, 6, 7 and 8.

Figure 1. a) A RNA electrophoresis gel with the tolerant ecotypes. The missing ecotypes exposed to UV-A (N22597, N22621, N22635, N22641 and N1454) is presented in Figure 2 and the missing ecotype exposed to UV-B (N22571) is presented in Figure 4. b) The matching membrane from the northern blot that was used in analysis of the gene MEB5.2.

Figure 2. a) The RNA electrophoresis gel with the tolerant ecotypes exposed to UV-A that were missing from the gel in Figure 1 (N22597, N22621, N22635, N22641 and N1454). b) The matching membrane from the northern blot that was used in analysis of the gene MEB5.2.
Figure 3. a) A RNA electrophoresis gel with the tolerant ecotypes exposed to UV-B. The missing ecotypes exposed to UV-A (all) is presented in Figure 4 and Figure 5. b) The matching membrane from the northern blot that was used in analysis of the gene CHS.

Figure 4. a) The RNA electrophoresis gel with the tolerant ecotypes missing from the gels in Figure 1 (N22571, UV-B) and in Figure 3 (N22641, N1284 and N1454, UV-A). b) The matching membrane from the northern blot that was used in analysis of the genes MEB5.2 (N22571, UV-B) and CHS (N22641, N1284 and N1454, UV-A).
Figure 5. a) The RNA electrophoresis gel with the tolerant ecotypes missing from the gels in Figure 3 (Col-0, Ler, N22571, N22583, N22597, N22615, N22621, N22635 and N22637, UV-A). The matching membrane from the northern blot that was used in analysis of the gene CHS.

Figure 6. a) A RNA electrophoresis gel with all the tolerant ecotypes. b) The matching membrane from the northern blot that was used in analysis of the gene PDX1.3.
Figure 7. a) A RNA electrophoresis gel with all the tolerant ecotypes. The missing ecotypes (N22583 and N22637, UV-B) are presented in Figure 8. b) The matching membrane from the northern blot that was used in analysis of the gene PR-5.

Figure 8. a) The RNA electrophoresis gel with the tolerant ecotypes missing from the gel in Figure 7 (N22583 and N22637, UV-B). b) The matching membrane from the northern blot that was used in analysis of the gene PR-5.
The gels from the first sowing of the sensitive ecotypes with matching membranes from the northern blots is showed in Figure 9, 10, 11 and 12.

Figure 9. a) The RNA electrophoresis gel with all the sensitive ecotypes. b) The matching membrane from the northern blot that was used in analysis of the gene MEB5.2.

Figure 10. a) The RNA electrophoresis gel with all the sensitive ecotypes. b) The matching membrane from the northern blot that was used in analysis of the gene CHS.
Figure 11. a) The RNA electrophoresis gel with all the sensitive ecotypes. b) The matching membrane from the northern blot that was used in analysis of the gene PDX1.3.

Figure 12. a) The RNA electrophoresis gel with all the sensitive ecotypes. b) The matching membrane from the northern blot that was used in analysis of the gene PR-5.

The gels from the second sowing with the tolerant ecotypes with matching membranes from the northern blots is showed in Figure 13, 14, 15 and 16.
Figure 13. a) The RNA electrophoresis gel with all the tolerant ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene MEB5.2.

Figure 14. a) The RNA electrophoresis gel with all the tolerant ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene CHS.

Figure 15. a) The RNA electrophoresis gel with all the tolerant ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene PDX1.3.
Figure 16. a) The RNA electrophoresis gel with all the tolerant ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene PR-5.

The gels from the second sowing with the sensitive ecotypes with matching membranes from the northern blots is showed in Figure 17, 18, 19 and 20.

Figure 17. a) The RNA electrophoresis gel with the sensitive ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene MEB5.2.
Figure 18. a) The RNA electrophoresis gel with the sensitive ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene CHS.

Figure 19. a) The RNA electrophoresis gel with the sensitive ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene PDX1.3.

Figure 20. a) The RNA electrophoresis gel with the sensitive ecotypes from the second sowing. b) The matching membrane from the northern blot that was used in analysis of the gene PR-5.