Regulation of Gene Expression by Low Levels of Ultraviolet-B Radiation in *Pisum sativum*: Isolation of Novel Genes by Suppression Subtractive Hybridisation

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**Introduction**

The influx of solar ultraviolet-B radiation (UV-B; 290–320 nm) to the surface of the earth has increased over the last several years due to the anthropogenic depletion of the ozone layer in the stratosphere (Madronich et al. 1998). Plants may thus be vulnerable to higher UV-B radiation levels than normal. It has been shown that UV-B radiation affects plants in different ways. Photosynthetic activity is reduced and total chlorophyll content in leaves declines (Strid et al. 1990), and a reduction of mRNA levels for chloroplast-localised proteins is evident after UV-B exposure (Strid 1993). Photodamage of DNA occurs, i.e. cyclobutane pyrimidine dimers and pyrimidine pyrimidone dimers are formed during irradiation of plants with UV-B (Britt 1999). Protective mechanisms, such as synthesis of UV-B-absorbing pigments and increases in antioxidative enzymatic activities, are induced (Strid and Porra 1992, A-H-Mackerness et al. 1998).

Studies of gene expression after UV-B exposure are one approach to identify both plant responses towards UV-B and signal transduction pathways contributing to development of stress tolerance. Several techniques are available when searching for unknown differentially regulated genes. Probably, the most effective one is the microarray technique where thousands of expressed sequence tags (ESTs) or cDNAs are spotted onto a chip and then hybridised to interesting samples of RNA. This method allows the expression levels of many genes to be analysed simultaneously (Brosché et al. 2002). For plants such as pea, where no EST or genome sequencing projects are in progress, other techniques have to be used. Differential display and suppression subtractive hybridisation (SSH) are examples of such methods that can be used to find differentially regulated genes (Liang and Pardee 1992, Diatchenko et al. 1996).

In this study, SSH was used to identify genes regulated by low levels of UV-B radiation with the aim to obtain previously unknown stress-regulated genes. The putative functions of these genes were established by sequence alignments with genes which have been sequenced in other plant species. Finally, in order to find out whether these genes were specifically regulated by UV-B radiation, the pattern of expression of these genes was also investigated after ozone exposure, a stress...

**Suppression subtractive hybridisation** was used to isolate genes differentially regulated by low levels of UV-B radiation (UV-B; 290–320 nm) in *Pisum sativum*. Six genes were regulated, two of which were novel. The mRNA levels for these two (*PsTSDC* and *PsUOS1*) were increased and depressed by UV-B treatment, respectively. Domains in the *PsTSDC* translation product was similar to TIR (Toll-Interleukin-1 receptor-similar) domains and a NB-ARC domain (nucleotide-binding domain in APAF-1, R gene products and CED-4). The *PsUOS1* translation product was similar to an open reading frame in Arabidopsis. Genes encoding embryo-abundant protein (*PsEMB*) and S-adenosyl-l-methionine synthase (*PsSAMS*) were induced by UV-B, whereas the transcript levels for genes encoding sucrose transport protein (*PsSUT*) or ribulose-5-phosphate 3-epimerase (*PsR5P3E*) were decreased. These regulation patterns are novel, and the *PsEMB* and *PsR5P3E* sequences are reported for the first time. The stress-specificity of regulation of these genes was tested by ozone fumigation (100 ppb O3). Qualitatively, the similarity of expression after both UV-B and ozone exposure suggests that, for these genes, similar stress-response pathways are in action.

**Key words:** Gene expression — Ozone — *Pisum sativum* — Suppression subtractive hybridisation — UV-B radiation.

Abbreviations: EMB, embryo-abundant protein; EST, expressed sequence tags; NB-ARC, nucleotide-binding domain shared by APAF-1, certain R gene products and CED-4; R5P3E, ribulose-5-phosphate-3-epimerase; SAM, S-adenosyl-l-methionine; SAMS, S-adenosyl-l-methionine synthase; SSH, suppression subtractive hybridisation; SUT, sucrose transport protein; TIR, toll-interleukin-1 receptor-similar domain; TSDC, TIR-similar-domain containing protein; UOS1, UV-B and ozone similarly-regulated protein 1; UV-A, ultraviolet-A radiation; UV-B, ultraviolet-B radiation; UV-B430, biologically effective ultraviolet-B radiation normalised to 300 nm.

The nucleotide sequences reported in this paper have been submitted to the GenBank under accession numbers AF369886 (*PsTSDC*), AF369887 (*PsR5P3E*), AF369888 (*PsUOS1*), AF369889 (*PsEMB*).

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that previously has been shown to give rise to responses similar to both biotic and abiotic stresses (Sandermann 1998, Sävenstrand et al. 2000).

**Results**

The forty-eight cDNA clones obtained by SSH were first analysed by slotblot hybridisation with radiolabelled driver cDNA (isolated from control tissue). This was performed in order to minimise the number of false positives by exclusion of clones either corresponding to rRNAs or to mRNAs present in the control plants. Thus, cDNAs from the slots where no hybridisation was seen were considered interesting for further study (not shown). Ten of these clones were chosen. The cDNAs for all ten were sequenced and five of them showed similarities with sequences in GenBank. One cDNA (UVB.55) was similar to ribulose-5-phosphate-3-epimerase genes from *Spinacia oleracea* (Nowitzki et al. 1995) and from *Arabidopsis thaliana* (Favery et al. 1998), cDNA UVB.18 and UVB.58 were both similar to the sucrose transport protein gene in *P. sativum* (Tegeder et al. 1999) and in *Vicia faba* (Weber et al. 1997). The cDNA clone UVB.53 was actually found to consist of two different cDNAs, and both were cloned separately and named UVB.53.1 and UVB.53.2. cDNA UVB.53.1 was similar to the sucrose transport protein (see UVB.18 and UVB.58 above), and cDNA UVB.53.2 was similar to the S-adenosyl-L-methionine synthase (SAMS) gene in *P. sativum* (Gómez-Gómez and Carrasco 1996). For the remaining six cDNAs no similarities were found in GenBank.

Northern blotting and hybridisation were performed with the nine different of the, in total, eleven sequenced cDNAs to verify the differential regulation of the genes by UV-B irradiation. Six of the nine genes were found to be truly regulated (Table 1). The three non-regulated cDNAs were not investigated any further. To be able to identify the five remaining regulated cDNAs, unknown so far, cloning of their entire coding sequences was necessary. Thus, rapid amplification of cDNA ends (RACE) was performed. The RACE products were sequenced in order to verify that the correct cDNAs had been amplified. The contigs corresponding to the full-length cDNAs were used for repeat searches in GenBank for identification of the genes.

The translated product of the cDNA corresponding to UVB.35 contained a domain involved in plant defence, the Toll-Interleukin-1 receptor-similar domain (TIR, Hammond-Kosack and Jones 1997), and was therefore named TIR-similar-domain-containing protein (TSDC), and the gene was named *PsTSDC*. TSDC showed similarities with TIR from two translated products of cDNA clones LM6 and MG13 from soybean. Forty-seven per cent identity and 57% similarity (LM6), and 41% identity and 59% similarity (MG13) were found, respectively, between the pea- and soybean-derived amino acid sequences (Graham et al. 2000). Thirty-nine per cent identity and 58% similarity on the amino acid level were shown between the TIR domain in the *Synchytium endobioticum* resistance protein NL27 from *Solanum tuberosum* and the corresponding domain in TSDC. A second domain similarity was found between NL27 and TSDC, and this domain was overlapping the TIR-domain in TSDC. Twenty-two per cent of the amino acids were identical, and 42% of the amino acids were similar in the two proteins (Hehl et al. 1999). This domain was an NB-ARC domain (nucleotide-binding domain shared by PAF-1, certain R gene products and CED-4; van der Biezen and Jones 1998). The three kinase motifs in NB-ARC that are part of the nucleotide binding can also be found in TSDC. Comparison of the amino acid sequences of the NB-ARC domain and TSDC showed 50% identity and 69% similarity for kinase 1a motif of NB-ARC, 25% identity and 43% similarity for the kinase 2 motif, and 19% identity and 38% similarity for the kinase 3 motif. The translational product for this cDNA is shown in Fig. 1 in an alignment with *S. tuberosum* NL27 protein, with the three kinase motifs and three other motifs of NB-ARC-containing proteins indicated (van der Biezen and Jones 1998).

The protein corresponding to the contig of cDNA UVB.48 showed high similarities, 53% identity and 70% similarity, to a putative embryo-abundant protein (EMB) in *A. thaliana* (Lin et al. 1999) and to an embryo-abundant protein in *Picea glauca*, with 42% identity and 59% similarity (Dong and Dunstan 1999, see Fig. 2a for alignment of the translated products from the three different plants).

The contig of UVB.53.2 was verified to consist of the cDNA for SAMS, which previously had been sequenced in *P. sativum cv. Alaska*. Three per cent of the amino acids were dif-

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**Table 1** Summary of the cDNAs isolated in this study and their expression pattern after UV-B irradiation and ozone exposure

<table>
<thead>
<tr>
<th>SSH clone</th>
<th>Gene name</th>
<th>Protein</th>
<th>Results Northern analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVB.35</td>
<td><em>PsTSDC</em></td>
<td>TIR-similar-domain containing protein</td>
<td>Up</td>
</tr>
<tr>
<td>UVB.48</td>
<td><em>PsEMB</em></td>
<td>Embryo-abundant protein</td>
<td>Up</td>
</tr>
<tr>
<td>UVB.53.1</td>
<td><em>PsSUT</em></td>
<td>Sucrose transport protein</td>
<td>Larger band down</td>
</tr>
<tr>
<td>UVB.53.2</td>
<td><em>PsSAMS</em></td>
<td>S-adenosylmethionine synthase</td>
<td>Up</td>
</tr>
<tr>
<td>UVB.55</td>
<td><em>PsRSP3E</em></td>
<td>Ribulose-5-phosphate-3-epimerase</td>
<td>Down</td>
</tr>
<tr>
<td>UVB.61</td>
<td><em>PsUOSI</em></td>
<td>UV-B and ozone similarly regulated protein</td>
<td>Down</td>
</tr>
</tbody>
</table>
Low UV-B level dependent gene regulation

Different according to the theoretical alignment of the translated cDNA sequences from the Alaska and Greenfeast cultivars (Gómez-Gómez and Carrasco 1996).

The protein corresponding to the UVB.55 cDNA showed high similarity to the ribulose-5-phosphate-3-epimerase cDNA from *A. thaliana* (89% identity and 94% similarities between the translated amino acid sequences, respectively, Favery et al. 1998) and *S. oleracea* (Nowitzki et al. 1995, again 89% identity and 94% similarities between the translated amino acid sequences, respectively). An alignment of the translated proteins from the three different species is shown in Fig. 2b.

The cDNA UVB.61 was considered to correspond to a novel protein since no similarities to any known protein was found. However, Fig. 2c shows the alignment (63% identity and 74% similarity) between the translation product of UVB.61 and the translation of an open reading frame from *A. thaliana*, chromosome 4 (GenBank accession number CAB37466 and AL035526). This protein was named UOS1 (UV-B and ozone similarly-regulated protein 1), and the gene was called *PsUOS1*.

For the cDNA UVB.53.1, cloning of any full-length cDNA was abandoned since this cDNA (for the sucrose transport protein) had already been sequenced in pea (Tegeder et al. 1999).

**Expression of the different genes after UV-B irradiation and ozone fumigation**

The expression for the cDNAs obtained with SSH was studied with Northern blotting to assure a truly differential gene regulation pattern after UV-B irradiation. In order to get indications on the stress-specificity of the regulation of the different genes, the cDNA abundance was studied after ozone fumigation, in addition to after UV-B treatment. Two different ozone experiments were performed, one in open-top chambers in the field (0–6 h of exposure) and one in environmentally controlled indoor cabinets (0–48 h of exposure).

For the TIR-similar-domain-containing protein (cDNA UVB.35) two bands of mRNA appeared on the blot, a larger one (approx. 2.4 kB, probably corresponding to the protein which is translated from a transcript of at least 1.7 kB in size) and a smaller one (approx. 1.4 kB). The abundance of both mRNAs was higher after 6 h of UV-B irradiation compared with the non-detectable level in the UV-A irradiated leaves, and the mRNA level increased even further for the smaller band until the 12 h time-point. After ozone fumigation in the open-top chambers, the two mRNAs were visible already after 4 h of exposure and increased further in plants exposed for 6 h. In the longer exposures the two mRNAs are present at 12 h of exposure in very low abundance and then more or less disappeared in the later time-points (Fig. 3a).

The Northern blot hybridised with cDNA for the embryo-abundant protein (UVB.48) showed two bands approximately 2.0 kB and 1.5 kB in size that both could correspond to functional mRNAs since the coding region was 786 bp in length. The size of both mRNAs was higher after 6 h of UV-B irradiation compared with the non-detectable level in the UV-A irradiated leaves, and the mRNA level increased even further for the smaller band until the 12 h time-point. After ozone fumigation in the open-top chambers, the two mRNAs were visible already after 4 h of exposure and increased further in plants exposed for 6 h. In the longer exposures the two mRNAs are present at 12 h of exposure in very low abundance and then more or less disappeared in the later time-points (Fig. 3a).

The Northern blot hybridised with cDNA for the embryo-abundant protein (UVB.48) showed two bands approximately 2.0 kB and 1.5 kB in size that both could correspond to functional mRNAs since the coding region was 786 bp in length. The larger mRNA was hardly affected at all by UV-B irradiation, whereas the smaller mRNA was clearly up-regulated after 12 h of exposure from a very low level (not detectable) in the leaves exposed to UV-A only. On the blots containing samples isolated from ozone-fumigated plants, the two mRNAs were visible already after 4 h of exposure and increased further in plants exposed for 6 h. In the longer exposures the two mRNAs are present at 12 h of exposure in very low abundance and then more or less disappeared in the later time-points (Fig. 3a).

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Low UV-B level dependent gene regulation

This expression pattern was most likely caused by diurnal rhythms (Fig. 3b).

For the sucrose transport protein (UVB.53.1), the corresponding coding region of which was approx. 2.0 kB, three different mRNAs were visible (2.8 kB, 1.8 kB and 1.4 kB in size). Obviously, only the larger message could correspond to the full-length transcript. The pattern of UV-B and ozone regulation was complex and differed between experiments. However, generally the larger message was decreased by both stresses, and the lowest band was increased by UV-B (not shown). Possibly, UV-B irradiation induced degradation of the full-length mRNA.

The mRNA levels for the SAMS (UVB.53.2; the mRNA and the coding region being approximately 2.0 and 1.5 kB, respectively) were clearly increased after UV-B radiation. After 12 h of UV-B irradiation the expression had increased with approx. 13-fold compared with the UV-A control. Ozone exposure, both in the field and in the environmentally controlled cabinets, altered the expression of SAMS but to a lesser extent than UV-B. An up-regulation could be seen after 6 h of fumigation in the field (approx. 4-fold of the controls). This up-regulation could also be seen after exposure in the cabinets. In the 12 h time-points the mRNA levels were approx. 3-fold higher than in the control. After 24 h of fumigation the levels of the transcripts were reduced to 42% of the control but again increased after 36 h of exposure to approx. 4-fold the control. At the 48 h time-point no difference in expression could be seen in control and exposed plants. Most probably, and similarly to the case with PsEMB, the PsSAMS gene was under...
Low UV-B level dependent gene regulation
diurnal control as well as regulated by UV-B and ozone stresses (Fig. 3c).

For ribulose-5-phosphate-3-epimerase (UVB.55), one band was found on the blots, 1.8 kB in size, compared with its coding region which was 843 bp (Fig. 4a). The mRNA levels were strongly reduced after 3 and 12 h of irradiation (to 37% and 28% compared with the controls) but not altered after 6 h of exposure. For leaves exposed to ozone, the mRNA abundance was also strongly reduced after 12 h of fumigation (to 18% of the control) and a minor reduction was also found after
24 h of ozone exposure (reduction to 65% of the transcript levels in the control). After 36 and 48 h of ozone fumigation the mRNA levels had returned to control levels showing that the down-regulation was transient. In the earlier time-points, with RNA from the field experiment, a slight reduction was found already after 2, 4 and 6 h of ozone exposure (reduction to 65%, 73%, 82% of the transcript levels in the controls).

For PsUOS1 (UVB.61), with a coding region of approx. 2.5 kB, three bands (2.7 kB, 1.8 kB and 1.4 kB) were regulated by UV-B irradiation (Fig. 4b). Both the larger and the two smaller bands were clearly down-regulated after 3 and 12 h of UV-B irradiation (a reduction by 65% and 40%, respectively, for the upper band, and by 65% and 50%, respectively, for the middle band). At the 6 h time-point, however, no regulation was seen, indicating that the diurnal control over-rides the stress-regulation of this gene. The same pattern of expression under UV-B radiation was seen in both UVB.55 and UVB.61 (i.e. regulation after 3 and 12 h, no regulation after 6 h). After 12 h of ozone fumigation the expression was also severely reduced (by 84% for the upper band and by 96% for the middle one) but not affected at the earlier time-points (open-top chambers) and only to a smaller extent at the later ones (environmentally controlled cabinets).

Discussion

In order to isolate and characterise genes expressed during UV-B irradiation, SSH was performed on cDNAs obtained from total RNA samples of UV-B-treated pea leaves. Out of forty-eight isolated clones, six turned out to be truly regulated by UV-B radiation (eight when considering the redundancy of three identical cDNAs encoding for the sucrose transport protein). This efficiency of the method was similar to the efficiency found in a previous study (Sävenstrand et al. 2000). In order to obtain a larger number of differentially regulated genes and to reduce the number of rRNA-derived clones (Sävenstrand et al. 2000), the driver population of the present study was spiked with some additional rRNA before the subtraction step. This was anticipated to remove the remaining rRNA-derived molecules present in the tester sample after any incomplete removal of rRNA during mRNA isolation. Twelve of the forty-eight cDNA clones were sequenced, and no mRNA cDNAs were found among these clones. Thus, the addition of rRNA did, in fact, improve the results in this respect.

SSH was performed with mRNA isolated from leaves exposed to both UV-A and UV-B radiation in the tester population and mRNA isolated from leaves exposed to UV-A alone in the driver population. This was done to assure that the genes obtained were regulated by UV-B only and not by UV-A. In order to find UV-B-specific regulation of genes, the plants were exposed to low levels of UV-B (2.8 kJ m$^{-2}$ in 6 h). This is in fact lower than a daily UV-B exposure under a clear sky in Lund, Southern Sweden, at midsummer (4.8 kJ m$^{-2}$, Yu and Björn 1997). The corresponding levels of UV-B irradiation were chosen for the SSH to be comparable to a previous study, where the expression of a low level UV-B-regulated gene (the sada gene) was shown (Brosché and Strid 1999). Also, the efficiency of the SSH had to be considered in this respect. Choosing an even lower UV-B level could have led to such a low expression level of the UV-B induced genes that difficulties in finding them with this type of method might have been encountered (Brosché et al. 2002).

UV-B-regulated genes

The novel TIR-similar-domain-containing protein, corresponding to the cDNA clone UVB.35, PsTSDC, carries a region of a structural domain associated with disease resistance function, the Toll-Interleukin-1 receptor-similar domain (TIR). This domain shows homology to the cytoplasmic Drosofila melanogaster Toll protein and to the mammalian interleukin-1 receptor (Hammond-Kosack and Jones 1997, Hehl et al. 1999). The NB-ARC domain (nucleotide-binding domain shared by APAF-1, certain R gene products and CED-4) is a motif shared by plant resistance genes from for example Arabidopsis and tomato and regulators of cell death in animals (van der Biezen and Jones 1998, Jaroszewski et al. 2000). The domain consists of kinase 1 (P-loop), kinase 2, and kinase 3 motifs and other short conserved motifs with unknown function. The TIR domain, together with the NB-ARC domain, is thought to be involved in the signal transduction after pathogen attack (Hammond-Kosack and Jones 1997). The mRNA levels of PsTSDC increased as early as after 6 h of UV-B exposure from low levels in the controls, and the response to ozone fumigation in the open-top chambers appeared even earlier. The response during UV-B and ozone exposure has previously been shown to be similar to the one after pathogen attack (Sävenstrand et al. 2000). Thus, it is possible that the PsTSDC protein could be involved in medium-early signal transduction during stress. When searching the A. thaliana genome for a PsTSDC homologue, no such gene was found. The lack of Arabidopsis TSDC proteins implies differences in stress responses between plant species.

The DNA sequence of the UVB.48 clone showed high similarity to a cDNA for embryo-abundant protein (EMB) in white spruce, in which species the gene was highly expressed in embryonic tissues and decreased to a lower expression level during development (Dong and Dunstan 1999). In untreated 3-week-old leaves from pea, mRNAs were present at low levels, and the expression was increased under UV-B irradiation and ozone fumigation (both in the open-top chamber and in the controlled cabinets). This is, to our knowledge, the first report on regulation of expression of any EMB gene during any stress condition.

The sucrose transport protein cDNA (UVB.53.1) had previously been isolated from a cotyledonary cDNA library from pea (Tegeder et al. 1999). However, the mRNA was expressed throughout the whole plant suggesting a more general role in sucrose transport than solely in developmental processes.
SAMS catalyses the formation of S-adenosyl-l-methionine (SAM) from methionine and ATP. SAM is the donor in most biological methylation reactions including methylation of DNA, RNA and proteins (Schröder et al. 1997). SAM is also active in the production of ethylene which has been identified as a signaling component in several different defense responses (A-H-Mackerness 2000, Tuomainen et al. 1997). Previously, two genes, SAMS1 and SAMS2, were cloned in pea, and differential expression of these two genes was previously reported during plant development (Gómez-Gómez and Carrasco 1998). The mRNA of UBV.53.2 (corresponding to SAMS1) was present at low levels in control plants and increased during UV-B irradiation, and the expression was also altered as a consequence of ozone fumigation (under both conditions used in this study). Accumulation of SAMS mRNA after salt stress and by bacterial elicitors has been observed (Gómez-Gómez and Carrasco 1998), in addition to induction after ozone exposure (Tuomainen et al. 1997). Increased levels of SAMS3 mRNA from tomato could be seen in addition to increased emission of ethylene (Tuomainen et al. 1997). No induction of SAMS after UV-B exposure has been reported so far although increased emission of ethylene from leaves in A. thaliana has been measured after UV-B irradiation (A-H-Mackerness et al. 1999). Interestingly, SHH (S-adenosyl-homocysteine hydrolase), another enzyme yielding SAM, showed a moderate induction in mRNA levels after exposure of parsley cells to UV radiation (Logemann et al. 2000). Increasing levels of SAMS after UV irradiation (as indicated in this study) might lead to an increased production of ethylene but may also indicate an increased need of activated methyl groups in general (Tuomainen et al. 1997).

The ribulose-5-phosphate-3-epimerase (corresponding to clone UBV.55) is an enzyme in the chloroplast-localised oxidative pentose phosphate pathway (OPPP) and also in the reductive Calvin cycle. R5P3E catalyses the reversible interconversion of ribulose-5-phosphate and xylose-5-phosphate (Favery et al. 1998). In our study, the mRNA levels of ribulose-5-phosphate-3-epimerase decreased during UV-B irradiation and also after ozone fumigation, most clearly in the later time-points from the fumigations performed in the environmentally controlled cabinets. Generally, reduction of mRNA levels for chloroplast-localised proteins is a known response to UV-B radiation (see above; Strid 1993, Brosché et al. 1999).

The translated product of clone UBV.61 (PsUOS1) was considered a novel protein since no similarities with previously known proteins were found by searching GenBank. The levels of mRNA corresponding to UBV.61 were reduced after UV-B irradiation, and a similar reduction could be seen after ozone exposure, especially after the longer exposure in the environmentally controlled cabinets. Further studies of the function of this gene during stress would be of great importance.

In addition to the regulation of gene expression exerted by UV-B and ozone stress, several of the genes were also under diurnal control (most clearly PsEMB, PsSAMS). This diurnal regulation led to a complex pattern of transcript abundance as a function of both the extent of stress exposure and of what time of day the leaf samples were collected. In fact, the daily rhythms were clearly more important as determinants of message levels than were the extent of UV-B irradiation or ozone fumigation. This was reminiscent of the developmental control of gene expression which was found for photosynthetic genes in pea during UV-B stress (Jordan et al. 1994) where the normally very clear decrease in transcript abundance in mature leaves was completely overridden by the developmentally controlled massive increase in photosynthetic mRNAs in developing leaf buds.

**Conclusion**

In this study, several genes were found to be differentially regulated by UV-B, most of which were not previously known to possess this type of regulation pattern. In order to study the stress-specificity of these genes, the expression was also monitored after ozone fumigation in two separate experimental set-ups. All genes that were expressed during UV-B irradiation turned out to be expressed in a similar way after ozone exposure, independent of if the fumigation was performed in the field or in controlled cabinets. This is in contrast to the findings of a previous study (Sävenstrand et al. 2000) where several of the genes tested were differently regulated by UV-B and ozone. Thus, the genes described herein are generally stress-regulated.

**Materials and Methods**

**Plant material**

For UV-exposure, *Pisum sativum* L. (cv. Greenfeast) were grown for 21 d in 12 h light/12 h darkness at 100 μmol photons m⁻² s⁻¹. On day 21, the population was, in addition to visible light, irradiated with 1.4 μmol photons m⁻² s⁻¹ UV-B or 0.49 μmol photons m⁻² s⁻¹ UV-A by covering the UV-B source (Philips TL40W/12UV) with one layer of either cellulose acetate (wavelength cut-off 292 nm) or mylar film (wavelength cut-off 315 nm). The biologically effective radiation was
For ozone treatment, the plants were grown in either environmentally controlled indoor cabinets or in open-top chambers in the field. In the environmentally controlled cabinets, 16 h light (8 a.m. until midnight) at 22°C, 170 µmol photons m⁻² s⁻¹ and 8 h of darkness at 20°C were applied. After 19 d, half of the plants were transferred to a corresponding cabinet supplied with 100 ppb of ozone. The exposure started at 09:00 a.m. and the plants were exposed for 12 h in each 24 h period. The last fully expanded pair of leaves was harvested after 12, 24, 36 and 48 h of treatment and immediately frozen in liquid nitrogen.

The open-top chamber experiment was performed at Östad säteri, 50 km north-east of Göteborg, Sweden from August until October. Due to the sun angle and the height of the chambers, no direct sunlight, and thus no UV-B radiation, reached the plants. The peas were grown for 3 weeks in the open-top chambers supplemented with charcoal-filtered air (approximately 5 ppb ozone). On day 21, half of the plants were transferred to chambers with charcoal-filtered air supplied with approximately 100 ppb of ozone. The second pair of leaves from the base of the plants was harvested after 2, 4, and 6 h of exposure and frozen in liquid nitrogen.

**RNA isolation, Northern blotting and hybridisation**

Total RNA was isolated from leaves harvested according to Strid et al. (1996) and stored at -80°C. RNA was separated on 1.2% agarose containing formaldehyde and transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Amersham, U.K.). cDNAs obtained by SSH (see below) were randomly labelled with [³²P]dCTP according to the manufacturer’s instructions and used as probes (Life Technologies, Rockville, MD, U.S.A.). Church buffer was used for prehybridisation and hybridisation (Church and Gilbert 1984). Equal loading of RNA was controlled using a randomly labelled 18S ribosomal RNA cDNA (pÅS3; Kalbin et al. 1997) to the same membrane. A-H-Mackerness, S. (1999) Molecular markers for UV-B stress in plants: alteration of the molecular expression of a small pea gene family regulated by low levels of ultraviolet B radiation under environmental stresses. Plant Growth Regul. 121: 479–487.

**Suppression subtractive hybridisation**

SSH was performed according to the manufacturer’s instructions (Clontech, Palo Alto, CA, U.S.A.). The tester population contained mRNA isolated from leaves exposed for 6 h to both UV-B and UV-A. The driver population contained mRNA and supplementary rRNA obtained by SSH (see below) were randomly labelled with [³²P]dCTP according to the manufacturer’s instructions and used as probes (Life Technologies, Rockville, MD, U.S.A.). Church buffer was used for prehybridisation and hybridisation (Church and Gilbert 1984). Equal loading of RNA was controlled using a randomly labelled 18S ribosomal RNA cDNA (pÅS3; Kalbin et al. 1997) to the same membrane after stripping. mRNA was isolated from total RNA using oligo dT Dynabeads according to the manufacturer’s instructions (Dynal A/S, Oslo, Norway).

**Cloning**

cDNAs obtained with SSH were cloned into the pCR-TOPO vector using the TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). A first screening was performed by transferring 48 cDNA clones to a nylon membrane Hybond-N by slot-blotting and by hybridisation of the resulting slot-blot to [³²P]dCTP randomly labelled driver. Clones that did not hybridise with the driver population were considered interesting and were further analysed. The Marathon amplification kit (Clontech, Palo Alto, CA, U.S.A.) was used to amplify the 5’- and the 3’-ends of the SSH cDNAs. The gene-specific primers used for amplification with the Marathon kit were as follows: UVB.53.2 5’ d(GAAGACCTCGTTGGATGATC); UVB.52.2 3’ d(TGATGAGACTCCTGAAGTGATG); UVB.53.5 5’ d(TCGACTCTCTGTGTTAGAATCT); UVB.53.5 3’ d(TGCTGTTAATCAATGTAAGAGCCT); UVB.61.5 5’ d(ATTATTTCCGGATGAACACACC); UVB.61.3 3’ d(GTCCGTCTAAAGGTGTGAGGCC).

For UVB.48 no 3’-RACE was performed, since the 3’-end was present in the SSH cDNA. The cDNAs obtained were cloned into the pEco vector (Invitrogen, Groningen, The Netherlands) and verified by sequencing.

**DNA sequencing**

Sequencing was performed by using the Amersham ThermoSequenase Fluorescent Sequencing kit (Amersham Pharmacia Biotech, Amersham, U.K.) and analysed with a Pharmacia ALF sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). The sequences were analysed with programs in the Lasergene software package (DNASTAR Inc, Madison, WI, U.S.A.), and the contigs obtained were compared with sequences in GenBank.

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**References**


Diatcchenko, L., Lau, Y-F.C., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Guriksaya, N., Sverdlov, E.D. and...


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