The Effects of Ultraviolet-B Radiation on Gene Expression in Douglas-fir

by

Anna-mary Schmidt
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[redacted]

Dr. S. Misra, Supervisor (Department of Biochemistry and Microbiology)

[redacted]

Dr. D.P. Ormrod, Co-Supervisor (Department of Biology)

[redacted]

Dr. C. Upton, Departmental Member (Department of Biochemistry and Microbiology)

[redacted]

Dr. W.E. Hintz, Outside Member (Department of Biology)

[redacted]

Dr. A. K. Mattoo, External Examiner (USDA, Maryland)

Supervisor: Dr. Santosh Misra

ABSTRACT

Depletion of the stratospheric ozone layer is leading to an increase in ultraviolet-B (UV-B) radiation reaching the earth's surface. The effects of UV-B on gene expression in Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) were examined during seed germination and early seedling development. Douglas-fir seeds were stratified, then germinated in a controlled environment chamber and exposed to UV-B doses of 0 or 7 kJ m^{-2} d^{-1}. Transcript levels for *hsp90*, *BiP* (luminal binding protein), *cpr* (NADPH:cytochrome P450 reductase) and ubiquitin increased in response to UV-B exposure. Correspondingly, UV-B treatment led to increases in HSP90, BiP and CPR protein levels. In contrast, *cab* (chlorophyll *a/b*-binding protein) transcript amounts were marginally reduced in response to UV-B. These results suggest a role for heat shock proteins, CPR and ubiquitin in the protection of Douglas-fir from the effects of UV-B radiation.

Examiners: [redacted]

Dr. S. Misra, Supervisor (Department of Biochemistry and Microbiology)

[redacted]

Dr. D.P. Ormrod, Co-Supervisor (Department of Biology)

[redacted]

Dr. C. Upton, Departmental Member (Department of Biochemistry and Microbiology)

[redacted]

Dr. W.E. Hintz, Outside Member (Department of Biology)

[redacted]

Dr. A. K. Mattoo, External Examiner (USDA, Maryland)

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BiP	luminal binding protein
CAB	chlorophyll *a/b*-binding protein
cDNA	complementary DNA
CHI	chalcone isomerase
CHS	chalcone synthase
CPD	cyclobutane pyrimidine dimer
CPR	NADPH:cytochrome P450 reductase
DEPC	diethyl pyrocarbonate
DFR	dihydroflavonol reductase
HSP	heat shock protein
HSP90	90-kDa heat shock protein
JA	jasmonic acid
kDa	kilodaltons
LMW HSP	low molecular weight heat shock protein
PAL	phenylalanine ammonia-lyase
PAR	photosynthetically active radiation, 400-700 nm
PR	pathogenesis-related
rRNA	ribosomal RNA
ROS	reactive oxygen species
Rubisco	ribulose 1,5-bisphosphate carboxylase/oxygenase
SA	salicylic acid
SOD	superoxide dismutase
Ubi	ubiquitin
UV-B	ultraviolet-B, 280-320 nm
$UV\text{-}B_{BE}$	biologically effective UV-B

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CHAPTER 1: LITERATURE REVIEW

I. INTRODUCTION

Wherever they grow, plants are constantly challenged by a great variety of stresses tending to restrict their chances of survival and development. In consequence, they have developed an impressive array of coping mechanisms to defend themselves against different forms of stress. In view of the diversity and changing climatic conditions of the global environment, comprehensive investigation of the impact of environmental stresses on plants is of paramount importance. This study focuses on the examination of the effects of ultraviolet-B (UV-B) radiation on gene expression in Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) during seed germination and early seedling development.

A. Stratospheric ozone depletion and increased solar UV-B

The evolution of terrestrial plant life was made possible by the presence of ozone within the stratosphere of the earth (20–50 km above the earth's surface). In fact, all life on earth is protected from ultraviolet radiation by this ozone, which absorbs all of the solar UV-C (<280 nm) and part of the solar UV-B (280–320 nm) radiation.

Diminution of stratospheric ozone is a well-documented phenomenon and will be very much an issue in the coming several decades. The destruction of ozone is so extensive that overall losses of 50% have been recorded over Antarctica, representing approximately 5% of global ozone (Austin *et al.*, 1992). Although the Antarctic may be a dramatic example of ozone reduction, in the last decade general stratospheric

ozone reduction at temperate latitudes has been much greater than predicted by atmospheric models (Gleason *et al.*, 1993; Caldwell and Flint, 1994). The destruction of the ozone layer is thought to be caused primarily by man-made chlorofluorocarbon (CFC) and other halons. The CFCs are particularly long-lived molecules and have been widespread in their use as refrigerants, aerosol propellants and in the manufacture of foam. Although damaging CFCs are being phased out, they will remain a significant problem due to their longevity and high levels of accumulation in the troposphere, allowing for slow permeation into the stratosphere. The recovery to pre-ozone depletion levels is expected to take place gradually over the next 50 years (Madronich *et al.*, 1995).

An inescapable consequence of the decreased ozone concentration in the stratosphere is that the levels of UV-B radiation reaching the earth's surface are increasing and will continue to do so in the foreseeable future (Kerr and McElroy, 1993; Madronich *et al.*, 1998). The primary concern of a global decline in stratospheric ozone is the increased penetration of the shorter, more energetic wavelengths within the UV-B range; despite the small quantities of UV radiation involved when compared to the total solar spectrum, even modest increases are significant since shorter UV-B wavelengths are particularly biologically damaging (Caldwell and Flint, 1994).

B. UV-B damage to plants

As photosynthetic organisms, plants categorically require sunlight. Because plants cannot avoid light (through behavioral responses), they are at particular risk to

enhanced UV-B exposure. The effects of UV-B radiation on plant growth and physiology have been studied extensively (reviewed in Caldwell and Flint, 1993; Ormrod and Hale, 1995; Jordan, 1996). A wide range of UV-B responses can occur, often injurious but sometimes beneficial, depending on species, nature of the UV-B treatment and the interaction of other environmental factors.

Of the many direct and indirect deleterious effects UV-B has on plants, the most important include damage to DNA, proteins and membranes (Table 1); alterations in photosynthesis and respiration; effects on flowering and reproduction; and changes in growth, development and morphology. These responses to UV-B radiation may lead to reduced productivity and yield and to significant changes in the nature of plant communities.

II. UV-B PROTECTIVE MECHANISMS IN PLANTS

Having an obligatory requirement of light for survival, how do plants cope with enhanced solar UV-B levels? Plants employ three fundamental strategies to counteract the effects of UV-B radiation: efficient DNA repair mechanisms, production of antioxidants, and the accumulation of UV-B absorbing compounds, or "sunscreens".

A. DNA Repair

Damage caused by UV-B penetration of cells can take a range of forms (Table 1), but perhaps the most pervasive UV-B-induced damage is the photo-oxidation of DNA.

Table 1. Molecular targets of UV-B radiation
(Adapted from Rozema *et al.*, 1997; Jansen *et al.*, 1998)

DNA
Formation of cyclobutane-pyrimidine dimers and (6-4) photoproducts
Induction of chromosome rearrangements
Indirect DNA damage caused by formation of free radicals
Photosynthetic machinery
Inactivation of photosystem II (PSII)
Degradation of the D1 and D2 proteins of PSII
Decreased thylakoid membrane integrity
Reduced activity of Rubisco and other enzymes
Decreased levels of chlorophyll and carotenoids
Down-regulation of photosynthetic genes
Changes in chloroplast ultrastructure
Membranes
Peroxidation of unsaturated fatty acids
Changes in membrane lipid composition
Phytohormones
Photo-oxidation of IAA (indolyl acetic acid)

Absorbance of UV-B photons by DNA triggers the formation of cyclobutane-pyrimidine dimers (CPDs) and, to a lesser extent, pyrimidine (6-4) pyrimidinone dimers [(6-4) photoproducts] (Britt, 1996). DNA polymerase II is unable to read through unrepaired dimers, leading to defects in DNA replication; as well, RNA polymerase II has been shown to stall at both CPDs and (6-4) photoproducts (preventing transcription) and remains bound to the damaged DNA, thereby reducing the overall concentration of free RNA polymerase (Donahue *et al.*, 1994). A small fraction of pyrimidine dimer sites create "hotspots" for (heritable) mutation, but in all likelihood the inhibitory effects of UV-B on transcription and replication in plant epidermal tissues are more significant (in terms of plant growth) than its mutagenic effects (Britt, 1996).

Repair of UV-B-damaged DNA is mainly through light-dependent photoreactivation. Photoreactivation is contingent upon the enzyme photolyase which, upon absorption of radiation at the appropriate wavelength (350–400 nm), directly reverses dimerization in an error-free manner, restoring the bases to their native form (Stapleton, 1992; Britt, 1996). Mutants deficient in photolyase activity have been isolated in rice (Hidema *et al.*, 1997) and *Arabidopsis* (Landry *et al.*, 1997); both are highly UV-B sensitive and are unable to repair CPDs. Photoreactivation itself appears to function most effectively with blue light and UV-A (Sancar, 1996); fortunately, in a natural environment, UV-B is always accompanied by considerably higher levels of UV-A (320–400 nm) and photosynthetically active radiation (PAR; 400–700 nm), making solar radiation very well suited to drive photoreactivation.

Photoreactivation is only one component of DNA repair in plants. A combination of photoreactivation and excision repair activities may be needed if plants are to survive higher levels of UV-B-induced damage. Excision repair pathways are widespread from bacteria to mammals, and include both base excision repair and nucleotide excision repair. Although photoreactivation is generally a more rapid and efficient repair method for UV-B-induced DNA damage, excision repair may be essential for the repair of minor, nondimer, UV-B-induced photoproducts (Britt, 1996).

B. Antioxidants

Certain cellular components (aromatic amino acids, NADH, and phenolic compounds) can be activated by the absorption of UV-B radiation and react with molecular oxygen to form highly reactive species such as singlet molecular oxygen, superoxide radicals, hydoxyl radicals and hydogen peroxide (Foyer *et al.*, 1994; Rao and Ormrod, 1995), collectively known as reactive oxygen species (ROS). To combat the resulting oxidative damage to cellular constituents, plant cells possess a range of low molecular weight antioxidants, such as ascorbate and glutathione. Additionally, the ability of plants to metabolize active oxygen species is largely dependent on the coordination of several interrelated antioxidant enzymes including superoxide dismutase, ascorbate peroxidase and glutathione reductase.

An up-regulation of the key antioxidants ascorbate and glutathione in response to UV-B has been demonstrated in *Arabidopsis* and cucumber (Rao and Ormrod, 1995; Takeuchi *et al.*, 1996). Furthermore, UV-B boosts the activities of superoxide

dismutase (Malanga and Puntarulo, 1995; Jansen *et al.*, 1996; Rao *et al.*, 1996), ascorbate peroxidase (Landry *et al.*, 1995; Rao *et al.*, 1996; Takeuchi *et al.*, 1996), and glutathione reductase (Strid, 1993; Jansen *et al.*, 1996; Rao *et al.*, 1996). Thus, UV-B-generated oxidative stress conditions trigger a protective antioxidant response that acts to increase plant cell tolerance to UV-B stress.

C. UV-B Absorbing Compounds

Analogous to the existence of the pigment melanin in human skin, plants accumulate unique protective UV-B-absorbing compounds, or pigments, in the epidermal and hypodermal layers of leaves and stems (the most illuminated layers). These compounds, including flavonoids, isoflavonoids, anthocyanins and sinapate esters, strongly absorb light in the UV-B wavelengths (but not in the PAR waveband, which would decrease photosynthetic yields), and increase in response to UV-B irradiation (Hashimoto and Shichijo, 1991; Strid and Porra, 1992; Lois, 1994; Landry *et al.*, 1995), thus providing selective attenuation of UV-B radiation and preventing penetration to the underlying mesophyll cells and sensitive photosynthetic tissues.

Flavonoids and sinapate esters are synthesized by the phenylpropanoid pathway (Figure 1). UV-B exposure induces a rapid and coordinated expression of genes encoding enzymes in the phenylpropanoid biosynthetic pathway (Chappell and Hahlbrock, 1984; Kubasek *et al.*, 1992; Brosché *et al.*, 1999), including phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). These two are very important regulatory enzymes; PAL is the first enzyme of general

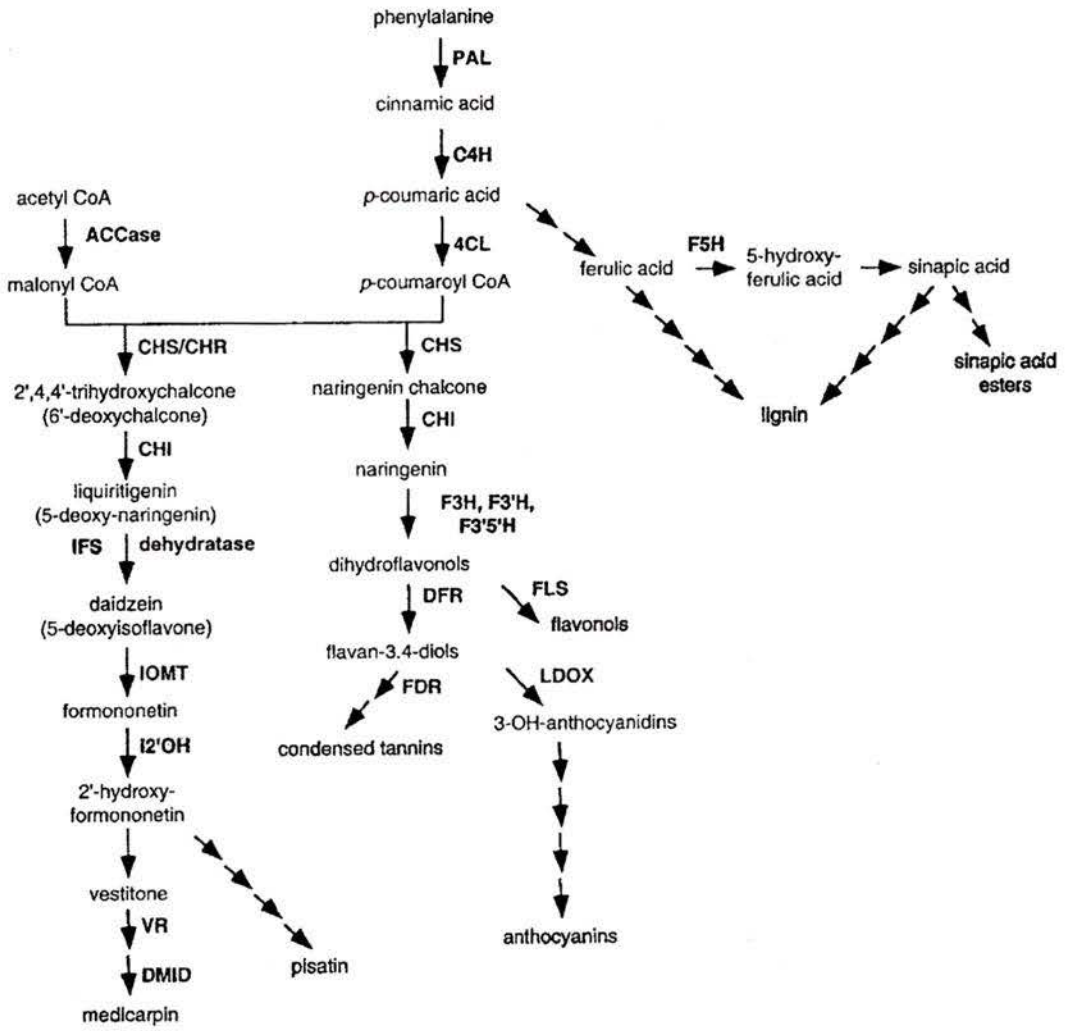

Figure 1. Schematic of the general phenylpropanoid pathway. Enzymes are indicated in bold, and abbreviated as follows: ACCase, acetyl-CoA carboxylase; C4H, cinnamate 4-hydoxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; DFR, dihdoflavonol 4-reductase; DMID, 7,2'-dihydroxy-4'-methoxy-isoflavonol dehydratase; FDR, flavan-3,4-diol reductase; F3H, flavanone 3-hydroxylase; F3'H and F3'5'H, flavonoid 3' and 3'5' hydroxylase; FLS, flavonol synthase, GST, glutathione S-transferase; IFS, isoflavone synthase; IOMT, isoflavone *O*-methyltransferase; I2'OH, isoflavone 2' hydroxylase; LDOX, leucoanthocyanidin dioxygenase; PAL, phenylalanine ammonia-lyase; VR, vestitone reductase. (Adapted from Winkel-Shirley, 1999)

phenylpropanoid metabolism, and CHS catalyses the committal step in flavonoid biosynthesis, being situated just before the point where the pathway branches into separate routes for the synthesis of different subclasses of flavonoids (and, ultimately, anthocyanins). The resulting accumulation of UV-B-absorbing compounds has clearly been shown to provide protection to normal plant functions such as photosynthesis (Tevini *et al.*, 1991; Wilson and Greenberg, 1993) and proper DNA function indicated by decreased pyrimidine dimer formation (Takakashi *et al.*, 1991). Consistent with this idea, UV-B irradiation of a flavonoid-deficient maize mutant caused increased accumulation of DNA damage products in leaf tissue (Stapleton and Walbot, 1994); as well, *Arabidopsis* mutants that do not accumulate flavonoids and/or sinapate esters are highly UV-B sensitive (Li *et al.*, 1993; Lois and Buchanan, 1994; Landry *et al.*, 1995).

Concomitant with their UV-B screening function, flavonoids also act as free-radical scavengers (Takeuchi *et al.*, 1996; Rice-Evans *et al.*, 1997), offering additional protection to cells accumulating these compounds. The activation of the flavonoid biosynthetic pathway is perhaps the best overall plant coping response to UV-B radiation, conferring a solid defence strategy to mitigate the harmful effects of UV-B.

III. EFFECTS OF UV-B ON GENE EXPRESSION IN PLANTS

The impact of increased UV-B on the physiological and morphological aspects of plants has been studied extensively; however, knowledge of the effect of UV-B at the biochemical and molecular levels is limited, focusing primarily on photosynthetic

genes or genes involved in flavonoid biosynthesis. Nevertheless, the information that is available indicates that this environmental stress has a dramatic influence on plant gene expression.

While genes encoding several important photosynthetic proteins are inhibited by UV-B radiation (Jordan *et al.*, 1991, 1992, 1994; Zhang *et al.*, 1994; Liu and White, 1998), some defence genes such as PAL, CHS and glutathione reductase are induced by UV-B (Kubasek *et al.*, 1992; Strid, 1993; Jordan *et al.*, 1994; Liu and McClure, 1995). Defence genes are up-regulated simultaneously with the down-regulation of photosynthetic genes (Strid, 1993; Jordan *et al.*, 1994; Mackerness *et al.*, 1997b; Brosché *et al.*, 1999), indicating that UV-B effects on gene expression in plants appear to be specific and not simply a result of random, non-specific damage to the DNA. In further support of this idea, some photosynthetic genes (chlorophyll *a/b* binding [*cab*] genes) are up-regulated in etiolated (light-deprived) buds while down-regulated in green leaves under the same UV-B treatments (Jordan *et al.*, 1994); additionally, the evidence for the existence of specific UV-B photoreceptors (e.g. Ballaré *et al.*, 1999) and specific UV-B signal transduction pathways (Christie and Jenkins, 1996; Fuglevand *et al.*, 1996; Frohnmeyer *et al.*, 1997; Mackerness *et al.*, 1999) also suggests that the UV-B down-regulation of gene expression may not be a result of non-specific DNA damage.

It is important to note that many factors likely influence UV-B-induced gene expression, as summarized in Table 2.

Table 2. Factors affecting UV-B induced changes in gene expression (Adapted from Jordan, 1996)

Perception of UV-B light

Perception could involve a specific photoreceptor (not yet identified) or a wide range of biologically active molecules. Recent studies consider the possibility that DNA lesions induced by UV-B (e.g. CPDs) have informational value and may regulate the transcription of specific genes (Ballaré, 1999).

Mechanisms of signal transduction

At present, these mechanisms are poorly understood. Recent research indicates the involvement of multiple signalling pathways mediating responses to UV-B, including those controlled by reactive oxygen species, jasmonic acid, salicylic acid and ethylene (Mackerness *et al.*, 1999).

Penetration of UV-B into the tissue

Penetration of UV-B largely depends on its attenuation by protective compounds (pigments) in the epidermis; mutants with reduced protective pigments show much more severe reductions in photosynthetic gene expression when exposed to UV-B (Jordan, 1996).

Damage to DNA

DNA is very susceptible to UV-B damage, and this may impair gene expression. Effective and efficient repair mechanisms are required to maintain a functional genome.

Developmental stage of tissue

Changes in gene expression are frequently dependent on the stage of development.

A. UV-B effects on photosynthesis-related gene expression

It is well established that a major site of UV-B damage is the chloroplast, leading to impairment of photosynthetic function (Bornman, 1989). In the last decade, a small number of studies have focused on the molecular mechanisms underlying photosynthetic sensitivity to UV-B radiation. Studies relating the effects of UV-B radiation on the expression of genes encoding chloroplast/photosynthetic proteins have mainly been carried out on pea (*Pisum sativum*), a highly UV-B-sensitive species. Interestingly, following UV-B treatment of pea plants, amounts of mRNA decline for all photosynthetic genes studied to date.

Chloroplast proteins are either nuclear- or chloroplast-encoded. Although the chloroplast genome codes for many proteins that participate in photosynthesis (particularly components of the thylakoid membranes), the majority of chloroplast proteins are nuclear-encoded and are synthesized in the cytosol, then transported to the chloroplast (Taiz and Zeiger, 1991).

The UV-B-induced down-regulation of several chloroplast-encoded photosynthetic genes has been reported. These genes include *psb A*, encoding the D1 protein of the photosystem II reaction centre complex (Jordan *et al.*, 1991); *rbc L*, encoding the large subunit of Rubisco (Jordan *et al.*, 1992); *pet B* and *pet D*, encoding cytochrome b and subunit IV of the cytochrome *b/f* complex, respectively (Strid *et al.*, 1994); and *atp B* and *atp E*, encoding subunit β and ε of the ATP synthase, respectively (Zhang *et al.*, 1994).

Reductions in mRNA levels for nuclear-encoded photosynthetic genes have also been detected following UV-B treatment. Among these are genes encoding for

the chlorophyll a/b-binding protein of photosystem II, *cab* (Jordan *et al.*, 1991); the small subunit of Rubisco, *rbc S* (Jordan *et al.*, 1992); and the γ subunit of the ATP synthase, *atp C* (Zhang *et al.*, 1994). Recently, Brosché *et al.* (1999) reported a UV-B-induced reduction of transcript levels for two putative chloroplast proteins (identified by differential display), one of which most likely encodes a chlorophyll *a/b*-binding protein of photosystem I.

Although both the chloroplast and nuclear genomes are affected by exposure to UV-B radiation, nuclear-encoded genes appear to be more sensitive than genes encoded in the chloroplast; thus, the chloroplast RNA levels are typically maintained for longer periods than nuclear-encoded genes (Jordan, 1996). For example, the reduction in transcripts for *cab* (nuclear-encoded) is very rapid, with an 80% reduction after only four hours; in contrast, reduction of *psb A* transcripts (chloroplast-encoded) is a slower process, in which transcript levels remain at 35% of the control level after three days of UV-B exposure (Jordan *et al.*, 1991). Furthermore, mRNA for the nuclear-encoded *atp C* gene declines more rapidly than mRNA for the chloroplast-encoded *atp B* and *atp E* genes (Zhang *et al.*, 1994); as well, transcript levels for the small subunit of Rubisco, encoded by the nuclear *rbc S* gene, decline ahead of transcript levels for the large subunit, encoded in the chloroplast by the *rbc L* gene (Jordan *et al.*, 1992).

Although specific receptors involved in the perception of UV-B have not been identified, and the key signaling pathways involved are not well understood, studies on UV-B-induced changes in gene expression for photosynthesis-related proteins

provide a molecular mechanism that can account, at least partially, for the UV-B-induced inhibition of photosynthesis.

B. UV-B effects on defence gene expression

In addition to changes in expression of genes involved in metabolism, exposure to UV-B leads to the induction of a range of genes involved in defence responses aimed at combating or reducing the impact of UV-B radiation.

Enzymes of the phenylpropanoid pathway

The first line of UV-B defence is to limit the penetration of UV-B within the tissue. Not surprisingly, most studies on UV-B-induced gene expression concern flavonoid biosynthetic genes from various plant species. As discussed earlier (*UV-B Protective Mechanisms in Plants: C. UV-B absorbing compounds*), exposure to UV-B results in the accumulation of flavonoids, sinapate esters and anthocyanins, compounds that selectively attenuate UV-B radiation. The increase in the level of these compounds is due to a coordinated UV-B-induced increase in the expression and activity of the enzymes of the phenylpropanoid pathway. UV-B-inducible phenylpropanoid genes include those encoding phenylalanine ammonia lyase (PAL), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) (Jordan, 1996, and references therein; see Figure 1).

Chappell and Hahlbrock (1984) were among the first to demonstrate that the production of flavonoids is preceded by the UV-B induction of coordinately regulated enzymes, reporting a small, but distinct, difference in the timing of induction of PAL and CHS. These early investigations were somewhat limited in their scope, as

parsley cell suspension cultures were regularly used as a model system. Presently, it is the general consensus that the time course of induction and the sequence in which different enzymes in the phenylpropanoid pathway are expressed are generally quite different when cell suspension cultures and seedlings of the same species are compared (Halbrock and Scheel, 1989); increasingly, therefore, studies have concentrated on whole plant systems using *Arabidopsis*, tobacco and pea as model plants.

Using *Arabidopsis* seedlings as a model system, Kubasek *et al.* (1992) reported the sequential UV-B induction of flavonoid genes in the order of the biosynthetic steps in the flavonoid pathway; that is, PAL mRNA began to accumulate before CHS and CHI mRNAs, which, in turn, began to accumulate before DFR mRNA (refer to Figure 1). Although most genes of the phenylpropanoid pathway can be activated by a variety of other environmental stimuli (wounding, pathogen attack, fungal elicitors, blue light, high-intensity white light), UV-B irradiation is most effective in the induction of this pathway (Kubasek *et al.*, 1992; Jordan *et al.*, 1994).

Antioxidants and antioxidant enzymes

Although the accumulation of UV-B absorbing compounds is a highly effective defence against UV-B radiation, no pigment can completely block all UV-B radiation penetration into the mesophyll. Plants also employ a number of tactics to reduce the immediate impact of UV-B damage within the tissues. For example, UV-B exposure leads to ROS generation and oxidative stress; in turn, low fluences of UV-B induce the expression and activity of a number of antioxidants and antioxidant enzymes including glutathione, ascorbate, ascorbate peroxidase, superoxide dismutase and

glutathione reductase (see *UV-B Protective Mechanisms in Plants: B. Antioxidants*). Although the signal transduction pathways by which UV-B regulates gene expression are at present poorly understood, recent studies suggest a potential role for ROS as signaling intermediates involved in regulation of gene expression in response to UV-B (Mackerness, 2000).

Pathogenesis-related proteins

Interestingly, a number of pathogenesis-related (PR) proteins have also been shown to increase in response to supplementary UV-B exposure. These include PR-1, -2, -3, and -5 (Green and Fluhr, 1995; Surplus *et al.*, 1998; Fujibe *et al.*, 2000), and a defencin protein (Mackerness *et al.*, 1999).

It is tempting to speculate that the induction of these PR proteins is another defence mechanism employed by plants under UV-B stress, since exposure to UV-B can increase the vulnerability of plants to infection by an array of pathogens (Panagopoulos *et al.*, 1992, and references therein); thus, production of general PR proteins could ensure defence against any potential opportunistic pathogens. However, it is difficult to determine exactly which stimulus is producing a particular response; most likely, UV-B signaling pathways share components of the pathogen-inducible pathway.

Other defence genes

A small number of disparate genes/proteins loosely classified in the "defence" category have shown increased expression in response to UV-B radiation.

a) Heat shock proteins (HSPs)

There are indications that the synthesis of heat shock-like proteins is induced by UV-

B exposure. Nedunchezhian *et al.* (1992) reported large increases in the levels of three groups of proteins in the range of 70, 53, and 16 kDa, similar to those observed after heat shock treatment, in *Vigna sinensis* seedlings grown at 30°C (natural growth temperature) and exposed to UV-B.

These proteins may play a protective role, although their exact function is presently unknown. Plants respond not only to elevated temperatures, but to many other stresses with the production of HSPs. The mechanism by which HSPs may afford protection has not been determined in detail, but it is known that several HSPs function as 'molecular chaperones'. As a group, molecular chaperones are quite diverse and serve a variety of functions that are specific to different chaperones and include: facilitating folding of nascent proteins as they exit the ribosome, promoting folding of proteins to their final native state, holding substrates in an unstructured form that is competent for membrane transport, maintaining proteins in specific conformations, preventing aggregation of unfolded proteins, and promoting renaturation of aggregated proteins (Boston *et al.*, 1996). The latter two functions are particularly important for cells experiencing stress.

b) NADPH:cytochrome P450 reductase (CPR)

Recent studies have shown a strong up-regulation by UV-B of two NADPH:cytochrome P450 reductases (CPRs): CPR1 from parsley (Koopmann and Hahlbrock, 1997) and *PsC450R1* from pea (Brosché *et al.*, 1999). Some of the steps in the flavonoid biosynthetic pathway are catalyzed by cytochrome P450 monooxygenases, which are dependent on the supply of electrons from an associated CPR (which catalyzes the transfer of electrons from NADPH via FAD and FMN to

the prosthetic heme group of the cytochrome P450 monooxygenase protein). Since many enzymes in the flavonoid pathway are regulated by UV-B, it makes sense that any CPR involved in the same biosynthetic pathway would also follow this pattern of induction by UV-B.

c) Ubiquitin

Ubiquitin is a protein found in all eukaryotes analyzed to date and is encoded by a family of polyubiquitin genes. The ubiquitin protein is used by the cell to label proteins for degradation, and its expression is often enhanced when cells have to cope with different stress conditions (von Kampen *et al.*, 1996). Two independent studies have shown that the polyubiquitin gene *PU1* of pea is up-regulated in response to UV-B (Brosché and Strid, 1999; Brosché *et al.*, 1999). The presence of ROS has been thought to induce protein ubiquitination in some plant species (Shimogawara and Muto, 1991); coupled with the conceivable degradation of certain proteins upon UV-B exposure, these factors may at least partially account for the increased expression of ubiquitin by UV-B.

IV. FACTORS INFLUENCING EFFECTS OF UV-B ON GENE EXPRESSION

Little is known about the interaction of UV-B with other stresses and the factors that affect the sensitivity or response of plants to UV-B radiation, and even less is known about the effects on gene expression. Plants respond to an array of stresses through common defence mechanisms, and therefore exposure to one stress may lead to protection from or tolerance of another (Jordan, 1996, and references therein). Although the underlying basis for this phenomenon is only partially characterized,

current research indicates that interactions between components of isolated pathways are responsible for the integration of responses to multiple environmental stimuli.

A. Multiple concurrent stress factors

Nature is not a controlled environment growth chamber – any outdoor plant during its life cycle is exposed to a wide range of environmental conditions. Several major environmental stresses may be implicated with UV-B stress responses, including light (high or low PAR), water deficit, CO_2, tropospheric ozone, temperature and pathogen attack.

A handful of studies have addressed the problem of potential interacting stress factors with an enhanced level of UV-B radiation. These investigations have shown that, in some instances, cross-tolerance was evident. For example, enhanced UV-B radiation increases the tolerance of *Arabidopsis* to water deficit (dehydration) stress (Schmidt *et al.*, 2000). In some species, this response is synergistic and the combined action of the two stresses leads to an increase in each other's effectiveness (Balakumar *et al.*, 1993). In other instances, an additional stress may cause a detrimentally additive or no interactive effect (Bornman and Teramura, 1993; Caldwell and Flint, 1994; Jordan, 1996). From the relatively few examples reported, it is clear that a combination of stress factors can bring about a variety of responses dependent upon species, stage of development and the particular stresses involved.

B. General defence responses and signal transduction

So far, none of the genes known to be regulated by UV-B radiation have been shown

to be exclusively so (Brosché and Strid, 1999). For example, genes that encode enzymes involved in flavonoid biosynthesis, including PAL and CHS, are induced by fungal elicitors, wounding and cold shock, as well as by UV-B (Hahlbrock and Scheel, 1989; Leyva *et al.*, 1995). In fact, it appears that genes uniquely regulated by only one stress factor are scarce. This implies a sharing of, or "cross-talk" between, components of different stress-inducible pathways.

Recent studies have begun to resolve some of the key signal pathways involved in the regulation of a number of stress-related genes, and this research has indicated that plant responses to UV-B share many common components with responses to other oxidative-type stresses such as pathogen infection, ozone exposure and wounding (Mackerness and Thomas, 1999; Mackerness, 2000). ROS, as well as a number of plant hormones, including salicylic acid (SA), jasmonic acid (JA) and ethylene, have been shown to be not only important factors in UV-B signaling, but common features of several stress responses (see Mackerness, 2000).

It is not surprising that recent studies have established a role for ROS in regulation of gene expression in response to UV-B radiation (Green and Fluhr, 1995; Mackerness *et al.*, 1998b; Surplus *et al.*, 1998). UV-B exposure leads to increases in ROS generation and therefore oxidative stress. The ROS then function not only as destructive radicals, but also as signaling components leading to changes in expression (down-regulation) of photosynthetic genes (Mackerness *et al.*, 1998b; Surplus *et al.*, 1998). Furthermore, the rise in ROS leads to the accumulation of the phenolic compound SA (Klessig and Malamy, 1994) and as predicted, SA levels rise in response to UV-B exposure (Surplus *et al.*, 1998). Making use of transgenic

Arabidopsis plants that are unable to accumulate significant levels of SA, Surplus *et al.* (1998) demonstrated that SA is an essential component of the signaling pathway leading to the regulation of pathogen-related (PR) genes in response to UV-B radiation.

Another signaling molecule implicated in plant responses to UV-B stress is JA. Increases in concentrations of JA are often associated with responses to wounding and pathogen infection (Blechert *et al.*, 1995; Vijayan *et al.*, 1998). However, exposure to abiotic stresses can also elicit JA induction. UV-B exposure leads to increases in JA concentrations in both tomato (Conconi *et al.*, 1996) and *Arabidopsis* plants (Mackerness *et al.*, 1999) and results in the induction of genes associated with wounding and pathogen responses, such as the *PDF1.2* defencin gene (Mackerness *et al.*, 1999). Induction of these genes by UV-B does not occur in mutant plants which are defective in the JA-synthesis pathway (Conconi *et al.*, 1996; Mackerness *et al.*, 1999).

In addition to ROS, SA and JA, the gaseous plant hormone ethylene has been identified as a signaling component in defence responses (see Morgan and Drew, 1997). Ethylene is synthesized in response to a diverse array of external stresses, including UV-B (Predieri *et al.*, 1995; Mackerness *et al.*, 1999). Furthermore, Mackerness *et al.* (1999) demonstrated that UV-B exposure resulted in the induction of the defence-associated gene *PDF1.2*, but induction did not occur in an *Arabidopsis* ethylene-insensitive mutant, implicating a role for ethylene in the UV-B-mediated *PDF1.2* induction. These authors additionally reported that application of JA and ethylene simultaneously resulted in a higher induction of *PDF1.2* than the two

compounds provided separately. Interestingly, ethylene is not directly involved in the UV-B-mediated induction of PR genes, but actually serves to increase the sensitivity of tissue to SA (Mackerness *et al.*, 1999).

Taken together, these studies have highlighted the importance of ROS, SA, JA and ethylene as key regulators of gene expression in response to a number of environmental stresses, including UV-B. There are also transcripts affected by UV-B radiation which are regulated through pathways that are ROS-independent, such as the induction of *Chs* transcripts (Long and Jenkins, 1998), indicating that other early signaling molecules, not yet identified, are also involved in responses to UV-B radiation. Figure 2 illustrates how ROS and the regulatory compounds SA, JA and ethylene may interact to regulate the expression of genes in response to UV-B exposure. It is clear that the effects of UV-B on gene expression are mediated through several distinct signaling pathways.

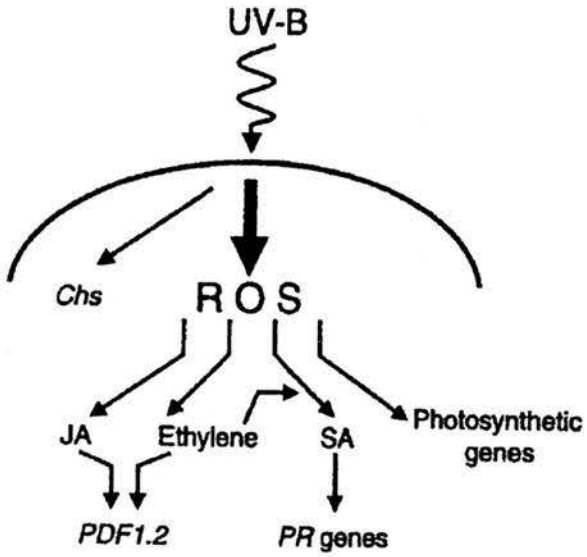

Figure 2. A scheme of the multiple signaling pathways mediating responses to UV-B. ROS play a pivotal role as a second messenger, and leads to the synthesis of SA, JA and ethylene. Induction of *PDFI.2* in response to UV-B requires both JA and ethylene, while ethylene and SA are involved in the pathway leading to the induction of *PR-1*. In contrast, ROS are the only components identified in the pathway leading to the down-regulation of photosynthetic genes. The UV-B-induction of *Chs* transcripts is regulated by pathways which are ROS-independent.
(Adapted from Mackerness, 2000)

V. UV-B AND CONIFERS

Forests contribute up to 80% of net productivity in global terrestrial ecosystems and are also of enormous economic importance, yet the UV-B sensitivity of very few conifer species has been examined (Laakso and Huttunen, 1999). Furthermore, the majority of studies have focused solely on growth parameters as a measure of the sensitivity of conifers to UV-B radiation, while information on the molecular basis of sensitivity towards UV-B in conifers is very limited.

Although relatively few studies addressing the effects of UV-B on conifers exist, the research available has unequivocally shown a remarkable range of responses, depending upon conifer species (Laakso and Huttunen, 1999). For example, some conifers, such as loblolly pine (*Pinus taeda*) are highly sensitive to UV-B (Sullivan and Teramura, 1988, 1992), while others are tolerant, such as high-elevation Douglas-fir ([*Pseudotsuga menziesii*]; Kossuth and Biggs, 1981), or even stimulated by UV-B (Norway spruce [*Picea abies*]; Baumbusch *et al.*, 1998).

A. UV-B screening properties in conifers

The amount of incident UV-B reaching the mesophyll differs considerably among plant species (Vogelmann, 1994), depending on epidermal transmittance, concentrations of UV-B absorbing compounds, and epidermal thickness of leaves or needles (Day, 1993; Day *et al.*, 1993). It is important to note that these determinants are not mutually exclusive, as transmittance is a function of both UV-B absorbing compounds and thickness.

It has been commonly accepted that conifers are particularly well equipped to filter out UV-B radiation due to their relatively thick epidermal layer; furthermore, reports suggesting that enhanced UV-B causes an increase in the thickness of the epidermal layer of Scots pine (*Pinus sylvestris*; Laakso and Huttunen, 1999), stone pine (*Pinus pinea*: Manetas *et al.*, 1997) and loblolly pine (*Pinus taeda*; Sullivan *et al.*, 1996) substantiate this idea. Measurements of UV-B penetration using a fibre-optic microprobe revealed that virtually all UV-B is absorbed in the epidermal layer of mature (≥1 year old) conifer needles (Day *et al.*, 1992, 1993; DeLucia *et al.*, 1992), whereas among a diverse group of herbaceous species examined, leaf epidermal transmittance ranged from 18–55%, with UV-B reaching well into the mesophyll (Day *et al.*, 1992, 1993). However, Day *et al.* (1992, 1993) argue that the UV-B screening effectiveness among conifers is primarily the result of different optical characteristics of their epidermis, and not thickness per se.

One such optical property is the location of UV-B absorbing compounds (phenolics) within the epidermis. In herbaceous species, which are highly UV-B sensitive, these screening compounds appear to occur primarily in the protoplast of epidermal cells; in contrast, conifers commonly contain significant amounts of phenolics bound within epidermal cell walls in addition to those in protoplasts (Strack *et al.*, 1989; Day *et al.*, 1992, 1993). Thus, the epidermis of conifer needles may provide a much more laterally complete or uniform UV-B screen than that of herbaceous species.

B. Conifer sensitivity to UV-B

Despite the effective absorption of UV-B in the epidermal layer of their needles, several conifer species, particularly those belonging to the genus *Pinus*, are sensitive to UV-B radiation (Laakso and Huttunen, 1999). Several factors may contribute to this sensitivity.

Native growth environment

An important determinant in conifer sensitivity to UV-B radiation is native habitat: species growing in high UV-B environments appear to attenuate UV-B more effectively than those in lower UV-B environments (Larson *et al.*, 1990; Sullivan *et al.*, 1992). Conifer species native to high altitudes are commonly more tolerant of UV-B, or even grow better under enhanced UV-B radiation than those from low elevation environments (Kossuth and Biggs, 1981; Sullivan and Teramura, 1988; L'Hirondelle and Binder, 1996; Robakowski and Laitat, 1999).

Lodgepole pine (*Pinus contorta*) is a species that shows UV-B sensitivity at sea-level but not at high altitudes (Sullivan and Teramura, 1988; Caldwell *et al.*, 1995). In experiments using a fibre-optic microprobe to measure the epidermal transmittance of high-elevation lodgepole pine needles, Turunen *et al.* (1999) concluded that both cotyledon and primary needles transmit very little radiation through the epidermis, as long as exposure to normal levels of UV-B radiation occurs during development. However, elimination of even a relatively small fraction of the normal UV-B exposure during development resulted in a substantial decline in epidermal UV-B screening, indicating that the normal development of UV-B screening in lodgepole pine needles is dependent on exposure to UV-B during early

seedling growth, which may not occur to the same extent at sea-level and could partly account for the greater sensitivity at low elevations.

The observed better tolerance of UV-B radiation in high altitude species might also be explained, in part, by the finding that plants at high elevations produce a consistently larger amount of UV-B absorbing compounds (such as flavonoids) than low elevation plants (Ziska *et al.*, 1992). In addition, Wildi and Lütz (1996) found that antioxidant concentration in plants increased as altitude increases. This enhancement was mainly caused by an elevated ascorbic acid content.

Early stages of needle development

To date, most research concerning UV-B effects on conifers has involved the use of first-year seedlings. During the early stages of needle development and growth, the efficient screening properties of the epidermis do not seem to be in place and, therefore, conifers are particularly susceptible to UV-B damage in this time. In post-emergent and elongating needles, epidermal cells have thin, less lignified walls (Connor and Lanner, 1991). Indeed, DeLucia *et al.* (1992) detected small but measurable quantities of UV-B in the mesophyll of post-emergent and elongating needles of conifers. Thus, shortly after emergence from bud scales in early to mid-summer, when UV-B doses are high, absorption of UV-B radiation by potentially sensitive chromophores in the mesophyll may disrupt the physiological and developmental processes in the young needles (DeLucia *et al.*, 1992; Bornman and Teramura, 1993).

Naidu *et al.* (1993) addressed the question of whether penetration of small amounts of UV-B radiation through the epidermis into the photosynthetic mesophyll

during early stages of needle development would impair the photosynthetic competence of conifer needles under field conditions. These researchers examined the effect of supplementary UV-B radiation on photosynthetic characteristics of different aged needles of 3-year-old loblolly pine (*Pinus taeda* L), and showed that the only reductions of photosynthesis were observed in the youngest, most recently emerged needles.

Cumulative exposure

It is thought that plants with long-lived foliage, such as conifers, may be especially vulnerable to long-term UV-B induced damage, since the degree of damage may depend on cumulative exposure (Caldwell *et al.*, 1989; Tevini and Teramura, 1989). Two reports suggest this may be the case with conifers. Sullivan and Teramura (1992) conducted a 3-year field study of loblolly pine, which showed plant biomass reductions of 12-20% after 3 years of supplemental UV-B radiation. As well, in a 3-year study of Norway spruce (*Picea abies*), Sprtová *et al.* (1999) reported an increased sensitivity to the photoinhibition of photosynthesis resulting from the long-term influence of enhanced UV-B radiation. Subsequent investigations implicated impairment of the photosystem II D1 protein turnover as a consequence of the long-term exposure to enhanced UV-B radiation (Sprtová *et al.*, 2000).

Beneficial aspects of UV-B on conifers

In some cases, the observed UV-B-induced changes in conifer growth have been positive and quite opposite to those commonly expected, as noted previously for species native to high altitudes. Other examples demonstrate the beneficial effects of UV-B on the multiple-stress tolerance of conifers. Enhanced UV-B improved water

relations in drought-stressed Mediterranean Aleppo pine and stone pine (Petropoulo *et al.*, 1995; Manetas *et al.*, 1997), resulting in less drought-induced needle loss and larger needle areas compared to drought-stressed seedlings which did not receive enhanced UV-B. Likewise, spruce seedlings grew better when grown under twice ambient ozone levels together with UV-B radiation compared with those grown under enhanced ozone alone (Baumbusch *et al.*, 1998); under the combined conditions, spruce needles showed the highest antioxidative protection (e.g. increased SOD activity) and showed no indication of lipid peroxidation.

C. Molecular aspects

To date, very few reports on the molecular effects of UV-B radiation in conifer species have been published; therefore, little is known about the molecular basis of protective mechanisms against UV-B in these species. The molecular research that is available, however, calls attention to an important and recurring aspect of conifer biology: direct comparisons between conifer (gymnosperm) and angiosperm species cannot be made.

For instance, based on UV-B studies involving various angiosperm species, increased amounts of phenylpropanoid pathway enzymes and/or mRNA would be expected in UV-B treated conifer species in the case of a coordinated induction of the pathway. However, Schnitzler *et al.* (1996) reported no such increase in the levels of chalcone synthase mRNA in UV-B treated Scots pine seedlings. Furthermore, no significant UV-B treatment-dependent increases in CHS protein activity were found (Schnitzler *et al.*, 1997). Similarly, irradiation of seedlings with UV-B caused no

induction of PAL activity in either cotyledon or primary Scots pine needles (Schnitzler *et al.*, 1997).

Stimulation of cellular antioxidant systems is an important defence against a plethora of environmental stresses which act on plants. In conifers, the picture as to how antioxidant systems respond to UV-B is not consistent. In Norway spruce needles, low UV-B levels induced a 1.5-fold increase in SOD activity; however, in Scots pine, UV-B-induced increases in protective antioxidant enzymes were not found (Baumbusch *et al.*, 1998). In fact, low UV-B exposure caused significant increases in thiobarbituric acid reactive compounds, pointing to increased lipid peroxidation in Scots pine (Baumbusch *et al.*, 1998). This research suggests that certain conifer species are less protected against reactive oxygen species and thus suffer higher oxidative stress.

Clearly, further research is required to assess the molecular consequences of enhanced UV-B on conifer species. Both the short-term effects and the potential for the long-term accumulation of UV-B effects must be recognized.

RESEARCH OBJECTIVES

Douglas-fir is a dominant tree species in the low-elevation forests of the Pacific coast of northwestern North America. Due to its favorable growth characteristics and superior wood quality, Douglas-fir is one of the most economically important conifer species in this region (Allen and Owens, 1972) and is, consequently, in high demand for use in reforestation.

Unfortunately, few studies are available to assess the magnitude of future UV-B levels on the productivity of Douglas-fir forests. The objective of this research was to determine how coastal (low-elevation) Douglas-fir seedlings, germinated and grown under enhanced UV-B conditions, would be affected by increased UV-B radiation in terms of alterations in gene expression.

Gene and protein expression was examined using both northern and western blot analyses to determine mRNA and protein amounts in young seedlings subjected to UV-B compared to control seedlings grown with no UV-B. The cDNA clones or antibodies used as probes were selected based on the potential propensity of their corresponding gene/gene product to be affected by UV-B radiation, and included 90-kDa heat shock protein (*hsp90*), luminal binding protein (*BiP*), NADPH:cytochrome P450 reductase (*cpr*), chlorophyll *a/b*-binding protein (*cab*), phenylalanine ammonia lyase (*pal*) and ubiquitin (*ubi*) cDNAs, as well as antibodies against HSP90, BiP and CPR polypeptides.

CHAPTER 2: MATERIALS AND METHODS

Plant material, growth and treatment conditions

Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) seeds (B. C. Ministry of Forests Tree Seed Centre, Surrey, B.C., Canada; seedlot 8192, coastal variety) were imbibed overnight with sterile distilled water at 4°C. The seeds were then blotted dry, placed into plastic bags and stored at 4°C for 3 weeks. This process, known as stratification, improves germination of several conifer species, including Douglas-fir (El-Kassaby *et al.*, 1992). Stratified seeds were germinated in trays in a controlled environment chamber (Conviron Model E15, Controlled Environments, Winnepeg, MB, Canada) at a day/night temperature of 30/20°C and an 8 h photoperiod with a photosynthetic photon flux density of approximately 300 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Germinating seeds were exposed to one of two intensities of UV-B from UV-B-emitting fluorescent lamps (UV-B 313, Q-Panel, Cleveland, OH, USA) added to the cool white fluorescent lamps. UV-B intensity was controlled by the use of frames covered with Mylar (0.127 mm thick, Johnston Industrial Plastics, Toronto, ON, Canada) or cellulose acetate (0.127 mm thick, McMaster-Carr, New Brunswick, NJ, USA) film (Figure 3). Mylar film provided near 0 UV-B radiation, and a single layer of cellulose acetate provided a UV-B dose of approximately 7 kJ m^{-2} d^{-1}. The daily ambient UV dose in Victoria, B.C. is approximately 5 kJ m^{-2} d^{-1} at sea level based on summer measurements on Saturna Island, B.C. (Internal Report, B.C. Forest Service, Research Division). The experimental UV-B intensity of ~7 kJ m^{-2} d^{-1} is an

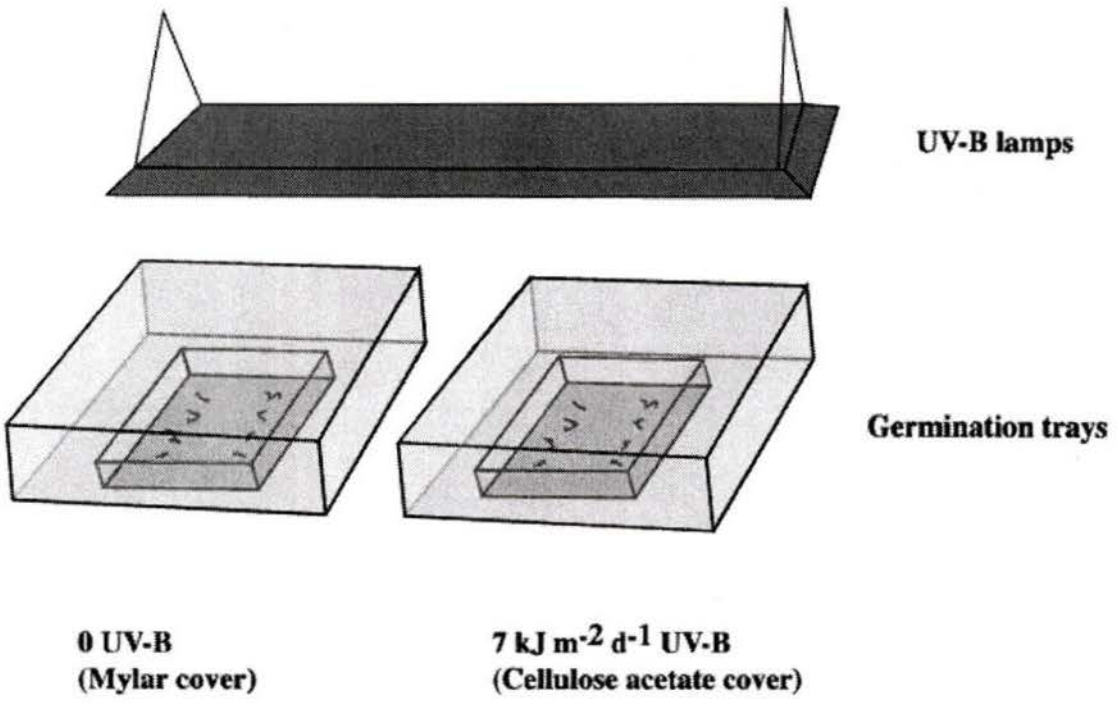

Figure 3. Representation of UV-B enclosures and experimental set-up. Douglas-fir seeds were germinated in trays in a controlled environment chamber. Germinating seeds were exposed to either 0 UV-B (under Mylar cover) or 7 kJ m^{-2} d^{-1} UV-B (under cellulose acetate cover).

ecologically relevant enhancement at temperate latitudes (Kerr and McElroy, 1993). Seeds for each UV-B treatment were in separate trays set under the appropriate film-covered frame.

UV-B-induced damage of proteins and nucleic acids increases exponentially as UV-B wavelength decreases (Caldwell and Flint, 1997). For this reason, UV-B wavelengths are multiplied by a weighting factor based on their biological effectiveness. Biologically effective UV-B ($UV\text{-}B_{BE}$) is an integration of all weighted UV-B wavelengths. UV-B irradiance was measured with a Photodyne Model 450 integrating radiometer (Opticon, Waterloo, ON, Canada) calibrated with an International Light IL 1700 radiometer (Harvard Apparatus, Saint-Laurent, PQ, Canada) with integrated estimates of $UV\text{-}B_{BE}$ between 290 and 320 nm measured with an International Light SED240 bulk sensor.

Samples were collected at 2 h, 12 h, 24 h, 2 d, 4 d, 6 d, 8 d, 10 d and 12 d after exposure to the differential UV-B treatments, immediately frozen in liquid nitrogen and stored at –80°C.

Preparation of BiP, CAB and CPR cDNA inserts

The germination-associated cDNAs encoding *BiP*, *cab* and *cpr* previously described by Tranbarger and Misra (1995) were used as probes in northern blot analyses. In brief, a cDNA library was constructed with mRNA pooled from 4- to 6-day-old Douglas-fir seedlings. Genes with increased expression during germination and early seedling development were identified by differentially screening the library with

cDNA probes synthesized using mRNA isolated from mature seeds and 6-day-old seedlings. Positive cDNA clones were sequenced and DNA similarity searches were performed. Based on partial nucleotide sequence data and predicted amino acid sequences, several clones were found to share identities with known plant genes, including *BiP*, *cab* and *cpr*.

Glycerol stocks (80%) of *Escherichia coli* cells containing the appropriate (*BiP*, *cab* or *cpr*) recombinant plasmids [pBluescript SK(-)] were streaked on Luria-Bertani-ampicillin (LB + amp; 100 µg/ml) plates and incubated overnight at 37°C. Single colonies were picked from plates and used to inoculate LB + amp (100 µg/ml) broth, which was then incubated overnight at 37°C with shaking (~250 rpm).

Plasmid DNA was isolated by the alkaline lysis method as described in Sambrook *et al.* (1989). To summarize, 1.5 ml of culture was centrifuged, the supernatant removed and the pelleted cells resuspended in 100 µl of solution I (50 mM glucose; 25 mM Tris-HCl, pH 8; 10 mM EDTA). After 5 minutes at room temperature, 200 µl of solution II (0.2 N NaOH; 1% SDS) was added and the contents of the tube were mixed by inverting 5 times. A 150 µl aliquot of cold solution III (60 ml 5M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml H_2O) was added, mixed well by inversion, and incubated on ice for 5 minutes. Following centrifugation, the supernatant was extracted with an equal volume of phenol:cholorform (1:1), vortexed, and centrifuged. The aqueous phase was removed and mixed with 2 volumes of 95% ethanol. The DNA was precipitated (2 minutes at room temperature), pelleted, washed with 70% ethanol, dried, and resuspended in 20 µl TE (10 mM Tris-HCl, pH8; 1 mM EDTA, pH 8) containing 20 µg/ml Rnase A.

Samples were stored at –20°C until needed.

Plasmid preparations were restriction digested with *Eco*RI (Life Technologies, Burlington, ON, Canada) at 37°C for 3 hours. Samples were then separated on 1% agarose gels containing ethidium bromide. The size of the digested plasmid was compared to a 1 kb ladder (Life Technologies); appropriate bands were excised from gels and purified using the Clontech (Palo Alto, CA, USA) Nucleospin Extraction® kit or QIAGEN (Mississauga, ON, Canada) QIAquick Gel Extraction® kit. Purified DNA (inserts) were quantified spectrophotometrically (A_{260}) and as a check, small aliquots were run on 1% agarose gels containing ethidium bromide to verify insert size when compared to a 1 kb ladder.

CPR restriction fragments

The Douglas-fir *cpr* clone has three *Eco*RI restriction sites which result in four fragments, three of which can be resolved on an agarose gel and visualized by staining with ethidium bromide (Tranbarger, 1998). Fragment A (~1.5 kb) corresponds to sections of the open reading frame (more conserved), while the B fragment (~0.9 kb) contains some of the 3' untranslated region (less conserved). Fragment A was used as a probe to detect Douglas-fir CPR in all northern blot analyses.

Preparation of HSP90 cDNA insert

A *Brassica napus* partial *hsp90* cDNA clone was a gift of Dr. Priti Krishna

(University of Western Ontario, London, Canada). Preparation of *hsp90* insert was performed by Kaukinen (1996) to the glycerol stock stage. In brief, high efficiency transformation of *Escherichia coli* with the *hsp90*-containing pUC18 plasmid was carried out by a modified Hanahan protocol. Plasmid was added to DH5α competent cells then incubated on ice for 30 minutes, heat shocked at 42°C for 2 minutes, then placed on ice for 90 seconds. Pre-warmed SOC medium (SOB with 20 mM glucose) was added to the transformation reaction and incubated with agitation for one hour at 37°C. Culture aliquots (100 µl) were plated on to LB + amp (100 µg/ml) plates and incubated overnight at 37°C. Single colonies were picked and cultured in LB + amp (100 µg/ml) broth overnight at 37°C. Several glycerol stocks (80%) were made and stored at -80°C.

Plasmid preparations were performed according to Sambrook *et al.* (1989) as previously outlined. The *Brassica napus hsp90* insert was excised from the plasmid with *Hin*dIII (New England Biolabs, Beverly, MA, USA). Insert DNA was separated, purified and stored as detailed above (*Preparation of BiP, CAB and CPR cDNA inserts*).

Preparation of Ubi cDNA insert

A white spruce (*Picea glauca*) partial ubiquitin cDNA (*Ubi4-2c2*; from a cDNA bank of stressed white spruce suspension cell cultures) was generously provided by Dr. Armand Séguin (Canadian Forest Service, Laurentian Forestry Centre, Sainte-Foy, Québec).

For transformation, 30 ng of the *Ubi4-2c2*-containing plasmid (pBluescript skII) was added to 100 µl *Escherichia coli* DH5α competent cells. The mixture was incubated on ice for 40 minutes, heat shocked at 42°C for 90 seconds, then cooled on ice. Pre-warmed LB medium was added to the transformation reaction and incubated at 37°C for one hour. Samples were centrifuged, and cells resuspended in fresh LB medium. Aliquots were spread onto LB + amp (100 µg/ml) plates and incubated at 37°C overnight. Single colonies were picked and cultured in LB + amp broth overnight at 37°C. Glycerol stocks (80%) were made and stored at -80°C.

Plasmid preparations were performed according to Sambrook *et al.* (1989) as previously outlined. The *Ubi4-2c2* cDNA insert was excised from the plasmid using *Eco*RI and *Xho*I (Pharmacia, Uppsala, Sweden). Insert DNA was separated, purified and stored as previously described.

Preparation of PAL insert

A PCR amplified genomic fragment of a jack pine (*Pinus banksiana*) phenylalanine ammonia lyase (*Pbpal1*) gene was kindly supplied by Dr. Brian Ellis (University of British Columbia, Vancouver, B.C., Canada). An LB plate with colonies growing on selection medium was provided. Plasmid purification was achieved as outlined previously, followed by excision of the insert with *Eco*RI and *Xba*I (Life Technologies). Insert DNA was separated, purified and stored as previously described.

Total RNA extraction

Total RNA was isolated according to Verwoerd *et al.* (1989) with minor modifications. Briefly, after grinding frozen seed and seedling tissue (150 mg) to a fine powder in liquid nitrogen, 500 µl of hot extraction buffer (100 mM LiCl; 100 mM Tris-HCl, pH 8; 10 mM EDTA; 1% SDS; in phenol at 80°C) and 250 µl chloroform-isoamyl alcohol (24:1) were added and vortexed. The aqueous phase was removed by centrifugation and mixed with one volume of 4 M LiCl. The RNAs were precipitated overnight at –20°C and collected by centrifugation. Pellets were dissolved in 250 µl DEPC-treated water and 0.1 volume of 3 M sodium acetate (pH 5.2); the RNAs were then reprecipitated with two volumes of cold ethanol (1 h at -20°C). Following centrifugation, pellets were washed with 70% ethanol (4°C) and resuspended in DEPC-treated water. The RNA concentrations were determined spectrophotometrically (A_{260}). Yields of between 50–100 µg of total RNA were routinely obtained from 150 mg of tissue. The RNA samples were stored at -80°C.

Northern blotting and hybridization

The RNA (20 µg total RNA per lane) was electrophoretically separated on agarose gels containing formaldehyde (Sambrook *et al.*, 1989), stained with ethidium bromide as a visual assurance of equal loading, and transferred to Zeta-Probe GT membranes (Bio-Rad, Mississuaga, ON, Canada) according to the manufacturer's instructions. After transfer, membranes were separated from gels, rinsed briefly, and vacuum dried at 80°C.

Prior to hybridization, membranes were moistened for 5 minutes in a

prehybridization solution (0.5 M Na_2HPO_4, pH 7.2; 7% SDS) at 65°C. The cDNA inserts were labeled by random priming with [α-^{32}P]dCTP (Life Technologies), and hybridized in prehybridization solution at 65°C overnight. Membranes were then washed twice for approximately 30 minutes at 65°C in wash solution #1 (40 mM Na_2HPO_4, pH 7.2; 5% SDS) and once or twice for 20 to 30 minutes in wash solution #2 (40 mM Na_2HPO_4, pH 7.2; 1% SDS). Washed membranes were either exposed to scientific imaging film (X-OMAT AR film; Eastman Kodak Company, Rochester, NY, USA) at –80°C and developed in an automatic developer, or exposed under a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) at room temperature and developed using the STORM 820 PhosphorImager system (Molecular Dynamics).

Membrane stripping, reprobing with 18S rRNA, and transcript quantification

Membranes were stripped by washing twice for 20 minutes each in a large volume of 0.1x SSC/0.5% SDS at 95°C, then checked by overnight exposure (see above). Membranes were then reprobed with a PCR amplified genomic fragment representing the Douglas-fir 18S ribosomal RNA (rRNA) gene. This probe was used to accurately determine RNA levels of the samples used in northern hybridization and to normalize values of transcript levels (thereby

ng as a control measure to account for differences in the amount of RNA loaded per lane).

Quantification of transcript accumulation of autoradiograms was assessed by

densitometric measurement using the ChemiImager™ 4000 system (Alpha Innotech Corporation, San Leandro, CA, USA). Variations in the total RNA loaded in each lane on a membrane were corrected by dividing the lowest rRNA densitometric value by each rRNA value to obtain a ratio. Each mRNA hybridization signal value was then multiplied by its respective rRNA ratio (calculation of integrated optical density). In some cases, adjustment for differences in the amount of RNA loaded per lane was done by dividing the mRNA signal density by the fluorescence value of the total ethidium bromide staining in each respective lane, then multiplying by 100. The results were represented graphically upon calculation of integrated optical density (IOD).

Quantification of transcript accumulation on membranes exposed under phosphor screens and developed using the STORM 820 PhosphorImager system was evaluated using ImageQuant (Molecular Dynamics) software.

Heat shock protein extraction (HSP90)

Cell extracts for heat shock protein isolation were prepared by a modification of the method of Lin *et al.* (1984). Seeds and seedlings were homogenized in microfuge tubes with the addition of cold extraction buffer (50 mM Tris-HCl, pH 8.5; 2% SDS; 2% β-mercaptoethanol; 1 mM PMSF). The homogenate was vortexed, then centrifuged at 12 000 x g for 20 minutes. The supernatant was decanted and precipitated with 5 volumes of acetone and stored at -20°C overnight. Precipitated proteins were pelleted by centrifugation at 16 000 x g for 10 minutes, dried and

resuspended in extraction buffer. Protein concentration was determined using the Bradford method (Bio-Rad). Protein samples were stored at -20°C.

Microsomal membrane isolation and protein extraction (BiP and CPR)

Microsomes were prepared according to the method of Shet *et al.* (1993) with minor modifications. Tissue samples were ground to a fine powder in liquid nitrogen with a mortar and pestle. Two volumes of Buffer A (100 mM Tris-HCl, pH 7.4; 250 mM sucrose; 1 mM EDTA; 1 mM PMSF; 1 mM benzamidine; 5 μM leupeptin; 0.3 μM aprotinin; 2.8 mM β-mercaptoethanol; 2% Polyclar AT) per gram of tissue was added to each ground sample. The slurry was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA, USA) and the filtrate centrifuged at 25 000 x g for 20 minutes at 4°C in a JA20 rotor. The supernatant (SN1) was collected and centrifuged at 100 000 x g for 60 minutes at 4°C in a Beckman Ti 70.1 fixed angle rotor using Beckman Polycarbonate thick wall centrifuge tubes. Supernatant (SN2) was removed; pellets (containing microsomes) were gently washed in Buffer A then air dried on ice and resuspended in cold Buffer B (50 mM sodium phosphate, pH 7.4; 20 % glycerol; 10 mM β-mercaptoethanol). Protein concentration was determined using the Bradford method (Bio-Rad). Samples were stored at -80°C.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Protein samples were resolved by SDS-PAGE according to Sambrook *et al.* (1989) using the Mini-Protean II (Bio-Rad) gel electrophoresis system. Extracts were mixed

1:1 (v/v) with 2X Laemmli sample buffer (Laemmli, 1970), heated at 90°C for 5 minutes and briefly centrifuged. Samples were then loaded onto duplicate SDS-PAGE gels (4% stacking gel; 11% separating gel) on an equal protein basis (either 10 or 15 μg per lane); gels were either stained or electroblotted to nitrocellulose.

Gels were stained in a Coomassie blue R-250 solution (2.5 g in 25% ethanol and 5% acetic acid) and set on a rotating platform overnight. Gels were then transferred to a destaining solution (25% ethanol; 5% acetic acid) and set on a rotating platform until adequate destaining had taken place. Destained gels were soaked in a gel pre-dry solution (10% glycerol; 5% acetic acid) for 20 minutes before being placed between two sheets of cellophane. Enclosed gels were air-dried overnight.

Duplicate gels were electroblotted to nitrocellulose using a Bio-Rad Mini-Trans-Blot cell. Proteins were transferred to nitrocellulose membranes slowly (20 V, overnight) in transfer buffer containing no SDS (39 mM glycine; 48 mM Tris base; 15% methanol; pH 8.3). After transfer, membranes were briefly rinsed twice in TTBS (0.5% Tween-20 in TBS: Tris-buffered saline, 20 mM Tris; 500 mM NaCl; pH 7.5) then blocked for 1–2 hours in TTBS at room temperature. Following blocking, membranes were incubated with either BiP-peptide antiserum (1:3000 dilution), CPR-peptide antiserum (1:2000), or HSP90-peptide antiserum (1:5000 dilution; gift of Dr. Priti Krishna, University of Western Ontario, London, Canada) in TTBS for 1–3 hours. Membranes were rinsed twice for 5 minutes in TTBS before being incubated for one hour in secondary goat anti-rabbit IgG-alkaline phosphatase antibody (Sigma, Oakville, ON, Canada) at a concentration of 1:20,000 in TTBS.

Membranes were then rinsed once in TTBS for 5 minutes and once in TBS for 5 minutes before development in alkaline phosphatase buffer (100 mM NaCl; 5 mM $MgCl_2$; 100 mM Tris-HCl; pH 9.5) containing NBT (nitro-blue tetrzolium, in 70% dimethylformamide) and BCIP (5-bromo-4-chloro-3-indolylphosphate, in 100% dimethylformamide). Development continued until bands appeared on nitrocellulose membranes; the reaction was stopped by rinsing with distilled water. Membranes were air-dried overnight.

Reproducibility of experiments

All experiments were repeated at least twice, and in each case, similar trends were observed. The data presented are from individual experiments that are representative of the results obtained.

CHAPTER 3: RESULTS

UV-B effects on germination and early seedling development

Following stratification, Douglas-fir seeds were germinated in trays in a controlled environment chamber and were exposed to either near 0 UV-B radiation or 7 kJ $m^{-2}d^{-1}$ UV-B as described in Chapter 2 (Materials and Methods). Two independent growth experiments were conducted to determine UV-B effects on seed germination and/or seedling development (Figure 4). In each experiment, three replicate germination trays containing 30 seeds each were subject to a UV-B dose of either 0 or 7 kJ m^{-2} d^{-1} for 12 days. After the 12-day period, the number of seeds which showed emerged radicles (Figure 4, day 4), established radicles (Figure 4, day 6-8), and emerged cotyledons (Figure 4, day 12) under each UV-B dose was noted. The total number of seeds germinated (at any stage) under each UV-B treatment was also compared.

The total number of seeds which germinated in the 12-day experimental period was similar between both UV-B treatments (Figure 5), with germination frequencies of approximately 70%. Likewise, the number of seeds that developed emerged and established radicles was consistent between treatments. However, there was a significant difference in the emergence of cotyledons between the treatments: a greater number of seedlings progressed to the point of cotyledon emergence under the near 0 UV-B treatment than those grown under UV-B.

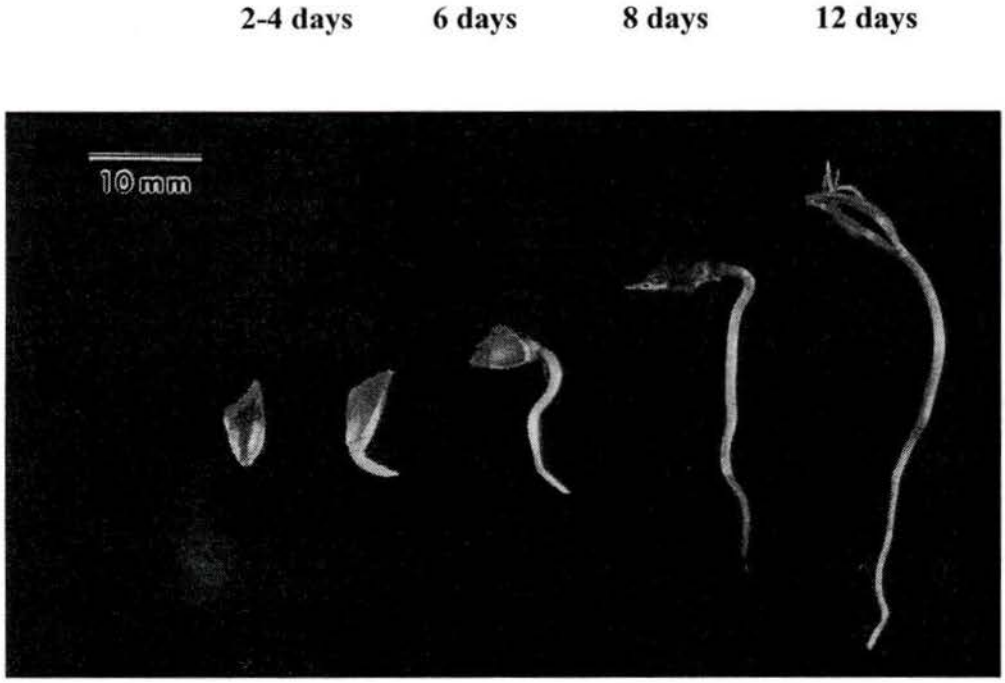

Figure 4. Time course of Douglas-fir seed germination and early seedling development. The germination phase of Douglas-fir occurs after an initial stratification treatment, lasts 2-4 days, and ends upon emergence of the radicle. During the following 5–12 days (post-germination phase), the radicle increases in length, the hypocotyl emerges and grows, the seed coat drops off and the cotyledons emerge. (Image used by permission.)

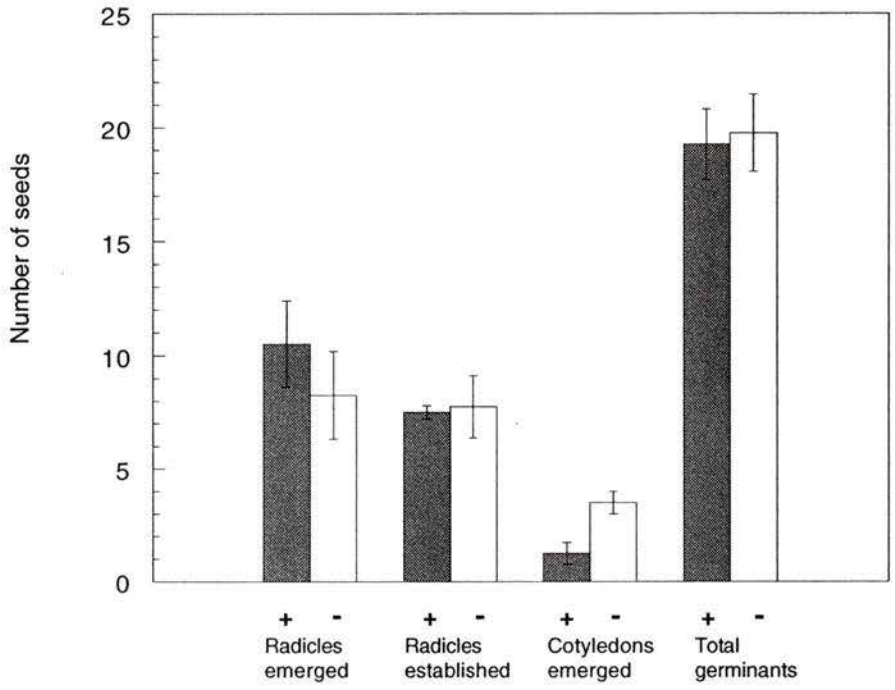

Figure 5. UV-B effects on germination and early seedling development in Douglas-fir. Filled bars represent seeds exposed to 7 kJ m^{-2} d^{-1} UV-B (+) for a 12-day period; white bars represent seeds exposed to near 0 UV-B (–) for the same time period. Means ± S.E. of three replicate germination trays, 30 seeds per tray, and two experiments.

UV-B effects on Douglas-fir hsp90 gene expression

To determine mRNA levels in response to UV-B at different time periods during germination and early seedling development, Douglas-fir seeds were germinated and grown in the presence (7 kJ m^{-2} d^{-1}) or absence of UV-B as described in Materials and Methods. Total RNA was extracted from seeds and/or seedlings. Equal amounts of total RNA were loaded per lane and visualized by staining with ethidium bromide prior to northern blot analysis (Figure 6).

The accumulation of heat shock-like proteins has been shown to be induced by UV-B in cowpea (*Vigna sinensis*) seedlings (Nedunchezhian *et al.*, 1992). Rape seed (*Brassica napus*) cDNA was used as a probe to determine if UV-B caused alterations in Douglas-fir *hsp90* gene expression. The heterologous probe successfully hybridized to Douglas-fir mRNA, and was detected as a single band of approximately 2.6 kb. Transcript abundance in UV-B treated samples was consistently higher than that of the corresponding controls, albeit not to a great extent (Figure 7). Quantitative analysis of changes in the abundance of Douglas-fir *hsp90* transcripts during exposure to UV-B showed percentages of control values between 104-122% (Figure 8). Transcript levels of both UV-B and control samples steadily increased up to 2d then, after 4d, fell to similar levels as the samples at 12h.

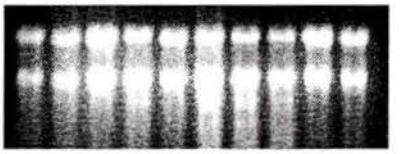

Figure 6. Ethidium bromide staining of total RNA showing 28S and 18S rRNA.

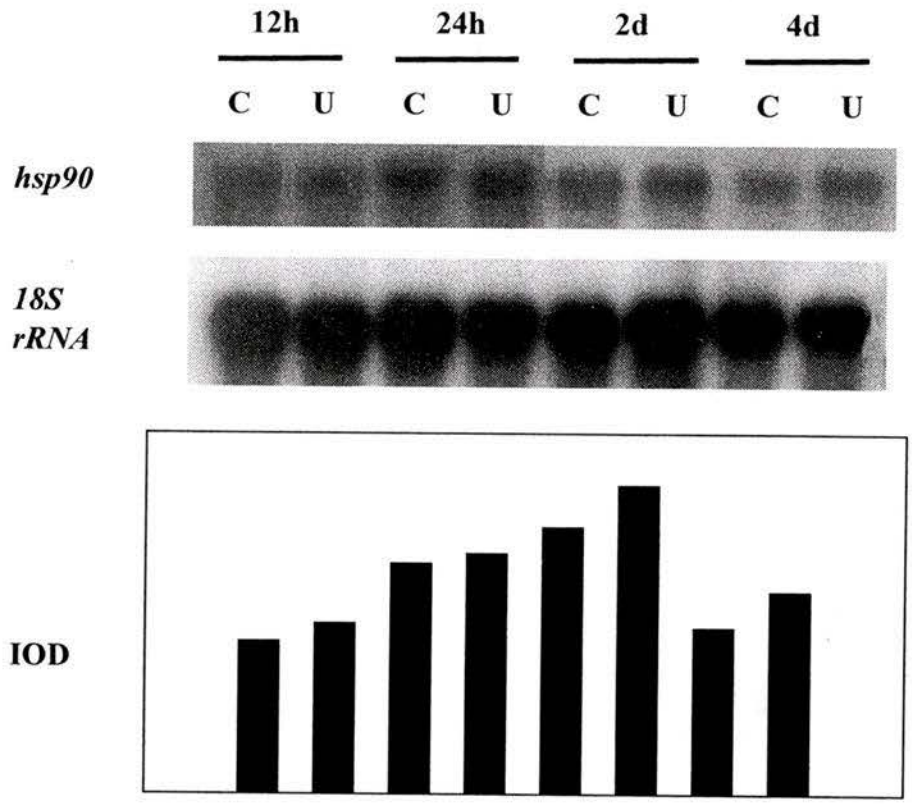

Figure 7. Northern blot analysis of *hsp90* expression in response to UV-B. Douglas-fir seeds were germinated and grown without UV-B (C) or irradiated with 7 kJ m^{-2} d^{-1} UV-B (U) for 12h, 24h, 2d or 4d. Total RNA was extracted from samples, then subjected to northern blot analysis (20 µg per lane) using a ^{32}P-labelled *Brassica napus hsp90* cDNA probe (upper panel; refer to Materials and Methods). The same membrane was stripped and rehybridized with a genomic DNA probe encoding a portion of the Douglas-fir 18S rRNA (mid panel) as a control for loading and transfer efficiency. The blots were scanned using the ChemiImager™ 4000 system. Integrated optical density (IOD) values were calculated and are represented graphically (lower panel; refer to Materials and Methods).

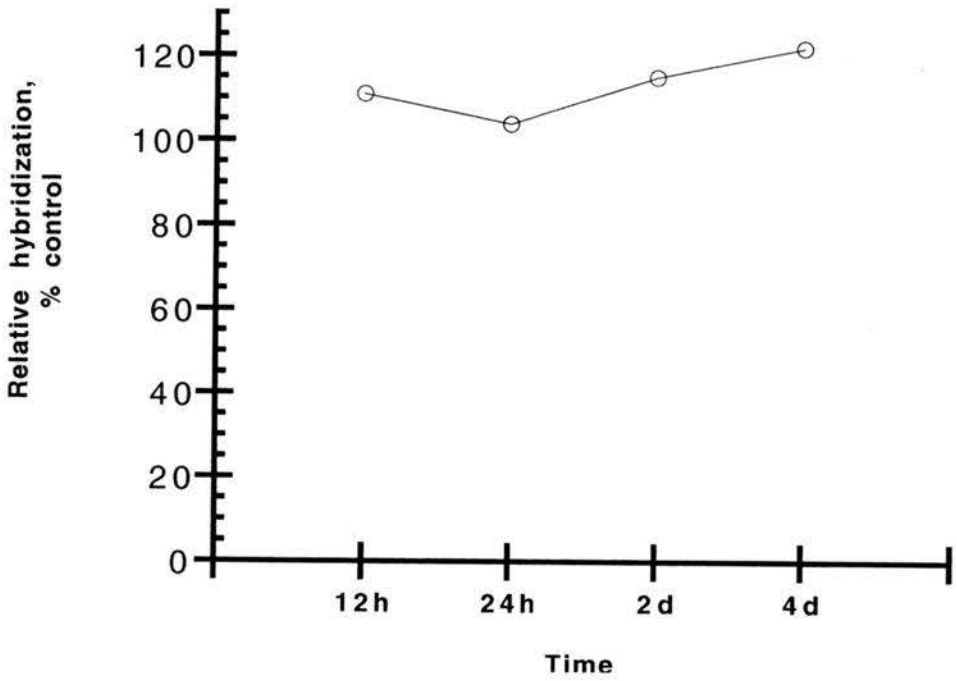

Figure 8. Changes in the abundance of *hsp90* mRNA during exposure to UV-B, relative to control values. Northern blots were quantified using the ChemiImager™ 4000 system. Values were normalized by the corresponding quantification of 18S rRNA from stripped and reprobed blots, and integrated optical density (IOD) values were calculated for each band. Relative hybridization is defined as the IOD values calculated from UV-B treated samples with respect to IOD values from control samples, which were treated under the same conditions, but in the absence of UV-B, at equivalent times. Results are given as percentages of control values.

UV-B regulation of Douglas-fir HSP90 protein

To determine if the accumulation of *hsp90* transcripts in Douglas-fir was accompanied by an increase in the protein levels of HSP90, protein was isolated from seeds or seedlings grown under the same conditions as previously outlined (Figure 7) and western analysis was done as detailed in Materials and Methods. Antibodies against a trpE-hsp90 fusion protein, which recognize members of the HSP90 family in several plants (Krishna *et al.*, 1995), were used as a probe.

The HSP90 protein expression, for the most part, paralleled the expression of *hsp90* transcripts (Figure 9). An exception to this correlation was seen at the 4d time point, in which a much higher level of the protein was observed compared to the transcript amounts in UV-B treated samples. Consistent with the expression of *hsp90* transcripts, a general increase of HSP in both UV-B treated and control samples was observed up to 2d; however, at 4d, HSP levels declined in control samples only, whereas HSP in UV-B treated samples increased almost 4-fold compared to the control.

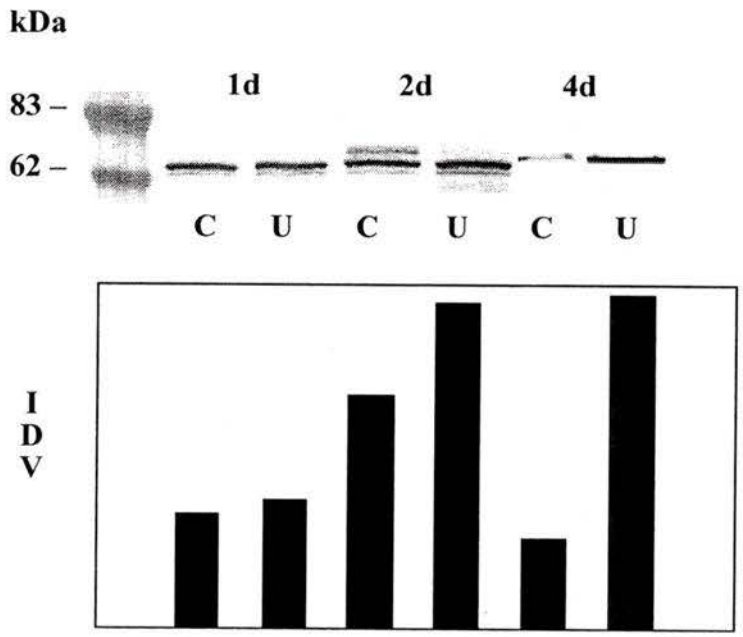

Figure 9. Western blot analysis of HSP90 protein accumulation in response to UV-B. Protein was isolated from control (C) or UV-B-irradiated (U) samples, which were grown under the conditions described in Figure 6. Equal amounts of protein (50 μg) were loaded per lane. Membrane was probed with antibodies against a trpE-hsp90 fusion protein, which recognize members of the HSP90 family in several plants (Krishna *et al.*, 1995). Proteins with an apparent molecular mass of ~65 kDa were detected (upper panel). The western blot was scanned using the ChemiImager™ 4000 system. Integrated density values (IDV) are represented graphically (lower panel).

UV-B effects on Douglas-fir BiP gene expression

BiP is a member of the HSP70 family of heat shock proteins and is thought to be involved in preventing the aggregation of misfolded proteins or protein domains. BiP mRNA and protein levels have been shown to increase in response to environmental stimuli (Boston *et al.*, 1996). To determine whether Douglas-fir *BiP* is regulated by UV-B, transcript amounts of UV-B treated or control seeds or seedlings at 2h, 12h, 24h, 2d, and 4d time points were examined in northern blot analysis.

As shown in Figure 10, a complex pattern of regulation was found for *BiP* transcripts in UV-B treated samples. *BiP* transcripts decreased after 2 hours of UV-B exposure compared to the control, and continued to be lower than controls for 24 hours. After 2d of UV-B treatment, *BiP* transcripts increased dramatically and after 4 days transcript accumulation in UV-B treated samples was higher than in the control. However, after 6 days, transcript amounts decreased in UV-B treated seedlings compared to controls (data not shown).

Data from quantitative northern blot analysis of *BiP* transcript amounts from UV-B-treated samples relative to those of the control samples at the same time points are plotted in Figure 11. Transcript amounts in UV-B treated samples decreased to 36% of the controls within 24h of treatment. After 24h of treatment, transcript amounts in the UV-B treated samples increased, reaching 68% of the control value after 2d and 186% of the control value after 4d.

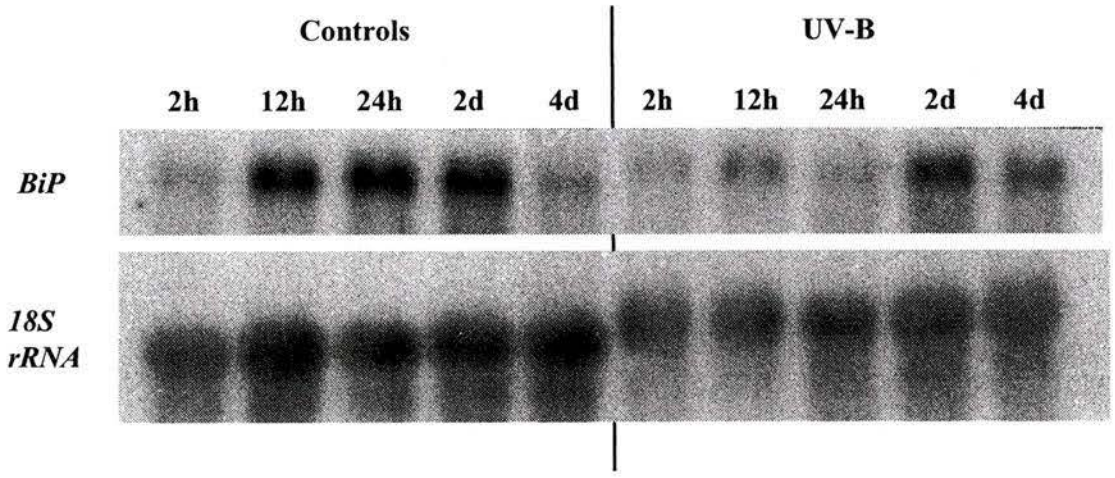

Figure 10. Northern blot analysis of *BiP* expression in response to UV-B. Douglas-fir seeds were germinated and grown without UV-B (controls) or irradiated with 7 kJ m^{-2} d^{-1} UV-B (UV-B) for 2h, 12h, 24h, 2d or 4d. Total RNA was extracted from seeds and seedlings, then subjected to northern blot analysis (20 μg per lane) using a ^{32}P-labelled Douglas-fir *BiP* cDNA probe (upper panel; refer to Materials and Methods). The same membrane was stripped and rehybridized with a genomic DNA probe encoding a portion of the Douglas-fir 18S rRNA (lower panel) as a control for loading and transfer efficiency.

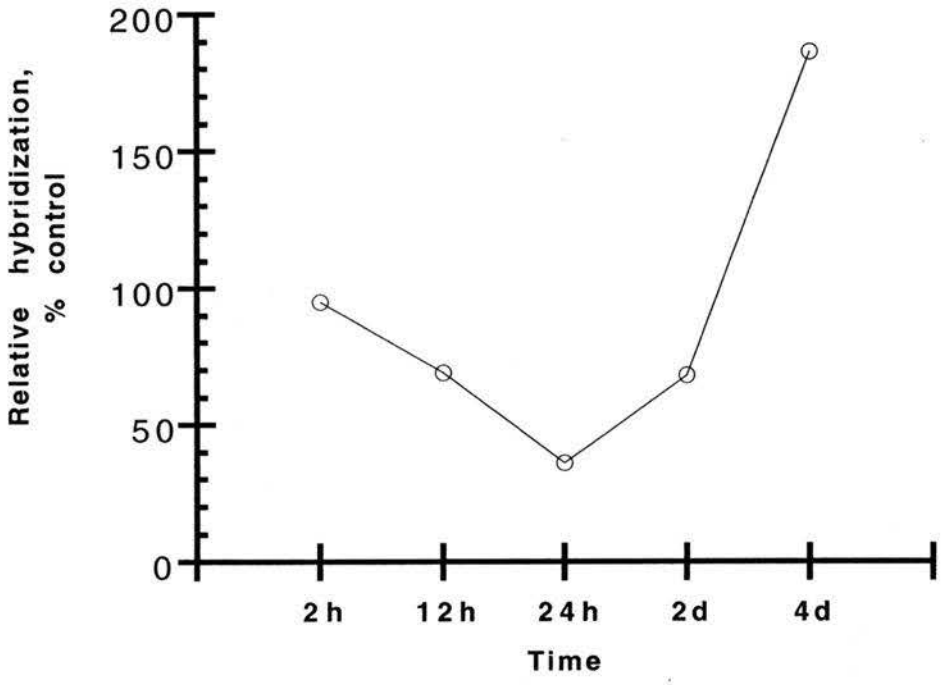

Figure 11. Changes in the abundance of *BiP* mRNA during exposure to UV-B, relative to control values. Northern blots were quantified using the ChemiImager™ 4000 system. Values were normalized by the corresponding quantification of 18S rRNA from stripped and reprobed blots, and integrated optical density (IOD) values were calculated for each band. Relative hybridization is defined as the IOD values calculated from UV-B treated samples with respect to IOD values from control samples, which were treated under the same conditions, but in the absence of UV-B, at equivalent times. Results are given as percentages of control values.

UV-B regulation of Douglas-fir BiP protein

Microsomal proteins (Forward, 2000) from seeds and seedlings grown as previously noted (Figure 10) were isolated, separated by SDS-PAGE, and blotted to nitrocellulose for western blot analysis as described in Materials and Methods. The amounts of BiP protein in UV-B treated samples remained at control levels after 12h and 24h exposure (data not shown) and, relative to controls, increased after 2d and 4d of UV-B exposure (Figure 12). The pattern of BiP protein induction followed that of *BiP* transcripts during the same time course and under the same UV-B treatment. The level of BiP protein from controls remained relatively constant throughout the experiment up to 2d, after which amounts declined slightly.

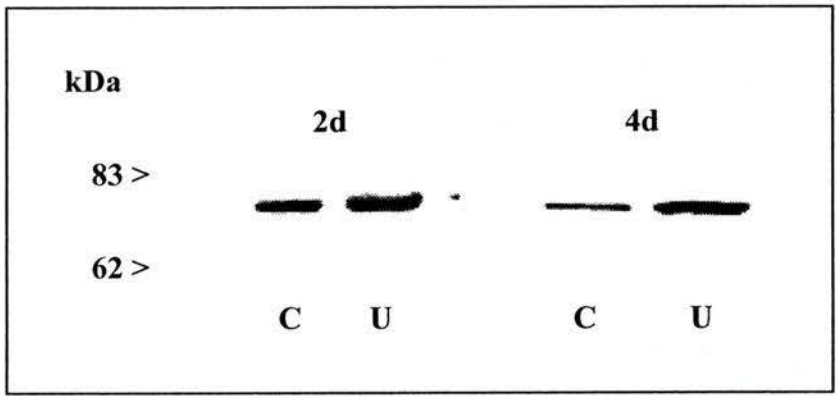

Figure 12. Western blot analysis of BiP protein accumulation in response to UV-B. Microsomal protein was isolated from control (C) or UV-B-irradiated (U) samples, which were grown under the conditions described in Figure 10. Equal amounts of protein (15 μg) were loaded per lane. Membrane was probed with antibodies against a synthetic peptide corresponding to the C-terminus of the Douglas-fir BiP protein (Forward, 2000). Numbers on the left indicate molecular mass markers in kDa.

UV-B effects on Douglas-fir CPR gene expression

Some of the steps in the flavonoid biosynthetic pathway are catalyzed by cytochrome P450 monooxygenases, which are dependent on the supply of electrons from an associated CPR. Since many enzymes in the flavonoid pathway are regulated by UV-B, it is probable that any CPR involved in the same biosynthetic pathway would also follow this pattern of induction by UV-B. To determine whether Douglas-fir *cpr* are regulated by UV-B, transcript amounts were examined using the same total RNA samples used in the northern blot analysis of *BiP* expression.

The Douglas-fir *cpr* clone has three *Eco*RI restriction sites which result in four fragments, three of which can be resolved on an agarose gel and visualized by staining with ethidium bromide (Figure 13, A). Fragments A (~1.5 kb) and B (~0.9 kb) were purified (Figure 13, B), and fragment A was used as a probe to detect Douglas-fir *cpr* in the northern blot analysis.

Transcript amounts of *cpr* were lower in UV-B treated samples up to 2d of exposure compared to controls (Figure 14). After 4d of exposure, UV-B activated the expression of *cpr* to a much greater extent than in the control. Control transcript levels decreased considerably by day 4 of the experiment.

Although the pattern of expression was very much the same for the *cpr* transcripts as for the *BiP* transcripts, quantitative analysis revealed a more extreme response to UV-B in *cpr* transcripts (Figure 15). Transcript amounts fell to a low of 26% of control values by 24h of treatment, then started increasing after this point. By 2d of treatment, transcript levels increased to 81% of control values, and by 4d of treatment a dramatic increase to 412% of the control was observed.

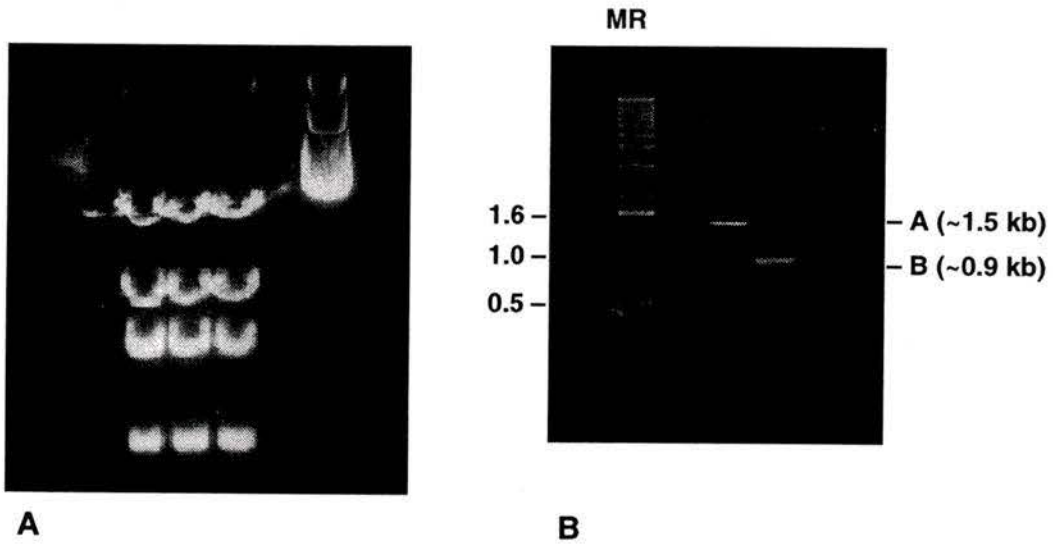

Figure 13. *Eco*RI restriction digest (A) and purified fragments (B) of Douglas-fir *cpr* cDNA. The restriction digest (A) shows released vector molecule (highest M_R band) as well as three coding fragments of approximately 1.5 kb, 0.9 kb and 0.3 kb. The last lane represents uncut plasmid. Fragments A and B, corresponding to the 1.5 kb and 0.9 kb bands, respectively, were purified (B). Fragment A was used as a probe to detect Douglas-fir *cpr* in all northern analyses.

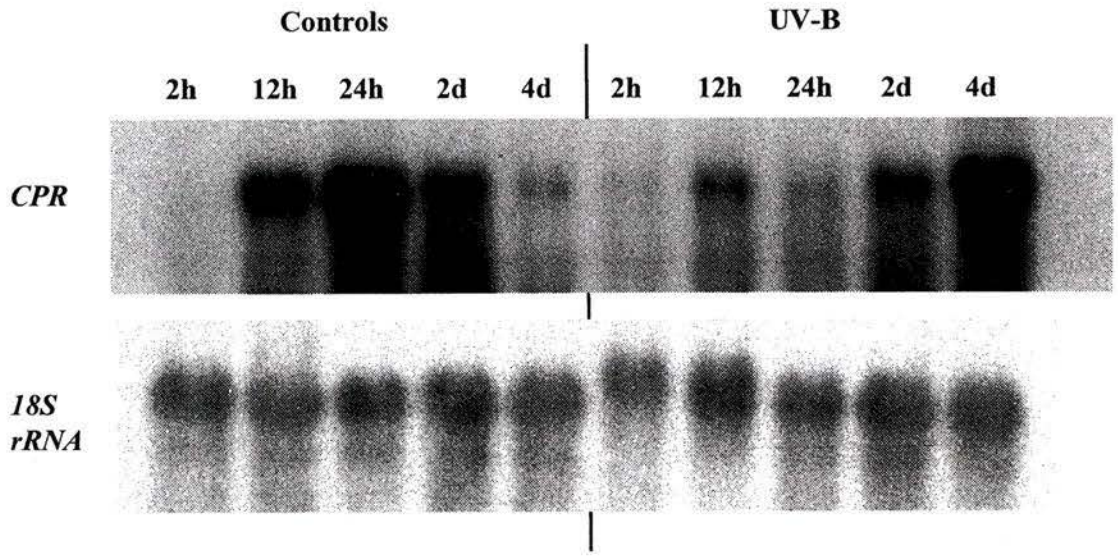

Figure 14. Northern blot analysis of *cpr* expression in response to UV-B. Total RNA was extracted from samples as in Figure 10, then subjected to northern blot analysis (20 μg per lane) using a ^{32}P-labelled Douglas-fir *cpr* DNA probe (upper panel; refer to Materials and Methods). The same membrane was stripped and rehybridized with a genomic DNA probe encoding a portion of the Douglas-fir 18S rRNA (lower panel) as a control for loading and transfer efficiency.

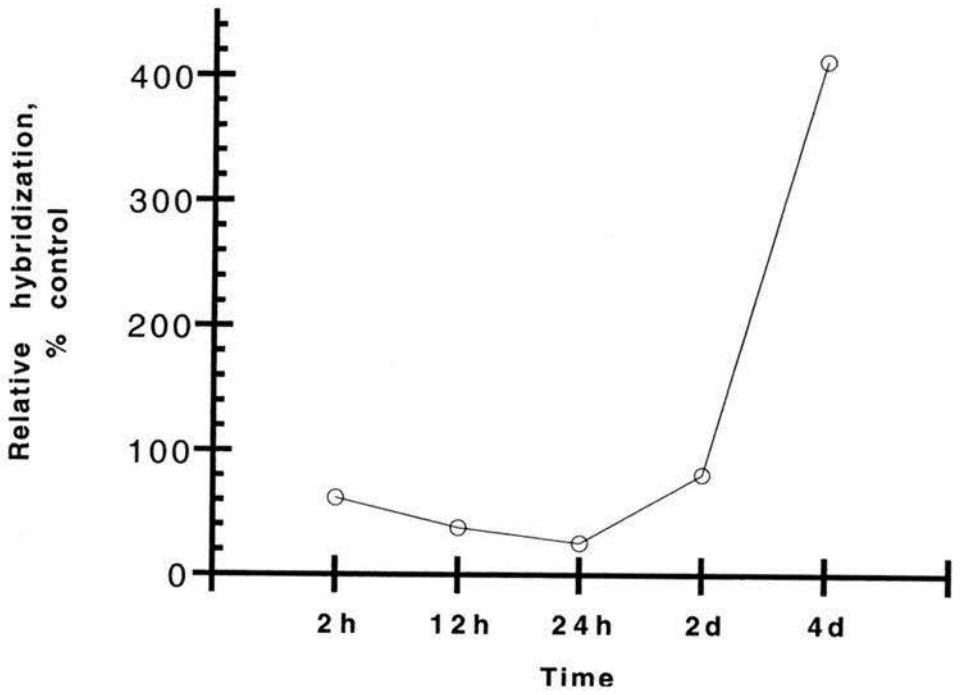

Figure 15. Changes in the abundance of *cpr* mRNA during exposure to UV-B, relative to control values. Northern blots were quantified using the ChemiImager™ 4000 system. Values were normalized by the corresponding quantification of 18S rRNA from stripped and reprobed blots, and integrated optical density (IOD) values were calculated for each band. Relative hybridization is defined as the IOD values calculated from UV-B treated samples with respect to IOD values from control samples, which were treated under the same conditions, but in the absence of UV-B, at equivalent times. Results are given as percentages of control values.

UV-B regulation of Douglas-fir CPR protein

To examine whether the increase in the amount of *cpr* transcripts was reflected in an increase in CPR protein accumulation, western blot analysis was performed as outlined in Materials and Methods. Relative to controls, CPR accumulation decreased slightly after 1d of UV-B exposure (Figure 16). However, after 2d of UV-B exposure, CPR accumulation increased compared to the control, and the increase was sustained 4d after the onset of UV-B exposure.

The pattern of CPR protein induction followed that of *cpr* transcripts to a certain extent, with differences occurring at the 1d and 2d time points: *cpr* transcripts decreased relative to the control, whereas CPR protein accumulation was the same or greater than that of the control after 1d and 2d of UV-B exposure, repectively. The amount of transcript at 2d was approaching that of the control (81% of the control value; Figure 15), but protein accumulation was ca. 150% of the control value (Figure 16).

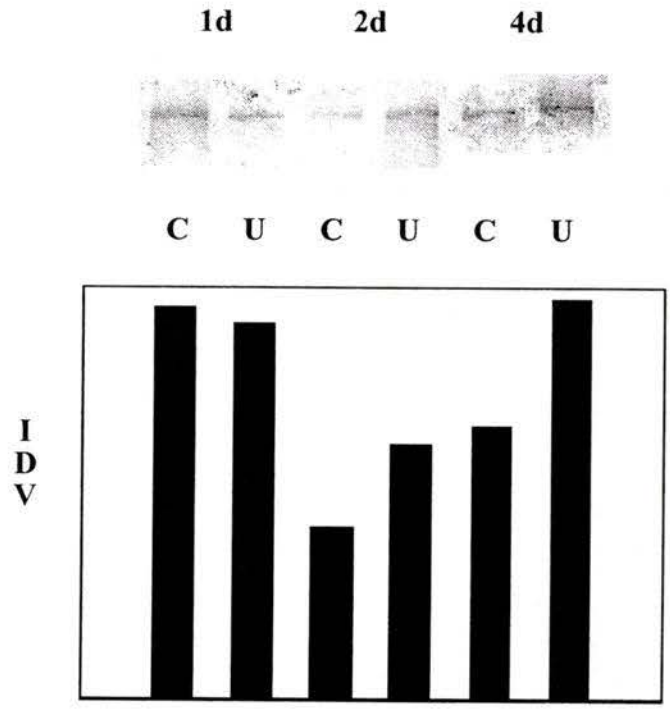

Figure 16. Western blot analysis of CPR protein accumulation in response to UV-B. Microsomal protein was isolated from control (C) or UV-B-irradiated (U) samples, which were grown under the conditions described in Figure 10. Equal amounts of protein (30 μg) were loaded per lane. Membrane was probed with antibodies against a synthetic peptide corresponding to a 14-amino acid region in the C-terminus of the Douglas-fir CPR protein (Tranbarger, 1998). Proteins with an apparent molecular mass of ~80 kDa were detected (upper panel). The western blot was scanned using the ChemiImager™ 4000 system. Integrated density values (IDV) are represented graphically (lower panel).

UV-B effects on Douglas-fir ubiquitin gene expression

The ubiquitin protein is used by the cell to label proteins for degradation, and several environmental stresses have been shown to increase its expression (Belknap and Garbarino, 1996). Recent studies have shown that the polyubiquitin gene *PU1* of pea is up-regulated in response to UV-B (Brosché and Strid, 1999; Brosché *et al.*, 1999). To determine whether Douglas-fir ubiquitin genes are regulated by UV-B, northern blot analysis was carried out using a white spruce (*Picea glauca*) partial ubiquitin cDNA as a probe.

Figure 17 shows the effect of UV-B on *ubi* mRNA transcripts in Douglas-fir seeds or seedlings after 2h, 12h, 24h or 48h of exposure. Transcript abundance was similar after 2h of exposure in UV-B-treated samples compared to the control, but was reduced to 68% of the control level after 12h of exposure (Figure 18). However, after 24h of exposure, a considerable increase in transcript amounts of UV-B treated samples was observed (134% of control), and was maintained in samples exposed for 48h.

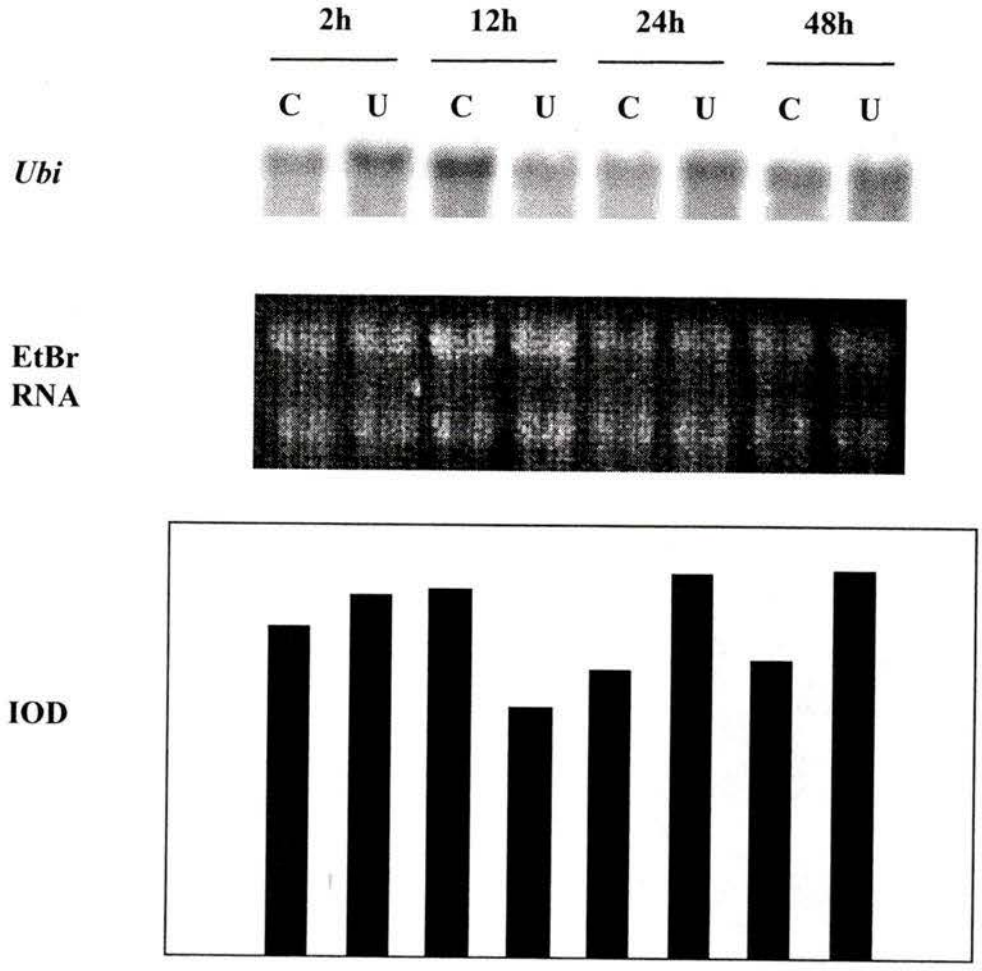

Figure 17. Northern blot analysis of *ubi* expression in response to UV-B. Total RNA was extracted from samples, then subjected to northern blot analysis (20 µg per lane) using a ^{32}P-labelled white spruce *ubi* cDNA probe (upper panel; refer to Materials and Methods). Ethidium bromide (EtBr) staining of total RNA was used to adjust for differences in the amount of RNA loaded per lane (mid panel). Differences in *ubi* mRNA amounts are represented graphically (integrated optical density, IOD) following adjustment in the amount of RNA loaded per lane (lower panel; refer to Materials and Methods).

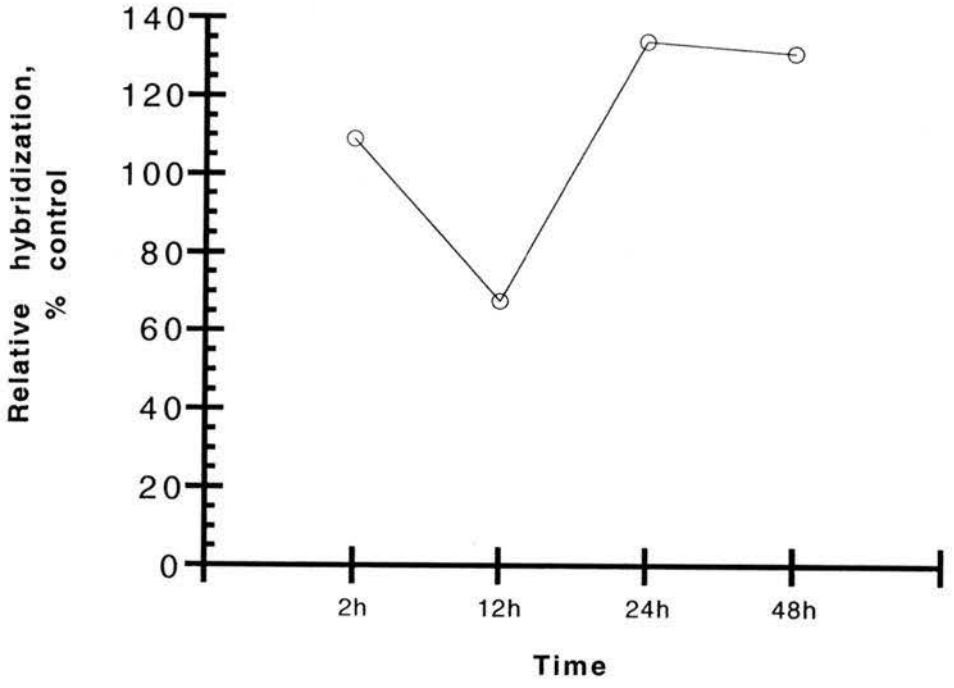

Figure 18. Changes in the abundance of *ubi* mRNA during exposure to UV-B, relative to control values. Northern blots were quantified using ImageQuant software. Values were normalized by the corresponding quantification of total RNA from the ethidium bromide stained gel, and integrated optical density (IOD) values were calculated for each band. Relative hybridization is defined as the IOD values calculated from UV-B treated samples with respect to IOD values from control samples, which were treated under the same conditions, but in the absence of UV-B, at equivalent times. Results are given as percentages of control values.

UV-B effects on Douglas-fir CAB gene expression

Several studies have shown that in pea (*Pisum sativum*) and *Arabidopsis*, UV-B causes a rapid and strong down-regulation of the expression of *cab*, a photosynthetic gene encoding the chlorophyll *a/b*-binding protein of the PS II light-harvesting complex (Kalbin *et al.*, 1997; Mackerness *et al.*, 1997a, 1997b, 1998a, 1999). To determine *cab* mRNA levels in response to UV-B in Douglas-fir, total RNA was isolated from seeds or seedlings grown in the absence or presence of UV-B for 2h, 12h, 24h or 48h, then subjected to northern blot analysis using a Douglas-fir *cab* cDNA probe.

Surprisingly, UV-B had very little effect on *cab* transcript levels in Douglas-fir (Figure 19). A maximum reduction in transcript abundance was detected after 2h of UV-B exposure, and even then the decrease amounted to only 24% of the control value (Figure 20). After 12h, transcript amounts of UV-B treated samples increased to control levels (104% of control) and remained at a relatively constant level thereafter: 89% of the control after 24h, and 96% of the control after 48h.

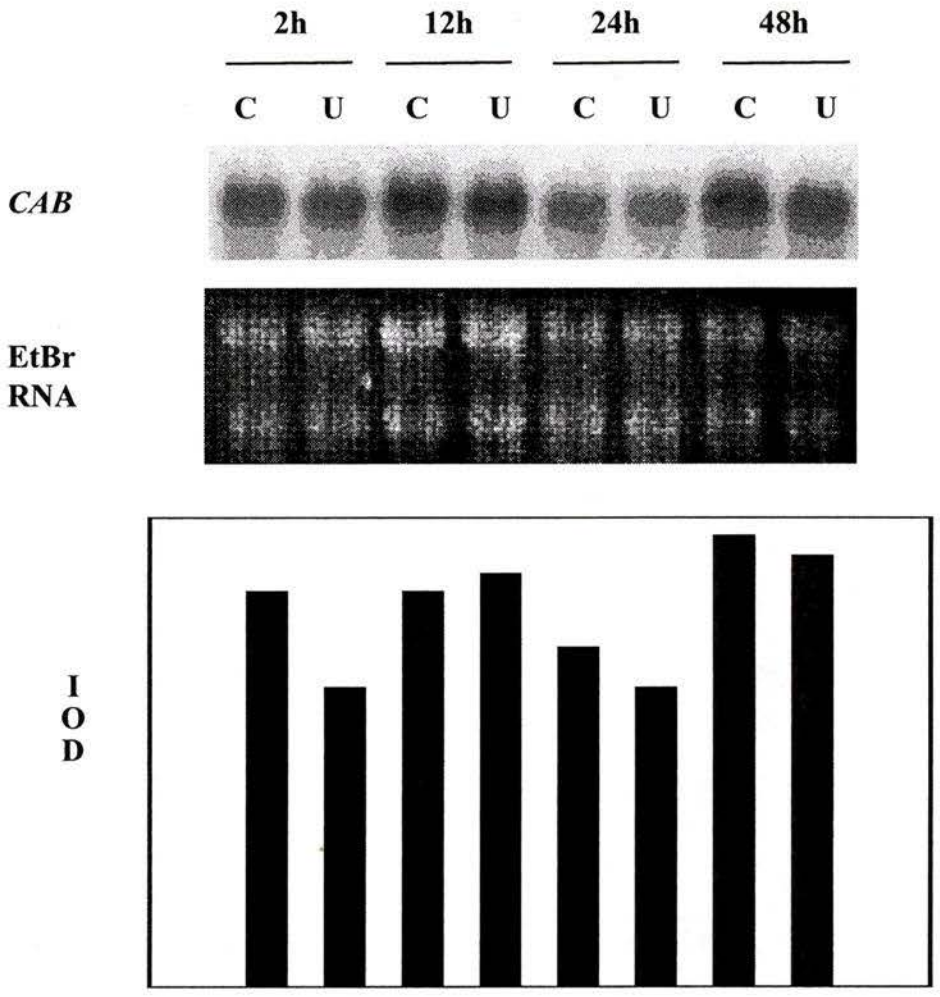

Figure 19. Northern blot analysis of *cab* expression in response to UV-B. Total RNA was extracted from samples, then subjected to northern blot analysis (20 µg per lane) using a ^{32}P-labelled Douglas-fir *cab* cDNA probe (upper panel; refer to Materials and Methods). Ethidium bromide (EtBr) staining of total RNA was used to adjust for differences in the amount of RNA loaded per lane (mid panel). Differences in *cab* mRNA amounts are represented graphically (integrated optical density, IOD) following adjustment in the amount of RNA loaded per lane (lower panel; refer to Materials and Methods).

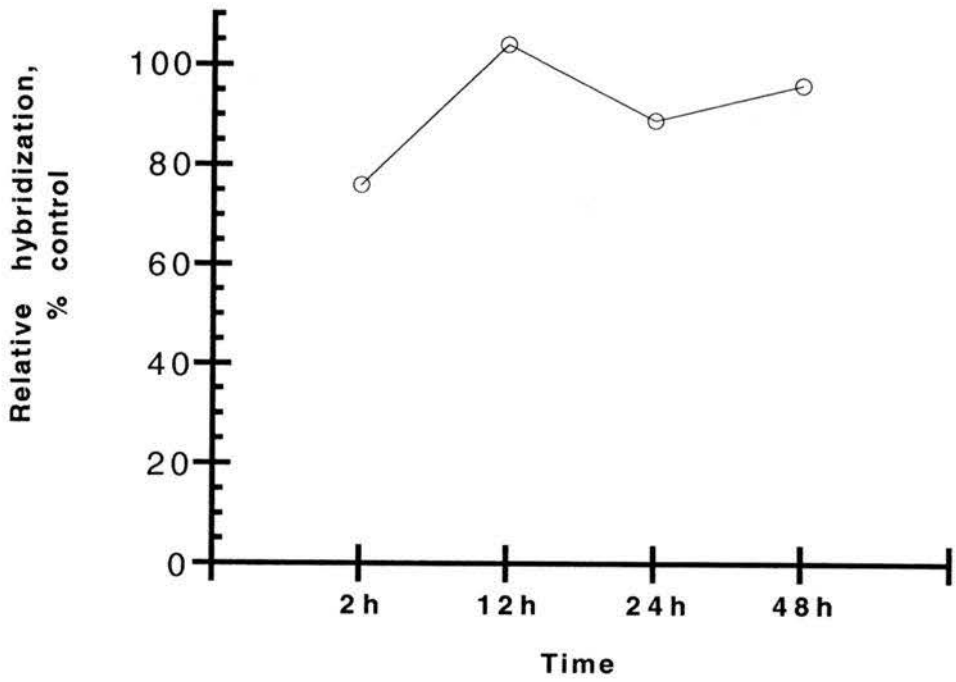

Figure 20. Changes in the abundance of *cab* mRNA during exposure to UV-B, relative to control values. Northern blots were quantified using ImageQuant software. Values were normalized by the corresponding quantification of total RNA from the ethidium bromide stained gel, and integrated optical density (IOD) values were calculated for each band. Relative hybridization is defined as the IOD values calculated from UV-B treated samples with respect to IOD values from control samples, which were treated under the same conditions, but in the absence of UV-B, at equivalent times. Results are given as percentages of control values.

PAL transcripts are not detected in Douglas-fir

A PCR amplified 1.1 kb genomic fragment of a jack pine (*Pinus banksiana*) phenylalanine ammonia lyase (*Pbpal1*) gene was kindly supplied by Dr. Brian Ellis (University of British Columbia, Vancouver, B.C., Canada). An LB plate with colonies growing on selection medium was provided. Plasmid purification was achieved as outlined in Materials and Methods, followed by excision of the insert with *Eco*RI and *Xba*I (Figure 21, A). Insert DNA was separated and purified as described in Materials and Methods (Figure 21, B).

The *Pbpal1* fragment was used as a probe in northern blot analyses of Douglas-fir mRNA, but no signal was detected. Three independent experiments were performed, each under progressively less stringent conditions. Although unlikely, the jack pine *pal1* fragment may not share adequate nucleotide identity with Douglas-fir *pal* to cross-hybridize. Alternatively, Douglas-fir *pal* corresponding to *Pbpal1* may not be expressed at the developmental stage examined.

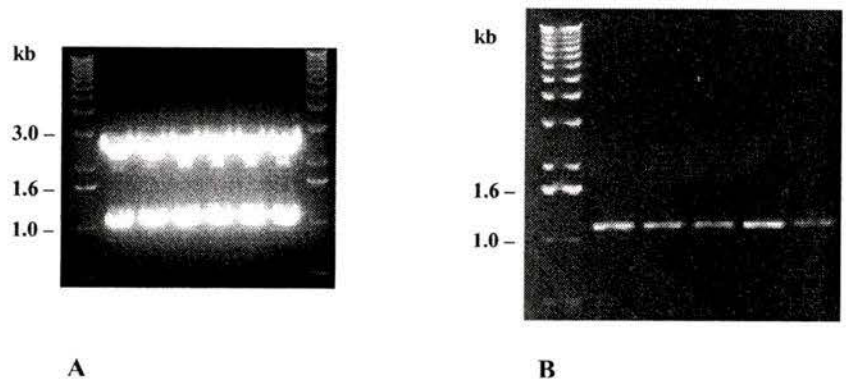

Figure 21. *Eco*RI and *Xba*I restriction digest (A) and purified fragment (B) of jack pine (*Pinus banksiana*) *Pbpal1*. The restriction digest (A) shows uncut plasmid (highest M_R band, ~2.6 kb) as well as one fragment of approximately 1.1 kb. Purified insert with a M_R of ~1.1 kb is shown in (B).

CHAPTER 4: DISCUSSION

Predictions based on expected rises in UV-B radiation in the next few decades have indicated that plant growth, development and likely yield will be affected. At present, the molecular mechanisms underlying UV-B effects are not well understood and, in particular, information on the molecular consequences of UV-B exposure in conifer species is very limited. Aside from a brief reference to chalcone synthase induction in Scots pine (Schnitzler, 1996), the results described here represent the first report on the effects of UV-B radiation on the expression of genes in a conifer species.

Genes for heat shock proteins

Plants respond not only to elevated temperatures, but to many other stresses with the production of HSPs, which function as 'molecular chaperones'. As a group, molecular chaperones are quite diverse and serve a variety of functions, some of which are particularly important for cells experiencing stress, including preventing aggregation of unfolded proteins and promoting renaturation of aggregated proteins (Boston *et al.*, 1996). The accumulation of heat shock-like proteins in the range of 70, 53 and 16 kDa has been shown to be induced by UV-B in cowpea (*Vigna sinensis*) seedlings (Nedunchezhian *et al.*, 1992). In the present study, two classes of molecular chaperones were induced by UV-B in Douglas-fir, HSP90 and BiP, indicating that an array of HSPs may be required by the plant to survive UV-B conditions by stabilizing protein folding and preventing protein aggregates.

HSP90

Exposure of Douglas-fir seeds and seedlings to UV-B led to a moderate increase in the amount of *hsp90* transcripts. Transcript levels of UV-B exposed samples were greater than those of controls as long as the plants were subjected to UV-B (4d). This suggests that HSP90 protein may be involved not only in the process of UV-B adaptation, but also in its maintenance. Consistent with this idea, a sustained increase in the level of protein during the 4d UV-B exposure was detected in western blot analysis.

Although the function of HSP90 and the nature of its interaction with other proteins have been well studied in mammalian cells (e.g. the interaction of HSP90 with steroid hormone receptors), knowledge of this important class of cytosolic chaperones in plant cells remains very limited. Genes encoding *hsp90*s along with their pattern of expression at the mRNA level have been reported in few plant species. Reddy *et al.* (1998) reported a high level of *hsp90* expression in developing seeds and germinating seedlings in the absence of stress in *Brassica napus*, suggesting that *hsp90* may play a functional role during these processes. Likewise, in the present study, *hsp90* transcripts were abundant and readily detectable in developing Douglas-fir seeds and germinating seedlings in the absence of UV-B. HSP90 may also play a role in protection against environmental stresses, as certain stimuli induce its expression. In *Brassica napus*, *hsp90* was induced by both high (42°C) and low (5°C) temperatures (Krishna *et al.*, 1995). Like UV-B, cold treatment induced a sustained increase in transcript levels throughout the experimental period (7d). As well, the heavy metals cadmium and arsenite induce the expression of *hsp90*

in *Arabidopsis* (Milioni and Hatzopoulos, 1997). While these observations suggest a role for *hsp90* during stress, the functional significance of these stress-induced expression patterns remains unknown.

Downs *et al.* (1999) have shown that the chloroplast small heat shock protein was induced in *Chenopodium album* (lamb's-quarters) and *Lycopersicum esculentum* (tomato) in response to UV-A radiation and oxidative stress (induced by methyl viologen). A common denominator among these stresses, as well as UV-B, is that all result in the production of ROS. The emerging concept implicating a role for ROS in the regulation of gene expression in response to UV-B radiation (Green and Fluhr, 1995; Mackerness *et al.*, 1998b; Surplus *et al.*, 1998) may extend to genes encoding various heat shock proteins.

Luminal binding protein: BiP

The expression of Douglas-fir *BiP* is modulated by UV-B and steady state transcript amounts appear to be transient and time-dependent: an initial decrease in transcript levels (up to 24 h of exposure) was followed by a substantial increase after 2d of exposure, and by 4d of exposure transcript amounts rose to 186% of the control value; however, by 6d of exposure, transcript levels fell back below those of the control. The pattern of BiP protein induction in Douglas-fir followed that of *BiP* transcripts during the same time course and under the same UV-B treatment. A similar induction of BiP by UV-B was found in *Arabidopsis*. Western blot analysis (using tomato anti-BiP antiserum) showed a slight increase in BiP accumulation in UV-B exposed *Arabidopsis* LER (wild-type) plants compared to controls (no UV-B), and a greater increase in BiP accumulation in UV-B exposed *Arabidopsis tt5*, a UV-

B-sensitive mutant defective in the ability to synthesize flavonoids, compared to controls (Appendix I, Figure AI-1). BiP mRNA and protein levels have been shown to increase in response to several environmental stimuli, such as desiccation (soybean; Figueiredo *et al.*, 1997), heat shock (*Arabidopsis*; Koizumi, 1996) and cold stress (Douglas-fir; Forward, 2000). BiP is a member of the HSP70 family of proteins and is thought to be involved in preventing the aggregation of misfolded proteins or protein domains (Boston *et al.*, 1996) which likely occurs under stress conditions.

The promoter region of Douglas-fir BiP was recently isolated and characterized (Forward, 2000). Interestingly, a group of cis-elements commonly found in the promoters of phenylpropanoid biosynthesis enzymes such as PAL and CHS and which interact with the Myb family of transcriptional activators were identified. Several Myb proteins have been implicated in the transcriptional activation of phenylpropanoid genes and it is thought that the Myb proteins may play a role in the activation of such genes in response to UV-B (Sablowski *et al.*, 1994). The observed increase in Douglas-fir *BiP* transcripts in response to UV-B lends supports to this contention.

Genes for polypeptides involved in protein degradation

Ubiquitin

Ubiquitin is one of the most conserved proteins known to date and is found in a wide variety of organisms including plants (von Kampen *et al.*, 1996). In most cases, ubiquitin acts as a marker for targeting a protein for its subsequent degradation, and

the system is therefore a major pathway for hydrolysis of short-lived, denatured, or abnormal proteins. It follows, then, that the expression of ubiquitin is often enhanced when plants are exposed to different stress conditions, such as wounding, heat stress and dehydration (Belknap and Garbarino, 1996). Detection of Douglas-fir *ubi* transcripts was achieved using a white spruce (*Picea glauca*) partial *ubi* cDNA as a probe. After 2h of UV-B exposure, transcript amounts were comparable to those of the control, followed by a short-lived declined to ca. 68% of the control after 12h. By 24h of exposure, transcript amounts increased to ca. 131% of the control and were maintained at this level after 2d of exposure. Recent studies have shown that the polyubiquitin gene *PU1* of pea is also up-regulated in response to UV-B (Brosché and Strid, 1999; Brosché *et al.*, 1999). In contrast to Douglas-fir, *PU1* of pea is more rapidly induced, increasing to over 200% relative to the control after only 12h of exposure. Given that some proteins are conceivably degraded upon UV-B exposure, it is reasonable to surmise that UV-B-damaged or degraded proteins may serve as signals to trigger the activation of ubiquitin genes.

Alternatively (or additionally), UV-B induced ubiquitin expression may be regulated by a ROS-mediated pathway. The presence of ROS has been thought to induce protein ubiquitination in some plant species (Shimogawara and Muto, 1991) and expression of *ubi* is induced by exogenously supplied JA and ethylene (Garbarino *et al.*, 1992, 1995). Taken together, these observations suggest that *ubi* may be regulated by the same pathway as the UV-B-mediated induction of PR genes, in which ROS act as secondary messengers leading to the synthesis of JA and ethylene which, in turn, stimulate the induction of PR genes (Mackerness *et al.*, 1999).

Defence genes

One powerful protective response against UV-B is the production of UV-B absorbing compounds, such as phenylpropanoids, flavonoids and sinapates. A UV-B-induced increase in the formation of certain enzymes involved in the synthesis of these protective compounds has been shown in several plant species.

NADPH:cytochrome P450 reductase: CPR

Some of the steps in the flavonoid biosynthetic pathway are catalyzed by cytochrome P450 monooxygenases, which are dependent on the supply of electrons from an associated CPR. Since many enzymes in the flavonoid pathway are regulated by UV-B, it is probable that any CPR involved in the same biosynthetic pathway would also follow this pattern of induction by UV-B. Indeed, Douglas-fir *cpr* expression was induced after 4d of UV-B exposure, and although the induction was rather long-delayed compared to the expression of other genes examined in this study, it was much stronger, with transcript amounts reaching over 400% relative to the control. Recent studies have shown a similar strong up-regulation by UV-B of two *cpr* genes: *CPR1* from parsley (Koopmann and Hahlbrock, 1997) and *PsC450R1* from pea (Brosché *et al.*, 1999).

A good correlation between CPR protein accumulation and the corresponding transcript amounts was generally found, with the exception of the 2d time point in which a higher level of protein was observed compared to the transcript level in UV-B treated samples. Perhaps this is a reflection of the mRNA versus protein stability of CPR after exposure to UV-B. Other studies demonstrate that the protein component is substantially more stable than the mRNA moiety under stress

conditions. For instance, Koopman and Hahlbrock (1997) reported similar discrepancies in CPR1 transcript and protein levels in elicitor-treated cultured parsley cells: transcript levels started to decline after 8h, whereas protein accumulation continued up to 20h. Furthermore, Rubisco protein is much more stable than its corresponding mRNA in UV-B treated pea plants (Jordan *et al.*, 1992).

Phenylalanine ammonia lyase: PAL

The increase in the level of UV-B protective compounds is due to a coordinated increase in the expression and activity of certain enzymes of the phenylpropanoid pathway, including PAL. Studies in pea, *Arabidopsis*, tobacco and barley have shown that there is an increase in *PAL* expression in response to UV-B exposure (Kuhn *et al.*, 1984; Kubasek *et al.*, 1992; Strid, 1993; Liu and McClure, 1995; Kalbin *et al.*, 1997; Fujibe *et al.*, 2000) and a subsequent increase in the composition of protective pigments (Day *et al.*, 1992; Jordan *et al.*, 1994; Mackerness *et al.*, 1997b). Although most genes of the phenylpropanoid pathway can be activated by a variety of other environmental stimuli (wounding, pathogen attack, fungal elicitors, blue light, high-intensity white light), UV-B irradiation is most effective in the induction of this pathway (Kubasek *et al.*, 1992; Jordan *et al.*, 1994).

A PCR amplified 1.1 kb genomic fragment of a jack pine (*Pinus banksiana*) phenylalanine ammonia lyase (*Pbpal1*) was used as a probe in northern blot analyses of Douglas-fir mRNA. Unfortunately, no signal was detected in any of three independent experiments performed, each under progressively less stringent conditions, even after several days of exposure under a storage phosphor screen. Although unlikely, the jack pine *pal1* fragment may not share adequate nucleotide

identity with the corresponding Douglas-fir *pal* to cross-hybridize. Alternatively, Douglas-fir *pal* corresponding to *Pbpal1* may not be expressed at the developmental stage examined. In jack pine, PAL is encoded by a multigene family of at least eight to ten loci, and it is thought that PAL in other conifer species is encoded by similar multigene families (Butland *et al.*, 1998). In angiosperms, *pal* genes vary in their spatial and temporal expression patterns; indeed, multigene families may exist in part so that gene family members might be specialized for expression at different times. It would be of interest to extend this research to determine how different members of the *pal* gene family respond to UV-B. In any case, it is interesting to note that in the only report to date of UV-B effects on PAL in a conifer species (Scots pine; Schnitzler *et al.*, 1997), PAL activity was only slightly induced by UV-B in mature needles of 3-week old seedlings, and no UV-B-induced PAL activity was observed in cotyledonary and primary needles.

Photosynthetic genes

Chlorophyll a/b-binding protein

One of the frequently reported responses to UV-B is a reduction in the rate of photosynthesis, and one of the factors contributing to this response is a decline in the level of transcripts for a number of key photosynthetic genes, including *cab* (reviewed in Mackerness and Jordan, 1999). CAB, or the chlorophyll *a/b*-binding protein of the light-harvesting complex, is involved in capturing light energy, which is the primary step of photosynthesis. The trapped energy is then transferred to the reaction centres of photosystems I and II.

An interesting result of this work is that, quite surprisingly, UV-B had very little effect on *cab* transcript levels in Douglas-fir: decreases in transcript abundance were minimal, if at all, in UV-B treated samples relative to controls at all time points examined. This is contrary to all reports of UV-B effects on *cab* expression to date, in which the reduction in *cab* transcripts is generally quite considerable and occurs rapidly (Jordan *et al.*, 1991, 1994; Kalbin *et al.*, 1997; Mackerness *et al.*, 1997b, 1998a; Liu and White, 1998; Brosché *et al.*, 1999). Having said that, all of these studies have been conducted on pea, which is one of the species most sensitive to UV-B (Jordan *et al.*, 1994). It is unknown whether the contradiction between Douglas-fir and pea is due to difference in species or other factors. It has been recently demonstrated that individual members of the pea *cab* multigene family respond differently to UV-B (Liu and White, 1998; Mackerness *et al.*, 1998a); although UV-B reduced transcript levels of all genes studied, some were much more sensitive to UV-B than others. In some cases, very slight decreases were detected; therefore, differences in UV-B responses may be dependent on the particular *cab* family member.

An interesting aspect of Douglas-fir (and most gymnosperm species) is that it is able to synthesize chlorophyll in the dark (Mariani *et al.*, 1990). Furthermore, it has been demonstrated that other gene products associated with photosynthesis are present in significant amounts in dark-grown gymnosperm tissue (Alosi *et al.*, 1990; Mukai *et al.*, 1991; Yamamoto *et al.*, 1991; Alosi and Neale, 1992). For example, dark-grown Douglas-fir shoots had significant levels (25-30% of the level that accumulated in light-grown seedlings) of *cab* mRNA (Alosi *et al.*, 1990; Alosi and

Neale, 1992) and, unlike angiosperms, *cab* expression is apparently not regulated by factors associated with circadian rhythms (Alosi *et al.*, 1990). Of course, light plays some role in development of the photosynthetic apparatus in Douglas-fir, since light-grown seedlings accumulate three or more times as much *cab* as do dark-grown seedlings; however, the substantial constitutive level of *cab* mRNA in the dark may mask any UV-B effect taking place during light (and UV-B) treatment.

CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

Conclusions

A number of conclusions can be made from this study on the effects of UV-B radiation on Douglas-fir gene expression. To start, the response of different genes to UV-B is varied, ranging from a very subtle down-regulation in the case of *cab*, to a considerable transcript increase over time in the case of *cpr*. Overall, these data show that UV-B changes gene expression in a specific manner that is dependent upon particular genes. Furthermore, the response of particular genes to UV-B in conifer species is not necessarily comparable to that of corresponding genes in angiosperm species; contrary to angiosperm species, *cab* gene expression was not markedly reduced in Douglas-fir.

An important aspect of the UV-B response is its similarity to that caused by other environmental stimuli, such as pathogen infection, ozone exposure and wounding (Mackerness and Thomas, 1999). The explanation for this could be that many stress factors, at least as one mode of action, cause oxidative stress, which in turn triggers the production of ROS. Recent studies have shown that ROS play a pivotal role as secondary messengers acting on several distinct pathways involved in the regulation of a number of genes (refer to Figure 2). The work presented here hints at the possible involvement of ROS in the regulation of ubiquitin and heat shock proteins (HSP90) in response to UV-B.

Future Studies

Differential display

Further studies on the alteration of gene expression will provide greater insight into the response of plants to UV-B radiation. For instance, additional biochemical pathways which are important for protection and stress responses may be identified. The approach taken in this study was to examine specific genes that were likely to be affected by UV-B through northern blot analyses. An alternative approach would be the use of differential display to identify UV-B-inducible genes. With this method, specific mRNA species, the levels of which are lowered or increased as a result of exposure of plants to UV-B, can be isolated. Genes that are differentially expressed can then be sequenced and identified. Since the differential display method is PCR based, its sensitivity is higher than that of hybridization procedures in which sensitivity may be limited, only allowing detection of relatively abundant mRNAs. In recent years, the differential display technique has been widely used for finding genes expressed only under certain conditions (Liang and Pardee, 1995). As well, differential display has been used as a highly efficient technique for expression analysis of multigene families, allowing the detection of expression of individual members of a multigene family at different developmental stages or in diverse tissues (Fischer *et al.*, 1995). A pitfall of the differential display approach is that the number of false positives obtained can be extremely high, reaching over 85% in some cases (Brosché *et al.*, 1999).

Signal transduction pathways in Douglas-fir

Several UV-B-induced alterations in gene expression have been described, but many appear as indirect consequences of exposure to UV-B rather than the primary response. It is therefore particularly important to determine the signal transduction pathways involved in these responses. Plant responses to UV-B share many common components with responses to other oxidative type stresses. To my knowledge, no studies on the effects of enhanced UV-B radiation on oxidative stress in conifers have been published yet, and further studies are needed. An interesting and important question is whether similar regulatory systems control gene expression in Douglas-fir and in angiosperm species. Although much work to date on angiosperm species involves the use of mutant plants defective in particular gene functions or pathways, this approach is not feasible with conifer species. As a start, however, simple spraying experiments could be conducted. For example, to determine if ROS play a role in the regulation of expression of heat shock proteins, ubiquitin or *cpr* genes, oxidants (such as 3-amino-1,2,4-triazole) could be applied to plants to determine if they mimic the effects of UV-B on gene expression. Following this, treatment of plants with antioxidants (such as ascorbic acid) prior to UV-B treatment could be done to determine if the effect of UV-B on the same set of genes is reduced. Similar experiments have been successfully conducted on *Arabidopsis* (Mackerness, 2000), pea (Mackerness *et al.*, 1998b) and tobacco (Green and Fluhr, 1995). The application of oxidants led to an increase in transcripts for particular genes that were also induced by UV-B, and the effect of UV-B on the same transcripts was reduced in the presence of the antioxidant ascorbic acid, indicating that removal of ROS reduced the effects of

UV-B on gene expression and, therefore, ROS must be an important intermediate in the pathway leading to the regulation of these genes by UV-B.

Future work should, therefore, include the identification and further characterization of these pathways and components in Douglas-fir, as they may be promising targets for future engineering of UV-B tolerance in important conifer species. Of course, identification of the receptors involved in perception of UV-B would be of great value and, perhaps, would provide a way of manipulating UV-B responses specifically without affecting responses to other stresses.

Transgenic studies

One decisive way to determine the importance of a particular defence response in relation to a stress is to examine its role in over- or under-expressing transgenic plants. The production of transgenic conifers has recently been achieved in several species, including black spruce (*Picea mariana*; Tian *et al.*, 1999), larch (*Larix* species; Pilate *et al.*, 1999), longleaf pine (*Pinus palustris*; Diner, 1999), radiata pine (*Pinus radiata*; Walter and Smith, 1999), loblolly pine (*Pinus taeda*; Wenck *et al.*, 1999) and Norway spruce (*Picea abies*; Wenck *et al.*, 1999). Transformation in these species has been carried out by the use of bacteria, namely *Agrobacterium*, or by the direct introduction of DNA into the plant cell using physical means such as particle acceleration or microprojectile bombardment. The ability to create transgenic conifer plants will open the door to a better understanding of the genetic regulation of important genes in response to UV-B.

Field studies

Realistically, it is not possible yet to predict whether the induction of genes by UV-B will potentially benefit important forestry (or agricultural) species. Most studies of responses of gene expression to UV-B have been conducted in controlled laboratory conditions. Whether or not similar changes in gene expression due to exposure to UV-B occur under natural conditions is not clear at present. This is indeed one of the most important questions to deal with in the future. Moreover, although several genes induced by UV-B have been identified, little is known about their environmental relevance.

Most of the knowledge on the responses of conifers to UV-B has come from studies conducted on young (<1-year-old) seedlings. These studies are certainly merited, since young seedlings may be more susceptible to UV-B than older trees; however, extrapolation of results obtained from small seedlings to predict the future of mature trees is questionable and may result in false assumptions. Therefore, in the future it will be important to conduct experiments on trees grown for several years (preferably the whole life-span of a needle) in natural conditions or field sites.

Additionally, the complex interaction between UV-B and other environmental parameters must be studied in greater detail, since a combination of stress factors may bring about a variety of responses that cannot be predicted on the basis of studying a single environmental parameter. Present predictions and scenarios can only touch upon the true complexity of the possible future situation. Consequently, unless multiple simultaneous stress factors are studied, the environmental impact of UV-B may not be correctly predicted.

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APPENDIX I: UV-B Effects on BiP Accumulation in *Arabidopsis thaliana*

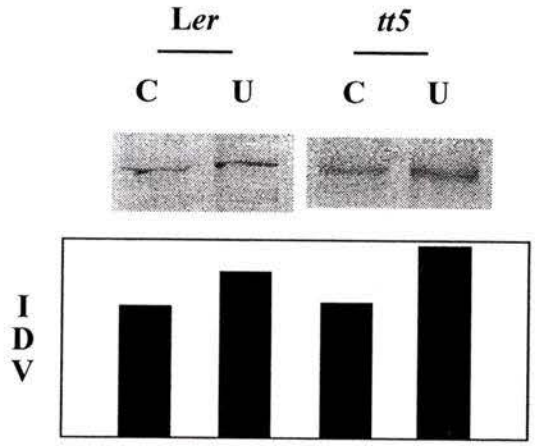

Figure AI-1. Western blot analysis of BiP protein accumulation in response to UV-B in two *Arabidopsis* genotypes. Microsomal protein was isolated[1] from control (C) or UV-B-irradiated (12 kJ m^{-2} d^{-1}; U) plants which were grown under the conditions described in Schmidt *et al.* (2000). *Arabidopsis* wild-type (L*er*) and the UV-B-sensitive transparent testa mutant (*tt5*) were analysed. Equal amounts of protein (20 µg) were loaded per lane. Membranes were probed with anti-BiP antibodies from tomato. Proteins with an apparent molecular mass of ~80 kDa were detected (upper panel). The blots were scanned using the ChemiImager™ 4000 system. Integrated density values (IDV) are represented graphically (lower panel).

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[1] Microsomal protein from *Arabidopsis* was isolated by a modification of the method of da Silva Conceição *et al.* (1997). All extraction steps were done at 4°C. Frozen plant tissue was ground in liquid nitrogen; 4 ml extraction buffer (100 mM Tris-HCl, pH 7.4; 250 mM sucrose; 1 mM EDTA; 1 mM PMSF; 2.8 mM ß-mercaptoethanol) was added per gram of tissue. Tissue was further ground, and debris was pelleted twice by centrifugations at 500 x g for 5 min. Organelles of high density were pelleted by centrifugation of the supernatant at 8 000 x g for 15 min. The supernatant was further centrifuged at 100 000 x g for 2 hr to pellet microsomal membranes. Pellets were resuspended in a buffer containing 50 mM sodium phosphate (pH 7.4), 20% glycerol and 10 mM ß-mercaptoethanol.

VITA

Surname: Schmidt Given Names: Anna-mary

Place of Birth: Calgary, Alberta, Canada

Educational Institutions Attended:

Camosun College	1988-1991
University of Victoria	1991-1993
University of Victoria	1996-2001

Degrees Awarded:

B.Sc. Biochemistry, University of Victoria 1993

Honours and Awards:

Brett Fisher Memorial Award, 1988
Academic Science Award, 1990
Canada Scholarship, 1990-1993
Graduate Teaching and Research Fellowship, University of Victoria, 1996-2000
Graduate Student Travel Grant, University of Victoria, 1997, 1998
Centre for Forest Biology Travel Grant, University of Victoria, 1997, 1998
Regional Directors' Award for best research presentation, Western Regional Meeting of the Canadian Society of Plant Physiologists,1998

Publications:

Ormrod, D. P., Schmidt, A. and Livingston, N. J. (1997) Effect of UV-B radiation on the shoot dry matter production and stable carbon isotope composition of two *Arabidopsis thaliana* genotypes. Physiologia Plantarum 101: 497-502

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Anna-mary Schmidt
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